Contents lists available at ScienceDirect

# Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

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

Juliana M.B. Ricci<sup>a,1</sup>, Emanuel R.M. Martinez<sup>a,1</sup>, Arno J. Butzge<sup>a</sup>, Lucas B. Doretto<sup>a</sup>, Marcos A. Oliveira<sup>a</sup>, Robie Allan Bombardelli<sup>b</sup>, Jan Bogerd<sup>c</sup>, Rafael H. Nóbrega<sup>a,\*</sup>

<sup>a</sup> Reproductive and Molecular Biology Group, Department of Morphology, Institute of Bioscience of Botucatu, São Paulo State University, Botucatu, São Paulo, Brazil

<sup>b</sup> Center of Engineering and Exact Sciences, Universidade Estadual do Oeste do Paraná, Rua da Faculdade 645, 85903-000 Toledo, PR, Brazil

<sup>c</sup> Reproductive Biology Group, Division Developmental Biology, Department of Biology, Faculty of Sciences, Utrecht University, Hugo R. Kruyt Building, Padualaan 8, 3584,

CH, Utrecht, The Netherlands

# ARTICLE INFO

Keywords: vasa Teleost Primordial germ cell Germ cell

# 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

E-mail address: nobregarh@ibb.unesp.br (R.H. Nóbrega).

<sup>1</sup> These authors contributed equally to this work.

https://doi.org/10.1016/j.gene.2018.02.029 Received 29 September 2017; Received in revised form 19 January 2018; Accepted 12 February 2018 Available online 14 February 2018 0378-1119/ © 2018 Elsevier B.V. All rights reserved.







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 \* Corresponding author at: UNESP - Campus de Botucatu, Instituto de Biociências, Rua Prof. Dr. Antonio Celso Wagner Zanin, s/n°, 18618-689 Botucatu, Brazil.

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<sup>2</sup> 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 2001 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 RqvasaRT-Fw and RqvasaRT-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
vasaFw3'-UPM (4156)	CAACCCACCCAAGGCTATCATGACAATCATGACATTTGAAGAA	RACE
vasaFw3'-NUP (4157)	GCCCAATTGTGTGAGACACTGAACAAAAATGTTGCTAAGT	RACE
vasaRv3'-NUP (4167)	ATACTTCTGAACAGGGGTAGGTTTCACGTATCCAGACTTA	RACE
vasaRv3'-UPM (4164)	TTCCCTGATCCAGTCTGGGCACAAGCCATGAGATC	RACE
vasaRv3'-NUP (4165)	CACAAGCCATGAGATCCCTCCCAGCAGATAT	RACE
vasaRv3'-NUP (4166)	CCCAGCAGATATGATGGGAATTCCATACTTCTGAACA	RACE
vasaRv3'-UPM (4163)	ACCTTCATTCATCAGATGCTGCAGAATAGGCAGC	RACE
vasaRv3'-NUP (4159)	CGTTGGTGCCTCCATAAACGACAACAGGACG	RACE
vasaRv3'-UPM (4158)	CACTTCTCGGATTGTAAATCCGACGTTGGTGC	RACE
RqvasaRT-Fw	GGCAGAGGTGGGCGTGGGGGGAAG	RT-PCR
RqvasaRT-Rv	CCACGACCAATAATGTCAAGCAATCTTCCAGGG	RT-PCR
RT-β-actin-Fw	TGACCTGACTGACTACCTCA	RT-PCR
RT-β-actin-Rv	AGCTCATAGCTCTTCTCCAGGGGCGGGGTG <u>TTATTAACCCTCACTAAA</u> GAGGCT	RT-PCR
RqvasaT3-Fw	ATCATGACATTTGAAGAAG	ISH
RqvasaT7-Rv	CCGGGGGGGTG <u>TAATACGACTCACTATA</u> GACACAGGTGCCATAAGCA	ISH
Rqvasa-qPCR-Fw	AGGCTATCATGACATTTGAAGAAG	qPCR
Rqvasa-qPCR-Rv	CCATACTTCTGAACAGGGGT	qPCR

were conducted using specific primers for *Rayasa* and  $\beta$ -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  $\Delta\Delta$ Ct 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 RqvasaT3-Fw and RqvasaT7-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 ( $3\mu$ m 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  $\pm$  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 Cterminal 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 Cterminal (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),

cacagcaggetcacaggaccage	t 24
cgacatttactatagccttcttcttagttcaaaccagtctcacagcttcgagagatgtcaca	.g 87
gaccagctcggcatttacagaccattattaaactatctgtatatctctcagctgtctgaaca	.c 150
<b>ATC</b> GAGGACTGGGAAGATGAACAGAGCCCAGTTGTAACCAGCTTAACTCTGGGCAAAGCAGA	A 213
M <b>EDWEDE</b> QSPVVTSLTLGKAE	21
AATGCATGGAACAGCAATGGACAACAAAACGGCAAGGACAATGAAGAAAGTTCTTGGAAACC	T 276
N A W N S N G Q Q N G K D N E E S S W K P	42
GGTGCTGGTTTTGGGACTCAGGGGGGAAACAGAGATCGGGGGTTTGGAAAAGTTGATGGAGA	C 339
G A G F G T Q G G N R D <b>R G</b> F G K V D G D	63
TTCAAGGGCTTCAGAACAGGAATTGATGAAAATGCAA <del>ATGA</del> AGGAGTTGATAATGGCCACTC	C 402
F K G F R T G I D E N A N E G V D N G H S	84
TGGAACACTGGTGGAGAGGGCTTTAGCGGACGAGGAGGCAGAGGACGAGGAGGACGAGGAG	A 465
W N T G G E G F S G <b>R G G R G R G G R G G</b>	105
GGAAGAGGATTTAGGAATTCCTTCAAATCTG <mark>ATGATGAAAATG</mark> CGAGTGATGAAGGTTTTAA	A 518
G <b>RG</b> FRNSFKSDDENASDEGFK	126
AGTGGCTTTAGTGGCAGAGGTGGGCGTGGAGGAAGAGGAGGCCGTGGAGCTTTCCAACAAGG	T 581
S G F S G <b>R G G R G G R G G R G A</b> F O O G	147
GGTGATGAAGAAGGCAAAGGACGCTTTGGTGGAGGCTATAGAGGACAGAACGAGGAGATATT	'T 644
G D E E G K G R F G G G Y <b>R G</b> O N E E I F	168
TCAAAGGGATCACCAAAGGATAACGAGGAAAAAGAAGATGGTGAGACTGCAGGGCCTAAGGT	'C 707
S K G S P K D N E E K E D G E T A G P K V	189
AACTATATTCCACCGCCACCGGGAGGAGGAGGAGCTCCATTTTTCTCACTACGCAACAGG	C 770
ΝΥΙΡΡΡΡΕΕΕΝSΙFSΗΥΑΤΘ	210
ATCAACTTTGACAGGTATGATGACATCCTGGTGGATGTAAGCGGAAGCAACCCACCC	T 833
INFDRYDDILVDVSGSNPPKA	231
ATCATGACATTTGAAGAAGCCCAATTGTGTGAGACACTGAACAAAATGTTGCTAAGTCTGG	A 896
IMTFEEAOLCETLNKNVAKSG	252
TACGTGAAACCTACCCCTGTTCÃGAAGTATGGAATTCCCATCATATCTGCTGGGAGGGATCT	'C 959
Y V K P T P V O K Y G I P I I S A G R D I	273
ATGGCTTGTGCCCAGACTGGATCAGGGAAAACGGCTGCCTTCCTGCTGCCTATTCTGCAGCA	T 1022
M A C A O T G S G K T A A F L L P I L O H	294
CTGATGAATGAAGGTTTAGCATCCAGCAAGTTCAGTGAGCTGCAGGAGCCTGAAGTCATCAT	'T 1085
L M N E G L A S S K F S E L O E P E V I I	315
GTTGCGCCCACTCGGGAACTCATTAATCAGATTTACCTAGAAGCCCGCAAGTTTGCTTATGG	C 1148
VAPTRELINOIYLEARKFAYG	÷ 336
ACCTGTGTGCGTCCTGTTGTCGTTGTCGTTTATGGAGGCACCAACGTCGGATTTACAATCCGAGAAGT	'G 1211
T C V R P V V Y G G T N V G F T I R E V	357
TTAAAAGGTTGCAATGTGCTGTGTGGGGGCCCCTGGAAGATTGCTCGACATTATTAACCGTGG	A 1274
LKGCNVLCGTPGRLLDIINRG	378
AAGGTTGGATTAAGTAAAATTCGTTTTTTGGTGCTGGATGAAGCTGATCGAATGTTGGATAT	'G 1337
KVGLSKIRFLVLDEADRMLDM	1 399
GGATTTGAGGCGGACATGCGAAAGCTGGTAAACTCTCCAGGAATGCCTTCTAAAGAAGAGGCG	A 1400
GFEADMRKLVNSPGMPSKEER	420
CAAACCCTTATGTTCAGTGCCACTTACCCGGAAGATATTCAGAAGCTGGCAGCCGACTTCCT	'A 1463
	441
AAGGTGGATTATCTGTTCCTGGCTGTGGGAGGAGGGGGGGG	G 1526
KVDYLFLAVGVVGGACNDTEC	462
	A 1589
нттоуто у сквекь во в кт	483
GGGACACAGAGAACAATGGTCTTTGTTGAAACGAAACGA	C 1652
G T O R T M V F V E T K R S A D F T A T F	' 502
	G 1715
L C O E K M P T T S T H G D R E O R E R F	
	יבכ ד 1779
	546

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 (<u>RG</u>) and arginine-glycine-glycine (<u>RG</u>) 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 (<u>aataaa</u>) 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

AGA	GGA	CTA	GAC.	ATT	GAG	CAT	GTC	CAG	CAI	GTG	GTO	GAAC	CTTI	GAC	CTG	GCCI	'AAA	GAA	ATT	GAT	1841
R	G	L	D	I	Ε	Η	V	Q	Η	V	V	Ν	F	D	L	Ρ	Κ	Ε	I	D	567
GAGTATGTGCACCGCATTGGGAGAACGGGCCGATGTGGAAACACAGGAAGAGCCGTGTCCTTT													1904								
Ε	Y	V	Η	R	I	G	R	Т	G	R	C	G	Ν	Т	G	R	Α	V	S	F	588
TTTGACCCGGAGGCCGATACTCCGTTGGCCCGCTCTCTGGTCAAAGTCCTTTCCGAGGCCCAG													1967								
F	D	Ρ	Ε	Α	D	Т	Ρ	L	Α	R	S	L	V	Κ	V	L	S	Ε	А	Q	609
CAG	GAA	GTT	CCT	TCA	TGG	CTG	GAG	GAA	ATT	GCA	ATTO	GGG	rgci	CAC	GGI	ACC	CACA	GGG	TTT	AAC	2030
Q	Ε	V	Ρ	S	W	L	Ε	Е	Ι	Α	L	G	A	Η	G	Т	Т	G	F	Ν	630
CCA	CGT	GGT	AAA	GTG	TTT	GCC	TCA	ACT	GAC	CTCI	CGC	CAG	GGGI	GGA	TCC	CTTI	ACA	AAG	AAC	CTG	2093
Ρ	R	G	Κ	V	F	А	S	Т	D	S	R	R	G	G	S	F	Т	Κ	Ν	L	651
GCA	CCA	CAA	CCA	GCT	GCA	CAA	AGC	ACC	GTC	CACI	GCI	rgco	CGCI	GAT	'GAC	CGAC	CGAC	TGG	GAG	TAG	2156
А	Ρ	Q	Ρ	А	А	Q	S	Т	V	Т	А	А	Α	D	D	D	D	W	Е		672
att	gac	ttt	ttt	ttt	gtt	ttt	ttg	ttg	tgc	ggt	att	cct	cto	gttc	att	ttt	tta	tac	ata	ttt	2219
tat	gtt	aaa	taa	gag	gcc	tat	ggg	ccc	aaa	agt	ata	aaaq	gaga	laga	gaa	atgo	gcag	gtc	ctg	agc	2282
tat	ggc	act	gaa	aga	gcc	aca	ggt	tca	acc	tga	agt	taa	aget	tca	caa	attt	aat	gtt	agt	aat	2345
ttt	gga	gtg	act	gac	ttt	aac	tgc	ttg	tgt	gtt	tgo	cact	taa	laga	ttt	ttt	ttg	rttt	gtt	ttg	2408
ctg	tga	gag	ctc	cta	gta	ggc	tag	aca	tca	acto	gtgt	cgga	atco	tgt	agt	aca	aaaa	att	aca	ttg	2471
agt	ttg	taa	aag	cct	tca	gct	cga	ggt	tgc	ato	ctgo	cact	ctt	tca	tca	aat	age	tct	ttt	ctg	2534
сса	cat	gtt	tcc	agg	ttg	tat	ttt	ggt	cac	tat	att	tgt	gto	Jaag	igca	igtt	gto	gaa	aaa	aaa	2597
aag	ttg	ttt	tgc	tat	tac	aat	aaa	ttc	aca	icaa	atac	yaaa	attt	aaa	aaa	aaaa	aaaa	aaa	aaa	aaa	2660
aaa	aaa	aaa	aa																		2671

# Fig. 1. (continued)

Rhamdia quelen EU532190\_Silurus\_meridionalis GU562470\_Clarias\_gariepinus AJ311625\_Danio\_rerio XP005248604\_Homo\_sapiens NP034159\_Wus\_musculus XP007053932\_Chelonia\_mydas NP\_90039\_Gallus\_gallus AAI61525\_Xenopus\_tropicalis CAA31405\_Drosophila\_melanogast

Rhamdia\_quelen EU532190\_S1lurus\_meridionalis GU562470\_Clarias\_gariepinus AJ311625\_Danio\_rerio XP005248604\_Homo\_sapiens NP034159\_Wus\_musculus XP007053932\_Chelonia\_mydas NP\_990039\_Gallus\_gallus AAI61525\_Xenopus\_tropicalis CAA31405\_Drosophila\_melanogast

Rhamdia\_quelen EU532190\_Silurus\_meridionalis GU562470\_Clarias\_gariepinus AJ311625\_Danio\_rerio XP085248604\_Homo\_sapiens NP084159\_YUNs\_musculus XP007053932\_Chelonia\_mydas NP\_900039\_Gallus\_gallus AAI61525\_Xenopus\_tropicalis CAA31405\_Drosophila\_melanogast

Rhamdia quelen EU532190\_Silurus meridionalis GU562470\_Clarias\_gariepinus AJ311625\_Danio\_rerio XP003248604\_Homo\_sapiens NP094159\_Wus\_musculus XP007053932\_Chelonia\_mydas NP\_990039\_Gallus\_gallus AL161525\_Xenopus\_tropicalis CAA31405\_Drosophila\_melanogast

Rhamdia\_quelen EU532190\_Silurus\_meridionalis GU562470\_Clarias\_gariepinus AJ311625\_Danio\_rerio XP005248604\_Homo\_sapiens NP004159\_Yus\_musculus XP007053932\_Chelonia\_mydas NP.990039\_Gallus\_Blus AAI61525\_Xenopus\_tropicalis CAA31405\_Drosophila\_melanogast

Rhamdia_quelen
EU532190_Silurus_meridionalis
GU562470_Clarias_gariepinus
AJ311625_Danio_rerio
XP005248604_Homo_sapiens
NP034159_Mus_musculus
XP007053932_Chelonia_mydas
NP_990039_Gallus_gallus
AAI61525_Xenopus_tropicalis
CAA31405 Drosophila melanogast

<pre>Decomparing the result of the result of</pre>	MEDWEDDQS-PW/TNTT-FGNAENSWNWGNQNSLKRITHDNKE-SSWKSAGDGFGSQDGDMMGSRKGSGDFKSFSSGVDENGNKE-VDGGNS-MN-GGDCFRGRGSRGRG-RGGRGSRGSRGSRGSRGSRGSRGS	109 116
	ADDRUGEDS - VYSESSI GESIGSGA IST INGARAN ISTOLE SSAM MORAN INFORMATION AND AND AND AND AND AND AND AND AND AN	107
Head of the second sec	MGDEDNEAELILKPIVSSVYPVFEKDVSSGANODTPNT3ASS-DIGESSK/KENTSTTGGFSRK/GFGNRGFLNNFFEG-DSSGFWKESNNDCEDNQTRSNGFSKRGQCQGOND MGDEPNIPTCD_DVSSGYPVFEKDVSGGANDPFNT3ASS-DIGESSK/KENTSTTGGFSRKGSFGNRGFLNNFFEG-DSSGFWKESNNDCEDNQTRSNGFSKRGQCQGOND	113
	-MEENDERLEGEAAASQGISEEQAMANISGRINSPSLRFSIRPSSPLSGFFGRINSPFFG-FSQNKGSLGANEGLINKSLPVQH-DIGGYS GSRESV/RPNREDQPVTRRGRISSSGSKDPQ	120
<pre>     the state is a state is</pre>	-MEENWOTELSTELYPTYIWFSSLETONTONYTAYSINDITNQIYQSERSFGMRGGYNSERGGPNINDIFOLFGGGEGGEGOLGAUGUSTERGEDAUGUSTERGETERGEDAUGUSTERGETAUUGUSTERGETAUUGUSTERGETERGETERGETERGETERGETERGETERGETERG	131
The start score is start is and scor	-INDAMORE TAD I ROYKOONZOAD I WZ-Z ZEFEORAG ZZOGZADA LODOWINOL AKTODOWODA WODINODOWU KOCK NEKO KOKO KOCKO KOKO KOKO KOKO KOKO KOKO	122
Provide Control Con	-FRNSFKSDDENASDEGFKSGFSGR GGRGGRGG-RGAFQQGG-DEEGKGRF-GGGYRGQNEE-IFSKGSPKDNEE KEDGETAGPKVNYIPPPPPEEENSIFSH-YATGINFDRYDDILVDV	223
Best State in the state is a state i		214
BLAGEPTRINGENES'INCLORENCE STORMAGE - DESCRIPTION OF THE SECTION OF THE SECTIO		266
Segeptize the set of t		220
Exercise in proceedings in the result of	SEASOFFRIGGROSFRIGCROGFGLGRPISESDQDQGTQRGGLFG SRKPASD-S0K0DTQSSGSGRGGYKGLNEEVITGSGKNSWSE TEGESSD-SQGPKVTYIPPPPPEDEDSIFAH-VQTGIN/F0KVDTILVEV TDDV/GEDBCGGE_DCOMMENEDPVOCEAUE_SDGFGGFGTGMISND_SSGESG_UCGVCVVVVVVVVPPPDPDNEEDSIFAH-VQTGIN/F0KVDTILVEV	251
Construction     C	ERISAL	226
Segmentation espectation with the service of the set of		242
Supervisiting Equication with a service provide transmission in AcQUIGS TAFELING UNDER STREED REPORT WITH EL DATA ACCOUNT OF THE LUCE UNDER STREED REPORT WITH ACCOUNT ACCOUNT OF THE LUCE UNDER STREED REPORT WITH ACCOUNT ACCOUNT OF THE LUCE UNDER STREED REPORT WITH ACCOUNT		255
SSIPPLATIFEERALCE LINEWASSOWER Phy OWSER PIETASSOUL MAC ATOS AN ALL LEI LAURANSA SERVE PEVILUATION AND AND AND AND AND AND AND AND AND AN		373
sopersummeread to the service of the	SGSNPPKAIMTFEEAQLCETLNRNVAKSGYVKPTPVQKHGIPIIFAGRDL MACAQTGSGKTAAFLLPILQQLMNDGVATSKFSEVQEPEVIIVAPTRELI SQIYLEARKFAYGTCVRPVVVY GGTSTGFTIREVLKGCNVLCGTPGRLLD	364
Security Treat Contract of the security of the security of the security of the second of the security of the second of the se	SGSIPPSATMTFEEAHLCETLININ/SAGYV/PIPVQ/YGTPIISAGROL MACADTGSG/TAAFLDTLIGRINDGAAASKFSEVGEPEVTIVAPTRELI NQTYLEARKFAYGTCVPIVVVA GGISTGYTTQEVLKGCNLCGTPGRLD GSGINDPJATMTFEEAHLCETLININ/SAGYV/PIPVQ/YGTPIISAGROL MACADTGSG/TAAFLDTLIGRINDGAAASKFSEVGEPEVTIVAPTRELI NQTYLEARKFAYGTCVPIVVA GGISTGYTTQEVLKGCNLCGTPGRLD	386
SidepParLifeEaut.cgTUNIALAGYTLLTURY, VESTEYLAGD, MACROSOFTAFLIPLELAMMONTASERUGGETASTRELEGETURYTELL NDTULEAUTORYTOCTANOTY OS OFFENSUEGUIGUCIALTARIUM OS AND	SHDAPPALITEEANLCOTLINNIAKAGYTKLIPVQXXPITILAGRDL MACQTGSGKTAAFLLPLAHWHDGTTASRKELQEPECIIVAPTRELVNQTVLEARKFSFGTCVRAVVI GGTQLGHSINQTVQCNILCATPGRLVD	370
Sicker State St	SGHDAPPAILTFEEANLCQTLNNNIAKAGYTKLTPVQKYSIPIVLAGRDL NAC4QTGSGKTAAFLLPTLAHMMRDGITASRFKELQEPECITVAPTRELI NQTVLEARKFSFGTCVRAVVT/GGTQFGHSVRQTVQGCHILLGAPGRI/DD	401
THEORY CELLS AND TRANSPORT AND	SoloPPPALIFEEMILG(ILKKI)AAAGYLKLPYQ(YSIP)IIABAD PACHTOSSA HAFLEYLMHTKUGYTSAFFAQGPECII/APTRELL NOFFEARKFSYGTCLPYVIGGTOTGUTGUGUGUGUGUGUGUGUGUGUGUGUGUGUGUGU	390
TSSUPPOLICY TSJALIDITIAWINGSFLIPPTICICSTIVISSERU MA APTEGO NA A	TGKDVPPAILTFEEANFCETLSRNTKAGYVKLTPVQKHSIPIILAGRDLMACAQTGSGKTAAFLLPILSHWWEGITASQFLPLQEPQAIIIAPTRELINQIYLDARKFSYGTCVRPVVVYGGTHPVHAMRDVERGCNILCATPGRLND	392
DEAD       A       B       CC         ITINGSVGESKIRFLV       DeAD       A       B       CC         ITINGSVGESKIRFLV       DeAD       A       B       CC         ITINGSVGESKIRFLV       DeAD       CC       CC <td>TGSDVPQPTQHFTSADLRDIIIDWW.SGFKIPTPIQKCSIPVISSGRDL MACAQTGSGKIAAFLLPILSKLLEDPHELELGRPQVIVSPTRELA IQINKARKFAFESYLKIGIV GGISFRHQNECLTRGCHVVIA TPGRLD</td> <td>381</td>	TGSDVPQPTQHFTSADLRDIIIDWW.SGFKIPTPIQKCSIPVISSGRDL MACAQTGSGKIAAFLLPILSKLLEDPHELELGRPQVIVSPTRELA IQINKARKFAFESYLKIGIV GGISFRHQNECLTRGCHVVIA TPGRLD	381
TINGS/VGLS/TEFLV) DEAD *NLDWEFEBORIL/WED/PROPERTIES/UNSPORTED/UP/LADPL/VV/LEAW/VGGAC/DIE/UT/VO/LEAW/VGGAC/DIE/UT/VO/LEAW/VGGAC/DIE/UT/VO/LEAW/VGGAC/DIE/UT/VO/LEAW/VGGAC/DIE/UT/VO/LEAW/VGGAC/DIE/UT/VO/LEAW/VGGAC/DIE/UT/VO/LEAW/VGGAC/DIE/UT/VO/LEAW/VGGAC/DIE/UT/VO/LEAW/VGGAC/DIE/UT/VO/LEAW/VGGAC/DIE/UT/VO/LEAW/VGGAC/DIE/UT/VGGAC/DIE/UT/VGGAC/DIE/UT/VGGAC/D		
TINGKVGLSUTFLVU DED MULDØRE FEMRIK VINSFØRPSEEDTU IFS AT YPEDDRILADELLVOVL-LVØVV GØGGSDEQTILIOTTISKE RULELLANTGENTMYVETKISSOFTAL-CQEVIPTISHOBIGE 534         TIGGENGUS SVIVVU DED MULDØRE FEMRIK VISSFØRPSEQRT. IFS AT YPEDDRILADELLEMVL-LVØVVGGGSDEQUTLIOTTISKE RULELLANTGENTMYVETKISSOFTAL-CQEVIPTISHOBIGE 536         TIGGENGUS SVIVVU DED MULDØRE FEMRIK VISSFØRPSEQRT. IFS AT YPEDDRILADELLEMVL-LVØVVGGGSDEQUTLIOTTISKE RULELLANTGENTMYVETKISSOFTAL-CQEVIPTISHOBIGE 536         TIGGENGUS SVIVVU DED MULDØRE FEMRIK VISSFØRPSEQRT. IFS AT FPEEDRILADELLEMVL-VISVGGGSDEQUTLIQVØVSKE RULELLANTGENTMYVETKISSOFTAL-CQEVIPTISHOBIGE 536         TIGGENGUS SVIVVU DED MULDØRE FEMRIK VISSFØRPSEQRT. IFS AT FPEEDRILADELLSVIVVUVOQGGGLDNOQTULQVØVSKE RULELLANTGENTMYVETKISKOFTAL-CQEVIPTISHOBIGE 536         TIGGENGUS SVIVVUV DED MULDØRE FEMRIK VISSFØRPSEQRT. IFS AT FPEEDRILADELLSVIVVUVOQGGGLDNOQTULQVØVSKE RULELLANTGENTMYVETKISKOFTAL-QEVILTSTISHOBIGE 540         TIGGENGUS SVIVVUV DED MULDØRE FEMRIK VISSFØRPSEDRIT. IFS AT FPEEDRILADELLSVIVVUVED ØR GASON GYTULGVØVSKE RULELLANTGENTMYVETKISKOFTAL-QEVILTSTISHOBIGE 540         TIGGENGUS SVIVVUV DED MULDØRE FEMRIK VISSFØRPSEDRIT. IFS AT FPEEDRILADELLSVIVVUVED ØR GASON GYTULGVØVSKE RULELLANTGENTMYVETKISKOFTAL-QEVILTSTISHOBIGE 540         TIGGENGUS SVIVVUV DED MULDØRE FEMRIK VISSFØRPENDELTING VISSFØRPENDELTINGVETKISKOFTAL-BEVRSTATISHOBIGE 550         TERKLØDENDE SVIDENT VISSFØRPENDELTING VISSFØRPENDELTINGVETKISKOFTAL SVIDENTMETHVETKISKOFTAL-BEVRSTATISHOBIGE 540         TIGGENGUS SVIVVU DED MULDØRE FEMRIK VISSFØRPENDELTINGVETKISKOFTAL SVIDENTMETHVETKISKOFTAL-BEVRSTATISHOBIGE 550         TERKLØDENDENDEN FERRICAL VISSFØRPENDELTINGVETKISKER VISSFØRPENDELTING SVIDENTMETHVETKISKOFTAL SVIDENTMETHVETKISKOFT		I
THE SERVICE SUMMAND AND AND AND AND AND AND AND AND AND	IIINGKVGLSKIRFLV, DEADWLDWSFEADWRLVWSFG9SKEERQTLWFSATYPEDIGKLAPFLVDVFLAVGWGGACNDICHTIQVTQYSK EKLLDLLKTGTQTTWFVETKSAAPTATFLCQEKVPTTSLHODREQRE TIGGEFUG SKIRFLV, DEADWLDWSFEADWRLVWSFG9SKEERQTLWFSATYPEDIGKLAPFLVDVFLAVGWGGACNDICHTIQVTQYSK EKLLDLLKTGTQTTWFVETKSAAPTATFLCQEKVPTTSLHODREQRE	523
Itels/CIGU/CUV/UDEAD/PL/D/SEPERVIC/USEPERVIC/	IIGR6KVGLSKVRVLVI DEAD WILDWGFETDMRKLVSSPGMPPKEQRQTLMFSAT YPEDIQRLAADFLKENYLFLWGWGGACSDIEQLIIQVTRYSKR EQLLEMLKTTGDERTMVFVETKRSADFIATFLCQEKVPTTSIHGDREQRE	536
LingerLandowner Dearbid version Lischer Preizur Lischer Preizur Landowner Version and Longersprechen Lischer Version and Lingersprechen Lischer Version a	LIGRECIG SIVERVIV DEAD RUDWEFEPERARULVASPORPSKEEROTL VE SATVPEDIGRMAADELVOVITILAVGVVGGACSOVEQTIVQVDQYSKE DQLIELLBATOBERTIMVEVETVRSADETATE LOGEVISTISTINGDREQRE	566
ITEGERITELAWYNY UD BEADHLOWEGE CONKULTS YEPERSON OT UN FAITPEETIQUL KAEFLUOY VU DEAL OWGALSON OT UT GAAF PERVIQUL KAEFLUOY VU DEAL ON UND VESTIGEN YN FOL AND PESTIGEN	11GKEXIGLKQVYTVU DEAD NILDWGPEPENKLISSCPEPSKEQNOL LF SATFPEEIGKLAGFLKSSVLFVAVQVGGGGACR0VQVTLQVQQTSKKELVEILINDGERTINFVETKKADFLATFLQEKSSTIFVAVG	551
Indextdy/with/basedumesultary       Bead and an action of the part Peer of the part Peeron Peer of the part Peer of the part Peer of the part Peer of the	IIGKEKIGLRIVAYLVI DEAD ANLDWGFGPENKKLISCPGVPSKDRRQTLWFAT PPEEDQRL&GEFLKPEYLFVAVGQVGGACSDVQQTILQVSQYSKR EKLVEILQSIRDERTMVFVETKKKADFIATFLCQEKIPTTSIHGDREQRE	540
FVDDTFITFEDTRFW, DEAD PULVAGSEDDWRRTHTHYTKP-E-BYDL NF SAT PREETORMAGEFLW- YYSVALGTVGGACSDV/QTTYEWIXVARSILLELSEQADG-TUYVETKVGADFLAGELSEKEEPTTSTKHORLOGO       527         Image: Deal of the second of the	11ERKK15LVEV/VTV DEU/#LIDK#FGLWK#FLISYPE#SKDK/QLC#F5ATPEEV@LLGFFL/DTFLVIGHICGACS/VQ/ALLQV#LSKD/L1E1LQ51GEEHINFVD/KKADUALA-LQEHI/S151HGDEQKE 11GREKTGLSVLVTV, DEU/#LDK#FGLWK#LISYPE#SKDK/QLC#F5ATPAEUALASYFLKSDHLFVVG/UGACS/VQ/ALLQV#LSKD/L1E1LQ51GEEHINFVD/KKADUALASYFLKSTJHEDEEXCO	526
BEAL OPERIOG CPU VATSVA ARGU ELEVOVINDE PYEDEVYN EL GRIG COL UTGAVSFFDPEADTPLARSLVIVU SEAQO EVPSMLEETALGAHGTTGFNPRGIVFASTDTRRGFPGUNSEPPAAG-TSADODD 668 REKALGDPRTGC/CVU VATSVA ARGU ELEVOVINDE PYEDEVYN EL GRIG COL UTGAVSFFDPESDTPLARALVIVU SAAQO EVPSMLEETA/SAHGTTGFSPHGIVFASTDTRRGFPGUNSEPPAAG-TSADODD 669 REKALSDRRTGC/CVU VATSVA ARGU ELEVOVINDE PYEDEVYN EL GRIG COL UTGAVSFFDPESDTPLARALVIVU SAAQO EVPSMLEETA/SAHGTTGFSPHGIVFASTDTRRGFPGUNSEPPAAG-TSADODD 669 REKALSDRRTGC/CVU VATSVA ARGU ELEVOVINDE PYEDEVYN EL GRIG COL UTGAVSFFDPESDTPLARALVIVU SAAQO EVPSMLEETA/SAHGTTGFSPHGIVFASTDTRRGFPGUNSEPPAAG-TSADODD 669 REKALSDRRTGC/CVU VATSVA ARGU ELEVOVINDE PYEDEVYN EL GRIG COL UTGAVSFFDPESDTPLARALVIVU SAAQO EVPSMLEETA/SAHGTTGFSPHGIVFASTDTRRGFPGUNSEPPGUNALSASCO EVPSMLEETA/SAHGTTGFSPHGIVFASTDTRRGFFGFUNSEPPGUALSASCO EVPSMLEETA/SAHGTTGFSPHGIVFASTDTRRGFFGFUNSEPPGUALSASCO EVPSMLEETA/SAHGTGFSPHGIVFASTDTRRGFFGFUNSEPPGUALSASCO EVPSMLEETA/SAHGTGFSPHGIVFASTDTRRGFFGFUNSEPPGUALSASCO EVPSMLEETA/SAHGTGFSPHGIVFASTDTRRGFFGFUNSEPPGUALSASCO EVPSMLEETA/SAHGTGFSPHGIVFASTDTRRGFFGFUNSEPPGUASCO EGPS REVELADOPRAGIC/VUVATSVA ARGU ELEVOVINDE PYEDEVYN EL GRIG COLTGAVASFFDPESDTPLARSLVIVUS TOAQO EVPSMLEETA/SAHGTGFSPHGIVFASTDTRRGFFGFSPHGIVFASTDTRRGFFGFSPHGIVFASTDTRRGFFGFSPHGIVFASTDRGFFGGUNSEPGGFSGSFGFUNGEVPGE GPS REGULDOPRAGIC/VUVATSVA ARGU ELEVOVINDE PYEDEVYN EL GRIG COLTGAVASFFDPESDTPLARSLVIVUS TOAQO EVPSMLEETA/SAHGTGFS	FVDRTFITFEDTRFVVLDEAD WILDMGFSEDMRRIMTHVTMRPEHQTLMFSATFPEEIQRMAGEFLKN-YVSVAIGIVGGACSDVKQTIYEVNKYAKR SKLIEILSEQADG-TIVFVETKRGADFLASFLSEKEFPTTSIHGDRLQSQ	527
I DEAD     II     DEAD     III     DEAD     IIII     DEAD     III     DEA     III     IIIIII		
EFALIOPRTGQCPVLVATSV4 AGLC IEHVQHVNPDLPHEIDEVVR KL GATGAC GITGAVSFFDPEADTPLARSLVIVLSEAQQ-EVPSNLEEKASAHGTGFNPRGIVFASTDSRGGSFTINLAPQAAQS-TSAADDDD 668 REALOPRTGQCPVLVATSV4 AGLC IEVQVNVNDDJPYIDEVX RL GATGAC GITGAVSFFDPESDTPLARSLVIVLSEAQQ-EVPSNLEEKASAHGTGFNPRGIVFASTDSRGGSFTINLAPQAAQS-TSAADDDD 668 REALOPRTGQCPVLVATSV4 AGLC IEVQVNVNDDJPSIDEVX RL GATGAC GITGAVSFFDPEDHLARSLVIVLSEAQQ-EVPSNLEEKASSAHGTGFNPRGIVFASTDSRGGSFTGNLEPAAQS-TSAADDDD 678 REALSDFRLGCPVLVATSV4 RGL IEVQVNVNDDJPSIDEVX RL GATGAC GITGAVSFFDPEDHLARSLVIVLSEAQQ-EVPSNLEEKASSAHGTGFNPRGIVFASTDSRGGSFTGSEPPSGTSAFSSAAAADDD 712 REQULOPRGCPVLVATSV4 RGL IEVQVNVNDDJPSIDEVX RL GATGAC GITGAVSFFDPESDTPLARSLVIVLSEAQQ-EVPSNLEEKASSFTGTFNPRGIVFASTDSRGGSFTGSEPPSGTSAFSSAAAADDD 712 REQULOPRGCPVLVATSV4 RGL IEVQVNVDDJPSTDEVX RL GATGAC GITGAVSFFDPESDTPLARSLVIVLSEAQQ-EVPSNLEEKASSFTGTFNPRGIVFASTDSRGGSFTGTASSS-QAPRVDDE 663 REQULOPRGCPVLVATSV4 RGL IEVQVNVDDJPSTDEVX RL GATGAC GITGAASFFDESDTPLARSLVIVLSEAQQ-EVPSNLEEKASSTTTPO-FSSTGGAVFASTDTRKIVGGGVTNDDFSSDEPPSGTSAFSSAAAADDD 712 REQULOPRGCPVLVATSV4 RGL IEVQVNVDDJPSTDEVX RL GATGAC GITGAASFFDESDMLAQPLIVLSEAQQ-EVPNLEEVX-SAHGTGFS-VASTTRKIVGGVTNDVDEGSS-QAPRVDDE 663 REQULOPRGCPVLVATSV4 RGL IEVQVNVDDJPSTDEVX RL GATGAC GITGAASFFDOESDMLAQPLIVLSEAQQ-EVPNLEEVX	DEAD Helicase C	
IEUALGDRITGGCULVATSVA ARGLI LEQUQWINEDUPYTY EIGTGG CENTGAVSFEDPESDPLAASULVULSGAVG-EVPSNLEEVXYSAHGTTGFSPHGUYASTDTRRGGFPQUASGASTSADDDDE 659 IEUALGDRITGGCULVATSVA ARGLI LEQUQHVNEDUPSTDEYVE IG GTGG CUTGAVSFEDPEDHALASULVULSGAVG-EVPSNLEEVXFSAHGTTGFNPHGUYASTDTRRGGFPQUASGASTSDEPPSOTSASSAAAADDE 72 IEUALGDRIGGCULVATSVA ARGLI LEQUQHVNEDUPSTDEYVE IG GTGG CUTGAVSFEDPESDPLAASULVULSGAVG-EVPSNLEETXFSAHGTGFNPHGUYASTDSRKGSFI'SDEPPSOTSASSAAAADDE 72 IEQULGDRIGGCULVATSVA ARGLI LEQUQHVNEDUPSTDEYVE IG GTGG CUTGAVSFEDPESDPLAASULVULSGAVG-EVPSNLEETXFSTTP-OFSGSTGGAVFASTDSRKGSFI'SDEPPSOTSASSAAAADDE 712 IEQULGDRIGGCCULVATSVA ARGLI LEQUQHVNEDUPSTDEYVE IG GTGG CUTGAVSFEDPESDPLAASULVULSGAVG-EVPSNLEETXFSTTP-OFSGSTGGAVFASVDTRKINGGKIHLITAGTSSOAPPRVDE 663 IEQULGDRIGGCCULVATSVA ARGLI LENQUKTIEDUPSTDEYVE IG GTGG CUTGAVSFEDUSODHALQULVULSGAVG-DVPANLEETASTTV-OFSFSSTGGAVFASVDTRKINGGKIHLITAGTSSOAPPRVDE 663 IEQULGDRISGCCULVATSVA ARGLI LENQUKTIEDUPSTDEVNEIG GTGG CUTGAVSFEDUSODHALQULVULSGAVG-EVPSNLEETASTTV-OFSFSSTGGAVFASVDTRKINGGKIHLITAGTSSOAPPRVDE 663 IEQULGDRISGCCULVATSVA ARGLI LENQUKTIEDUPSTDEVNEIG GTGG CUTGATSFEDUSODHALQULVULSGAVG-EVPSNLEETASTTV-OFSFSSTGGAVFASVDTRKINGGKIHLITAGTSSOAPPRVDE 663 IEQULGDRISGCCULVATSVA ARGLI LENQUKTIEDUPSTIEDUSYNEIG GTGG CUTGATSFEDUSODHALQULVULSGAVG-EVPSNLEETASTTV-OFSFSSTGGAVFASVDTRKINGGAVGHPEININS-SSET-TFK 660 IEEALTDRISGCCULVATSVA SGGLI LENQUKTINDIPSTDEVENEIG GTGG CUTGATSFEDUSODAGUVGATFFDUNEDAVAAPULVILIDAHQ-EVPANLEETAST V ****** * :: *: * : * : * : * : * : * :	RETALRDFRTGQCPVLVATSVA ARGLI I EHVQHV/NFDLPKEIDEYV/RI GRTGRIGGITGRAVSFFDPEADTPLARSLVKVLSEAQQ-EVPSNLEEIALGAHGTTGFNPRGKVFASTDSRRGGSFTKNLAPQPAAQS-TVTAAADDD	668
REIALSOFILIGIEVULATSVA ARGLI LEVQVINPUPSLEEVY RLUKIGI CUIGANSFIDEPEDERLASUVULSGAQ: EVYSALEELASARGI IDENPROVATASUDIKOSFINGAVARABDE 712 REQALGOFRGICEVULATSVA ARGLI EVQVINPUPSLEEDEVY RLUKIGI CUIGANSFIDEPEDERLASUVULSGAQ: EVYSALEELASARGI IDENPROVATSUDIKOSFINGAVARABDE 712 REQALGOFRGICEVULATSVA ARGLI EVQVINPUPSLEDEVYR IG RIGGI CUIGANSFIDEPEDERLASUVULSGAQ: EVYSALEELAS SARGI IDENPROVATSUDIKOSFINGAVARABDE 712 REQALGOFRGICEVULATSVA ARGLI EVQVINPUPSLEDEVYR IG RIGGI CUIGANSFIDEPEDIASUDIALAQU.VIVI TDAQDVPAALEELAS STYTP-FSSIGGAVASVOTIKUGISLASTIAGSSQAPIPVDDE 669 REEALGOFRSGICEVULATSVA ARGLI EVQVINPUPSLIDEVYR IG RIGGI CUIGANSFIDESDOMLAQU.VIVI TDAQDVPAALEELAS STYTP-FSSIGGAVASVOTIKUTYGGUNITATSUSSQAPIPVDDE 669 REEALGOFRSGICEVULATSVA ARGLI EVQVINTDU PSTIDEVYR IG RIGGI CUIGANSFIDOSOMILAQU.VIVI TDAQ-EVPAALEENESTYPPS-FSGQAVASVOTIKUTYGGUNITGALTISSQAPIPVDDE 669 REEALGOFRSGICEVULATSVA ARGLI EVQVINTDU PSTIDEVYR IG RIGGI CUIGANSFIDOSOMILAQU.VIVI TDAQ-EVPAALEENESTYPPS-FVPSFESSIGGAVASVOTIKUTYGGUNITGGUNITASUSSQAPIPVDE 663 REEALGOFRSGICEVULATSVA ARGLI EVQVINTDU PSTIDEVYR IG RIGGI CUIGANSFIDOSOMILAQU.LIVISADQQEVPSNLEELAS-SARGI CONTINUTSGORMINGGUNITGGUNITETTAS VOTIKITYGGUNITGGUNITASSA CONTINUE REEALGOFRSGICEVULATSVA ARGLI EVQVINTDU PSTIDEVYR IG RIGGI CUIGANSFIDOSOMILAQU.LIVISADQQEVPSNLEELAS-SARGI CONTINUTGGUNITGGUNITGGUNITASS- REEALGOFRSGICEVULATSVA ARGLI EVQVINTDUPIPSTIDEVYR IG RIGGI CUIGANSFIDOSOMILAQU.LIVISADQQEVPSNLEELAS-SARGI CONTINUTSGORMINGGUNITASS- REEALGOFRSGICEVULATSVA ARGLI EVQVINTDUPIPSTIDEVYR IG RIGGI CONGINASFIPPENDANARANU VII TDAVO-EVPAALEELAS REEALGOFRSGICEVULATSVA ARGLI EVQVINTDUPYR IG RIGGI CONGINASFIPPENDANARANU VII LOSAGO VII PSTIDEV REEALGOFRSGICEVULATSVA ARGLI EVQVINTDUPYR IG RIGGI CONGINASFIPPENDANARANU VII TDAVO-EVPAALEENAS- REEALGOFRSGICEVULATSVA ARGLI EVQVINTDUPYR IG RIGGI CONGINASFIPPENDANARANU VII LEGSGO VII PSTIDEVINGUNATINE REEEQ VECK VII VII VII VII VII VII VII VII VII VI	REKALGDERTGQCPVLVATSVA ARGLE IEQVQMVINEDLPKYDEVVRI GRTGRCG/TGRAVSFEDPESDTPLARALVIVLSGAVQ-EVPSNLEEVAYSAHGTTGFSPHGKVFASTDTRRGGFFQKNSEPQPAAQS-TSAGDDDDE	659
EQULGDRARGIC CVU VATSVA AGULT ENVQAVITNEDI PSTIDEYY R IG ATGIS CONTIGALIS FEDU ESDMELAGY LIVU. TDAQČ. DVPANLEELAPS TYTI- OFSQSTIGG. MIS ASUDTRIX:GISLITATAGESSQAPRIPUDDE 663 EQULGDRAGGIC VVU VATSVA AGULT ENVQAVITNEDI PSTIDEYY R IG ATGIS CONTIGALIS FFDI SDMELAQU LIVU. SDAQQ EVPANLEELAPS TYTI- OFSQSTIGG. MIS ASUDTRIX:	KEKALSDERTGICEVELVATSVA ARGLETERVORVNI DE KSTEEVVERT GATGA COTTGAAVSEPDPEPDAREARSEVIVESGAQQ-EVPSNEEETAF SARGTGANPHGKVEASTDTRKGSFMTQAPQ-AAPS-NVAG-ADED	
REQUEDREGIC (PULVATSVA RAGU I ENVOYMEND PSTIDERYNE I GREGIC GUEGALSFEDDISONHLAGY UNU SDAQ-DVPANLEELAFS TYVPPSFSSTRGGAVFASUDTIKHYGGI HTLITASTSS- QAPRPVDDE 699 REBLADERSGIC (PULVATSVA RAGU I ENVOYMEND PSTIDERYNE I GREGIC GUEGALSFEDDISONHLAGY LLIVU SDAQC PVPANLEELAFS TYVPPSFSSTRGGAVFASUDTIKHYGGI HTLITASTSS- QAPRPVDDE 699 REBLADERSGIC (PULVATSVA RAGU I ENVOYMEND PSTIDERYNE I GREGIC GUEGALSFEDDISONHLAGY LLIVU SDAQC PVPANLEELAFS TYVPPSFSSTRGGAVFASUDTIKHYGGI SMPHARABUS RETALEDRESGIC (PULVATSVA RAGU I ENVOYMEND PSTIDERYNE I GREGIC GUEGALSFEDDISONHLAGY LLIVU SDAQC PVPANLEELAFS REGULDERSGIC (PULVATSVA RAGU I ENVOYMEND PSTIDERYNE I GREGIC GUEGALSFEDDISONHLAGY LLIVU SDAQC PVPANLEELAFS REQUEDRESGIC (PULVATSVA RAGU I ENVOYMEND PSTIDERYNE I GREGIC GUEGALSFEDDISONHLAGY LLIVU SDAQC PVPANLEELAFS REQUEDRESGIC (PULVATSVA RAGU I ENVOYMEND PSTIDERYNE I GREGIC GUEGALSFEDDISONHLAGY LLIVU SDAQC PVPANLEELAFS REQUEDRESGIC (PULVATSVA RAGU I ENVOYMEND PSTIDERYNE I GREGIC GUEGALSFEDDISONHLAGY LLIVU SDAQC PVPANLEELAFS REQUEDRESGIC (PULVATSVA RAGU I ENVOYMEND PSTIDERYNE I GREGIC GUEGALSFEDDISONHLAGYN LLITASS STORGAVINGDATINVEELED REGIL THE RETAIL TO REGIL (PULVATSVA RAGU I ENVOYMEND PSTIDERYNE I GREGIC (PULVATSVA RAGU I ENVOYMENCE) NUT E 62 EVE E 61 EVE E 715 SVD 666 SVD 702 SVD 688 SVE 663 SVD 678 - VD 661	REKALSUERLOREPVEVATSVA AKOLE LEUVURVVNEUPPSSTUERVERTORTORTORTORTORTORTORTORTORTORTORTORTOR	678 712
EELLIDPROGUCULVATSVA SAGLI EENQAVIINED HITTEDYN HITGATGA CEMIGIAASFEDDOSOGHUVYELLIVU SEAQO EVPANLEENAVQTATUVASLGAQ-NAVAGAGAMPPEENAN-SYSETTFK 660 EELIDPROGUCULVATSVA SAGLI EENQAVIINDUPHEIDEYN HITGATGA CEMIGIAASFEDDOSOGHUVYEDALAADLVAILEELAFG	HEALOPHERG(CVLVATSV4 ARGL 1ENQ/MYNDPD PSTLDEVFELGVIG GRIGANSFPMESDIFLAGUULUSANQC-VVFALLEEVAFSARDI 104777480153000-7501500-75000-75000-75000-75000-75000-75000-75000-75000-75000-75000-75000-75000-75000-75000-75000-75000-75000-7500	678 712 663
REALTOPERSGIC/DVTUCTAVA ARGLI LINUQH/INTOPYEIDDYYN EI GETGG CGW/GVATSFFMMEDHVAAPU/ULI TDAVQ-EVPAMLEELAFGGHGALHSLVAADSVGGEAGE(YVSAPSS-AQEEEA 675 REQULDYN SAGLI LINUUH/INTOPYEIDDYYN EI GETGG CGW/GVATSFFMMEDHVAAPU/ULI TDAVQ-EVPAMLEELAFGGHGALHSLVAADSVGGEAGE(YVSAPSS-AQEEEA 675 REQULDYN SAGLI LINUUH/INTOPYEIDDYYN EI GETGG CGW/GVATSFFMMEDHVAAPU/ULI TDAVQ-EVPAMLEELAFG	ECALOPERCENT VALVATSVA AND LEDVORTVINTPOLISSIDETVER ON TOURISTIC CONTRACTS FOR STATUS AND LEDVORTVINT DE SUBJECT SUBJECT STATUS AND LEDVORTVINT DE SUBJECT SUBJECT SUBJECT SUBJECT STATUS AND LEDVORTVINT DE SUBJECT SUBJECT SUBJECT STATUS AND LEDVORTVINT DE SUBJECT	678 712 663 699
IV         Helicase C         V           DNE 671         DME 661           SND 663         SND 661	ECALOPERSGNCQULVATSVA ANGLIENQQUTWINDUTSDUETVER GITAG LIGHTANSFETWESDIFLANGLIVIV. TDAQO-VIVIV. TDAQO-VIVIVIV. TDAQO-VIVIVIVIVIVIVIVIVIVIVIVIVIVIVIVIVIVIVI	678 712 663 699 685 660
Image: Weight of the sector of the	EEALOFERGE/EVI/ATSVA KOLLEEU/QURVIN/EVI/SSUDEV/ELG/UGA/STEM/SSUDEV/ELG/UGA/SSUDEV/SSUDEV/ELG/UGA/SSUDEV/ELG/UGA/SSUDEV/ELG/UGA/SSUDEV/SSUDEV/ELG/UGA/SSUDEV/SSUDEV/ELG/UGA/SSUDEV/SSUDEV/ELG/UGA/SSUDEV/SSUDEV/ELG/UGA/SSUDEV/SSUDEV/ELG/UGA/SSUDEV/SSUDEV/ELG/UGA/SSUDEV/SSUDEV/ELG/UGA/SSUDEV/SSUDEV/SDU	678 712 663 699 685 660 675
DVE 671           DVE 662           EVE 715           SVD 666           SVD 782           SVD 678           SVE 663           SVD 661	IE ALS DIR LEARCY UNA IS WARDLE LEWY OWN PUP SSILLEW FLOW IN CONTRANS FEW PS/DIR LAGY UNV TADAGAD STUDIES AND THE CHARGE STUDIES AND THE	678 712 663 699 685 660 675 659
DVE     671       DVE     662       EVE     681       SVD     666       SVD     688       SVE     663       SVD     678       -VD     661	HEALGOFRIGHT/ULATSVA NOLL ELGVQUTVINDUFSDLEVIN LGITIGLIGHTGANSFEMPESDIPLANSLIVINU TDAQE UVPALLEELAPSTVPPSTSSTIGGAVFASUDTIKINGGIFLDEPPSQLSAPSAAAADDE HEALGOFRIGUPLANSVA NOLL ELGVQUTVINDUFSDLEVING GITIGGATGASEPEDISOMALAQELIVINU TDAQE UVPALLEELAPSTVPPSTSSSTIGGAVFASUDTIKINGGIFLDITTAGISSS- QAPIPVDDE HEALGOFRIGUPLANSVA NAGL ELGVQUTVINDUPSDLSTIDEVN HEI GITIGGGITGALSFEPTDISOMALAQELIVINU TDAQE UVPALLEELAPSTVPPSTSSSTIGGAVFASUDTIKINGGIFLDITTAGISSS- QAPIPVDDE HEALGOFRIGUPLANSVA NAGL ELGVQUTVINDUPSDLSTIDEVN HEI GITIGGGITGALSFEPTDISOMALAQELIVINU SDAQQE DVPALLEELAPSTVPPSTSSSTIGGAVFASUDTIKINGGIFLTITTAGISSS- QAPIPVDDE HEALGOFRIGUPLANSVA NAGL ELGVQUTVINDUPSDLSTIDEVN HEI GITIGGGITGALSFEPTDISOMALAQELIVINU SDAQQE DVPALLEELAPSTVPPSTSSSTIGGAVFASUDTIKINGGIFLTITTAGISSS- QAPIPVDDE HEALGOFRIGUPLANSVA NAGL ELGVQUTVINDUPSDLSTIDEVN HEI GITIGGGITGANSFEPDDISOMALAQELIVINU SDAQQE DVPALLEELAPST HEALGOFRIGUPLANSVA NAGL ELGVQUTVINDUPSDLSTIDEVN HEI GITIGGGITGANSFEPDDISOMALAQELIVINU SDAQQE DVPALLEELAPST HEALGOFRIGUPLANSVA NAGL ELGVQUTVINDUPSDLST HEALGOFRIGUPLATSVA SIGLI ELIVINUTVINDUPSDIST DVN HEI GITIGG GITGANSFEPDDISOMALAQELIVINU SEAQU EVPALLEELAPST HEALGOFRIGUPLATSVA SIGLI ELIVINUTVINDUPSDIST DVN HEI GITIGG GITGANSFEPDDISOMALAQELIVINU SEAQU EVPALLEELAPST HEALGOFRIGUPLATSVA SIGLI ELIVINUTVINDUPSDIST DDVN HEI GITIGG GITGANSFEPDDISOMALAQELIVINU SEAQU EVPALLEELAPST HEALGOFRIGUPLATSVA SIGLI ELIVINUTVINDUPST HEALGOFRIGUPLATSVA SIGLI ELIVINUTVINTUPST HEALGOFRIGUPLATSVA SIGLI EL	678 712 663 699 685 660 675 659
EVE 681 EVE 715 SND 666 SND 702 SND 688 SNE 663 SND 678 -ND 661	EEALGOFRIGUPULATSVA VAGLI ELOUGUPUNDUFSUEDUSTI ELOVIA LIGHTIGAAUSETMISSIOTULAGULUVULTDAGO, DYMALEELAS STUTIOFISSISSI SAGAVASVDTRAINOGISKUETUSSISSI CAPPAVDOE EEALGOFRIGUPULATSVA VAGLI ELOUGUPUNDUSSI DEVIN ELIGITAGI GATGALSEPTDISSIONALAQULUVULTDAGO DYMALEELAS STUTIOFISSISSISSISSISSISSISSISSISSISSISSISSISS	678 712 663 699 685 660 675 659
EWE 715 SND 666 SND 702 SND 688 SNE 663 SND 678 -ND 661	IECALOBERGEVULATISMA NOLL LEWOURDWINDUTSULEUM KLONDAUGUTUAASTERINGSDUTCASCUVIUS DAAQQC VVPALLEELAYST VPPSKISSTINGAAVFASODISUS USAKOGAN SESSE CAPPANDOLE IECALOBERGEVULATISMA NAGLI EUWOURDUTSDUTSUTEN KLONDAUGUTUAASTERINGADUS USAKOGAN VVTDAAVELAYST VVPPSKISSTINGAAVFASODISUS USAKOGAN VVTDAAVAADUSE IECALOBERGEVULATISMA NAGLI EUWOURDUTSDUSTIDEN KLONDAUGUTUKUS USAKOGAN VVTDAAVELAYST VVPPSKISSTINGAAVFASODISUS USAKOGAN VVTDAAVAADUSE IECALOBERGEVULATISMA NAGLI EUWOURDUTSDUSTIDEN KLONDAUGUTUKUS USAKOGAN VVTDAAVELAYST VVPPSKISSTINGAAVFASODISUS USAKOGAN VVTDAAVAADUSE IECALOBERGEVULATISMA NAGLI EUWOURDUSTIDEN KLONDAUGUTUKUSTEROSODISOBALAQULUKUS DAAQQE VVPALLEELAYST VVPPSKISSTINGAAVFASODISUS UNITAKISSS- QAPIAVDODE IEEALOBERGIK (VPULVATSMA NAGLI EUWOURDUSTIDEN KLONDAUGUTUKUSTEROSODISOBALAQULUKUS DAAQQE VVPALLEELAYST VVPPSKISSTINGAAVFASODISOBANI VVTDAAVELAYST VVPPSKISSTINGAAVFASODISOBANI VVTDAAVELAVELAYST IEEALOBERGIK (VPULVATSMA NAGLI EUWOURDUSTIDEN KLI GINTAKI CGINVAKATSFFENDOSODALAQULUKUS DAAQQE VVPALLEELAYST VVPPSKISSTINGAAVFASODISOBANI VVTDAAVELAVELAYST IEEALOBERGIK (VPULVATSMA NAGLI EUWOUNDUSTIDEN KLI GINTAKI CGINVAKATSFFENDOSODALAQULUKUS DAAQQE VVPALLEELAYST VVPPSKISSTINGAAVFASODISOBANI VVTDAAVELAVELAYST IEEALOBERGIK (VPULVATSMA NAGLI EUWOUNDUSTIDEN KLI GINTAKI CGINVAKATSFFENDANDAUANUS UNIT LIDAKE VVPALLEELAYST VVPPSKISSTINGAAVFASODISOBANI VVTDAAVELAVELAYST IEEALOBERGIK (VPULVATSMA NAGLI LINULTINDUSTIDEN KLI GINTAKI SEPTIDOSODIALAQULUKUS DAAQO VVTDAAVISAUGUTUST IEEALOBERGIK (VPULVATSMA NAGLI LINULTINDUSTIDEN KLI GINTAKI SEPTIDASINA VILLI EGGQO VVPOFLITICGAAGEDGG IEEALOBERGIK (VPULVATSMA NAGLI LINULTINDUSTIDEN KLI GINTAKI SEPTIDASINA VILLI EGGQO VVTDAAVISAUGUTUSTEELAYST VVEDAVISAUGUTUST IEEALOBERGIK (VPULVATINDUSTIDEN KLI GINTAKI GINTAKI SEPTIDASINA VILLI EGGQO VVPOFLITICGAAGEDGG IEEALOBERGIK (VILLIANSINA ANAKI LI MULTINDUSTIDEN KLI GINTAKI SEPTIDASINA VILLI EGGQO VVEDAATINEELAYST VILLISAGO IEEALOBERGIK (VILLIANSINA ANAKI LI MULTINDUSTIDEN KLI GINTAKI SEPTIDASINA VILLI EGGQO VVEDAATINEELAYST VILLISAG	678 712 663 699 685 660 675 659
SND 702 SND 688 SVE 663 SND 678 -ND 661	IE CALOBERGE VULATSVA SOLLE LEVOURTUME DUEST DE VERLON IGUIDANSE FEMENSOLU CALOUR SANCE - VMPALLE ELAPS STATISTICA SOLLA SOLUTION SOLUTIA SOLUTIA SOLUTIA SOLUTIA SOLUTIA SOLUTIA SOLUTIA SOLUTIA SOLUTIA	678 712 663 699 685 660 675 659
SMD 688 SWE 663 SWD 678 -WD 661	HEALGOFRGEVULATIVA ISVA ANGLE LEVIQUETVINTOUTSUBETVI LGI TIG GATIGATISTEMESDIPLASULVIVULASUAGE VVIPALEELAPSTVPPSTSSTAGGAVFASUDTRAVGORTUVTASUSA AAAAADDE HEALGOFRGEVPLATIVA MAGLE LEVIQUETVINTOUTSUBETVINTE GI TIG GATIGATASTEPTDISONILAQUELVIVULTDAQE DVIPALEELAPSTVPPSTSSTAGGAVFASUDTRAVGOI: HITTAGISSS- QAPHPVDDE HEALGOFRGEVPLATIVA MAGLE LEVIQUETVINTEDUSTI DEVINTE GI TIG GATIGATISTEPTDISONILAQUELVIVULTDAQUEDVIVULTDAGISSSTAGGAVFASUDTRAVGOI: HITTAGISSS- QAPHPVDDE HEALGOFRGEVPLATIVA MAGLE LEVIQUETVINTEDUSTI DEVINTE GI TIG GATIGATISTEPTDISONILAQUELVIVULTOSAQUE DVIPALEELAPSTVPPSTSSSTAGGAVFASUDTRAVGOI: HITTAGISSS- QAPHPVDDE HEALGOFRGEVPLATIVA MAGLE LEVIQUETVINTEDUSTI DEVINTE GI TIG GATIGATISTEPTDISONILAQUELVIVULTI DAGISAPSTVPPSTSSSTAGGAVFASUDTRAVGOI: HITTAGISSS- QAPHPVDDE HEALGOFRGEVPLATIVA MAGLE LEVIQUETVINTEDUSTI DEVINTE GI TIG GATIGATISTEPTDISONILAQUELVIVULSAQUE VEVALEELAPSTVPPSTSSSTAGGAVFASUDTRAVGOI: HITTAGISSS- QAPHPVDDE HEALGOFRGEVPLATIVA MAGLE LEVIQUETVINTEDUSTI DEVINTE GI TIG GATIGAVFASUDTRAVGOI AND HITTAGISSS- QAPHPVDDE HEALGOFRGEVPLATIVA MAGLE LEVIQUETVINTEDUSTI DEVINTE GI TIG GATIGAVISTEPTDOSONILAQUELVIVULSAQUE VEVALEELAPST HEALGOFRGEVPLATIVA MAGLE LEVIQUETVINTEDUSTI DEVINTE GI TIG GATIGAVGATISTEPTDE DAGIALQUELVIVUSE VEVALEELAPST HEALGOFRGEVPLATIVA MAGLE LEVIQUETVINTEDUSTI DEVINTE GI TIG GATIGAVISTEPTDOPEDQEDGALVIQUELLIVULSEAQUE VEVALEELAPST HEALGOFRGEVPLATIVA MAGLE LEVIQUETVINTEDUSTI DEVINTE GI TIG GATIGAVISTEPTDE PEDQEDGALVISELUVUSE DAGIA MAGLI DEVICTIONINTE GUELVE AAAAAADUE HEALGOFRGEVPLATIVA MAGLE LEVIQUETVINTEDUSTI DEVINTE GI TIG GATIGAVISTEPTDE PEDQEDGALVISELUVUSE AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	678 712 663 699 685 660 675 659
SWE 003 SWD 678 -WD 661	HEALGOFRIGHT/UNISWA WOLL EUQURINIPOURSULENT RUGING GITTAGISTER GITAL GITAL GITTAGISTER GITAL GIT	678 712 663 699 685 660 675 659
-WD 661	REALGERREVULATION AND LEQUERVINEOURS DUE VELGENDU GUIDANDER HERSTER SUBJOARD VERAUE DE VERAUS AND TENTE - PROVINCE AND THE CHART	678 712 663 699 685 660 675 659
	IE CALOBER CALVATSVA NACL E LEVOQUTIONE DUE YTE LIG IT CALOTIONAUS FEMESDIP CASULY VI, TDAQO MYALE ELAY STAND TEATHPRAVTAS DIRK CASES - GAPPRVDDE EQAL GDFR CGLCPULATSVA HAGL E EWVQH THEOL STIDE VN FLG IT CALCIFICATION CONTRALSE CALSTAND	678 712 663 699 685 660 675 659

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 sustentiation 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  $\pm$  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  $\pm$  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 fulllength 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 DEADbox 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. In *terestingly*, 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; C, 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 =  $250 \,\mu$ m; B, E =  $100 \,\mu$ m; C, F =  $25 \,\mu$ m.

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

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, 14/07620-7 and 12/00423-6) and Coordenação de Aperfeiçoamento de Pessoal de Nível superior, Graduate Student Program (JMBR scholarship). JB is supported by the Norwegian Research Council BIOTEK2021 project SALMOSTERILE (221648). The authors would like to thank the Aquaculture Center of Sao Paulo State University (CAUNESP), Institute of Biosciences of Botucatu (IBB-UNESP) and Institute for Research in Environmental Aquaculture (InPAA). The authors are also grateful to Dr. Cesar Martins, Dr. Danillo Pinhal, Dr. Ivan de Godoy Maya, and Dra. Maeli Dal Pai for their technical assistance in this work. The authors would like to thank Msc. Sheryll Yohana Corchuelo Chavarro for drawing the Graphical abstract.

# References

- Albamonte, M.I., Albamonte, M.S., Stella, I., Zuccardi, L., Vitullo, A.D., 2013. The infant and pubertal human ovary: Balbiani's body-associated VASA expression, immunohistochemical detection of apoptosis-related BCL2 and BAX proteins, and DNA fragmentation. Hum. Reprod. 28, 698–706.
- Blazquez, M., Gonzalez, A., Mylonas, C.C., Piferrer, F., 2011. Cloning and sequence analysis of a vasa homolog in the European sea bass (*Dicentrarchus labrax*): tissue distribution and mRNA expression levels during early development and sex differentiation. Gen. Comp. Endocrinol. 170, 322–333.
- Braat, A.K., Zandbergen, T., van de Water, S., Goos, H.J., Zivkovic, D., 1999. Characterization of zebrafish primordial germ cells: morphology and early distribution of vasa RNA. Dev. Dyn. 216, 153–167.
- Filby, A.L., Ortiz-Zarragoitia, M., Tyler, C.R., 2014. The vas::egfp transgenic zebrafish: a practical model for studies on the molecular mechanisms by which environmental estrogens affect gonadal sex differentiation. Environ. Toxicol. Chem. 33, 602–605.
- Ghiraldelli, L., Machado, C., Fracalossi, D.M., 2007. Desenvolvimento gonadal do jundiá, Rhamdia quelen (Teleostei, Siluriformes) em viveiros de terra, na região sul do Brasil. Acta Sci. Biol. Sci. 29, 349–356.
- Gruidl, M.E., Smith, P.A., Kuznicki, K.A., McCrone, J.S., Kirchner, J., Roussell, D.L., Strome, S., Bennett, K.L., 1996. Multiple potential germ-line helicases are components of the germ-line-specific P granules of *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. U. S. A. 93, 13837–13842.
- Hartung, O., Forbes, M.M., Marlow, F.L., 2014. Zebrafish vasa is required for germ-cell differentiation and maintenance. Mol. Reprod. Dev. 81, 946–961.
- Hay, B., Ackerman, L., Barbel, S., Jan, L.Y., Jan, Y.N., 1988. Identification of a component of *Drosophila* polar granules. Development 103, 625–640.
- Herpin, A., Rohr, S., Riedel, D., Kluever, N., Raz, E., Schartl, M., 2007. Specification of primordial germ cells in medaka (*Oryzias latipes*). BMC Dev. Biol. 7, 3.
- Hickford, D.E., Frankenberg, S., Pask, A.J., et al., 2011. DDX4 (VASA) is conserved in germ cell development in marsupials and monotremes. Biol. Reprod. 85 (4), 733–743.
- Hu, C.J., Wu, F.R., Liu, Z.H., Huang, B.F., Zhang, Y.G., Wang, D.S., 2008. Molecular cloning and expression of two isoforms of vasa gene in southern catfish Silurus meriodinalis. Acta Zool. Sin. 54 (6), 1051–1060.
- Ikenishi, K., Tanaka, T.S., 1997. Involvement of the protein of *Xenopus vasa* homolog (*Xenopus vasa*-like gene 1, *XVLG1*) in the differentiation of primordial germ cells. Develop. Growth Differ. 39 (5), 625–633.
- Ikenishi, K., Tanaka, T.S., 2000. Spatio-temporal expression of *Xenopus vasa* homolog, XVLG1, in oocytes and embryos: the presence of XVLG1 RNA in somatic cells as well as germline cells. Develop. Growth Differ. 42, 95–103.
- Kane, D.A., Kimmel, C.B., 1993. The zebrafish midblastula transition. Development 119, 447–456.
- Knaut, H., Pelegri, F., Bohmann, K., Schwarz, H., Nusslein-Volhard, C., 2000. Zebrafish vasa RNA but not its protein is a component of the germ plasm and segregates asymmetrically before germline specification. J. Cell Biol. 149, 875–888.
- Kobayashi, T., Kajiura-Kobayashi, H., Nagahama, Y., 2000. Differential expression of vasa homologue gene in the germ cells during oogenesis and spermatogenesis in a teleost fish, tilapia, *Oreochromis niloticus*. Mech. Dev. 99, 139–142.
- Komiya, T., Itoh, K., Ikenishi, K., Furusawa, M., 1994. Isolation and characterization of a novel gene of the DEAD box protein family which is specifically expressed in germ cells of *Xenopus laevis*. Dev. Biol. 162, 354–363.
- Krovel, A.V., Olsen, L.C., 2002. Expression of a vas::EGFP transgene in primordial germ cells of the zebrafish. Mech. Dev. 116, 141–150.
- Kuznicki, K.A., Smith, P.A., Leung-Chiu, W.M., et al., 2000. Combinatorial RNA interference indicates GLH-4 can compensate for GLH-1; these two P granule components are critical for fertility in *C. elegans*. Development 127 (13).
- Lacerda, S.M., Batlouni, S.R., Costa, G.M., Segatelli, T.M., Quirino, B.R., Queiroz, B.M., Kalapothakis, E., Franca, L.R., 2010. A new and fast technique to generate offspring after germ cells transplantation in adult fish: the Nile tilapia (*Oreochromis niloticus*) model. PLoS One 5, e10740.
- Lasko, P.F., Ashburner, M., 1988. The product of the Drosophila gene vasa is very similar to eukaryotic initiation factor-4A. Nature 335, 611–617.
- Li, M., Hong, N., Xu, H., Yi, M., Li, C., Gui, J., Hong, Y., 2009. Medaka vasa is required for migration but not survival of primordial germ cells. Mech. Dev. 126, 366–381.
- Linder, P., Lasko, P.F., Ashburner, M., Leroy, P., Nielsen, P.J., Nishi, K., Schnier, J., Slonimski, P.P., 1989. Birth of the D-E-A-D box. Nature 337, 121–122.
- Mochizuki, K., Nishimiya-Fujisawa, C., Fujisawa, T., 2001. Universal occurrence of the vasa-related genes among metazoans and their germline expression in hydra. Dev. Genes Evol. 211, 299–308.
- Nagai, T., Yamaha, E., Arai, K., 2001. Histological differentiation of primordial germ cells in zebrafish. Zool. Sci. 18, 215–223.
- Nagasawa, K., Fernandes, J.M., Yoshizaki, G., Miwa, M., Babiak, I., 2013. Identification and migration of primordial germ cells in Atlantic salmon, *Salmo salar*: characterization of vasa, dead end, and lymphocyte antigen 75 genes. Mol. Reprod. Dev. 80, 118–131.
- Nakamura, A., Seydoux, G., 2008. Less is more: specification of the germline by transcriptional repression. Development 135, 3817–3827.
- Nobrega, R.H., Greebe, C.D., van de Kant, H., Bogerd, J., de Franca, L.R., Schulz, R.W., 2010. Spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish. PLoS One 5.
- Okutsu, T., Yano, A., Nagasawa, K., Shikina, S., Kobayashi, T., Takeuchi, Y., Yoshizaki, G., 2006. Manipulation of fish germ cell: visualization, cryopreservation and transplantation. J. Reprod. Dev. 52, 685–693.
- Presslauer, C., Nagasawa, K., Fernandes, J.M.O., Babiak, I., 2012. Expression of vasa and

## J.M.B. Ricci et al.

nanos3 during primordial germ cell formation and migration in Atlantic cod (Gadus morhua L.). Theriogenology 78, 1262–1277.

- Raghuveer, K., Senthilkumaran, B., 2010. Cloning and differential expression pattern of vasa in the developing and recrudescing gonads of catfish, *Clarias gariepinus*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 157, 79–85.
- Raz, E., 2003. Primordial germ-cell development: the zebrafish perspective. Nat. Rev. Genet. 4, 690–700.
- Reitzel, A.M., Pang, K., Martindale, M.Q., 2016. Developmental expression of "germline" and "sex determination" related genes in the ctenophore Mnemiopsisleidyi. EvoDevo 7, 17.
- Rocak, S., Linder, P., 2004. Dead-box proteins: the driving forces behind RNA metabolism. Nat. Rev. Mol. Cell Biol. 5, 232–241.
- Saffman, E.E., Lasko, P., 1999. Germline development in vertebrates and invertebrates. Cell. Mol. Life Sci. 55, 1141–1163.
- Saito, T., Otani, S., Fujimoto, T., Suzuki, T., Nakatsuji, T., Arai, K., Yamaha, E., 2004. The germ line lineage in ukigori, Gymnogobius species (Teleostei: Gobiidae) during embryonic development. Int. J. Dev. Biol. 48, 1079–1085.
- Saito, T., Otani, S., Nagai, T., Nakatsuji, T., Arai, K., Yamaha, E., 2002. Germ cell lineage from a single blastomere at 8-cell stage in shiro-uo (ice goby). Zool. Sci. 19, 1027–1032.
- Schupbach, T., Wieschaus, E., 1991. Female sterile mutations on the second chromosome of *Drosophila melanogaster*. II. Mutations blocking oogenesis or altering egg morphology. Genetics 129, 1119–1136.
- Shang, P., Baarends, W.M., Hoogerbrugge, J., Ooms, M.P., van Cappellen, W.A., de Jong, A.A., Dohle, G.R., van Eenennaam, H., Gossen, J.A., Grootegoed, J.A., 2010. Functional transformation of the chromatoid body in mouse spermatids requires testis-specific serine/threonine kinases. J. Cell Sci. 123, 331–339.
- Silfvergrip, A.M.C., 1996. A Sistematic Revision of the Neotropical Catfish Genus Rhamdia (Teleostei, Pimelodidae). pp. 156 Stockholm, Sweden, (PhD Thesis) -Department of Zoology, Stockholm University and Department of Vertebrate Zoology, Swedish Museum of Natural History.
- Silva, M.A., Costa, G.M.J., Lacerda, S.M.S.N., Brandão-Dias, P.F.P., Kalapothakis, E., Silva Júnior, A.F., Alvarenga, E.R., França, L.R., 2016. Successful xenogeneic germ cell transplantation from Jundia catfish (*Rhamdia quelen*) into adult Nile tilapia (*Oreochromis niloticus*) testes. Gen. Comp. Endocrinol. 230–231, 48–56.
- Takeuchi, Y., Yoshizaki, G., Takeuchi, T., 2003. Generation of live fry from intraperitoneally transplanted primordial germ cells in rainbow trout. Biol. Reprod. 69, 1142–1149.
- Takeuchi, Y., Yoshizaki, G., Takeuchi, T., 2004. Biotechnology: surrogate broodstock produces salmonids. Nature 430, 629–630.
- Tanaka, S.S., Toyooka, Y., Akasu, R., Katoh-Fukui, Y., Nakahara, Y., Suzuki, R.,

Yokoyama, M., Noce, T., 2000. The mouse homolog of *Drosophila* vasa is required for the development of male germ cells. Genes Dev. 14, 841–853.

- Thisse, C., Thisse, B., 2008. High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat. Protoc. 3, 59–69.
- Tonelli, Lacerda, Tonelli, Costa, de Franca, Resende, 2017. Biotechnol. Adv. 35 (6), 832-844.
- Tsunekawa, N., Naito, M., Sakai, Y., Nishida, T., Noce, T., 2000. Isolation of chicken vasa homolog gene and tracing the origin of primordial germ cells. Development 127, 2741–2750.
- Vischer, H.F., Teves, A.C., Ackermans, J.C., van Dijk, W., Schulz, R.W., Bojerd, J., 2003. Cloning and spatiotemporal expression of the follicle-stimulating hormone ß subunit complementary DNA in the African catfish (*Clarias gariepinus*). Biol. Reprod. 8, 1324–1332.
- Voronina, E., Seydoux, G., Sassone-Corsi, P., Nagamori, I., 2011. RNA granules in germ cells. Cold Spring Harb. Perspect. Biol. 3.
- Wang, Z., Gao, J., Song, H., Wu, X., Sun, Y., Qi, J., Yu, H., Wang, Z., Zhang, Q., 2014. Sexually dimorphic expression of vasa isoforms in the tongue sole (*Cynoglossus semilaevis*). PLoS One 9.
- Wolke, U., Weidinger, G., Koprunner, M., Raz, E., 2002. Multiple levels of posttranscriptional control lead to germ line-specific gene expression in the zebrafish. Curr. Biol. 12, 289–294.
- Wu, X., Wang, Z., Jiang, J., Gao, J., Wang, J., Zhou, X., Zhang, Q., 2014. Cloning, expression promoter analysis of vasa gene in Japanese flounder (*Paralichthys olivaceus*). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 167, 41–50.
- Xu, H., Gui, J., Hong, Y., 2005. Differential expression of vasa RNA and protein during spermatogenesis and oogenesis in the gibel carp (*Carassius auratus gibelio*), a bisexually and gynogenetically reproducing vertebrate. Dev. Dyn. 233, 872–882.
- Xu, H., Lim, M., Dwarakanath, M., Hong, Y., 2014. Vasa identifies germ cells and critical stages of oogenesis in the Asian seabass. Int. J. Biol. Sci. 10, 225–235.
- Yan, M., Sui, J., Sheng, W., Shao, M., Zhang, Z., 2013. Expression pattern of vasa in gonads of sea cucumber *Apostichopus japonicus* during gametogenesis and reproductive cycle. Gene Expr. Patterns 13, 171–176.
- Yoon, C., Kawakami, K., Hopkins, N., 1997. Zebrafish vasa homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells. Development 124, 3157–3165.
- Yoshizaki, G., Takeuchi, Y., Sakatani, S., Takeuchi, T., 2000. Germ cell-specific expression of green fluorescent protein in transgenic rainbow trout under control of the rainbow trout vasa-like gene promoter. Int. J. Dev. Biol. 44, 323–326.
- Yuan, Y., Li, M., Hong, Y., 2014. Light and electron microscopic analyses of vasa expression in adult germ cells of the fish medaka. Gene 545, 15–22.