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Research Article

Regulation of early spermatogenesis in the giant prawn *Macrobrachium rosenbergii* by a *GCL* homolog†

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Abstract

The *germ cell-less* gene is crucial for gonad development in various organisms. Early interventions in its expression suggested a regulatory role at the mitotic stages of spermatogenesis, and its early knockout resulted in complete sterility in *Drosophila*. Genomic and transcriptomic data available for the catadromous giant prawn *Macrobrachium rosenbergii* enabled the identification of a *germ cell-less* homolog for this species, which we termed *MroGCL* (mRNA accession number OQ533056). An open reading frame containing 494 amino acids and a typical evolutionarily conserved BTB/POZ domain suggests possible protein–protein interaction functions in keeping with the *Drosophila* germ cell-less protein. Genomic mapping of *MroGCL* showed a full length of 120 896 bases. Analysis of the temporal expression of *MroGCL* showed constant expression in early prawn embryonic and larval stages, but a significant increase 10 days after metamorphosis when crucial sexual differentiation processes occur in prawns. In adult animals, high expression was detected in the gonads compared to the somatic tissues. RNAi-based knockdown experiments showed that both the silenced and control groups reached advanced spermatogenic stages, but that there was a significant decrease in the yield of spermatozoa in about half of the silenced animals. This finding supports our hypothesis that *MroGCL* is crucial for mitosis during early stage spermatogenesis. In conclusion, this study contributes to the understanding of crustacean gonad development and provides a stepping stone in the development of environmentally valuable sterile crustacean populations.

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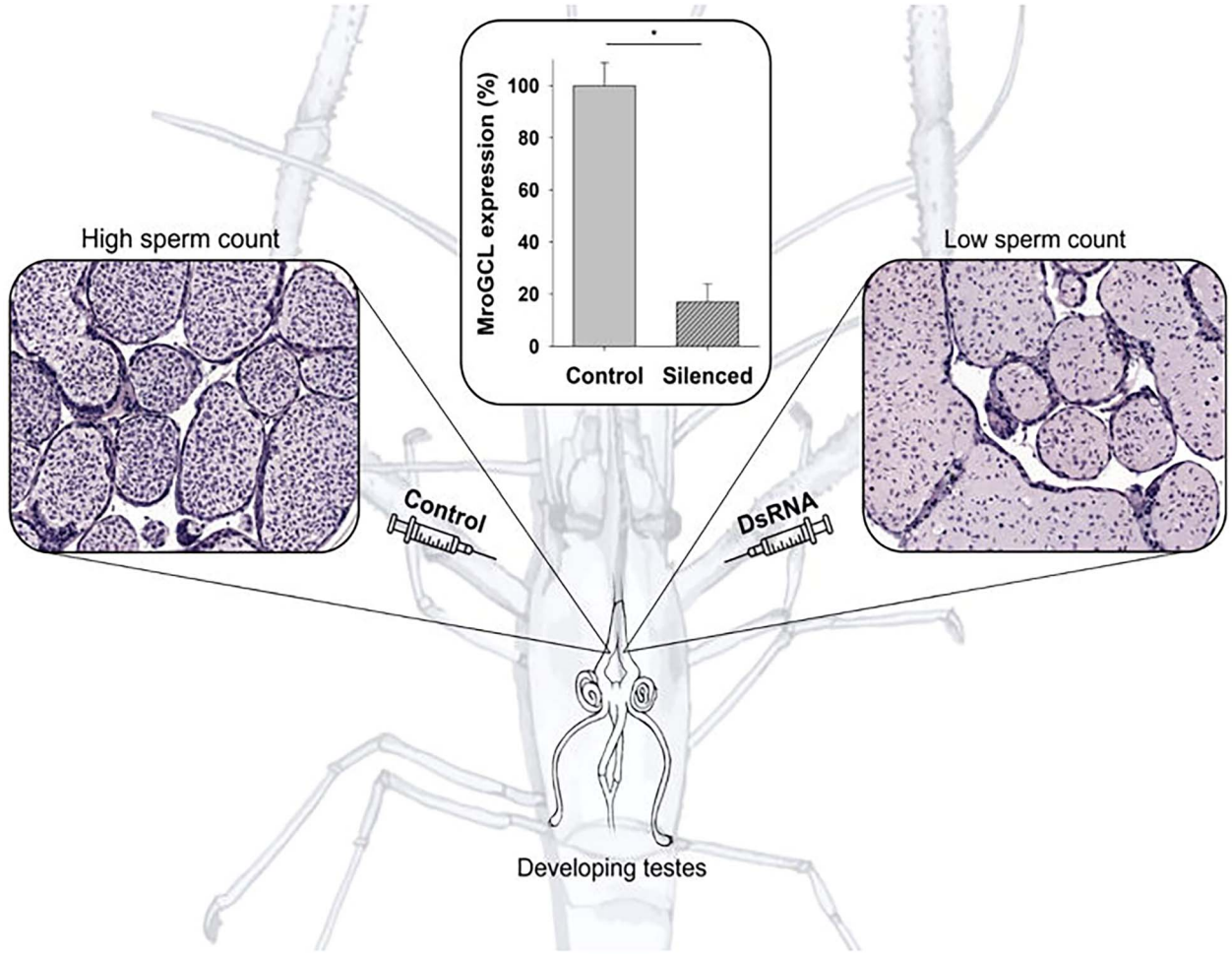
Graphical Abstract



Key words: Crustacea, Decapoda, germ-cell less, gonad development, Macrobrachium rosenbergii, spermatogenesis

Introduction

The *germ cell-less* (*GCL*) gene has been shown to be tightly linked to the development of the gonads. Studies on *GCL*, which began in the 1990s, have indicated that it is a highly conserved BTB/POZ (broad-complex, tramtrack, and bric-a-brac/poxvirus and zinc finger) protein localized in the nuclear envelope [1]. In the fruit fly *Drosophila melanogaster*, it operates as part of the Cullin3-RING ubiquitin ligase complex substrate-specific adaptor for *Torso* [2], a receptor tyrosine kinase and unknown determinant of somatic cell fate [3, 4]. Maternal *GCL* mRNA is concentrated at the posterior end of the developing *Drosophila* embryo, where it promotes the division of pole cells into primordial germ cells (PGCs) [5]. In previous studies, it has been shown that knock-out mutations in flies led to a reduction in pole cells and subsequent fertility problems, while the absence of maternal *GCL* resulted in sterile offspring [1]. In *Drosophila*, *GCL* was shown to be crucial for the development and formation of pole cells, which are the precursors of germ cells [5]. Lerit, Shebelut [6] showed that *GCL* regulates the spatial organization of centrosomes to promote the formation of pole buds [2]. Disruption of the function of *GCL* in developing early *Drosophila* embryos interfered with the normal development of the PGCs by expressing genes that are normally transcribed only in somatic cells [7], resulting in sterility [5]. Furthermore, the offspring of *GCL* mutants did not develop gonads [1]. In mammals, *GCL* homologs were found to be active in the testes of mice and humans [8, 9]. For example, *GCL* knocked-out mice showed impaired fertility due to severe sperm defects [10]. Similarly, human *GCL* was associated with male sterility, with the absence of *GCL* being associated with defective sperm motility [8]. Despite these extensive studies on *GCL*, knowledge about its role in crustaceans is lacking, especially in the widely studied species that are of aquacultural importance.

The giant prawn *Macrobrachium rosenbergii* is a catadromous crustacean living in fresh water but swimming to the sea to spawn. The species, which is indigenous to Southeast Asia, has become a popular aquacultural product—over 11 million tons of prawns were harvested in 2020 at an estimated worth of 1.7 billion USD [11]. With the general motivation of increasing yields and protecting the environment from invasions by aquacultural escapees and biocontrol agents [12], two types of monosex population of *M. rosenbergii* have been established, namely, all-male and all-female populations, each with its own benefits for aquaculture and environmental protection [13]. However, in these populations, the gonads mature normally [14], and the potential for unwanted

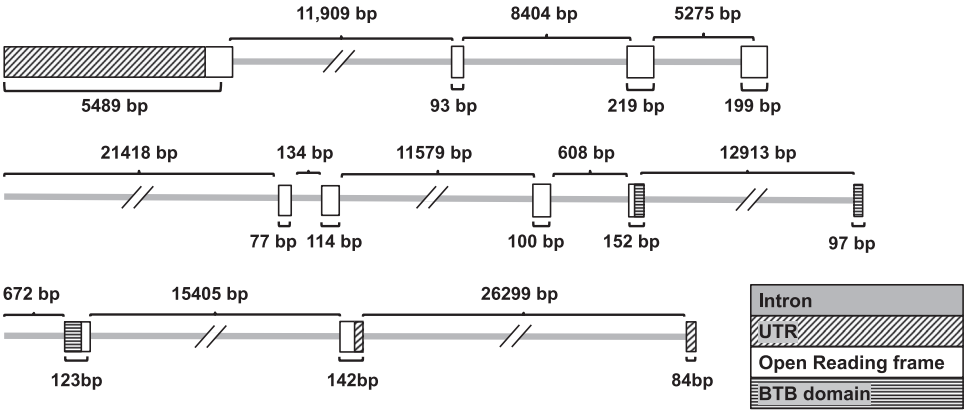


Figure 1. Gene map of the 120 896-bp long *MroGCL* gene characterized by using transcriptome and genome libraries. The ORF is shown as empty rectangled boxes, the UTR as diagonal stripes rectangled box, introns, as solid lines, and the BTB domain, as horizontal stripes rectangled boxes. Sequence length is written above and below the sequence for introns and exons, respectively.

reproduction still exists. For this reason, a deeper understanding of early gonad development and spermatogenesis is required, and the first step is to identify key genes upstream to these processes. It is noteworthy that a large group of genes regulates reproduction. The mining process, which led to the selection of *GCL*, was initiated by a wide mining effort in the *D. melanogaster* genome and literature that suggested potential genes. Candidates being explicitly expressed in gonads or during critical periods in prawn development and temporal expression patterns that are closely linked to gonad development were mined, e.g. *Nanos*, *Vasa*, and *Wnt4* [1, 10, 15–17]. *GCL* was the first to be thoroughly studied due to its reported knockout effects on gonad development in fruit flies and mice [1, 7, 10].

Detailed knowledge of the process of spermatogenesis in *M. rosenbergii* [18] has been applied in several studies for spermatogenic staging [19, 20] through 12 morphologically distinct stages. From these studies, it is known that the testes display two distinct regions: the region adjacent to the epithelial cells at the periphery of the lobule contains dividing spermatogonia (germline/maturing sperm cells), which begin differentiating into spermatozoa, while the lumen at the center of the lobule contains spermatozoa (mature sperm cells), some still in the process of spermatogenesis.

In the present study, we identified a *GCL* homolog in *M. rosenbergii*, which we designated *MroGCL*. We studied its expression pattern during early development and its spatial expression in various adult prawn tissues. We also verified its involvement in spermatogenesis in males, with the temporal expression pattern suggesting possible roles for *MroGCL* during early development in prawns. Our working hypothesis for this study was that *MroGCL* functions in the prawn as it does in the known model organism *D. melanogaster*, exhibiting high expression in mature gonads and during early gonad differentiation, but knockdown of *MroGCL* will disrupt normal gonad function.

Results

Structural analysis of *MroGCL*

A *GCL* homolog—termed *MroGCL*—was found in the *M. rosenbergii* embryonic transcriptome by using the orthologous isoform A protein from *D. melanogaster* (NP_724708.1). By using the novel transcriptomes [21] and the high-quality genome [22] developed in our lab, we characterized the *MroGCL* structure (Figure 1). The full-length Mro*GCL* gene is 120 896 bp long (**GenBank accession number** OR820902), and it contains 11 introns, ranging in size from 134 to 26 299 bp. A schematic representation of the gene shows that the BTB domain of the protein is split between exons 8, 9, and 10 (Figure 1).

Sequence analysis of *MroGCL*

The *MroGCL* transcript is 7070 bp long (accession number OQ533056) and contains an open reading frame (ORF) of 494 amino acids (Figure 2). The 5′-untranslated region (UTR) is 90 bp long, and the 3’-UTR is 5315 bp long. *MroGCL* encodes a typical BTB/POZ domain (Figure 2, marked in red).

Multiple sequence alignment and phylogeny

Multiple sequence alignment of *GCL* proteins of different species (Figure S1; Table 1) showed high conservation in certain regions of the protein, particularly the BTB domain (Figure 3, top). Several cysteines were shown to be conserved, implying a conserved structure of the BTB domain. Most of the differences were at the two ends of the protein. A phylogenetic tree of *GCL* (Figure 3, bottom), constructed using the same sequences and organisms as those presented in Table 1, showed a clear separation of vertebrates and invertebrates and an additional subdivision of decapod crustaceans and *D. melanogaster*. Interestingly, the human and mouse *GCL*s did not branch together.

Temporal expression patterns of *MroGCL* in different larval and post larval stages

Figure 4 shows the temporal expression patterns of *MroGCL* in different larval and PL stages. No significant differences were observed between males and females up to PL1, and both sexes showed a significant increase in *GCL* expression from PL1 to PL10, namely, from 90.7 ± 15.1 to 282.1 ± 22.2 in females ($p < 0.001$) and from 89 ± 13.6 to 220.9 ± 38.1 in males ($p = 0.001$).

Spatial expression of *MroGCL* in adult tissues

Initial PCR test of the primers on several tissues showed *MroGCL* expression as a 434 bp fragment in all tested tissues of both sexes (Figure S2A). The fragment was sequenced and found to be identical to the transcriptomic sequence (Figure S2B). The spatial expression pattern of

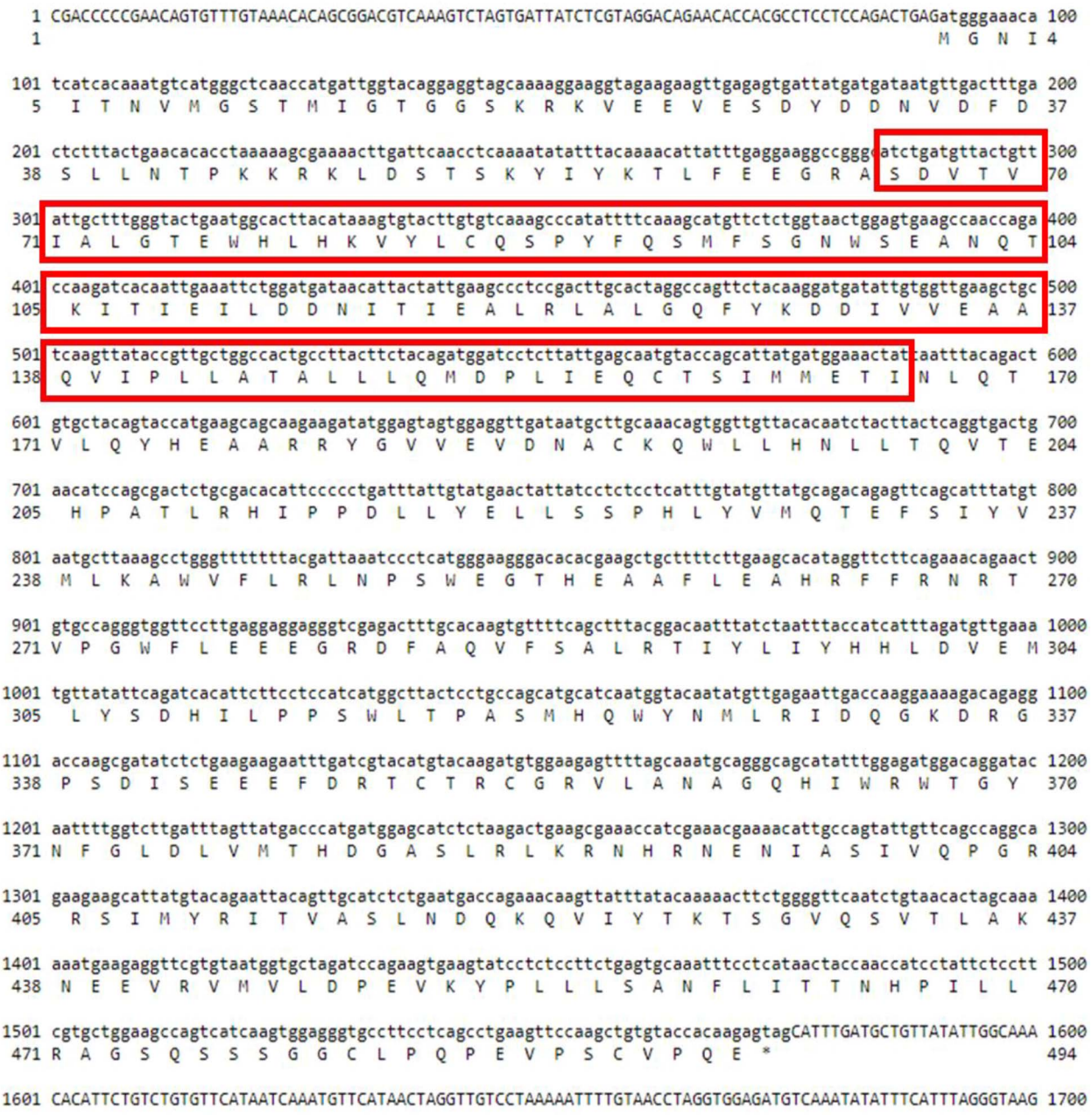


Figure 2. Nucleotide and deduced amino acid sequence of *MroGCL*. 5′ UTR and 3′ UTR are indicated with capital letters. ORFs are shown in lowercase letters. The translation start codon (ATG) and termination codon (TAG) are underlined. The BTB domain is indicated with rectangled boxes.

MroGCL in various *M. rosenbergii* tissues was determined by qRT-PCR of cDNA derived from specific tissues (Figure 5). In males, the relative expression was low in most tissues (cuticle 5.55 ± 2.94; eye 39.83 ± 25.85; heart 3.07 ± 1.46; hepatopancreas 6.17 ± 1.42; muscle 2.6 ± 0.78; and sperm duct 56.53), with the exception being the testis (1333.62 ± 369.09). The female animals exhibited higher expression than the males in all tissues (cuticle 429.95 ± 26.95; eye 585.46 ± 172.26; heart 322.35 ± 70.41; hepatopancreas 134.08 ± 7.17; muscle 168.4 ± 37.88), with the highest expression in the ovary (2384.68 ± 337.22).

RNAi induced knock-down of *MroGCL* in sexually active small males

MroGCL fragment inserted to the pGEM vector was sequenced (Figure S2B). The average expression level of *MroGCL* in the control animals was set as 100%, as measured by qRT-PCR (Figure 6). A significant decrease in expression levels was observed in the silenced prawns in comparison to the controls ($p = 0.004$, df = 13), with expression levels (±SE) of $17.0 \pm 6.8\%$ and $100.0 \pm 8.9\%$, respectively.

Histology of sexually active small males with RNAi knock down of *MroGCL*

According to the spermatogenic staging of Poljaroen et al. (2010), both control and silenced animals showed advanced stages of spermatogenesis, with the control group showing stages VI and VIII, and the silenced group showing slightly more advanced stages, namely, VIII and IX (Figure 7A). Nonetheless, there was a significant reduction in average sperm count in the silenced group (33.3 ± 5.7 spermatids/50 μm^2) vs. the control group (58.9 ± 3.8

Table 1. GCL protein sequences

Organism	Protein sequence	NCBI sequence ID:
Drosophila melanogaster (isoform A)	MGQIVGSMHMNVAEVFSNRRKRKRSTDSSLGKDDPAQLDTTQPKKKKLLT TTQYIYKALFKEEKNSDVAVMALDKVWHLHKVYLSQSPYFYTMFNGTWRE AQQNFIQITILDDRITVASLDAVFGSMYSDEIEIESADVISVLATATLFHLDGIID KCAEVMVDNISPETAIQYYEAACQYGVVGVKKSTFQWFQINLLSIYSKQPNL LRHISIELMSALTASPDLYVMQTEFSLYTLLRTWMFLRLHPDYDPEDPVQRAE ALKTQELLVNAGVETHAPSGDVVQWTYFTSRSEERSFLATPEGQPYVKVFQK LRTQYLTNHYMDLKIIYNDNIIPKEWLYRHIHNHWDALLRIDHGQEDCSPQ QLDDEQFFENCMRCGRMLLEPGYQKWRWTGFNFGMDLILIMDSRRLNIRR HHRHEHERVLSLQTKRKFMVRTTVTSINAQRQAVFTQTSEICSLSLEKNEEVP LMVLDPKLVHPLLISINMLVVMPPNQSFKEIVPLSEEATTSLSIPISEIGANSDRPL SPSSADDSAVFIGDSEPSTPSSPAPRPRIAWSASETGAICGQLAC	NP_724708.1
Homo sapiens (isoform X1)	MGSLSSRVLRQPRPALAQQAQGARAGGSARRPDTGDDAAGHGFCYCAGSHK RKRSSGSFCYCHPDSETDEDEEEGDEQQRLLNTPRRKKLKSTSKYIYQTLFLN GENSDIKICALGEEWSLHKIYLCQSGYFSSMFSGSWKESSMNIIELEIPDQNIDV EALQVAFGSLYRDDVLIKPSRVVAILAAACLLQLDGLIQQCGETMKETVNVKT VCGYYTSAGTYGLDSVKKKCLEWLLNNLMTHQNVELFKELSINVMKQLIGS SNLFVMQVEMDIYTALKKWMFLQLVPSWNGSLKQLLTETDVWFSKQRKEW LSSVYKQQWFAMLRAEQDSEVGPQEINKEELEGNSMRCGRKLAKDGEYCWR WTGFNFGFDLLVTYTNRYIIFKRNTLNQPCSGSVSLQPRRSIAFRLRLASFDSSG KLICSRTTGYQILTLEKDQEQVVMNLDSRLLIFPLYICCNFLYISPEKKN	XP_011531335.1
Mus musculus	MGALSSRVLRPAGRTEQPEPTPGAGGAARRSDAGEDAGHSFCYCPGGRKRKR SSGTFCYCHPDSETDDDEDEGDEQQRLLNTPRRKKLKSTSKYIYQTLFLNGE NSDIKICALGEEWSLHKIYLCQSGYFSSMFSGSWKESSMNIIELEIPDQNIDIEA LQVAFGSLYRDDVLIKPSRVVAILAAACMLQLDGLIQQCGETMKETISVRTVC GYYTSAGTYGLDSVKKKCLEWLLNNLMTHQSVELFKELSINVMKQLIGSSNL FVMQVEMDVYTALKKWMFLQLVPSWNGSLKQLLTETDVWFSKWKKDFEGT TFLETEQGKPFAPVFRHLRLQYIISDLASARIIEQDSLVPSEWLAAVYKQQWLA MLRAEQDSEVGPQEINKEELEGNSMRCGRKLAKDGEYCWRWTGFNFGFDL LVTYTNRYIIFKRNTLNQPCSGSVSLQPRRSIAFRLRLASFDSSGKLICSRATGY QILTLEKDQEQVVMNLDSRLLIFPLYICCNFLYISPEKRTESNRHPENPGH	NP_035948.3
Penaeus monodon	MGNLISNLMGSHVMGSGGSKRKVEDLESEDEDAADFDSLLHTPKRRRLNS TSQYIYKTLFEEGCASDVTVVALGREWHLHKVYLCQSPYFGSMFSGNWTET NQSKINIEILDDNINIDALHLALGQFYKDDIVVEPAQVIPLLATALLLQMDPLI DQCTSIMMETINLQTVLQYHEAARRYGVLEVDKACKQWLLHNLLTQVTE HPATLRHIPSDLMTELLTSPHLYVMQTEFSVYVMLKAWVFLRLNPSWDGSHE TAFLEAHKHFRNRAERGWFLEEEGREFSPVFSALRLVYLIYHHLDVEMLYSD RILPPSWLTPASMHQWYNMLRIDQGKDRGPSEISEEEFDRSCTRCGRVLGNT GQHIWRWTGYNFGLDLVMTHDGASLRLKRNHRTENIASIAQPARRNIMYR TVASLDDQKQVVYTKSTGVQSVSLAKNEEIRVMVLDPDVKYPLLLSANFLITT NHSLITSHPGGSSGTSTSPSAIASASNLSARAPGQPSRE	XP_037788887.1
Penaeus vannamei	MGNLISHLMGSHVMGSGGSKRKVEDLESEDEDATDFDSLLHTPKRRRLNS TSQYIYKTLFEEGCASDVTVVALGREWHLHKVYLCQSPYFGSMFSGNWTET NQSKINIEILDDNINIDALHLALGQFYKDDIVVEPAQVIPLLATALLLQMDPLI DQCTSIMMETINLQTVLQYHEAARRYGVLEVDKACKQWLLHNLLTQVTEH PATLRHIPSDLMTELLTSPHLYVMQTEFSVYVMLKAWVFLRLNPSWDGSHET AFLEAHKHFRNRAERGWFLEEEGREFSPVFSSLRLVYLIYHHLDVEMLYSDRIL PPSWLTPASMHQWYNMLRIDQGKDRGPSEISEEEFDRSCTRCGRVLGNSGQH IWRWTGYNFGLDLVMTHDGASLRLKRNHRTENIASIAQPARRNIMYRVTVAS LDDQKQVVYTKSTGVQSVSLAKNEEIRVMVLDPDVKYPLLLSANFLITTNHSLI TSHPGSSSGTSTSPSAIASASNLSARAPGQPSRE	XP_027224867.1
Cherax quadricarinatus	MGNLLKGFMGSHVMGSGGSKRKVEEVESDDDDGMDLNSLLHTPKKRKLNS TSKYIYKTLFEDGRSSDVSVVALGHEWNLHKVYLCQSPYFGSMFSGNWTETN QSQITIEILDDNINIDALHLALGQFYKDDIEVEAAQVIPLLATSLLLQMDPLIDQ CTCIMLETINLQTVVQYHEAARRYGVVEVDKACKQWLLHNLLTQVTEHPAT LRHIPPDLLYELLSSPHLYVMQTEFSVYVMLKAWVFLRLNPSWEGSHEEAFLEA HKYFRNRTDSGWFLEEEGREFSLVFSALRLVYLIYHHLDVEMLYSDRILPPSWLT PASMHQWYNMLRIDQGKDKGPSEISEQEFDKSCTRCGRVLANSGQHIWRWTG YNFGLDLVMTHDGASLRLKRNHRTENIASIIAQPGTRNIMYRVTVASLDEQKQ VVYTKTTGVQAASLAKNEEIRVMLLDPEVKYPLLLSANFLITTNHQLTVQGAT TSATAGPPCQPSKE	Internal library

spermatids/50 μm^2, $p = 0.0023$, df = 14; Figure 7B). While the entire control group showed a relatively uniform sperm count, about half of the silenced group (designated group A) exhibited sperm count similar to those of the controls. The other half of the silenced group (designated group B) had a dramatic reaction to the treatment, with significantly lower sperm count (Figure 7C). With this separation, the average sperm counts for groups A and B were 46.1 ± 3.5 and 20.6 ± 5.7 spermatids/50 μm^2, respectively. A quantitative comparison of the sperm counts for each of the above groups

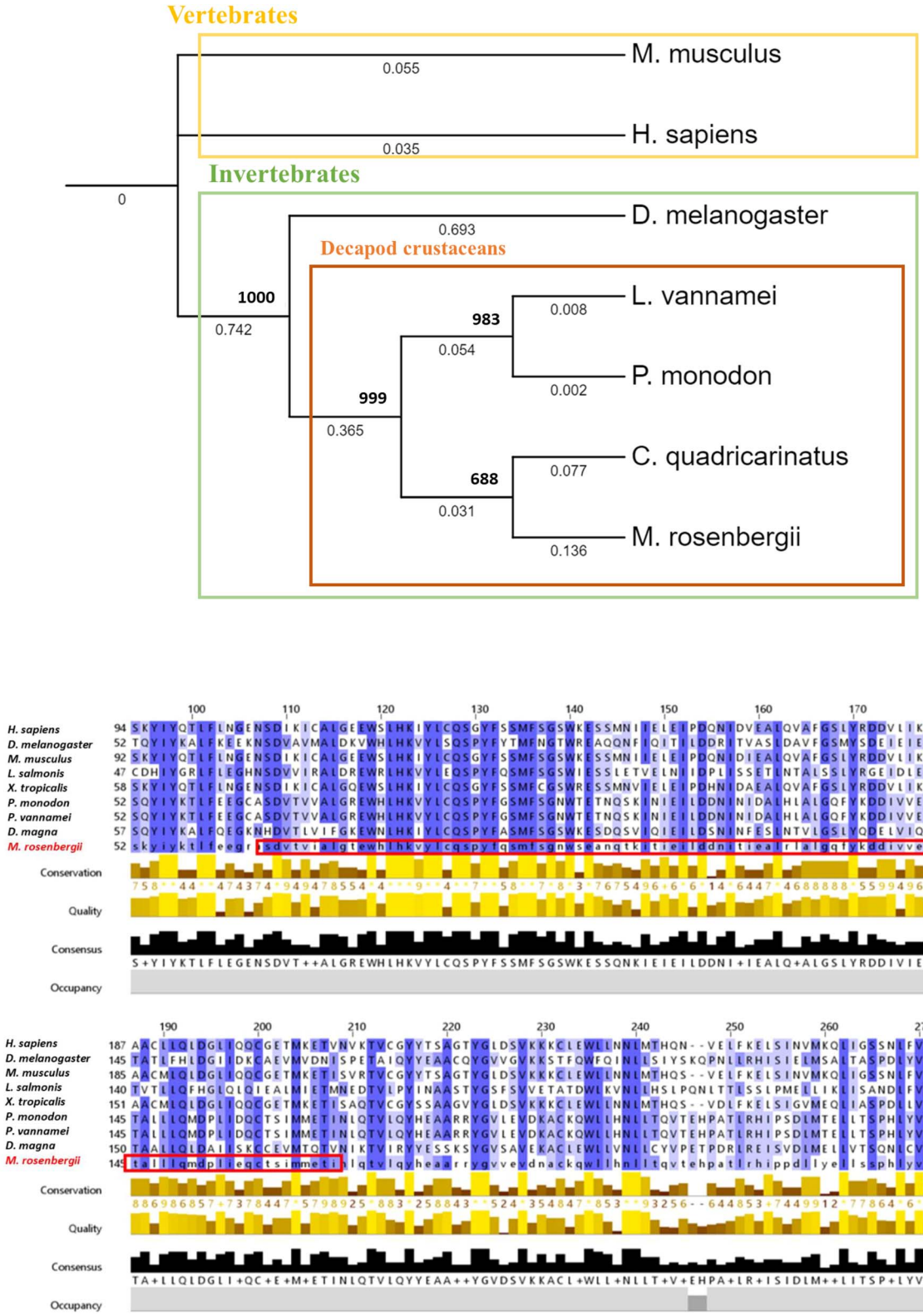


Figure 3. Comparison of homologous amino acid sequences of GCL from various organisms (Table 1). Top: phylogeny of representative GCL proteins with phylum and order separation indicated with rectangled boxes. Bottom: multiple sequence alignment of BTB domain with consensus amino acids highlighted. The putative MroGCL sequence is shown in lowercase letters (last row), and the BTB domain is marked by a square rectangled box. Branch lengths are given below each branch, and supporting values are above the junction (in bold).

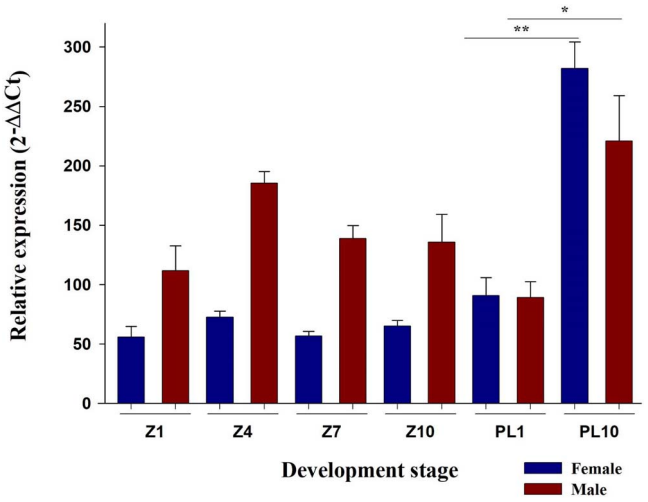


Figure 4. Mean temporal expression of *MroGCL* in *M. rosenbergii* larvae and post-larvae, as shown by RT-qPCR. Statistically significant differences between PL1 and PL10 are indicated with $*0.01 > p > 0.001$ in females and $**p \leq 0.001$ in males. Error bars represent standard error.

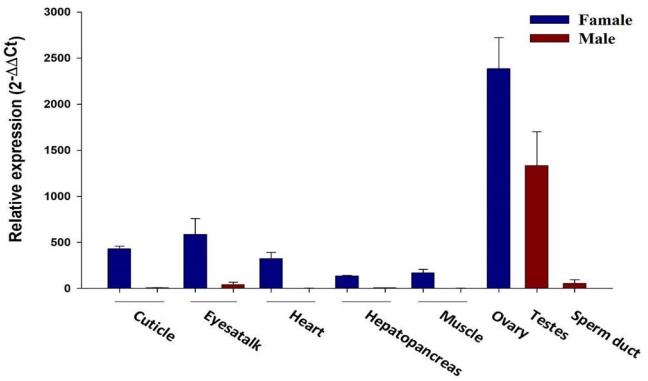


Figure 5. Mean spatial expression of *MroGCL* in tissues of sexually mature prawns ($n = 6$ for all tissues). Error bars represent standard error.

separately vs. the control group showed no significant differences between group A and the control group ($p = 0.14$), but a significantly lower sperm count in group B ($p = 0.00026$) (df = 13 for both, Figure 7D).

Discussion

It is known that *GCL* has evolutionarily conserved functions related to gonad development and the maintenance of germline characteristics. Extensive studies of *GCL* in a range of model organisms (*Drosophila*, mice, fish, and even humans) have shown that *GCL* is involved in gonad development and spermatogenesis [1, 8, 10]. With this knowledge in hand, we leveraged the catadromous giant prawn *M. rosenbergii* genome [22] to find a *GCL* homolog in *M. rosenbergii* with close resemblance in its deduced protein sequence to previously published models.

The identification of the *MroGCL* gene and the conservation of its structure in several crustacean species suggest that its function might also be conserved in the prawn. The genomic structure of *MroGCL* includes long introns, spanning from 134 up to 26 000 bp. Such long and diverse introns are common in the *M. rosenbergii* genome [21]. The MroGCL protein contains the BTB/POZ domain [23], typically found

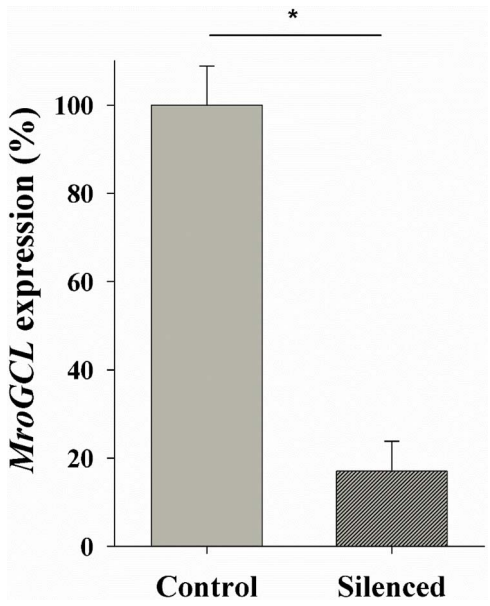


Figure 6. Mean expression of *MroGCL* in testes of prawns after 12 weeks of RNAi injections. The average expression level for the control group was set as 100%. The control group ($n = 7$; right column) was injected with dsRNA of GFP, while the silenced group ($n = 7$; left column) was injected with dsRNA of *MroGCL*. Bars indicate one standard error. A *t* test showed the difference in expression levels to be statistically significant ($p = 0.018$, df = 12).

in *GCL* genes of other species, and the amino acid similarity of this domain to that of model organisms suggests that its function is probably conserved. In the present study, clustering of these domains across phyla demonstrates a clear evolutionary link with both vertebrates and invertebrates and with all crustaceans investigated and aligned thus far.

In *M. rosenbergii*, sexual differentiation and the development of primary and secondary sexual characteristics start ~20 days after metamorphosis [24], with the PL10 stage being the stage at which this process can still be manipulated. Therefore, as expected, we found that *MroGCL* expression started rising at PL10, probably in concert with the preparation of the PGCs for differentiation into testes or ovaries. Expression of *MroGCL* does not peak at the stage of PGC development, like in the fruit fly, but rather during gonad development, similar to mice [25]. Similarly, zebrafish embryos also show a rise of *GCL* mRNA expression levels at late stages of embryogenesis [26]. The spatial expression patterns in representative tissues of *M. rosenbergii* adults showed that *MroGCL* is expressed mostly in the gonads of both sexes: in females, expression in the ovaries was high vs. comparatively low in other tissues, and in males [similar to mice [9]], Mro*GCL* was expressed almost exclusively in the testes, with only very low expression in all the somatic tissues examined. These findings support the argument that *MroGCL* is crucial for gonad development and spermatogenesis.

In view of the strong supporting evidence presented above, functional experiments were carried out on sexually active small males that exhibited a high knockdown response of over 80% to *MroGCL* RNAi. The results showed that *MroGCL* silencing disrupted sperm production in the testis, resulting in a reduced sperm count but with no apparent disruption of sperm maturation. Since sperm cells in this species are not motile and the external fertilization act is mediated by active transfer of oocytes over the sperm mass by the female [27],

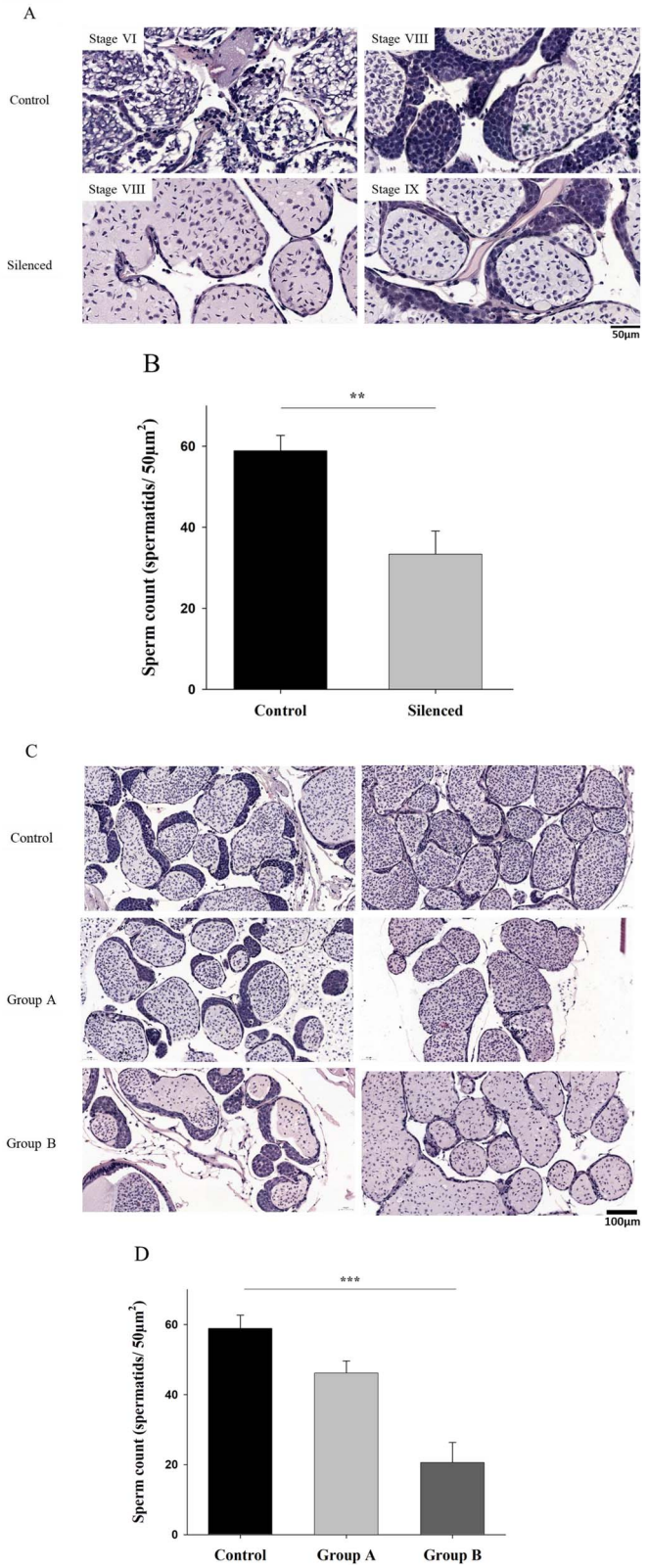


Figure 7. Histological sections of the testes and sperm counts of *MroGCL* silenced and control animals. (A) Hematoxylin-Eosin stained sections of representative spermatogenic stages in silenced (stages VIII and IX) and control (stages VI and VIII) animals. (B) Sperm counts in control and silenced groups showing a clear reduction of in the numbers of spermatozoa within the lumen of the seminiferous tubules of the silenced animals ($p = 0.0023$, df = 14). (C) Histological sections showing two distinct groups of silenced prawns – group A with no significant reduction in sperm count ($p = 0.14$, df = 13) and group B with a marked drop in sperm count vs. the control group ($p = 0.00026$, df = 13) (D).

the common evaluation of sperm viability in the species is based on morphological observations [18]. Indeed, advanced stages of sperm maturation were observed in both silenced and control groups, despite a significant decrease in sperm count—up to 65% decrease in mature sperm. This is most noted in the treated prawns in which half exhibited a dramatic reduction in sperm count, a variance that could reflect physiological differences such as molt stage [28, 29] and initial spermatogenic stage [18] at the time of treatment. These findings imply that *MroGCL* is involved in the mitotic proliferation of early spermatocytes. However, future mechanistic insights are needed to verify this hypothesis. Decreased mitotic cycles, along with normal maturation of the spermatozoa, resulted in decreased numbers of mature sperm cells, as seen in *MroGCL*-silenced males. Known functions of the GCL protein in *Drosophila* imply a similar function in the proliferation and formation of pole cells by regulating centrosome separation [6]. Previous studies have shown that knocking out *GCL* in *Drosophila* led to an absence or reduction in pole cells [5], and *GCL*-overexpressing mothers produced progeny with increased numbers of pole cells [30]. A high knockdown effect method [13, 24] was used in the present study on mature *M. rosenbergii* males with 83% knockdown effect on average. Testing early embryo knockdown was not possible, due to the lack of an appropriate knockout platform in *M. rosenbergii* in which, unlike other CRISPR platforms in crustaceans [31, 32] edited larvae did not survive till metamorphosis yet. Thus, we were able to show only the effects of *MroGCL* knockdown on spermatogenesis. With future completion of the development of such a platform for *M. rosenbergii* [see initial attempts by [33]], studies on gonad development will be possible in crustaceans, as has previously been done for classical model organisms through knockout experimentation [1].

In this work, we revealed a *GCL* homolog in *M. rosenbergii*, with clear evidence of its effect on the miotic stage of early spermatogenesis and supporting evidence for its possible function in gonad development. With these insights and further research into gonad development attending more genes of the gonad regulating toolkit, we hope to shed additional light on this understudied mechanism of early gonad development in crustaceans, resulting in the possibility of inducing sterility in cultured crustaceans, with the attendant environmental benefits.

Materials and methods

Identification and characterization of *M. Rosenbergii GCL*

To identify the *M. rosenbergii GCL* homolog, a known GCL sequence from *D. melanogaster* (accession number NP_724708.1; Table 1) was used to search for homologs in several transcriptomic libraries available in our lab [21]. These libraries cover embryonic, larval, and post-larval (PL) stages as well as several adult tissues of *M. rosenbergii* [21, 34]. Transcriptomic libraries were constructed from Monosex pools of *M. rosenbergii* prawns in different developmental stages. ZZ males and WW females were collected in three replicates as described previously [35, accession number PRJNA906315], sequenced using Illumina technology and assembled using HISAT2 with default parameters. In addition, *GCL* homologs were searched in a high-quality prawn genome established in our laboratory. The genome was sequenced out of a WZ female with a second and third-generation sequencing using Illumina technologies. Data were processed and assembled using the DeNovoMAGIC assembler application version 3.0 [22]. The homologs found were examined for meaningful annotations using pfam (https://pfam.xfam.org/), and putative protein sequences were obtained using the ExPASy Translate tool on the *MroGCL* mRNA (https://web.expasy.org/translate/). The protein sequence was placed into NCBI conserved domain search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Comparisons to similar sequences were performed by BLAST for both mRNA and protein sequences. Introns and exons were calculated using the alignment of the mature mRNA sequence to the full genomic sequence found in the genome.

Search for relevant GCL sequences

A search of public transcriptomes and articles that refer to GCL proteins revealed three crustacean GCLs, and human, mouse, and *D. melanogaster* GCLs (Table 1). These sequences were converted to proteins (if the protein sequence was not available) using the ExPASy Translate tool (https://web.expasy.org/translate/). All comparative studies were performed using these proteins, since *GCL* genes exhibit only minor similarities in their DNA or RNA sequences, even within a single species with several orthologs, while the translated proteins exhibit more similarities. The sequences of these seven proteins were submitted to multiple sequence alignment with MAFFT version 7.511 [36]. To find the best phylogenetic model to select, we used the Smart Model Selection (SMS) function with Bayesian information criterion (BIC) in PhyML [37]. Finally, the evolutionary history was inferred by the maximum likelihood method and the MtZOA model [38, 39]with bootstrapping 1000 replicates. The evolutionary phylogenetic analysis was conducted and visualized using iTOL [Interactive Tree of Life; 40].

Animals

The *M. rosenbergii* prawns, all of the BGU line, from ZZ all-male [24, 41], WZ all-female [14], and WW all-female [22, 42] populations (produced as described below), were held in our laboratory in separate tanks (from hatching) to prevent unwanted breeding. Larvae and post-larvae were obtained from breeding adults according to the sex and stages required for the experiments. Larval development staging from zoea 1 to zoea 11 stage (Z1-Z11) was performed according to Uno [43]. In *M. rosenbergii*, males are homogametic (ZZ) and females are heterogametic (WZ). All-male progenies were obtained by crossing males with neo-females (ZZ females lacking the W chromosomes), guaranteeing only male (ZZ) offspring. ZZ all-male and ZZ neo females were obtained from the Tiran group through their subcontractor, Colors Ltd. WW females were supplied by Enzootic Genetics and Innovation Ltd. All-female populations were obtained by crossing ZZ and WW prawns in house generating normal WZ females.

In vitro expression of *MroGCL* in larvae and post-larvae

Total RNA was extracted using an EZ-RNA II Isolation Kit (Biological Industries, CT, USA), and cDNA was synthesized using a qScript cDNA Synthesis kit (Quantabio, MA, USA). Total RNA was extracted from four distinct larval stages: (Z1, Z4, Z7, Z10). In addition, two PL stages were sampled, the first collected right after metamorphosis (PL1) and

the second 10 days after metamorphosis (PL10). PL10 was selected as a critical point of sex differentiation in *M. rosenbergii*. RNA samples were extracted from the above larval and PL stages both, from allmale (ZZ) and allfemale (WZ) progenies ($n = 5$ from each stage). Relative quantification of transcript levels of *MroGCL* was performed using SYBR Green. The following primers were used: qMroGCL F (5'-AAGCATGTTCTCTGGTAACTGGA-3′) and qMroGCL R (5'-CAGAATTTCAATT GTGATCTTGGT-3′). *M. rosenbergii 18S*, which was used for normalization, was also quantified by means of qPCR using the primers, qMr-*18S* F (5'-CCCTAAACGATGCTGACTAGC-3′) and qMr-*18S* R (5'-TACCCCCGGAACTCAAAGA-3′). One sample (Z7 female), exhibiting the lowest expression level of *MroGCL* was chosen to serve as the 0-reference point for relative quantification measurements of *MroGCL*. This sample was diluted 1:100, to insure a very low concentrations of mRNA and used as control. The qPCR reactions were performed in the QuantStudio 1 Real-Time PCR System, Applied Biosystems (Foster City, CA, USA).

In vitro expression of *MroGCL* in specific tissues in mature males and females

RNA was extracted using an EZ-RNA II Isolation Kit, and cDNA was synthesized, using a qScript cDNA Synthesis kit, from six tissues (heart, eye, muscle, cuticle, hepatopancreas (HPT), and ovaries of different stages or testis & sperm duct). The cDNA was then amplified by PCR, using MroGCL F (5'-TATTGAAGCCCTCCGACTTG-3′) and MroGCL R (5'-TCAAGAAAAGCAGCTTCGTG-3′) primers. PCR products were electrophoresed in 1% agarose along an ExcelBand 100 bp DNA ladder (SMOBiO Technology Inc, Taiwan) and visualized by exposure to UV light and SYBR Safe DNA Gel Stain (ApexBio, TX, USA) for *MroGCL* sequence verification by Sanger sequencing and initial spatial expression test. After, similar tissue samples from six females and males were surgically collected. Total mRNA was extracted for each tissue sample and cDNA synthesized as described above (EZ-RNA II Isolation Kit, qScript cDNA Synthesis kit) and qRT-PCR preformed as described above (Syber Green using qMroGCL F/R and qMr-*18S* F/R primers, QuantStudio 1 Real-Time PCR System). Three male muscle samples were diluted 1:100 and used as baseline comparison as they express the least *MroGCL* from initial results.

In vivo *GCL* silencing in small mature males and PL10

A fragment of *MroGCL* (using MroGCL F/R primers detailed in the section above) was inserted into the pGEM Teasy vector system I (Promega), sequenced using Sanger, and aligned to *MroGCL* mRNA for verification. This template was amplified with specific primers as mentioned above (*MroGCL* F/R) in two separate reactions. Each reaction contained a primer extension of a T7 recognition site on the 3′/5′ ends separately. RNA was transcribed with the TranscriptAid T7 High Yield Transcription Kit (Thermoscientific, Waltham, MA, USA) and purified by phenol-chloroform extraction.

MroGCL silencing in sexually mature small males

An initial RNAi experiment was conducted to ensure double-strand RNA efficiency. Fourteen males were selected by weight (12–20 g) and split randomly into two groups: a negative control group and an *MroGCL* silenced group. The prawns of the negative control group were injected with ds*GFP* RNA, and the silenced group was injected with dsRNA of *MroGCL*. Each prawn in the control and treatment groups was injected with 5 μg dsRNA/g body weight. Each group was injected twice, with a 48-h gap between injections. Twenty-four hours after the second injection, the prawns were anesthetized in ice-cold water for 5 min, and the testes were surgically removed. RNA extraction and cDNA synthesis were performed on the testis as mentioned above. *MroGCL* expression levels were calculated in qRT-PCR as mentioned above, using the qMroGCL primers. Average *MroGCL* expression in the control group considered 100% expression level and the expression level of *MroGCL* in the treatment group was calculated relative to the control group. For the long-term in vivo experiment, the same procedure was repeated with 56 males (each weighing 12–20 g), which were assigned to two equal-sized groups, one injected with *MroGCL* dsRNA, and the other with saline. The injection regime comprised 12 injections, given once a week over 12 weeks.

Histology and sperm count

The testes of seven prawns of each of the above two groups were removed as described above. The testes were fixed in a 4% formalin PBS solution for 24 h. Dehydration was performed over 8 h with increasing concentrations of ethanol, and the testes were then embedded in Paraplast. Sections of 5 μm were cut. After rehydration and staining with Hematoxylin–Eosin, slides were scanned using a PANNORAMIC MIDI panoramic scanner (3DHISTECH, Hungary) and representative sections were selected for sperm counting. In each testicular section, four squares (50 μm^2) were placed to fit inside random lobules, and the spermatids within each square were counted with the naked eye.

Statistical analysis

Data used for statistical analysis were first tested for the normality of the residuals using the Shapiro–Wilk test and for homogeneity of the variance using Levene test. For the qRT-PCR relative transcript levels of *MroGCL* and *Mr18S*, the data were first logarithmically transformed. The transformed data were then analyzed using one-way ANOVA, followed by a post hoc Tukey HSD test with a significance value of $p < 0.05$. Sperm counts of silenced and control prawns were compared using a T-test and one-way ANOVA with a significance value of $p < 0.05$. All statistical analyses were performed using Statistica v13.3 software (StatSoft, Ltd, Tulsa, OK, USA).

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Author contributions

This study was conceived and designed by JM, AS, and EDA. JM, DA, and RM collected the bioinformatics data and performed the alignment. Temporal and spatial expression of *MroGCL* was determined by JM, DA, and TL. JM, DA, and RM designed primers for qPCR and dsRNA synthesis. Prawn injections were the task of JM, DA, and RM.

Histological sectioning of testes were performed by JM and EDA, and analyzed by JM, EDA, AS, and JA. The manuscript was written by JM, DA, and AS and reviewed and approved by all co-authors.

Supplementary Data

Supplementary data are available at *BIOLRE* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material. This data can be shared on reasonable request to the corresponding author.

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