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Sexual plasticity of *Hippolyte inermis* Leach (Crustacea, Decapoda): Gene expression of vitellogenin and insulin-like androgenic gland hormone

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ABSTRACT

Hippolyte inermis is a protandric hermaphroditic shrimp widespread in most Mediterranean seagrass meadows. It has a complex life cycle, because an unknown compound present in benthic diatoms (*Cocconeis* spp.) induces an early sex reversal in its post-larvae upon ingestion. This change occurs due to the disruption of the androgenic gland (AG) germ cells. To elucidate the molecular mechanisms behind this unique example of sexual plasticity, we isolated sex-specific genes from the transcriptomes of shrimp collected at various stages of their life cycle. Using Real-Time qPCR, we measured, in wild adults, the expression levels of the *Insulin-like Androgenic Gland hormone* (*Hi-IAG*), *Vitellogenin-1* (*Hi-VG1*), two *uncharacterized male* and *female genes* (*Hi-UCM* and *Hi-UCF*), and two *uncharacterized male and female eyestalk* genes (*Hi-UCMe* and *Hi-UCFe*). Further, the expression of these genes was evaluated in shrimp cultured in the laboratory, after the ingestion of bioactive diatoms, to identify potential molecular markers. Only *Hi-VG1* and *Hi-UCM* showed a consistent relationship with the phenotype of adults, according to their sex. Subsequently, we investigated the physiological and developmental expression patterns of *Hi-VG1* and *Hi-UCM*, along with several *Vitellogenin* variants, across a range of developmental stages, to reveal the mechanisms of sex regulation. While the results revealed complex response patterns, they lay the groundwork for understanding the link between key gene expression and sexual determination and maturation in decapod crustaceans.

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1. Introduction

Reproductive strategies in crustaceans vary from gonochorism to hermaphroditism, including protandric and protogynic patterns. Although crustaceans are usually gonochoristic, i.e., their male or female sex is determined *a priori* and kept throughout their adult lives (Juchault, 1999), protandric hermaphroditism is widespread in several decapod crustaceans (Bauer, 2000). The protandric sex change from male to female generally starts with the formation of an "*ovotestis*", proceeding from the posterior end of the testis, which is transformed into an ovary, thereby forming an intermediate organ which contains both testicular and ovarian components (Vogt, 2020; Zupo and Hodgson, 2022). The sex transition continues until the establishment of a female phenotype and the total disappearance of male traits (complete protandry), or the development of a simultaneous hermaphrodite (Chiba, 2007; Abaci et al., 2009).

The determination of sex in decapod crustaceans is regulated by hormones playing the role of a switch for the differentiation of the gonads and the secondary sexual traits. Among these, the Insulin-like Androgenic Gland hormone (IAG), produced by the androgenic gland (AG- a small masculine endocrine organ located near the spermatic ducts and the ejaculatory bulbs), acts within the endocrine axis between eyestalk and testis (Huberman, 2000; Khalaila et al., 2002), regulating the development and the maintenance of primary and secondary male sexual characters (Charniaux-Cotton, 1954; Nagamine and Knight, 1980; Sagi et al., 1997; Manor et al., 2004, 2007). The X-organ-sinus gland complex (an organ analogous to the hypothalamus of vertebrates) releases neuropeptides that regulate the synthesis and secretion of the IAG hormone, which is considered the essential switch (IAG switch) for male differentiation (Khalaila et al., 2002).

The IAG switch has already been studied in 29 decapod species (Mareddy et al., 2011; Ventura et al., 2011, 2015; Chung, 2014; Huang et al., 2014; Levy and Sagi, 2020). Externally, the sexual phenotype of several shrimps is often determined by morphological assessment of the presence (in males) or absence (in females) of the *appendix masculina* in the inner part (endopodite) of the second pleopods. The secondary sexual characters, however, are absent in immature individuals (e.g., penaeids, alpheids, etc.; Banner and Banner, 1979; Felder, 1982; Dardeau, 1984). In these cases, the inspection of gonopores and their shape and location on the pleopods permits an early determination of the sex (Saito, 2002; Tóth and Bauer, 2007; Dardeau, 1984). In protandric species such as *Hippolyte inermis*, the sex is also correlated with size, because individuals with a total length (TL) larger than 10–13 mm are usually sex-reverted females (Zupo et al., 1994). *H. inermis* is a protandric sequential hermaphrodite (Reverberi, 1950; Veillet et al., 1963; Yaldwyn, 1966), whose life cycle is characterized by two yearly reproductive cycles occurring in spring and fall, respectively (Veillet et al., 1963; Zupo, 1994) synchronous with the seasonal cycle of *Posidonia oceanica* L. Delile (Buia et al., 1992) and its epiphytes. After hatching, *H. inermis* developmental cycle begins with a planktonic phase consisting of several (from 9 to 12) zoeal stages. The duration of all larval phases varies, in total, from 25 to 30 days, after which the post-larvae settle on the seagrass canopy (Zupo and Buttino, 2001) reaching sexual maturity 1–2 months later (Zupo et al., 2008). During the fall reproductive burst, individuals grow slowly and when they reach 5–7 mm they differentiate as males, reverting their sex into *alpha* females, about 12 months after hatching (in the spring reproductive cycle), when their size usually ranges between 10 and 13 mm. Conversely, in spring both male and female shrimps are present in the recruits, and they grow faster than shrimps born in fall.

In spring, the presence of early (smaller, also called *beta*) females is due to the ingestion of diatoms of the genus *Cocconeis*, seasonally abundant (Zupo, 2000; Zupo and Messina, 2007; Mazzella, 1984). This diatom contains a metabolite (hypothesized to be a poly-unsaturated fatty acid; PUFA) which, upon ingestion after settlement, induces ferroptotic cell death of the AG germ cells (Zupo et al., 2023). In contrast, the regression of the androgenic gland in the fall generation is simply induced by aging. In *H. inermis*, the disruption of testes consequent to the fast regression of the AG is particularly rapid (Reverberi, 1950) and an ovotestis phase has never been demonstrated (Reverberi, 1950; Cobos et al., 2005). The ingestion of diatoms after the first ten days of post-larval life is ineffective because apparently the bioactive compounds present in diatoms are effective only on undifferentiated AG cells (Zupo and Messina, 2007; Zupo et al., 2023). This peculiar plant-animal interaction has increased the interest of scientists, also due to implications for marine biotechnologies (Sagi et al., 1986; Sagi and Aflalo, 2005). In addition, this distinctive strategy of sex determination may be helpful for the understanding of the mechanisms of sex determination in decapod crustaceans and in other invertebrates.

To shed light on these physiological mechanisms of development, involving high sexual plasticity and a reproductive strategy regulated by both intrinsic and extrinsic factors, we analysed the sex-related genes of *H. inermis* by evaluating their expression patterns in a range of individuals, both cultured and collected in the field at various stages of development.

2. Materials and methods

2.1. Sample collection and experimental plan

Adult shrimps were collected using a 400 μm mesh net (50 cm diameter), hand-towed by scuba divers through a shallow *Posidonia oceanica* meadow located off Calopezzati (Ionian Sea, Southern Italy; 39.56°N, 16.83°E) during its spring reproductive burst, in April 2024. Specimens were sorted from 5 samples and transported to the laboratory in plastic bags containing clean seawater. They were further examined under an MZ16 dissecting microscope (Leica Microsystems, Milan, Italy) for species confirmation, according to Zupo and Messina (2008). An initial group of 10 individuals was fixed in *RNA*later (Qiagen, Hilden, Germany). Their second pleopods were dissected, mounted on slides and observed under the optical microscope for sex determination (Zupo and Messina, 2007; Zupo et al., 2014, 2008), by checking the presence/absence of an appendix masculina. After the identification of the sex, each shrimp was returned to RNAlater and stored at −80°C for subsequent RNA extraction.

2.2. Ethical Statement

All animal procedures were performed following the ARRIVE guidelines and in compliance with the U.K. Animals (Scientific Procedures) Act 1986, EU Directive 2010/63/EU, and the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). Both male and female specimens were used, with sex indicated in the study, and all efforts were made to minimize animal suffering. Ethical permission was not required, according to the European Laws and the rules of Stazione Zoologica, for investigations on decapod crustaceans.

2.3. Post-larval cultures and tests

The remaining ovigerous females were reared in 2 L Erlenmeyer flasks containing 1.5 L of filtered seawater, kept in a thermostatic chamber and provided with continuous aeration. The containers were checked daily for the presence of larvae and water was renewed with the addition of *P. oceanica* leaves, to provide shelter and a source of epiphytic food. Larvae hatched from each ovigerous female were collected and reared (1 ind. 10 mL^{-1}) in 1 L Erlenmeyer flasks. Each flask contained 800 mL of 0.22 μm filtered seawater. The vessels were gently aerated and kept at 18°C in a thermostatic chamber. Larvae were fed just-hatched *Artemia salina* nauplii (SHG group, Ovada, Italy; 4 ind. mL^{-1}) during the first seven days. Subsequently, they were fed *Artemia salina* nauplii enriched with Algamac Biomarine (Hawthorne, CA, USA). Water was renewed daily with filtered seawater at the same temperature. Larval metamorphosis was monitored under a stereomicroscope, as larvae passed through 9–12 typical zoeal stages (Zupo, 1994), until settlement (Zupo and Messina, 2008). Immediately after settlement, post-larvae were transferred to 500 mL crystallizing dishes containing 400 mL of filtered seawater.

2.4. Production of benthic diatoms

A strain of *Cocconeis scutellum* var. *parva* (Grunow) Cleve 1895 was obtained from the culture collection of the Stazione Zoologica Anton Dohrn. Large-scale cultures were established in sterile Petri dishes (140 mm diameter) containing 150 mL of F/2 medium. A verification of the strain purity was performed by examining each Petri dish under an inverted microscope (Leica DMi8, Leica Microsystems, Milan, Italy), to ensure the absence of contaminants (both bacteria and other microorganisms), the correct shape of diatoms and the color and size of the chloroplast, along with the complete confluency of the bottom. After this verification, benthic diatoms were collected with a sterile Pasteur pipette and inoculated into each plate, to be cultured for two weeks at 18°C in a thermostatic chamber. Illumination was provided by Gro-Lux neon lamps (Sylvania Group, Milan, Italy) under a 12/12-hour light/dark cycle, with an irradiance of 150 μE. Culturing continued until the diatoms reached full confluence, completely covering the bottom of the Petri dishes. To obtain a sufficient quantity of benthic diatoms for the feeding treatments (described below), a large-scale production of *C. scutellum* var. parva was obtained in F/2 medium (Merck Life Science S.r.l., Milan, Italy), in 400 Petri Dishes. The diatoms collected from all dishes were subsequently freeze-dried, scraped off and pooled, for further incorporation into the experimental feeds. In fact, previous research (Zupo and Messina, 2007) demonstrated that the ingestion of this diatom induces feminization in shrimp by triggering early sex reversal in post-larvae.

2.5. Feeding treatments

Feeding treatments were performed on post-larvae after their settlement. Immediately after settlement, post-larvae (PL) derived from different females were pooled to randomize any variation due to maternal influences. Batches of 25 PL per replicate were transferred to 500 mL crystallizing dishes containing 400 mL of filtered seawater. Each of three experimental groups was fed 5 mg day^{-1} of basic feed (containing equal proportions of dried spirulina, dried *A. salina*, and SHG micro-pearls with high-protein content (Microperle, Super High Group, Ovada, Italy) added with 25% (in weight) of freeze-dried *C. scutellum parva,* starting from the first day of the post-larval stage. These experimental replicates were defined "FD" (Feeding on Diatoms). Three additional batches of post-larvae were cultured in crystallizing dishes to serve as the negative controls. In particular, each of three control replicates consisted of 25 PL (cultured in identical conditions in 400 mL crystallizing dishes), but these control groups were fed on a basic feed (containing equal proportions of dried spirulina, dried *A. salina*, and SHG micro-pearls with high-protein content) without diatom additions. These control replicates were defined "NFD" (Not Feeding on Diatoms) and received daily doses of 5 mg of control feed.

Treatments and controls were both maintained in thermostatic chambers at 18°C. Seawater was daily replaced with all replicates. Size and health status of the post-larvae were periodically monitored under a light microscope (Leica MZ16) according to the technique described by Zupo and Messina, (2008) and Zupo et al. (2014); (2023). Two post-larvae per replicate were collected on the 15th day (PL_{15}) from each control dish and each treatment dish and fixed in *RNA*later. In total, 6 PL_{15} were obtained to be used for molecular analyses. The remaining shrimp were reared for 45–60 days, until they reached a mature stage (a minimum size of 7.5 mm was considered sufficient to reach sexual maturity). Collected shrimp were moved on a cold box (to reduce stress) and immediately fixed in *RNA*later. Further, each fixed specimen was measured using millimetric paper under a dissecting macroscope (Leica Z16 APO), and its sex was assessed based on the presence/absence of a masculine appendix on the second pleopod.

2.6. Gene isolation and identification

Hi-IAG, *Hi-Vitellogenin-1*, *Hi-UCM* (*Uncharacterized Male Gene*), *Hi-UCF* (*Uncharacterized Female Gene*), *Hi-UCMe* (*Uncharacterized*

Male Gene – eye) and *Hi-UCFe* (*Uncharacterized Female Gene – eye*) were previously isolated by Levy et al. (2021) through analysis of the *H. inermis* transcriptome, starting from the ortholog genes of *Pandalus platyceros*. In addition, three *Vitellogenin* variants (*Hi-VG2, Hi-VG3, Hi-VG4*) were isolated in the *H. inermis* transcriptome from the ortholog genes of origin from *Macrobrachium rosenbergii*. The four *Hi-VG* amino acidic sequences were aligned with Clustal Omega online software (available at: https://www.ebi.ac.uk/jdispatcher/msa/clustalo?stype=protein, accession date: 15/09/2025) to assess their degree of sequence identity and evaluate the extent of their divergence. Specific primers for each gene were designed based on the nucleotide sequence by using Primer3 input (available at https://primer3.ut.ee/) (Table 1).

2.7. RNA extraction and cDNA synthesis

Adult shrimp previously fixed were cut into two or three segments (max 30 mg per sample) and each part was lysed in 350 μL of RLT/2-ME buffer (10 μL β-mercaptoethanol for each mL of RLT buffer) and homogenized with TissueLyser (Qiagen, Austin, TX, USA) at 20.0 Hz for 3 min using 3 mm sterile aluminium beads (Glaviano et al., 2024). High quality RNA was extracted through *RNeasy Mini Kit* (Qiagen, Austin, TX, USA), following the protocol supplied. RNA was eluted with 30 μL RNase-free water, and then stored at −80 ∘C. The quantity of the extracted RNA was assessed measuring the absorbance at 260 nm. Additionally, the quality was assessed by measuring the ratio of absorbance 260/230 nm and 260/280 nm using a Nanodrop (ND-1000 UV Vis, NanoDrop Technologies, Wilmington, DE, USA). The A260/A280 ratios were between 1.8 and 2.0, indicating no protein contamination, while A260/A230 ratios were between 2.0 and 2.2, indicating no contamination by organic compounds, salts, or reagents.

About 100–200 ng of RNA was loaded on 0.8% agarose gel to check its integrity. About 600 ng of RNA for each sample was retrotranscribed through *iScript cDNA synthesis kit* (Bio-Rad, Milan, Italy), following the manufacturer's instructions. cDNA was then amplified by *Polymerase Chain Reaction* (PCR) with a Xtra Taq Pol (GeneSpin) in 30 μL final volume containing 3 μL of 10 × PCR reaction buffer, 0.5 μL of 5 U/μL Taq, 3 μL of 10 × 2 mM dNTP, 25 pmol/μL of each primer specific for *Hi-UCMe*, *Hi-UCFe*, *Hi-VG2, Hi-VG3,* and *Hi-VG4* and nuclease-free water, according to the protocol as follows: 95°C for 5 min, then 35 cycles at 95 °C for 45 s, 55°C for 1 min, and 72°C for 30 s, and then a final extension step at 72 °C for 10 min. Control reactions were performed without cDNA template. The amplified fragments were purified in 1.5% agarose gel using *QIAquick Gel Extraction kit* (Qiagen, Milan, Italy) and sequenced to assess their specificity. The obtained sequences were aligned with complete coding sequences of the gene by means of MultAlin (available at http://multalin.toulouse.inra.fr/multalin/).

2.8. Gene expression by Real-Time qPCR

For each pair of primers, the specificity of the amplification for the genes *Hi-UCMe*, *Hi-UCFe*, *Hi-VG2, Hi-VG3,* and *Hi-VG4* was assessed by melting curve analysis by *Real Time qPCR*. The theoretical efficiency (E) of each primer pair was calculated according to a standard function, as follows:

$$E = 10^{-1/\text{slope}}$$

Where "slope" is the slope of the standard curve, plotted with the y axis as Ct (Cycle Threshold, number of PCR cycles it takes for the fluorescence signal to cross a set threshold, which reflects the point at which detectable amplification begins) and the x axis as log (quantity).

Five serial dilutions (1:1, 1:5, 1:10, 1:50, 1:100) were prepared to generate standard curves, and Ct values were determined for each dilution by plotting Ct values against the logarithm of the corresponding dilution factor. cDNA (1 μL) was used as a template in a reaction containing a final concentration of 0.7 μM for each primer and 2 × Quantitative Master Mix with SYBR Green low ROX (total volume of 10 μL). PCR amplifications were performed in a thermal cycler CFX384 Real Time System using the following thermal profile: 95°C for 20 s, one cycle for cDNA denaturation; 95°C for 5 s and 60°C for 20 s, 40 cycles for amplification; 95°C for 15 s and 60°C for 15 s for final elongation. Control reactions were performed without cDNA template. The fluorescence was measured using Bio-Rad CFX Maestro software (Bio-Rad Laboratories, Inc., Milano, Italy).

The relative expression ratios were calculated from quantification cycles and undiluted cDNA was then chosen as the template to compare the expression of the newly isolated genes together with all the previously isolated sex-specific genes (*Hi-VG-1*, *Hi-IAG*, *Hi-*

Table 1
Sex-related genes ID, accession number (GenBank NCBI) and specific primers (forward and reverse) sequences (in 5' – 3'), and amplicon length in base pairs (bp).

Gene ID	Accession number	Forward primer	Reverse primer	Amplicon length (bp)
Hi-IAG	MZ222391	5'-AGATGAGAGCATCACCCAAGA – 3'	5'-ATCGAATCGACACAGAAGAAGT- 3'	97
Hi-UCM	MZ222393	5'- ACATCTTGGCCATGCTTACC-3'	5'-GAAAACTTTATCAGCAACACATG-3'	67
Hi-UCF	MZ222394	5'-TCTGGTACTGAGATTGTTGATGG-3'	5'-TCAGATCCAGACCCACTTCC-3'	82
Hi-UCMe	MZ995266	5'-GATGTCATATTAGTACTCTTGC-3'	5'- GGTGATGACTCAACTGGAAC −3'	155
Hi-UCFe	MZ995265	5'-GATGATGGTCGATTGACTGC-3'	5'- GCATCATCAGATCTACTGCATG −3'	188
Hi-VG1	MZ222391	5'-GAAGAGAAAAGAGGAGTATCA-3'	5'GGATGGTACTGGCTTCTAGTACTTTT-3'	76
Hi-VG2		5'-CAGTGTTGAGACTGCTATCCAG-3'	5'-GCTGGAGTGCTAAGCTCAATC-3'	171
Hi-VG3		5'-CAATGGTGTCTACGCGTCTTA-3'	5'-CACTGCCACGACTGCGATC-3'	204
Hi-VG4		5'-CGTTATGCTCGACGGAGTGTC-3'	5'-CACATCACAAGGACTCAAC-3'	182

UCM, *Hi-UCF*) which were already tested by Levy et al. (2021) – considering *Hi-Actin* as reference gene - in 9 wild adult males (WMs) and 9 wild adult *alpha* females (WFs). The expression of genes considered as sex markers was then tested in the following groups:

1. Wild younger males (WSMs) and *beta* females (WSFs) (8 mm of TL)
2. Cultured young males (AMs) and *beta* females (AFs) (8 mm TL)
3. Post larvae fed on *C. scutellum parva* fixed 15 days after settlement (PL_{15+}) corresponding to the end of the cell death process triggered by the benthic diatom
4. Post larvae fed on control food (NFD) fixed 15 days after settlement (PL_{15-})

The expression was checked in three replicates per sample. Relative gene expression analysis was performed using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). For comparisons between males and females (as well as between young and adult individuals) no one-to-one pairing between individual samples was applied. Instead, the mean Ct value of the entire control group was calculated and used as a single calibrator reference for all individual samples. This approach was adopted to minimize potential bias arising from inter-individual variability in gene expression among control samples and to ensure that relative expression levels were standardized against a representative baseline. Consequently, all ΔΔCt values were computed using the group-average calibrator rather than individual samples, in accordance with the assumptions of the method.

The expression level of each gene was normalized by REST software (Relative Expression Software Tool, Weihenstephan, Germany), using the housekeeping gene and reported the values of the expression levels of the genes under analysis as compared to the control (Pfaffl, 2001; Pfaffl et al., 2002). Differences in gene relative expression higher than 2 were considered significant (Glaviano et al., 2024; Levy et al., 2021). All Real time qPCR experiments were conducted in accordance with the MIQE guidelines (Bustin et al., 2009).

2.9. Phylogenetic analyses

The NCBI protein database was searched for *VG* sequences within the Crustacean infraorder of Caridea, the clade comprising *M. rosenbergii* and *Exopalaemon carinicauda*, two species with the highest number of *Vitellogenin* genes described so far (Jiang et al., 2023; Wang et al., 2024b), to compare the *VG* genes found in *H. inermis* with other related *VG*s. A Maximum Likelihood tree was calculated through IQ-Tree 1.6.12 (Nguyen et al., 2015) using the web server (http://iqtree.cibiv.univie.ac.at; Trifinopoulos et al., 2016) with ultra-fast bootstrapping (Hoang et al., 2018) and an integrated Model finder (Kalyaanamoorthy et al., 2017), incorporating 44 Caridean shrimp sequences, including the four *Hi*-VGs identified in *H. inermis*. The final tree was visualized using the iToL platform (https://itol.embl.de). The alignment was built with MUSCLE (Edgar, 2004).

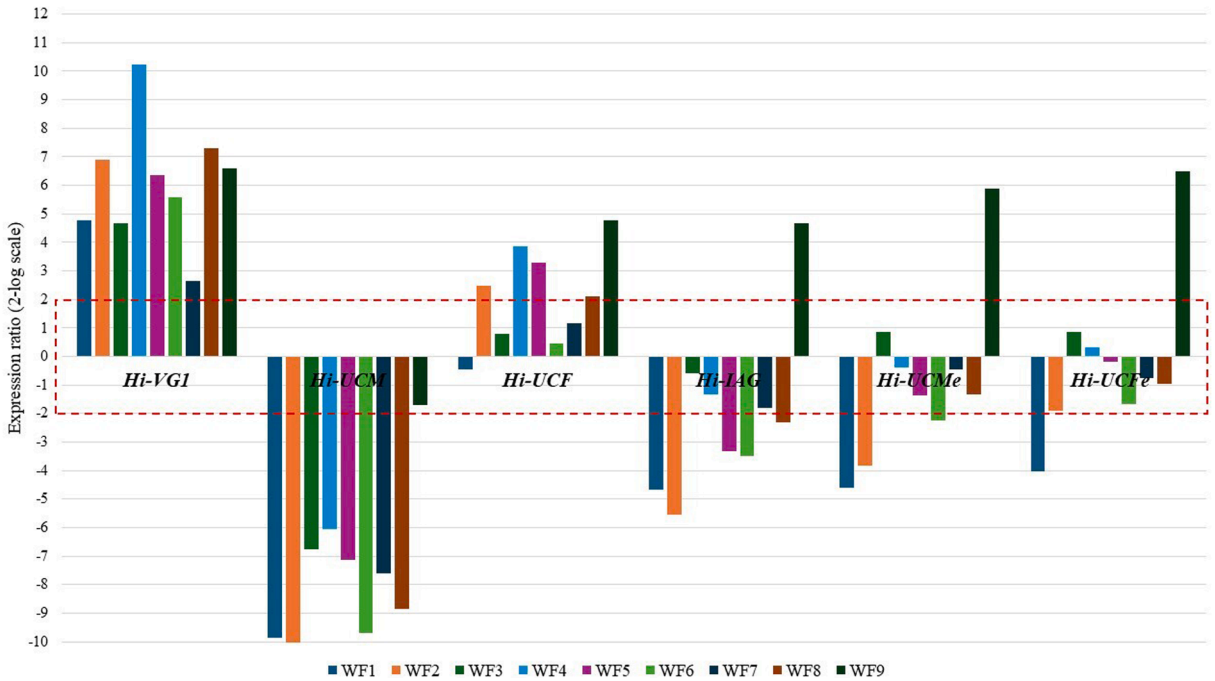


Fig. 1. Relative expression of sex-related genes in wild adult *alpha* females (WFs; 17–21 mm TL) using wild adult males (WMs; 11–13 mm TL) as the negative control. Reference gene: *Hi-Actin*. Expression difference values ±2 compared to the control gene are considered significant. The bars represent results from individual animals.

2.10. Statistical analyses

The average total length (TL) of males and females was calculated while determining their sex in adult shrimp. The relative expression of sex-related genes was computed in wild *alpha* females by considering wild adult males as the negative controls. The expression levels of different genes were compared adopting *Hi-Actin* as a reference gene. Expression difference values higher than ±2 (compared to the reference gene) were considered significant. The sequences of 44 Caridean shrimp including four Hi-VGs of *H. inermis* were used to build a phylogenetic tree using Maximum Likelihood analysis and adopting IQ-tree V 1.6. with 1000 bootstrap steps (Clustal Omega Software, access 15/09/2025).Expression level data were analyzed using a non-parametric Kruskal-Wallis test, followed by Dunn's post-hoc test. Graphical representations were obtained using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

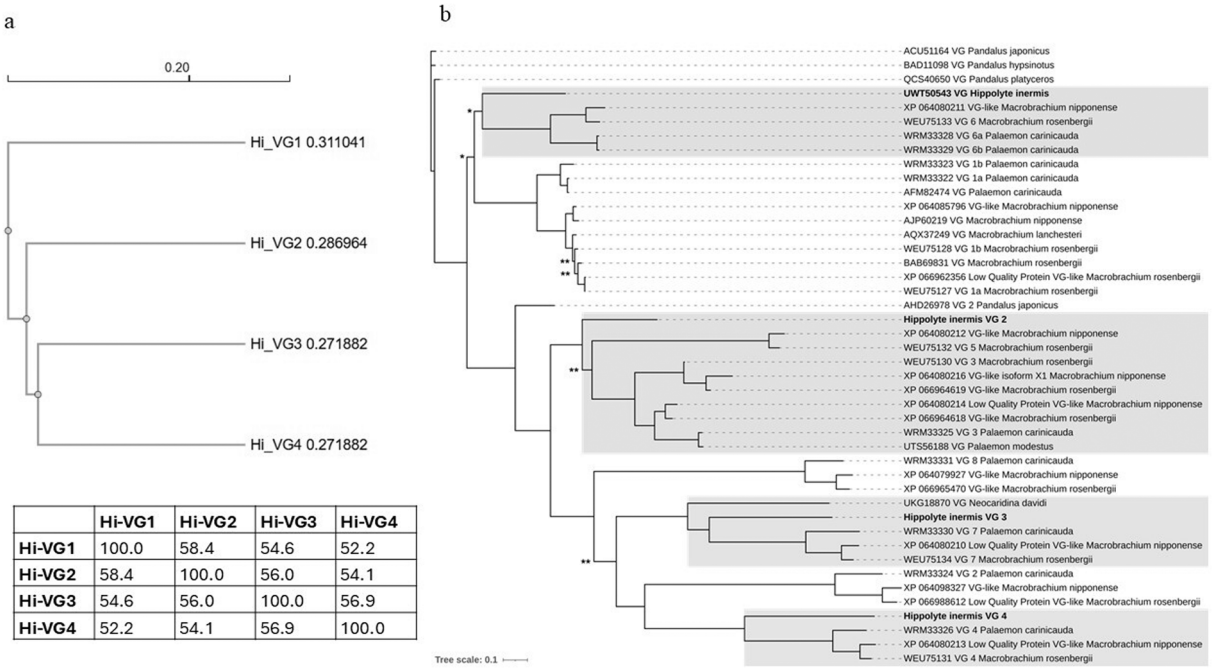
3. Results

3.1. Sex-related genes in wild adult shrimp

The genes *Hi-VG1* and *Hi-UCM* were validated for a molecular determination of sex in adult *H. inermis* specimens. *Hi-VG1* was upregulated in all wild adult *alpha* females (WFs) which had a total length (TL) between 17 and 21 mm and downregulated in all wild adult males (WMs; 11–13 mm TL) while *Hi-UCM* was upregulated in eight out of nine wild adult males. *Hi-IAG* was upregulated in only five WMs, while *Hi-UCF, Hi-UCMe* and *Hi-UCFe* were generally not differentially expressed between males and *alpha* females (Fig. 1; Table S1 for numerical values).

3.2. Sex-related genes in younger individuals

The expression of *Hi-IAG* and *Hi-UCM* was not significantly different when PL_{15+} and PL_{15-} were compared (Figure S1). Similarly, the expression of *Hi-IAG* did not differ significantly between WSMs and WSFs (8 mm TL) (Figure S2). A similar result was observed for



	Hi-VG1	Hi-VG2	Hi-VG3	Hi-VG4
Hi-VG1	100.0	58.4	54.6	52.2
Hi-VG2	58.4	100.0	56.0	54.1
Hi-VG3	54.6	56.0	100.0	56.9
Hi-VG4	52.2	54.1	56.9	100.0

Fig. 2. a) Phylogenetic Tree and identity (reported as percentage in the table below) matrix of Hi-VG1, Hi-VG2, Hi-VG3, Hi-VG4 amino acid sequences, made with Clustal Omega Software (access 24/02/2024). The scale bar at the top (with the value 0.20) indicates the scale for evolutionary distances along the branches. For example, a branch length of 0.20 corresponds to about a 20% difference in the sequences analyzed (such as nucleotide or amino acid differences, depending on the data). The numbers next to each label represent the evolutionary distance of each sequence from their nearest common node, which is the length of the terminal branch, showing how much that sequence has evolved from its closest divergence point. Hi-VG3 and Hi-VG4 are more closely related to each other than to Hi-VG1 or Hi-VG2, since their terminal branches are shorter. b) Phylogenetic relationships of the four different *H. inermis* VGs within different VGs of other Crustaceans of the order Caridea. The phylogenetic tree was constructed by Maximum likelihood analysis using IQ-tree V 1.6. with 1000 bootstrap steps. The bootstrap values percentages of support) of individual branches are > 97% except when indicated: * 40–60%; ** 60–90%. lusters containing *Hippolyte* VGs are shaded grey. The tree scale represents 0.1 substitutions per site.

Hi-UCM, which was downregulated only in WSF2. In comparing AFs and AMs (both 8 mm TL), *Hi-IAG* expression was not significantly different, with the only exception of AF6, which showed downregulation. For *Hi-UCM*, significant differences in this group were only observed in AF3 and AF6 (Figure S3). The expression of both genes was downregulated in PL_{15} relative to WMs (Figure S4). *Hi-IAG* was not differentially expressed in WMs (negative control) and AFs, except for AF4 and AF6, where downregulation was observed. A similar pattern was exhibited by *Hi-UCM*, with the only exception of AF1 and AF6 (Figure S5). The expression of *Hi-IAG* in WMs (as the negative control) and AMs was upregulated in AM3, downregulated in AM4, and not differentially expressed in the other four AMs. In the same samples, *Hi-UCM* was upregulated in AM1, downregulated in AM4, AM5 and AM6, and not differentially expressed in AM2 and AM3 (Figure S6).

3.3. Phylogenetic analysis and expression of Vitellogenin genes

Percent identity matrix between the four Hi-VG amino acid sequences, along with the phylogenetic tree showed higher identity between Hi-VG1 and Hi-VG2 compared to Hi-VG3 and Hi-VG4 (Fig. 2a). The different Hi-VG genes appeared in clearly separated clusters with the VG genes of other species on the phylogram suggesting an early origin of VG diversity within the Caridea (Fig. 2b).

The relative expression of *Hi-VG2, Hi-VG3* and *Hi-VG4* was initially evaluated in WFs, considering WMs as the negative controls and *Hi-Actin* as a reference gene. *Hi-VG3* was upregulated in most WFs, while *Hi-VG2* and *Hi-VG4* were overall had not significant differential expression (Fig. 3, Table S2 for numerical values).

The relative expression of the four *Hi-VGs* in cultured small (younger) adults (8 mm TL) showed no significant differences between males (negative control) and *beta* females, as shown in Fig. 4a (see also Table S3 for numerical values). The relative expression of the four *Hi-VGs* in most WSFs did not differ significantly from WSMs (negative control), with the exception of *Hi-VG3* downregulated in WSF2 and WSF3, and *Hi-VG4* downregulated in WSF2, as shown in Fig. 4b (see also Table S4 for numerical values).

When *Hi-VG2* was used as the reference gene, the relative expression of *Hi-VG1* and *Hi-VG3* was upregulated in most WFs and, conversely, downregulated in most WMs. In contrast, *Hi-VG4* did

not show significant differences in relative expression between WMs and WFs in most of samples (Fig. 5, Table S5 for numerical values).

The relative expression of *Hi-VGs* between younger adult *beta* females (both cultured and wild ones; 8 mm TL) and WFs (negative control, 17–21 mm) showed a downregulation of *Hi-VG1, −2* and *-3* only in WSFs while AFs had no significant differential expression (with the only exception of AF6). Conversely, *Hi-VG4* was upregulated in five out of six AFs when using WFs as the negative control, whereas it was not differentially expressed in any of the WSFs (Fig. 6, Table S6 for numerical values).

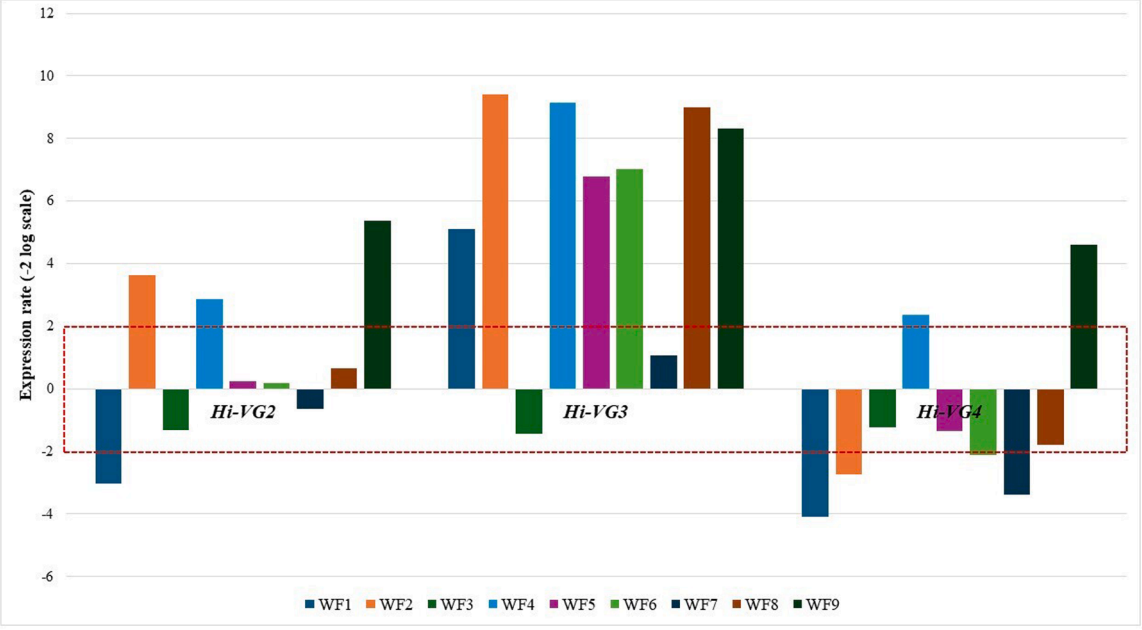


Fig. 3. Relative expression of *Hi-VG2-4* variants in wild adult *alpha* females (17–21 mm TL) compared to wild adult males (11–13 mm TL), used as the negative control. Data were calculated using the Relative Expression Software Tool (REST). Reference gene: *Hi-Actin*. The threshold value of ±2 was considered as a significant expression difference compared to the control gene. The bars represent results from individual animals.

Fig. 4. a) Relative expression of *Hi-VG* variants in cultured young *beta* females (AFs; 8 mm TL) compared to cultured young males (AMs; 8 mm TL) considered as the negative control. Data were calculated using the Relative Expression Software Tool (REST). Reference gene: *Hi-Actin.* The threshold value of ±2 was considered as a significant expression difference compared to the control gene. The bars represent results from individual animals. b) Relative expression of *Hi-VG* variants in wild young *beta* females (WSFs; 8 mm TL) compared to wild young males (WSMs; 8 mm TL), used as the negative control. Data were calculated using the Relative Expression Software Tool (REST). Reference gene: *Hi-Actin.* The threshold value of ±2 was considered as a significant expression difference compared to the control gene. The bars represent results from individual animals.

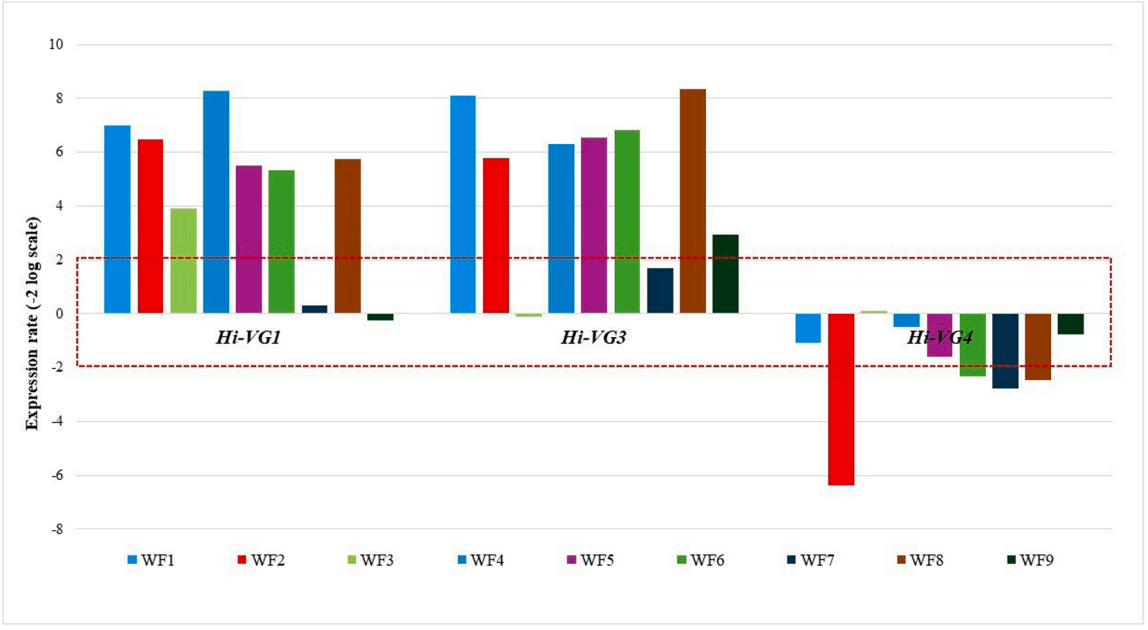


Fig. 5. Relative expression of *Hi-VG1*, *Hi-VG3*, and *Hi-VG4* in wild adult *alpha* females (WFs; 17–21 mm) compared to wild adult males (WMs; 11–13 mm), used as the negative control. Data were calculated using the Relative Expression Software Tool (REST). Reference gene *Hi-VG2.* The threshold value of ±2 was considered as a significant expression difference compared to the control gene. The bars represent results from individual animals.

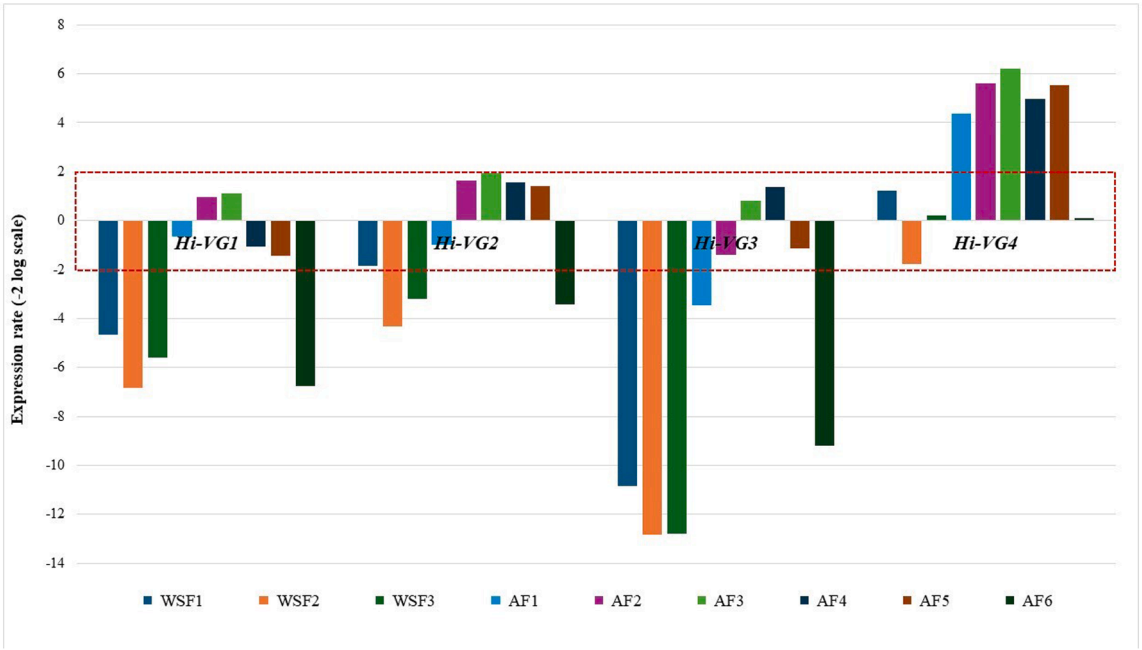


Fig. 6. Relative expression of *Hi-VG* variants in *beta* females (WSFs, and AFs; both 8 mm TL) compared to wild adult *alpha* females (WFs; 17–21 mm TL), used as the negative control. Data were calculated using the Relative Expression Software Tool (REST). Reference gene: *Hi-Actin*. The threshold value of ±2 was considered as a significant expression difference compared to the control gene. The bars represent results from individual animals.

4. Discussion

4.1. Molecular mechanisms of sex regulation

Gonochorism is the predominant reproductive strategy in decapod crustaceans. Several lineages have evolved alternative sexual systems, exhibiting remarkable plasticity in their reproductive function (Charniaux-Cotton and Payen, 1985; Bauer and Newman, 2004). Protandric hermaphroditism is among the most widespread and ecologically significant forms of sequential hermaphroditism in decapods (Bauer, 1986; 2006). While the primary genetic determinant of sex in decapod crustaceans remains unidentified, recent studies highlighted the involvement of multiple regulatory elements, including sex-related genes, endocrine signals, neurochemical factors and hormones, in orchestrating sexual differentiation (Wang et al., 2024b; Zupo and Hopkins, 2022).

Among these, the insulin-like androgenic gland hormone (IAG) functions as a pivotal endocrine regulator of male sexual differentiation, orchestrating the development of sex-specific traits (Ventura and Sagi, 2012). Its role was thoroughly investigated in several decapod species, including shrimp, prawns, crayfish and crabs, where targeted manipulation of IAG expression has proven effective in controlling phenotypic sex (Ford, 2008). Such techniques as androgenic gland (AG) ablation and *IAG* gene knock-down in males have been employed to induce the formation of functional neo-females, while the implantation of AG tissue or injection of AG-derived cells into females successfully produced neo-males. These approaches enabled the production of mono-sex populations, primarily for aquaculture applications, but also highlighted the exceptional role of IAG in decapod crustaceans (Aflalo et al., 2006; Levy et al., 2020, Levy and Sagi, 2020).

In *M. rosenbergii*, *IAG* gene expression has been detected at multiple stages of development, initiating the male differentiation early in the developmental timeline, well before external sexual traits become visible (Ventura and Sagi, 2012). On the other hand, in the protandric hermaphrodite *P. platyceros*, *IAG* transcript levels are highest during early juvenile stages and subsequently decline as individuals approach sexual maturity.

During the protandric sex transition, *IAG* expression becomes negligible, while *Vitellogenin* (*VG*) expression in the hepatopancreas increases remarkably, continuing until the complete transformation of the ovotestis into a functional ovary (Levy and Sagi, 2020). This temporal regulation highlights the critical role of this hormone throughout ontogeny, not only in the initial sexual differentiation but also in maintaining male phenotypes in the adult stages (Sagi et al., 1990), acting as a repressor for vitellogenesis and *VG* gene transcription (Sagi et al., 2002).

4.2. Influence of developmental stage and physiological conditions

In this study, the expression of the *Insulin-like Androgenic Gland hormone* gene (*Hi-IAG*) in the protandric hermaphrodite *H. inermis* was found to be increased in adult males and significantly reduced in adult *alpha* females, consistent with previous observations by

Levy et al. (2021). Interestingly, *Hi-IAG* levels remained relatively constant in adult males as compared to earlier developmental stages, suggesting sustained IAG production by the androgenic gland throughout ontogeny. The expression of *Hi-IAG* did not differ significantly between juvenile males and *beta* females showing completely formed external sexual characters, nor between post-larvae fed on benthic diatoms (known to induce direct feminization of young individuals) and those fed on a diet without diatoms (Zupo, 2000; Zupo et al., 2023). These results indicate that the expression of *Hi-IAG* is not a reliable marker of early-stage sex differentiation. The absence of significant differences in *Hi-IAG* transcript levels between PL_{15+} (diatom-fed) and PL_{15-} post-larvae does not necessarily contradict the previously demonstrated AG germ cell depletion induced by *C. scutellum parva*. The PUFA-like compound produced by the benthic diatom is known to trigger ferroptotic degeneration of undifferentiated AG germ cells during the early post-settlement window. This mechanism, however, may primarily affect cellular integrity rather than directly suppressing *IAG* transcription. Moreover, since gene expression was measured at 15 days post-settlement and on whole-body RNA extracts, transient early transcriptional changes or localized alterations within the small AG tissue may not have been detectable. Diatom-induced feminization in *H. inermis*, consequently, involves structural and cellular disruption of the AG preceding or occurring independently from measurable changes in *Hi-IAG* mRNA levels. The low levels of *Hi-IAG* expression observed both in young PL_{15+} (diatom-fed) and PL_{15-} (NFD) indicate that *Hi-IAG* transcription is not yet activated at an early developmental stage. This evidence might support the hypothesis that Hi-IAG does not act as the only primary trigger of sex differentiation in *H. inermis* but rather contributes to the maintenance of the male phenotype in mature specimens. Accordingly, PL_{15+} individuals must be interpreted as a treatment group undergoing early AG disruption rather than as a negative control for *Hi-IAG* expression. Consequently, IAG is not a reliable marker of early sex differentiation in juveniles. Instead, our results demonstrate that the diatom-induced feminization proceeds through AG tissue destruction, before detectable changes in IAG mRNA levels occur. This indicates that structural and cellular mechanisms precede and may be partially uncoupled from transcriptional regulation of IAG.

A comparable pattern was observed for an *Uncharacterized Male Gene* (*Hi-UCM*), identified from the male body transcriptome of *H. inermis* (Levy et al., 2021), which was upregulated in adult males when compared with adult *alpha* females, but it showed no differential expression between male and (*beta*) female younger individuals. These two male-specific genes are likely to have a more complex expression pattern in *H. inermis* with respect to other species, suggesting that their regulation may depend not only on gender and developmental stage, but also on specific physiological factors.

Recent studies indicate that the insulin-like androgenic gland hormone may regulate not only the sex differentiation, but also the molting cycle, the cuticle formation and the post-ecdysal recovery of growth, possibly modulating energy allocation or tissue proliferation during ecdyses (Huang et al., 2014, Farhadi et al., 2021).

On the other hand, ovarian maturation is a critical process for female differentiation, ruled by vitellogenesis, which is the biosynthesis and subsequent accumulation of *VG* in the ovary (Thongda et al., 2015). In many decapod species, the hepatopancreas, however, was revealed to be the site of *VG* synthesis, with tissue-specific expression patterns varying according to species and potentially reflecting a range of functional roles, like oocyte maturation, antioxidant defence, antibacterial activity, and stress response (Dietrich et al., 2022; Li et al., 2017; Shen et al., 2014; Sun and Zhang, 2015; Wang et al., 2024b).

Recent studies identified the presence of multiple *Vitellogenin* genes within single species of decapod crustaceans characterized by gonochoristic reproductive strategies. For instance, two *VG* genes have been reported in *Metapenaeus ensis* and *Penaeus japonicus*; three were reported for *Fenneropenaeus merguiensis* and *Litopenaeus vannamei*; four were reported for *Procambarus clarkii*; seven were reported for *M. rosenbergii* and *Macrobrachium nipponense*; remarkably, as many as ten were reported for *E. carinicauda* (Kung et al., 2004; El-Desoky, 2023; Zhao et al., 2021; Wang et al., 2020; Manfrin et al., 2021; Jiang et al., 2022; Jiang et al., 2023; Wang et al., 2024b). These studies reported that the expression site of *VG* isoforms may vary according to presumed functional roles, species-specific physiology, conserved gene regions and the stage of maturation of analysed individuals. The expression patterns of *VGs* isoforms were detected predominantly in the ovary or the hepatopancreas, but also in other tissues such as gill, muscle eyestalk, intestine,

Table 2
Vitellogenin genes and expression sites identified in different decapod species, as reported in literature until now.

Sub-order	Species	Reproductive strategy	*VGs*	Expression site	Reference
Dendrobranchiata	*Metapenaeus ensis*	Gonochoristic	*Me-Vg1, MeVg2*	Hepatopancreas	Kung et al., (2004)
Dendrobranchiata	*Fenneropenaeus merguiensis*	Gonochoristic	*FmVg1,* *FmVg2* *FmVg3*	Ovary Hepatopancreas Ovary	Zhao et al., (2021)
Dendrobranchiata	*Litopenaeus vannamei*	Gonochoristic	*LvVg1, LvVg2, and LvVg3*	Hepatopancreas, ovary	Wang et al., (2020)
Dendrobranchiata	*Penaeus japonicus*	Gonochoristic	*Maj-Vg1* *Maj-Vg2*	Hepatopancreas Ovary	El-Desoky et al., (2023)
Pleocyemata	*Procambarus clarkii*	Gonochoristic	*VTG1_Pcl* *VTG2_Pcl, VTG3_Pcl, VTG4_Pcl*	Ovary Hepatopancreas	Manfrin et al., (2021)
Pleocyemata	*Macrobrachium rosenbergii*	Gonochoristic	*MrVg1a, MrVg1b and MrVg2–7*	Hepatopancreas	Jiang et al., (2023)
Pleocyemata	*Macrobrachium nipponensis*	Gonochoristic	*Vtg1a, Vtg1b* *Vtg2–7*	Ovary Hepatopancreas	Jiang et al., (2022)
Pleocyemata	*Exopalaemon carinicauda*	Gonochoristic	*EcVtg1a, EcVtg1b, EcVtg2–5, EcVtg6a, EcVtg6b, EcVtg7*	Hepatopancreas	Wang et al., 2024b

stomach and heart in *E. carinicauda* although in much lower expression rates (Wang et al., 2024a). In 2021, the first *VG* gene (*Hi-VG1*) was isolated from the transcriptome of the protandric hermaphrodite shrimp *H. inermis* by Levy et al. (2021). This gene was identified as an ortholog of a *VG* from the protogynous hermaphrodite *P. platyceros* and was found to be expressed in the ovary in both species.

Here, we isolated three additional *Vitellogenin* variants (*Hi-VG2*, *Hi-VG3*, and *Hi-VG4*) from the *H. inermis* transcriptome, all sharing orthology with *VG* genes previously characterized in *M. rosenbergii*. Even though all *VG* in *M. rosenbergii* were expressed in the hepatopancreas (Jiang et al., 2023), although with very different expression rates, this was not a universal trait across crustaceans (Hoeger and Schenk, 2022). Indeed, *Vitellogenin* variants may differ in both site of expression and functional role across families (Table 2). For example, a *VG* designated *VG3* is predominantly expressed in the ovary in *L. vannamei* and *F. merguiensis*, whereas *VG2* is typically hepatopancreatic in origin, with the notable exception of *P. japonicus*. Similarly, *VG4*, where present, shows species-specific expression profiles (El-Desoky et al., 2023; Jiang et al., 2022; Kung et al., 2004; Manfrin et al., 2021; Wang et al., 2020, 2024b; Zhao et al., 2021). It should be noted that the designations *Hi-VG2*, *Hi-VG3*, *Hi-VG4* given by the respective authors only denote different *VG* genes within a given species without necessarily implying a relationship with the same names assigned to the *VG* in other species. In the phylogenetic analysis (Fig. 2b), the different *Hi-VGs* clustered in distinct clades together with other caridean shrimps indicating an early event of gene duplication during evolution as suggested for decapod crustaceans (Jeon et al., 2010).

Since our data are based on transcriptomic sequences and not on a fully assembled genome, we cannot completely exclude the contribution of alternative splicing without genomic structure analysis. In fact, current phylogenetic and sequence evidence supports gene duplication as the most parsimonious explanation. The possibility that some of the *Hi-VG* isoforms could arise from alternative splicing rather than from independent gene duplication events is indeed a relevant consideration. Several lines of evidence suggest, however, that four *VGs* most likely represent distinct paralogous genes rather than splice variants. The four *Hi-VG* sequences were assembled as independent full-length transcripts, each showing distinct coding regions rather than sharing common exon structures with variable internal segments, as typically observed in alternative splicing. The percent identity matrix shows moderate divergence among the four Hi-VGs (approximately 52–58% identity). This level of divergence is higher than generally expected for splice variants of the same gene, which typically retain very high sequence similarity outside alternatively spliced regions. In the Maximum Likelihood analysis, the four Hi-VGs clustered into distinct, well-supported clades together with orthologous VGs from other caridean species. If these isoforms were generated by alternative splicing of a single ancestral gene, we would expect them to cluster together as species-specific variants rather than grouping with different orthologous VG clades. Finally, multiple independent *VG* genes have been described in several decapod species (e.g., *M. rosenbergii*, *E. carinicauda*), where gene duplication rather than alternative splicing accounts for *VG* diversity. The moderate sequence divergence observed among *Hi-VG1*–*Hi-VG4*, together with their phylogenetic placement, is consistent with functional divergence following duplication, a process widely recognized as a driver of molecular innovation in multigene families. Such early duplication events may have enabled subfunctionalization or neofunctionalization of *VG* paralogs, allowing partitioning of roles in ovarian maturation, lipid transport, immune defence, or stress response, as reported in other decapods. In this framework, the female-biased expression of *Hi-VG1* and *Hi-VG3* and the broader expression of *Hi-VG2* and *Hi-VG4* may reflect differential evolutionary constraints acting on paralogs after duplication. The phylogenetic structure of the VG family in *H. inermis* therefore not only supports an ancient origin of VG multiplicity within Caridea but also provides a mechanistic basis for the functional complexity observed in this species' reproductive and physiological regulation.

Our findings revealed the specific expression patterns of *Hi-VG1* and *Hi-VG3* restricted to adult *alpha* females, suggesting an ovary-specific role for these *Vitellogenin* genes. Importantly, the present data indicate that *Hi-VG1* and *Hi-VG3* function more reliably as markers of ovarian maturation in fully developed adult females than as early indicators of sex differentiation. In both wild and cultured small individuals, including *beta* females and juveniles, *VG* expression patterns were more variable and not consistently sex specific. This suggests that these genes reflect active vitellogenesis and reproductive physiological status rather than primary sex determination. The applicability of *Hi-VG1* and *Hi-VG3* as molecular markers therefore is strongest in mature individuals undergoing ovarian development, particularly in natural populations where seasonal reproductive dynamics are well defined. In contrast, *Hi-VG2* and *Hi-VG4* were expressed by both sexes and across multiple developmental stages. Notably, the consistently stable expression levels of *Hi-VG2* in most analysed individuals, along with the unchanged relative expression of *Hi-VG1*, *Hi-VG3*, and *Hi-VG4* when *Hi-VG2* was used as a reference gene, supports the hypothesis that *Hi-VG2* may be useful to explore intra-family normalization within the *VG* gene set to better highlight potential relative shifts among *VG* paralogs. Given that *Hi-VG2* and *Hi-VG4* were expressed at similar levels, both in males and females, they cannot be considered reliable sex-specific markers. This pattern of expression indicated that their site of synthesis is likely the hepatopancreas, rather than the ovary but perhaps even other tissues as found for *E. carini*cauda (Wang et al., 2024b). The expression of *Hi-VG2* in both sexes and independent of the developmental stage is unusual and has not been reported before. The presence of *VG* genes in both sexes has been reported only in a few other crustaceans so far. Jeon et al. (2010) found two *VGs* (termed *Pj-VG1* and *Pj-VG2*) in both sexes of *Pandalopsis japonica*. The authors suggested that this was related to the protandric hermaphroditism in this species, with initially functional males developing into functional females. *VG* genes were also expressed in males of *M. rosenbergii* (Jiang et al., 2023) although at rates much lower than the dominant *VG* (*Mr-VG1*) expressed mainly in the hepatopancreas. An enigmatic finding is the presence of a *VG* gene exclusively found in the spermatozoa and not in any other tissues in the gonochoristic mud crab *Scylla paramosain* (Yang et al., 2016). It was identified as a splice variant of the ovarian and hepatopancreatic *VG*. The role of this VGs was seen as a molecule involved in immune protection during the spermatozoon maturation since its levels were induced upon challenge with bacterial lipopolysaccharides. In the other species, the biological function of male VGs is presently unknown. It remains to be determined whether the other Hi-VG isoforms (Hi-VG2–Hi-VG4) are incorporated into eggs like Hi-VG1 or if they perform distinct, yet unknown functions in *H. inermis* and other crustaceans. Comparative analyses of the relative expression levels of *alpha* females vs. both wild and cultured *beta* females, demonstrated considerable variability in the transcriptional profiles of these sex-related genes. Interestingly, cultured *beta* females exhibited *Hi-VG1* and *Hi-VG3* expression levels comparable to

those observed in *alpha* females, whereas wild *beta* females showed downregulation of the same genes. The marked differences observed in *Hi-VG*s expression between wild *alpha* and *beta* females are consistent with the known seasonal regulation of sexual maturation of *H. inermis*. It is essential to consider the timing of specimen collection—in this case, late April—as the reproductive status of this species is linked to its seasonal cycle.

Despite the early development of external sexual characteristics, individuals originating from the spring reproductive cohorts typically reach sexual maturity by the late summer and participate in the subsequent reproductive event in the autumn (Zupo, 1994). These temporal dynamics must be considered when interpreting gene expression patterns related to sexual differentiation and maturation. On the other hand, the differential expression pattern of wild and cultured *beta* females may reflect an advanced reproductive maturation stage in cultured individuals, potentially attributable to enhanced nutritional status, suggesting that reproductive maturation in *H. inermis* may progress independently from the body size. In contrast, the expression of *Hi-VG4* was consistent in wild *alpha* and *beta* females but significantly upregulated in cultured *beta* females. This observation may indicate a divergent functional role for *Hi-VG4*, potentially associated with responses to environmental stressors, bacterial infections, or oxidative stress (Sun et al., 2015; Dietrich et al., 2022; Li et al., 2017). These factors may represent a concrete issue under rearing conditions (Wuertz et al., 2023). Consequently, despite the maintenance of cultured shrimp assured under stable conditions (18°C with daily water exchange and regular feeding) the presence of additional, unmonitored stressors affecting gene expression cannot be excluded. As in the case of *Hi-VG1*, *Hi-VG3* and *Hi-VG4* in females, the relative expression of *Hi-IAG* and *Hi-UCM* in adult males appears to be independent of individual size but closely associated with the stage of sexual maturation, and consequently, with the seasonal life cycle and the time at which the specimens were collected. These gene expression profiles are important molecular indicators of the reproductive state and are key regulators of developmental transitions in *H. inermis*.

5. Conclusions

Our findings indicate that the expression patterns of the initially isolated sex-related genes are highly variable and influenced by multiple physiological and environmental factors. Notably, our results also revealed that the body size is not a reliable indicator of sexual maturation stage in young adults of *H. inermis*. In contrast to other hermaphroditic decapod species, the notable absence of an "ovotestis" in *H. inermis* (Reverberi, 1954) may facilitate the interpretation of the genetic mechanisms underlying protandric sexual shift. Despite the complexity of the observed expression patterns, our results provide a foundation for developing molecular tools for sex identification in specific age classes. Furthermore, they offer insights to understand the relationships between gene expression, gene function, and sexual maturation in crustacean decapods, thanks to the remarkable degree of sexual plasticity in *H. inermis* as compared to other decapods. Sex shift as part of a tightly regulated life cycle, influenced by both intrinsic maturation and extrinsic seasonal cues, reflects an evolutionary adaptation that allows a species to optimize reproductive output under varying seasonal and environmental conditions. Understanding the molecular basis of these processes will clarify broader regulatory networks governing sex differentiation and reproductive strategy in decapod crustaceans.

CRediT authorship contribution statement

Ulrich Hoeger: Writing – review & editing, Writing – original draft, Conceptualization. **Takashi Gojobori:** Writing – review & editing, Conceptualization. **Anna Italiano:** Writing – review & editing, Methodology. **Roberta Esposito:** Writing – review & editing, Methodology. **Amir Sagi:** Writing – review & editing, Conceptualization. **Eliahu D. Aflalo:** Writing – review & editing, Conceptualization. **Rachele Macirella:** Writing – review & editing, Methodology. **Elvira Brunelli:** Writing – review & editing, Conceptualization. **Bruno Pinto:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Valerio Zupo:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Conceptualization. **Marialuisa Lusito:** Writing – review & editing, Formal analysis, Data curation. **Maria Costantini:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Conceptualization. **Amalia Amato:** Writing – review & editing, Methodology. **Robert Hoehndorf:** Writing – review & editing, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no competing financial interests or personal relationships that could be perceived to have influenced the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anireprosci.2026.108211.

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