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Could Crustaceans' Ovary Synthesize Ecdysone? First Evidence in a Crab Species During Intermolt

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The function of ecdysone in promoting molting is widely known in arthropods. Moreover, in insects, ecdysone synthesis in the ovary is involved in reproduction and development. In crustaceans, some studies conducted in species with the molt linked to mating have shown that ecdysone produced by the Y organ stimulates ovarian maturation, but scarce evidence about the capacity of the ovary itself to synthesize ecdysone is currently available. The present study provides the first evidence of the expression of a putative *shadow* gene (sad, cytochrome P450 CYP315A1), involved in the ecdysone synthesis pathway, in the ovary of the estuarine crab *Neohelice granulata*, considered a derived species with the molt not linked to mating. The obtained results provide the first evidence for future studies testing the hypothesis that during the intermolt period, the mature ovary of females synthesizes ecdysone to locally act on the female vulvae to promote its decalcification, allowing mating. These results are discussed considering phylogenetic relationships among Arthropoda.

Keywords: Ecdysone, Ovary, Molt, Reproduction, Crustaceans

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BACKGROUND

Some physiological roles of ecdysteroids have been well-studied in crustaceans, involving the regulation of molting, development, and reproduction (Asazuma et al. 2007; Hyde et al. 2019). Among ecdysteroids, ecdysone was defined as the key hormone mediating various physiological and behavioral changes necessary for molting such as the decalcification of the exoskeleton (Chang and Mykles 2011; Marchal et al. 2010). However, although the molecular mechanisms involved in both molting and gonadal maturation of crustaceans have received special attention in recent years, the current research is still scant compared to that made on insects (Xie et al. 2016). Interestingly, the ecdysone synthesis route has been described to be similar in insects and crustaceans, suggesting its presence in the common ancestor of arthropods, unique to this phylum (Grieneisen 1994; Rewitz and Gilbert 2008). In this respect, it is interesting to highlight that crustaceans are thought to represent the ancestral arthropods from which insects originated (Glenner et al. 2006).

The genes encoding the cytochrome P450 mono-oxygenases (CYPs) involved in the ecdysone synthesis pathway were deeply studied in insects, and their orthologs are present in crustaceans (Rewitz and Gilbert 2008). The genes for these enzymes are called Halloween genes (Mykles and Chang 2020), including *spook* (Spo; CYP307A1), *phantom* (Phm: CYP306A1), *disembodied* (Dib: CYP302A1), *shadow* (Sad: CYP315A1), and *shade* (Shd: CYP314A1). The CYP315A1 (*shadow*) 2-hydroxylase converts 2-deoxyecdysone into ecdysone, while the CYP314A1 (*shade*) 20-hydroxylase converts ecdysone into 20-hydroxyecdysone (Rewitz et al. 2007; Xie et al. 2016). In both insects and crustaceans, *shade* is expressed in several peripheral tissues to convert the circulating ecdysone into a biologically active hormone, while *shadow* would be only expressed in endocrine tissues that produce and secrete ecdysone, as has been verified in the prothoracic gland of insects and the Y organ of crustaceans (Grieneisen 1994; Mykles 2011).

In Brachyura (i.e., true crabs), the reproductive context is variable, since mating can be linked to female molting, or instead occur during intermolt when both sexes have hard exoskeletons; the first case is considered the ancestral condition, while the latter is a derived condition (McLay and López Greco 2011). Cancridae and Portunidae crabs are considered ancestral groups, since they

mate when the female is soft, immediately after molting. In some species belonging to these families, an increased expression of the nuclear ecdysone receptor (EcR) has been reported in the ovary, in coincidence with high ecdysone levels in hemolymph and advanced vitellogenesis in the ovary (Girish et al. 2015; Gong et al. 2015; Lu et al. 2018). On the contrary, crabs belonging to the Majidae, Hymenosomatidae, Varunidae, Grapsidae, and Ocypodidae are derived species that mate during the intermolt period, when both sexes are hard-shelled.

In the derived species, the entire vitellogenesis and the ovarian maturation occur during the intermolt period, at very low hemolymphatic ecdysteroid levels. Moreover, in Majidae crabs, molting ceases after a pubertal molt and the Y organ begins to undergo atrophy; however, in the Majid crab *Acanthonyx lunulatus*, the ecdysteroids levels increased in the ovary in correlation with the onset of secondary vitellogenesis (Chaix and De Reggi 1982). Therefore, given that in those Majid species the Y organ degenerates after the puberty molt, the source of ecdysteroids should be the ovary, as hypothesized by Rotllant et al. (2018). On the other hand, the receptive period in females that copulate during the intermolt period is associated with the vulvae form. In Grapsoidea crabs, the vulva is covered by an operculum attached to the sternum through a hinge (McLay and Sal Moyano 2016). In receptive females (i.e., having a mature ovary), the operculum becomes temporarily mobile during the intermolt period, allowing male gonopod insertion and copulation (McLay and Sal Moyano 2016). In the crab *Neohelice granulata* (Varunidae), it was demonstrated that local softening of the hinge was stimulated by injecting ecdysone during the intermolt period (Sal Moyano et al. 2017). Given that during the intermolt period, the Y organ only maintains basal circulating levels of ecdysone (Chen et al. 2012), the ovary could be synthesizing ecdysone in Brachyura-derived species, to promote local decalcification of the vulvae hinge, therefore allowing copulation.

Neohelice granulata has been taken for decades as a model species for a wide variety of research lines (Luppi and Rodriguez 2020). As a typical brachyuran, this species presents a derived condition, i.e., mating not linked to molting. Therefore, the current study hypothesizes that the mature ovary (i.e., in secondary vitellogenesis) synthesizes ecdysone, which is directly involved in the local decalcification and mobility of the vulvae hinge previously observed during the female intermolt receptive period. In this context, the present study aimed to identify, in the mature ovary of *N. granulata*, the expression of the *shadow* gene encoding the enzyme (sad, cytochrome P450 CYP315A1) involved in the ecdysone biosynthetic pathway.

MATERIALS AND METHODS

Biological material

Crabs were captured from Mar Chiquita Coastal Lagoon (37°45'S; 57°19'W, Buenos Aires Province, Argentina) during the molting season (end of March–April). Once in the laboratory, they were placed in glass aquaria (30 × 35 × 25 cm, 26 L capacity, filled with 3 L of seawater), at a density of four crabs/aquarium, under a controlled photoperiod of 12:12 (L:D), and continuous aeration. Room temperature was maintained at 23 ± 1.5°C. Crabs were fed daily with rabbit pellet food (15% protein content), and water was changed just after feeding. To stimulate the ecdysone production by the Y organ, one eyestalk was ablated after two days of acclimation to laboratory conditions, following the same methodology used by Rodriguez Moreno et al. (2003). Five days later, the other eyestalk was ablated following the same procedure. No mortality was observed in ablated crabs. Seven days after the second ablation, crabs were sacrificed by placing them at -20°C for approximately 30 min; the Y organs were dissected and immediately fixed in liquid nitrogen for further analysis.

During the reproductive period (September–March), non-ovigerous mature females were captured from the same site and transported to the laboratory. According to Sal Moyano et al. (2020), an ovarian cycle in *N. granulata* includes five developmental stages based on both macroscopic and microscopic features of the ovary: 1: filiform and translucent; 2: ovarian branches are tubular and narrow, orange color; 3: ovarian branches are tubular and wider, brown color (these three stages belong to primary vitellogenesis); 4: the anterior part expanded but not covering the cardiac stomach and hepatopancreas, orange to dark violet color; and 5: ovary completely covering the cardiac stomach and hepatopancreas, always granular, dark red to dark violet color (these two stages belong to secondary vitellogenesis). Only females in stage 5 (*i.e.*, the female is ready to spawn mature oocytes) decalcify the vulvae hinge, and mating occurs. However, given that the local decalcification of the hinge might be potentially stimulated by the ecdysone synthesized by the mature ovary in secondary vitellogenesis, females with mature ovaries in secondary vitellogenesis (stages 4 and 5) were selected. These females were sacrificed by placing them at -20 °C for approximately 30 min; the ovary was then dissected and fixed in liquid nitrogen for further analysis.

Alignment of Decapoda sequences and primer design

Shadow (sad, cytochrome P450 CYP315A1) mRNA sequence from the swimming crab *Portunus trituberculatus* (Accession No. KM880023, Xie et al. 2016) was used to find homologous sequences using BlastN (www.blast.ncbi.nlm.nih.gov) in Transcriptome Shotgun Assembly (TSA) database, limited to Decapoda taxon. Among the homologous sequences retrieved, sequences of the

Chinese mitten crab *Eriocheir sinensis* (Accession No. XM_050871681), the Jonah crab *Cancer borealis* (Accession No. GEFB01021314), the green crab *Carcinus maenas* (Accession No. GFYW01138202), the Christmas Island red crab *Gecarcoidea natalis* (Accession No. GFXJ01003614) and the southern rock lobster *Jasus edwardsii* (Accession No. GGHM01060911) were selected for alignments. Additionally, CYP315A1 mRNA sequences from the orange mud crab *Scylla paramamosain* (Accession No. MN782364), the spiny lobster *Sagmariasus verreauxi* (Accession No. MH536668, Ventura et al. 2018), and *Portunus trituberculatus* were used for alignments. Degenerate primers were designed from highly conserved regions using Unipro Ugene v 47.0 software (Okonechnikov et al. 2012) and analyzed to avoid hairpins and homodimers with Oligo Analyzer (Integrated DNA technology). Two forward primers: sadF1 (5-GGA[AC]T[CT]TTCTTCATGGATGG-3), sadF2 (5-ACACGGCATCTTCAAGGAGA-3) and three reverse primers: sadR1 (5-GTGTGGGATGT[CT]GTGTCT-3), sadR2 (5-[CT]GCC[GA]AA[GC]GGGA[AT]GAAGG-3), sadR3 (5-CATGCGGATCACCATGTC-3) were designed and synthesized.

RNA isolation, reverse transcription (RT), and polymerase chain reaction (PCR)

Total RNA from both Y organs and ovary was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions and was resuspended in nuclease-free H_2O with a ribonuclease inhibitor. The quantity and quality of RNA were determined using an Epoch2 spectrophotometer with a Take3 micro-volume plate (BioTek). RNA integrity was verified by visualization of clear bands of the 18S and 28S ribosomal RNA in 1.5% agarose gel. For each sample, RNA was treated with DNase I (RNase-free DNase I, Invitrogen) to eliminate potential genomic DNA contamination. Reverse transcription was performed by TransScript Reverse transcriptase kit (Transgene) using 1 μg total RNA, oligodT, and random primers according to the manufacturer’s protocol. For PCR analysis cDNA was diluted tenfold. Ovary and Y-organ samples were processed in the same manner.

PCR amplifications were carried out using EasyScript Taq (Transgene). After an initial denaturation step of 4 min at 95°C, 40 amplification cycles were performed. Each cycle involved denaturation at 94°C for 30 s, annealing at 46°C for 60 s, and extension at 72°C for 60 s. A final extension step of 10 min at 72°C was done to complete the reaction. Amplification with sadF1 and sadR3 primers produced a main PCR product of the expected size (1150 bp), that was used as the template for nested PCR reactions using sadF2-sadR1 and sadF2-sadR2 primer pairs. For nested PCR, the sample was diluted 100-fold, and the same cycling conditions were used. Single PCR products of the expected sizes (300 bp for sadF2-sadR1 and 720 bp for sadF2-sadR2) were obtained.

Since the same forward primer was used for both amplifications, the 300 bp product overlaps with the 720 pb product. Amplicons obtained from Y-organ and ovary were purified and sequenced (Macrogene Inc).

RESULTS

Homologous sad cDNA sequence in *Neohelice granulata*

To identify sad mRNA sequence in *N. granulata*, total RNA was purified from the Y organs dissected from three eyestalkless crabs. Subsequently, cDNA was synthesized and used as a template for PCR amplifications with primers designed from conserved regions of the sad transcript, as described in the Materials and Methods section. PCR amplicons of the expected sizes (300 and 720 bp) were subjected to sequencing. Since the 300 bp sequence is entirely contained within the 720 bp sequence, the latter was used for subsequent analysis. The obtained DNA sequence was aligned with previously characterized sequences of sad (CYP315A1) mRNA from *Eriocheir sinensis*, *Gecarcinus lateralis*, *Portunus trituberculatus* (Xie et al. 2016), *Scylla paramamosain* and *Sagmariasus verreauxi* (Ventura et al. 2018). The high similarity between the six sequences strongly suggests that the PCR product was derived from *N. granulata* sad mRNA. The homologous regions were highlighted with black and grey boxes in the alignment display (Fig. 1). Interestingly, predicted CYP315A1 mRNA from *Eriocheir sinensis* (Accession No. XM_050871681), a crab that belongs to the same family that *Neohelice* (Varunidae) exhibits a 95% identity with the cDNA amplified from *N. granulata.* Additionally, the putative sad mRNA sequence of *N. granulata* exhibits 85% identity with the shadow mRNA sequence from the crab *Geocarcinus lateralis* and 70% identity with that from *Portunus trituberculatus* (not shown).

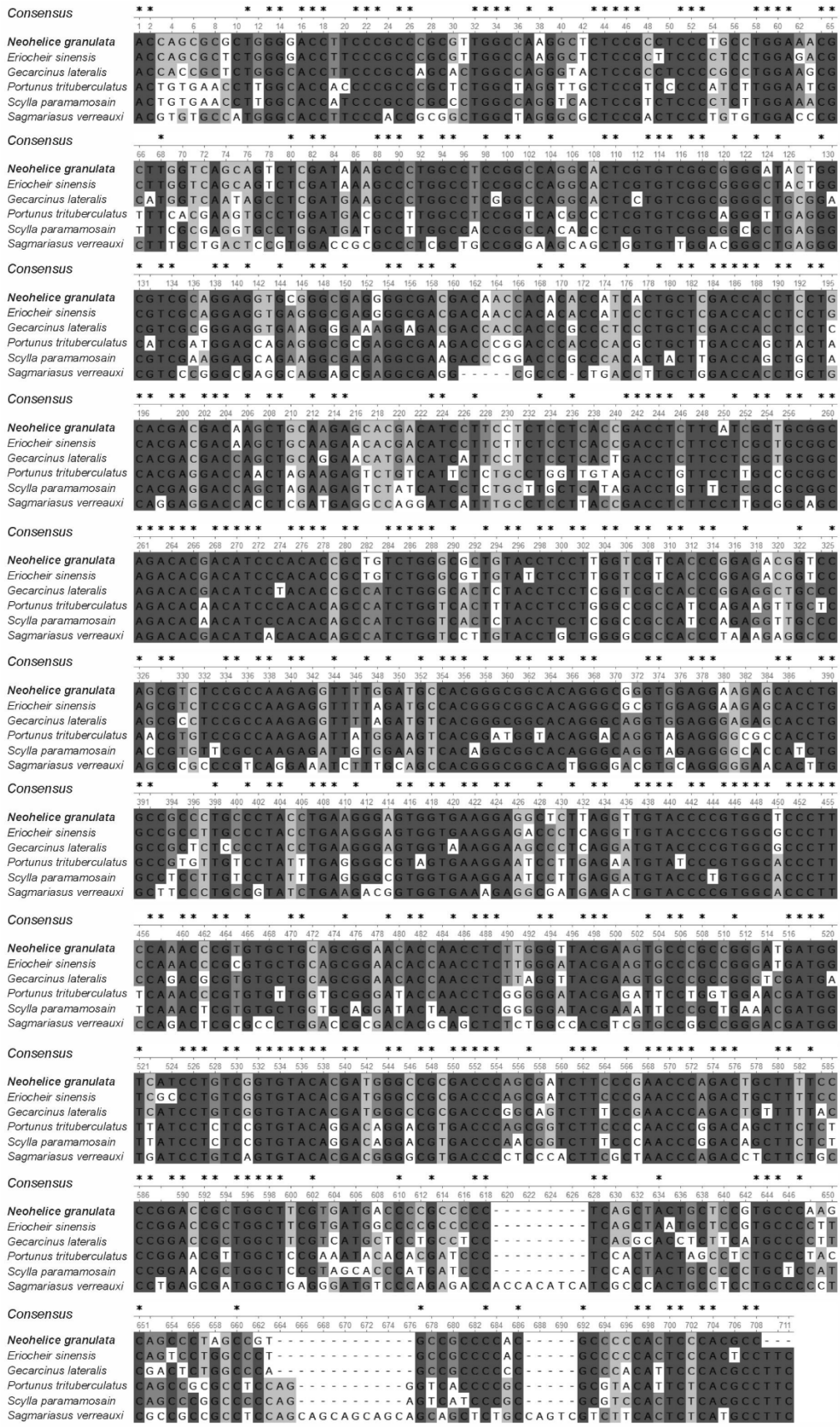

Fig. 1. Homology of sad (CYP315A1) mRNA sequences from five Decapoda and partial putative sad mRNA sequence from *N. granulata* (681 bp). For alignments, *Eriocheir sinensis* (1653–2072 bp, Accession No. XM_050871681), *Gecarcinus lateralis* (1653–2333 bp, Accession No. OP555909), *Portunus trituberculatus* (962–1645 bp, Accession No. KM880023, Xie et al. 2016), *Scylla paramamosain* (334-1017 bp, Accession No. MN782364) and *Sagmariasus verreauxi* (784–

1485 bp, Accession No. MH536668, Ventura et al. 2018) sequences were used. Sequence alignment was carried out using Unipro Ugene v 47.0 software. Dark grey boxes indicate 83–100% consensus, grey boxes denote 66% consensus, and light grey boxes represent 50% sequence consensus among the aligned sequences.

The amino acid sequence was deduced from the PCR-amplified sequence, and the predicted protein sequence was used as a query in a BLASTp search against the NCBI RefSeq database (Sayers et al. 2021). A high identity percentage with sequences from shadow proteins of other crustacean species was obtained: 93% identity with shadow protein from *Eriocheir sinensis* (XP_050727638.1), 80% identity with that of *Geocarcinus lateralis* (WGJ63592.1), 66% identity with sequences from *Portunus trituberculatus* (AJF94636.1) and *Scylla paramamosain* (QNQ17530.1), and 64% identity with shadow protein from *Sagmariasus verreauxi* (QBJ27553.1). Multiple protein sequence alignment of the predicted *N. granulata* putative shadow protein and these sequences was performed using T-Coffee (Di Tommaso et al. 2011) and is shown in figure 2. Analysis of conserved domains within the predicted protein, using the Conserved Domain Database search tool at NCBI (Wang et al. 2023), identified it as belonging to the cytochrome 450 superfamily, aligning with the conserved protein domain family CYP24A1-like, which includes *Drosophila melanogaster* shadow protein.

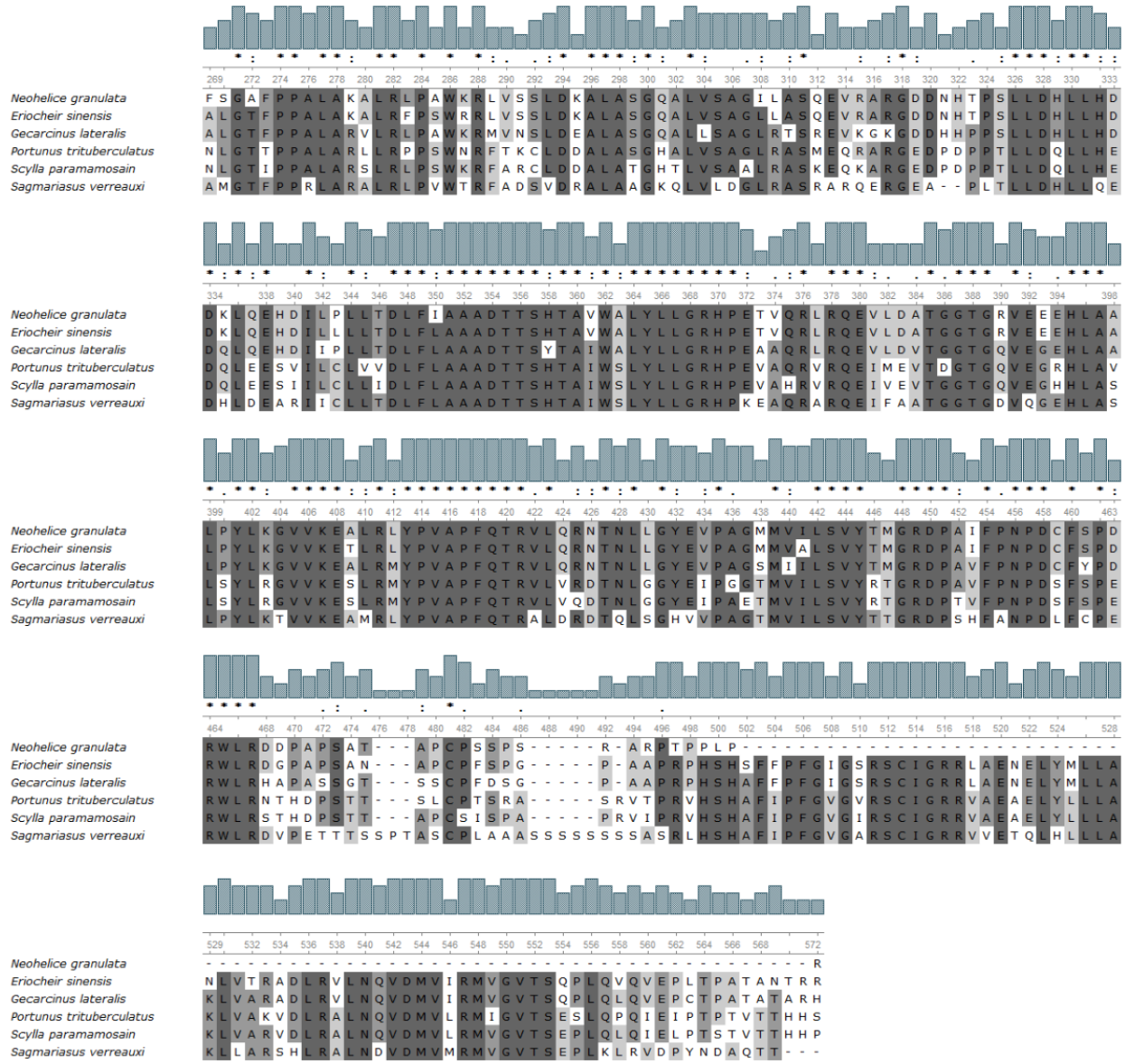

Fig. 2. Homology of the shadow (CYP315A1) protein sequences from five Decapoda and the predicted partial putative sad protein sequence from *N. granulata*. For alignments, *Eriocheir sinensis* (Accession No. XP_050727638.1), *Gecarcinus lateralis* (Accession No. WGJ63592.1), *Portunus trituberculatus* (Accession No. AJF94636.1), *Scylla paramamosain* (Accession No. QNQ17530.1) and *Sagmariasus verreauxi* (Accession No. QBJ27553.1) sequences were used, as they showed the highest percentage of sequence identity with *N. granulata* upon a BLASTp search against the NCBI database. Sequence alignment was carried out using T-Coffee and the graph was designed with Unipro Ugene v 47.0 software. In the alignment, an asterisk (*) indicates positions with fully conserved residues across all sequences, a colon (:) indicates conservation between groups of strongly similar chemical properties, and a period (.) indicates conservation between groups of weakly similar chemical properties. Gaps introduced to optimize the alignment are represented by dashes (-). Dark grey boxes indicate 83-100% consensus, grey boxes denote 66% consensus, and light grey boxes represent 50% sequence consensus among the aligned sequences. Bars above each position are proportional to the number of sequences containing the same residue in that position.

Detection of Sad transcript in ovary tissue

To analyze the presence of sad transcript in ovary tissue, total RNA was isolated from pooled ovaries of four mature females and used for cDNA synthesis. PCR analysis revealed the presence of sad transcript in ovary tissue since both single amplicons of 300 and 720 bp were detected (Fig. 3). The same reaction with no addition of reverse transcriptase was performed to confirm that no genomic DNA was amplified. As expected, amplification from the Y organ was also observed (Fig. 3). Sequencing of PCR amplicons derived from ovary tissue showed complete identity with sequences obtained from the Y organ. No amplicons were detected in the negative control, *i.e.*, performed using claw-closer muscle tissue.

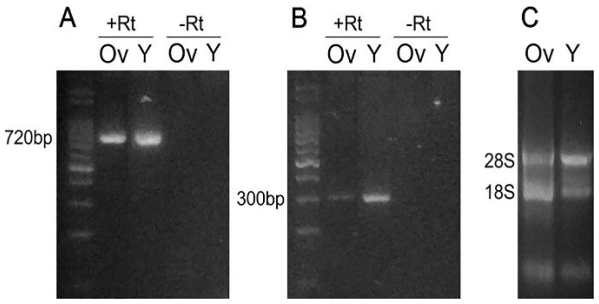

Fig. 3. Sad expression in ovary tissue. Y-organs (Y) were dissected from eyestalkless crabs, and mature ovaries (Ov, stages 4 and 5) were dissected from non-ovigerous intermolt female crabs, as detailed in the Material and Methods. Total RNA was extracted and RT-PCR reaction was performed with (+Rt) and without (-Rt) addition of reverse transcriptase enzyme. Primers were used to amplify a 720 bp (A) and a 300 bp (B) region of *N. granulata* sad transcript. (C) Total RNA from ovary tissue and Y organ in 1.5% agarose gel electrophoresis.

DISCUSSION

In the present study, we have identified one putative member of the Halloween gene family (*shadow*: sad, cytochrome P450 CYP315A1) involved in the ecdysone synthesis pathway, whose expression was verified in both the ovary and the Y organ of the crab *N. granulata*. Moreover, the expression of this gene was also found in the mature vitellogenic ovary of females during intermolt. Although the potential capacity of the crustaceans' ovary to synthesize ecdysone has been previously proposed (Subramoniam 2000; Rotllant et al. 2018), scarce evidence is currently available. For instance, the expression of some Halloween genes, such as *spook*, *disembodied* and *shade* and has been reported in several tissues of both copepods and prawns (Sanlund et al. 2018; Yuan et al. 2021, respectively). However, as far as we know, the current study provides the first evidence of the expression of a putative *shadow* gene, which encodes the enzyme that catalyzes the final synthesis of ecdysone, in the ovary of crustaceans. Nevertheless, further assays to quantify the

levels at which *shadow* is expressed in both the ovary and Y organ during the entire ovarian cycle would be necessary to elucidate the physiological role of ovarian ecdysone.

The production of ecdysone by follicular cells of the ovary has been widely reported in insects to have important roles in reproduction, such as the synthesis of the yolk proteins necessary for embryonic development (Bellés 1998; Freeman et al. 1999; Chávez et al. 2000). The proposed function of ecdysteroids in crustacean female reproduction was mainly based on correlations between vitellogenesis and hemolymph ecdysteroid titer, in ancestral type species (Subramonian 2016), in which the ovary is a probable target of the ecdysone synthesized by the Y organ, as reported for some prawns, lobsters, and peracarids; in such species, the increased expression in maturating ovaries of the nuclear receptor of ecdysone (EcR) has been observed during molting (reviewed by Okumura et al. 1992; Suzuki et al. 1996; Tiu et al. 2006; Subramonian 2016). Given that most of these studies were conducted in species with the molt linked to mating, the ecdysone production was attributed to the Y organ, disregarding the synthesis by an alternative organ, such as the ovary. The current study, through the identification of a gene expression involved in the ecdysone synthesis pathway, provides first evidence of the possible synthesis of ecdysone by the ovary itself, in a crustacean species with a derived condition, such as a decapod crab during the intermolt period.

The fact that *N. granulata* females mate in intermolt, i.e. with basal titers of circulating ecdysone (Rodríguez Moreno et al. 2003), indicates that another source of ecdysteroid production may be involved. The synthesis of ecdysteroids by the mature ovary of *N. granulata* might occur during secondary vitellogenesis, i.e., in the transition between stages 4 and 5 of ovary development. Our current results suggest that the ovary at this stage might produce a high titer of ecdysone that could promote the decalcification of the vulvae hinge, in coincidence with the end of stage 5, when oocytes are ready to ovulate (Sal Moyano et al. 2017). The receptivity of females could be also induced by ecdysone, given the direct connection of the ovary to the vulva through the vagina (Sal Moyano et al. 2012). As mentioned previously, further studies quantifying the sad gene expression in the different developmental stages of the ovary, immature and mature, will be useful to elucidate this hypothesis.

The arthropod steroidogenic CYPs would have become functionally specialized in the common ancestor of arthropods (Rewitz and Gilbert 2008). Phylogenetic analysis of arthropods has found the Hexapod clade to be an ancient divergent of the Pancrustacea, thus, insects could be considered as derived crustaceans (Misof et al. 2014). The evolutionary relationship between insects and crustaceans is evident from the common growth strategy that involves molting (Rewitz and Gilbert 2008). However, crustaceans have continuous somatic growth through periodical molting even in adults (Subramonian 2000). Despite the evolutionary relationship between both groups of

arthropods, the knowledge of crustacean endocrine regulation is lesser compared to that of insects (Hyde et al. 2019). In this context, the results of the present study represent a relevant contribution, especially considering that the studied species has the derived condition, i.e. molting and growth not linked to mating, similar to the condition of insects. Therefore, our results reinforce the idea that insects and derived brachyuran crustaceans share an intermediate common ancestor. Finally, several crustacean species with a derived condition are used for aquaculture purposes, stressing the importance of deepening into all aspects of their reproductive biology to optimize their production.

CONCLUSIONS

The current study presents new evidence about gene expression in the ecdysone synthesis pathway in the mature ovary of a crustacean species, the crab *N. granulata*. Ecdysone synthesis would promote the decalcification of female vulvae allowing mating during the intermolt period. Previous research conducted in crustacean species with the molt link to mating showed that the Y organ produced ecdysone involved in reproduction. However, the results of the present study indicate that in derived crustacean species with the molt not linked to mating, the ovary would synthesize ecdysone, as insects do, thus reinforcing the hypothesis that both groups share an intermediate common ancestor.

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Authors' contributions: Conceptualization, investigation, methodology, writing-original draft: Sal Moyano M.P., methodology, writing-original draft: Graziano M., methodology: Canosa I.S., Da Cuña R., Silveyra G.R., conceptualization, writing - review & editing, funding acquisition: Rodríguez E.M., investigation, funding acquisition: Luppi T.A.

Competing interests: All authors declare they have no conflict of interest.

Availability of data and materials: The datasets generated and analyzed during the current study are available on request, from either the first or the corresponding author.

Consent for publication: Not applicable.

Ethics approval consent to participate: Authors declare they have followed the ethical standards of the responsible committee on laboratory animal experimentation (CICUAL Universidad Nacional de Mar del Plata).

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