Neuroendocrine pathways at risk? Simvastatin induces inter and transgenerational disruption in the keystone amphipod *Gammarus locusta*

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Highlights

- Simvastatin Inter and transgenerational effects are not well understood in aquatic organisms
- It´s unexplored if simvastatin effects are associated with neuroendocrine system disruption
- Simvastatin inter/transgenerational exposure severely impacts *G.locusta* neuroendocrine regulation
- Ecdysteroid, Dopamine, NO/cGMP/PKG, GABA and Cholinergic signaling pathways are affected
- These findings improve risk assessment of biological active compounds, such as Simvastatin

ABSTRACT

The primary focus of environmental toxicological studies is to address the direct effects of chemicals on exposed organisms (parental generation – F0), mostly overlooking effects on subsequent non-exposed generations (F1 and F2 – intergenerational and F3 transgenerational, respectively). Here, we addressed the effects of Simvastatin (SIM), one of the most widely prescribed human pharmaceuticals for the primary treatment of hypercholesterolemia, using the keystone crustacean *Gammarus locusta*. We demonstrate that SIM, at environmentally relevant concentrations, has significant inter and transgenerational (F1 and F3) effects in key signalling pathways involved in crustaceans´ neuroendocrine regulation (Ecdysteroids, Catecholamines, NO/cGMP/PKG, GABAergic and Cholinergic signalling pathways), concomitantly with changes in apical endpoints, such as depressed reproduction and growth. These findings are an essential step for improving hazard and risk assessment of biological active compounds, such as SIM and highlight the importance of studying the transgenerational effects of environmental chemicals in animals' neuroendocrine regulation.

Keywords: Simvastatin; *Gammarus locusta*; Neuroendocrine signalling pathways; Transcriptomic; Inter and Transgenerational effects; Regulatory agencies.

1.INTRODUCTION

Aquatic environments are the ultimate reservoirs for most anthropogenic chemicals. Accordingly, aquatic organisms are chronically exposed during critical periods in early life stages or even for an entire life cycle (Capela et al., 2016; Groh et al., 2015; Major et al., 2020). Knowledge of the hazards and risks associated with chronic exposure to anthropogenic chemicals has grown considerably over the last decades. However, contrasting with the extensive literature dealing with the direct effects of chemicals on parental exposed organisms (F0), little is known about the effects that parental exposure (F0) exerts on indirectly exposed (F1 and F2) or truly non-exposed (F3) generations – intergenerational and transgenerational effects (Bhandari et al., 2015; Kalichak et al., 2019; Neuparth et al., 2020a).

Yet, given the potentially broad ecological impacts of such effects in aquatic ecosystems, a growing scientific interest has emerged (Shaw et al., 2017; Van Cauwenbergh et al., 2020). Still, only a limited number of inter and transgenerational studies are available in the literature, focusing on a few endocrine disrupting chemicals (EDCs; e.g. Bisphenol A, some pesticides and phthalates), known for their ubiquity in the environment and associated with reproductive endocrine disorders (Brevik et al., 2018; Li et al., 2020; Santangeli et al., 2019). However, many other natural and synthetic substances, including pharmaceuticals, are suspected to hijack endocrine functions and disrupt reproduction, development, neuronal processes and/or other important physiological responses; not only in exposed individuals, but also in subsequent non-exposed generations (Gillette et al., 2018; Tuscher and Day,

2019; Walker and Gore, 2011). One of such pharmaceuticals is the hypocholesterolaemic pharmaceutical simvastatin (SIM). SIM is among the most prescribed human pharmaceuticals. Hovever, only a few studies have examined its concentrations in surface waters. According with the available information, SIM can reach the aquatic environments, in the vicinity of urban areas, with concentrations above 100 ng/L (Norway 108 ng/L - Langford and Thomas, 2011; India 414.9 ng/L - Khan et al., 2021 and South Africa 1585 ng/L - Tete et al., 2020). SIM mode of action operates through inhibition of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), a rate-limiting step of the mevalonate pathway (MP), responsible for the *de novo* synthesis of cholesterol in vertebrates and the synthesis of the methyl farnesoate hormone (MF) in crustaceans, with a key role in the regulation of reproduction and molting (Bellés X. et al., 2005; Fent et al., 2006; Li et al., 2010; Santos *et al.*, 2016). Although the final steps of the mevalonate pathway differ between vertebrates and crustaceans, most of the enzymatic cascade is highly conserved among metazoans (Santos *et* al., 2016). In our previous research (Neuparth *et* al., 2020a), *G. locusta* was exposed for 4 consecutive generations (F0 to F3) to environmentally relevant concentrations of SIM (64 and 320 ng/); simultaneously, the offspring of the F0-exposed generation were raised in SIM-free water during three subsequent generations (non-exposed F1 to F3). Our findings showed that reproduction and growth were particularly impacted by environmentally relevant concentrations of SIM, not only in directly exposed animals (exposed F0 to F3), but also in the generations raised in SIM-free water (non-exposed F1 and F2, intergenerational, and non-exposed F3, transgenerational effects). In addition, a significant decrease in MF levels was recorded in exposed and non-exposed females during four generations (F0 to F3). Although the molecular mechanism(s) underlying the observed transgenerational effects of SIM are not fully defined, epigenetic remodeling stands as the most plausible explanation for these findings, since most of environmental chemicals do not have the ability to alter DNA sequences or produce direct genetic mutations in such a short timeframe. In agreement, Alves et al. (2021) reported a significant downregulation of DNA methyltransferase 1 (DNMT1) gene expression upon exposure of *G. locusta* to 320 and 8000 ng/L of SIM, concomitantly with global DNA hypomethylation.

Although severe effects on reproduction, growth, and development are known to occur in arthropods, including amphipods, following exposure to SIM or other statins (Dahl et al., 2006; Liu et al., 2019; Neuparth et al., 2014; Ortiz de García et al., 2014; Zapata et al., 2003), the absence of mechanistic association between exposure and observed effects limits our understanding, notably regarding the contribution of endocrine disruption processes.

Crustaceans utilize a variety of neuroendocrine signaling cascades to regulate molting, growth, and reproduction, with most being unique to crustaceans and arthropods in general (Rodríguez et al., 2007; Waye and Trudeau, 2011). The crustacean X-organ–sinus gland complex is the major neuroendocrine structure, responsible for the release of an array of neuropeptides hormones: including MIH (molt inhibiting hormone), GIH (gonad inhibiting hormone), MOIH (mandibular organ inhibiting hormone)

and CHH (crustacean hyperglycemic hormone), hormones that have been extensively studied over the last decades and demonstrated to have a central role in homeostasis (Gismondi and Joaquim-Justo, 2019; Hyne, 2011). With the recent advances in next-generation sequencing, as well as the growing availability of arthropods transcriptomes and genomes, new neuropeptides genes have been cataloged in different crustacean species, some of which with a foreseen role in reproduction and development, such as Allatostatins, SIFamide, Prohormones, among others (Nguyen et al., 2016). Besides X-organ–sinus gland neuropeptides hormones, the sesquiterpenoid hormone MF is synthetized in the mandibular organ, whereas various types of steroidal hormones implicated in molting, such as ecdysteroids, are produced by the Y-organ. These hormones, involved in the endocrine system of crustaceans, are physiologically linked to the X-organ–sinus gland complex (Subramoniam, 2000). Apart from these arthropod-specific neuropeptides, sesquiterpenoid and steroidal hormones, other neurotransmitters, like biogenic amines (e.g. serotonin, dopamine), are associated with the endocrine regulation of physiological functions that control upstream neurosecretory processes shared by both arthropods and vertebrates (Campbell et al., 2004; Vaudry and Kah, 2017). In crustaceans, the biogenic amines are linked to the X-organ–sinus gland complex by controlling the release of the neuro-hormones MIH and CHH.

In addition to disrupting the homeostasis of endocrine hormones, different EDCs were also reported to produce multiple actions in the neural system, being neurotoxic particularly in early life stages (Gu et al., 2019; Sun et al., 2016; Xu and Yin, 2019). Several studies in humans and rodents have provided evidence for positive associations between pre- /post-natal exposure to certain EDCs (e.g., bisphenol A, phthalates and pesticides) and impaired neurodevelopmental outcomes in offspring (e.g. emotional, cognitive, motor and muscular disorders) leading to lifelong or even transgenerational dysfunctions (Lupu et al., 2020; Repouskou et al., 2020). In fact, recent epidemiological and toxicological studies established a robust link between human neurological diseases and exposure to certain EDCs (Trasande et al., 2015). Given the importance of neuroendocrine control for the maintenance of homeostasis, further efforts should be made to address the effects of EDCs at this level. Yet, it is not clear whether the observed effects occur as direct neurotoxicity of EDCs or result from an indirect effect through disruption of endocrine functions (Knigge *et al.*, 2021; Waye and Trudeau, 2011).

The emergence of new genomic tools, which can provide a high throughput screening of gene expression changes, are revolutionizing the way ecotoxicologists address the impact of environmental stressors on model organisms (Chen and Li, 2016; Merrick, 2019; Wang and Chang, 2018). These "omics" technologies provide robust approaches to further advance the mechanistic linkage between exposure and effects of potential endocrine disruptors like SIM, in crustaceans. Therefore, considering the findings of Neuparth *et al.* (2020a), reporting a marked reproductive and growth effect in *G. locusta* after inter and transgenerational exposure to SIM, the present study performed a comprehensive investigation that simultaneously assessed the inter and transgenerational (F1 and F3) molecular

changes of genes/pathways related with the regulation of the endocrine system, with a special focus on the neuroendocrine system and brain functions. In order to improve our knowledge on the molecular mechanisms related with the potential endocrine/neurotoxic efects of SIM throughout *G. locusta* generations, we addressed the effects particularly in five key signalling pathways involved in crustaceans´ neuroendocrine regulation, i.e., Ecdysteroids, Catecholamines, NO/cGMP/PKG, GABAergic and Cholinergic signalling pathways.

2. Material and Methods

2.1. Experimental design

To thoroughly analyze the effects of environmentally relevant concentrations of SIM on endocrine and neural systems of *G. locusta* in an inter and transgenerational context (non-exposed F1 and F3), we took advantage of the experiment conducted by Neuparth *et al.* (2020a). Briefly, the experiment started with one-week-old *G. locusta* offspring (parental generation - F0) continuously exposed to an environmentally relevant concentration of SIM (320 ng/L prepared in 0.0005% acetone), plus control (0.0005% acetone in filtered natural seawater). Fifty offspring, from our laboratory culture, were randomly allocated in 7L aquaria (four per treatment) and exposed to SIM up to adulthood (55-65 days). In each aquarium, with a 1cm layer of natural clean sediment, the amphipods were kept in filtered natural saltwater (33-35‰ salinity) with a temperature and photoperiod set to 20°C and 16:8 hours (light:dark) and feed *ad libitum* with *Ulva sp*. Water renewal in each aquarium occurred every three days and final SIM concentration was re-established after the SIM solution was spiked directly in the aquaria and properly stirred. The actual SIM concentration was monitored in the course of the assay (Neuparth *et al.*, 2020b). In order to evaluate the putative inter and transgenerational effects of SIM, the F0 offspring of each treatment were allocated in SIM-free water (natural filtered seawater) for three consecutive generations (F1, F2 and F3). Each generation was initiated with fifty offspring of the previous one and kept for 55 to 65 days. At the end of each generation, Females were sampled immediately after the third reproduction after maturity to determine growth, reproduction and methyl-farnesoate (MF) levels; the last measured according to the methods developed by Montes et al. (2017). Furthermore, three randomly selected females from control groups (F1 and F3) and from 320 ng/L SIM inter/transgenerational groups (F1 and F3), were preserved in RNA later at −80°C until individual use in RNA-sequencing analyses. Further methodological details are available in Neuparth *et al.*, (2020a,b).

2.2. Analyses of the disrupted endocrine and neural genes in F1 and F3 *G. locusta* females' transcriptome

The comprehensive transcriptome assembly produced in Neuparth *et al.* (2020a,b) was used in the current study to thoroughly evaluate the functional annotated genes of *G. locusta* females from inter and transgenerational exposure to SIM (non-exposed F1 and F3 generations, respectively). Briefly, to produce the *G. locusta* females transcriptome reported in Neuparth *et al.* (2020a,b), RNA was extracted from 3 randomly selected *G. locusta* females per condition [control and SIM inter/transgenerational groups (F1 and F3)] and individually sequenced using the Illumina HiSeq2500paired-end (2x150) system. Notably, the whole amphipod body was used rather than specific organs, because *G. locusta* females are too small to fully separate the organs of interest without contamination from other tissues. The RNA-Seq datasets were *de novo* assembled using the Trinity assembler and functionally annotated with the Trinotate suite. Lastly, the differential gene expression (DEGs) analyses (False Discovery Rate – corrected (FDR) p-value < 0.05, log2|fold change| ≥ 2 and blast e-value less than 1×10^{-29}) were performed using the Degust platform and KEGG pathways scrutinized with the KAAS webserver (detailed information available in Neuparth *et al.*, 2020b, sections 2.3 to 2.7).

In the present study, all the F1 and F3 functional annotated genes from the Neuparth et al. (2020b) transcriptome were first manually analized and a high number of genes involved in neuroendocrine control were identified. Based on these findings, we performed a data-driven approach searching for the main molecular components of the neuroendocrine network from all functional annotaned genes. We focused particulary on the metabolic pathways related with molting, reproduction and neural regulation (Ecdysteroid, Catecholamines, NO/cGMP/PKG, GABAergic and Cholinergic signalling pathways). Considering that crustacean neuroendocrine data is limited in KEGG databases, to properly study these specialized physiological processes in *G. locusta*, the functional annotated genes related with the neuroendocrine system were systematically reviewed using a manual approach, thus avoiding misrepresentation of *G. locusta* transcriptomic data due to annotation omission.

Thus, to perform a gene functional analysis, we first searched known protein names within several platforms such as Uniprot (https://www.uniprot.org/ accessed in March 2020), Genecard (https://www.genecards.org/ accessed in March 2020), NCBI (https.//www.ncbi.nlm.nih.gov/ accessed in March 2020) and Nextprot (https://www.nextprot.org/ accessed in March 2020). When available, the information was obtained from species with the closest taxonomy to *G. locusta*. We then used different databases (NCBI-https://www.ncbi.nlm.nih.gov/gene accessed in April 2020; Reactome-https://reactome.org/ accessed in April 2020; Biogrid v4.4-https://thebiogrid.org/ accessed in April 2020; STRING v11.5- https://string-db.org/ accessed in April 2020; IntAct-https://www.ebi.ac.uk/intact/ accessed in April 2020 and Rhea- https://www.rhea-db.org/ accessed in April 2020) to retrieve potential direct and/or indirect biological interactions between proteins encoded by selected DEGs related to the neuroendocrine system. All putative interactions recorded by the databases above were confirmed through a detailed review of functional studies in the

213 literature with a special focus on crustaceans and when the information for crustaceans was scarce,
214 on insects (e.g. Hyne, 2011; Sterkel and Oliveira, 2017; Covi et al., 2009; Mykles et al., 2010; Zhang et
215 al., 2014b; Nako et al., 2018; Takesian and Hensch, 2013). Additionally, we used arthropods´ dedicated
216 functional studies that were available in the literature to find proteins encoded by the selected DEGs
217 of interest related with ecdysteroids and molting processes that were not found in the databases used
218 (e.g. Qian et al., 2014; Street et al., 2019; Webster, 2015a,b; Zhou et al., 2019; Guittard et al., 2011).
219 All this data was used to manually design the neuroendocrine pathways presented in the present study
220 (Figure 1).

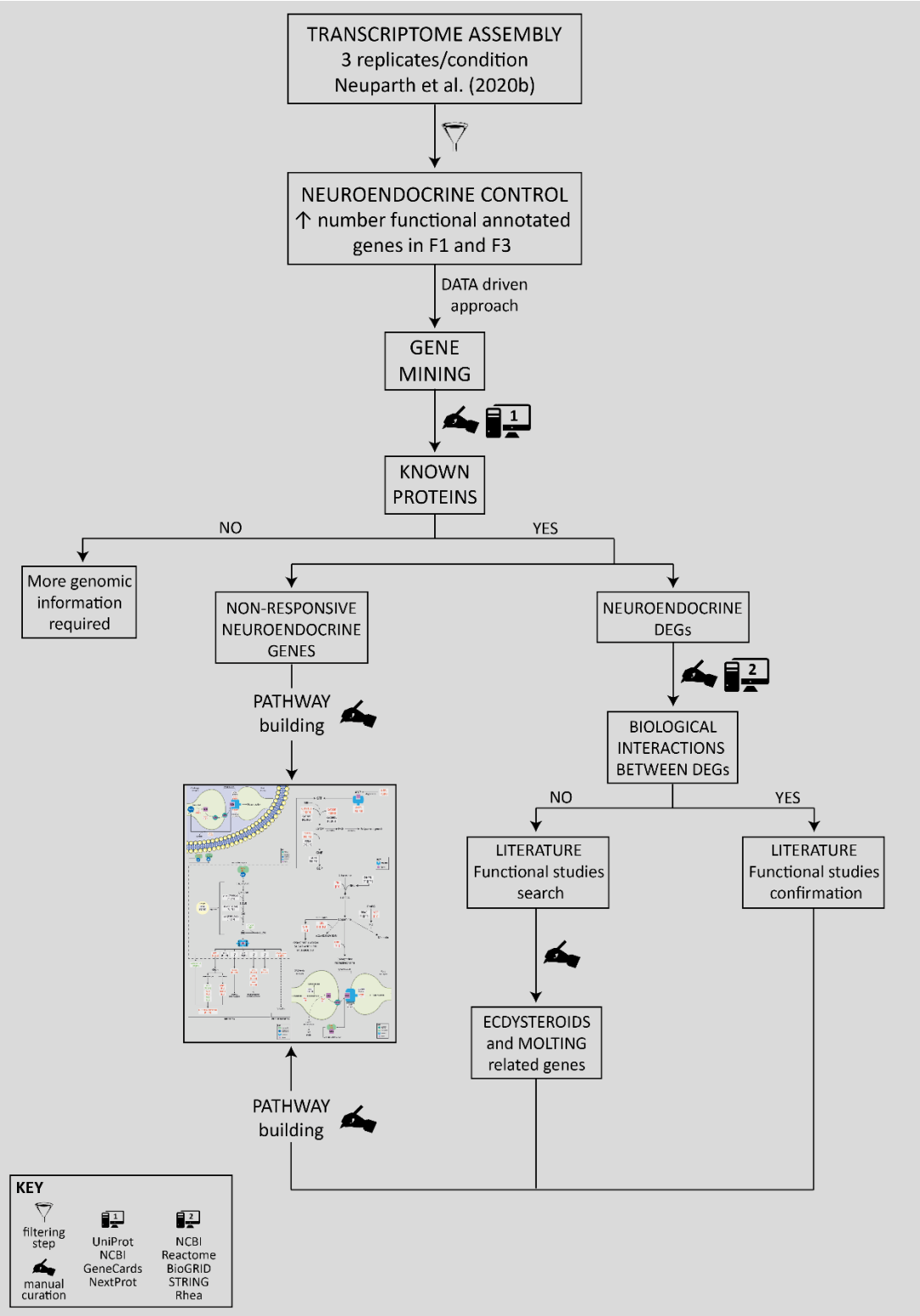

Figure 1 – Study workflow

3. Results and discussion

Due to the extensive use of industrial, agricultural and pharmaceutical products, tens of thousands of chemicals are released annually into aquatic environments (Evans et al., 2019; Rzymski, 2017; Wang and Yang, 2016). However, to date, only an infinitesimal fraction of environmental chemicals has been assessed for their endocrine-disrupting potential in wildlife (Brack et al., 2018; Santos et al., 2018).

Therefore, more studies are needed to screen for the endocrine disruption potential of environmental contaminants that are prevalent in aquatic environments, like SIM. Whereas most studies associated with EDCs focus on reproduction, other systems can also be impacted by EDCs (Lupu *et al.*, 2020; Yilmaz et al., 2020). The neuroendocrine control is of particular relevance given its central role in the general homeostatic regulation (Kalra and Kalra, 2010; Papathanasiou et al., 2019; Toni, 2004).

In a previous work (Neuparth *et al.*, 2020a,b), it was demonstrated that an environmentally relevant concentration of SIM (320 ng/L) produces marked inter and transgenerational effects in the keystone amphipod *G. locusta*, an invertebrate used as a test species in aquatic risk assessment (Figure 2). These findings are particularly relevant due to the persistence of the observed effects on reproduction/MF levels and growth: starting from an exposed parental generation (F0), these effects were truly inherited by the following non-exposed generations at least up to F3 (inter and transgenerational effects, F1 and F3 respectively) (Figure 2). Although the inter and transgenerational reproductive/growth effects of SIM were well characterized in *G. locusta*, one key question emerged: are neuroendocrine signaling pathways, that control key physiological functions in *G. locusta*, inter and transgenerationally modulated by SIM?

Therefore, to study the potential interplay between neuroendocrine regulation at the molecular level and apical effects, we manually analyzed and curated the list of functional annotated genes of *G. locusta* females in the F1 and F3 generations (Figure 2, Annexes 1, 2 and 3).

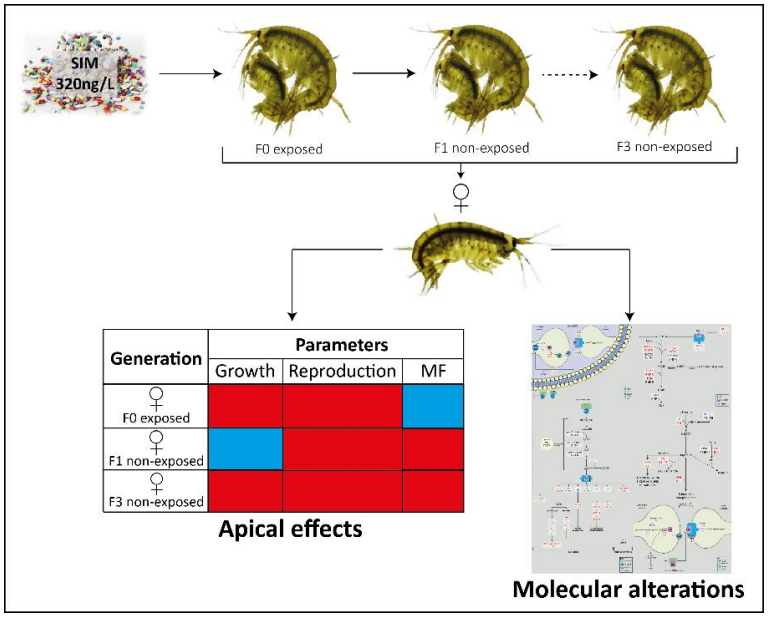

Figure 2 – Schematic representation of apical effects and molecular alterations produced by an environmentally relevant concentration of SIM (320 ng/L) on *G. locusta* exposed females from F0 and on *G. locusta* non-exposed females from F1 and F3 (intergenerational and transgenerational effects, respectively); Red rectangles indicate a significant decrease and blue rectangles indicates no effect.

3.1. The Ecdysteroids pathway and its role in downstream molting and vitellogenesis related processes

The reproductive cycle of female crustaceans (vitellogenesis/embryogenesis) is closely related with molting, since ovulation and mating are restricted to the early post-molt period; and females molt after embryos have fully hatched (Geffard et al., 2010; Hyne, 2011). Therefore, we first analyzed the main endocrine pathway, in crustaceans, deeply involved in the regulation of molting, ovarian development, and reproduction, the ecdysteroids cascade (Gong et al., 2015; Nagaraju, 2011). We aimed to evaluate whether the transcriptional shift of genes from this pathway could be associated with the observed dysregulation of reproduction, growth and impaired MF levels caused by the exposure to SIM, previously reported in Neuparth *et al.* (2020a) (Figure 2). In the ecdysteroids cascade, several key genes with functions related with cholesterol transport (Niemann-Pick protein 2—NPC2), 20-hydroxyecdysone degradation (CYP 2L1), molting regulation genes and vitellogenin were differentially expressed in F1 and F3 females when compared with the respective control. Moreover, genes involved in the biosynthesis of ecdysone—E (MIH, Phanton—phm, Disemboldied—dis, and Shadow—sad) were found non-responsive to SIM (Figure 3).

Arthropods utilize cholesterol as a precursor of ecdysteroids (ecdysone—E and its derivative 20-hydroxyecdysone—20E) (Yoshiyama-Yanagawa et al., 2011). However, arthropods cannot synthesize cholesterol *de novo*, and therefore, for ecdysteroid biosynthesis, they must incorporate cholesterol from food that is transported by a cholesterol-binding protein (NPC2) to acceptor membranes (Huang et al., 2007). Ecdysone (E), under the negative control of MIH, is synthesized from cholesterol in the Y-organ and then is secreted into the hemolymph in a physiologically active form, 20E (Priya et al., 2010; Webster, 2015a,b). 20E interacts with and activates the ecdysone receptor/retinoid-X-receptor heterodimer (EcR/RXR), initiating a downstream cascade of gene regulatory events that triggers the expression of vitellogenin and ecdysone early responsive genes, such as ecdysone inducible protein 75 (E75), ecdysone inducible protein 74EF (E74) and Broad-complex core protein (BRC), which in turn regulates the expression of late responsive genes that participate in various developmental processes during crustaceans molting. E75, for example, regulates cuticle,chitin andmuscle genes related with different enzymatic activities, including the degradation of old cuticle and the formation of a new cuticle in the molting process (King-Jones and Thummel, 2005; Qian et al., 2014). The *G. locusta* transcriptome analysis depicted a modulation of several genes in this pathway (Figure 3). A decrease in NPC2 expression was observed in F1 which could imply a lower cholesterol transport for ecdysteroid synthesis. Also, an upregulation of CYP-2L1 in F1 and F3, a steroidogenic enzyme from the CYP clan 2, was observed. CYP-2L1 displays similarity with the previously characterized insect homolog CYP18a1; thus, we hypothesized that CYP-2L1 could possibly metabolize the subtract of 20E in *G. locusta*; yet, decisive functional analysis is still needed. In insects, the CYP18a1 regulates the catabolism of 20E through its conversion into 20,26-dihydroxyecdysone (20,26E) (Guittard et al., 2011). The hypothesized CYP-2L1-dependent decrease of ecdysteroid titers would affect the transcription of the above-mentioned downstream cascade events that regulate the molting process. A downregulation of

vitellogenin and E75 genes, the primary targets of EcR/RXR receptors, was observed in F1 and F3, but E74 and BRC were non-responsive (Figure 3, Table 1). Interestingly, several studies in arthropods reported that E75, which is positively regulated under the control of 20E levels, has a key role in the ecdysteroid cascade acting on late responsive genes vital for proper molting and survival (Qian et al., 2014; Song et al., 2017; Street et al., 2019; Zhou et al., 2019). Furthermore, Priya et al. (2010) showed that E75 silencing in the crustacean *Fenneropenaeus chinensis*, leads to molting arrest due to an epidermal retraction and poor development of new cuticle.

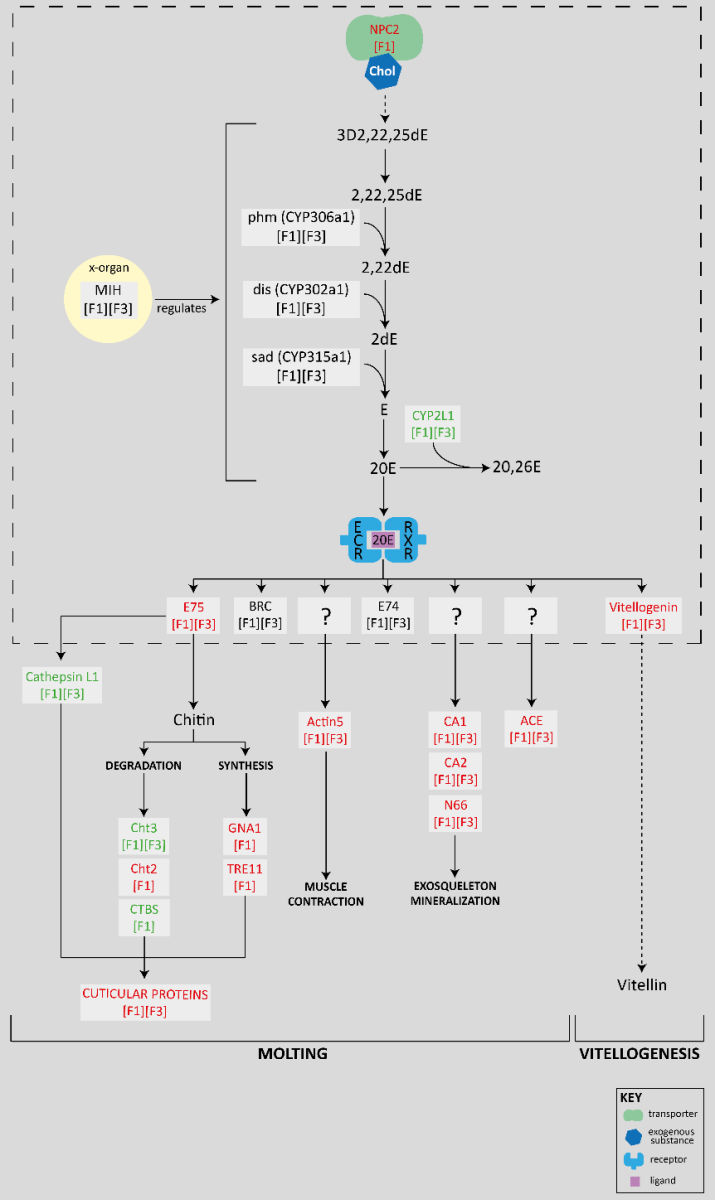

Figure 3 – Ecdysteroid pathway and its role in downstream molting and vitellogenesis related processes. Genes modulated by SIM and non-responsive genes in F1 and F3 generations are presented in boxes. Red text inside the boxes indicates gene expression downregulation in F1 and/or F3 generations; green text inside the boxes indicates gene expression upregulation in F1 and/or F3 generations and black text inside the boxes indicates unchanged gene expression in F1 and F3 generations. According to the actual knowledge in the literature (Hyne et al., 2011; Qian et al., 2014; Gong et al., 2016; Song et al., 2017; Street et al., 2019; Zhou et al., 2019), we have delimitated the Ecdysteroids pathway from the downstream molting and vitellogenesis related processes with a dashed line. Chol – cholesterol; NPC2 – Niemann-Pick protein 2; phm – phantom (cytochrome P450 306a1); dis – disembodied (cytochrome P450 302a1); sad – shadow (cytochrome P450 315a1); MIH – molt-inhibiting hormone; 3D2,22,25dE – 5β-Diketol; 2,22,25dE – 5β-Ketodiol; 2dE – 2-deoxy-ecdysone; E – ecdysone; 20E – 20-hydroxyecdysone; 20,26E – 20,26-dihydroxyecdyson; CYP2L1 – cytochrome p450 2L1; EcR – ecdysone receptor; RXR – retinoid X receptor; E75 – ecdysone-inducible protein E75; BRC – broad-complex core protein; E74 – ecdysone-induced protein 74EF;

CA1- Carbonic anhydrase 1; CA2 – Carbonic anhydrase2; N66 – N66 matrix protein; ACE – angiotensin converting enzyme; GNA1 - glucosamine 6-phosphate N-acetyltransferase; TRE11 - facilitated trehalose transporter; Cht2 – chitinase 2; Cht3 – chitinase 3; CTBS – N-acetylchitobiase.

The results of the present study also show that several genes characterized as late responsive genes were differentially expressed in F1 and/or F3 (Table 1, Figure 3). These genes are involved in new cuticle formation (cuticle proteins and precursor genes of chitin synthesis: glucosamine 6-phosphate N-acetyltransferase – GNA1 and facilitated trehalose transporter–TRE11), chitin degradation (chitinase 2–Cht2, chitinase 3–Cht2 and N-acetylchitobiase–CTBS), and cuticle protein degradation (Cathepsin-L1), with most being downregulated in F1 and F3 (Figure 3 and Table 1). Moreover, the expression of an actin gene (Actin 5, muscle specific) was also depressed in F1 and F3 (Figure 3 and Table 1). Actin participates in muscle contraction needed to split the old cuticle during molting and is a component of cytoskeleton (Lažetić and Fay, 2017; Zhang et al., 2014a). Interestingly, it has been hypothesized that actin genes are late responsive genes in the ecdysteroid cascade with potential to be targeted by ecdysteroids, but its early responsive gene is unknown (Qian *et al.*, 2014). Furthermore, Carbonic anhydrase genes (CA1, CA2) and N66 matrix protein (N66), which are suggested to be under the direct or indirect control of E20 and targeting exoskeletal mineralization (Calhoun and Zou, 2016), were also found to be dysregulated in F1 and F3 (Figure 3 and Table 1). Still, our transcriptome analyses shown a downregulation of angiotensin converting enzyme (ACE), which has an important role in insects´ metamorphosis and is under the direct or indirect control of 20E (Isaac et al., 2007; Macours et al., 2004). The precise role of ACE in metamorphosis/molting of arthropods is not fully understood, but the study of Isaac et al. (2007) found that *Manduca sexta* larvae failed to complete the shedding of their old cuticle during the molt from fourth to fifth instar and died within 10 days.

Table 1 - Late responsive genes related with EcR/RXR pathway and their differential expression in F1 and F3 generations

Gene	F1 status	F3 status	E value/ bitscore	Reference ID Blast (n/u/p) DB (NT/NR)
pro-resilin			3.4e-32 / 147.9	XP_027228565.1
larval cuticle protein A2B			4.1e-41 /177.2	XP_018010163.1
cuticle protein 8			2.0e-30 / 141.7	XP_018022126.1
larval cuticle protein 8			6.0e-55 / 223.4	XP_018028050.1
cuticle protein AM/CP1114			1.6e-54 / 222.2	XP_018028051.1
cuticle protein CP575			7.25e-38 / 172	XM_018172370.1
cuticle protein AMP1A			1.9e-45 / 192.2	XP_018028055.1
cuticle protein 18.6			1.2e-34 / 155.6	XP_018010162.1
cuticle protein AM1199			8.6e-48 / 199.5	XP_018025034.1
adult-specific cuticular protein ACP-20			2.5e-30 / 141.4	XP_018017515.1
larval cuticle protein LCP-22			6.0e-37 / 163.7	XP_018018467.1
cuticle protein AMP5			1.4e-52 / 215.7	XP_018011663.1
pupal cuticle protein 36a		-	3.07e-32 / 152	XM_027360260.1
cuticle protein 19.8			1.3e-30 / 142.5	XP_018014406.1
cuticle protein 7		-	1.2e-33 / 153.3	XP_018010161.1
cuticle protein 19			3.1e-39 / 171.0	XP_018010165.1
cuticle protein AM1159			6.4e-44 / 186.8	XP_018015052.1

endocuticle structural glycoprotein SgAbd-2			3.5e-37 / 164.1	XP_018015001.1
larval cuticle protein 14		-	4.8e-41 / 177.9	XP_018012764.1
Chitinase 3			6.9e-303 / 1050.4	XP_018025864.1
N-acetylchitobiase		-	8.9e-112 / 413.7	XP_018021134.1
Chitinase 2, partial		-	4.6e-180 / 641.0	XP_018008554.1
glucosamine 6-phosphate N-acetyltransferase		-	8.9e-49 / 202.6	XP_018023774.1
facilitated trehalose transporter Tret 1-2		-	1.2e-121 / 446.8	XP_018013786.1
Cathepsin-L1 like			1.6e-117 / 432.6	XP_018009840.1
N66 matrix protein			4.4e-39 / 171.0	XP_018018908.1
actin-5, muscle-specific-like			8.5e-70 / 272.7	XP_027220903.1
Carbonic anhydrase 1			1.8e-55 / 226.1	XP_027209605.1
Carbonic anhydrase 2			1e-66 / 261.9	XP_018019224.1

Red indicates downregulation, green indicates upregulation; (-) indicates no changes in gene expression. All sequences used as reference were from *Hyalella azteca* except for XP_027228565.1, XM_027360260.1, XP_027209605.1 and XP_027220903.1 what were from *Penaeus vannamei.*

Taken together, the data here presented suggests that the inter and transgenerational SIM exposure interferes with the main endocrine signalling pathway in crustaceans, the ecdysteroid cascade, which may produce a delay in *G. locusta* molting. Although SIM effects on growth and reproduction have been previously demonstrated (Neuparth *et al.*, 2020a; Neuparth *et al.*, 2014) (Figure 2), these findings suggest that it would be important to perform dedicated functional experiments to evaluate the effects of SIM in the molt cycle of *G. locusta* in the sense of yield further insights to support a potential mechanistic linkage between SIM exposure and effects on molting.

It is well known that endocrine signalling processes regulate molting, growth and reproduction (Reddy et al., 2004; Xie et al., 2015). In order to grow, crustaceans must periodically change the exoskeleton in a process called ecdysis or molting. Furthermore, the crustacean's reproductive cycle is coordinated with molting because females´ mate and spawn occur immediately after ecdysis (Gao et al., 2015; Hyne, 2011). Thus, the findings of a potential molting cycle delay here proposed integrate well and may provide an explanation for the apical deleterious effects on growth and reproduction on *G. locusta* females and the gonad maturation delays reported in (Neuparth *et al.*, 2020a; Neuparth *et al.*, 2014). Neverthless, dedicated functional studies should be performed to explore this potential link. Moreover, the decrease of methyl farnesoate (MF) levels observed on *G. locusta* females (Neuparth *et al.*, 2020a; Neuparth *et al.*, 2014) integrates well with the downregulation of E75 described above. Several studies in crustaceans suggested that MF binds with high affinity to RXR and that MF synergizes with ecdysteroids to enhance gene transcription of early responsive genes mediated by RXR-EcR heterodimer, i.e., E75 (Hyde et al., 2019; Hyne, 2011; LeBlanc, 2007; Nagaraju, 2011; Wang and LeBlanc, 2009). At least in insects, E75 is a common element in both Juvenile Hormone (JH) and ecdysteroid signaling pathways. Dubrovskaya et al. (2004) reported that the simultaneous transcriptional activation of JH and ecdysone in insects suggests that E75 may have an important role in the developmental cross-talk of the two hormones.

3.2. Catecholamines pathway and its role in cuticle sclerotization and pigmentation

The crustaceans´ reproductive cycle is regulated by a complex endocrine regulatory network with other regulators beyond the ecdysteroids and MF. Other endogenous molecules, such as neuropeptides and biogenic amines, also have a central role in the homeostatic regulation of molting and reproduction (Hyne, 2011; LeBlanc, 2007; Swetha et al., 2011). Dopamine (DA) is an important catecholamine neurotransmitter in the central nervous system with a conserved role in several behavior processes in metazoans, such as feeding behavior, aversive learning, movement control, social interactions and development. In arthropods, DA is also involved in cuticle tanning (sclerotization and pigmentation), an essential process for exoskeleton formation, normal molt development, growth, and reproduction, but also for protection from exogenous physical injury (Hiruma and Riddiford, 2009). The first and rate-limiting step in DA biosynthesis is the hydroxylation of L-Tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA), catalyzed by tyrosine 3-monooxygenase, also known as Tyrosine Hydroxylase (TH) (Daubner et al., 2011). At least in insects, the expression and regulation of TH was shown to affect cuticle tanning. Functional studies revealed that, in several insects, TH knockdown leads to decreased cuticle hardness and pigmentation, corroborating its key role in cuticular tanning and molting (Gorman and Arakane, 2010; Qiao et al., 2016; Sterkel and Oliveira, 2017). Given that arthropods growth and reproduction are closely coordinated with molt cycles, and cuticle maintenance and molts are tightly associated, TH-dependent downregulation of several cuticle proteins (CPs) and cuticle sclerotization defects can result in growth and reproduction disruption. Another important enzyme for arthropod cuticle pigmentation is the serine protease Bi-vsp (VSP) involved in the synthesis of melanin used not only for exoskeletal pigmentation but also for cuticular hardening, wound healing, and innate immune responses (Arakane et al., 2016; Sugumaran and Barek, 2016). The transcriptome analysis performed in the present study shows that TH is downregulated in F1 and CPs are downregulated in both F1 and F3 (Figure 4, Table 1). Moreover, venom serine protease Bi-vsp (VSP), that activates phenol oxidase (PO) involved in the melanin biosynthesis, was found to be downregulated in F1 (Figure 4). However, this downregulation does not disrupt the transcription level of Prophenoloxidase activating enzyme (PPAE). The same can be observed for dihydropteridine reductase (DHPR) found to be non-responsive gene (Figure 4). These findings match well with the potential *G. locusta* molting cycle delay hypothesized above and with the observed growth and reproduction defects reported in *G. locusta* females (Neuparth *et al.*, 2020a) (Figure 2). However, as highlighted above, dedicated functional studies should be undertaken to study in detail this issue.

Other catecholamines, such norepinephrin (NEP) and epinephrine (EP), are also important neuroregulators (Tong et al., 2020). NEP and EP are synthesized in neuroendocrine tissues and are responsible for the regulation of metabolic, cardiovascular and immune responses in crustaceans

(Avramov et al., 2013; Chang et al., 2015; Gallo et al., 2016). Catecholamines, particularly DA, mediate the release of several critical neuro-hormones, such as Crustacean Hyperglycaemic Hormone (CHH) and alterations on CHH levels can lead to disruption of important biological processes such as sugar and lipid metabolism (Bulau et al., 2003; Chang, 2005) and osmoregulation (Chung and Webster, 2006). In our transcriptome, besides the downregulation of TH and VSP mentioned above, we also detected, in F1, a downregulation of Dopamine B- hydroxylase (DBH) that catalyzes the conversion of DA to NEP/EP, and a downregulation of CHH.

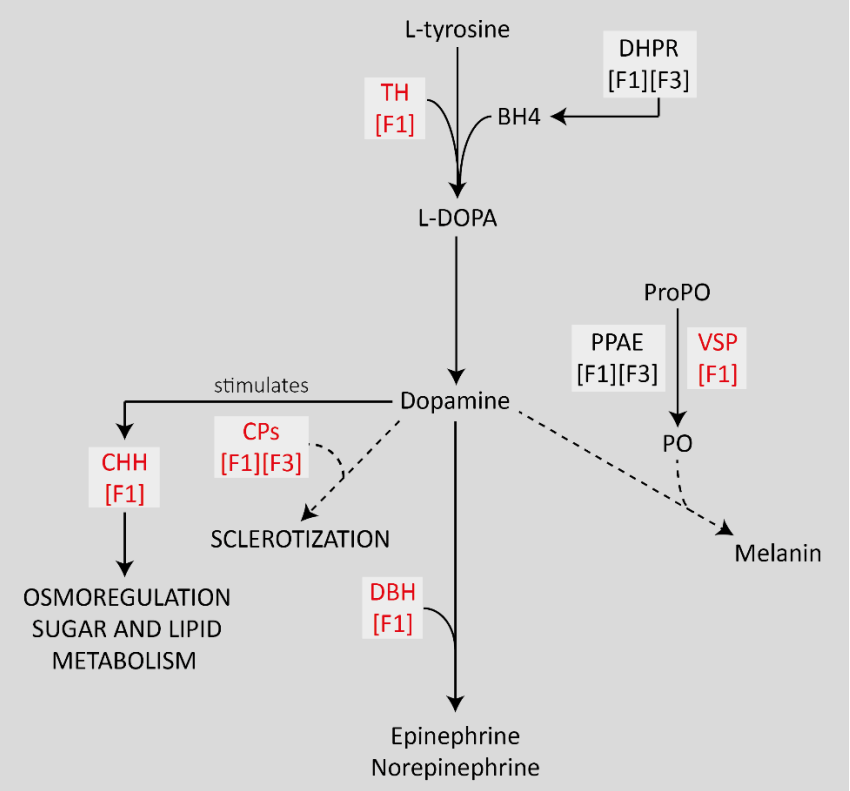

Figure 4 – Catecholamines pathway in Crustaceans. Genes modulated by SIM and non-responsive genes in F1 and F3 generations are presented in boxes. Red text inside the boxes indicates gene expression downregulation in F1 and/or F3 generations and black text inside the boxes indicates unchanged gene expression in F1 and F3 generations. No gene was found to be upregulated in this pathway. . TH –Tyrosine hydroxylase; DHPR – dihydropteridine reductase; BH4 – tetrahydrobiopterin; L-DOPA – L-3,4-dihydroxyphenylalanine; CPs – cuticle proteins; ProPO – Pro phenol oxidase; PO – Phenol oxidase hyperglycemic hormone; VSP – venom serine protease Bi-vsp; PPAE – prophenoloxidase activating enzyme; CHH – crustacean hyperglycemic hormone; DBH – dopamine beta-hydroxylase.

3.3. NO/cGMP/PKG signalling pathway

As aforementioned, MIH can negatively control the synthesis of ecdysteroids. MIH regulates the NO/cGMP/PKG signalling, critical for ecdysis (Lee and Mykles, 2006). There is consensus that cyclic guanosine monophosphate (cGMP) acts as an intracellular signalling messenger of MIH-mediated action (Chen et al., 2018; Covi, 2012; Francis et al., 2010; Nagai et al., 2009; Xu et al., 2019), leading to an activation of cGMP-dependent protein kinase G (PKG) and subsequent inhibition of ecdysteroidogenesis (Covi et al., 2009; Mykles et al., 2010). Covi *et al.* (2009) found that MIH induces a sustained increase in cGMP, by the activation of membrane-bound guanylyl cyclases (GYC32E and

GUCY1B1) that convert guanosine triphosphate (GTP) to cGMP. In the present study, we observed a downregulation of GYC32E and GUCY1B1 in F1 and F3, indicative of a decrease in cGMP, althougth the receptor-type guanylate cyclase Gyc76C (GYC76C) and the soluble guanylate cyclase 89Db (GYC89DB) remained unchanged (Figure 5). Interestingly, a downregulation of cGMP-specific 3',5'-cyclic phosphodiesterase (PDE5A), that prevents the conversion of cGMP to its inactive form guanosine monophosphate (GMP), was also downregulated in F1 and F3, suggesting a cGMP triggered action (Figure 5), even though 3',5'-cyclic phosphodiesterase pde-4 (PDE4) was non-responsive. Also, the guanylate kinase-like isoform X1 (GUK1), involved in cGMP and GMP recycling, was unchanged. However, a downregulation of neprilysin-1 (NEP1) that promotes the degradation of atrial natriuretic peptide (ANP) was observed in F1 and F3 which indicates an activation of cGMP, since more ANP is available for cGMP synthesis. However, the atrial natriuretic peptide receptor B (ANR) was found to be downregulated in F1 and F3, suggesting a decrease of cGMP, since the binding of ANP to its receptor activates the synthesis of cGMP (Chen et al., 2008; Piggott et al., 2006) (Figure 5). Thus, with these findings taken together, we hypothesize that SIM triggers a feedback mechanism to control cGMP levels and subsequent inhibition of ecdysteroidogenesis through the balances between the action of GUCYB1/GYC32E and PDE5A and between NEP1 and ANR in F1 and F3**.**

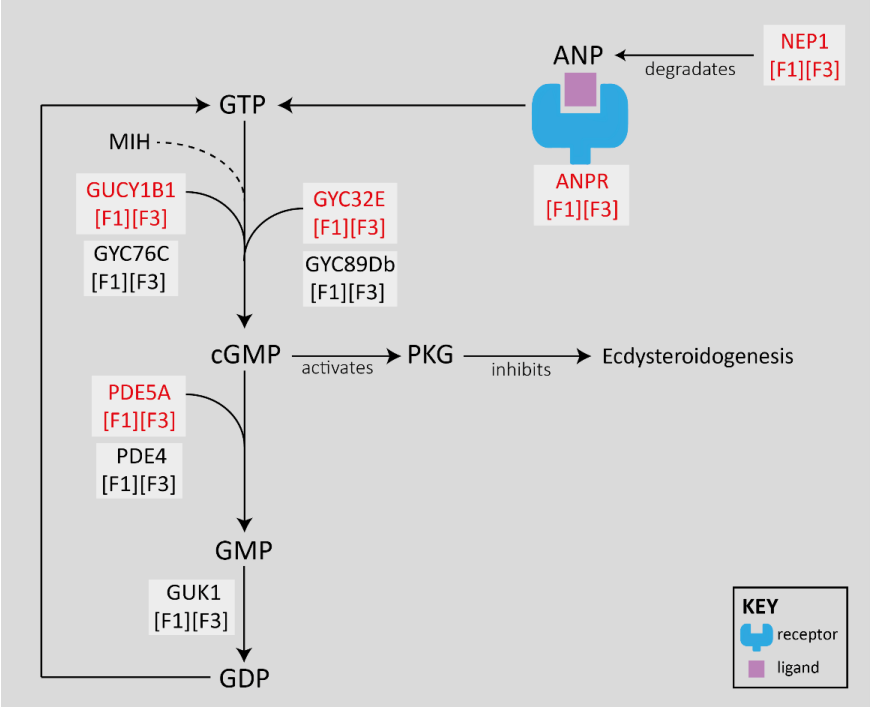

Figure 5 – NO/cGMP/PKG signalling pathway in Crustaceans. Genes modulated by SIM and non-responsive genes in F1 and F3 generations are presented in boxes. Red text inside the boxes indicates gene expression downregulation in F1 and/or F3 generations and black text inside the boxes indicates unchanged gene expression in F1 and F3 generations. No gene was found to be upregulated in this pathway.. MIH – molt inhibiting hormone; GTP – guanosine triphosphate; cGMP – cyclic guanosine monophosphate; PKG – cGMP-dependent protein kinase; GYC32E – guanylate cyclase 32E; GUCY1B1 – guanylate cyclase soluble subunit beta-1; GYC76C – receptor-type guanylate cyclase Gyc76C; GYC89DB – soluble guanylate cyclase 89Db; PDE5A – cGMP-specific 3',5'-cyclic phosphodiesterase; PDE4 – 3',5'-cyclic phosphodiesterase pde-4; GMP – guanosine monophosphate; GUK1 – guanylate kinase isoform X1; GDP – guanosine diphosphate; NEP1 – neprilysin1; ANP – atrial natriuretic peptide; ANPR – atrial natriuretic peptide receptor B.

3.4. Cholinergic synapse pathway

Given the importance of neural regulation for the homeostatic control (Legradi et al., 2018), we also assessed whether additional pathways/genes related with neural/behavioral regulation were modulated by SIM. Interestingly, the transcriptomic analysis showed that several genes related with the synthesis, transport, and release of the neurotransmitter acetylcholine (Ach), which plays a key role in the regulation of behavior, cognition, and control of muscle contraction, is downregulated in F1 and/or F3 (Figure 6). The cholinergic synapse pathway is a highly conserved evolutionary pathway in eukaryotes, vital for the normal function of the sensorial and neuromuscular systems (Bossy et al., 1988; Zhang et al., 2014b). Even though the choline transporter (CHT2) and some acetylcholine receptors subunits (ACRb1 and CHRNAa4) were found to be non-responsive to SIM, we observed that SIM severely disrupts the cholinergic synapse pathway, as choline O-acetyltransferase (ChAT), choline (Ch) and acetylcholine transporters (SLC5A7 and VAChT, respectively), acetylcholine receptors - nicotinic α7-like subunits (CHRNA7 and ACR-16) and Acetylcholinesterase (AChE) are all downregulated in F1 and ChAT, SLC5A7, VAChT, CHRNA7 and AChE are also downregulated in F3 (Figure 6). Since this pathway is critical for the normal function of cholinergic transmission—i.e., the communication of neurons in the nervous system, including motor neurons, that will activate muscle contraction—we hypothesize that inter and transgenerational exposure to SIM could modulate the muscle system of *G. locusta*. Interestingly, previous studies reported the occurrence of adverse neuromuscular reaction upon administration of SIM, possibly mediated by the nicotinic acetylcholine receptor (nAChRs) affecting nerve transmission across the synapse and resulting in muscle weakness and pain by neuromuscular degeneration (Grajales-Reyes et al., 2013). Our findings also suggest that the observed SIM modulation of the cholinergic synapse pathway could potentially disturb the molting cycle of *G. locusta* in F1 and F3 generations. In fact, simultaneous contractions of specialized muscle in arthropods are required to split the old cuticle during molting and generate pressure pulses for body expansion (Nako et al., 2018). Molting, as described above, is a key process for crustaceans´ growth and reproduction. Therefore, the inter and transgenerational disruption of the cholinergic synapse pathway could be potentially linked with the observed effects of SIM on *G. locusta* growth and reproduction previously reported (Figure 2) (Neuparth *et al.*, 2020a; Neuparth *et al.*, 2014) and be potentially related with the ecdysteroid cascade disturbance described above, also involved in the regulation of molting, reproduction and growth.

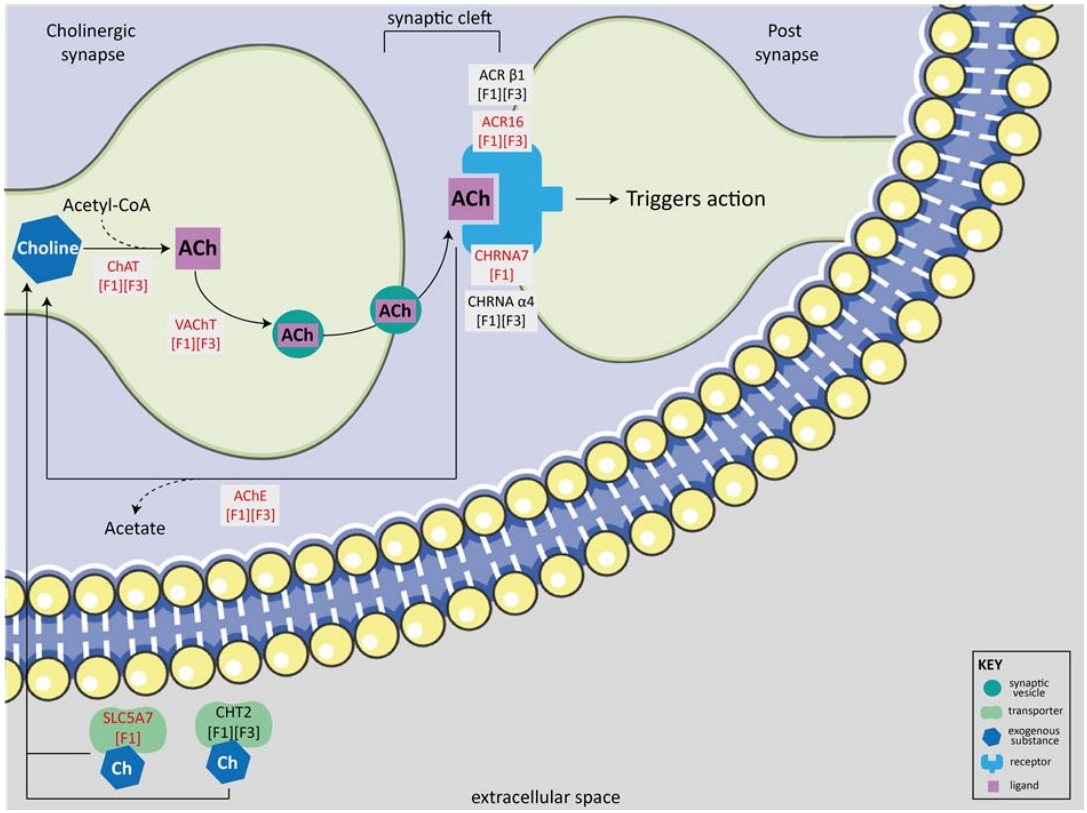

Figure 6 – Cholinergic synapse pathway in Crustaceans. Genes modulated by SIM and non-responsive genes in F1 and F3 generations are presented in boxes. Red text inside the boxes indicates gene expression downregulation in F1 and/or F3 generations and black text inside the boxes indicates unchanged gene expression in F1 and F3 generations. No gene was found to be upregulated in this pathway. . Ch – Choline; Ach – Acetylcholine; ChAT – choline O-acetltransferase; ACR16 – acetylcholine receptor subunit alpha-type ACR-16-like; ACR b1 – acetylcholine receptor subunit beta; CHRNA7 – acetylcholine receptor subunit alpha-7-like; CHRNA α4 – acetylcholine receptor subunit alpha-4; SLC5A7 – high affinity choline transporter 1; CHT2 – choline transporter protein 2; VAChT – vesicular acetylcholine transporter 1; AChE – acetylcholinesterase.

3.5. GABAergic signalling pathway

A downregulation of several proteins involved in the neurotransmitter γ-aminobutyric acid (GABA) cycle was also observed. GABA is primary involved in hyperpolarising inhibitory synaptic transmission by counterbalancing the depolarization of neuronal membranes (Crowley et al., 2016) and is synthesized from L-glutamate, the principal excitatory neurotransmitter involved in processes such as synaptic memory and plasticity (Benarroch, 2010; Fagg and Foster, 1983; Fonnum, 1984). Its action reduces the excessive glutamatergic signalling and subsequent excitotoxicity, acting as a feedback control system (Gonzalez-Burgos and Lewis, 2008; Lau and Tymianski, 2010), regulating neural activity and energy metabolism and thus, modulating brain homeostasis (Takesian and Hensch, 2013; Xu et al., 2011). Figure 7 displays the transcriptomic results of GABA signalling pathway where downregulation of genes coding for key proteins in F1 was observed: i.e., glutaminase liver, mitochondrial-like (GLS), 4-aminobutyrate aminotransferase (ABAT), GABA receptor delta (GABA-R) and glutaminase, sodium and chloride dependent GABA transporter 1-like (GAT1) that are involved in the action, transport and degradation of GABA. On the other hand, it was also observed that some genes related with the interaction, anchoring and organization of GABA receptors with the cytoskeleton, such as gephyrin

(GPHN) and gamma-aminobutyric acid receptor-associated protein (GABARAP), and the formation of ketoglutarate from glutamate, i.e., mitochondrial glutamate dehydrogenase 1 (GAD), were non-responsive to SIM.

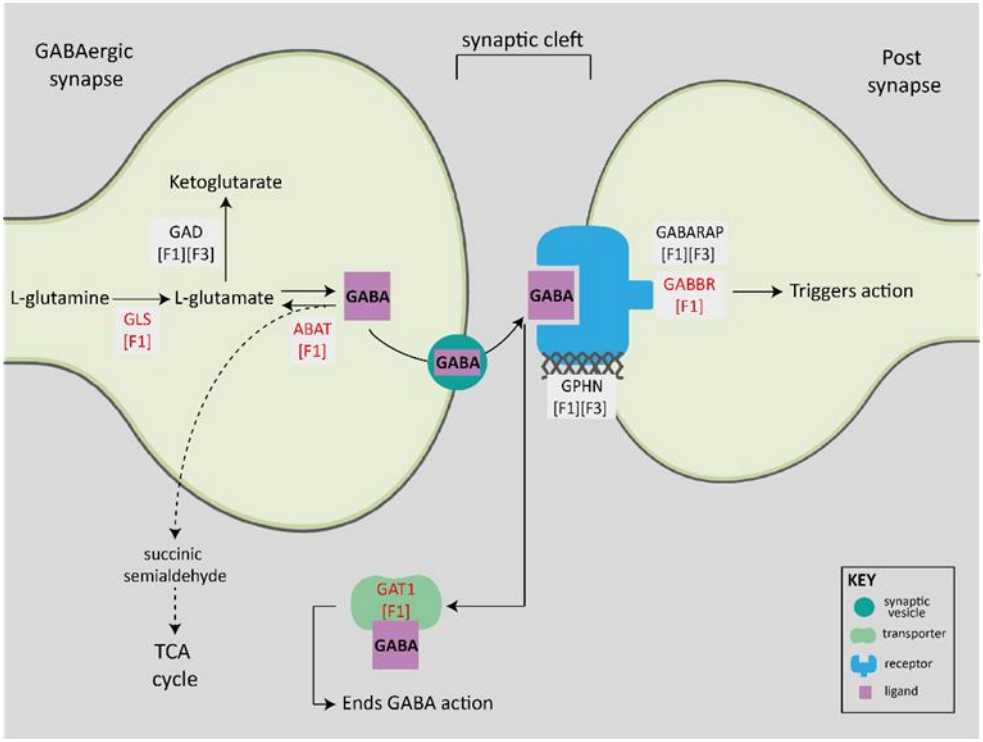

Figure 7 – GABAergic signalling pathway in Crustaceans. Genes modulated by SIM and non-responsive genes in F1 and F3 generations are presented in boxes. Red text inside the boxes indicates gene expression downregulation in F1 and/or F3 generations and black text inside the boxes indicates unchanged gene expression in F1 and F3 generations. No gene was found to be upregulated in this pathway.. GLS – glutaminase liver mitochondrial-like; GAD – mitochondrial glutamate dehydrogenase 1; ABAT – 4-aminobutyrate aminotransferase; GPHN – gephyrin; GABA-R – GABA receptor delta; GABARAP – gamma-aminobutyric acid receptor-associated protein; GAT1 – sodium and chloride dependent GABA transporter-1-like.

Besides the neuroendocrine pathways discussed above, table 2 displays additional genes involved in the regulation of nervous and neuromuscular functions that showed a shift in gene expression profile after inter and transgenerational SIM exposure.

Table 2 –Genes related with neurotoxic effects and their respective function

Gene	F1 status	F3 status	Evalue/ Bit score	Function	Species
prohormone-4-like isoform X1		-	1.3e-86 329.3	Cleaved into the following six chains: Brain peptides IDLSRFYGHFNT, IDLSRFYGHFN, IDLSRFYGHF, DLSRFYGHFNT, DLSRFYGHFN or Brain peptide DLSRFYGHF	*Ame*
putative lachesin-like			2.9e-94 356.7	Functions as a homophilic cell-adhesion molecule. May play a role in early neuronal differentiation and axon outgrowth	*Dme*
prohormone-3-like		-	1.6e-34 156.0	Cleaved into Brain peptide ITGQGNRIF	*Ame*
prohormone-1-like			3.1e-31 144.8	Cleaved into three chains: Brain peptide LRNQLDIGDLQ, Brain peptide LRNQLDIGDL and Brain peptide SYWKQCAFNAVSCF-amide	*Ame*
synaptobrevin-1-like isoform X4		-	1.1e-48 202.6	Acts in neuronal exocytosis of synaptic transmission. Potential role in cholinergic transmission	*Cbr*
protein unc-79 homolog			8.5e-299 1036.9	Component of the NALCN channel is responsible for Na(+) leak currents and activated by neuropeptides	*Hsa*
neuropilin-1-like isoform X1			2.1e-99 372.5	Receptor involved in the formation of certain neuronal circuits and in organogenesis outside the nervous system.	*Hsa*
neprilysin-1-like			5.9e-109 405.2	Metalloendoprotease which functions in fertility and memory formation.	*Dme*

zwei Ig domain protein zig-8-like			3.2e-98 368.6	Required postembryonically to maintain the position of several neuron cell bodies and ventral nerve cord axons of specific neurons	*Cel*
neurotrophin 1-like			1.3e-90 342.4	Ligand for the Toll-related receptors, promoting motor axon targeting and neuronal survival	*Dme*
putative neural-cadherin 2			2.9e-261 912.5	Cell adhesion proteins involved in the transmission of developmental information	*Dme*
contactin-6-like			0.0e+00 1128.6	Mediate cell surface interactions. Participates in oligodendrocytes generation by acting as a ligand of NOTCH1.	*Hsa*
potassium voltage-gated channel subfamily B member 1-like isoform X1			1.3e-54 223.4	Mediates transmembrane transport in excitable membranes Regulation of action potential in neurons, muscle and endocrine cells	*Hsa*
irregular chiasm C-roughest protein-like			2.5e-308 1067.8	Required for correct axonal pathway formation in the optic lobe and for programmed cell death in the developing retina.	*Dme*
putative neurotrimin-like			9.2e-69 271.2	Neural cell adhesion molecule	*Hsa*
protein RUFY3-like isoform X1		-	1.0e-176 630.2	Plays a role in the generation of neuronal polarity formation and axon growth	*Hsa*
Down syndrome cell adhesion molecule-like protein Dscam2			0.0e+00 1729.9	Cell adhesion molecule. Involved in axon guidance.	*Dme*
ninjurin-2-like isoform X2		-	6.0e-39 170.6	Homophilic cell adhesion molecule that promotes axonal growth. May play a role in nerve regeneration and in the formation and function of other tissues	*Hsa*
protein turtle homolog B-like isoform X7		-	1.3e-53 220.7	Abundantly expressed in interneurons, where it may regulate inhibitory synapse development	*Hsa*
Down syndrome cell adhesion molecule-like protein 1 homolog, partial			1.4e-196 696.8	Cell adhesion molecule that plays a role in neuronal self-avoidance.	*Hsa*
sodium-coupled monocarboxylate transporter 1-like		-	2.8e-210 741.5	May play a critical role in an electrochemical Na^+ gradient on neurons. Maintenance of the energy status and function of neurons.	*Hsa*
putative neurotrypsin-like		-	3.0e-274 954.5	Plays a role in neuronal plasticity and is associated with learning and memory operations	*Hsa*
neuroligin-4, Y-linked-like		-	0.0e+00 1387.1	Neuronal cell surface protein involved in cell-cell-interactions	*Hsa*
neuroligin-4, X-linked like			1.8e-295 1025.4	Neuronal cell surface protein involved in cell-cell-interaction	*Hsa*
neuroligin-2-like, partial			1.4e-219 772.3	Involved in cell-cell interactions via its interactions with neurexin family members. Mediates cell-cell interactions both in neurons and in other types of cells	*Hsa*
synaptotagmin-11-like isoform X2			5.7e-179 637.1	Plays an important role in dopamine transmission by regulating endocytosis and the vesicle-recycling process	*Hsa*
arylsulfatase B-like			2.8e-78 302.0	Regulator of neurite outgrowth and neuronal plasticity	*Hsa*
RNA-binding protein Musashi homolog Rbp6-like			4.3e-66 261.2	May play a role in the proliferation and maintenance of stem cells in the central nervous system	*Dme*
ionotropic receptor 25a-like		-	0.0e+00 1389.4	Integral part of various neural sensory systems in the antenna that provide the neural basis for the response to environmental changes	*Dme*
serine/threonine-protein kinase BRSK2-like isoform X2		-	3.4e-296 1027.7	Serine/threonine-protein kinase that plays a key role in polarization of neurons and axonogenesis,	*Hsa*
innexin inx1-like isoform X2		-	1.2e-31 145.6	Structural component of the gap junctions	*Dme*
myelin P2 protein-like		-	4.5e-49 203.8	May play a role in lipid transport protein in Schwann cells	*Hsa*
calcium-activated potassium channel slowpoke-like			1.9e-252 881.3	Potassium channel activated by both membrane depolarization or increase in cytosolic Ca^{2+} that mediates export of K^+. Its activation dampens the excitatory events that elevate the cytosolic Ca^{2+} concentration and/or depolarize the cell membrane. It therefore contributes to repolarization of the membrane potential.	*Dme*
peptidyl-glycine alpha-amidating monooxygenase B-like		-	4.9e-32 147.5	Bifunctional enzyme that catalyzes the post-translational modification of inactive peptidylglycine precursors to the corresponding bioactive alpha-amidated peptides, a terminal modification in biosynthesis of many neural and endocrine peptides	*Hsa*
peptidyl-alpha-hydroxyglycine-alpha-amidating lyase I		-	1.8e-76 296.2	Probable lyase that catalyzes an essential reaction in C-terminal alpha-amidation of neuropeptides	*Dme*
peptidyl-alpha-hydroxyglycine alpha-amidating lyase 2-like		-	2.8e-88 335.5	Probable lyase that catalyzes an essential reaction in C-terminal alpha-amidation of neuropeptides	*Dme*
neuropeptide SIFamide receptor-like		-	2.8e-159 573.5	Controls different reproductive behaviours, *e.g.*, sexual receptivity in females, dominance and aggression behaviours	*Dme*

521 Red indicates downregulation, green indicates upregulation; (-) indicates no changes in gene expression; Hsa – *Homo sapiens*;
522 Ame – *Apis mellifera*, Dme – *Drosophila melanogaster*, Cbr - *Caenorhabditis briggsae*, Cel - *Caenorhabditis elegans*
523

4. Conclusions

Overall, we demonstrate here that inter and transgenerational exposure to SIM, at environmentally relevant levels, has significant effects in the regulation of key signaling pathways involved in crustacean neuroendocrine regulation, concomitantly to changes in apical endpoints, such as depressed reproduction and growth. The neuroendocrine system plays a major role in metazoan homeostatic control, particularly in *taxa* displaying a central neuroendocrine system: regulating behavior, reproduction, molting, among other major biological processes. The neuroendocrine metabolic pathways here designed provide additional genomic information in this poorly annotated species and will represent a reference source for further and more focused neuroendocrine molecular analysis on this and other amphipod species.

Yet, despite the biological importance of neuroendocrine regulation, the impact of environmental chemicals at this level has been poorly studied. Therefore, the findings reported here support the importance of addressing the effects of environmental chemicals in neuroendocrine regulation, as a step towards improving hazard and risk assessment of biological active compounds. Importantly, the disruption in signaling pathways reported here was not only observed in F1 but also in the F3 generation, which implies transgenerational effects. Neuparth et al. (2020a,b) reported the modulation of several genes involved in the regulation of the epigenome in F3, which may explain the observed transgenerational effects. A detailed evaluation of the epigenetic machinery involved in this disruption should be disclosed in the future, given that environmental chemicals inducing transgenerational effects over several non-exposed generations are of major concern.

5. Authorship contribution statement

T. Neuparth: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition. **N. Alves**: Conceptualization, Methodology Formal analysis, Investigation, Data curation, Data curation, Writing – original draft, Writing - review & editing. **A.M. Machado**: Formal analysis, Investigation, Data curation. **M. Pinheiro:** Formal analysis, Investigation, Data curation, Writing - review & editing. **R. Montes**: Investigation, Writing - review & editing. **R. Rodil:** Investigation, Writing - review & editing. **S. Barros**: Formal analysis, Investigation, Data curation, Writing - review & editing. **R. Ruivo**: Formal analysis, Investigation, Data curation, Writing - review & editing. **L. Filipe C. Castro:** Validation, Writing - review & editing. **J.B. Quintana:** Investigation, Writing - review & editing, Funding acquisition. **M.M. Santos:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

6. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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