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STUDY OF INVERTEBRATE-SPECIFIC EFFECTS OF ENDOCRINE DISRUPTING
CHEMICALS IN THE ESTUARINE MYSID *NEOMYSIS INTEGER* (LEACH, 1814)

Thesis submitted in fulfillment of the requirements
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Dutch translation of the title:

Studie van invertebraat-specifieke effecten van endocrien-verstorende stoffen in de estuariene aasgarnaal *Neomysis integer* (Leach, 1814)

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CHAPTER 9

GENERAL CONCLUSIONS AND RESEARCH PERSPECTIVES

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Despite the many studies on endocrine disruption using vertebrate models published over the past decade, few studies on invertebrates are available. This is surprising since 95% of all animal species do not have a backbone. Two main reasons for this observation can be given. First, initial studies with invertebrates were directly based on those with vertebrates thereby ignoring major differences in hormonal control strategies between both groups. Second, basic understanding of hormonal regulation in most invertebrates is still largely lacking. Some groups of invertebrates like the arthropods offer major advantages in this perspective as their hormone systems have been characterized in detail. For example, it has been well established that in arthropods and other ecdysozoa (animals that grow through molting) ecdysteroids (molting hormones) are major endocrine signaling molecules involved in the control of physiological processes such as molting, embryonic development, metamorphosis, and reproduction. To date, the uniqueness of this hormonal regulation is not reflected in proposed regulatory screening and testing programs that only focus on estrogen, androgen, and thyroid signaling which are not functional hormones in arthropods. Since arthropod models have been proposed for inclusion in many regulatory screening and testing programs for endocrine disruptors, assays are urgently needed to assess chemical interference with ecdysteroid signaling as a way of identifying invertebrate-specific endocrine toxicity. Specifically, mysids are the only invertebrates that have been proposed by the US Environmental Protection Agency in their endocrine disruptor testing and screening program.

The aim of this doctoral thesis is to specifically address the issues raised above by developing novel methods to evaluate invertebrate-specific endocrine disruption using the mysid shrimp *Neomysis integer* (Crustacea: Mysidacea). A number of crucial physiological processes that

are under ecdysteroid control were selected for this purpose, **vitellogenesis**, **molting**, and **embryonic development**.

Vitellogenesis involves the production of the egg yolk protein vitellin. This protein is the major source of nourishment during embryonic development of egg-laying invertebrates and vertebrates. Upregulation of vitellogenin, the precursor of vitellin, has been a reliable way of quantifying estrogenic exposure in fish. In ecdysozoans, vitellogenesis is known to be under ecdysteroid control. However, little research has been done on crustacean vitellogenesis following exposure to endocrine disrupting chemicals. In this study, we purified and characterized vitellin from the mysid *Neomysis integer* (Chapter 2) and subsequently developed a quantitative enzyme-linked immunosorbent assay (ELISA) (Chapter 3). The availability of a mysid vitellin ELISA is of particular importance for two reasons: (1) as a research tool to study the hormonal control of vitellogenesis in a crustacean (2) as a potential assay to study chemically-induced disruptions in mysid vitellogenesis and how this relates to effects on well-established reproductive endpoints. In Chapter 4 we evaluated the usefulness of the *N. integer* vitellin ELISA to detect potential effects of three suspected endocrine-disrupting chemicals on mysid vitellogenesis. From these studies we can conclude that while developing an ELISA method is time-consuming, once established it is relatively rapid and easy to use. Our studies determined the size of *N. integer* vitellin (~700 kDa) and found that it is associated with a carotenoid moiety that facilitates the identification of vitellin during gel filtration. A protein elution profile will show two peaks with absorptions at both 474 nm (carotenoid) and 280nm (protein). Our purified vitellin was injected into rabbits to produce polyclonal antibodies. With the purified vitellin and the polyclonal antibodies, the ELISA was optimised. The time needed to optimise the final ELISA assay depends on the accuracy that one wants to achieve. The production of the polyclonal antibodies took a few months and the development of the mysid vitellin ELISA took approximately 3 to 4 weeks to complete.

In the next step, the ELISA was used to study the effects of methoprene (insecticide), nonylphenol (surfactant and xeno-estrogen) and estrone (natural estrogen) on the vitellogenesis of *N. integer* (Chapter 4). These studies further added to the weight-of-evidence that estrogens appear to be less effective in causing disruption of normal vitellogenesis in crustaceans than in oviparous vertebrates. Most likely, this is a result of the different hormonal control strategies for vitellogenesis in crustaceans compared with oviparous vertebrates. Future studies should therefore be aimed at the identification and quantification of the hormones, the hormone receptors and downstream hormone-responsive genes and gene

products involved in the control of vitellogenesis and other hormone-regulated processes in crustaceans. Overall, such studies should lead to a better understanding of the mode-of-action of chemicals on crustacean hormone-regulated processes.

In Chapter 8, preliminary steps were taken to validate the developed mysid vitellin ELISA method in the field by quantifying levels in ovigerous mysids collected from the Scheldt estuary. This study led to the first reported levels of vitellin in a mysid field population. Significant difference in vitellin levels were observed in females at different sampling sites in April 2005, and these could not be correlated with differences in either brood size or standard length. Similarly, no obvious correlations were observed between the abiotic factors (temperature, salinity, and dissolved oxygen) and the *in situ* vitellin levels. The most striking finding was significantly lower levels of vitellin in animals collected from the most upstream sites (S12, S15 and S22) compared to the most downstream site (S09). Verslycke et al. (2004b) previously studied seasonal and spatial patterns in energy allocation of *N. integer* in the Scheldt estuary at the same sites. *N. integer* in the latter study had less energy at the more upstream sites S15, and S22, where pollution was highest. Lower energy levels would be expected to impact a high energy-demanding process such as vitellogenesis. This would lead to eggs with lower vitellin levels that would be of lower quality (Arcos et al., 2003). While these spatial differences were significant in the spring campaign, no such differences were observed during the sampling campaign of July 2005. Although our study in the Scheldt estuary demonstrated the usefulness of the mysid vitellin ELISA to quantify vitellin in mysid field populations, a more comprehensive dataset needs to be compiled in the future to confirm the spatial trends observed in these preliminary studies.

Molting of *N. integer* was also studied as an invertebrate-specific endpoint to evaluate chemical interference with ecdysteroid signaling. The effects of the insecticide methoprene, a juvenile-hormone analog, on mysid molting were examined in Chapter 5. Crustaceans are an ecologically important part of the aquatic fauna, making this an essential group for assessing potential non-target effects of many pesticides - such as the mosquitocidal agent methoprene - that end up in aquatic ecosystems. Our study demonstrated that methoprene can indeed significantly affect mysid molting and growth at a sublethal concentration of 100 µg/l. This potential for invertebrate-specific endocrine toxicity of chemicals to non-target organisms is presently not addressed in regulatory screening and testing programs and could lead to a significant underestimation of the actual environmental risk of chemicals. Future studies should focus on measuring the hormones and receptors involved in mysid ecdysteroid

signaling to provide insights into the mode-of-action of juvenile hormone analogues and other pesticides in non-target arthropods and how this compares to what is known in insects.

Fockedey et al. (2005a) recently described **embryogenesis** in *N. integer*. Subsequently, we evaluated embryonic development in mysids as a tool to study the potential effects of endocrine disruptors (Chapters 6,7). In this study, we examined mysid embryonic development from oviposition to free-living juveniles following exposure to methoprene, nonylphenol, and estrone. Embryos exposed to 1 and 100 µg methoprene/l had a significantly lower hatching success and lower survival rates than embryos in the control treatment. Nonylphenol had no effect on the duration of the three different developmental stages, but it significantly reduced survival and hatching at the highest tested concentration (100 µg/l) compared to the control. Estrone only affected hatching at the highest tested concentration of 1 µg/l. In addition, we observed that hatched embryos exposed to 1 and 100 µg nonylphenol/l were more lethargic, and higher mortality was noted at these concentrations during the first two days after the final embryonic molt to free-living juveniles. Behavioral changes should be further examined in future studies and might provide sensitive and alternative ways of evaluating endocrine disruption.

In conclusion, the juvenoid methoprene was capable of interfering with all three ecdysteroid-regulated processes in *N. integer*. Methoprene affected mysid molting at 100 µg/l, embryogenesis at 1 µg/l, and lowered vitellin levels at all concentrations tested, although this latter effect was not statistically significant. It should be noted that differences in effect concentrations between these endpoints are to some extent a reflection of the exposure duration (i.e., 96h for vitellogenesis compared to 3 weeks for molting and 2 weeks for embryonic development) and therefore are not a direct indication of endpoint sensitivity. Interestingly, methoprene is a juvenile-hormone analog and not an ecdysteroid-analog. However, our studies and an increasing number of other studies have demonstrated that there is significant cross-communication between the juvenile hormone and ecdysteroid regulatory pathways (Mu and LeBlanc, 2004). Future studies could use a mechanistic approach to unravel further this crosstalk between regulatory pathways by quantifying the hormones in question, selecting chemicals with a known mode-of-action, by including other endpoints, and by doing lifecycle exposures in which all processes are quantified in a single exposure experiment.

Overall, this doctoral study produced data on chemical interference with three invertebrate-specific endpoints in a standard toxicity invertebrate model, the mysid *N. integer*. While a previous doctoral study by Verslycke (2003) highlighted the use of mysid models in endocrine disruption research, this doctoral work points out the need for a shift in paradigm away from vertebrate-type approaches toward an assessment of invertebrate-specific endocrine disruption. Much is still unknown about invertebrate endocrine systems and fundamental studies are urgently needed to get a mechanistic understanding of the effects of endocrine disruptors in invertebrates. Our results can provide a guide as to which processes and which chemicals can be used in these types of mechanistic studies. Specifically, ongoing studies are using molecular approaches to characterize hormone receptors involved in mysid ecdysteroid/juvenoid signaling (Soin et al., unpublished data; Verslycke et al., unpublished data). These studies will provide a crucial next step in our understanding of ecdysteroid disruption in mysids. However, not all chemicals with molt-interfering potency will exert their effect at the receptor level. As such, a combination of *in vivo* and *in vitro* assays will continue to be needed for screening effects of chemicals on crustacean. The endpoints developed in this doctoral study could be an important and integral part of an *in vivo* mysid assay to evaluate endocrine disruption.