Promotors: Prof . Dr. C. JANSSEN
Department of Applied ecology and environmental biology
Laboratory of Environmental Toxicology and Aquatic Ecology
Dr. Ir. T. VERSLYCKE
Woods Hole Oceanographic Insitution, Biology Department, USA

Dean: Prof. Dr. Ir. H. VAN LANGENHOVE

Rector: Prof. Dr. P. VAN CAUWENBERGE

AN GHEKIERE

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

Thesis submitted in fulfillment of the requirements
For the degree of Doctor (PhD) in Applied Biological Sciences

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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# LIST OF ABBREVIATIONS

| | |
|---|---|
| APE | alkylphenol ethoxylate |
| BCIP/NBT | 5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium |
| BSA | bovine serum albumin |
| CMP | cypris major protein |
| CSTEE | Scientific Committee on Toxicity, Ecotoxicity and the Environment |
| DNA | deoxyribonucleic acid |
| 20E | 20-hydroxyecdysone |
| EAC | ecotoxicological assessment criteria |
| EcR | ecdysteroid receptor |
| EDC | endocrine-disrupting compound |
| ELISA | enzyme-linked immunosorbent assay |
| EPA | Environmental Protection Agency |
| EXO | length of exopodite |
| GIH | gonad-inhibiting hormone |
| GR | growth rate |
| GSH | gonad-stimulating hormone |
| IgG | immunoglobulin G |
| IGR | insect growth regulator |
| IMP | intermolt period |
| JH-III | juvenile hormone III |
| $K_R$ | retardation coefficient |
| MCD | molt cycle duration |
| MF | methyl farnesoate |
| MI | molt increment or stepwise increase in size at ecdysis |
| MIH | molt-inhibiting hormone |
| MOIF | mandibular organ-inhibiting factor |
| NSB | non-specific binding |
| OECD | Organization for Economic Cooperation and Development |
| OPD | *o*-phenylenediamine dihydrochloride |
| OSPAR | Oslo and Paris Commission |
| PAGE | polyacrylamide gel electrophoresis |

| | |
|---|---|
| PAH | polycyclic aromatic hydrocarbons |
| PAS | periodic acid schiff |
| PBDE | polybrominated diphenyl ethers |
| PBS | phosphate buffered saline |
| PBS-T | phosphate buffered saline-tween20 |
| PCB | polychlorinated biphenyl |
| $R_f$ | electrophoretic mobility |
| SDS | sodium dodecyl sulfate |
| SETAC | Society of Environmental Toxicology and Chemistry |
| SL | standard length |
| TBT | tributyltin |
| USEPA | United States Environmental Protection Agency |
| USP | ultraspiracle protein |
| Vg | vitellogenin |
| Vt | vitellin |
| VIH | vitellogenesis-inhibiting hormone |
| VSH | vitellogenesis-stimulating hormone |
| VSOH | vitellogenesis-stimulating ovarian hormone |

# CHAPTER 1

## GENERAL INTRODUCTION

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## GENERAL INTRODUCTION

Many human-introduced and natural compounds in the environment can influence the endocrine system of animals (Colborn et al., 1993; Oberdörster and Cheek, 2000) and have been termed "endocrine-disrupting compounds" (EDCs). There has been increasing attention to the problem of EDCs ever since the hypothesis was put forward that possible declines in fertility and increases in specific cancers in humans could be due to the ubiquitous presence in our environment of chemicals with hormone-mimicking capacities. These compounds can disrupt major hormone-regulated processes, including growth, reproduction and sexual differentiation. Given the presence of thousands of anthropogenic compounds in today's environment, and given the complexity and diversity of hormone-regulatory pathways in animals, the possible mechanisms for disruption and the range of effects are enormous. As such, only a small fraction of this potential endocrine-disrupting capacity has been investigated so far.

To date, most studies have focused on endocrine disruption in vertebrates, including mammals, fish, birds and reptiles. The most cited examples include thyroid dysfunction in birds and fish; decreased fertility, metabolic abnormalities, masculinization and feminization in birds, fish, and mammals; decreased hatching success in birds, fish and turtles; behavioral abnormalities in birds; and compromised immune systems in birds and mammals (Krimsky. 2000).

Invertebrates constitute about 95% of all animal species and occupy an important position in many foodwebs. Still, relatively little research has been directed at understanding the potential effects of EDCs on this group of species. This is mainly due to a shortage in fundamental understanding of endocrine regulation in many invertebrate species. Among the invertebrates,

there is only one well-documented example of environmental endocrine disruption to date. Imposex (the superposition of male characteristics in female snails) is caused by exposure to a compound used in antifouling paints on ships, tributyltin or TBT (Fent , 1996) and it has been observed in 150 different species of marine snails (Matthiessen et al., 1999). It is estimated, for example, that all populations of dogwhelk (*Nucella lapillus*) in the coastal areas of the North Sea are affected to some extent by imposex, leading to complete population loss in some areas (Vos et al., 2000). An international SETAC (Society of Environmental Toxicology and Chemistry) workshop on endocrine disruption in invertebrates, held in the Netherlands in 1998 (DeFur et al., 1999), identified insects and crustaceans as potential organisms for evaluating EDCs because of the 'wealth' of information available on their endocrinology compared to other invertebrates (Chang, 1993; Downer and Laufer, 1983; Laufer and Downer, 1988; LeBlanc, 1999; Oberdörster and Cheek, 2000, Verslycke et al., 2004a).

This chapter gives a brief introduction on the crustacean endocrine system, with special reference to the endocrine regulation of molting, vitellogenesis and reproduction, processes that were explored in the mysid crustacean *Neomysis integer* for this doctoral research. An introduction is given to the biology and ecology of the test species *N. integer*, as well as on its use in genereal toxicity and endocrine-disruption testing. In the final part of this chapter, a literature overview on the specific endpoints selected for this doctoral study is presented.

## 1.1 CRUSTACEAN ENDOCRINOLOGY – A BRIEF INTRODUCTION

Hormonal regulation of physiological processes is common to all animals but some of these processes, such as molting, are unique to specific groups of invertebrates. Several reviews have been published on crustacean endocrinology, some dating back to the early 1920s and 1930s. Among the relatively recent reviews, Quackenbush (1986) presented a literature overview on the four types of compounds that play a role in the regulation of crustacean physiology: peptides, steroids, terpenoids, and biogenic amines (Fig. 1.1). Later reviews approached crustacean endocrinology by focusing on specific physiological processes, particularly growth and reproduction (Chang, 1997a; Charmantier et al., 1997; Fingerman, 1997; Subramoniam, 2000).

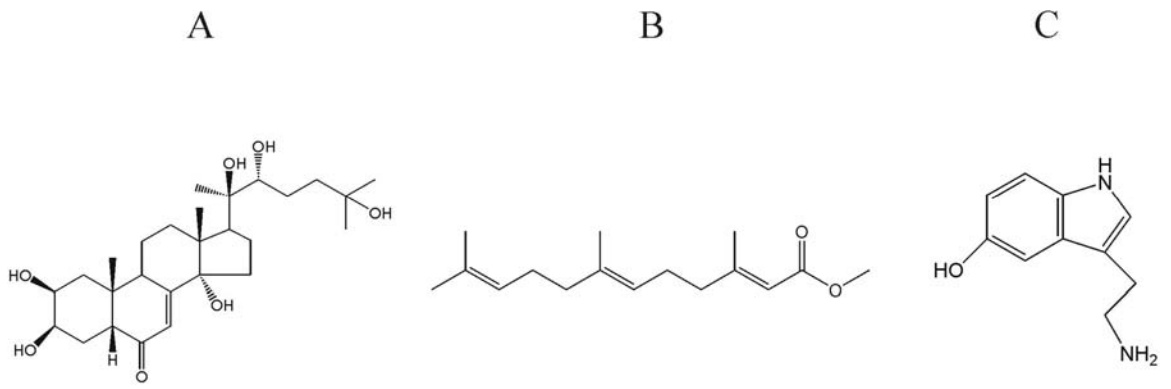


**Figure 1.1:** Four groups of compounds play an important rol in regulating crustacean physiology: steroids (A; the ecdysteroid 20-hydroxyecdysone), terpenoids (B; methyl farnesoate), biogenic amines (C; serotonin), and peptides.

Figure 1.2 depicts the main endocrine centers in crustaceans, including the Y-organ, X-organ, sinus gland, and androgenic gland, which will be discussed further in this chapter. Basically, environmental inputs are integrated by a central nervous system. This crustacean 'brain' contains neurotransmitters that govern the release of neuropeptides. These peptides regulate the production of hormones by the different endocrine glands (Cuzin-Roudy and Saleuddin, 1989).

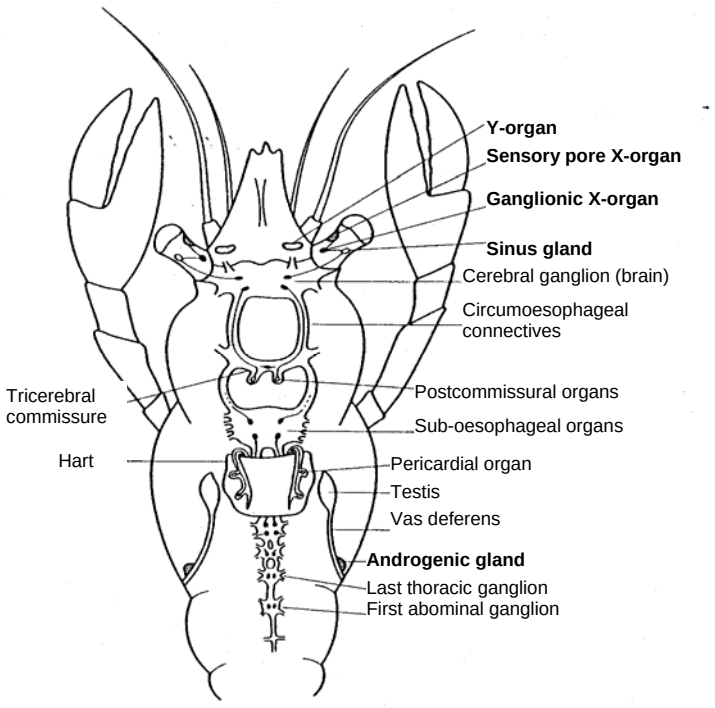


**Figure 1.2:** General overview of the endocrine system of a male crustacean (from Highnam and Hill, 1969).

As mentioned above, a number of endocrine-regulated processes are unique to invertebrates, or more specifically to Ecdysozoans (animals that molt). These processes provide ways of evaluating potential endocrine disruption that is unique to the invertebrates. Growth through periodic molting is a clear example of an ecdysozoan-specific endocrine-regulated process. The hormonal control of this process in crustaceans will be further explained, as well as the endocrine regulation of crustacean vitellogenesis, reproduction, sex determination, and a number of other hormone-regulated processes.

### 1.1.1. Crustacean growth and molting

Increase in size in all arthropods can only occur after shedding of the hard exoskeleton and before the new cuticle is hardened. Similarly, crustaceans grow through periodic molting or ecdysis. The increase in size and weight during ecdysis is not considered growth. Growth in crustaceans is defined as the increase in dry body weight which occurs in the periods between molts, when the absorbed water is gradually replaced by protein. Consequently, although ecdysis, increase in size, and increase in total weight are all markedly discontinuous, crustacean growth is a continuous process (Highnam and Hill, 1969). The molt cycle, i.e. the period between two subsequent ecdyses or molts (Fig. 1.3) is generally divided into four major phases: postmolt, intermolt, premolt and ecdysis. These periods have been given the stage designations of A-B, C, D and E, respectively (Gorokhova, 1999; Passano, 1960; Subramoniam, 2000).

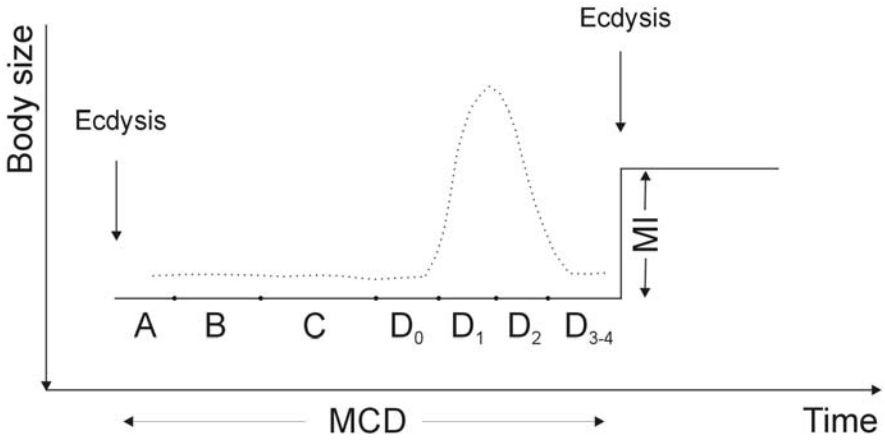


**Figure 1.3:** The crustacean molt cycle. Adapted from Gorokhova (1999) and Subramoniam (2000). MCD: molting cycle duration; MI: molt increment or stepwise increase in size at ecdysis; A-B: postmolt; C: intermolt; $D_0$, $D_1$, $D_2$, $D_{3-4}$: premolt; ⋯⋯: ecdysteroid concentration.

Highnam and Hill (1969) described the four stages of the molt cycle as follow:
*Stage E*: Ecdysis is a short period during which the animal sheds the remains of the old cuticle. There is a rapid uptake of water and the organism does not feed.
*Stage A-B*: Postmolt begins with the newly molted animal, its exoskeleton is still soft as water uptake continues. Initially, the animal still does not feed, continuing to utilize reserves in the hepatopancreas. During the latter half of postmolt, feeding recommences, the production of the exoskeleton is completed, and tissue growth occurs, replacing the absorbed water. Both protein and DNA have high turnover rates during this time, and the tissues double their dry mass, losing water proportionally.
*Stage C*: During the intermolt both exoskeletal formation and tissue growth have been completed, but feeding continues and metabolites in excess of current requirements are stored in the hepatopancreas. Lipid is the major reserve, but some glycogen and protein are also stored. The intermolt period is often referred to as the period of normality, but the specific accumulation of reserves in preparation for the next molt is no more normal than any other part of the molt cycle.
*Stage D*: Premolt is the preparation for molting. The first signs of premolt are activation of the epidermal cells and hepatopancreas. The epidermal cells separate from the cuticle, a process known as apolysis, and then divide. Almost immediately the epidermal cells begin to secrete the new exoskeleton. At the same time calcium is removed from the old cuticle, resulting in an increased blood calcium concentration. As these processes continue, the animal stops feeding and becomes inactive: during this time the reserves of the hepatopancreas are utilized. Splitting of the old cuticle marks the end of the premolt stage.
The different molt stages in mysids have been described for *Siriella armata* (Cuzin-Roudy et al., 1989), *Mysis mixta* (Gorokhova, 2002) and *Neomysis integer* (Gorokhova, 2002).

### 1.1.2. Endocrine control of molting in crustaceans

Molting in crustaceans is regulated by a multi-hormonal system (Fig. 1.4) and provides an excellent example of the involvement of all four types of crustacean hormones, i.e., **peptides, steroids, terpenoids,** and **biogenic amines**. Molting is under immediate control of the **steroid** molting hormones called ecdysteroids (Chang et al., 1993). The Y-organ (homologue of the prothoracic gland in insects) secretes ecdysone which, on release in the hemolymph, is converted into active 20-hydroxyecdysone (Fig. 1.1A) by a 20-hydroxylase activity (Huberman, 2000; Wang et al., 2000). Several studies have shown that the Y-organ in some

crabs also secretes 3-dehydroxyecdysone and 25-deoxyecdysone. 25-deoxyecdysone is the precursor to ponasterone A, the primary circulating ecdysteroid in the premolt stage of crabs (reviewed by Subramoniam, 2000). The Y-organ is located in the anterior branchial chamber in crustaceans (Huberman, 2000), which is the space between the inner body and the outer wall of the carapace enclosing branchia or respiratory organs. Other sources for ecdysteroids are the ovary and epidermis (Delbecque et al., 1990).

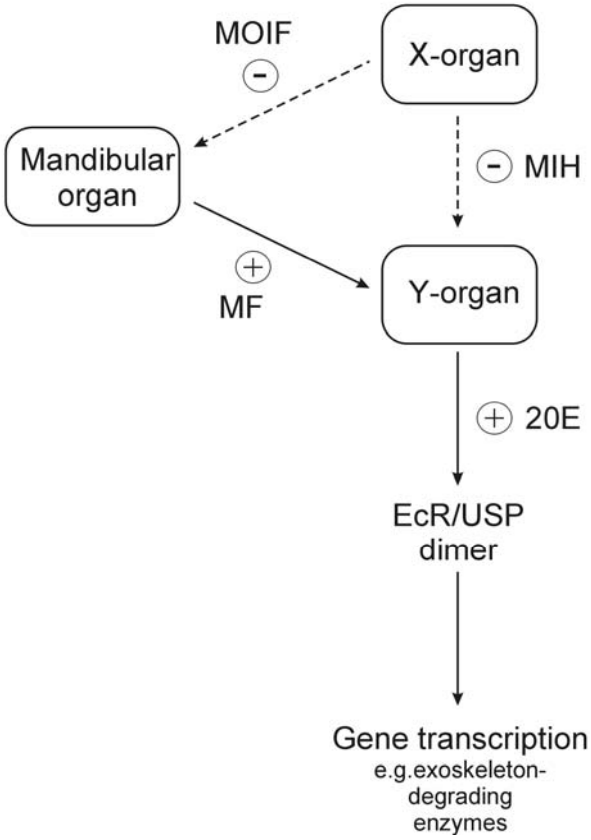


**Figure 1.4:** Hormonal control of molting in crustaceans. Adapted from DeFur et al. (1999) and Zou (2005). Interrupted arrows (-) represent inhibition and full arrows (+) stimulation. The following hormones play an important role in regulating crustacean molting: 20E, 20-hydroxyecdysone, the active molting hormone; MF, methyl farnesoate; MIH, molt-inhibiting hormone; MOIF, mandibular organ-inhibiting factor. See Figure 1.1 for structures of 20E and MF.

The circulating titer of 20-hydroxyecdysone varies along the molt cycle. Immediately after ecdysis, the titer is low and generally remains so during intermolt. A major increase occurs at

stage $D_1$-$D_2$ followed by a precipitous drop just before the actual molt (Fig. 1.3; Chang, 1992). Crustaceans obtain cholesterol, the precursor to ecdysone, from their diet.

Ecdysone secretion by the Y-organ is under negative control of the **neuropeptide**, molt-inhibiting hormone (MIH) (Nakatsuji and Sonobe, 2004; Soumoff and O'Connor, 1982), which is stored in the X-organ, a group of neurosecretory cells in the eyestalks of crustaceans. These cells send the majority of their axons to a neurohaemal organ, called the sinus gland. Virtually all aspects of crustacean physiology are affected by eyestalk removal (Quackenbush, 1986). A peptide that is similar to the insect hormone allatostatin is secreted by the X-organ to negatively control the mandibular organ, the mandibular organ-inhibiting factor. The mandibular organ (homologue of corpora allata in insects) secretes methyl farnesoate (Fig. 1.1B), a **terpenoid** which is the crustacean analogue of the insect juvenile hormone (for review, see DeFur et al., 1999).

Ecdysteroids regulate gene activities at the transcriptional level through binding with the ecdysteroid receptor (EcR), which then heterodimerizes with ultraspiracle protein (USP) (Oberdörster and Cheek, 2000). This EcR/USP dimer binds to specific DNA response elements in the genes regulated by the molting hormones. The EcR is a nuclear hormone receptor in the same gene family as the vertebrate thyroid receptor and USP is homologous to the vertebrate retinoid X receptor, which makes EcR/USP closely comparable to the vertebrate thyroid receptor/retinoid X receptor complex (Laudet, 1997). Among the products of ecdysteroid-regulated genes are the enzymes responsible for exoskeleton degradation. For instance, chitobiase (N-acetyl-β-glucosaminidase) is required for complete degradation of exoskeletonal chitin and the activities of chitinolytic enzymes have been used as markers for ecdysteroid action (Zou, 2005).

Serotonin, a **biogenic amine** (Fig. 1.1C), is involved in regulating important aspects of behavior and a variety of systemic physiological functions in both vertebrates and invertebrates (Sosa et al., 2004). Moreau et al. (2002) documented its presence in mysids, although they did not study its specific function.

### 1.1.3. Crustacean reproduction and vitellogenesis

In crustaceans both sexual differentiation and gonadal activity can be influenced by hormones and this, to some extent, resembles the situation in the vertebrates (Highnam and Hill, 1969). Unlike insects, reproductive physiology of crustaceans is greatly influenced by continued somatic growth, permitted by periodical molting in the adults. The resulting relationship

between molting and reproduction is much more evident in females. Vitellogenesis in female crustaceans, i.e. production of the yolk protein vitellin, as well as secretion of a new cuticle during molting, affect the organisms' physiology by their competitive utilization of reserve materials from storage organs.

The relationship between molting and reproduction is diverse throughout the crustacean phylum (Adiyodi and Subramoniam, 1983) and their integration is regulated via complex, and still largely unknown, endocrine signals (Quackenbush, 1986). Most crustaceans can be placed into three groups based on the organization of gonadal and somatic growth (Adiyodi and Subramoniam, 1983, Charniaux-Cotton, 1985, Subramoniam, 2000). Crabs and lobsters fit into type 1 where reproduction takes place during the relatively long intermolt period. Isopods, amphipods, and shrimps fit into type 2 where gonadal and somatic growth occur simultaneously. Type 3 includes the rapid molting cirripedes where reproduction may require several molt cycles. These groupings describe extremes as many species tend to fall somewhere in between two of these general groupings.

In mysids, the embryonic and post-embryonic development occurs in the marsupium (Fig. 1.6) and include five consecutive stages from oviposition to the juvenile stage (Mauchline, 1980; Wittmann, 1981a,b; Wortham-Neal and Price, 2002). Although the main neurosecretory centers and the sinus glands in mysids resemble these from decapods, mysid reproduction is more like those of amphipods and isopods and strictly linked to the molt cycle (Cuzin-Roudy and Saleuddin, 1989). Until now, the marsupial development in *Neomysis integer* had not been described in detail (see Chapter 6).

### 1.1.4. Endocrine control of crustacean reproduction and vitellogenesis

Vitellogenesis is the formation of the yolk protein vitellin which is the major nutrient source for the developing embryo. Vitellin is derived from a precursor called vitellogenin that can be synthesized in extraovarian tissues or in the ovaries (Huberman, 2000). In many species, vitellogenin is transported through the hemolymph to developing oocytes, where it is sequestered and modified with the addition of polysaccharides and lipids into vitellin. The synthesis of yolk proteins is a good indicator of female reproductive activity. In addition, the presence of yolk proteins has been used frequently to study hormonal control of reproduction (Tsukimura, 2001). Similar to molting, crustacean reproduction and vitellogenesis are regulated by a complex system that involves steroids, peptides, terpenoids, and amines.

Classical eyestalk ablation experiments, for instance, have demonstrated that crustacean reproduction is under sinus gland control (Fig. 1.5; Brown and Jones, 1947; Carlisle, 1953; Gomez, 1965; Panouse, 1943; Stephens, 1952). These and more recent studies have been extensively reviewed e.g. by Adiyodi (1985), Chang (1992), De Kleijn and Van Herp (1995), Fingerman (1987), and Okumura (2004). Briefly, ablation of the sinus gland led to the discovery of a vitellogenesis-inhibiting hormone (VIH, also called gonad-inhibiting hormone, GIH) (Aguilar et al., 1992; Gohar et al., 1984; Soyez et al., 1987). VIH/GIH has also been detected in the male sinus gland (Azzouna et al., 2003; Martin et al., 1999) and it is probably involved in androgenic gland growth (Martin and Juchault, 1999). Other **neuropeptides** that regulate crustacean reproduction are vitellogenesis-stimulating ovarian hormone (VSOH) in the follicular layers of oocytes, vitellogenesis-stimulating hormone (VSH, also named gonad stimulating hormone, GSH) in the brain and thoracic ganglia (Eastman-Reks and Fingerman, 1984; Gomez, 1965; Otsu, 1960; Takayanagi et al., 1986), and methyl farnesoate (MF) in the mandibular organ (Meusy and Payen, 1988).

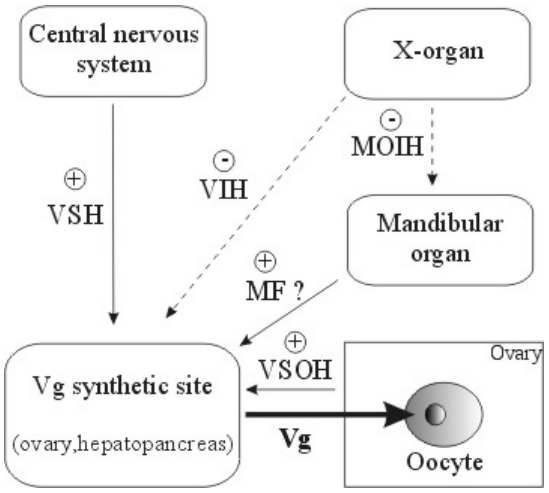


**Figure 1.5:** Hormonal control of vitellogenesis in crustaceans. Adapted from Okumura (2004). Interrupted arrows (-) represent inhibition and full arrows (+) stimulation. The following hormones are believed to play an important role in regulating crustacean vitellogenesis: MF, methyl farnesoate; MOIF, mandibular organ-inhibiting hormone; Vg, vitellogenin; VIH, vitellogenesis-inhibiting hormone; VSH, vitellogenesis-stimulating hormone; VSOH, vitellogenesis-stimulating ovarian hormone.

The role of the **terpenoid** MF in crustacean reproduction was originally inferred by correlating oocyte size and MF levels in the hemolymph (Borst et al., 1987; Borst et al., 1995;

Laufer et al., 1987). Subsequent experimental studies led to conflicting results on the role of MF in stimulating oocyte development (Tsukimura, 2001). Incubation of ovarian tissue with MF, and dietary administration of MF, have both been shown to stimulate ovarian development in the white shrimp *Penaeus vannamei* (current name *Litopenaeus vannamei*) and in the crayfish *Procambarus clarkii* (Laufer et al., 1998; Tsukimura and Kamemoto, 1991). However, no significant effects were detected in American lobster *Homarus americanus* and in the freshwater prawn *Macrobrachium rosenbergii* when MF was injected into senescent females (Tsukimura et al., 1993; Wilder et al., 1994). With a half-life in water of less than one hour, it is possible that the incidental presence of MF was insufficient to reinitiate reproduction. Conversely, MF incubation experiments using fully active tadpole shrimp (*Triops longicaudatus*) ovarian tissues might not have been effective because vitellogenesis was already near maximal capacity (Riley and Tsukimura, 1998). Laufer et al. (1987) suggested that MF may act as a juvenile hormone-like compound that, as in insects, maintains juvenile morphology and enhances reproduction in adults. Linder and Tsukimura (1999) have reported that MF sigificantly reduced the number of developing oocytes when administered continuously to juvenile tadpole shrimp. These findings support the initial hypothesis of Laufer and colleagues (1987) that MF may act as a juvenilizing agent in crustaceans. Recently, Laufer et al. (2002) have further provided support for the interpretation that ecdysteroids and low MF concentrations promote allometric growth.

Chen et al. (2003) reported the effects of the **biogenic amines** dopamine and serotonin on ovarian development in the crayfish *Macrobrachium rosenbergii*. Dopamine depressed vitellogenin synthesis while serotonin enhanced the process. Since dopamine is able to inhibit vitellogenin synthesis in eyestalk-ablated prawns in a similar manner as in intact prawns, the inhibitory action of dopamine is at the thoracic ganglia through inhibition of VSH release, but not at the eyestalk level through stimulation of VIH release from the X organ-sinus gland complex.

As discussed earlier, molting and reproduction are closely connected hormone-regulated processes in crustaceans, and much research has been done on the role of **ecdysteroids** in crustacean reproduction. Subramoniam (2000) published a review on the role of crustacean ecdysteroids in reproduction and embryogenesis. This author reported that there is evidence that the ovary sequesters ecdysteroids from the hemolymph and the presence of ecdysteroids in the ovary has led to the proposition that they have a role in reproduction and embryonic development. Ecdysteroids have been shown to stimulate vitellogenesis in the ovaries of some crustaceans (Gohar and Souty, 1984; Gunamalai et al., 2004; Okumura et al., 1992; Steel and

Vafopoulou, 1998), while inhibiting or having no effect on vitellogenesis in others (Chaix and De Reggi, 1982; Chan, 1995; Fyhn et al., 1977; Okumura and Aida, 2000; Young et al., 1993). In conclusion, while a role for ecdysteroids in crustacean vitellogenesis is clearly evident, their precise function remains to be determined and the endocrine control of vitellogenesis is likely to vary from species to species (DeFur et al., 1999; Gunamalai et al., 2004; Subramoniam, 2000).

### 1.1.5. Crustacean androgenic gland

Sexual differentiation in decapod crustaceans (i.e., crabs, lobsters, shrimp) and other malacostracans is under the regulatory control of the androgenic hormone (Olmstead and Leblanc, 2000). This hormone is the product of the androgenic gland, which is typically associated with the terminal region of the male gamete ducts or *vas deferens*. Ablation of the androgenic gland causes feminization in male prawns *Macrobrachium rosenbergii* (Nagamine et al., 1980) and shrimp *Penaeus indicus* (Mohamed and Diwan, 1991). Conversely, implantation of the gland into females causes masculinization. Vitellogenin synthesis has also been shown to be under negative regulatory control of the androgenic hormone in the isopod *Armadillidum vulgare* (Suzuki et al., 1990). Recently, the effects of androgenic gland implantation and ablation have been studied in the crayfish *Cherax quadricarinatus* (Manor et al., 2004; Sagi et al., 2002). In these studies, the vitellogenin gene was found to be induced in the hepatopancreas of androgenic gland-ablated individuals suggesting that the androgenic gland represses transcription of this gene in intact individuals. Cui et al. (2005) recently reported the inhibitory effect of the androgenic gland on ovarian development in the mud crab *Scylla paramamosian*.

The androgenic gland has not been described in lower crustaceans, such as cladocerans and mysids. However, a comparable organ or cell type may be responsible for sexual differentiation in these animals. Interestingly, a recent study has suggested the presence of sex-determining genes in daphnids that may possess regulatory elements that interact with a putative MF receptor (Rider et al., 2005). This could indicate that MF plays a role in sexual determination in daphnids. At this point, it is not known whether this a reproductive strategy only found in asexually reproducing cladocerans, or a more general strategy that is also present in mysids.

### 1.1.6. Other hormonal-regulated processes in crustaceans

*Pigmentation*: The sinus gland and other parts of the crustacean central nervous system are storage sites for neurosecretory material that regulates color change, the chromatophorotropins. There are two types of pigmentary effectors in crustaceans: the chromatophores and retinal pigment cells. Chromatophores are pigment-containing cells that occur, not only on the surface of crustaceans, but also in some internal tissues. Their function is to adjust body color with respect to it surrounding environment. The retinal pigments are located in the eyes. They regulate the amount of light impinging on the rhabdome, which is the light-sensitive portion of each ommatidium (the functional unit) of the compound eye. The physiology and morphology of these two types of pigmentary effectors are quite different, although both are subject to endocrine regulation. There are several excellent reviews on this topic (DeFur et al., 1999; Fingerman, 1985; Highnam and Hill, 1969; Kleinholz, 1985; Kleinholz and Keller 1979; Rao et al., 1985).

*Limb regeneration*: Crustaceans possess a remarkable ability to regenerate limbs and other appendages. The actual factors responsible for the growth of a new limb are still largely unknown. However, it has been observed that there is a precise interplay between the molt cycle and regenerative events (DeFur et al., 1999). Although the observation that multiple limb losses affect the duration of the molt interval had been made earlier (as reviewed by Skinner, 1985), this phenomenon was not thoroughly defined until later work by Skinner and Graham (1970, 1972). Their studies with crabs (*Gecarcinus lateralis*) demonstrated that multiple limb autonomy was, in some ways, a more effective stimulus for molting than eyestalk removal. They further hypothesized that, when a threshold number of limbs are lost, a molt-promoting factor acts to initiate the molting process. Skinner (1985) termed this molt-promoting substance the “limb autotomy factor, anecdysial”. Both the chemical nature and the source of this factor are unknown at present.

In summarizing about 75 years of crustacean endocrinological studies, Fingerman (1997) concluded that despite the many significant advances, work in the field “has really just begun”. This is especially true considering the tasks ahead in examing the potential disruption of crustacean endocrine systems by anthropogenic compounds (OECD, 2005).

## 1.2. TEST ORGANISM: *NEOMYSIS INTEGER*

### 1.2.1. Biology and distribution

Mysids (Crustacea: Peracardia) are shrimp-like crustaceans, often referred to as 'opossum shrimp' due to oostegites forming a marsupium or brood pouch used by females to carry their developing embryos (Fig. 1.6c). This marsupium also distinguishes mysids from other shrimp-like crustaceans. Male mysids are distinguished from females by an elongated 4$^{th}$ pleopod (abdominal limb, Fig. 1.6a). Mysids are identified from other peracarids (Amphipoda, Isopoda, Cumacea, Tanaidacea) by the presence of a statocyt on the proximal part of the uropodal endopod. Beside the marsupium and statocyt, mysids are characterized by a shield-like carapax which covers the greater part of the cephalothorax, but is not attached to it in the last thoracal segments. For a more detailed description of mysids, we refer to Tattersall and Tattersall (1951).

*Neomysis integer* (Leach, 1814) is a mysid that grows up to about 17 mm in length (Fig. 1.6). It is a hyperbentic, euryhaline and eurythermic species that occurs in various aquatic environments, mainly estuaries (Tattersall and Tattersall, 1951). *N. integer* is one of the most common mysid species along the Atlantic coasts of Western Europe and is found along the Atlantic coastline of Britain and between the longitudes 68°N (coast of Norway) and 36° (South coast of Spain), as well as in the Baltic Sea (Fig. 1.7). Fockedey (2005) recently published an extensive literature review on the distribution, feeding, behavior, physiology and energetics of *N. integer*.

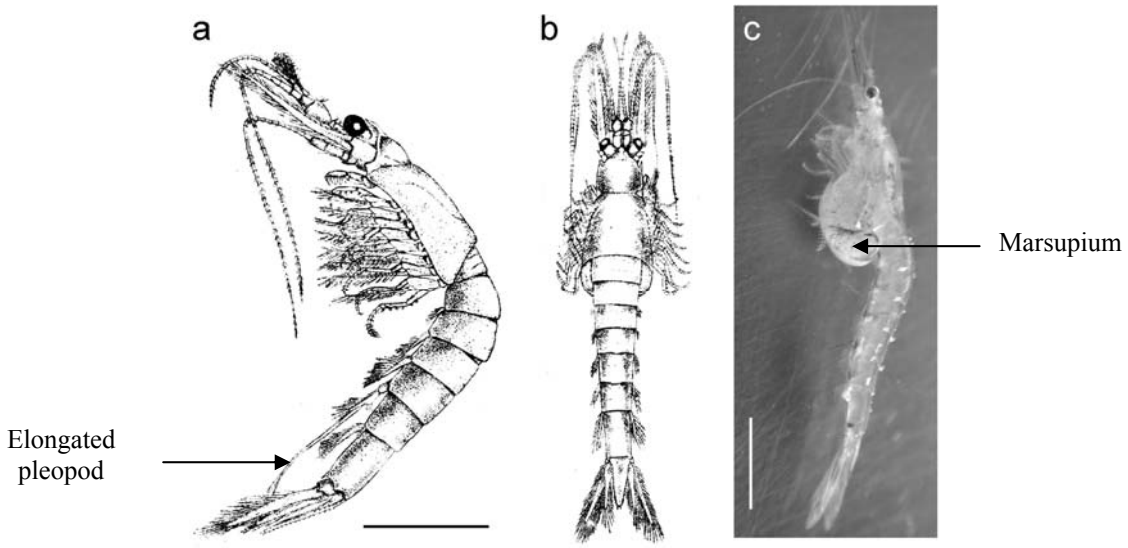


**Figure 1.6:** *Neomysis integer* (Crustacea: Mysidacea). a, adult male; b, adult female and c, ovigerous female (scale bar 5 mm). Drawings (a & b) are from Tattersall and Tattersall (1951) and photo (c) from Fockedey (2005).

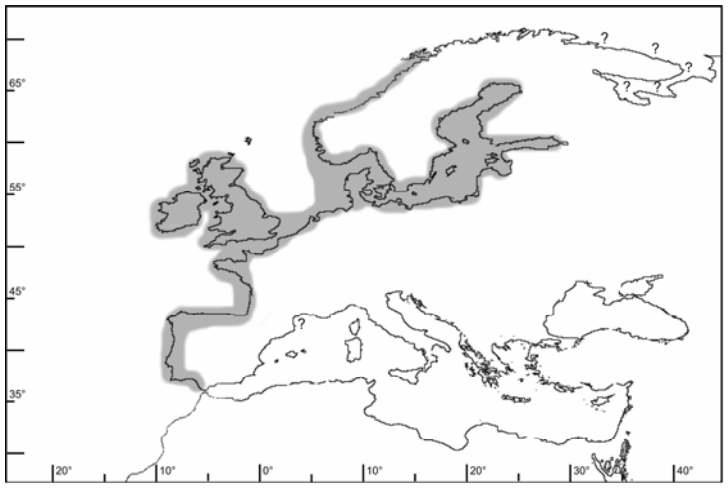


**Figure 1.7:** Distribution of *Neomysis integer* (gray areas) based on records in literature (Remerie, 2005).

### 1.2.2. *Neomysis integer* as a test species for evaluating endocrine disruption

Of the crustaceans, mysid shrimp have been proposed as suitable test organisms to assess endocrine disruption (CSTEE, 1999; DeFur et al., 1999; LeBlanc, 1999). *N. integer* is easily collected in the field throughout the year and can be maintained in the laboratory. *N. integer* has a relatively short life cycle which allows multi-generation exposures. In addition, ovigerous females carry their developing embryos in a marsupium, allowing various aspects of their reproductive biology to be studied. Their size allows for the individual measurement of hormones and other biochemical fractions. *N. integer* is an important part of estuarine food webs, e.g. in the brackish part of the Scheldt estuary. As a predator it can structure zooplankton populations and as a detrivore it can also affect the detrital chain (Fockedey and Mees, 1999; Mees et al., 1994). *N. integer* is also an important prey for demersal and pelagic fish and larger epibenthic crustaceans in the Scheldt estuary. *N. integer* has a strong tolerance for temperature and salinity changes, characteristic of many North-European estuaries. Herefore, it can be used in cold water and estuarine testing, which is not possible with the standard American mysid test species, *Americamysis bahia*. Verslycke et al. (2004a) published an excellent review on mysid crustaceans as potential test organisms for the evaluation of environmental endocrine disruption.

An important advantage for the use of *N. integer* as a test species to study endocrine disruption is available information on its biology, ecology and ecotoxicology (Fockedey and Mees, 1999; Mees and Hamerlynck, 1992; Mees et al., 1993 a,b; 1994; 1995a,b; Verslycke et al., 2004b; 2005). In addition, Roast and co-workers (1998a,b; 1999a,b,c; 2000a,b,c; 2001a,b; 2002; 2004) have demonstrated the successful use of this species in sublethal toxicity testing. Finally, this species has been cultured in our laboratory for a long time and recently it has been used extensively as a model for endocrine disruption research (Heijerick, 1994; Poelmans et al. 2005; Verslycke et al., 2002; 2003a,b,c; 2004c). Most of the recent studies on endocrine disruption using *N. integer* are an integral part of the doctoral dissertation of Tim Verslycke, published in 2003, which focuses on the energy and steroid metabolism of this species. In addition, Stephen Roast (University of Plymouth, UK) used *N. integer* as a test species to evaluate chemical effects on it's energy metabolism and swimming behavior (Roast et al., 1998b; 1999c; 2000a,c; 2001b).

To date, few studies have evaluated the potential effects of EDCs on hormone-regulated processes that are specific to the invertebrates, such as molting. While vertebrate-type steroids (e.g., testosterone) have been measured in mysids (Verslycke et al., 2002) and other

crustaceans (DeFur et al., 1999), the function of these hormones remains unclear. On the other hand, it has been well established that ecdysteroids and juvenile hormones are the major endocrine regulators of molting, embryonic development, metamorphosis, reproduction, and pigmentation in arthropods (insects, crustaceans, and some minor groups) (DeFur et al., 1999). Moreover, many pesticides are specifically designed to mimic the action of invertebrate-specific hormones, such as ecdysteroids and juvenoids. This unique potential for chemicals to disrupt invertebrate-specific processes is presently not being addressed in regulatory programs for EDCs, generally because of a lack of fundamental understanding of hormone regulation in many invertebrates. As such, there is an urgent need to better understand the potential impact of chemicals on invertebrate-specific hormone-regulated processes. Within this context, we selected three known ecdysteroid-regulated processes in the mysid *N. integer*; molting, embryonic/marsupial development, and vitellogenesis. A fundamental study of the effects of temperature and salinity on molting and embryonic development of *N. integer* were performed as part of the doctoral dissertation work of Fockedey (2005). These studies were highly complementary to the studies that are part of this doctoral research.

## 1.3. FIELD STUDY: THE SCHELDT ESTUARY

This doctoral study was carried out within a large interdisciplinary research project, ENDIS-RISKS, which focuses on endocrine disruption in the Scheldt estuary (Belgium/The Netherlands, Fig. 1.8) (project website: http://vliz.be/projects/endis). The Scheldt estuary is known to be one of the most polluted estuaries in the world and from an ecological point of view it is an important tidal river systems in Europe (Verslycke et al., 2004b). It is an important passing, overwintering and feeding area for waterbirds, and a nursery for fish and shrimp. Within the context of ENDIS-RISKS, water, sediment, suspended solids and biota were sampled three times a year for a period of four years (2002-2006) using the RV Belgica (Fig.1.8). In all these matrices, seven groups of suspected endocrine disruptors were analyzed (hormones, phenols, pesticides, organotins, flame retardants and PCBs, PAHs and phtalates). This allowed for the identification of priority substances which could be further tested in the laboratory to evaluate their effects on the estuarine mysid *N. integer*. For this purpose, several invertebrate-specific endpoints needed to be developed for *N. integer* in the laboratory. The development of methods to evaluate effects on molting, vitellogenesis, and embryogenesis are

described in Chapters 3 to 6. These and future laboratory and field studies will lead to an integrated risk assessment for endocrine disruptors in the Scheldt estuary.

The initial phases of the ENDIS-RISKS project led to the first publication on concentrations of potential endocrine disruptors in *N. integer* of the Scheldt estuary (Verslycke et al., 2005). This study reported high concentrations of flame retardants, surfactants (alkylphenols) and organotins in sediment and mysids of the Scheldt estuary. More recent measurements have confirmed very high levels of endocrine disruptors in mysids, i.e. up to 3000 µg TBT/kg mysid dw, up to 1119 µg nonylphenol ethoxylates/kg mysid dw, up to 1400 µg sum of 7 PCBs/kg mysid dw and up to 210 µg polybrominated diphenyl ethers (47, 100, 119 and 99 PBDE)/kg mysid dw (Monteyne et al., in preparation). In addition, concentrations of organochlorine pesticides in mysids vary from 5 to 35 µg/kg mysid dw and the highest concentrations are found for dieldrin and hexachlorobenzene (Monteyne et al., in preparation). All measured body burdens for TBT, PCBs and PBDEs in mysids exceeded the Ecotoxicological Assessment Criteria (EAC, for blue mussel) as put forward by OSPAR. Within the ENDIS-RISKS project, we also found significant levels of estrogen in water samples from the Scheldt estuary, e.g. up to 8 ng/l for estrone (Noppe et al., 2005). Of the organonitrogen pesticides analysed in Scheldt water samples, atrazine (up to 736 ng/l) has been detected most frequently.

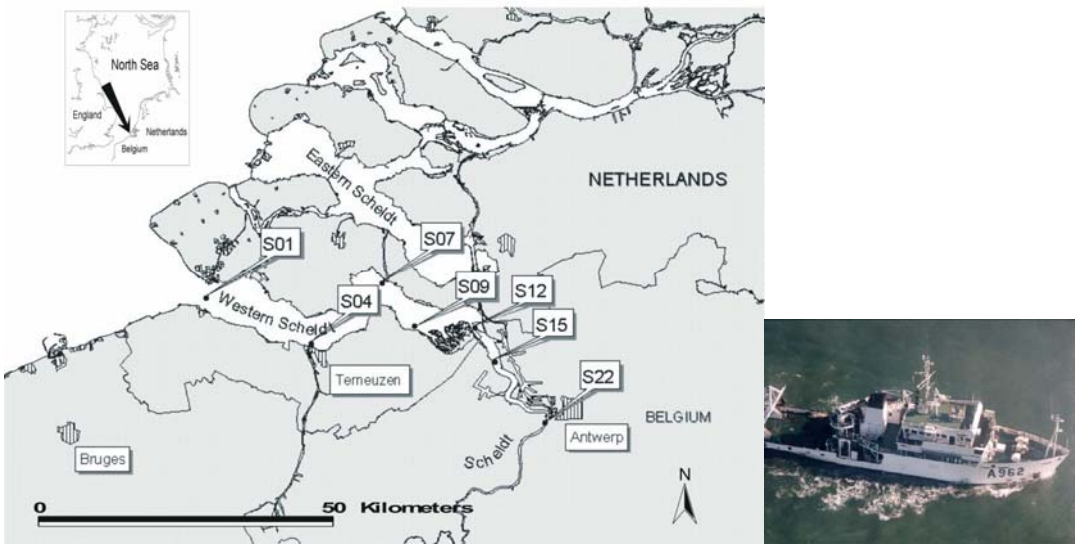


**Figure 1.8:** Left: map of the Scheldt estuary with location of the different sampling sites (S01, Vlissingen; S04, Terneuzen; S07, Hansweert; S09, Saeftinge; S12, Bath; S15, Doel and S22, Antwerp). Right: research vessel, RV Belgica.

Three different invertebrate-specific physiological processes were studied in this doctoral research and evaluated for their use as evaluation tools to detect the potential effects of endocrine disruptors in *N. integer*: vitellogenesis (Chapter 4), molting (Chapter 5), and embryogenesis or marsupial development (Chapter 6,7). More specifically, the effects of nonylphenol and estrone, both known to be present at high levels in field-collected mysids, were evaluated on the vitellogenesis and embryogenesis of *N. integer* (Chapter 7 and 4, respectively). In addition the effects of methoprene (a juvenile hormone analog) on vitellogenesis, molting and embryonic development of *N. integer* were evaluated in Chapters 4, 5 and 6, respectively.

Our ongoing and future reserach goals are to validate, in the Scheldt estuary, the use of the endpoints we developed in the laboratory. An initial study by Verslycke et al. (2004b) looked at seasonal and spatial patterns in cellular energy allocation in *N.integer* of the Scheldt estuary. As part of this doctoral thesis, vitellin levels in *N. integer* of the Scheldt estuary were quantified (Chapter 8) using a newly developed mysid vitellin immunoassay (Chapter 3).

## 1.4. LABORATORY STUDIES: HORMONE-REGULATED PROCESSES SELECTED FOR THIS STUDY

In section 1.1, we presented a brief introduction to crustacean endocrinology. We refer to a comprehensive review by Verslycke et al. (2004a) which describes different hormone-regulated endpoints in mysids and their potential value in evaluating endocrine disruption. As discussed in the previous sections, there is an urgent need for the development of invertebrate-specific endpoints to evaluate endocrine disruption. For the purpose of this doctoral research, we selected a number of physiological processes that are known to be regulated by invertebrate-specific hormones. These processes and their use as biomarkers is discussed below.

### 1.4.1. Mysid growth and molting

In crustaceans, significant growth can only occur through molting, therefore, disruption of molting will result in effects on growth (Toda et al., 1984; USEPA, 2002). Furthermore, disruption of the molt cycle can have profound effects on many other aspects of organismal function like reproduction and embryogenesis (Gorokhova, 2002; Subramoniam, 2000).

Many pesticides, generally classified as IGRs (Insect Growth Regulators), have been developed to specifically target insect development. Because insects and crustaceans use both molting and juvenile hormones to regulate growth, metamorphosis, metabolism, and reproduction, IGRs can cause adverse effects in non-target animals, such as crustaceans. The IGRs include ecdysteroid agonist insecticides, juvenile hormone analogs, and insecticides with chitin synthesis inhibitory activity. For reviews on IGRs, we refer to Dhadialla et al. (1998), Hoffmann and Lorenz (1998), and Staal (1975). Bisacylhydrazines (e.g., tebufenozide and halofenozide) are **non-steroidal agonists** of 20-hydroxyecdysone and exhibit their activity via interaction with the ecdysteroid receptor complex (Smagghe et al., 2002; 2004). One of the first effects of bisacylhydrazine ingestion by susceptible larvae is feeding inhibition (Retnakaran et al., 1997; Smagghe et al., 1996). Exposed larvae ultimately die as a result of their inability to complete molting and starvation. The unsuccessful lethal molt is a result of the presence of bisacylhydrazines in the hemolymph which inhibits the release of eclosion hormone (Truman et al., 1983).

The second group of IGRs are the **juvenile hormone analogs**. The major function of juvenile hormone is the maintenance of the larval status or the so-called juvenilizing effect in insects. The mode-of-action of juvenile hormone and their analogs in crustaceans are not well understood (Tuberty and McKenney, 2005). Methoprene is by far the most thoroughly studied juvenile hormone analog. Extensive data collected by the US Environmental Protection Agency (EPA) have demonstrated that this pesticide is relatively non-toxic to most non-target organisms (Dhadialla et al., 1998). However, methoprene has been shown to affect growth in the mysid *Americamysis bahia* (McKenney and Celestial, 1996), *Palaemonetes pugio* (McKenney and Matthews, 1990), and in the cladoceran *Daphnia magna* (Olmstead and LeBlanc, 2001). Other juvenile hormone agonists, such as fenoxycarb and pyriproxyfen, have been reported to affect energy metabolism and development in mud crabs and mysids (Nates and McKenney, 2000; Tuberty and McKenney, 2005; Verslycke et al., 2004c).

The last group of IGRs are **chitin synthesis inhibitors**. These compounds disrupt cuticle formation process in insects, which leads to mortality. Two types of insect regulatory chitin synthesis inhibitors have been developed and are used as commercial compounds for controling agricultural pests: the benzoylphenyl ureas, and buprofezin/cyromazine (Londerhausen, 1996; Palli and Retnakaran, 1999; Retnakaran and Oberlander, 1993; Spindler et al., 1990). These pesticides may also adversely affect non-target organisms including benificial insect species and crustaceans (Miyamoto et al., 1993), but to the best of our knowledge little research has been done on this topic.

Vertebrates and non-arthropod invertebrates appear considerably less susceptible to IGRs due to their intrinsic mode-of-action. However, detailed information regarding the effects of IGRs is still lacking for many arthropod species, limiting an overall assessment of their environmental impact (Miyamoto et al., 1993). As such, Sumpter and Johnson (2005), suggested the necessary precaution when assuming that IGRs are a group of highly specific EDCs. The potential invertebrate-specific endocrine-disruptive effects of chemicals such as IGRs to non-target organisms are presently not specifically addressed in regulatory screening and testing programs and this could lead to significant underestimations of the actual environmental risk of these compounds.

In addition to IGRs, molting can also be disrupted by other EDCs. For example, molting is inhibited by heavy metals (Kang et al., 1997; Moreno et al., 2003; Weis et al., 1992), polychlorinated biphenyls (PCBs) (Fingerman and Fingerman, 1977), brominated flame retardants (Wollenberger et al., 2005), benzene (Cantelmo et al., 1981), methoxychlor (Baer and Owens, 1999), and vertebrate steroid hormones (Baldwin et al., 1995; Mu and LeBlanc, 2002; Zou and Fingerman, 1997a,b).

Zou and Bonvillain (2004) have used chitinase activity as an *in vivo* screen for molt-interfering xenobiotics. Since environmental chemicals could in theory affect any step in the endocrine cascades of the multi-hormonal system for molting, the effect of such a molt-interfering agent should be reflected in the activities of chitinolytic enzymes since these enzymes are the final step in ecdysteroid signaling (see Fig. 1.4). These authors reported no effects for the juvenile hormone analog methoprene on chitinase activity in the fiddler crab, *Uca pugilator*.

Most toxicological studies on crustacean physiology have not examined cellular effects or effects on hormone titers (DeFur et al., 1999). However, to understand or distinguish between general toxicological and endocrine-mediated effects, mechanistic studies are needed. For example, Dinan et al. (2001) and Smagghe et al. (2002) used *in vitro* assays to determine whether a chemical has (anti-)ecdysteroidal activity. This activity is based on the affinity of the chemical to an insect ecdysteroid receptor complex that has been cloned into a cell line. Recently, Yokota et al. (2005) developed an *in vitro* binding assay with the ecdysone receptor from *Americamysis bahia* which holds promise as a rapid *in vitro* screen of chemical interaction with the mysid ecdysteroid receptor complex. Similar to previous attempts by other authors, we have not been able to develop a stable crustacean cell line (in our case, of *Neomysis integer*). A crustacean cell line would allow *in vitro* mechanistic studies that are specifically relevant to crustaceans. Methods for quantifying the different crustacean

hormones would greatly advance our mechanistic understanding of endocrine disruption. Recent studies have quantified ecdysteroids in the mysid *A. bahia* (Tuberty and McKenny, 2005) and efforts are ongoing to quantify ecdysteroid levels in *N. integer* by adding extracts to a transformed insect cell line with a sensitive ecdysone reporter construct (Soin et al., in preparation). Establishing a basic understanding of hormonal titers and receptor-mediated hormone regulation in mysids will greatly improve our ability to assess and predict endocrine disruption in crustaceans and other invertebrates. In this perspective, ongoing studies are characterizing the receptors involved in ecdysteroid/juvenoid signaling of *N. integer* (Soin et al., unpublished data; Verslycke et al., unpublished data). These studies are a first step in developing a transcriptional activation or receptor binding assay to screen chemicals based on a crustacean hormone receptor complex. However, not all chemicals with molt-interfering potency will exert their effect at the receptor level. Thus, a combination of *in vivo* and *in vitro* assays will continue to be needed for screening effects of chemicals on crustacean molting. In Chapter 5, we describe the development of an *in vivo* molting assay with *N. integer*. This assay was validated in the laboratory using a methoprene exposure experiment.

### 1.4.2. Mysid reproduction and vitellogenesis

There are several measures of reproductive performance that can be used to assess sublethal responses in crustaceans. For example, sexual maturity, the time to first brood release, the time required for egg development, brood size, and hatching have all been used as endpoints in experiments with cladocerans and mysids (Kast-Hutchenson et al., 2001; LeBlanc et al., 2000; McKenney and Celestial, 1996). Generally, few studies have evaluated the potential effects of endocrine disruptors on embryogenesis of crustaceans and no such studies exist for mysids. Fockedey et al. (2005a) developed a methodology to study the embryonic development of *N. integer in vitro*, and evaluated the combined effects of temperature and salinity on its embryogenesis. In Chapter 6, this marsupial development assay with *N. integer* is evaluated as a potential research tool to detect the potential effects of endocrine disruptors on mysid early development.

Occurrence of vertebrate-type steroid hormones such as 17β-estradiol, progesterone, and 17α-hydroxyprogesterone, has been reported in the hemolymph and ovaries of several crustacean species (Fingerman et al., 1993; Subramoniam, 2000). It is well established that these circulating steroid hormones induce oocyte growth in oviparous vertebrates such as fish (Mommsen and Walsh, 1988). Fairs et al. (1990) suggested that 17β-estradiol might possibly

control ovarian development in the shrimp *Penaeus monodon*. Recent studies, however, have reported that these hormones do not play a role in crustacean ovarian development (Okumura, 2004; Okumura and Sakiyama, 2004). Thus, the role and presence of vertebrate-type hormones in crustaceans remains unclear.

Upregulation of vitellogenin, the precursor of the egg yolk protein vitellin, has been a reliable way of measuring estrogenic exposure in fish (Oberdörster and Cheek, 2000). However, little research has been done on the expression of vitellin in crustaceans after exposure to EDCs. A review paper by USEPA on mysid life cycle testing (2002) suggested that differences in vitellin production among treated and non-treated mysids could provide evidence of endocrine system disruption and should be explored. During this doctoral research, we purified and characterized vitellin from the mysid *Neomysis integer* (Chapter 2), and subsequently developed a quantitative enzyme-linked immunosorbent assay (ELISA) (Chapter 3). In Chapter 4 we further describe the use of the *N. integer* vitellin ELISA to detect potential effects of three reported endocrine-disrupting chemicals on mysid vitellogenesis.

## 1.5. RESEARCH NEEDS AND CONCEPTUAL FRAMEWORK OF THE STUDY

From the literature review in this introductory chapter, it is obvious that relatively little information exists on the endocrine system of many invertebrates. As such, more fundamental studies are needed to understand or distinguish between general toxicological and endocrine-mediated toxic effects. More mechanistically-driven approaches, such as those used in this doctoral research, should lead to a better understanding of hormone regulation in mysids. Further, there is a clear need for invertebrate-specific endpoints to study endocrine disruption. This will lead to a more relevant risk assessment with respect to EDCs and invertebrates. Ecdysteroid- and juvenoid-regulated processes are an excellent example of invertebrate-specific hormone-regulated processes that can be disrupted by chemicals. This is important as many insecticides are specifically designed to disrupt these processes in insects, and have been shown to cause non-target effects in crustaceans. This could lead to serious understimation of the risk these chemicals pose to our ecosystems.

The scope of this doctoral thesis is to address a number of fundamental research needs as identified in the literature review given in this introductory chapter. More specifically, the goal of this research is a fundamental study of the invertebrate-specific hormone-regulated

processes molting, vitellogenesis and embryogenesis in the mysid *N. integer*. These invertebrate-specific processes will be evaluated for their usefulness as endpoints to evaluate endocrine disruption following exposure to environmentally-relevant chemicals as identified during the field studies in the Scheldt estuary. Finally, the endpoints developed in the laboratory are also used in field validations in the Scheldt estuary. The outline of the different chapters is as follows:

**Chapter 2** describes the purification and charcterization of vitellin in *N. integer*. Vitellin was purified from eggs using gel filtration and characterized by electrophoresis and differential staining techniques. Specific polyclonal antibodies were produced in rabbit against the purified *N. integer* vitellin.

**Chapter 3** describes the development of an enzyme-linked immunosorbent assay (ELISA) to quantify vitellin in *N. integer* based on the vitellin purified in Chapter 2.

**Chapter 4** evaluates the effects of methoprene, nonylphenol and estrone on the vitellogenesis of *N. integer* using the vitellin ELISA developed in Chapter 3.

**Chapter 5** evaluates the non-target effects of the insecticide methoprene on molting in *N. integer*. Preliminary studies were performed to develop invertebrate-specific molting assay to evaluate the effects of EDCs.

**Chapter 6** describes the marsupial development of *N. integer* as an endpoint to evaluate the effects of environmental chemicals. The fundamental knowledge on the marsupial development is included in this chapter.

**Chapter 7** descibes the effects of nonylphenol and estrone on the marsupial development of *N. integer*.

**Chapter 8** reports vitellin levels in resident *N. integer* of the Scheldt estuary based on two sampling campaigns in April and July 2005. In addition, population parameters of *N. integer* are described.

In **Chapter 9**, general conclusions are drawn and future research needs are formulated.

# CHAPTER 2

## PURIFICATION AND CHARACTERIZATION OF VITELLIN FROM *NEOMYSIS INTEGER*

Redrafted after:

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# CHAPTER 2

## PURIFICATION AND CHARACTERIZATION OF VITELLIN FROM *NEOMYSIS INTEGER*

**ABSTRACT**----------

Invertebrates account for roughly 95% of all animals, yet surprisingly little effort has been invested to understand their value in signaling potential environmental endocrine disruption. There has, however, been much recent attention to vitellogenin induction in egg-laying invertebrates and vertebrates as indicators of exposure to estrogenic xenobiotics. Mysid shrimp (Crustacea: Mysidacea) have been put forward as suitable test organisms for the evaluation of environmental endocrine dispution by several researchers and regulatory bodies (e.g. USEPA). In view of developing sensitive assays to study endocrine disruption in the estuarine mysid *Neomysis integer*, we isolated and characterized vitellin, the major yolk protein in eggs. Vitellin was purified using gel filtration and characterized by electrophoresis using different staining procedures. Specific (as shown by Western blotting) polyclonal antibodies against the purified vitellin of *N. integer* were produced in rabbit. These antisera will be used to develop immunoassays to study vitellogenesis in mysids and to detect potential stimulatory or inhibitory effects of endocrine disruptors on the production of vitellin.

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### 2.1. INTRODUCTION

Invertebrates have received little attention in signaling potential environmental endocrine disruption compared to vertebrates (e.g. Billinghurst et al., 2000; LeBlanc and Bain, 1997; DeFur et al., 1999; LeBlanc, 1999; Oberdörster and Cheek, 2000). This can be attributed largely to the shortage of fundamental knowledge of their endocrine systems. The relatively

large body of information on insect and crustacean endocrinology makes them excellent candidates for evaluating environmental consequences of chemically-induced endocrine disruption. Of the crustaceans, mysid shrimp have been put forward as suitable test organisms for the evaluation of endocrine disruption by several researchers (DeFur et al., 1999; LeBlanc, 1999; Verslycke et al., 2004a) and regulatory instances (CSTEE, 1999; USEPA, 2002).

*Neomysis integer* (Leach, 1814) is one of the most common mysids in European coastal waters. It is a hyperbenthic, euryhaline and eurythermic species, typically occurring in high numbers in estuarine and brackish water environments (Tattersall and Tattersall, 1951). Mysids are omnivorous species and form important links in the food webs of aquatic ecosystems (Mees and Jones, 1997). Furthermore, a growing number of studies has been published on the use of *N. integer* in general toxicity testing (Roast et al., 1999a,c; 2001b; Verslycke et al., 2003a,b,c) and endocrine disruptor evaluations (for a review, we refer to Verslycke et al., 2004a). While effects of chemicals on growth, molting, behavioral endpoints, energy and steroid metabolism, sexual maturity and reproduction have been reported, no studies have been published on the effects of xenobiotics on gonadal maturation and vitellogenesis in mysid shrimp (Verslycke et al., 2004a).

Vitellogenin, the precursor of the egg yolk protein vitellin, has proved to be a valuable endpoint to assess exposure of fish to environmental estrogens (Fenske et al., 2001; Korsgard and Pedersen, 1998; Sumpter and Jobling, 1995). Control of vitellogenesis is being studied intensively because it is an excellent model for studying mechanisms of hormonal control at the cellular and molecular levels (Billinghurst et al., 2000; Tuberty et al., 2002). Recently, an increasing number of studies have been published on vitellogenesis in egg-laying invertebrates (e.g. Lee et al., 1997; Oberdörster et al., 2000; Tsukimura, 2001; Tsukimura et al., 2002; Vazquez Boucard et al., 2002). Crustacean vitellin is a high molecular weight lipo-glyco-carotenoprotein (Kerr, 1969). It is the major yolk protein of mature crustacean eggs and it is vital to the nutritional needs of the developing embryo (Lee, 1991). To assess potential adverse effects of xenobiotics on crustacean reproduction, it is imperative to measure accurately vitellogenin and vitellin in crustacean models (Tsukimura et al., 2000; Tuberty et al., 2002). Recent studies have focused on the identification and purification of vitellin from well-established standard toxicity test species such as mysids (Tuberty et al., 2002), prawns (Chang et al., 1996; Chen and Kuo, 1998; Kawazoe et al., 2000) and shrimp (Oberdörster et al., 2000).

In line with our previous work on endocrine disruption in the European estuarine mysid *N. integer*, the aim of the present study was to purify and characterize vitellin from this species.

Vitellin was purified from eggs of adult female *N. integer* and characterized by electrophoresis using different staining procedures. The purified protein was used to immunize rabbits for polyclonal antibody production. The specificity of the antisera was tested by Western blotting.

## 2.2 MATERIAL AND METHODS

### *2.2.1. Test organisms*

*N. integer* were collected from the shore by hand net in the Galgenweel, a brackish water near the river Scheldt in Antwerp (Belgium). After a 24h acclimation period to the maintenance temperature, the organisms were transferred to 200-l glass aquaria. Culture medium was artificial seawater (Instant Ocean®, Aquarium Systems, France), diluted with aerated deionized tap water to a final salinity of 5 psu. A 14h light:10h dark photoperiod was used during culturing and water temperature was maintained at 15°C. Cultures were fed daily with 24-48h old *Artemia* nauplii *ad libidum*. Hatching of the *Artemia* cysts was performed in 1-l cylinder-conical vessels under vigorous aeration and continuous illumination at 25°C.
Initial animals of the sub-tropical American mysid species *Americamysis bahia* were obtained from Aquatic Research Organisms (Hampton, USA). The animals were cultured under similar conditions as those of *N. integer* in the laboratory, except for a salinity of 30 and a temperature of 20°C.

### *2.2.2. Purification of vitellin*

Egg masses were taken from ovigorous females, weighed (335 mg, the total wet weight of eggs used for purification) and homogenized on ice in 1.8 ml buffer (136 mM NaCl, 10 mM $Na_2HPO_4$, 2.7 mM KCl, 1.8 mM $KH_2PO_4$, 2% glycine ethyl ester, 0.03% EDTA, 1 mM PMSF, 0.2% aprotinin, 2 mM leupeptin, and 0.02% sodium azide; pH 7.3). Homogenates were centrifuged at 4°C for 15 min at 11000 g. The supernatant was centrifuged a second time (15 min, 11000 g). Crude homogenates were stored at 4°C until further purification.
The supernatant (1.8 ml) was subjected to gel filtration using an Äkta system (Amersham Biosciences, Uppsala, Sweden) by injecting the sample from a 2 ml sample loop onto a HiLoad 16/60 200 prep grade Superdex column (Amersham Biosciences). The column was equilibrated with 20 mM TBS, 5 mM EDTA, pH 7.6, during two column volumes. Elution of

the sample was performed with 1 column volume at a flow rate of 1 ml/min. Eluates were monitored at 280 and 474 nm for concomitant maximum protein and carotenoid pigment absorption, respectively. Fractions of 1.5 ml were automatically collected. The diluted fractions that eluted with concomitant peaks at both 280 and 474 nm were pooled and concentrated in an ultrafiltration device with a 10.000 molecular weight cut-off membrane (Vivascience, Hannover, Germany).

### *2.2.3. Gel electrophoresis and staining*

Purity of the isolated vitellin was analyzed using native polyacrylamide gel electrophoresis (native PAGE), consisting of a 12% polyacrylamide separating gel with a 4% polyacrylamide stacking gel (Ready Gel Tris-HCl Gels, Biorad). The electrophoresis was performed with an electrophoresis buffer (25 mM Tris, 192 mM glycine, pH 8.3) in a vertical Ready Gel® Precast Gel System (BioRad, Eke, Belgium). The gels were stained with Coomassie brilliant blue R250 and Silver staining (rapid silver staining kit, Sigma).
Gels were also stained for carbohydrates and lipids with Periodic Acid Schiff's reagent (Sigma, Bornem, Belgium) and Sudan Black B (Sigma), respectively, after separating the subunits on a 7.5% sodium dodecyl sulfate (SDS) PAGE (Clausen, 1988).

### *2.2.4. Determination of molecular weight*

The molecular weight of native vitellin was estimated by two methods. One method consisted of electrophoresis on 4.5-10% native PAGE using a nondenatured protein molecular weight marker kit (Sigma). The standard molecular mass markers in this kit were urease hexamer (545 kDa), urease trimer (272 kDa), bovine serum albumin dimer (132 kDa), bovine serum albumin monomer (66 kDa), chicken egg albumin (45 kDa), bovine erythrocytes carbonic anhydrase (29 kDa), and bovine milk α-lactalbumin (14.2 kDa). Eight gels of different polyacrylamide content (4.5-10%) were run to determine the the electrophoretic mobility ($R_f$) of the protein in each gel relative to the position of the tracking dye. The slope of the plot presenting log ($R_f$ x 100) against the percent gel concentration, yielding the Retardation Coefficient ($K_R$) for each protein. The $K_R$ was plotted against the logarithm of the molecular weight of each protein, this allowed to determine the molecular weight of vitellin. The other method used was gel filtration on an Äkta system using a HiLoad 16/60 200 prep grade Superdex column (Amersham Biosciences). The standard molecular mass markers used in the

latter method were blue dextran (2000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa).

The molecular weights of the different vitellin subunits were determined using 7.5 % SDS-PAGE gels. The molecular weight marker (All Blue) ranged from 250 kDa to 10 kDa and was purchased from Bio-Rad (Eke, Belgium). Protein gels were scanned with a GelDoc 2000 system (Bio-Rad) and analyzed using Quantity One® software (Bio-Rad).

### *2.2.5. Preparation of antiserum against vitellin*

Polyclonal antibodies against vitellin were produced in New Zealand white rabbits by Eurogentec (Seraing, Belgium). The antiserum was stored in aliquots at –80°C until further use.

### *2.2.6. Western blotting*

All products were purchased from Sigma (Bornem, Belgium) except where indicated differently.

Proteins were transferred onto a nitrocellulose membrane (0.45 µm) from native- and SDS-PAGE gels using a Mini Trans-Blot® system (BioRad). Transfer was conducted at 100 V for 90 min in TGM buffer (3.03 g/l Trisma base, 14.4 g/l Glycine, 200 ml/l Methanol, pH 8.3). The membrane was blocked overnight with 5 % nonfat dehydrated milk in 150 mM phosphate buffered saline (PBS), pH 7.2. The membrane was then washed 3 times for 10 min in PBS containing 0.1% Tween20 (PBS-T) and incubated with anti-vitellin polyclonal antisera (1:10000 in PBS-T) for 1 h at room temperature. The membrane was washed 3 times with PBS-T and incubated for 2 hours with the secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG) diluted 1:5000 in PBS-T. The membrane was then washed 3 times 10 min in PBS-T. The antigen-antibody complexes were identified by addition of color developing solution (fast BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets). Washing the membrane in several changes of deionized water terminated the reaction.

## 2.3. RESULTS

### *2.3.1. Purification of vitellin*

The elution profile of the crude egg homogenate from gel filtration on a HiLoad 16/60 200 prep grade Superdex column is presented in Fig. 2.1. During separation of the crustacean egg homogenates, two peaks with absorptions at both 474 nm and 280nm, eluted after 40 min and 60 min (40 and 60 ml of buffer volume respectively). These peaks were assumed to be vitellin because the compound is known to be associated with carotenoid pigments (i.e. absorption at 474 nm). Additional protein peaks eluted from the column after 100-120 min, but these did not show absorption at 474 nm.

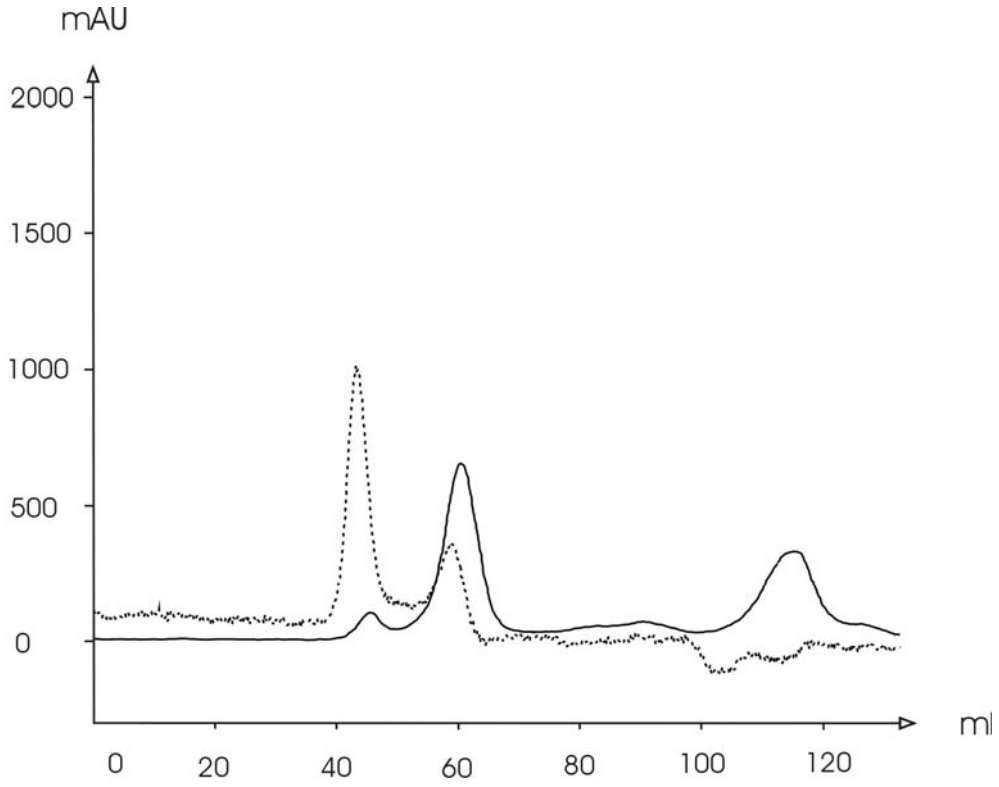


**Figure 2.1:** Gel filtration chromatography of crude egg homogenate from *N. integer* on a HiLoad 16/60 200 prep grade Superdex column. Eluates were monitored at 280 nm (full line) and 474 nm (interrupted line). Elution of the sample was performed at a flow rate of 1 ml/min.

*2.3.2. Characterization of vitellin*

The molecular mass of native vitellin of *N. integer*, determined by electrophoresis on 4.5-10% native PAGE gels with standard molecular mass markers and by gel filtration, was approximately 700 kDa.

Electrophoresis of purified vitellin by SDS-PAGE under reducing and denaturing conditions produced the breakdown of the native form into 6 subunits, visible after staining the gel with Coomassie Blue (Fig. 2.2; 51, 55, 62, 66, 84, and 89 kDa). Some minor bands were detected after staining the gel with Silver staining. These extra bands had molecular masses of 25, 27, 30, 99, 123, 156, and 192 kDa.

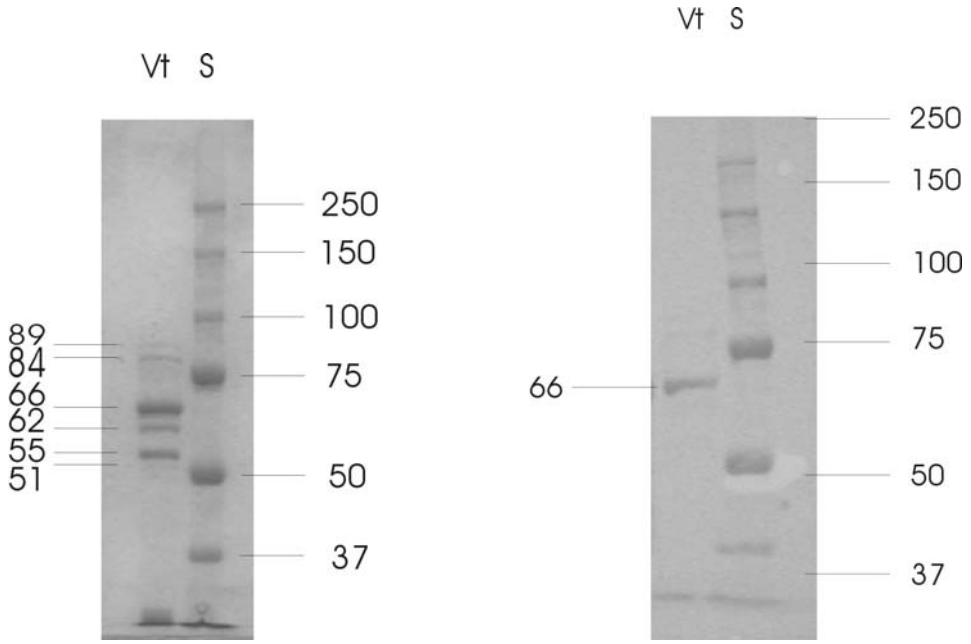


**Figure 2.2:** SDS-PAGE showing the subuntis of purified vitellin of *N. integer* (left). Gel was stained with Coomassie brilliant blue R250. Western blot with antiserum against vitellin of *N. integer* (right). Vt, vitellin; S, standard in kilodaltons (kDa).

The purified vitellin contained carbohydrate and lipid moieties based on the staining with Periodic Acid Schiff's reagent and Sudan Black B reagent, respectively.

Western blots of denatured SDS-PAGE gels using antisera against mysid vitellin produced one band, corresponding to the most concentrated subunit of 66 kDa (Fig. 2.2). Western

blotting on native PAGE loaded with egg extract, vitellin and male homogenate showed no cross-reactivity of the antisera against other proteins than vitellin (Fig. 2.3).

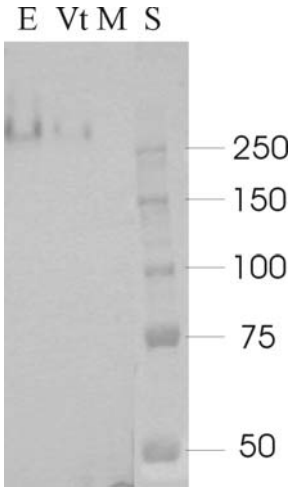


**Figure 2.3:** Western blot on native PAGE with antiserum against vitellin of *N. integer*. E, egg extract; Vt, vitellin; M, male homogenate; S, standard in kilodaltons (kDa).

*2.3.3. Antisera against vitellin of N. integer and A. bahia*

Antiserum against purified vitellin of *N. integer* was compared to the antiserum against purified vitellin of *Americamysis bahia* (courtesy of Dr. S. Tuberty, University of West Florida, FL, USA). Western blots with antiserum of *N. integer* showed that the antibody reacted specifically with vitellin of *N. integer*, but that no cross-reactivity was found with proteins of male *N. integer* or proteins of *A. bahia* (Fig. 2.4). In the eggs of *N. integer*, the antiserum reacted also with lower molecular mass proteins than the 66 kDa subunit of vitellin. Contrary to this, the antiserum of *A. bahia* was less specific and also reacted with proteins of male *A. bahia* and with proteins of *N. integer*.

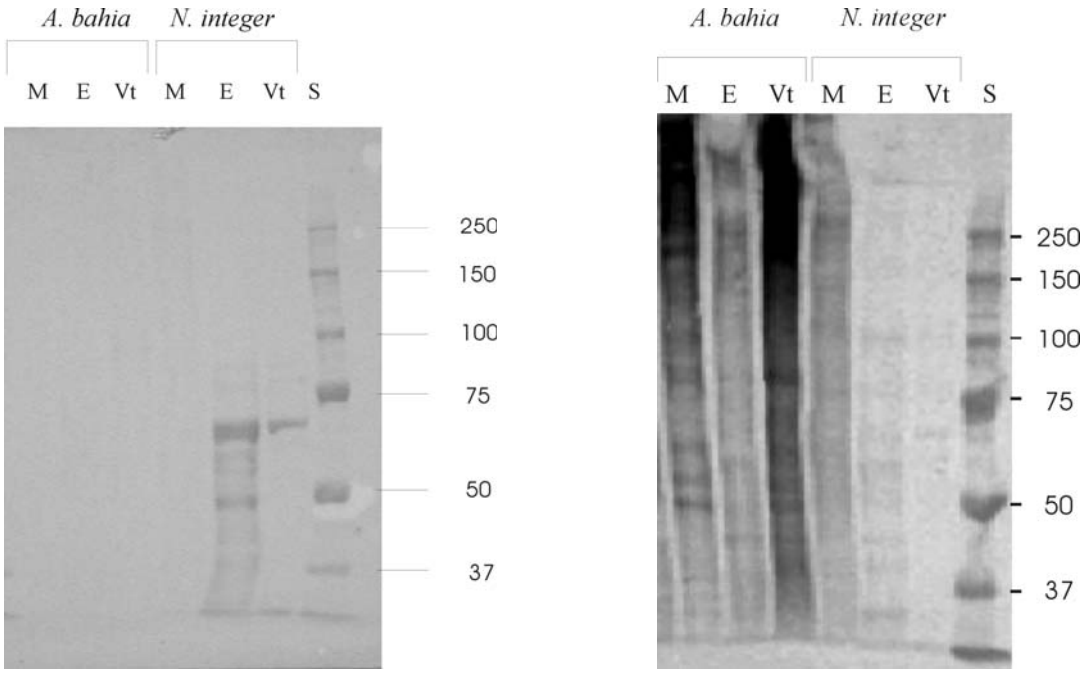


**Figure 2.4:** Comparison of antisera against vitellin of *N. integer* (left) and *A. bahia* (right) using Western blots . Vt, vitellin; E, egg extract; M, male homogenate; S, standard in kilodaltons (kDa).

## 2.4. DISCUSSION

The current investigations were triggered by our attempts to search for a suitable biomarker in *Neomysis integer* indicating exposure to endocrine disruptors. Reproductive endpoints are considered as good biomarkers for evaluating environmental endocrine disruption. A promising biomarker is vitellogenesis which can be studied by measuring accurately vitellogenin and vitellin (an overview of crustacean species from which vitellin or vitellogenin has been isolated, or partially characterized, is given in Tuberty et al., 2002). The initial steps in identifying vitellin in *N. integer* used differential staining techniques and Western Blotting with an antibody against vitellin of *Americamysis bahia*. However, differential staining (PAS and Sudan black) of the proteins in *N. integer* did not allow the identification of vitellin, and Western blots with antibodies against vitellin of *A. bahia* produced cross-reactivity. It was therefore decided to purify vitellin of *N. integer* and produce polyclonal antibodies. Because vitellin is the major protein in crustacean eggs and since it has a relatively high molecular weight, the protein can be purified easily by gel filtration and anion exchange chromatography (Kawazoe et al., 2000; Tuberty et al., 2002).

Other methods reported in literature for the isolation of vitellin of crustaceans are ultracentrifugation (Vazquez Boucard et al., 2002) and high-performance liquid chromatography (Chen and Kuo, 1998; Yang et al., 2000). A preliminary experiment demonstrated that a second purification step was redundant. In this experiment, the pooled fractions were loaded onto a prepacked HR 5/5 anion exchange column (Amersham Biosciences) connected to an Äkta system. Following electrophoresis, the vitellin was as pure as the vitellin purified by gel filtration alone, i.e. only one band was observed on native PAGE, so this additional purification step was omitted in the future in order to avoid loss of protein. The elution profile of *N. integer* by gel filtration (Fig. 2.1) was very similar to the one of *A. bahia* (Tuberty et al., 2002). In the elution profile two peaks showed absoptions at both 474 (carotenoid) and 280 (protein) nm. These were assumed to be vitellin, as vitellin is known to be associated with carotenoid pigments. Fractions from the second peak were pooled to avoid contamination in the final product. The first peak may indicate the presence of vitellogenin, the precursor of vitellin, which has a higher molecular weight. Alternatively, the first peak could result from multimerisation of vitellin. Like fish vitellogenin, crustacean vitellin is sensitive to degradation. Frozen crustacean vitellin samples produced two to five protein bands on a native gel (Tuberty et al., 2002). For the present study, mysid egg masses were stored at 4°C for a minimum amount of time (a few hours) before purification to minimize degradation. Native PAGE of the purified extracts showed that no protein degradation had taken place using this procedure.

Polyclonal antibodies against vitellin of other crustaceans have been produced for different purposes. For instance, antibodies have been used to study fundamental aspects of vitellogenesis, like elucidating the mechanisms of control and synthesis of the egg yolk production (Lee and Chang, 1997; Tsukimura, 2001; Vazquez Boucard et al., 2002). In addition, induction of vitellin by chemicals has been studied in crustaceans (Riffeser and Hock, 2002; Volz and Chandler, 2004) and specific immunoassays have been developed (Lee and Watson, 1994; Tsukimura et al., 2000). The goal of the present study corresponds to the latter type of studies. The antisera will be used to develop an Enzym-Linked Immunosorbent Assay (ELISA) for investigating effects of xenobiotics on vitellogenesis. Western blots demonstrated that the polyclonal antibody against vitellin of *N. integer* is specific and produces no cross-reactivity against other male proteins or proteins of *A. bahia*. In the eggs of *N. integer*, the antiserum also reacted with lower molecular mass proteins than the 66 kDa subunit of vitellin. The egg sample may contain vitellogenin that produces other subunits than vitellin as shown by Lee et al. (1997) in the Indian white prawn *Ferreropenaeus indicus*. On

the contrary, in our study, the antibody against vitellin of *A. bahia* showed high cross-reactivity against high-molecular weight proteins (Fig. 2.4). Differences in antisera specificity could be due to antigen purity, immunization conditions or blotting conditions. The Western blotting conditions were the same as the conditions described by Tuberty et al. (2002).

Most studies indicate that crustacean vitellin has a molecular mass ranging from 300 to 500 kDa (e.g. Lee and Chang, 1997 and references therein), although other researchers reported higher molecular masses for vitellin (e.g. Nakagawa et al., 1982; Quinitio et al., 1989). In the present study it was found that vitellin from the mysid *N. integer* has a molecular mass of approximately 700 kDa. Electrophoresis of purified vitellin by SDS-PAGE produced the breakdown of the native form into 6 subunits with molecular masses of 51, 55, 62, 66, 84, and 89 kDa (Fig. 2.2). There appears to be considerable variability in the subunit composition of vitellin in crustacean species. Tuberty et al. (2002) isolated vitellin from 5 different estuarine crustacean species and depending on the species these authors detected 6 to 12 subunits. *A. bahia* vitellin formed 8 subunits.

To conclude, in the present study we purified and characterized vitellin of *N. integer* using gel filtration and PAGE with differential staining. *N. integer* vitellin has a molecular mass of 700 kDa and is composed of 6 subunits. Polyclonal antibodies were produced and Western blotting demonstrated that these were specific against vitellin of *N. integer*. The produced antibodies will be used to develop an ELISA which will be an essential research tool to investigate vitellogenesis and its disruption by chemicals in mysid shrimp.

# CHAPTER 3

## ELISA DEVELOPMENT FOR VITELLIN OF *NEOMYSIS INTEGER*

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# CHAPTER 3

## ELISA DEVELOPMENT FOR VITELLIN OF *NEOMYSIS INTEGER*

**ABSTRACT** ----------

Mysid crustaceans have been put forward by several regulatory bodies as suitable test organisms to screen and test the potential effects of environmental endocrine disruptors. Despite the well-established use of mysid reproductive endpoints such as fecundity, egg development time, and time to first brood release in standard toxicity testing, little information exists on the hormonal regulation of these processes. Control of vitellogenesis is being studied intensively because yolk is an excellent model for studying mechanisms of hormonal control, and because vitellogenesis can be chemically disrupted. Yolk protein or vitellin is a major source of nourishment during embryonic development of ovigorous egg-laying invertebrates. The accumulation of vitellin during oocyte development is vital for the production of viable offspring.

In this context, we developed a competitive enzyme-linked immunosorbent assay (ELISA) for vitellin of the estuarine mysid *Neomysis integer*. Mysid vitellin was isolated using gel filtration, and the purified vitellin was used to raise polyclonal antibodies. The ELISA was sensitive within a working range of 4 to 500 ng vitellin / ml. Serial dilutions of whole body homogenates from female *N. integer* and the vitellin standard showed parallel binding curves, validating the specificity of the ELISA. The intra- and interassay coefficients of variation were 8.2 and 13.8%, respectively. Mysid vitellin concentrations were determined from ovigorous females and eggs at different developmental stages. The availability of a quantitative mysid vitellin ELISA should stimulate further studies on the basic biology of this process in mysids. Furthermore, it could provide a means to better understand and predict chemically-induced reproductive effects in mysids.

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## 3.1. INTRODUCTION

Most of the current knowledge of crustacean endocrinology is based on studies with decapods such as crabs, lobsters, crayfish and shrimp (Carlisle and Knowles, 1959; Chang, 1997a; Charmantier et al., 1997; DeFur et al., 1999; Fingermann, 1987; Lafont, 2000; Quackenbush, 1986). We recently published a comprehensive review on the use of mysid shrimp as potential models to study hormonal regulation and its disruption by chemicals (Verslycke et al., 2004a). In addition, mysids have been put forward as suitable test organisms by several other researchers (DeFur et al., 1999; LeBlanc, 1999) and regulatory authorities (CSTEE, 1999; USEPA, 2002) for the evaluation of endocrine disruptors. Despite the well-established use of mysid reproductive endpoints such as fecundity, egg development time, and time to first brood release in standard toxicity testing, little information exists on the hormonal regulation and basic biology of these processes (Verslycke et al., 2004a). Few studies have examined the effects of contaminants on gonadal maturation in crustaceans, and the lack of knowledge of invertebrate endocrinology in general, is one of the main reasons for the very limited progress that has been made regarding endocrine disruption research in invertebrates (Oetken et al. 2004). Much attention has recently been given to vitellogenin, the precursor to the yolk protein vitellin in egg-laying vertebrates and invertebrates, as an indicator of exposure to endocrine disruptors (Billinghurst et al., 2000; Fenske et al., 2001; Tsukimura, 2001). Vitellogenesis involves the production of yolk proteins that act as nutrient sources for developing embryos. Consequently, any event that affects the synthesis of the yolk precursor vitellogenin will also modify reproductive success. Studies on the hormonal regulation of vitellogenesis in mysids at this point are nonexistent because assays to measure the relevant hormones are not available. In a recent study, we purified and characterized vitellin from the mysid *Neomysis integer* (Chapter 2). *N. integer* is the dominant hyperbenthic mysid in the upper reaches of European estuaries. It is sensitive to many toxicants at environmentally relevant concentrations, and has been suggested as a more ecologically relevant alternative to high-latitude and low-saline systems than the standard toxicity test species *Americamysis bahia* (Emson and Crane, 1994; Mees et al., 1995b; Mees and Jones, 1997; Roast et al., 1999a, 2001a; Verslycke et al., 2003b; Wildgust and Jones, 1998).

Hormonal control of vitellogenesis in crustaceans is closely linked with the molt cycle (Fig. 3.1). Molting, a well-studied hormonally regulated process, is critical in the development and maturation of every arthropod (DeFur, 2004). There are several feedback mechanisms for the control of molting hormones (e.g., ecdysone) and a number of peptide hormones that regulate

vitellogenesis and molting in crustaceans (Chang, 1993; DeFur et al., 1999; Meusy and Payen, 1988; Oberdörster and Cheek, 2000). The production of vitellin is under direct control of peptide hormones like the 'vitellogenesis-inhibiting hormone' (VIH) produced by the X-organ located in the eyestalk, and the 'vitellogenesis-stimulating hormone' (VSH). The complex hormonal regulation of vitellogenesis makes it an excellent model for studying mechanisms of hormone signalling at the cellular and molecular level (Billinghurst et al., 2000; Tuberty et al., 2002).

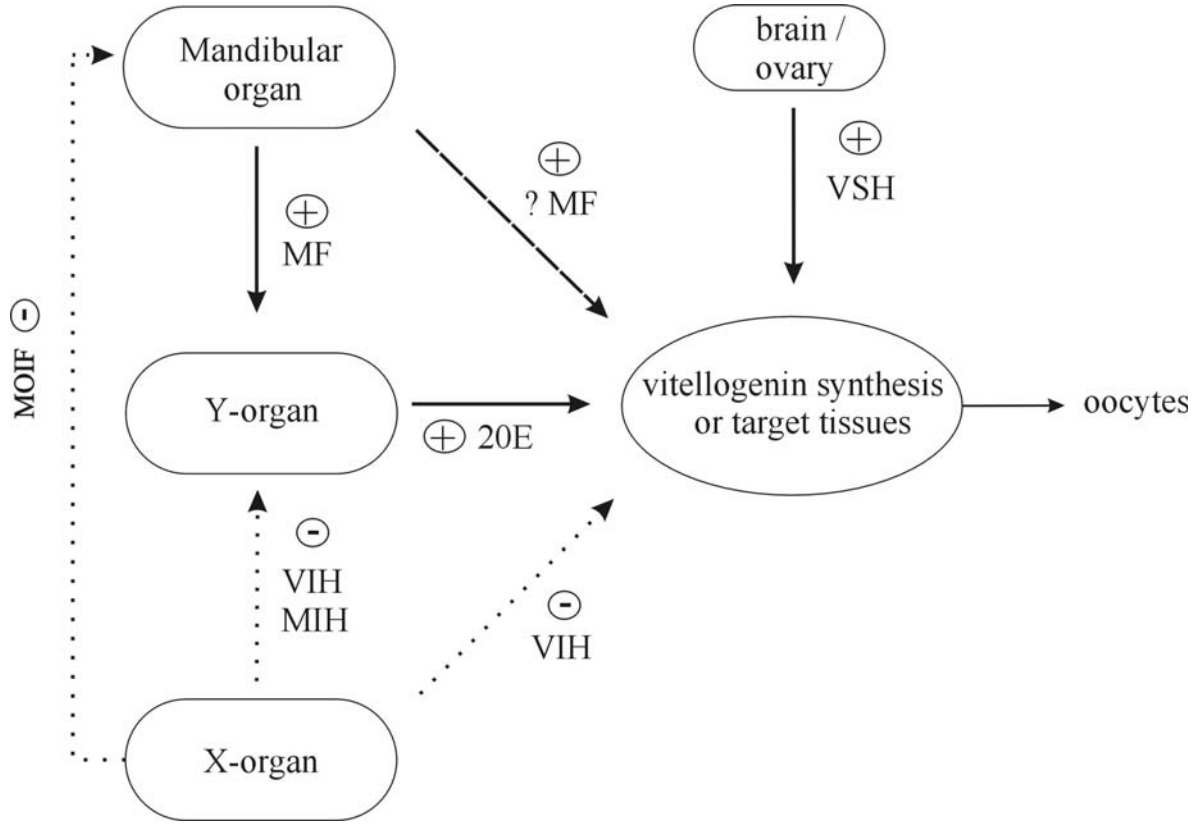


**Figure 3.1:** Simplified scheme of the hormonal control of the crustacean molt cycle and vitellogenesis. Adapted from Defur et al., 1999; Meusy and Payen, 1988; Oberdörster and Cheek, 2000. Interrupted arrows (-) represent inhibition and full arrows (+) stimulation. The following hormones play an important role in regulating crustacean molting and vitellogenesis: 20E, 20-hydroxyecdysone, the active molting hormone; MF, methyl farnesoate; MOIF, mandibular organ-inhibiting factor; VIH, vitellogenesis-inhibiting hormone; VSH, vitellogenesis-stimulating hormone.

Vitellogenesis involves two phases, primary and secondary vitellogenesis. Primary vitellogenesis is continuous and primary follicles have endogenous vitellin. The secondary

vitellogenesis takes place during the reproductive season. The prominent feature of secondary vitellogenesis is the uptake of exogenous vitellogenin in the oocytes (Charniaux-Cotton, 1985). In mysids, juveniles are released from the marsupium immediately before ecdysis of the mother, shortly after which she lays a new batch of eggs in the marsupium. A secondary vitellogenic cycle starts for a new batch of oocytes on the second day of the molt cycle, offering an example of the type 2 pattern for the regulation of simultaneous gonadal and somatic growth as seen in Decapoda, Amphipoda and Isopoda (Adiyodi and Subramoniam, 1983; Charniaux-Cotton, 1985).

In this study, we developed a competitive ELISA to measure vitellin concentrations in *N. integer,* which will allow for future investigations into hormonal regulation of mysid vitellogenesis and its potential disruption by chemicals.

## 3.2. MATERIAL AND METHODS

### *3.2.1. Test organisms*

*N. integer* were collected from Braakman , a brackish water (10 psu) near the Schelde estuary in Hoek (The Netherlands) in summer of 2004 and cultured in the laboratory as described in Chapter 2 (§ 2.2.1.).

### *3.2.2. Vitellin purification*

Vitellin was purified from egg masses taken from ovigorous females as described in Chapter 2 (§ 2.2.2.).

### *3.2.3. Production of polyclonal vitellin antibodies*

Polyclonal antibodies against vitellin were produced in New Zealand white rabbits by Eurogentec (Seraing, Belgium). The antiserum was stored in aliquots at –80°C until further use.

### *3.2.4. Development of a homologous competitive ELISA for Neomysis integer vitellin*

The assay is based on a competition for the vitellin antibody between vitellin coated on the

wells of a microtiter plate and free vitellin molecules in the sample solution. The antigen-antibody complex bound to the plate is detected by a secondary antibody directed against the primary vitellin antibody. This secondary antibody is conjugated with the enzyme horseradish peroxidase. The enzyme activity is revealed by adding a suitable substrate and hydrogen peroxide, and is measured colorimetrically.

### *3.2.5. General ELISA protocol*

#### *3.2.5.1. Coating the plates*

Purified vitellin was thawed on ice and diluted in coating buffer (0.05 M sodium carbonate buffer, pH 9.6). The wells of 96-well microtiter plates (Nunc F96 Maxisorp™ Immuno Plate) were coated with 100 µl of vitellin solution (100 ng vitellin/ml coating buffer), sealed and incubated overnight at 4°C. For determination of non-specific binding (NSB) effects, three wells per plate were treated with coating buffer only.

#### *3.2.5.2. Preincubation of samples/standards*

For the standards, purified vitellin was diluted in PBS-T blocking buffer (0.01 M phosphate-buffered physiological saline solution with 0.05 % Tween 20 and 1 % fatty acid-free Bovine Serum Albumin (BSA) to a concentration of 2000 ng vitellin/ml. From this stock solution, serial dilutions were prepared in PBS-T blocking buffer. In parallel, samples with an unknown vitellin content were diluted in PBS-T blocking buffer. The vitellin standards and unknown samples (60 µl/well) were incubated in non-coated 96-well microtiter plates with vitellin antibody (60 µl/well, 1:10 000 in PBS-T blocking buffer). Our vitellin standard was quantified using the Bradford method with BSA as reference protein. For the NSB, 60 µl/well of blocking buffer was mixed with 60 µl of the antibody solution only. The incubates were mixed on a rotary shaker, and the plates were sealed and incubated overnight at 4°C.

#### *3.2.5.3. Antibody incubation*

The coated plates were washed three times with 100 µl PBS-T washing buffer (0.01 M phosphate-buffered physiological saline solution with 0.05 % Tween 20, pH 7.4). To reduce background, the plates were blocked with 150 µl of PBS-T blocking buffer/well for 30 min at

37°C. After this blocking step, the plates were washed another three times with PBS-T, before 100 µl of the sample/antibody or standard/antibody incubates were pipetted into the wells. The plates were sealed and incubated for 120 min at 37°C. The first antibody incubates were then removed and the plates were washed three times with PBS-T. Second antibody (125 µl) against rabbit IgG (goat anti-rabbit IgG, whole molecule, peroxidase conjugate; Sigma) was added to each well at a dilution of 1:2000 in PBS-T blocking buffer and the plates were sealed and incubated at 37°C for 60 min.

*3.2.5.4. Detection*

The plates were washed three times with PBS-T and then 125 µl of the enzyme substrate solution was added to each well. This solution was prepared by dissolving 0.5 mg/ml of *o*-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich) in 0.05 M phosphate-citrate buffer, pH 5.0 (0.051 M dibasic sodium phosphate, 0.024 M citric acid). After addition of 0.5 µl/ml of $H_2O_2$ (30%; Merck), the substrate solution was immediately pipetted into the plates (125 µl/well). The enzyme reaction was allowed to proceed for 10 min in the dark, at which point the color reaction was stopped by the addition of 30 µl of 3 N $H_2SO_4$. The absorbance of the reaction product was read at 490 nm using a microtiter plate reader (Multiskan Ascent®, Thermo Labsystems). The absorbance values obtained in the ELISA were inversely proportional to the amount of vitellin present in the sample. Vitellin content in samples was quantified from the log-transformed standard curve.

*3.2.6. Quantification of vitellin in eggs and whole body homogenates*

Eggs in the marsupium of gravid females were staged under a microscope. Mauchline (1980) gives a description of the different developmental stages of the embryos. After decapitation of the gravid females, mysid embryos were removed with a fine spatula while submerged in Tris-HCl pH 7.2. Individual eggs were placed in 60 µl Tris-HCl pH 7.2, and further diluted 100 times using the same buffer to quantify vitellin. Vitellin was quantified in 10 replicate eggs of each developmental stage. Gravid females with embryos of stage I were homogenized in 200 µl Thris-HCl pH 7.2 and diluted 10,000 times to quantify vitellin. Ten replicates were used.

## 3.3. RESULTS

### *3.3.1. Purification and characterization of vitellin*

We purified and characterized vitellin of *N. integer* using gel filtration and polyacrylamide gel electrophoresis (PAGE) with different stainings. Polyclonal antibodies were produced and Western blotting demonstrated that these were specific against vitellin of *N. integer*. Details are given in Chapter 2 (§ 2.3.).

### *3.3.2. Development and validation of a competitive ELISA*

Dilutions of the homologous antiserum between 1:5000 and 1:30 000 (data not shown), together with a second antibody titer of 1:2000, produced the best and most reproducible assay conditions. The secondary antiserum dilution was chosen on the basis of other vitellin/vitellogenin ELISAs which use dilutions between 1:3000 and 1:1000 (Fenske et al., 2001; Sagi et al., 1999; Vazquez Boucard et al., 2002). The effect of different coating concentrations (100, 200 and 500 ng/ml) on the standard curve is shown in Fig. 3.2. The standard curve with a coating concentration of 100 ng/ml showed the largest working range. For routine applications of the assay, a primary antibody titer of 1:10 000, a secondary antibody dilution of 1:2000 and a vitellin coating concentration of 100 ng vitellin/ml were chosen. The working range for the assay was between 4 and 500 ng vitellin/ml (Fig. 3.3A). Serial dilutions of whole body homogenate of female *N. integer* showed a good parallelism or similar curves with the standard within the working range of the assay (Fig. 3.3B).

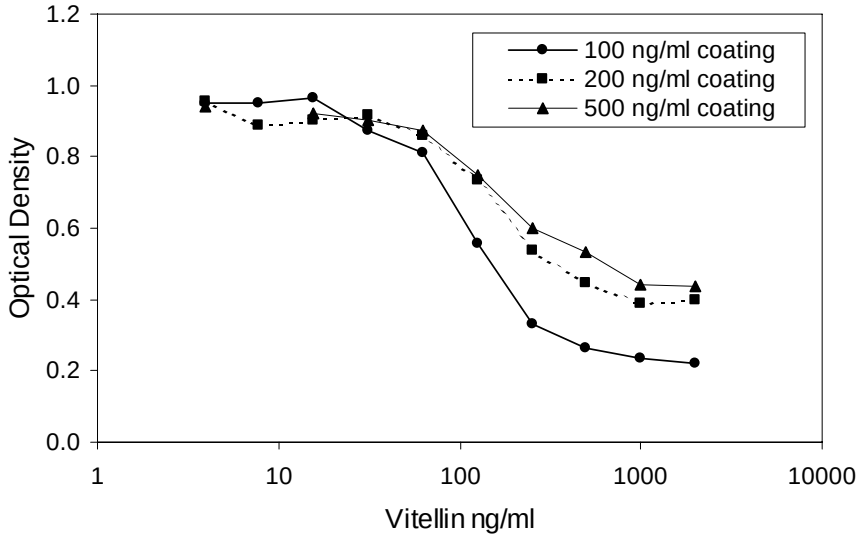


**Figure 3.2:** The effect of different coating concentrations (100, 200 and 500 ng/ml) on the standard curve of *N. integer* vitellin with a primary antibody dilution of 1:10000 and a secondary antibody dilution of 1:2000.

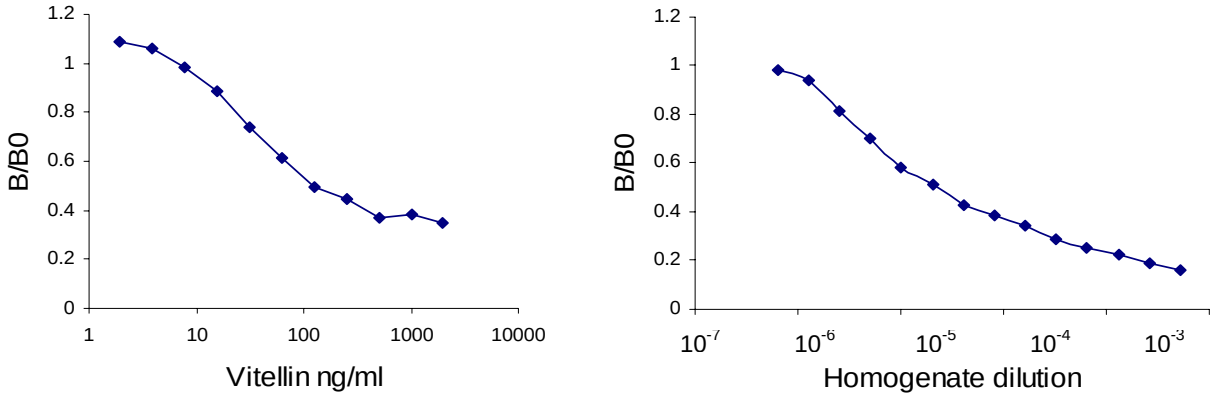


**Figure 3.3:** A: ELISA standard curve of vitellin from *N. integer* with serial dilutions from 2000 to 1.95 ng/ml. B: Serial dilutions of whole body homogenate from female *N. integer*. B/B0, is the optical density of the sample divided by optical density of the saturated well.

The reproducibility of the assay was evaluated. Egg samples with low to high vitellin levels were analyzed multiple (4-5) times in the same and in separate assays. The intra- and

interassay coefficients of variation were 8.2 and 13.8%, respectively.

### *3.3.3. Vitellin levels in eggs and whole body homogenates*

The quantitative ELISA allowed us to measure vitellin levels in a single egg of *N. integer*. Fig. 3.4 shows vitellin levels of eggs at different developmental stages. The development of the eggs within the marsupium can be divided into three stages, which correspond with the stages described by Mauchline (1980) as eggs (stage I), eyeless larvae (stage II) and eyed larvae (stage III). For more information about the embryogenesis of *N. integer* see Chapter 6. Eggs of stage I, II and III have vitellin levels of 104.6 (± 41.0), 40.2 (± 23.6) and 11 (± 8.6) µg/ml respectively. Vitellin levels are expressed in µg/ml since we had to dilute one egg to measure vitellin concentrations, therefore these results are µg/ml from a single egg. The results shown in Fig. 3.4 are from ten replicates. Vitellin levels were also quantified in gravid female animals. Females with eggs of stage I in their marsupium have vitellin levels of 542 (± 120.3) µg/ml.

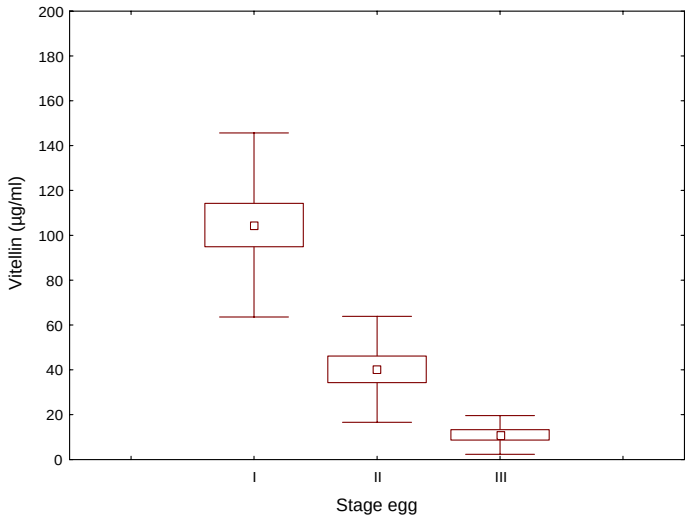


**Figure 3.4:** Vitellin levels in eggs at different developmental stages. Box-plot shows the mean (small square), standard error (box) and the standard deviation (whisker) of 10 replicate measurements.

## 3.4. DISCUSSION

Accurate methods to quantify vitellogenin and vitellin in crustaceans can contribute to

elucidate crustacean reproduction and its potential disruption by endocrine disrupting chemicals. Previous studies often relied on oocyte size or ovarian weight (Anilkumar and Adiyodi, 1980, 1985; Eastman-Reks and Fingerman, 1984). While such measurements have provided important insights into vitellogenesis, they are time-consuming and indirect. Specific immunoassays are generally rapid, precise, reproducible, and therefore provide distinct advantages over other bioassays (Chard, 1987; Lee and Watson, 1994). Vitellin has been measured mostly in crustacean hemolymph and ovaries (Table 1). Ovarian vitellin and protein concentrations are closely correlated with the ovarian stage, and the accumulation of vitellin is linked with an increase in ovarian weight (Lee and Chang, 1997). Vitellin levels in the ovaries of white prawn *Fenneropenaeus indicus* increased from 5.9 to 372.3 mg per ovary during ovarian development (Vazquez Boucard et al., 2002). Tsukimura (2001) reports that vitellogenin levels in crustacean hemolymph ranges from 0.03-10 mg/ml.

The present study is the first to report vitellin concentrations in eggs of a well-established crustacean test species, the mysid *N. integer*. Vitellin levels decreased with the development of the egg, in accordance with the role of vitellin as a major source of nourishment for the developing embryo. It has been shown that vitellin concentrations in eggs are an important and useful indicator of egg quality, and vitellin levels can be used to predict female reproductive performance (Arcos et al., 2003). The ELISA developed in this study was capable of detecting vitellin levels in *N. integer* from 4 to 500 ng/ml. This range is comparable to the ELISA developed for ridgeback shrimp *Sicyonia ingentis* vitellin (0.3-300 ng/ml; Tsukimura et al., 2000) and for Chinese mitten-handed crab *Eriocheir sinensis* vitellin (7.8-500 ng/ml; Chen et al., 2004). Our ELISA is slightly more sensitive than the one developed for blue crab *Callinectes sapidus* (62-1500 ng/ml; Lee and Watson, 1994) and for the copepod *Amphiascus tenuiremis* (31-1000 ng/ml; Volz and Chandler, 2004). In the ELISA standard curve (Fig.3A) the value of B/B0 generally exceeds 0.3. This observation could be due to unspecificity of the secondary antibody, the ratio between primary to secondary antibody, and/or the ratio between coating and primary antibody, i.e., antibody titre too high and/or high coating concentration could encourage unwanted binding of antibody to plate despite high concentration of free vitellin in plate. However, our results indicate that our ELISA was sufficiently sensitive and similar to previously published ELISAs to quantify vitellin have similar results (Fenske et al., 2001; Lee and Watson, 1994; Tsukimura et al., 2000).

The induction of vitellogenin in male fish has been used extensively as a biomarker of estrogen exposure (Fenske et al., 2001; Heppel et al., 1995; Tyler et al., 1996; Versonnen and

Janssen, 2004). Several researchers have looked at using vitellogenesis in egg-laying invertebrates in a similar way. However, standardized quantitative assays to study vitellogenesis in invertebrates are largely unavailable. Vitellin or vitellogenin has been isolated or partially characterized in several crustacean species (for an overview refer to Tuberty et al., 2002). Still, only a limited number of enzyme linked immunosorbent assays (ELISAs) to quantify vitellogenin or vitellin in freshwater and marine crustaceans have been developed (Table 3.1).

**Table 3.1:** Overview of available ELISAs to quantify vitellogenin or vitellin in freshwater and marine crustaceans

| **Species** | **Tissue** | **References** |
|---|---|---|
| Blue crab, *Callinectes sapidus* | Ovary, hemolymph | Lee and Watson, 1994 |
| Freshwater prawn, *Macrobrachium rosenbergii* | Hemolymph, ovary, hepatopancreas | Lee and Chang, 1997 |
| Crayfish, *Cherax quadricarinatus* | Hemolymph | Sagi et al., 1999 |
| Tiger shrimp, *Penaeus monodon* | Hemolymph | Vincent et al., 2001 |
| Ridgeback shrimp, *Sicyonia ingentis* | Hemolymph | Tsukimura et al., 2000 |
| Lobster, *Homarus americanus* | Hemolymph | Tsukimura et al., 2002 |
| Penaeid prawn *Fenneropenaeus indicus* | Hemolymph, ovary, hepatopancreas | Vazquez Boucard et al., 2002 |
| Copepod, *Amphiascus tenuiremis* | Whole body homogenate | Volz and Chandler, 2004 |
| Crab, *Eriocheir sinensis* | Ovary | Chen et al., 2004 |
| Mysid shrimp, *Neomysis integer* | Whole body homogenate, eggs | Present study |

The complex and still poorly understood regulation of vitellogenesis in many crustaceans, limits its use as a biomarker of endocrine disruption at this time. Future laboratory and field studies with crustaceans, using assays like the one developed in this study, will help to unravel the hormonal regulation of crustacean vitellogenesis and will also allow for the assessment of the potential impact of endocrine disruptors on the reproduction of crustaceans.
In this study, a competitive ELISA to quantify vitellin in the estuarine mysid *Neomysis integer* was successfully developed and allows accurate quantification of vitellin from whole-body homogenates as well as single eggs. 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. The development of suitable standard invertebrate test methods for endocrine disrupting compounds remains an urgent need and mysid crustaceans could provide unique opportunities through their routine use in regulatory screening and testing programs world-wide.

# CHAPTER 4

## EFFECT OF METHOPRENE, NONYLPHENOL AND ESTRONE ON THE VITELLOGENESIS OF THE MYSID *NEOMYSIS INTEGER*

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# CHAPTER 4

## EFFECTS OF METHOPRENE, NONYLPHENOL AND ESTRONE ON THE VITELLOGENESIS OF THE MYSID *NEOMYSIS INTEGER*

**ABSTRACT**----------

The induction of the female-specific protein, vitellogenin, in male fish is a well-established endpoint to assess exposure to estrogen-like chemicals. The use of vitellogenesis as a biomarker for xenobiotic exposure in egg-laying invertebrates, however, is still relatively unexplored. Recently, we developed a quantitative enzyme-linked immunosorbent assay (ELISA) for vitellin in *Neomysis integer* (Crustacea: Mysidacea) to study mysid vitellogenesis and its potential disruption by xenobiotics. In this study, gravid mysids were exposed to methoprene, nonylphenol, and estrone for 96h. All methoprene-exposed (0.01, 1, 100 µg/l) animals had lower vitellin levels compared to the control animals, though this effect was not statistically significant. Exposure to nonylphenol resulted in significantly increased vitellin levels in the lowest exposure concentration (0.01 µg/l), whereas no effects were observed at higher concentrations. Estrone significantly decreased vitellin levels at the highest test concentration (1 µg/l). These results indicate that mysid vitellogenesis can be disrupted following chemical exposure. Difficulties in the interpretation of the observed chemical-specific and concentration-specific responses in this study highlight the need for a better understanding of hormone regulation of crustacean vitellogenesis.

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### 4.1. INTRODUCTION

Vitellogenesis involves the production of the yolk protein vitellin that acts as a nutrient source for the developing embryo. Consequently, any event that affects the synthesis of vitellin and the yolk precursor vitellogenin will also modify reproductive success. A number of

anthropogenic chemicals are known to have the potential to disrupt vitellogenesis in vertebrates. A well known example of endocrine disruption is the induction of vitellogenin in male fish exposed to xeno-estrogens (Fenske et al., 2001; Tyler et al., 1999; Versonnen and Janssen, 2004). Little is known about the potential effects of endocrine-disrupting chemicals on vitellogenesis in invertebrates, and few studies have evaluated endocrine toxicity to vitellogenesis in crustaceans (Billinghurst et al., 2000; Lee and Noone, 1994; Oberdörster et al., 2000; Sanders et al., 2005; Tsukimura, 2001; Volz and Chandler, 2004). Recently, 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 present study validates the use of the *N. integer* vitellin ELISA to detect potential effects of three reported endocrine-disrupting chemicals on mysid vitellogenesis.

ENDIS-RISKS is a multidisciplinary project that studies the occurrence, distribution and potential effects of endocrine disruptors in the Scheldt estuary (Belgium/The Netherlands), one of the most polluted estuaries in the world (ENDIS-RISKS project, http://www.vliz.be/projects/endis). Our first studies found high exposure to endocrine-disrupting substances and potential effects on the resident mysid population in this estuary (Noppe et al., 2005; Verslycke et al., 2004b; Verslycke et al., 2005). A number of priority substances have been identified based on these field studies, and their potential effects on hormone-regulated processes in the mysid *N. integer* are being evaluated through laboratory studies. To that end, we have been researching a number of hormone-regulated processes in mysids that could be used as endpoints to evaluate endocrine disruption, e.g. energy and steroid metabolism (Verslycke et al., 2004b, 2004c), molting (Chapter 5), embryogenesis (Chapter 6,7), and vitellogenesis (present study). Mysid shrimp have been used extensively in regulatory toxicity testing, and it is the only invertebrate species included in USEPA's endocrine disruptor screening and testing program (Verslycke et al., 2004a).

One of the test compounds that has been used in validation studies of these endpoints is the insecticide methoprene. Methoprene is an insect growth regulator that is generally used to control mosquitos. This insecticide has been shown to disrupt normal development in non-target organisms, such as crustaceans (Celestial and McKenney, 1994; McKenney and Celestial, 1996; McKenney and Matthews, 1990; Mu and LeBlanc, 2004; Olmstead and LeBlanc, 2001; Templeton and Laufer, 1983, Walker et al., 2005). The two other chemicals used in the present study are nonylphenol (a breakdwon product of alkylphenol ethoxylates, APEs) and estrone, which have been reported to be present in the Scheldt estuary and are known endocrine disruptors (Noppe et al., 2005; Verslycke et al., 2005). APEs are synthetic

surface-active agents (surfactants), commonly used in industrial detergents and plastic manufacturing (Blackburn et al., 1999). Around 80% of all manufactured APEs are nonylphenol ethoxylates (Naylor, 1998), which degrade to nonylphenol in sewage treatment plants (Ahel et al., 1994). Reported nonylphenol concentrations in U.K. rivers are <0.2-12 µg/l, although concentrations as high as 180 µg/l have been detected in water receiving effluent directly from sewage treatment works (Blackburn et al., 1999; Blackburn and Waldock, 1995; Allen et al., 2002). These levels of nonylphenol correspond with concentrations measured in the Scheldt estuary (Verslycke et al., 2005; Vethaak et al., 2002). From the large group of substances that are suspected or known to be environmental endocrine disruptors, the natural and synthetic estrogens are suggested to have high estrogenic potency (Noppe et al., 2005). Synthetic estrogens are used in birth-control pills and for the management of menopausal syndromes, and cancer (De Alda and Barcelo, 2001). Of the natural female sex hormones, estrone is detected most frequently in the Scheldt estuary at concentrations of up to 8 ng/l (Noppe et al., 2005, Vethaak et al., 2002).

Natural estrogens and xeno-estrogens like APEs cause a number of well-documented estrogenic effects in fish, such as disruption of vitellogenesis (Fenske et al., 2001; Korsgard and Pedersen, 1998; Sumpter and Jobling, 1995). The effect of (xeno-) estrogens on the reproduction of crustaceans, however, remains controversial and is still poorly understood (Billinghurst et al., 2000; Sanders et al., 2005; Tsukimura, 2001). This study is the first to report effects of environmental endocrine disruptors on the vitellogenesis of the mysid *N. integer*.

## 4.2. MATERIAL AND METHODS

### *4.2.1. Chemicals*

Methoprene (CAS # 40596-69-8) and estrone were obtained from Sigma-Aldrich (Bornem, Belgium). Nonylphenol was obtained from Acros Organics (Geel, Belgium). Stock solutions of the test compounds were prepared in absolute ethanol. The ethanol concentration in the solvent control and in the different test concentrations was 0.01%.

### *4.2.2. Test organisms*

The mysid crustacean, *Neomysis integer*, was collected in March 2005 by handnet in the

Braakman, a brackish water (10 psu) near the Scheldt estuary in Hoek (The Netherlands). The mysids were cultured in the laboratory as described in Chapter 2 (§ 2.2.1.).

### *4.2.3. Acute toxicity test with estrone*

96h LC50s for methoprene and nonylphenol to juvenile *N. integer* were previously published by Verslycke et al. (2004c). Juvenile mysids of similar size (visual selection of organisms with a size of 2-4 mm) were taken from the laboratory culture and randomly distributed to 400 ml glass beakers containing 200 ml of the desired test concentration in water with a salinity of 5 psu and temperature of 15°C. For each test concentration, 2 replicate beakers containing 5 mysids each were used. Mysids were exposed for 96h to 1-100-1,000-10,000 µg estrone/l. Exposure solutions were renewed every 24h and juveniles were fed daily with 24h old *Artemia* nauplii *ad libidum*.

### *4.2.4. Test design for vitellin assessment*

Gravid females of approximately the same size (27.5 ± 4.9 mg wet weight), carrying stage I embryos in their marsupium, were selected and exposed to the test compounds. Stage I carrying females were used as this stage can be determined easily and it is a short embryonic stage (±4 days), minimizing the intra-stage variability between individual animals. A detailed description of the different developmental stages of *N. integer* embryogesis is given in Fockedey et al. (2005a) and Chapter 6. Females were exposed to the sublethal concentrations 0.01, 1, and 100 µg methoprene/l; 0.01, 1, and 100 µg nonylphenol/l; 10, 100, and 1,000 ng estrone/l. Females were randomly put in 400 ml beakers containing 200 ml of the desired test concentration in water with a salinity of 5 psu and a temperature of 15 °C. For each test concentration, 2 replicate beakers with 6 females were used and the mysids were exposed for 96h. Exposure solutions were renewed every 24h and test organisms were fed daily with 24h old *Artemia* nauplii *ad libidum*. After 96h, the females were shock-frozen in liquid nitrogen and kept at –80°C until analysis of the vitellin levels using the ELISA. All vitellin analyses were performed within 2 weeks after exposure to reduce the risk of vitellin degradation.
All individual animals were homogenized in 200 µl Tris-HCl pH 7.2 and diluted 10,000 times in this buffer for vitellin quantification. Concentrations are expressed in 1 ml of this homogenate.

*4.2.5. Competitive enzyme-linked immunosorbent assay for Neomysis integer vitellin*

The *N. integer* vitellin ELISA assay was recently developed (Ghekiere et al., 2005). The general ELISA protocol is described in Chapter 3 (§ 3.2.5.)

*4.2.6. Statistics*

All data were checked for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene's test respectively, with an $\alpha = 0.05$. The effect of the treatment was tested for significance using a one-way analysis of variance (Dunnett's test; Statistica™, Statsoft, Tulsa, OK, USA). All box-plots were created with Statistica™ and show the mean (small square), standard error (box), and the standard deviation (whisker).

## 4.3. RESULTS

*4.3.1. Acute toxicity of methoprene, nonylphenol and estrone*

To establish relevant test concentrations for subsequent sublethal vitellogenesis testing, the acute toxicity of estrone was determined. No significant mortality was observed at any of the tested exposure concentrations of estrone, i.e. the 96h-LC50 of estrone to *N. integer* is > 10 mg/l. Mortality in the controls was ≤ 20 %. The 96h-LC50 of methoprene and nonylphenol to juvenile *N. integer* were previously determined to be 320 and 590 µg/l, respectively (Verslycke et al., 2004c).

*4.3.2. Sublethal effects of methoprene, nonylphenol and estrone on vitellogenesis*

The effect of 96h exposure to sublethal concentrations of methoprene, nonylphenol, and estrone on the vitellin levels in gravid *N. integer* are shown in Fig. 4.1. Although methoprene-exposed females exhibited lower vitellin levels than control animals, these reductions were not statistically significant. Only animals exposed to the lowest nonylphenol exposure concentration, 10 ng nonylphenol/l, had significantly induced vitellin concentrations. Finally, only the highest estrone exposure concentration (1000 ng/l) resulted in sigificantly lower vitellin concentrations.

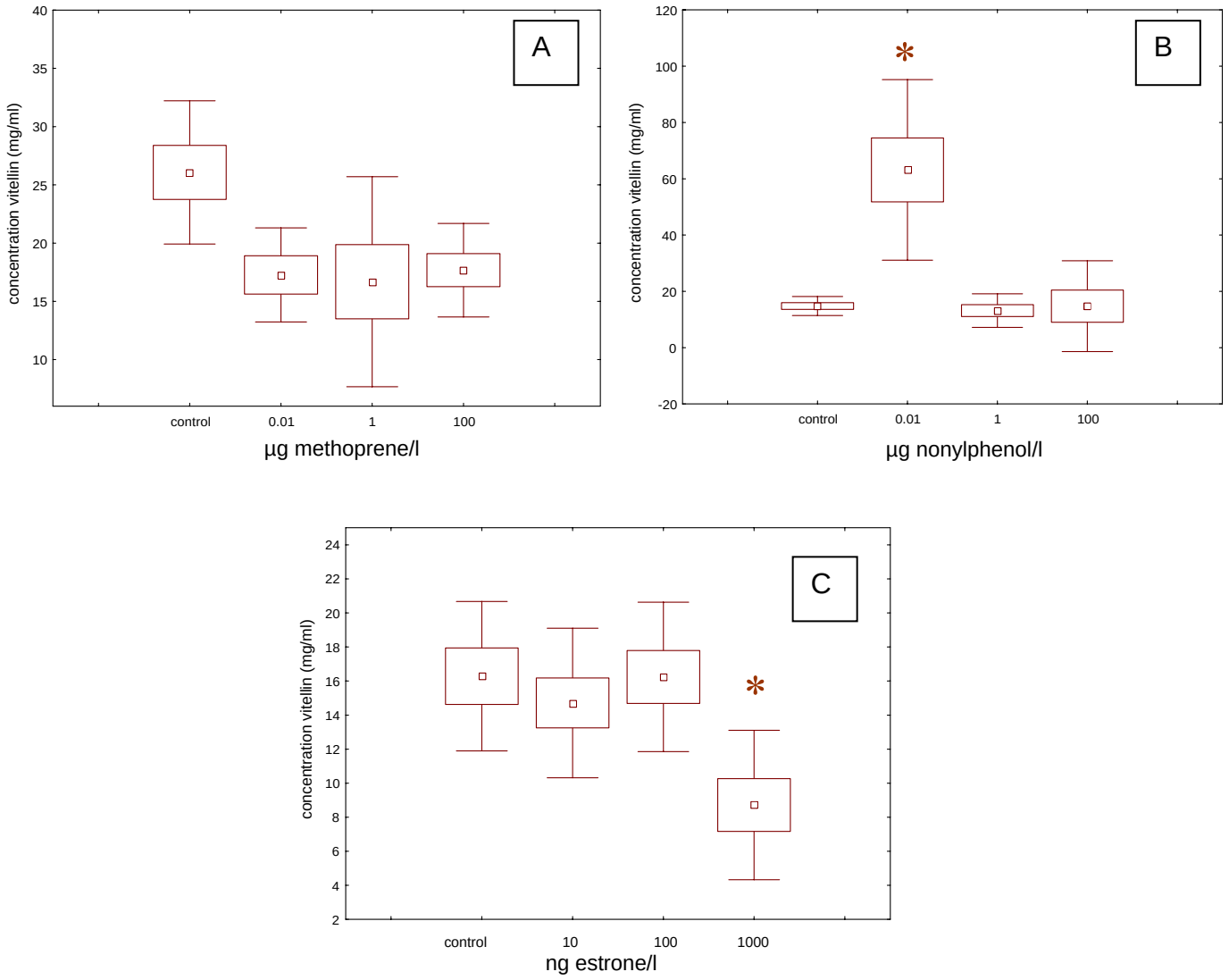


**Figure 4.1:** Levels of vitellin (mg/ml) in females exposed to different concentrations methoprene (A), nonylphenol (B), and estrone (C). * significantly different from control (Dunnett's; $p<0.05$).

## 4.4. DISCUSSION

The best documented examples of endocrine disruption in the aquatic environment are the estrogenic effects of discharges of treated sewage effluents on fish (Harries et al., 1996; Harries et al., 1997; Vos et al., 2000). Alkylphenols, natural hormones and synthetic hormones, amongst others, have been suggested as the most likely responsible for the 'feminization' detected in male fish (Desbrow et al., 1998; Vos et al., 2000). Existing studies on the effects of endocrine disruptors on crustacean vitellogenesis, however, are fragmented

and contradictory. Billinghurst et al. (2000) reported that cypris major protein (CMP), which is related to barnacle vitellin, is elevated in larvae of the barnacle *Balanus amphitrite* exposed to both nonylphenol and 17β-estradiol at a concentration of 1.0 µg/l. They concluded that CMP and perhaps other vitellin-like proteins are potential biomarkers of low level estrogen exposure in crustaceans. In another study by Tsukimura (2001), ridgeback shrimp *Sicyonia ingentis* were injected with 1.0 µg of 17β-estradiol, but no significant changes in hemolymph vitellogenin levels were observed. A recent study by Sanders et al. (2005) found that 17β-estradiol and nonylphenol had contrasting effects on the expression of a vitellin-like protein in the glass prawn *Palaemon elegans*. Relatively high concentrations of 17β-estradiol (0.2 µg/l) significantly reduced expression of the protein, while nonylphenol produced a concentration-independent increase. The lowest concentration of nonylphenol tested, 0.2 µg/l, exerted the most consistent stimulatory effect.

In an effort to further explore the potential effects of chemicals on crustacean vitellogenesis, we recently developed a quantitative vitellin enzyme-linked immunosorbent assay (ELISA) for the mysid shrimp *Neomysis integer* (Chapter 3). Here, we present the first validation study of the *N. integer* vitellin ELISA following exposure to toxicants with suspected endocrine activity. All methoprene-exposed (0.01, 1, 100 µg/l) mysids had lower vitellin levels compared to the control animals, but this effect was not statistically significant. A siginificant increase in mysid vitellin was observed at the lowest nonylphenol exposure concentration (0.01 µg/l), whereas mysid vitellin levels decreased in the highest estrone exposure (1 µg/l), compared to vitellin levels in the controls. These effect levels on mysid vitellogenesis are above environmental concentrations determined in the Scheldt estuary for estrone (8 ng/l; Noppe et al., 2005), and within the same range for nonylphenol (<0.2-12 µg/l; Verslycke et al., 2005; Vethaak et al., 2002). As such, nonylphenol concentrations in this estuary are determined through our field studies are likely to effect mysid vitellogenesis.

We have recently developed *in vivo* assays to study growth and embryonic development in *N. integer* and have evaluated the potential disruption of these processes by methoprene at the same concentrations used in the present study (Chapter 5 and 6). In these studies, methoprene caused a concentration-dependent decrease in hatching succes (significant at 1 and 100 µg/l), whereas growth of *N. integer* was significantly reduced at 100 µg/l. As such, embryogenesis and growth of *N. integer* seem to be more sensitive to the effects of methoprene than vitellogenesis. The observed differences in the effect concentration of methoprene on these physiological processes in mysids could be due to differences in the developmental stage of the test organisms, differences in the exposure duration, and differences in the mode-of-action

or hormonal regulation of the respective physiological process. Females with stage I embryos, eggs, and <24h-old juveniles were used for the vitellogenesis, embryogenesis and growth assay, respectively. Animals in the vitellogenesis study were exposed for 96h, whereas animals in embryogenesis study were exposed from oviposition until hatching (~2 weeks). Finally, animals in the growth assay were exposed during 5 successive molts (~3 weeks). With respect to the toxic mode-of-action, methoprene is known to mimic juvenile hormone and can directly disrupt early stages of embryonic development in developing insects (Dhadialla et al., 1998; Hoffmann and Lorenz, 1998), whereas nonylphenol and estrone are estrogenic. Effects on crustacean growth through molting are most likely an indirect effect that is caused through cross-communication between the ecdysteroid and juvenoid hormone regulatory pathways as recently suggested by Mu and Leblanc (2004) in daphnids. While a juvenile hormone receptor has not been identified to date in arthropods, the anti-ecdysteroidal activity of juvenile hormone, or chemicals with juvenile hormone activity, has been demonstrated (Celestrial and McKenney, 1994; McKenney and Matthews, 1990; Olmstead and LeBlanc, 2001; Templeton and Laufer, 1983). Finally, we previously examined the effect of methoprene on the energy and testosterone metabolism of *N. integer* (Verslycke et al., 2004c). Mysids exposed to 100 µg methoprene/l had significantly altered energy and steroid metabolism. Based on the above studies with methoprene and mysids, we suggest that chronic exposure to juvenoids in mysids could result in effects on reproduction via different pathways, i.e., by interaction with vitellogenesis (as demonstrated in this study), by interaction with energy allocation and the metabolic machinery of mysids (Verslycke et al., 2004c), by disruption of embryonic development (Chapter 6), and by disruption of molting and growth (Chapter 5) leading to reduced fecundity as size and fecundity are linked in mysids. A recent transgenerational exposure study by McKenney Jr. (2005) found that second generation adult mysids, which were exposed to the juvenile hormone analog phenoxycarb only as embryos, produced fewer young and had altered sex ratios. Future chronic exposure studies should focus on determining which life stages and/or physiological processes are critical in leading to reproductive and ultimately population effects in mysids.

The present study further adds to the weight-of-evidence that (xeno)estrogens appear to be less effective in causing disruption of normal vitellogenesis in crustaceans than they are 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 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. These studies will lead to a better understanding of the mode-of-action of chemicals on crustacean hormone-regulated processes.

While it can be concluded that vitellogenesis is an interesting physiological process to study endocrine toxicity in crustaceans, future studies should focus on understanding hormonal regulation of vitellogenesis and other hormone-regulated processes (vitellogenesis, molting, embryogenesis, energy metabolism, steroid metabolism) in mysids and other invertebrates. In addition, priority should be given to exposures with chemicals that are more likely to interact with hormones that are unique to invertebrates, such as ecdysteroids and juvenile hormones. To date, the uniqueness of hormonal regulation in invertebrates as compared to vertebrates, is not reflected in proposed regulatory screening and testing programs that only focus on vertebrate estrogen, androgen, and thyroid hormones.

# CHAPTER 5

## NON-TARGET EFFECTS OF METHOPRENE ON MOLTING IN *NEOMYSIS INTEGER*

Redrafted after:

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# CHAPTER 5

## NON-TARGET EFFECTS OF METHOPRENE ON MOLTING IN *NEOMYSIS INTEGER*

**ABSTRACT** ----------

Ecdysteroids, the molting hormones in crustaceans and other arthropods, play a crucial role in the control of growth, reproduction and embryogenesis of these organisms. Insecticides, such as methoprene - a juvenile hormone analog, are often designed to target specific endocrine-regulated functions such as molting and larval development.

The aim of this study was to examine the effects of methoprene on molting in a non-target species, i.e. the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea). Mysids have been proposed as standard test organisms for evaluating the endocrine disruptive effect of chemicals. Juveniles (< 24h) were exposed for 3 weeks to the nominal concentrations 0.01, 1 and 100 µg methoprene/l. Daily, present molts were checked and stored in 4% formaldehyde for subsequent growth measurements. Methoprene significantly delayed molting at 100 µg/l by decreasing the growth rate and increasing the intermolt period. This resulted in a decreased wet weight of the organism. The anti-ecdysteroidal properties of methoprene on mysid molting were also evaluated by determining the ability of exogenously administered 20-hydroxyecdysone, the active ecdysteroid in crustaceans, to protect against the observed methoprene effects. Co-exposure to 20-hydroxyecdysone did not mitigate methoprene effects on mysid molting. This study demonstrates the need for incorporating invertebrate-specific hormone-regulated endpoints in regulatory screening and testing programs for the detection of endocrine disruption caused by man-made chemicals.

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## 5.1. INTRODUCTION

It is increasingly recognized that the assessment of the ecological impact of potential endocrine disrupters relevant hormonal mechanisms for both invertebrates and vertebrates need to be studied. Invertebrates account for roughly 95% of all animals (Barnes, 1980), yet surprisingly little effort has been invested to understand their value in signaling potential environmental endocrine disruption. Since the hormones produced and used in invertebrates are different from those of vertebrates, it is essential to incorporate invertebrate-specific hormone-regulated endpoints in studies aimed at evaluating potential endocrine disruption.

Mysid crustaceans have been traditionally used in standard marine/estuarine toxicity testing because of their ecological importance, wide geographic distribution, year-round availability in the field, ease of transportation, ability to be cultured in the laboratory, and sensitivity to contaminants. In addition, mysids have been proposed as potential test organisms for the regulatory screening and testing of endocrine disruptors by several agencies such as USEPA, OECD and the Ministry of the Environment of Japan (Verslycke et al., 2004a).

Molting is regulated by a multihormonal system, but is under the immediate control of molt-promoting steroid hormones, the ecdysteroids, secreted by the Y-organ (Fig. 3.1). The Y-organ secretes ecdysone which upon release in the hemolymph is converted into active 20-hydroxyecdysone. Ecdysteroids also play a fundamental role in the control of reproduction and embryogenesis (Subramoniam, 2000). One major advantage of using ecdysteroid metabolism as an endpoint is that it provides a means for evaluating the impact of environmental chemicals on crustaceans (and potentially other arthropods); chemicals which may not necessarily affect vertebrates (Verslycke et al., 2004a). Juvenile hormones regulate metamorphosis and reproduction in insects. With the discovery of the chemical structure of insect juvenile hormone in 1967 (Roller et al., 1967), attempts were made to produce synthetic analogs for use as “third generation” insecticides (Williams, 1956). Methoprene is such an insecticide which acts as a juvenile hormone analog and disrupts normal development of insects by inhibiting developing pupae from molting and passing into the adult stage. Methoprene is one of the most widely used and succesful insect growth regulators. One of the main applications of methoprene is mosquito control. Methoprene can enter estuarine environments by either direct application for controling aquatic-borne pests or indirectly through land-drainage or erosion from adjacent pesticide-treated agricultural lands (Dhadialla et al, 1998; Retnakaran et al., 1985). Methoprene degrades rapidly in sunlight (Quistad et al., 1975) and in water (Schaefer and Dupras, 1973). Methoprene may have broken down during

the bioassay, but methoprene breakdown products are also known to be bioactive (Harmon et al., 1995; LaClair et al., 1998). It was beyond the scope of this study to determine whether the effects observed were mediated by methoprene itself or by its breakdown products such as methoprenic acid. The use of methoprene at recommended application rates is expected to result in environmental concentrations of ~10 µg/l (Ingersoll et al., 1999). Methoprene concentrations in natural water of the US ranged from 0.39 to 8.8 µg/l (Knuth, 1989), which is in the concentration range where laboratory effects were observed on endocrine regulated processes in crustaceans (McKenney and Celestrial, 1996; McKenney and Matthews, 1990; Peterson et al., 2001). However, USEPA has not reported any specific ecological effects indicating a significant risk associated with methoprene (USEPA, 2001).

Similarities between the endocrinology of molting in crustaceans and insects led to the discovery of a crustacean analog (methyl farnesoate, the unepoxidated form of juvenile hormone III) to the insect juvenile hormone. Figure 5.1 represents the chemical structures of juvenile hormone III, methyl farnesoate and methoprene.

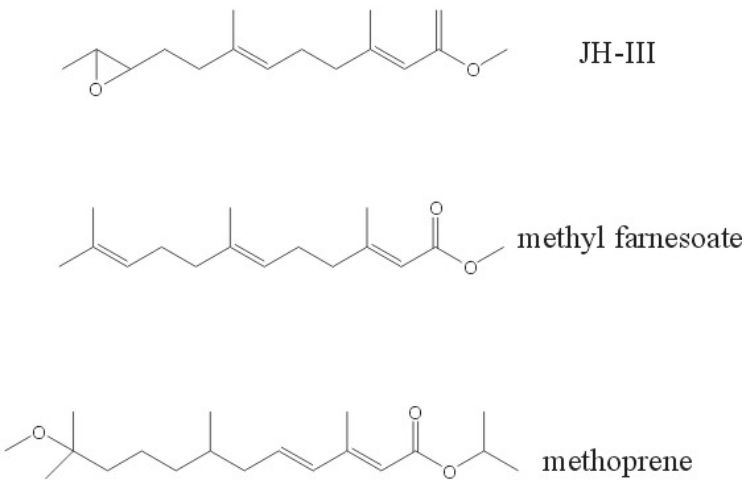


**Figure 5.1:** Chemical structures of juvenile hormone III (JH-III) present in insects, methyl farnesoate in crustaceans and the juvenile hormone analog methoprene.

We previously developed assays to evaluate chemical effects on steroid and energy metabolism in *Neomysis integer* (Verslycke et al., 2002; Verslycke and Janssen, 2002). The purpose of this research is to evaluate molting of *N. integer* as invertebrate-specific endpoint. To this end, we exposed *N. integer* to the test compound methoprene. Methoprene has been shown to reduce mysid fecundity (McKenney and Celestrial, 1996), interfere with juvenile crustacean development (Celestrial and McKenney, 1994; McKenney and Matthews, 1990;

Olmstead and LeBlanc, 2001; Templeton and Laufer, 1983) and act as an anti-ecdysteroid in daphnids (Mu and LeBlanc, 2004). In a recent study, Mu and LeBlanc (2004) demonstrated that juvenile hormones - and their chemical analogues - interfere with normal ecdysteroid signaling in daphnids, probably via a receptor-based process. Although other crustaceans most likely have similar cross talk between juvenoid and ecdysteroid signaling pathways, this has not been studied yet in mysids. We performed a co-exposure using the juvenile hormone analog methoprene and the active ecdysteroid 20-hydroxyecdsyone.

## 5.2. MATERIAL AND METHODS

### *5.2.1. Chemicals*

Methoprene (CAS # 40596-69-8) and 20-hydroxyecdysone were obtained from Sigma-Aldrich (Bornem, Belgium). Stock solutions of methoprene and 20-hydroxyecdysone were prepared in absolute ethanol and stored in a dark refrigerator. The ethanol concentration in the solvent control and in the different test concentrations was 0.01%.

### *5.2.2. Test organisms*

The mysid crustacean, *Neomysis integer*, was collected by handnet in the Braakman, a brackish water (10 psu) near the Schelde estuary in Hoek (The Netherlands). The mysids were cultured in the laboratory as described in Chapter 2 (§ 2.2.1.).

### *5.2.3. Chronic toxicity test*

Gravid females were collected from the culture and individually transferred to aquaria. The aquaria were examined daily for newly released juveniles. Juveniles <24h old were placed individually in 80 ml glass recipients containing 50 ml of the desired test concentration at a salinity of 5 psu and a temperature of 15°C. The juveniles were randomly distributed between the different test vessels containing 0-0.01-1-100 µg methoprene/l and 100 µg methoprene/l + 0, 24, 77, 240 mg/l 20-hydroxyecdysone (=0, 0.05, 0.16, 0.5 µM 20-hydroxyecdysone). These concentrations are based on previous studies with *N. integer* (Verslycke et al., 2004c) and *Daphnia magna* (Mu and LeBlanc, 2002). All concentrations reported in this study are nominal, based on dilutions of the stock solutions. Exposure lasted 5 molts (~3 weeks) and 15

replicates per concentration were used. Exposure solutions were renewed every 48h and juveniles were fed daily with 24- to 48h-old *Artemia* nauplii *ad libitum*. Daily, dead food was removed and molts were stored in 4% formaldehyde for subsequent growth measurements.

### *5.2.4. Growth and Molting*

Toxicological endpoints include time (days) between two successive molts (intermolt period; IMP), and length incresase (growth rate, µm/day) during IMPs. The standard length of *N. integer* or the distance from the base of the eyestalks to the posterior end of the last abdominal segment (Fig. 5.2), cannot be measured directly on the exuvia since the molt is too fragile and easily brakes during manipulation. Therefore, well-defined rigid parts of the molts were measured using conventional light microscopy (Fig. 5.2). Preferably, the length of the exopodites of the uropod (EXO) were used. The standard length (SL) can subsequently be calculated from the exopodite length (EXO) using the linear regression: SL (mm)= 1.085566 + 4.081793 * EXO(mm); $R^2$= 0.9569, n=97 (Fockedey et al., 2005b).

### *5.2.5. Statistics*

All data were checked for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene's test respectively, with an $\alpha = 0.05$. The effect of the treatment was tested for significance using a one-way analysis of variance (Dunnett's test; Statistica™, Statsoft, Tulsa, OK, USA). All box-plots were created with Statistica™ and show the mean (small square), standard error (box), and the standard deviation (whisker).

## 5.3. RESULTS

In a preliminary study, we exposed subadults (average length 7 mm) to the test compound methoprene (0.01, 1, 100 µg/l) over the course of 5 molts (data not shown). Because of the high individual varability in mysid subadult intermolt period (IMP) and growth rate (GR), we decided to work with freshly released juveniles (<24h) to minimize individual variability. The duration of the first intermolt stage was equal for all animals of the same brood and occured 3 to 4 days after release from the marsupium (Fockedey et al., 2005b). Animals of the same brood were randomly distributed over the different exposure treatments which significantly

decreased the individual varability of the IMP and GR as compared to the preliminary study with subadults.

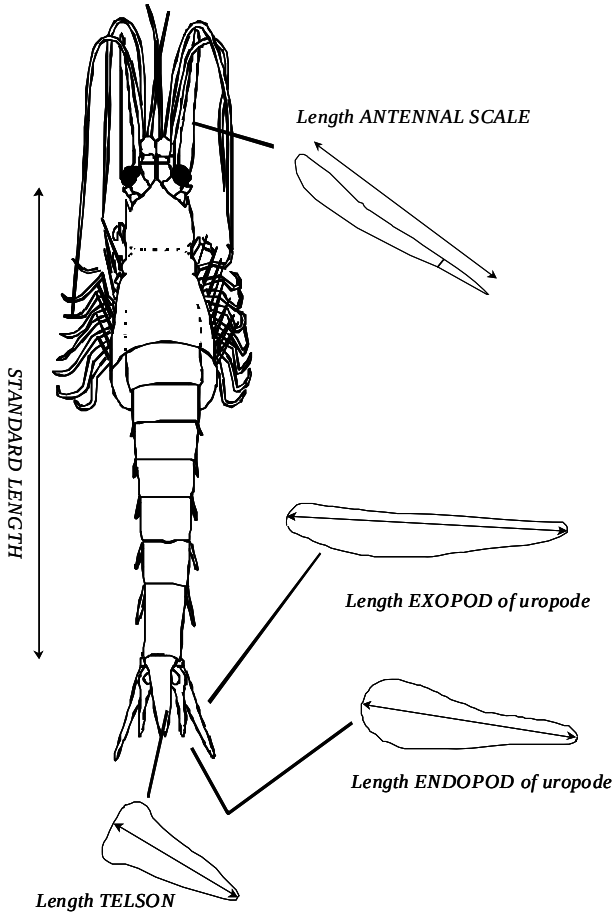


**Figure 5.2:** Schematic representation of *Neomysis integer* with indication of the rigid parts of the molts measured in order to calculate the standard length: length of antennal scale, length of endopod and exopod of the uropod and telson length (Fockedey, 2005).

### *5.3.1. Effect of methoprene on mysid intermolt period (IMP)*

Figure 5.3A shows the effect of methoprene on the IMP during five successive molts. Generally, the growth of *N. integer* is characterized by successively increasing IMPs (Fockedey et al., 2005b). In the controls, the first IMP (1-2) takes 3.4 ± 0.63 days on average, whereas the last IMP (4-5) takes about 4.8 ± 1.12 days. Except for IMP(4-5), all the IMPs were significantly longer in the highest exposure concentration (100 µg methoprene/l)

compared to the respective controls. Although the first three IMPs appeared to be longer in the 1 µg methoprene/l treatment, these differences were not statistically significant. Only the third IMP (3-4) was significantly longer in *N. integer* exposed to 0.01 µg methoprene/l.

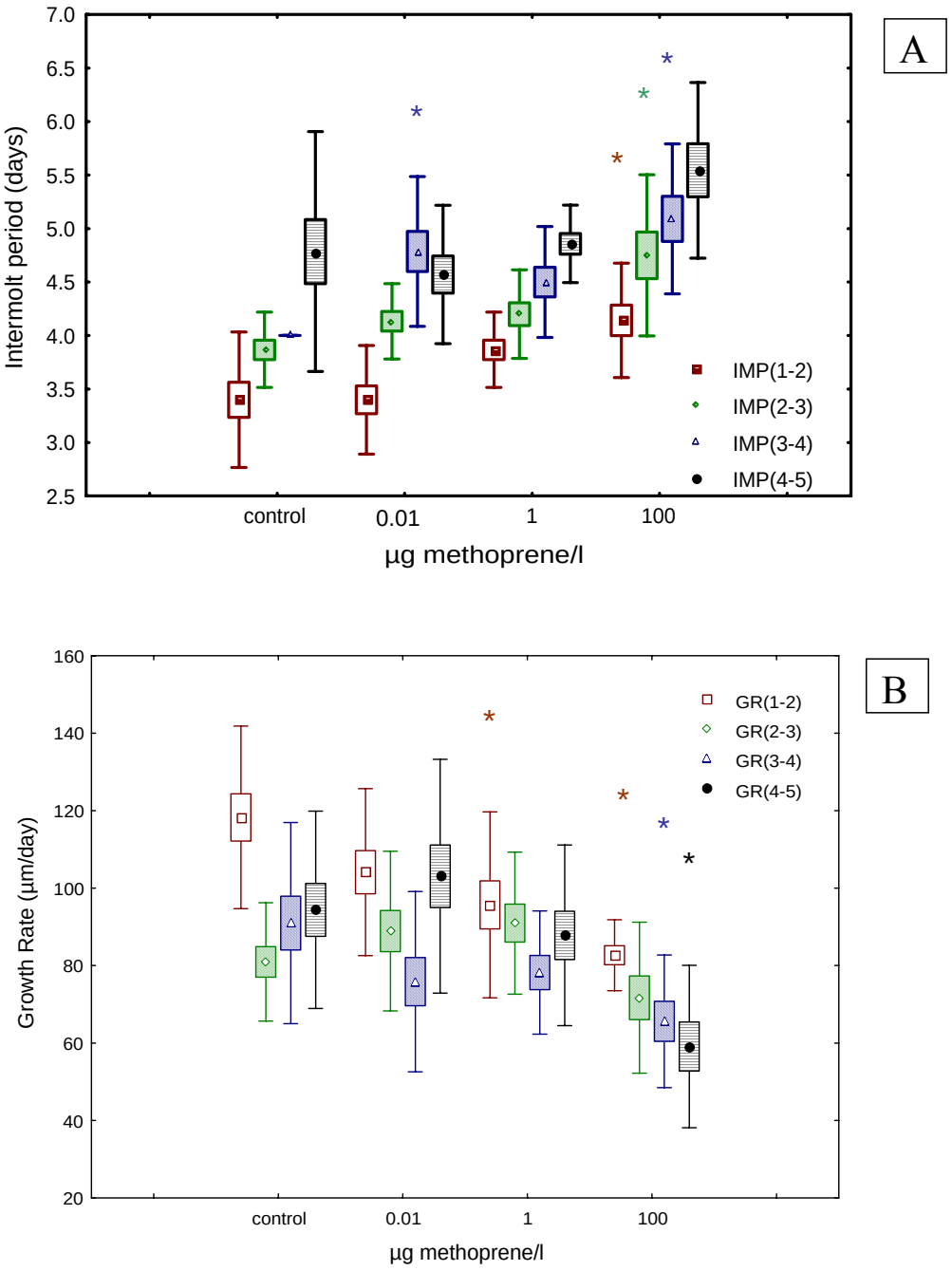


**Figure 5.3:** Effect of methoprene on A) intermolt periods (IMP) and B) growth rates on five successive molts of *Neomysis integer*. * significantly different from control (Dunnett's; $p<0.05$).

*5.3.2. Effect of methoprene on mysid growth rate*

Figure 5.3B shows the effect of methoprene on mysid growth rate during the first five molts. Generally, mysid growth rate is highest during the first molt GR (1-2) and subsequently decreases (Fockedey et al., 2005b). Significant effects were seen on mysid growth rate of juveniles exposed to 100 µg methoprene/l for all molts (GR (1-2), (3-4), (4-5)), except the second GR (2-3). Exposure to 1 µg methoprene/l reduced the growth rate at the first molt only. When growth rate is calculated as total growth (µm) over the total exposure time (day), a significant decrease is found in the 100 µg methoprene/l treatment (data not shown).

*5.3.3. Effect of methoprene on mysid wet weight*

After the fifth molt, all organisms were weighed. Figure 5.4 shows the effect of methoprene on mysid wet weight. There was a significant decrease in wet weight at the highest exposure concentration compared to control animals. The average wet weight of control organisms was 1.43 ± 0.32 mg, almost double of organisms in the 100 µg methoprene/l treatment (average wet weight of 0.75 ± 0.17 mg).

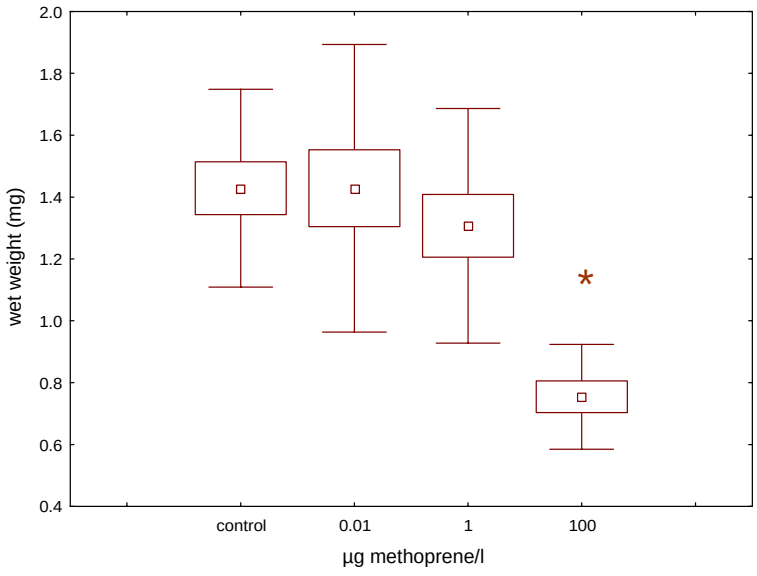


**Figure 5.4:** Wet weigth of *Neomysis integer* after the fifth molt, following exposure to methoprene.

*5.3.4. Combined effects of methoprene and 20-hydroxyecdysone*

At 100 µg/l methoprene significantly reduced mysid growth rate by delaying the IMPs (Fig. 5.3). To further investigate the anti-ecdysteroidal effects of methoprene, mysids were co-exposed to the active ecdysteroid, 20-hydroxyecdysone, to establish whether the observed methoprene effect (IMP delay and decreased growth rate) could be mitigated. Figure 5.5 shows the growth rate expressed as total growth (µm) during the total exposure time (day) to 100 µg methoprene/l and increasing concentrations of 20-hydroxyecdysone (0.05, 0.16 and 0.5 µM 20E). 20-hydroxyecdysone did not mitigate the putative anti-ecdysteroidal effects on growth rate caused by methoprene. The effects of methoprene on mysid growth reduction were confirmed in this second study.

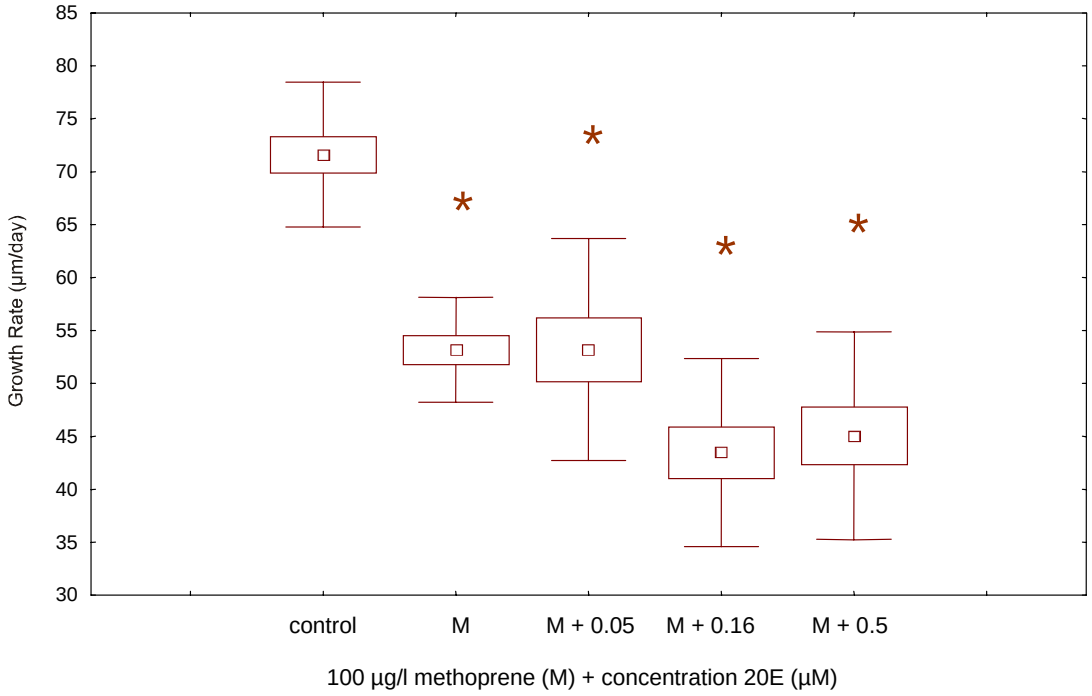


**Figure 5.5:** Growth rate expressed as total growth over the total exposure time (in µm.day$^{-1}$), following exposure to 100 µg methoprene/l (M) spiked with increased concentrations of 20-hydroxyecdysone (0.05, 0.16 and 0.5 µM 20E).

## 5.4. DISCUSSION

Ecdysteroids (molting hormones) and juvenoids (juvenile hormones) represent two classes of hormones in arthropods that regulate many aspects of their development, growth, and reproduction. Therefore, chemicals that disrupt normal ecdysteroid/juvenoid signaling could have profound effects on many aspects of invertebrate function. During their development, insects undergo changes at specific times (such as pupation) which are mediated by endogenous hormones. The active molting hormone 20-hydroxyecdysone, triggers larva-to-larva molts as long as the juvenile hormone is present. In its absence, ecdysone promotes the pupa-to-adult molt. Thus, juvenile hormone present at specific times during insect development leads to normal metamorphosis, however, if present at other times it will lead to morphogenetic abnormalities. This is the basic theory behind the use of methoprene and other juvenile hormone analogues (e.g. pyriproxyfen and fenoxycarb) as insect growth regulators (Dhadialla et al., 1998; Hoffmann and Lorenz, 1998). Methoprene is therefore not directly toxic to insects, but as it disrupts the development of the insect it causes death or reproductive failure at a specific time during the insect life-cycle.

A large portion of the aquatic fauna are crustaceans, making the group important for assessing the non-target effects of many pesticides - such as the mosquitocidal agent methoprene - that end up in aquatic ecosystems (McKenney and Celestrial, 1996; Olmstead and LeBlanc, 2001; Peterson et al., 2001; Templeton and Laufer, 1983). As the potential invertebrate-specific endocrine-disruptive effects of chemicals to non-target organisms are presently not specifically addressed in regulatory screening and testing programs, this could lead to significant underestimations of the actual environmental risk of these chemicals.

While growth through molting of *Neomysis integer* has been described in the laboratory (Astthorsson and Ralph, 1984; Fockedey et al., 2005b; Winkler and Greve, 2002), its disruption by chemicals through specific hormone-regulated mechanisms has not been studied. Methoprene effects on growth of *N. integer* were observed after the first molt, which should therefore allow the use of shorter exposure periods in future studies. Methoprene is acutely toxic (96h) to *Neomysis integer* at 320 µg/l (Verslycke et al., 2004c) and to *Americamysis bahia* at 125 µg/l (McKenney and Celestrial, 1996). McKenney and Celestrial (1996) examined the influence of methoprene on survival, growth and reproduction of *A. bahia* during a complete life cycle, from one-day-old juvenile through juvenile growth and maturation and production of young as an adult. The most sensitive response was a significant reduction in the number of young produced per female at concentrations ≥ 2 µg/l. The mysids

weighed significantly less at exposure concentration of 62 µg methoprene/l as compared to the controls, which is in the same range as what we found in this study (*N. integer* weighed significantly less at 100 µg/l). Our results also corroborate effect concentrations reported for other non-target crustaceans. Methoprene significantly reduced completion of larval metamorphosis in the estuarine grass shrimp *Palaemonetes pugio* at a concentration of 100 µg/l (McKenney and Matthews, 1990). Methoprene adversely affected molting and reproduction in *Daphnia magna* at concentrations higher than 30 nM (~10 µg/l) (Olmstead and LeBlanc, 2001). Recently, we found that methoprene adversely affects the energy and steroid metabolism of *N. integer* at 100 µg/l (Verslycke et al., 2004c). Recently, we also tested the effect of ecdysone agonists, the bisacylhydrazines tebufenozide, halofenozide and methoxyfenozide on the molting of the non-target organism *Neomysis integer* and found that halofenozide and tebufenozide inhibited growth at 1mg/l and 0.1 mg/l, respectively (Soin et al., in preparation).

The present study demonstrates that methoprene significantly affects mysid molting and growth at sublethal concentrations. However, previously reported methoprene effect on mysid reproduction were noted at lower concentrations (McKenney and Celestrial, 1996). As juvenoids and ecdysteroids play a crucial role in the regulation of mysid growth, reproduction and development, comparative approaches that look at a range of ecdysteroid/juvenoid regulated processes in crustaceans should be informative in selecting which endpoints are most sensitive. In addition, measuring the hormones and receptors involved in mysid ecdysteroid/juvenoid signaling will 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. In an effort to improve our understanding of ecdysteroid/juvenoid signaling in mysids, we have recently developed assays to study mysid vitellogenesis (Ghekiere et al, 2005; Chapter 3), embryonic development (Fockedey et al., 2005a), ecdysteroid receptor interaction (Verslycke, personal communication) and are validating these assays in exposure studies with methoprene and other pesticides (Chapters 4,6).

Although the ecdysteroid hormone 20-hydroxyecdysone acts as a EcR ligand and activates transcription through EcR/USP heterodimers, the activity of juvenoids and juvenile hormone-analogs such as methoprene remains unclear. Recently, Maki and co-workers (Maki et al., 2004) have demonstrated that JH III- and methoprenic acid-bound USP markedly repressed ecdysone-dependent EcR transcription.

In the second part of the present study, we evaluated the anti-ecdysteroidal activity of methoprene in *N. integer* by exogenously administrating 20-hydroxyecdysone. We found no

mitigation of the inhibiting effect on growth. This could indicate that methoprene did not exert its effect through ecdysteroid receptor antagonism. Mu and LeBlanc (2002) demonstrated that testosterone had an anti-ecdysteroidal activity in *Daphnia magna* by delaying the molt frequency and this effect was mitigated by co-exposure to 20-hydroxyecdysone. They proposed ecdysteroid receptor antagonism as one possible mechanism by which testosterone caused these effects. We previously tested the effect of 20-hydroxyecdysone on molting of *N. integer* and found no effects on the molting frequency. Raising the concentration to $10^{-5}$ M was associated with premature death caused by incomplete ecdysis. These results correlate with the findings of Baldwin and co-workers (Baldwin et al., 2001). Although recent studies with daphnids indicate that juvenoids modulate ecdysteroid signaling through a mechanism that may involve reduced availability of the receptor partner protein ultraspiracle (the ecdysone receptor is functional only as a heterodimer with ultraspiracle), the exact mechanism of action of juvenoids and methoprene remains unclear (Mu and LeBlanc, 2004). In this respect, the increasing availability of sequences for the different receptors involved in crustacean ecdysteroid/juvenoid signaling may be very valuable. Recently, the EcR and USP has been isolated from the fiddler crab *Uca pugilator* and the mysid *Americamysis bahia* (Chung et al., 1998, Yokota et al., 2005). The deduced amino acid sequences of both EcR and USP share 40-60% homology with insect counterparts.

The endocrine system of an invertebrate differs from that of a vertebrate organism both in the type of endocrine glands present and in the chemical structure of specific hormones that are produced. As such, assessing the impact of endocrine disrupting chemicals on invertebrates, requires an approach that is specifically directed at invertebrates. In this context, we are exploring a range of endocrine-regulated processes in invertebrates that could be specifically disrupted by chemicals. This approach should lead to both a better understanding of hormone regulation and its disruption by chemicals in invertebrates.

# CHAPTER 6

## MARSUPIAL DEVELOPMENT OF *NEOMYSIS INTEGER* TO EVALUATE THE EFFECTS OF ENVIRONMENTAL CHEMICALS

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And

Fockedey, N., Ghekiere, A., Bruwiere, S., Janssen, C.R., Vincx, M., 2005. Effect of salinity and temperature on the *in vitro* embryology of the brackish water mysid *Neomysis integer* (Crustacea: Mysidacea). *Marine Biology* (in press).

# CHAPTER 6

## MARSUPIAL DEVELOPMENT OF *NEOMYSIS INTEGER* TO EVALUATE THE EFFECTS OF ENVIRONMENTAL CHEMICALS

**ABSTRACT** ----------

Embryonic development is a crucial time window within an organism's life history. Relatively few studies have focused on understanding the potential effects of endocrine disruptors on embryogenesis in invertebrates. Mysids (Crustacea: Mysidacea) have been used extensively in regulatory toxicity testing and it is the only invertebrate model currently included in the USEPA's Endocrine Disruptor Screening and Testing Program. We developed a method to study mysid marsupial development of the embryos until the release of free-living juveniles. This method was used to evaluate the potential effects of the insecticide methoprene, a juvenile hormone analog, on mysid embryogenesis. Embryos were exposed to the nominal concentrations 0.01, 1 and 100 µg methoprene/l. Average percentage survival, hatching success, total development time and duration of each developmental stage were analyzed. Embryos exposed to 1 and 100 µg methoprene/l had a significantly lower hatching success and lower survival rates. Our study indicates that *in vitro* embryogenesis can be used as a valuable tool to study the effects of endocrine disruptors in mysids.

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### 6.1. INTRODUCTION

The occurrence of endocrine disruptors in the environment and their potential effects on wildlife species is receiving increased public attention. Although invertebrates account for roughly 95% of all animals, little research has been performed to understand effects of endocrine disruptors on these organisms, compared to the vertebrates. Hormones involved in growth, development and reproduction differ between vertebrates and invertebrates (Chang,

1997b; Charmantier et al., 1997; Huberman, 2000; Hutchinson, 2002; Subramoniam, 2000; USEPA, 2002; Verslycke et al., 2004a). There is a need to develop sensitive and relevant assays that evaluate endocrine toxicity in invertebrates, based on their unique signaling pathways. Unfortunately, knowledge of endocrine-regulated processes and their potential disruption by chemicals in invertebrates is limited. The relatively large body of information on arthropod endocrinology makes insects and crustaceans good models for evaluating chemically-induced endocrine disruption. Recent studies have highlighted the need for more studies on the impact of endocrine disruptors on the reproduction and development of estuarine invertebrates (Lawrence and Poulter, 2001). Mysid crustaceans have been proposed by several regulatory bodies (e.g. USEPA, OECD) as suitable test organisms to evaluate the potential effects of environmental endocrine disruptors (Verslycke et al., 2004a).

Ecdysteroids (molting hormones) and juvenoids (juvenile hormones) represent two classes of hormones in arthropods that regulate many aspects of their development, growth and reproduction. However, the regulation, function and potential chemical disruption of ecdysteroid/juvenoid-regulated processes in crustaceans remains largely unknown. Increased understanding of the endocrine system of insects has led to the introduction of insecticides known as insect growth regulators, with the largest group being juvenile hormone analogues (Keeley et al., 1990; Riddiford, 1994). These insect growth regulators elicit highly specific effects on target insects based on their ability to interact with insect hormone receptors (Dhadialla et al., 1998). As ecdysteroid-like and juvenoid-like compounds function in crustaceans in a manner similar to that seen in insects, they may have a role in the regulation of crustacean reproduction and development (Charmantier et al., 1997; Laufer and Borst, 1988; Laufer et al., 1993; Walker et al., 2005). Consequently, chemicals with ecdysteroid/juvenoid activity are potentially adversely affecting susceptible non-target animals, such as crustaceans (OECD, 2005; Tuberty and McKenney, 2005).

A recent study by McKenney Jr. (2005) found that juvenile mysids released by adults exposed to the juvenile-hormone analog phenoxycarb and reared through maturation without further exposure produced fewer young and had altered sex ratios. This study indicates that pesticides with ecdysteroid/juvenoid activity, may be acting like other EDCs with exposure during developmental periods (in this case during ovarian, embryonic and larval development) producing irreversible reproductive dysfunction in adults. In addition, we have demonstrated the ability of a number of insecticides to disrupt hormone-regulated processes in mysids at very low concentrations, i.e. vitellogenesis (Chapter 4), molting (Chapter 5), and steroid and energy metabolism (Verslycke et al., 2004c). To further explore the potential effects of EDCs

on ecdysteroid/juvenoid-regulated processes in mysids, we developed a mysid embryogenesis assay. In this study, we validated our embryogensis assay through an exposure experiment with the mysid *Neomysis integer* and the juvenile-hormone analog methoprene. Methoprene is an insect growth regulator that is generally used to control mosquitos. Methoprene degrades rapidly in sunlight (Quistad et al., 1975) and in water (Schaefer and Dupras, 1973). Methoprene may have broken down during the bioassay, but methoprene breakdown products are also known to be bioactive (Harmon et al., 1995; LaClair et al., 1998). It was beyond the scope of this study to determine whether the effects observed were mediated by methoprene itself or by its breakdown products such as methoprenic acid. The use of methoprene at recommended application rates is expected to result in environmental concentrations of ~10 µg/l (Ingersoll et al., 1999). Methoprene concentrations in natural water of the US ranged from 0.39 to 8.8 µg/l (Knuth, 1989), which is in the concentration range where laboratory effects were observed on endocrine regulated processes in crustaceans (Celestrial and McKenney, 1994; DeFur et al., 1999; McKenney and Celestrial, 1996; McKenney and Matthews, 1990; Mu and LeBlanc, 2004; Olmstead and LeBlanc, 2001; Peterson et al., 2001, Templeton and Laufer, 1983; Walker et al., 2005).

*Neomysis integer*, like all mysids, carries its embryos in a marsupium where the entire embryonic development takes place from oviposition to the release of free-living juveniles (Wittmann, 1984). Unfortunately, studying the embryonic development *in vivo* is difficult due to the semi-transparent oostegites (Fockedey, personal observation) and requires anaesthetization (Irvine et al., 1995). Recently, Fockedey et al. (2005a) developed a methodology to study the *in vitro* embryogenesis of *N. integer* and evaluated the combined effects of temperature and salinity on mysid embryogenesis. A few studies on *in vitro* embryogenesis in mysids and the effects of temperature and salinity were previously published by Greenwood et al. (Greenwood et al., 1989), Johnston et al. (Johnston et al., 1997) and Wortham-Neal and Price (Wortham-Neal and Price, 2002). In addition, some studies have evaluated the effects of endocrine disruptors on embryonic development in other crustaceans (Kast-Hutchenson et al., 2001; Lawrence and Poulter, 2001; LeBlanc et al., 2000). To date, no studies have evaluated embryonic development in mysids as a tool to study the potential effects of endocrine disruptors.

## 6.2. MATERIAL AND METHODS

### *6.2.1. Chemicals*

Methoprene (CAS # 40596-69-8) was obtained from Sigma-Aldrich (Bornem, Belgium). Stock solution of methoprene was prepared in absolute ethanol and stored in a refrigerator. The ethanol concentration in the solvent control and the exposure concentrations was 0.01%.

### *6.2.2. Experimental animals*

The mysid crustacean, *Neomysis integer*, was collected from the dock B3 in the harbor of Antwerp (Belgium), situated at the right bank of the river Scheldt. The dock B3 is in open connection with the river Scheldt through the Berendrecht and Zandvliet sluices. Animals are extracted over a 1x1 mm sieve. Salinity and temperature conditions during the sampling period (weekly from 30$^{th}$ March to 16$^{th}$ April 2004) were 5 psu and 11°C on average. *N. integer* were collected with a handnet (2x2 mm mesh size) and transported within 2 hours after sampling in 15-L bins containing environmental water. The animals were kept in a 16°C climate room for a maximum of 7 days at a concentration of ± 50 ind/L and at a salinity of 5 psu (made from artifical seawater, Instant Ocean®, Aquarium Systems, France). They were fed *ad libitum* with <24h old *Artemia* nauplii and water was replaced every 2-3 days.

### *6.2.3. Test design*

Non-gravid females with well developed ovaries were selected and placed with 2 adult males in a 400 ml glass beakers filled with 350 ml artificial seawater (5 psu) to allow fertilization. The ovary is situated in the posterior dorsal lateral regions of the thorax and can be easily observed through the carapax (Fig. 6.1). Mature males were distinguished by their elongated 4$^{th}$ pleopods that are stretched to the end of the last abdominal segment (Fig. 1.5). A 12h light: 12h dark photoperiod was used and the water temperature was maintained at 16°C. Daily, excess food (<24h old *Artemia* nauplii), faeces, molts and dead animals were removed. Dead individuals were replaced by new animals and 80% of the medium was renewed and fresh food was added. Mating takes place at night (Mauchline, 1980) and coincides with the molting of the female (Wittmann, 1984). Upon fertilization, gravid females were placed in individual beakers for two more days before removal of their embryos from the marsupium on

day three. Before day three, the embryos are too fragile and removal of the embryos causes damage. Non-fertilized embryos disintegrate within 24h and are not included in the test. After decapitation of the gravid females, the embryos were removed with a fine spatula while submerged in artificial seawater medium (15 psu, 15°C).

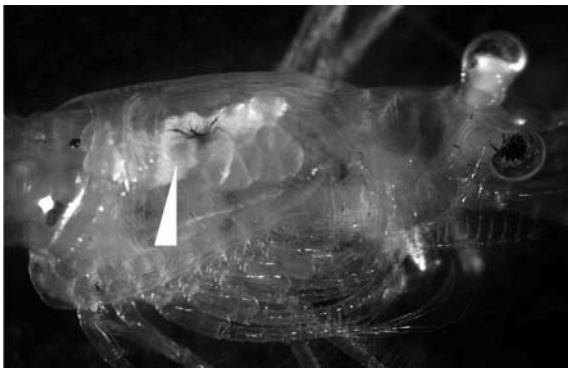

**Figure 6.1:** Ovarium of *Neomysis integer* (Fockedey et al., 2005a). The ripe ovary fills the posterior dorsal lateral regions of the thorax (white arrow).

Using a glass pipette, the embryos were individually transferred at random to each of the wells of a 12-cell plate containing 4 ml of the different exposure concentrations of methoprene (dilution water is artificial seawater of 15 psu). Embryos were exposed to 0-0.01-1-100 µg methoprene/l. All concentrations reported in this study are nominal, based on dilutions of the stock solutions. Twelve replicates per concentration and at least 7 embryos per replica were used. Multiwells were placed on an orbital shaker (80 rpm) and covered from the light. Daily, survival, developmental stage and hatching were recorded, dead embryos were removed, and 75% of the medium was replaced.

### *6.2.4. Description of the embryology*

The intra-marsupial development of *Neomysis integer* was divided into 3 substages in the present study, while generally for mysids a subdivision into 3 to 12 substages is common (de Kruijf, 1977; Mauchline, 1973; Wittmann, 1981b). Table 6.1 and Figure 6.2 summarize the terminology used by the different authors, including the one used in the present study, and applied to the observed morphology in the intra-marsupial development of *Neomysis integer* (with supporting pictures).

The early embryos (stage I) are spherical or sub-spherical (Fig. 6.2a). Rudiments of antennae and abdomen are developing (Fig. 6.2b) and observable under low magnification (25x) as a lighter coloured disk. The abdominal rudiment is ventrally bent and develops anteriorly towards the cephalic appendix. Stage I ends with the hatching from the egg membrane by puncturing it with the developing abdomen. The shed egg membrane quickly disintegrates, but is sometimes visible in the wells.

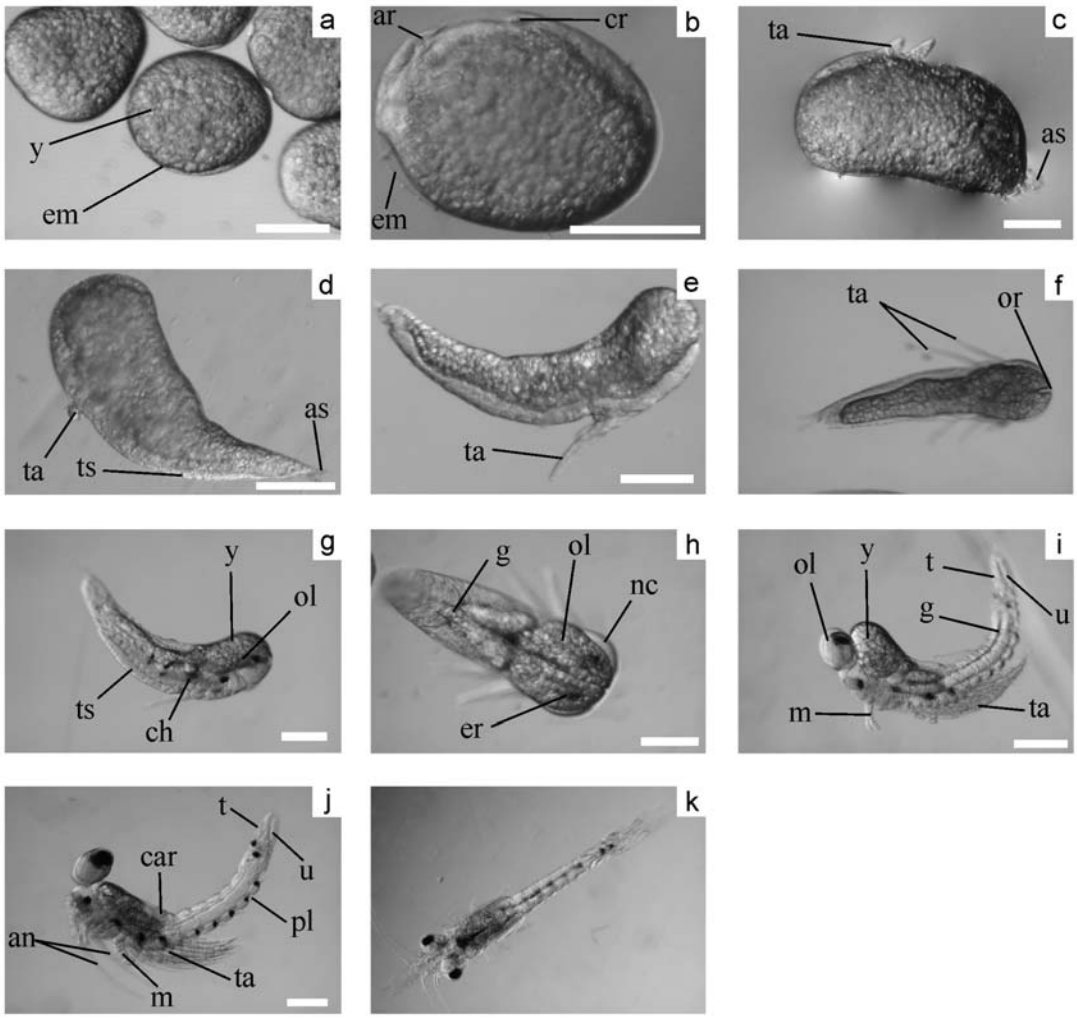


**Figure 6.2:** Intra-marsupial development of *Neomysis integer* (Fockedey et al., 2005a): stage I (a,b), stage II (c-h), stage III (i,j) and the free-living juvenile (k). (an: antennae; ar: abdominal rudiment; as: abdominal setae; car: carapace; c: naupliar cuticle; cr: cephalic rudiment; ch: thoracic chromatophore; er: eye rudiment; em: egg membrane; g: gut; m: mouth parts; nc: naupliar cuticle; or: optic rudiment; ol: optic lobe; pl: pleopods; t: telson; ta: thoracic appendages; ts: thoracic segmentation; u: uropods; y: yolk granules). Scale bar = 250µm.

**Table 6.1**: Morphological and activity characteristics of the intra-marsupial development of *Neomysis integer* (Fockedey et al., 2005).

| **Morphology** | **Yolk** | **Activity** | PRESENT STUDY<br>Kinne (1955)<br>Mauchline(1973) | De Kruif (1977) | Wittmann (1981) |
|---|---|---|---|---|---|
| ● Egg-like, (sub)spherical (Figure 2a); first 2 days in 2 packages within a tertiary egg membrane<br><br>● Later: cephalic and abdominal rudiments developing (Figure 2b) | Yolk granules spread all over embryo | Inactive | stage I<br>= 'egg' | stage I | E1 – half E5 |
| ● Shedding of egg membrane | | | | | |
| ● Comma-shaped habitus with rudimentary pointed abdomen clearly distinguished from rounded anterior; appearance of two pair of rudimentary thoracic appendages and abdominal setae (Figure 2c)<br><br>● Later: the beginning of abdominal segmentation, without appendages (Figure 2d) | Yolk granules homogeneously spread all over embryo | Inactive | stage II<br>= naupliar stage<br>= 'eyeless' larva | stage II<br>stage III<br>stage IV | Half E5<br>+ N1 to N4 |
| ● Further extension of the body and elongation thoracic appendages (Figure 2e); appearance of cleft at optic rudiment (Figure 2f) | Yolk migrates dorsally | Inactive | | | |
| ● Development of head; optic lobes with pigmented eye rudiments; rudiments of telson and uropods visible; further segmentation of abdomen; brown chromatophores appearing laterally (Figure 2g and 2h) | Yolk diminishes and migrates dorsally in the anterior part | Rhythmic contractions of the gut and beating of the heart | | stage V | |
| ● Moulting from naupliar cuticle | | | | | |
| ● Distinct eye projections; development of uropods and pleopods; developing 8 thoracic appendages, mouthparts and antennae; developing carapace and elongated abdomen (Figure 2i and 2j) | Yolk disappears | Very active flexing and stretching of the body; moving of the appendages | stage III<br>= post-naupliar stage<br>= 'eyed' larva | stage VI | P1 – P3 |
| ● Moult | | | | | |
| ● All (except sexual) characteristics similar to adult (Figure 2k) | No yolk left; actively feeding | Freely swimming | Juvenile | Juvenile | Juvenile |

The stage II larvae are dorsally bent and have a comma-like appearance. Initially, a rudimentary abdomen with a clear distinction between the rounded anterior and the pointed posterior of the larva can be observed together with two thoracic appendages (Fig. 6.2c). In a later phase, the abdomen shows the clear beginning of segmentation, however, without any appearance of appendages (Fig. 6.2d). Later on, the body is further extended and the thoracic appendages more elongated (Fig. 6.2e). The larvae have globules of the yolk protein vitellin within their tissues. These globules are homogeneously distributed throughout the body in stage I embryos and early stage II larvae, but as the yolk volume decreases relative to the body volume, the yolk becomes more concentrated in the anterior dorsal regions at the end of stage II. Dorsally the optical rudiment is visible as an anterior cleft (Fig. 6.2f). As the larva grows, the naupliar cuticle is stretched and the uropods and telson are formed. Eight abdominal segments are clearly visible. Lateral chromatophores appear, mainly in the anterior part (Fig. 6.2g). The optical lobes are visible with pigmented eye rudiments (Fig. 6.2h). A rhythmic beating of the heart and contractions of the gut are visible. The naupliar stage II terminates with the moulting from the naupliar cuticle.

The post-naupliar stage III larvae (Fig. 6.2i) have stalked eyes, a developed telson and uropods without lith in the statocyst of the inner ramus. The thoracic appendages, mouth parts and antennae are developing. All over the body, darkly pigmented chromatophores appear. Near the end of this stage a carapace can be observed (Fig. 6.2j). The larvae are very actively moving by a longitudinal dorsal flexing and stretching of the body. Also an active rhythmic moving of the thoracic appendages is observed. Stage III terminates in a moult, leading to free-living young juveniles (Fig. 6.2k) that are, except for the sexual characteristics, morphologically similar to the adults. The gradually disintegrating yolk is completely consumed.

### *6.2.5. Statistics*

All data were checked for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene's test respectively, with an $\alpha = 0.05$. The effect of the treatment was tested for significance using a one-way analysis of variance (Dunnett's test; Statistica™, Statsoft, Tulsa, OK, USA). All box-plots were created with Statistica™ and show the mean (small square), standard error (box), and the standard deviation (whisker).

## 6.3. RESULTS

### *6.3.1. Survival*

The percentage embryo survival/day was calculated for each of the exposure concentrations: 0, 0.01, 1 and 100 µg methoprene/l (Fig. 6.3). The highest mortality generally occured within the first 6 days of embryonic development, i.e. during stage I. Survival did not change from day 7 until day 12, however, survival was lower in organisms exposed to 1 and 100 µg methoprene/l. From day 13 onwards, mysid survival was affected in all methoprene exposures. Average daily survival was 69.4 ± 22.0 % and 70.8 ± 15.3 % for the control and exposure to 0.01 µg methoprene/l, respectively. Exposure to 1 and 100 µg methoprene/l resulted in average daily survival of 55.4 ± 19.0 % and 56.4 ± 17.9 %, respectively. Although average survival was affected in a concentration-dependent way, this effects was not siginificant (one-way ANOVA between the control and any of the treatments (p=0.090).
Major hatching occured on day 15. Due to this hachting we observed a higher variation of survival % on day 16.

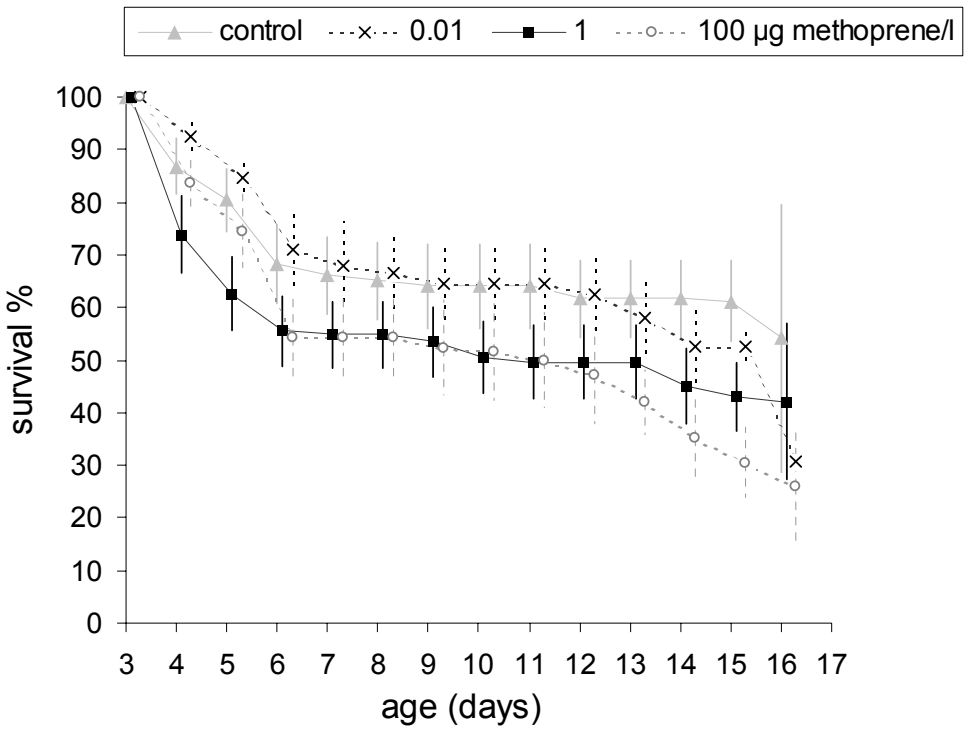


**Figure 6.3:** Percentage survival of the embryos exposed to 0.01, 1 and 100 µg methoprene/l.

*6.3.2. Duration of the different developmental stages*

Figure 6.4 shows the duration of the different developmental stages of the embryos exposed to methoprene. The total length of *N. integer* embryonic development was about 15 days and this length was not significantly different between treatments. However, significant effects were seen between treatments on stage-specific length. The duration of the stage I was between 4 and 5 days and not significantly different between treatments. Stage II embryos had a development time between 6 and 7 days and embryos exposed to 0.01, 1 and 100 µg methoprene/L had a significantly longer development time than that of control embryos. Stage III embryos had a development time between 3 and 4 days and embryos exposed to 0.01 µg/L methoprene had a significantly shorter development time than control animals.

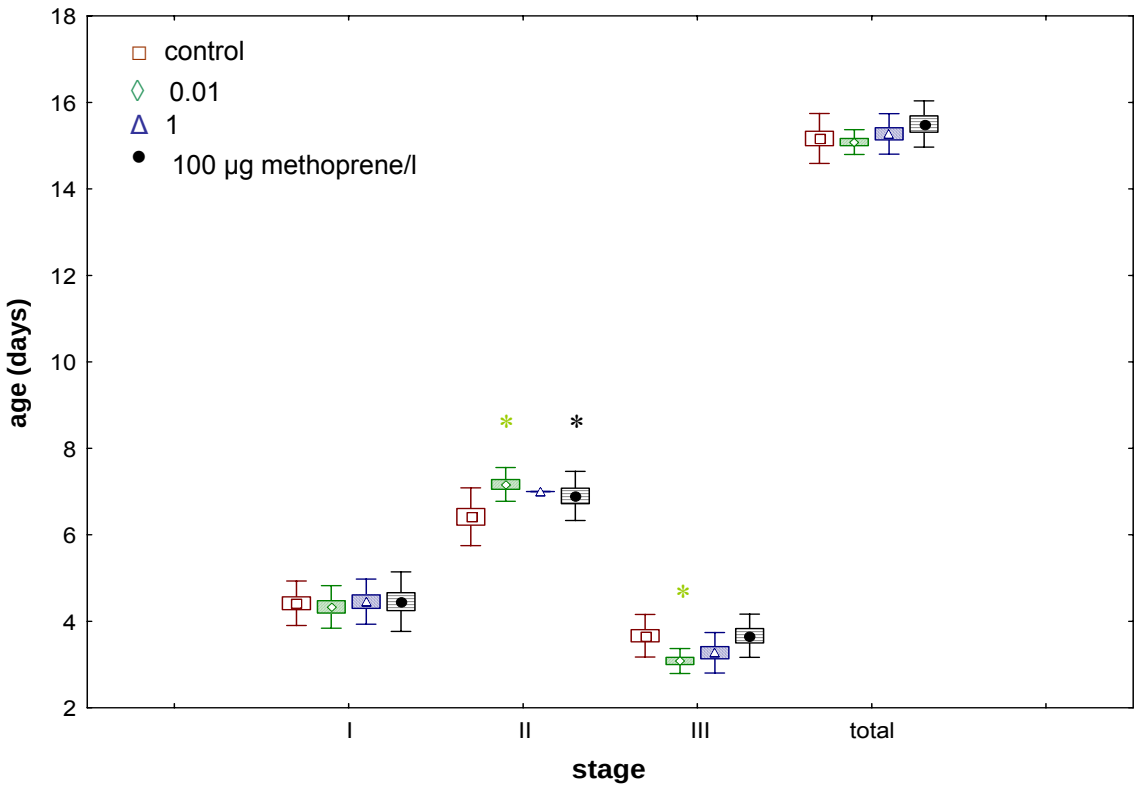


**Figure 6.4:** Duration of the different developmental stages of embryos (days) exposed to 0.01, 1 and 100 µg methoprene/l. Total= duration of the total development time. (Anova, Dunnett; *p<0.05, significance from control)

*6.3.3. Hatching*

The most obvious effects of methoprene were seen on mysid embryonic hatching success. The average hatching percentages were 59.7 ± 26.0 %, 47.8 ± 20.6 %, 40.2 ± 23.8 % and 23.3 ± 21.8 % for the control and 0.01, 1 and 100 µg methoprene/l treatments, respectively (Fig. 6.5). Exposure to 1 and 100 µg methoprene/l resulted in significantly less embryos that hatched compared to the control.

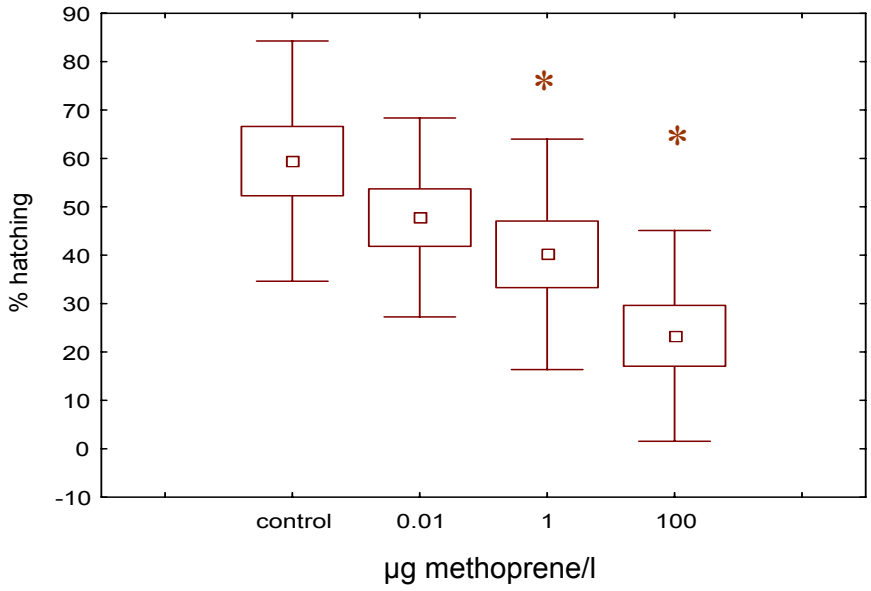


**Figure 6.5:** Percentage hatching of embryos exposed to 0.01, 1 and 100 µg methoprene/l. (Anova, Dunnett; *p<0.05, significance from control)

## 6.4. DISCUSSION

Ongoing studies in our laboratory are aimed at understanding the regulatory role of ecdysteroids and juvenoids in mysids, and how this regulation can be chemically disrupted. We have developed several new assays that quantify processes controlled by ecdysteroids and juvenoids in mysids. More specifically, we have developed a quantitative mysid vitellogenesis assay (Chapter 3), as well as a mysid *in vivo* assay to quantify effects on molting (Chapter 5). Using these assays, we were able to demonstrate that the juvenile hormone analogue methoprene significantly affects mysid vitellogenesis and molting

(Chapters 4, 5). In the present study, we describe marsupial development in mysids as a research tool to evaluate potential endocrine toxicity on embryonic development. Given the known sensitivity of this particular life stage in arthropods and many other animals, we expect mysid embryonic development to be a particularly sensitive life stage for the effects of endocrine disruptors. Very few studies have evaluated the effect of endocrine disruptors on the *in vitro* embryogenesis of crustaceans. Indeed, only for the amphipod *Chaetogammarus marinus* (Lawrence and Poulter, 2001) and the cladoceran *Daphnia magna* (Kast-Hutchenson et al., 2001; LeBlanc et al., 2000) have this type of studies been reported.

The marsupial development of 16 mysid species has been previously described (Greenwood et al., 1989; Johnston et al., 1997; Wortham-Neal and Price, 2002). The opaque marsupium of the living female makes it difficult to study embryonic development in mysids (Fockedey et al., 2005a). However, mysid embryos can be removed from the marsupium to allow for an easy assay to evaluate mysid embryonic development. In a previous study, we evaluated the effects of salinity and temperature on the *in vitro* embryogenesis of *Neomysis integer* to determine the optimal abiotic conditions for our assay: a salinity of 15 psu and a temperature of 15°C (Fockedey et al., 2005a). These conditions were used in the present exposure study with the juvenoid methoprene.

Percentage survival, stage duration and hatching were all easily quantified and were affected at different concentrations by methoprene in *N. integer*. In short, methoprene had no effect on the duration of the first stage, prolonged the second stage and shortened the last stage. Interestingly, the mysid embryos molt after stage II, so a possible explanation for the prolonged second stage is that methoprene interferes with the process of molting/metamorphosis in the mysid embryo. An increase in larval development time is a common sublethal response in crustacean larvae exposed to juvenoids (Celestrial and McKenney, 1994; McKenney and Celestrial, 1996; McKenney and Matthews, 1990; McKenney et al., 2004). In a recent study, we evaluated the effects of methoprene on the molting success of juvenile *N. integer* (< 24h old) through five successive molts. Methoprene delayed juvenile molting at 100 µg/l demonstrating that this chemical can interfere with normal molt success (Chapter 5). Similarly, Lawrence and Poulter (2001) reported that copper, pentachlorophenol, and benzo[a]pyrene only affected specific embryonic stages of the amphipod *C. marinus*. Indeed, stage I was not affected, but stages II to IV -in which the embryo undergoes development of e.g. the germinal disc, dorsal organ rudiments, eye and heart- were all prolonged in the toxicant exposures. Stage V was generally shortened in these studies. These and a growing number of studies are adding to the body of evidence that

juvenoids can cross communicate with the ecdysteroid pathway in ecdysozoans (animals that molt) (Mu and LeBlanc, 2004; Tuberty and McKenney, 2005). The mechanisms involved in the disruption of ecdysteroid/juvenoid signaling in crustaceans remain to be discovered. It is likely that ecdysteroid/juvenoid disruption in crustaceans is caused, at least to a certain extent, by the interaction of insect growth regulators with the nuclear receptors for the endogenous ecdysteroids/juvenoids.

A study by McKenney and Celestial (1996) in which *Americamysis bahia* were exposed during a complete life cycle to methoprene, showed that fecundity was significantly altered at concentrations ≥ 2 µg/l. They reported a lower number of young produced per female. This corroborates our findings that methoprene causes lower hatching rates at 1 and 100 µg/L compared to the control. Other studies have focused on comparing responses between crustacean embryos and larvae to juvenoids (McKenney et al., 2004). Concentrations of methoprene ≥ 8 µg/l resulted in significant mortality in larval grass shrimp *Palaemonetes pugio* (McKenney and Celestrial, 1993), whereas embryos successfully hatched under exposure to 1 mg/l methoprene (Wirth et al., 2001). While these studies demonstrate the differential sensitivity between lifes stages in one species, it also shows significant differences in sensitivity to juvenoids between crustacean species. We found that methoprene is acutely toxic to juvenile *N. integer* at 320 µg/l (96h-LC50) (Verslycke et al., 2004c), whereas hatching in this study was affected at 1 µg/l.

Finally, we also measured the length of the embryos during this exposure experiment (data not shown). We found no differences in length of the embryos exposed to the different methoprene concentrations.

In conclusion, the juvenoid methoprene is capable of interfering with many aspects of mysid growth, development and reproduction. In the present study, we described the effects of this chemical on embryonic development in *N. integer*. The mysid embryogenesis assay provides a novel and interesting addition to existing and proposed assays for endocrine disruptor testing with mysids. In chronic studies (partial or full life cycle, multigenerational), this assay would focus on the effects during embryonic development and how these are correlated with effects in later life. Such studies would provide important insights into critical time-windows of exposure and the chemical mode-of-action. Finally, mysid embryos are easily recorded through image documentation, and a library of normal and abnormal development could be produced for future reference in toxicity studies.

# CHAPTER 7

## EFFECT OF NONYLPHENOL AND ESTRONE ON MARSUPIAL DEVELOPMENT OF *NEOMYSIS INTEGER*

# CHAPTER 7

## EFFECT OF NONYLPHENOL AND ESTRONE ON MARSUPIAL DEVELOPMENT OF *NEOMYSIS INTEGER*

ABSTRACT----------

Recently, marsupial development of *Neomysis integer* (Crustacea: Mysidacea) was described in detail by Fockedey et al. (2005a). Subsequently, we reported on the use of mysid marsupial development as a sensitive assay to evaluate endocrine disruption by exposure to the juvenile hormone analog methoprene. The present study further evaluates the potential use of this assay by exposing mysid embryos to the alkylphenol degradation product nonylphenol and the natural hormone estrone. Stage I embryos were exposed to 0.01, 1, and 100 µg nonylphenol, and 10, 100, and 1000 ng/l estrone, until hatching to a free-living juvenile. Duration of the different developmental stages, survival and hatching succes were recorded. Nonylphenol exposure had no effect on the duration of the different developmental stages but did significantly reduce survival and hatching at the highest test concentration. Estrone affected hatching at the highest exposure concentration, but had no effects on embryonic development at lower concentrations.

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### 7.1 INTRODUCTION

Embryonic development is generally regarded as a sensitive time window for toxic effects during an organism's life history. *Neomysis integer*, like other mysids, carries its embryos in a marsupium where the entire larval development takes place from oviposition to the release of free-living juveniles (Wittmann, 1984). The marsupial development of *N. integer* was recently described in detail by Fockedey et al. (2005a). Marsupial development in mysids was subsequently shown to be a sensitive endpoint to evaluate the effects of the insecticide,

methoprene (Chapter 6). Our ongoing and future efforts are to further explore mysid early embryonic development as a sensitive target to assess endocrine disruption.
ENDIS-RISKS is a multidisciplinary project that has studied the occurrence, distribution and potential effects of endocrine disruptors on mysids in the Scheldt estuary (Belgium/The Netherlands), one of the most polluted estuaries in the world (ENDIS-RISKS project, http://www.vliz.be/projects/endis). We earlier reported high levels of organotins, surfactants and flame retardants in mysids of the Scheldt estuary (Verslycke et al., 2005). More recent studies found significant levels of estrone in water of the Scheldt estuary (Noppe et al., 2005). The effect of nonylphenol and estrone on the vitellogenesis of *N. integer* are described in more detail in Chapter 4 (§ 4.1). This chapter describes the effects of nonylphenol and estrone on marsupial development of *N. integer*.

## 7.2 MATERIAL AND METHODS

### *7.2.1. Chemicals*

Estrone was obtained from Sigma-Aldrich (Bornem, Belgium) and nonylphenol from Acros Organics (Geel, Belgium). Stock solutions of the test compounds were prepared in absolute ethanol. The ethanol concentration in the solvent control and in the different test concentrations was 0.01%.

### *7.2.2. Test organisms*

Mysid crustaceans, *Neomysis integer*, were collected in March 2005 by handnet in the Braakman, a brackish water (10 psu) near the Scheldt estuary in Hoek (The Netherlands). Mysids were taken to the laboratory and cultured as described in Chapter 2 (§ 2.2.1.).

### *7.2.3. Test design*

A detailed description of the marsupial development assay in *N. integer* is described in Chapter 6 (§ 6.2.3). In short, stage I embryos were transferred at random to each of the wells of a 12-cell plate each containing 4 ml of the different exposure concentrations of nonylphenol and estrone (dilution water is artificial seawater of 15 psu). Embryos were exposed to 0, 0.01, 1, and 100 µg nonylphenol/l, and 0, 10, 100, and 1000 ng estrone/l. All

concentrations reported in this study are nominal, based on dilutions of the stock solutions. Fifteen replicates per concentration, and a minimum of 6 embryos per replica, were used. Multiwells containing the embryos were placed on an orbital shaker (80 rpm), and survival, developmental stage and hatching were recorded daily. Concurrently, dead embryos were removed and 75% of the medium was replaced.

The different developmental stages of the mysid embryo were distinguished as described in Chapter 6 (§ 6.2.4.). Stage I embryos are spherical and stage I ends with hatching from the egg membrane (Fig 7.1). Stage IIa embryos are dorsally bent and have a comma-like appearance. In stage IIb, the body is further extended and the thoracic appendages become more elongated. Stage II ends with molting of the embryo. Stage III embryos have stalked eyes and a developed telson and uropods. Stage III terminates with a final molt, leading to free-living juveniles.

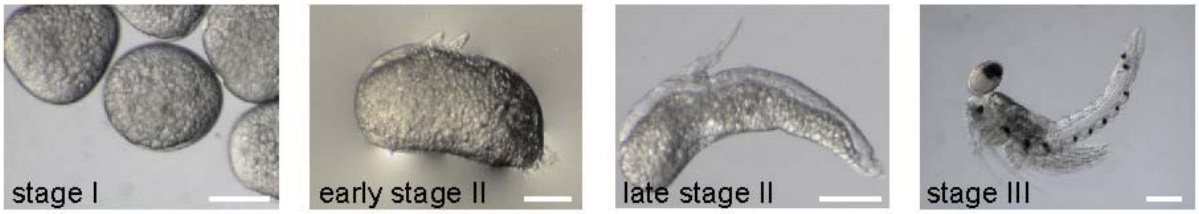


**Figure 7.1:** Different developmental stages of *Neomysis integer* embryo. Scale bar = 250 µm.

*7.2.4. Statistics*

All data were checked for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene's test respectively, with an $\alpha = 0.05$. The effect of the treatment was tested for significance using a one-way analysis of variance (Dunnett's test; Statistica™, Statsoft, Tulsa, OK, USA). All box-plots were created with Statistica™ and show the mean (small square), standard error (box), and the standard deviation (whisker).

## 7.3. RESULTS

### *7.3.1. Survival*

Percentage embryo survival was calculated each day for the different exposure concentrations (Fig. 7.2). Survival was highest during stage I for both chemicals. Exposure to all test concentrations of estrone had no significant effect on survival in all three different developmental stages of the embryo. Exposure to the highest concentration nonylphenol (100 µg/l), however, resulted in significant mortality on embryos of stage II and III.

### *7.3.2. Duration of the different developmental stages*

Figure 7.3 shows the duration of the different developmental stages of the embryos exposed to nonylphenol and estrone.

Duration of stage I was approximately 3 days, while stage II and III lasted about 6 and 3.5 days, respectively. The total embryonic development time of *N. integer* embryos development was around 12.5 days. Nonylphenol and estrone had no effect on the duration of the different developmental stages and the total embryonic development time of *N. integer*.

### *7.3.3. Hatching*

Exposure to 100 µg nonylphenol/l, and 1000 ng estrone/l resulted in significantly lower hatching rates compared to the control (Fig. 7.4). Average hatching percentages were 81.4 ± 19.4 %, 78.1 ± 14.5 %, 82.1 ± 13.8 % and 11.1 ± 16.2 % for the control, 0.01, 1 and 100 µg nonylphenol/l treatments, respectively. For estrone, average hatching percentages were 81.1 ± 15.2 %, 75.5 ± 19.8 %, 76.2 ± 16.9 % and 64.2 ± 11.0 % for the control, 10, 100, 1000 ng estrone/l treatments, respectively.

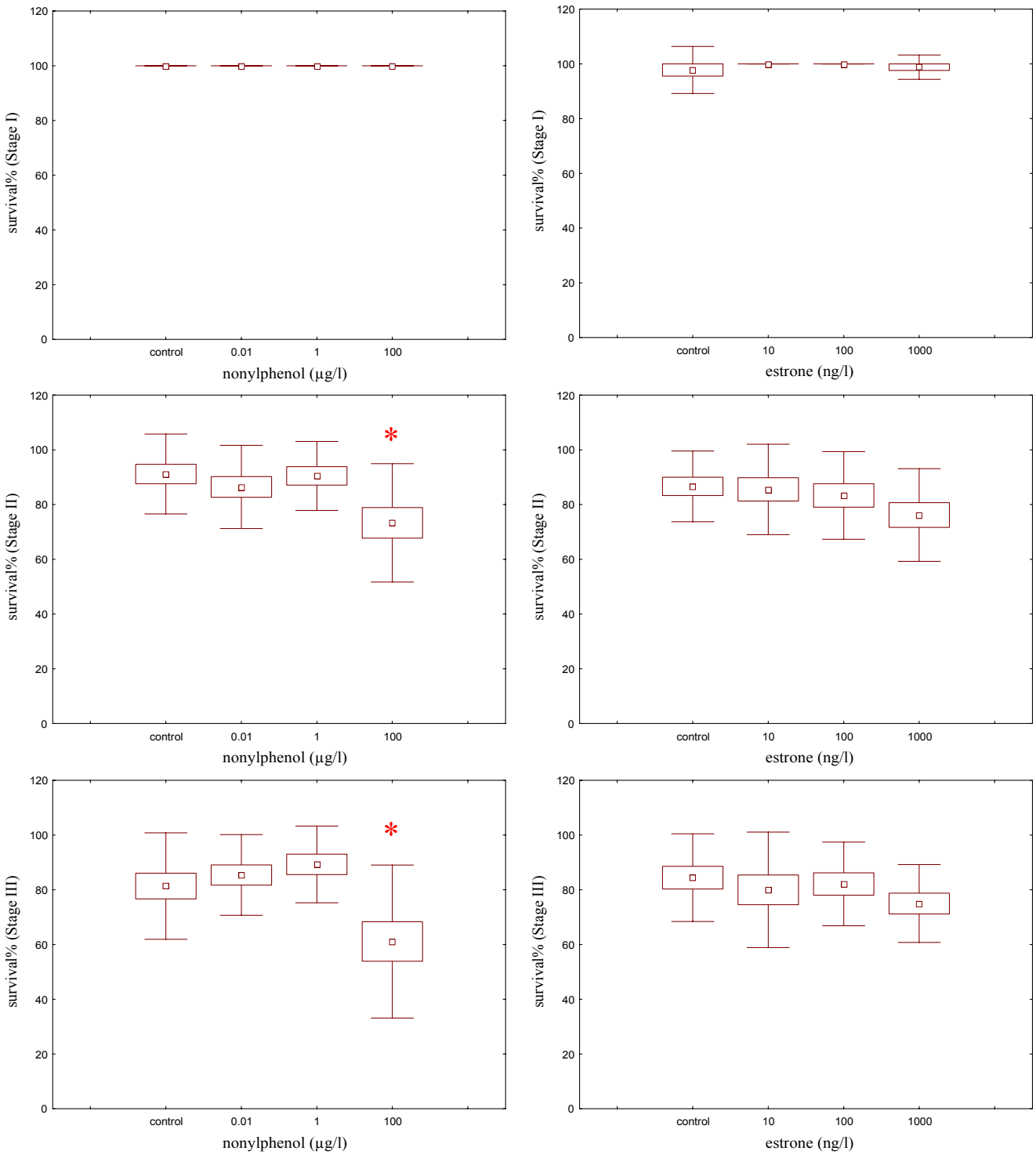


**Figure 7.2:** Percentage survival of *Neomysis integer* embryos during the different developmental stages exposed to 0.01, 1, 100 µg nonylphenol/l (left), and 10, 100, 1000 ng estrone/l (right).(Anova, Dunnett; *p<0.05, significance from control).

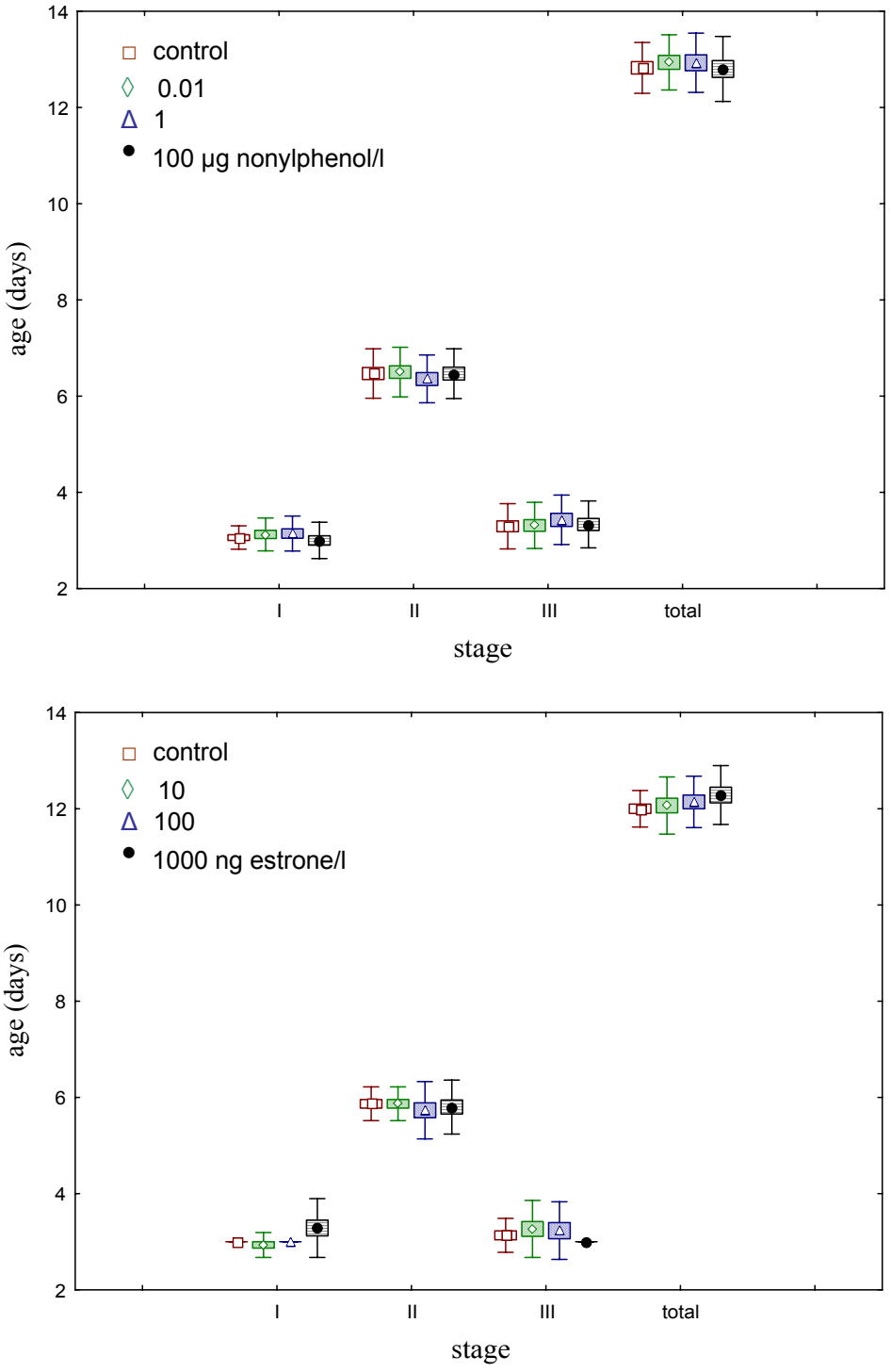


**Figure 7.3:** Duration of the different developmental stages of *Neomysis integer* embryos (days) exposed to 0.01, 1, 100 µg nonylphenol/l (above), and 10, 100, 1000 ng estrone/l (below). Total= duration of the total development time.

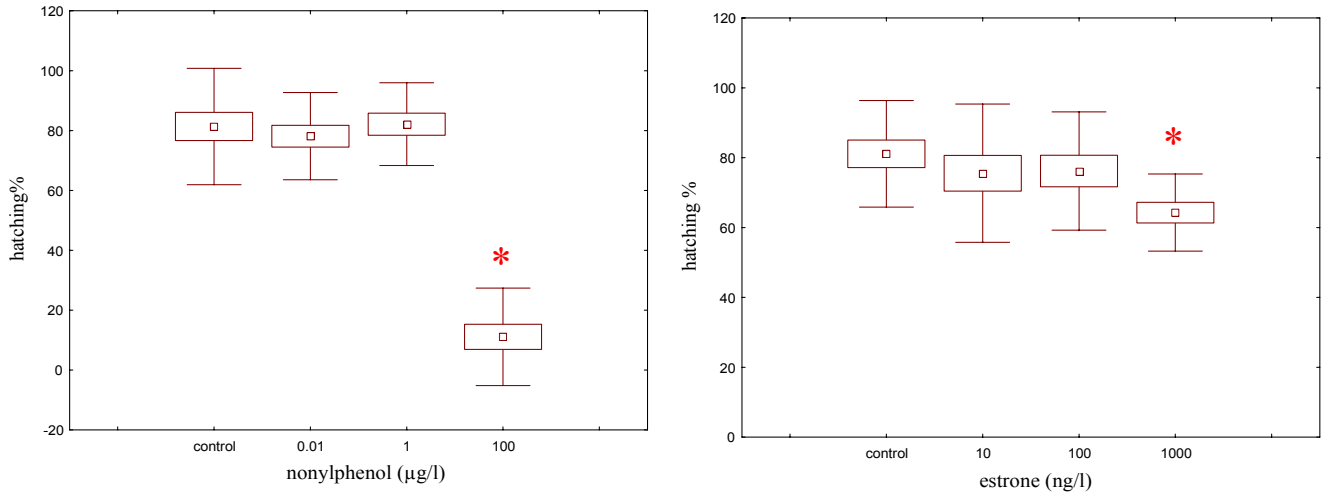


**Figure 7.4:** Percentage hatching of *Neomysis integer* embryos exposed to 0.01, 1, 100 µg nonylphenol/l (left), and 10, 100, 1000 ng estrone/l (right) from oviposition till free-living juveniles. (Anova, Dunnett; *p<0.05, significance from control)

## 7.4 DISCUSSION

In this doctoral study, we described the development of three physiological processes in *Neomysis integer* that are regulated by invertebrate-specific hormones, the ecdysteroids (Chapter 1). These three processes, vitellogenesis (Chapter 4), molting (Chapter 5), and embryogenesis (Chapter 6) were subsequently evaluated as endpoints to assess endocrine disruption through exposure with the test compound methoprene. These experiments demonstrated that methoprene lowers vitellin levels in *N. integer*, but this effect was not statistically significant. Methoprene did affect mysid molting at 100 µg/l, and embryogenesis at 1 µg/l. These results indicate that embryogenesis was the most sensitive endpoint at detecting the endocrine-disruptive effects of methoprene.

In this chapter we evaluated the potential effects of nonylphenol and estrone on the embryogenesis of *N. integer*. Estrone is frequently detected in water of the Scheldt estuary at concentrations of up to 8 ng/l (Noppe et al., 2005, Vethaak et al., 2002). Nonylphenol concentrations in the Scheldt estuary are similar to levels found in U.K. rivers and are around 10 µg/l (Verslycke et al., 2005; Vethaak et al., 2002). Nonylphenol and estrone did not affect embryogenesis of *N. integer* at environmentally relevant concentrations. However,

nonylphenol did significantly reduce survival and hatching at 100 µg/l when compared to the control. Estrone significantly lowered hatching at 1000 ng/l. Estrone was previously shown to affect mysid vitellogenesis at 1000 ng/l by lowering vitellin levels in females (Chapter 4). In the same study, nonylphenol increased vitellin levels at 0.01 µg/l.

We also observed that hatched embryos exposed to 1 and 100 µg nonylphenol/l were more lethargic, and higher mortality was observed at these concentrations during the first two days after final embryonic molt to free-living juveniles. Behavioural changes will be examined in future studies and might provide a sensitive way for evaluating endocrine disruption. Forget-Leray et al. (2005) found that nonylphenol significantly reduced molting success in the copepod *Eurytemora affinis* (LOEC of 15 µg/l). Molting from one stage to another was often incomplete. Animals from unsuccesfull molt were deformed and displayed a mix of naupliar and copepodid characteristics, and were also unable to move.

Alkylphenols, natural hormones and synthetic hormones, have been suggested as the most likely candidate chemicals responsible for the 'feminization' detected in male fish (Desbrow et al., 1998; Vos et al., 2000). Effects of these vertebrate (xeno-) estrogens on crustaceans, however, remains controversial and poorly understood giving the current knowledge on the presence of vertebrate-type steroids, such as estrogens, in these animals (Billinghurst et al., 2000; Sanders et al., 2005; Tsukimura, 2001). As such, existing studies on the effects of (xeno-)estrogens on crustacean vitellogenesis remain fragmented and contradictory (Chapter 4, § 4.4). However, the present study further adds to the weight-of-evidence that (xeno)estrogens appear to be less effective in causing endocrine disruption in crustaceans than in oviparous vertebrates. This also supports the growing consensus that invertebrate-specific approaches are needed for a relevant assessment of the risk of endocrine disrupting chemicals to invertebrates as they are most likely affected through hormonal pathways that are different from those of vertebrates.

# CHAPTER 8

## VITELLIN LEVELS OF *NEOMYSIS INTEGER* IN THE SCHELDT ESTUARY (BELGIUM/THE NETHERLANDS)

# CHAPTER 8

## VITELLIN LEVELS OF *NEOMYSIS INTEGER* IN THE SCHELDT ESTUARY (BELGIUM/THE NETHERLANDS)

ABSTRACT----------

Vitellogenesis involves the production of yolk protein, vitellin, which acts as a nutrient source for developing embryos. Consequently, any event that affects vitellin synthesis will likely impact reproductive success. Recently, we developed a quantitative enzyme-linked immunosorbent assay (ELISA) for vitellin in the mysid shrimp, *Neomysis integer*, to study vitellogenesis and its potential disruption by xenobiotics (Ghekiere et al., 2005). The present study was aimed at quantifying vitellin levels of *N. integer* collected in the Scheldt estuary. Mysids were collected during two sampling campaigns in April and July of 2005. Vitellin levels of females carrying stage I embryos from four different sites in this estuary were measured. Also, vitellin levels of females carrying broods in different developmental stages were quantified at one site. Abiotic (temperature, salinity, and dissolved oxygen) and biotic parameters (brood size and standard length) were recorded at all sites. Significantly lower vitellin levels were observed in the more upstream sites during the sampling canmpaign of April 2005 but no such differences were found in July 2005. No obvious correlations were found between mysid vitellin levels and the abiotic or biotic parameters. Contrary to significant differences in vitellin levels in eggs of different developmental stages, no significant differences were found in vitellin levels of field-collected animals carrying broods in different stages of development. This study is the first to report vitellin levels in field populations of mysid crustaceans.

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## 8.1. INTRODUCTION

*Neomysis integer* is a hyperbentic, euryhaline, and eurythermic species that occurs in brackish water environments, mainly estuaries. Its biology, ecology, and use in ecotoxicology have been well documented (see Chapter 1, § 1.2.). We have been using the Scheldt estuary as our field site to evaluate the presence, distribution, and potential effects of environmental endocrine disruptors on the resident mysid population. Part of these studies were incorporated into a four-year field project, ENDIS-RISKS (http://www.vliz.be/projects/endis). In the laboratory, we have been studying several hormone-regulated processes in *N. integer*, with specific attention to processes that are regulated by hormones that are unique to invertebrates, notably the ecdysteroids (Chapter 3 through 7).

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. In ecdysozoans (animals that molt), vitellogenesis it is under ecdysteroid control. The accumulation of vitellin during oocyte development is vital for the production of viable offspring. As such, disruption of vitellogenesis will result in effects on reproduction. This has led to the use of vitellogenesis – mainly in fish - as an important biomarker to evaluate exposure to chemicals that mimic the hormones involved its control. Specifically, plasma vitellogenin induction can be used as a sensitive biomarker of exposure to estrogen-mimics in many fish species (Fenske et al., 2001; Sumpter and Jobling, 1995). Several immunoassays have been developed to quantify vitellin in crustaceans, both for the fundamental study of hormone control (Lee and Chang, 1997; Tsukimura et al., 2002; Vazquez Boucard et al., 2002) and for use as biomarkers (Billinghurst et al., 2000; Sanders et al., 2005; Tsukimura, 2001). Studies on mysid vitellogenesis have been very limited because assays to measure the relevant hormones were lacking. In a recent study, we purified and characterized vitellin from the mysid *Neomysis integer* (Chapter 2) and developed a quantitative enzyme-linked immunosorbent assay (ELISA) for vitellin in this organism (Chapter 3).

While only a few studies have reported on using immunoassays to quantify vitellin in field populations of decapod crustaceans (Martin-Diaz et al., 2005), to the best of our knowledge, no such studies have been published with lower crustaceans like mysids. The goals of the present study were: (1) to quantify vitellin levels in *N. integer* of the Scheldt estuary at different sites and at different times; (2) to examine spatial and temporal trends in mysid vitellogenesis in the field; and (3) to evaluate potential relationships between mysid vitellin

levels and biotic (standard length, brood size) or abiotic (temperature, salinity, and dissolved oxygen) factors.

## 8.2. MATERIAL AND METHODS

### *8.2.1. Study area*

The river Scheldt (Fig. 8.1) takes its rise in the northern part of France (St. Quentin), and flows into the North Sea near Vlissingen (The Netherlands). Total length of the river is 355 km and its mean depth is about 10 m. The estuarine zone of the tidal system is about 70 km long and extends from the North Sea to the Dutch-Belgian border near Bath. From an ecological point of view, the Scheldt estuary is one of the most important tidal river systems in Europe. It is an important overwintering and feeding area for birds and an important nursery for several North Sea fish and shrimp species. The Scheldt estuary has been described as one of the most polluted estuaries world-wide, based on contaminant concentrations in the dissolved as well as the particulate phase (Baeyens, 1998). The physico-chemistry and ecology of the Scheldt estuary have been described by several authors (Heip, 1988, 1989; Herman et al., 1991; Van Eck et al., 1991; Baeyens et al., 1998).

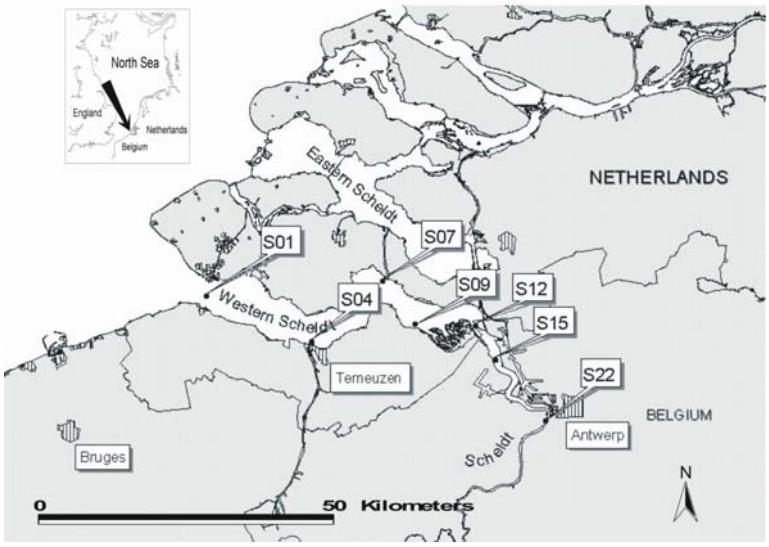


**Figure 8.1:** Map of the Scheldt estuary with location of the different sampling sites used in the ENDIS-RISKS project (S01, Vlissingen; S04, Terneuzen; S07, Hansweert; S09, Saeftinge; S12, Bath; S15, Doel and S22, Antwerp). Samples from S09, S12, S15, and S22 were analyzed in the present study.

*8.2.2. Mysid sampling*

Mysid were sampled during spring (April) and summer (July) of 2005 at four different sites in the Scheldt estuary (Saeftinge, S09; Bath, S12; Doel, S15; Antwerp, S22; see Fig. 8.1). Mysids were collected with a hyperbenthic sledge of 3m long, 1.7m wide and 1.4m high with two pairs of nets (71 cm wide, 3 m long) mounted on the sledge next to each other (Fig. 8.2, Verslycke, 2003; Verlycke et al., 2004b). Each net (mesh sizes 1x1 mm) was equipped with a collector at the end, which is fixed onto the sledge's frame at an angle of 45°. This prevents the collected fauna from escaping by swimming back or getting damaged by the strong flow. All samples were taken during daytime when hyperbenthic animals are known to concentrate near the bottom. Gravid *N. integer* specimens were sorted out on board. These animals were staged according to the three major embryonic developmental stages, as described in Chapter 6. Females with broods in a specific developmental stage were individually placed into an eppendorf and shock-frozen in liquid nitrogen. Samples were kept at -80 °C until analysis of the vitellin levels. Fifteen females with stage I embryos were used for each sampling point and per sampling campaigns (April and July 2005). Stage I animals were used because this developmental stage is easily distinguished and is also a short embryonic stage (± 4 days), minimizing intra-stage variability. At sampling point S15 (Doel, see Fig. 8.1), animals were collected carrying broods of the three major different developmental stages (15 animals were collected per stage). All vitellin analyses were performed within two weeks of collection to reduce the risk of vitellin degradation. Salinity, dissolved oxygen concentrations, and temperature were measured at all sites (depth around 5 m) using a Sea-Bird SBE21 thermosalinograph (Sea-Bird Electronics, Bellevue, WA, USA) and a Sea-Bird SBE19 'SeaCat' CTD profiler (Table 8.1).

**Figure 8.2:** Hyperbentic sledge (left) used for sampling of mysid shrimp in the Scheldt estuary. The sledge was operated from the research vessel Belgica (right).

### *8.2.3. Quantification of vitellin*

All animals were individually homogenized in 200 µl Tris-HCl (pH 7.2), and diluted 10,000 times in this buffer for vitellin quantification. All concentrations were calculated in 1 ml of this homogenate and normalized for the wet weight of the animal. Vitellin levels were measured as described in detail in Chapter 3.

### *8.2.4. Brood size and standard length determination*

Standard length was measured as described in Chapter 5 (§5.2.4.). Brood size was determined by taking the eggs out of the masupium en subsequently counting.

### *8.2.5. Statistical analyses*

All data were checked for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene's test respectively, with an $\alpha = 0.05$. The effect of the treatment was tested for significance using a one-way analysis of variance (Tukey's Honestly Significant Difference test; Statistica™, Statsoft, Tulsa, OK, USA). All box-plots were created with Statistica™ and show the mean (small square), standard error (box), and the standard deviation (whisker).

## 8.3. RESULTS

### *8.3.1. Abiotic measurements*

Table 8.1 shows temperature, salinity, and dissolved oxygen of the water at the different sampling sites during the two campaigns. Sites are representative of a transect along the salinity gradient, as is obvious from the salinity measurements at the different sites.

**Table 8.1**: Temperature, salinity, and dissolved oxygen during April and July 2005 at the different sampling sites (see Fig. 8.1) in the Scheldt estuary.

| Parameter | Station | Spring (April) | Summer (July) |
|---|---|---|---|
| Temperature (°C) | S09 | 11.3 | 21.2 |
| | S12 | 11.4 | 21.4 |
| | S15 | 12.0 | 22.0 |
| | S22 | 12.3 | 21.8 |
| Salinity (PSU) | S09 | 10.8 | 18.4 |
| | S12 | 8.2 | 12.6 |
| | S15 | 7.6 | 7.9 |
| | S22 | 4.0 | 5.0 |
| Dissolved oxygen (mg/l) | S09 | 10.45 | 8.09 |
| | S12 | 12.55 | 6.33 |
| | S15 | 11.90 | 6.71 |
| | S22 | 13.45 | 2.99 |

### *8.3.2. Vitellin levels in mysids collected from different sites*

Vitellin levels were quantified in *N. integer* carrying stage I broods in their marsupium (Fig. 8.3). Average vitellin concentrations in April 2005 were 0.32 ± 0.21 mg/ml*mg ww, 0.40 ± 0.23 mg/ml*mg ww, and 0.31 ± 0.21 mg/ml*mg ww for S12, S15, and S22, respectively. The average vitellin level in ovigorous mysids collected at the most downstream site (S09) was 1.09 ± 0.34 mg/ml*mg ww, which is sigificantly higher than average levels in mysids collected at the three most upstream sites (S12, S15, and S22). In July 2005, female mysids had avarage vitellin concentrations of 0.83 ± 0.49 mg/ml*mg ww at S09, 0.47 ± 0.42 mg/ml*mg ww at S12; 1.01 ± 0.92 mg/ml*mg ww at S15, and 0.95 ± 1.19 mg/ml*mg ww at S22. These levels were not significantly different between sites. The overall vitellin level of ovigorous mysids collected during the spring campaign was significantly lower than that of organisms collected during the summer.

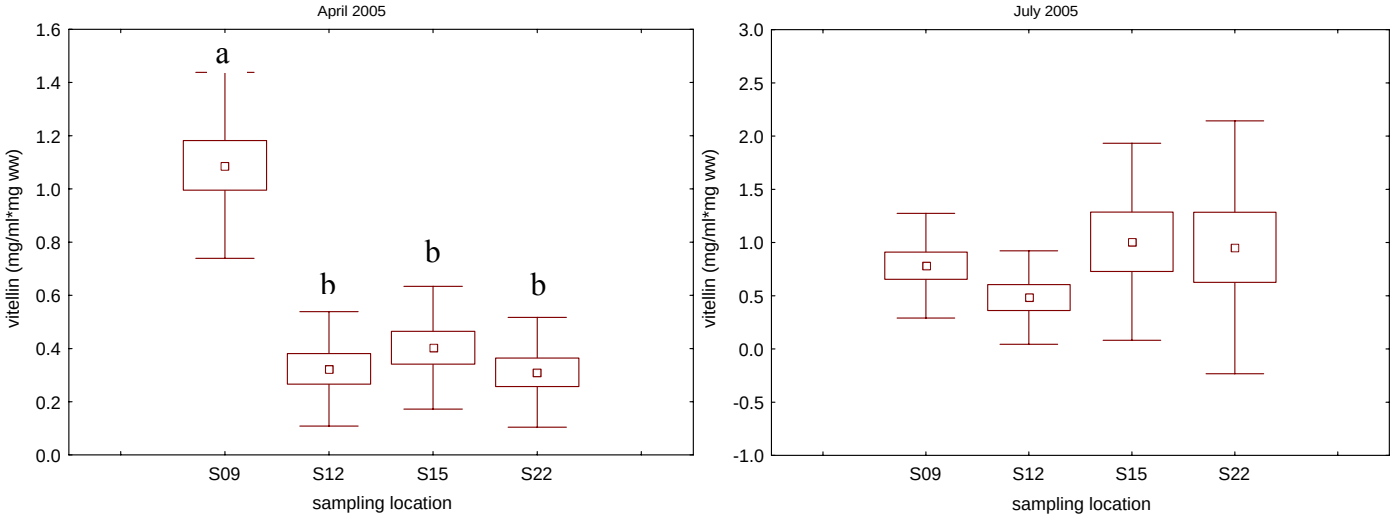


**Figure 8.3:** Vitellin levels in ovigorous *N. integer* of the Scheldt estuary carrying stage I broods during the sampling campaigns in April (left) and July (right) 2005. Different letters indicate significant differences ($p<0.05$). Refer to Figure 8.1 for sampling locations.

### *8.3.2. Brood size and standard length*

Brood size (amount of stage I embryos per female) was determined during the sampling campaign of April 2005 as shown in Figure 8.4. Average brood sizes were 56.3 ± 13.3; 49.0 ± 18.5; 60.6 ± 14.8; 41.2 ± 15.1 in animals collected at S09, S12, S15, and S22, respectively. No brood size data was collected in July 2005 due to sample loss.

In addition to brood size, the standard length of the collected females carrying stage I broods was determined. Mysids collected during April 2005 had standard lengths of 14.1 ± 1.2; 14.5 ± 1.4; 13.5 ± 1.2; 13.8 ± 1.2 mm at S09, S12, S15, and S22, respectively (Fig. 8.4). In July 2005, standard lengths were 9.9 ± 1.6; 8.4 ± 0.5; 8.0 ± 0.6 mm at S12, S15, and S22, respectively (no females from S09 were available in July 2005 to measure the standard length). No significant differences are found between standard length of different sampling locations in the two sampling campaigns.

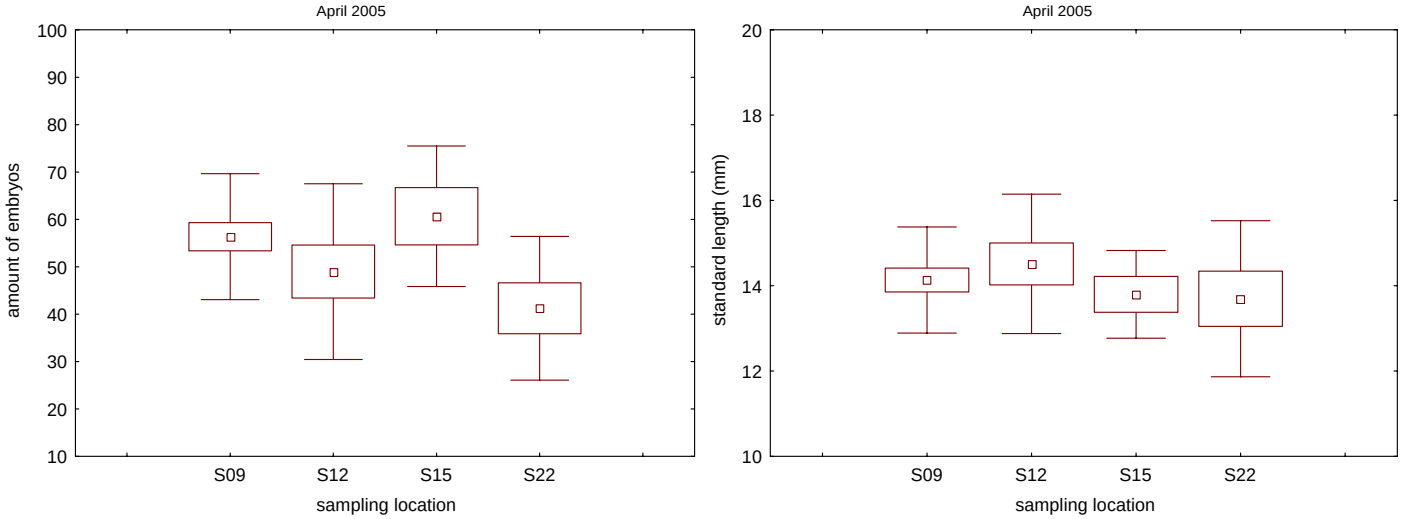


**Figure 8.4:** Brood size (left) and standard length (right) of female *N. integer* of the Scheldt estuary carrying stage I embryos in April 2005. Refer to Figure 8.1 for sampling locations.

### *8.3.3. Vitellin levels in ovigerous females with different developmental stages*

At one site (S15, Doel), we also collected female mysids carrying broods in the three different developmental stages and compared vitellin levels (Fig. 8.5). No significant differences were found in vitellin levels of females carrying embryos in different developmental stages.

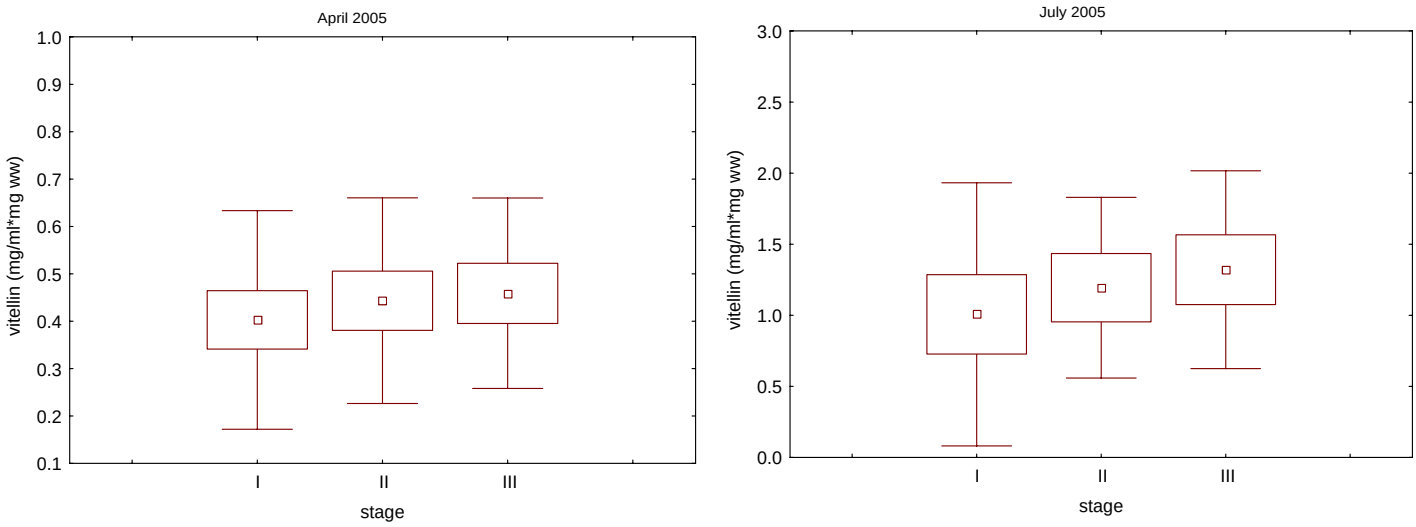


**Figure 8.5:** Vitellin levels of *N. integer* of the Scheldt estuary carrying embryos in three different developmental stages. Animals were collected at the site S15 (Doel) during the April (above) and July (below) campaigns in 2005. The different developmpental stages are described in detail in Chapter 6.

## 8.4. DISCUSSION

Recently, we purified vitellin from the mysid *N. integer* (Chapter 2), produced antibodies against mysid vitellin and subsequently developed an immunoassay to quantify this protein (Chapter 3). The present study is our first attempt at validating the *N. integer* vitellin ELISA in the field by quantifying levels in ovigorous mysids collected from the Scheldt estuary. This estuary has been our field site for research on mysids for more than 6 years, and is currently being sampled for the presence of endocrine disruptors in sediment, water, suspended solids, and in mysids (ENDIS-RISKS project). Vitellogenine induction has been used as a sensitive biomarker of exposure to xeno-estrogens in field studies with fish (Jobling et al., 1998; Luizi et al., 1997; Tyler and Routledge, 1998). While the hormonal control of vitellogenesis in crustaceans is different from that in vertebrates, laboratory studies have demonstrated that chemicals are equally capable of disrupting normal vitellogenesis in crustaceans (Billinghurst et al., 2000; Lee and Noone, 1994; Oberdörster et al., 2000; Sanders et al., 2005; Tsukimura, 2001; Volz and Chandler, 2004). Specifically, we demonstrated that nonylphenol and estrone disrupt vitellogenesis in *N. integer* (Chapter 4). Effect concentrations for nonylphenol as determined in the latter study were similar to concentrations found in water samples of the Scheldt estuary (Vethaak et al., 2002). Consequently, we wanted to evaluate mysid vitellogenesis in this estuary with respect to spatial and temporal changes in the biotic and abiotic environment. The present study describes our preliminary results, and are the first reported levels of vitellin in a mysid field population.

Spatial differences in vitellin levels of ovigorous mysids carrying stage I embryos were evaluated by sampling animals from different sites (S09, S12, S15, S22; see Fig. 8.1) along a salinity gradient in the Scheldt estuary. At these sites, we also collected abiotic (temperature, salinity, and dissolved oxygen) and biotic (brood size and standard length) information. Significant difference in vitellin levels were observed 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 measurements (see Table 8.1) 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 this study had less energy at the more upstream sites S15 and S22, where pollution is 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 and 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 in July 2005. A more comprehensive dataset needs to be compiled in the future to confirm the spatial trends observed in this preliminary study.

We also examined vitellin levels in ovigorous mysids carrying eggs at different developmental stages at one site (S15-Doel) during both campaigns. We recently analyzed vitellin levels in eggs of different developmental stages (Ghekiere et al., 2005; Chapter 3), and found that eggs in later developmental stage had significantly lower vitellin levels. In the present study, vitellin levels in homogenates of females carrying eggs of different developemental stages were not significantly different. Differences between vitellin levels in eggs that are isolated from the mother (Chapter 3) and vitellin levels in a homogenate containing the whole animal (this study) could be related to vitellin derived from developing oocytes in the ovarium. We did indeed observe that the ovarium of animals carrying later stage broods were more developed. For future studies, it might therefore be better to isolate the eggs from the collected individuals to minimize interference of developing eggs in the ovarium. On the other hand, whole body homogenates provide more biomass and should be suitable for evaluating potential effects on mysid vitellogenesis.

In conclusion, we reported baseline data on vitellin levels of *N. integer* in the field. Significantly lower vitellin levels were observed in the more upstream sites during the sampling campaign of April 2005, corresponding to earlier observed effects on mysid energy metabolism at these sites. While the present study demonstrates the use of our ELISA to quantify vitellin in mysid field populations, more extensive field sampling will be needed to assess spatial and temporal differences of vitellin levels observed in the field. Our ongoing studies are creating a unique dataset on the exposure of *N. integer* in the Scheldt estuary and on the potential effects of this exposure to the resident population by looking at energy and steroid metabolism, and now, vitellogenesis in these animals. These studies will ultimately lead to an overall risk assessment for endocrine disruptors in this estuary specifically focusing on the risk to the mysid population.

# CHAPTER 9

## GENERAL CONCLUSIONS AND RESEARCH PERSPECTIVES

# CHAPTER 9

## GENERAL CONCLUSIONS AND RESEARCH PERSPECTIVES

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 (cartenoid) 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 ovigorous 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.

# SUMMARY

# SUMMARY

This doctoral thesis is situated in the field of aquatic toxicology, a sub-discipline of ecotoxicology. Aquatic toxicology aims at studying the fate and effects of natural and anthropogenic substances on aquatic ecosystems. Recently, it has been shown that certain natural and man-made chemicals, called endocrine disruptors, can mimic and dirsupt hormone signaling in a large number of organisms. Endocrine disruption has received a lot of attention due to a series of alarming reports that point to increases in certain reproductive cancers and declines in male fertility in humans, and feminization in fish and alligators. To date, most studies have focused on endocrine disruption in the vertebrates, including mammals, fish, birds, and reptiles. Although, invertebrates constitute about 95% of all animal species and occupy an important position in many foodwebs, relatively little research has been directed at understanding the potential effects of endocrine disrupting chemicals (EDCs) on this group of species. Moreover, few studies have evaluated the potential effects of EDCs on invertebrate-specific hormone-regulated processes, such as molting. It has been well established that ecdysteroids and juvenile hormones are the major endocrine regulators of molting, embryonic development, metamorphosis, and reproduction in more than 75% of all invertebrates, the ecdysozoans (animals that molt). Furthermore, many pesticides are specifically designed to mimic the action of invertebrate-specific hormones. To date, regulatory action is based on vertebrate hormone disruption and therefore risks to seriously underestimate the potential effects of endocrine disruptors on our ecosystems. This is partly due to a lack of understanding of hormone regulation in many invertebrate models, and the lack of sensitive and targeted assays to evaluate invertebrate-specific endocrine disruption

The aim of the present doctoral study was to assess endocrine disruption in the mysid shrimp *Neomysis integer* (Crustacea: Mysidacea) using invertebrate-specific processes that are regulated by the action of ecdysteroids, molting, vitellogenesis and embryonic development.

**Chapter 1** starts with a brief introduction on the crustacean endocrine system, with special reference to the endocrine regulation of molting, vitellogenesis and reproduction. An introduction to the biology and ecology of the test species *N. integer* is presented, as well as its use in standard toxicity testing and in endocrine-disruptor testing. The final part of this chapter provides a literature overview on the specific processes in *N. integer* that were selected for this doctoral study.

**Chapter 2** describes the purification and characterization of the yolk protein vitellin in *N. integer*. Mysid vitellin was purified from eggs using gel filtration. The molecular mass of *N. integer* vitellin is approximately 700 kDa as determined by electrophoresis on 4.5-10% native polyacrylamide gels using standard molecular mass markers and by gel filtration. The purified vitellin contained carbohydrate and lipid moieties identified using staining with Periodic Acid Schiff's reagent and Sudan Black B reagent, respectively. Specific polyclonal antibodies against the purified *N. integer* vitellin were produced in rabbit.

**Chapter 3** describes the development of an enzyme-linked immunosorbent assay (ELISA) to quantify vitellin in *N. integer*. The ELISA was sensitive within a working range of 4 to 500 ng vitellin/ml. Serial dilutions of whole body homogenates from female *N. integer* and the vitellin standard showed parallel binding curves, validating the specificity of the ELISA. The intra- and interassay coefficients of variation were 8.2 and 13.8%, respectively. Mysid vitellin concentrations of ovigorous females and eggs at different developmental stages were determined.

**Chapter 4** describes the application of the ELISA for mysid vitellin developed in **Chapter 3** through a series of laboratory exposures with potential endocrine disruptors. Gravid mysids were exposed to methoprene (insecticide), nonylphenol (surfactant and xeno-estrogen) and estrone (natural estrogen) for 96h. All methoprene-exposed (0.01, 1, 100 µg/l) animals had lower vitellin levels compared to the control animals, though this effect was not statistically significant. Exposure to nonylphenol resulted in significantly induced vitellin levels in the lowest exposure concentration (0.01 µg/l), whereas no effects were observed at higher concentrations. Estrone significantly decreased vitellin levels at the highest test concentration (1 µg/l). While these expsosure studies validated the usefulness of the mysid vitellin ELISA to detect chemical interference with mysid vitellogenesis, future studies should also focus on using this assay to get a better understanding of the hormonal regulation of vitellogenesis.

**Chapter 5** evaluates the non-target effects of the insecticide methoprene on molting in *N. integer*. First, a series of preliminary studies were performed to develop an *in vivo* assay with *N. integer* that allow the assessment of chemical intereference with mysid molting. Next, juveniles (< 24h) were exposed for 3 weeks to three nominal methoprene concentrations (0.01, 1 and 100 µg /l) and a control treatment.Daily, treatments were chekced for newly

shedded exoskeletons which were stored in 4% formaldehyde for subsequent growth measurements. Methoprene significantly delayed molting at 100 µg/l by decreasing the growth rate and increasing the intermolt period. Methoprene-exposed animals also had a lower wet weight compared to control animals. The potential anti-ecdysteroidal properties of methoprene on mysid molting were evaluated by determining the ability of exogenously administered 20-hydroxyecdysone, the active ecdysteroid in crustaceans, to protect against the observed methoprene effects. Co-exposure to 20-hydroxyecdysone, however, did not mitigate methoprene effects on mysid molting. This study clearly demonstrates the non-target effects of methoprene on a hormone-regulated process in mysids.

**Chapter 6** describes embryonic development of *N. integer* as a hormone-regulated process potentially useful to evaluate the effects of environmental chemicals. First, a detailed description of the different developmental stages of *N. integer* embryos is provided. Next, mysid embryos were exposed to three nominal concentrations of methoprene (0.01, 1, and 100 µg /l) and a control. Average percentage survival, hatching success, total development time, and duration of each developmental stage were analyzed during the full duration of the embryonic development. 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. This study indicates that in the early developmental stages of mysid sensitive hormone-regulated processes occur that can be targeted by chemicals at environmentally relevant concentrations.

**Chapter 7** descibes the effects of nonylphenol and estrone on the embryonic development of *N. integer*. Stage I embryos were exposed to nonylphenol (0.01, 1, and 100 µg /l), estrone (10, 100, and 1000 ng/l), and a control until hatching to free-swimming juveniles. Duration of the different developmental stages, survival and hatching succes were examined. Nonylphenol had no effect on the duration of the three different developmental stages, but it significantly lowered survival and hatching at the highest test concentration 100 µg/l compared to the control. Estrone only affected hatching at the highest test concentration. This study further adds to a series of reports which indicate that estrogens are less effective in crustaceans than they are in vertebrates in causing endocrine disruption. Most likely, this is a result of the different hormonal control strategies in crustaceans compared with oviparous vertebrates.

**Chapter 8** reports on the vitellin levels of field-collected *N. integer* occuring in the Scheldt estuary (Belgium/The Netherlands). Vitellin levels of females carrying stage I embryos were

collected in April and July of 2005 from different sites in the estuary. During the sampling campaign of April 2005, significantly lower vitellin levels were observed in the more upstream sites, corresponding to the more polluted part of this estuary and to earlier reported effects on mysid energy metabolism at these sites. However, these spatial differences were not confirmed in July 2005. In addition to quantifying vitellin levels at the different sites, we also measured a number of abiotic (temperature, salinity, and dissolved oxygen) and biotic parameters (brood size and standard length). No obvious correlations were found between mysid vitellin levels and the abiotic and biotic parameters. Finally, vitellin levels were quantified in females carrying eggs of the three different developmental stages. Contrary to significant differences in vitellin levels in single eggs of the different developmental stages, no such differences were observed in the field in whole animal/embryo homogenates. While the present study demonstrates the use of the developed ELISA to quantify vitellin in mysid field populations, more extensive field sampling is needed to assess spatial and temporal differences of vitellin levels observed in the field.

In **Chapter 9**, general conclusions and future perspectives of this doctoral study were formulated.

# SAMENVATTING

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Deze doctoraatsthesis is gesitueerd in het domein van de aquatische toxicologie, een subdiscipline van de ecotoxicology. Het doel van aquatische toxicology is het gedrag en de effecten te bestuderen van natuurlijke en antropogene stoffen voor aquatische ecosystemen. Onlangs werd er aangetoond dat bepaalde natuurlijke en xenobiotische stoffen, endocriene verstoorders genoemd, capaciteiten bezitten om hormonen na te bootsen en te verstoren. Endocriene verstoring staat sterk in de publieke belangstelling wegens een reeks van alarmerende berichten over verhoging van bepaalde kankers aan het voortplantingstelsel, verminderde vruchtbaarheid bij de mens en vervrouwelijking van vissen en alligators. Tot op heden, hebben de meeste studies zich gericht op het bestuderen van endocriene verstoring bij de gewervelden zoals zoogdieren, vissen, vogels en reptielen. Ongewervelden vertegenwoordigen ongeveer 95% van alle organismen en spelen een belangrijke rol in vele voedselketens. Er zijn echter relatief weinig studies beschikbaar i.v.m. de potentiële effecten van endocrien verstorende stoffen (EVS) op ongewervelden. Bovendien evalueren weinig studies de potentiële effecten van EVS op invertebraat-specifieke hormoon gereguleerde processen, zoals vervelling. Het is gekend dat ecdysteroïden en juveniel hormonen de voornaamste endocriene regulators zijn van vervelling, embryonale ontwikkeling, metamorfose en reproductie in meer dan 75% van alle invertebraten, de ecdysozoa (organismen die vervellen). Verder zijn vele pesticiden speciaal ontwikkeld om de werking van invertebraat-specifieke hormonen na te bootsen. Tot op heden zijn regulatorische maatregelen enkel gebasseerd op kennis van de hormoonverstoring bij vertebraten en daarom bestaat het gevaar dat de potentiele effecten van hormoonverstoorders op onze ecosystemen zwaar worden onderschat. Dit is voornamelijk te wijten aan een gebrekkige kennis van de hormonale regulatie in vele invertebraten en het ontbreken van gevoelige en gerichte testen om invertebraat-specifieke endocriene verstoring te bestuderen.

Het doel van de huidige doctoraatsstudie is het bestuderen van endocriene verstoring in de aasgarnaal *Neomysis integer* (Crustacea: Mysidacea) gebruikmakende van invertebraat-specifieke processen die gereguleerd worden door de ecdysteroïden. Deze processen zijn vervelling, vitellogenese en embryonale ontwikkeling.

**Hoofdstuk 1** begint met een korte introductie over het endocrien systeem van crustaceeën met bijzondere verwijzing naar de endocriene regulatie van vervelling, vitellogenese en

reproductie. Ook wordt een beknopt overzicht gegeven van de biologie en ecologie van de testorganisme *N. integer*, evenals zijn gebruik in standaard toxiciteitstesten en assays gericht op de evaluatie van endocrien verstorende stoffen. Het laatste deel van dit hoofdstuk wordt een literatuuroverzicht gegeven over de specifieke processen van *N. integer* die geselecteerd werden voor dit doctoraatsonderzoek.

**Hoofdstuk 2** beschrijft de opzuivering en karakterisatie van het dooiereiwit vitelline in *N. integer*. Vitelline wordt opgezuiverd uit eitjes met gelfiltratie. De moleculaire massa van *N. integer* vitelline is ongeveer 700 kDa, zoals bepaald door elektroforese op 4,5-10% natieve polyacrylamide gels met standaard moleculaire massa merkers en door gelfiltratie. De opgezuiverde vitelline bevat saccharide en lipide delen, bepaald met respectievelijk de kleuringen 'Periodic Acid Schiff's reagent' en 'Sudan Black B reagent'. Specifieke polyclonale antilichamen worden geproduceerd in konijn tegen opgezuiverd vitelline van *N. integer*.

**Hoofdstuk 3** beschrijft de ontwikkeling van een 'enzyme-linked immunosorbent assay' (ELISA) om vitelline in *N. integer* te kwantificeren. De ELISA is gevoelig en heeft een werkend gebied van 4 tot 500 ng vitelline/ml. Seriële verdunningen van homogenaten van het volledig lichaam van vrouwelijke *N. integer* en de vitelline standaard vertoont parallelle bindings curven wat de specificiteit van de ELISA valideert. De intra- en interassay variatiecoëfficiënten waren respectievelijk 8,2 en 13,8 %. Vitellineconcentraties worden bepaald in gravide vrouwtjes en eitjes uit verschillende ontwikkelingsstadia.

**Hoofdstuk 4** beschrijft de toepassing van de ELISA ontwikkeld in **Hoofdstuk 3** door het uitvoeren van een reeks van laboratoriumblootstellingen met potentiële endocriene verstoorders. Gravide aasgarnalen worden blootgesteld aan methopreen (insecticide), nonylfenol (surfactant en xeno-oestrogeen) en oestrone (natuurlijke oestrogeen) voor 96 uur. Alle methopreen-blootgestelde (0,01; 1; 100 µg/l) organismen hebben een lager vitelline gehalte ten opzichte van controle-organismen, maar dit effect is niet statistisch significant. Blootstelling aan nonylfenol resulteert in significant toegenomen vitellinegehaltes in de laagste blootstellingsconcentratie (0,01 µg/l), terwijl geen effect zichtbaar is bij hogere concentraties. Oestrone verlaagt significant de vitellinegehaltes bij de hoogste testconcentratie (1 µg/l). Terwijl deze blootstellingsstudies het nut van de ELISA aantoont om verstoring van vitellogenese bij de aasgarnaal te detecteren, zouden toekomstige studies zich ook moeten

richten op het gebruik van deze test om een beter inzicht te krijgen in de hormonale regulatie van vitellogenese.

**Hoofdstuk 5** evalueert de effecten van de insecticide methopreen op de vervelling van *N. integer*. Eerst wordt een reeks van preliminaire studies uitgevoerd met als doel een *in vivo* test met *N. integer* te ontwikkelen die ons zal toelaten om chemische interferentie met de vervelling van mysids te bestuderen. Vervolgens worden juvenielen (<24u oud) blootgesteld voor drie weken aan drie methopreen concentraties (0,01; 1 en 100 µg/l) en een controlebehandeling. Dagelijks wordt gecontroleerd of er vervellingen aanwezig waren. Deze worden bewaard in 4% formaldehyde voor de groeimetingen. Methopreen vertraagt het vervellingsproces bij 100 µg/l door de groeisnelheid te verlagen en de intermolt periode te verlengen. Methopreen-blootgestelde organismen hebben ook een lager gewicht vergeleken met controle-organismen. De potentiële anti-ecdysteroïdale eigenschappen van methopreen op de vervelling van de aasgarnalen worden geëvalueerd door het bepalen of exogeen toegediende 20-hydroxyecdysone, de actieve ecdysteroïd in crustaceeën, bescherming kan bieden tegen de effecten veroorzaakt door methopreen. Simultane blootstelling aan methopreen en 20-hydroxyecdysone doen de effecten van methopreen op de vervelling niet verdwijnen. Hoewel methopreen ontwikkeld is tegen pestorganismen, toont deze studie aan dat ook op andere organismen ongewenste effecten worden veroorzaakt.

**Hoofdstuk 6** beschrijft de embryonale ontwikkeling van *N. integer* als een hormoon gereguleerd proces die mogelijk bruikbaar zou kunnen zijn om de effecten van chemicaliën te evalueren. Eerst wordt een gedetailleerde beschrijving van de verschillende ontwikkelingsstadia van het embryo gegeven. Vervolgens worden embryo's blootgesteld aan drie concentraties van methopreen (0,01; 1 en 100 µg/l) en een controle. Gemiddelde percentage overleving, ontluiking, totale ontwikkelingstijd en duur van elk ontwikkelingsstadium worden geanalyseerd gedurende de volledige duur van de embryonale ontwikkeling. Embryo's blootgesteld aan 1 en 100 µg methopreen/l hebben een significant lagere ontluiking en een lagere overleving dan embryo's in de controle. Deze studie toont aan dat in vroege ontwikkelingsstadia van aasgarnalen gevoelige hormoon gereguleerde processen aanwezig zijn die kunnen verstoord worden door chemicaliën bij relevante omgevingsconcentraties.

**Hoofdstuk 7** beschrijft de effecten van nonylfenol en oestrone op de embryonale ontwikkeling van *N. integer*. Embryo's uit stadium I worden blootgesteld aan nonylfenol (0,01; 1 en 100 µg/l), oestrone (10, 100 en 1000 ng/l) en een controle tot het ontluiken tot een vrij-levende juveniel. Duur van de verschillende ontwikkelingsstadia, overleving en ontluiking worden onderzocht. Nonylfenol heeft geen effect op de duur van de drie verschillende ontwikkelingsstadia, maar veroorzaakt een significant lagere overleving en ontluiking bij de hoogste test concentratie (100 µg/l) ten opzichte van de controle. Oestrone heeft enkel een effect op de ontluiking bij de hoogste testconcentratie. Deze studie versterkt de stelling dat oestrogenen minder effectief zijn in crustaceeën dan dat ze zijn in vertebraten in het veroorzaken van endocriene verstoring. Dit is waarschijnlijk het resultaat van verschillende hormonale controle strategiën in crustaceeën vergeleken met vertebraten.

**Hoofdstuk 8** rapporteert vitellinegehaltes van veld-gecollecteerde *N. integer* uit het Schelde estuarium (België/Nederland). Vitellinegehaltes worden bepaald van vrouwtjes met stadium I embryos gecollecteerd in april en juli 2005 op verschillende plaatsen in het estuarium. Significant lagere vitellinegehaltes worden waargenomen in de meest stroomopwaartse staalnameplaatsen van de campagne van april 2005. Deze plaatsen zijn de meer vervuilde gebieden van het estuarium waar ook reeds vroeger effecten op het energiemetabolisme van de aasgarnalen werden waargenomen. Deze plaatsgebonden verschillen worden nochtans niet bevestigd in juli 2005. Naast het kwantificeren van de vitellinegehaltes op verschillende plaatsen, worden ook een aantal abiotische (temperatuur, saliniteit en opgelost zuurstof) an biotisch parameters (broedsel grootte en standaard lengte) gemeten. Er worden geen duidelijke correlaties gevonden tussen vitellinegehaltes en de abiotische en biotische parameters. Tenslotte worden de vitellinegehaltes gekwantificeerd in vrouwtjes met eitjes van de drie verschillende onwikkelingsstadia. In tegenstelling met significante verschillen in vitellinegehaltes in eitjes van verschillende ontwikkelingsstadia worden geen dergelijke verschillen geobserveerd in het veld in volledige organisme/embryo homogenaten. Terwijl de huidige studie de bruikbaarheid van de ontwikkelde ELISA om vitelline te kwantificeren in aasgarnaal veldpopulaties aantoont, zijn meer uitgebreide veldbemonsteringen nodig om plaatsgebonden en tijdsgebonden verschillen in vitellinegehaltes te bestuderen in het veld.

In **Hoofdstuk 9**, werden de algemene conclusies en toekomstperspectieven van dit doctoraatsonderzoek geformuleerd.

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# CURRICULUM VITAE

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## Personalia

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|---|---|
| Name: | An Ghekiere |
| Date of Birth: | March 19th, 1979 |
| Place of Birth: | Roeselare, Belgium |

## Education

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|---|---|
| 1997-1999: | Sports Sciences (Bachelor)<br>Ghent University |
| 1999-2001: | Biochemistry (Master)<br>Ghent University<br>Thesis: Mutagenesestudie van de aminozuren betrokken in de redoxfunctie van cytochroom *b-558* uit de fototrofe bacterie *Ectothiorhodospira vacuolata*<br>Promotor: Prof. Dr. J. Van Beeumen |
| 2002-2006: | PhD student. Laboratory of Environmental Toxicology and Aquatic Ecology.<br>Ghent University, Faculty of Bioscience Engineering<br>Thesis: Study of invertebrate-specific effects of endocrine disrupting chemicals in the estuarine mysid *Neomysis integer* (Leach, 1814)<br>Promotor: Prof. Dr. Colin Janssen; Co-promotor: Dr. Tim Verslycke |

## Publications

Verslycke, T., Ghekiere, A., Janssen, C.R., 2004. Seasonal and spatial pattern in energy allocation in the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) of the Scheldt estuary. *Journal of Experimental Marine Biology and Ecology* 306(2), 245-267.

Ghekiere, A., Verslycke, T., De Smet, L., Van Beeumen, J., Janssen, C.R., 2004. Purification and characterization of vitellin from the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea). *Comparative Biochemistry and Physiology, Part A 138, 427-433*.

Ghekiere, A., Fenske, M., Verslycke, T., Tyler, C., Janssen, C.R., 2005. Development of an enzyme-linked immunosorbent assay for vitellin of the mysid *Neomysis integer*. *Comparative Biochemistry and Physiology, Part A* 142(1), 43-49.

Fockedey, N., Ghekiere, A., Bruwiere, S., Janssen, C.R., Vincx, M., 2005. Effect of salinity and temperature on the *in vitro* embryology of the brackish water mysid *Neomysis integer* (Crustacea: Mysidacea). *Marine Biology* (in press).

Ghekiere, A., Verslycke, T., Fockedey, N., Janssen, C.R., 2005. Non-target effects of the insecticide methoprene on molting in the estuarine crustacean *Neomysis integer* (Crustacea: Mysidacea). *Journal of Experimental Marine Biology and Ecology* (in press).

Ghekiere, A., Fockedey, N., Verslycke, T., Vincx, M., Janssen, C.R., 2006. Marsupial development in the mysid *Neomysis integer* (Crustacea: Mysidacea) to evaluate the effects of environmental chemicals. *Ecotoxicology and Environmental Safety* (accepted).

Ghekiere, A., Verslycke, T., Janssen, C.R., 2006. Effect of methoprene, nonylphenol and estrone on the vitellogenesis of the mysid *Neomysis integer*. *General and Comparative Endocrinology* (accepted).

Verslycke, T., Ghekiere, A., Raimondo, S., 2006. Mysid crustaceans as standard models for the screening and testing of endocrine-disrupting chemicals. Special Issue of Ecotoxicology on 'Endocrine Disruption in Aquatic Invertebrates' (submitted-invited paper).

Noppe, H., Ghekiere, A., Polfliet, K., Verslycke, T., De Wulf, E., Poelmans, S., Verheyden, K., Monteyne, E., Van Caeter, P., Janssen, C.R., De Brabander, H. The occurrence of organonitrogen herbicides in the Scheldt estuary (B-Nl): Ecotoxicological evaluation using *Neomysis integer* (Crustacea: Mysidacea) (in preparation).

## Oral presentations

Poelmans, S., De Wasch, K., Noppe, H., Verslycke, T., Ghekiere, A., Van Hoof, N., De Brabander, H.F., Janssen, C.R. (2002). Derivatisation in LC-$MS^n$ and GC-$MS^n$: a necessary approach to meet the low detection limits in environmental analyses. SETAC Europe 13th Annual Meeting, April 27th - May 1st 2003, Hamburg, Germany.

Verslycke, T., Ghekiere, A., Fockedey, N., Roose, P., De Wasch, K., Vethaak, D., Mees, J., Monteyne, E., Noppe, H., Deneudt, K., Vanden Berghe, W., Vincx, M., De Brabander, H., Janssen, C.R. (2003). The ENDIS-RISKS project: Endocrine disruption in the Scheldt estuary; distribution, exposure and effects. March 31st-April 1st, 2003, Conference: SETAC U.K./SETAC Europe meeting on endocrine disruptors in the environment - Linking research and policy, York, U.K.

Verslycke, T., Ghekiere, A., Fockedey, N., Roose, P., De Wasch, K., Vethaak, D., Mees, J., Monteyne, E., Noppe, H., Deneudt, K., Vanden Berghe, W., Vincx, M., De Brabander, H., Janssen, C.R. ( (2003). Endocrine disruption in the Scheldt estuary; distribution, exposure and effects (ENDIS-RISKS). Kick-off meeting PODO II: Global change, ecosystems and biodiversity, April 8th 2003, Brussels, Belgium.

Ghekiere, A., Fockedey, N., Verslycke, T., Fenske, M., Soin, T., Smagghe, G., Janssen, C.R. (2005). Invertebrate-Specific Assessment of the Effect of Endocrine Disrupting Chemicals Using the Estuarine Mysid *Neomysis integer*. In Vitro Biology Meeting, June 4th-7th 2005, Baltimore, USA.

Ghekiere, A., Verslycke, T., Fockedey, N., Monteyne, E., Noppe, H., Roose, P., Vethaak, D., Deneudt, K., Janssen, C.R. (2005). Endocrine disruption in the Scheldt estuary (Belgium/The Netherlands): distribution, exposure and effects on the mysid *Neomysis integer* (ENDIS-RISKS). SETAC North America 26th Annual Meeting, November 13th-17th, Baltimore, USA.

Ghekiere, A., Janssen, C.R. (2005). Hormoonverstoring. Aware: Eco-Health. November 28th, Roeselare, Belgium.

## Poster presentations

Verslycke, T., Ghekiere, A., Fockedey, N., Roose, P., De Wasch, K., Vethaak, D., Mees, J., Monteyne, E., Noppe, H., Deneudt, K., Vanden Berghe, W., Vincx, M., De Brabander, H., Janssen, C.R. (2003). The ENDIS-RISKS project: Endocrine disruption in the Scheldt estuary; distribution, exposure and effects. VLIZ Young Scientists' Day, February 28th 2003, Sint-Andries (Brugge), Belgium.

Ghekiere, A., Verslycke, T., Janssen, C.R. (2003). Ecdysteroid metabolism in *Neomysis integer* and its future perspectives in invertebrate endocrine disruption research. VLIZ Young Scientists' Day, February 28th 2003, Sint-Andries (Brugge), Belgium.

Ghekiere, A., Verslycke, T., Janssen, C.R. (2003). Ecdysteroid metabolism in Neomysis integer and its future perspectives in invertebrate endocrine disruption research. SETAC Europe 13th Annual Meeting, April 27th - May 1st 2003, Hamburg, Germany.

Ghekiere, A., Verslycke, T., Janssen, C.R. (2004). Non-target effects of the insecticide methoprene on molting of mysid shrimp (Crustacea, Mysidacea). VLIZ 4th Annual Young Scientists' Day, March 5th 2004, Sint-Andries (Brugge), Belgium.

Ghekiere, A., Verslycke, T., Janssen, C.R. (2004). Non-target effects of the insecticide methoprene on molting of mysid shrimp (Crustacea, Mysidacea). SETAC Europe 14th Annual Meeting, April 18th- 22nd 2004, Prague, Czech Republic.

Ghekiere, A., Fenske, M., Verslycke, T., Tyler, C.R., Janssen, C.R. (2004). A competitive ELISA for vitellin in *Neomysis integer* as a tool to assess endocrine disruption in crustaceans. CREDO Cluster Workshop, July 5th- 7th 2004, University of Exeter, Exeter, U.K.

Verslycke, T., Ghekiere, A., Fockedy, N., McKenney, C., Janssen, C.R. (2004). Mysid crustaceans as potential test organisms for the evaluation of environmental endocrine disruption. e.hormone October 27th- 30th , New Orleans, USA.

Ghekiere, A., Fockedey, N., Verslycke, T., Polfliet, K., Bruwiere, S., Janssen, C.R. (2005). Impact of endocrine disruptors on the molting and embryogenesis in mysid shrimp (Crustacea; Mysidacea). February 25th 2005, Sint-Andries (Brugge), Belgium.

Fockedey, N., Bruwiere, S., Ghekiere, A., Janssen, C.R., Vincx, M. (2005) *In vitro* embryogenesis of *Neomysis integer* (Crustacea; Mysidacea) as a potential indicator of endocrine disruption. February 25th 2005, Sint-Andries (Brugge), Belgium.

Ghekiere, A., Fockedey, N., Verslycke, T., Polfliet, K., Bruwiere, S., Janssen, C.R. (2005). Impact of endocrine disruptors on the molting and embryogenesis in mysid shrimp (Crustacea; Mysidacea). SETAC Europe 15th Annual Meeting, May 23th- 26th 2005, Lille, France.

Doggett, M., McCarthy, I., Kaiser, M., Mudge, S., Janssen, C.R., Ghekiere, A. (2005). Cellular energy allocation in the early life stages of the European plaice, *Pleuronectes platessa*, following exposure to Aroclor 1254. 29th Annual Larval Fish Conference of the American Fisheries Society, July 11th –14th , Barcelona, Spain.

Soin, T., Ghekiere, A., Janssen, C.R., Smagghe, G. (2005). Comparative toxicity and disturbace of molting by non-steroidal ecdysone agonists in the mysid shrimp, *Neomysis integer*. 4th International conference on arthropods: chemical, physiological and environmental aspects, September 18th-23th, Zakopane, Poland.

Ghekiere, A., Fockedey, N., Verslycke, T., Janssen, C.R. (2005) Invertebrate-specific effects of endocrine disruptors on molting, embryogenesis, and vitellogenesis in mysids. SETAC North America 26th Annual Meeting, November 13th- 17th 2005, Baltimore, USA.

## Foreign research visits

United Kingdom, Exeter University, Environmental and Molecular Fish Biology group, Prof. Dr. Charles Tyler: October 3th-12th, 2003. Developing ELISA with Dr. Martina Fenske.

USA, MA, Woodshole Oceanographic institution: June 2nd-5th, 2005.