

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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CHAPTER 1

GENERAL INTRODUCTION

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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 general 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).

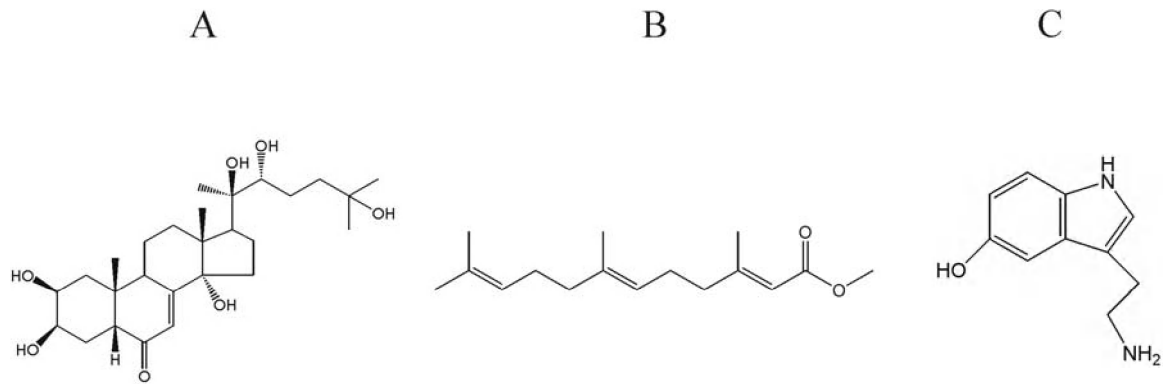


Figure 1.1: Four groups of compounds play an important role in regulating crustacean physiology: steroids (A; the ecdysteroid 20-hydroxyecdysone), terpenoids (B; methyl farnesoate), biogenic amines (C; serotonin), and peptides.

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).

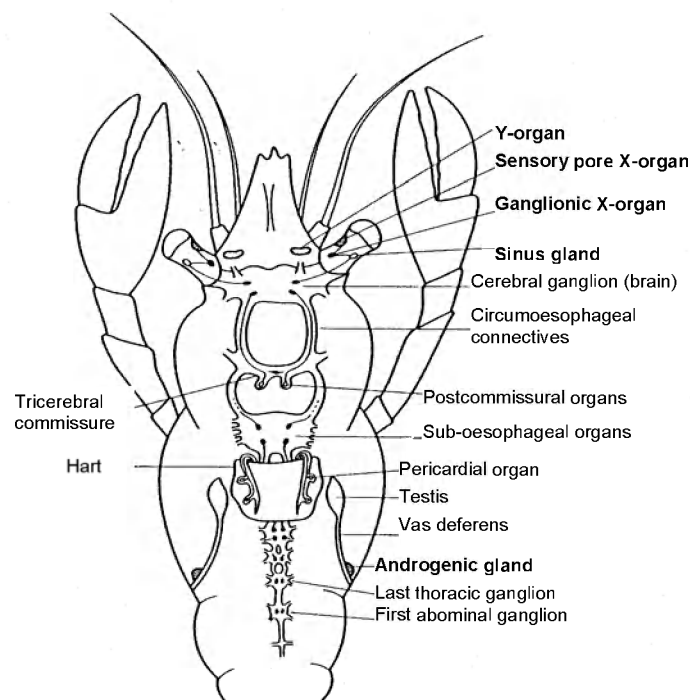


Figure 1.2: General overview of the endocrine system of a male crustacean (from Highnam and Hill, 1969).

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).

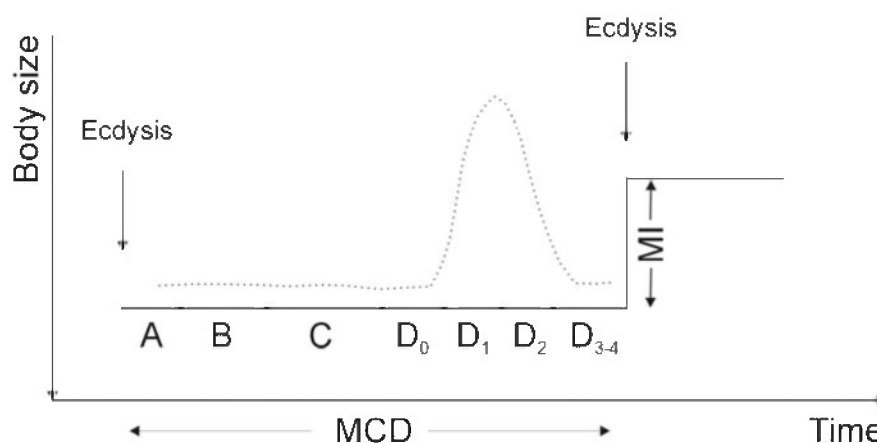


Figure 1.3: The crustacean molt cycle. Adapted from Gorokhova (1999) and Subramoniam (2000). MCD: molting cycle duration; MI: molt increment or stepwise increase in size at ecdysis; A-B: postmolt; C: intermolt; D₀, D₁, D₂, D₃₋₄: premolt;: ecdysteroid concentration.

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 hepatopaneas. 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 hepatopaneas. 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 hepatopaneas. 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 hepatopaneas 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 secrete 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 (Delbecq et al., 1990).

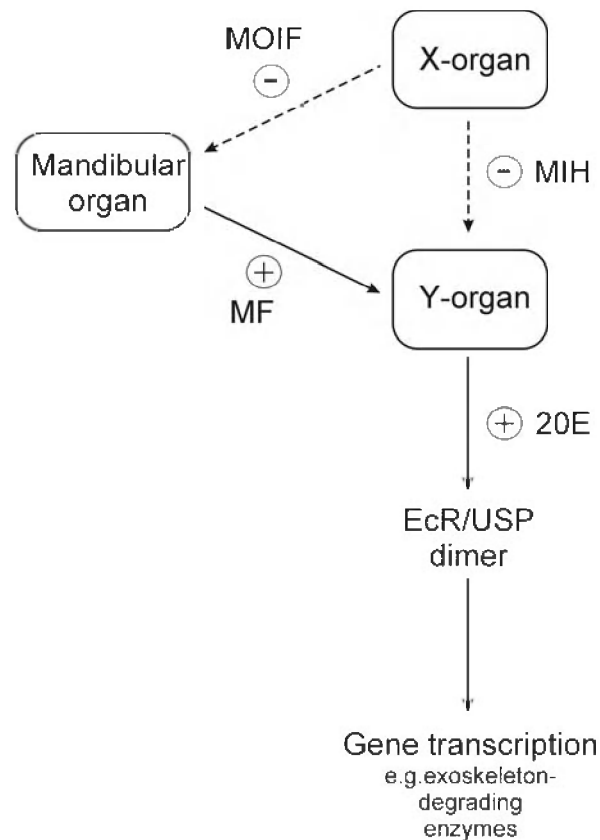


Figure 1.4: Hormonal control of molting in crustaceans. Adapted from DeFur et al. (1999) and Zou (2005). Interrupted arrows (-) represent inhibition and full arrows (+) stimulation. The following hormones play an important role in regulating crustacean molting: 20E, 20-hydroxyecdysone, the active molting 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 D₁-D₂ 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, chitinase (N-acetyl- β -glucosaminidase) is required for complete degradation of exoskeletal 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; Soye 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).

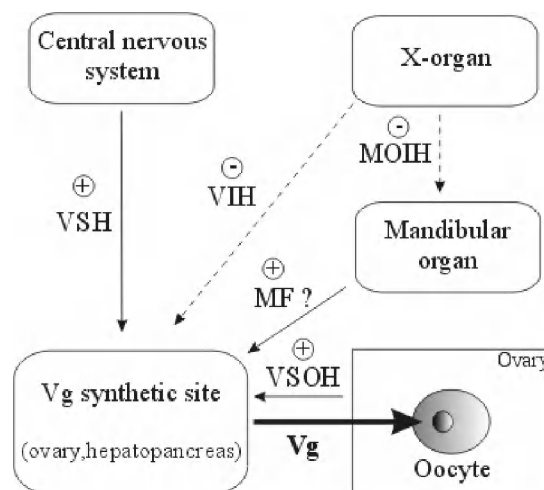


Figure 1.5: Hormonal control of vitellogenesis in crustaceans. Adapted from Okumura (2004). Interrupted arrows (-) represent inhibition and full arrows (+) stimulation. The following hormones are believed to play an important role in regulating crustacean vitellogenesis: MF, methyl farnesoate; MOIF, 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 significantly 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 *Armadillidium 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 its 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 autotomy 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 examining 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 4th 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*.

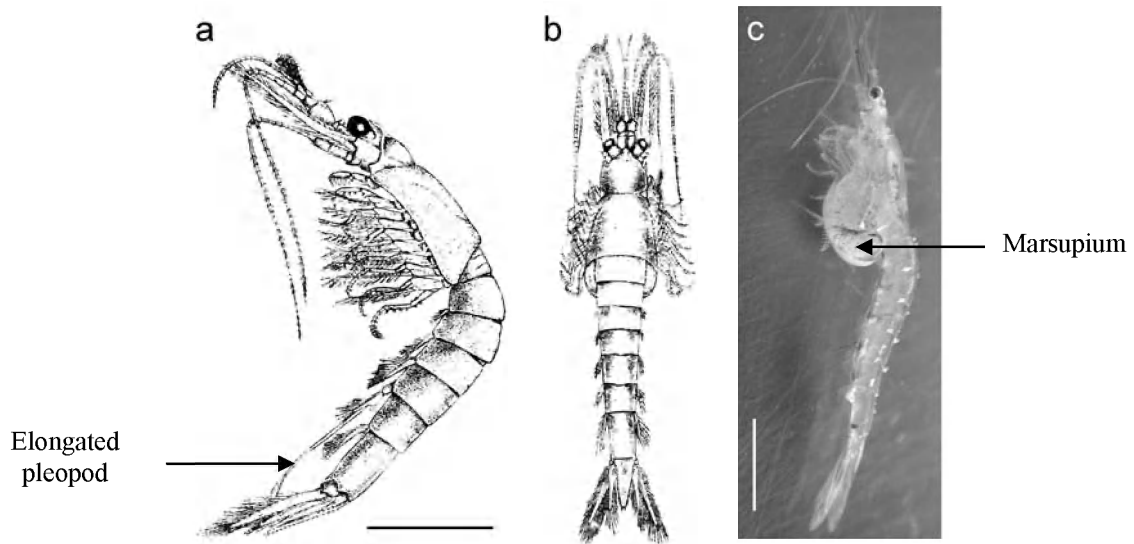


Figure 1.6: *Neomysis integer* (Crustacea: Mysidacea). a, adult male; b, adult female and c, ovigerous female (scale bar 5 mm). Drawings (a & b) are from Tattersall and Tattersall (1951) and photo (c) from Fockedey (2005).

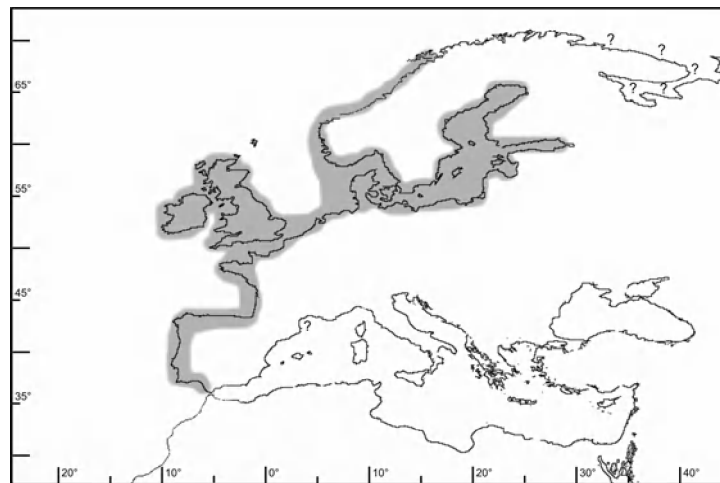


Figure 1.7: Distribution of *Neomysis integer* (gray areas) based on records in literature (Remerie, 2005).

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 detritivore 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 its 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 Fockedeey (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.

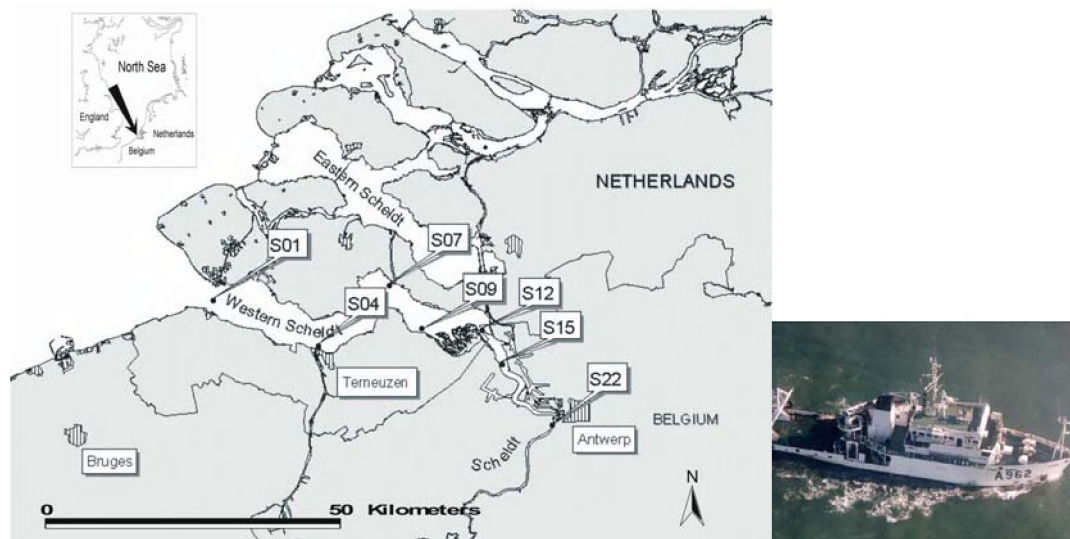


Figure 1.8: Left: map of the Scheldt estuary with location of the different sampling sites (S01, Vlissingen; S04, Terneuzen; S07, Hansweert; S09, Saeftinghe; S12, Bath; S15, Doel and S22, Antwerp). Right: research vessel, RV Belgica.

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 controlling 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 beneficial 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. Fockede 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 17 β -estradiol, progesterone, and 17 α -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 17 β -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 characterization 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 describes 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.