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Toegepaste Biologische Wetenschappen

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# ENDOCRINE DISRUPTION IN THE ESTUARINE INVERTEBRATE *NEOMYSIS INTEGER* (CRUSTACEA: MYSIDACEA)

# ENDOCRIENE VERSTORING BIJ DE ESTUARIENE INVERTEBRAAT *NEOMYSIS INTEGER* (CRUSTACEA: MYSIDACEA)

door

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Thesis submitted in fulfillment of the requirements for the degree of Doctor (Ph.D.) in Applied Biological Sciences

Proefschrift voorgedragen tot het bekomen van de graad van Doctor in de Toegepaste Biologische Wetenschappen

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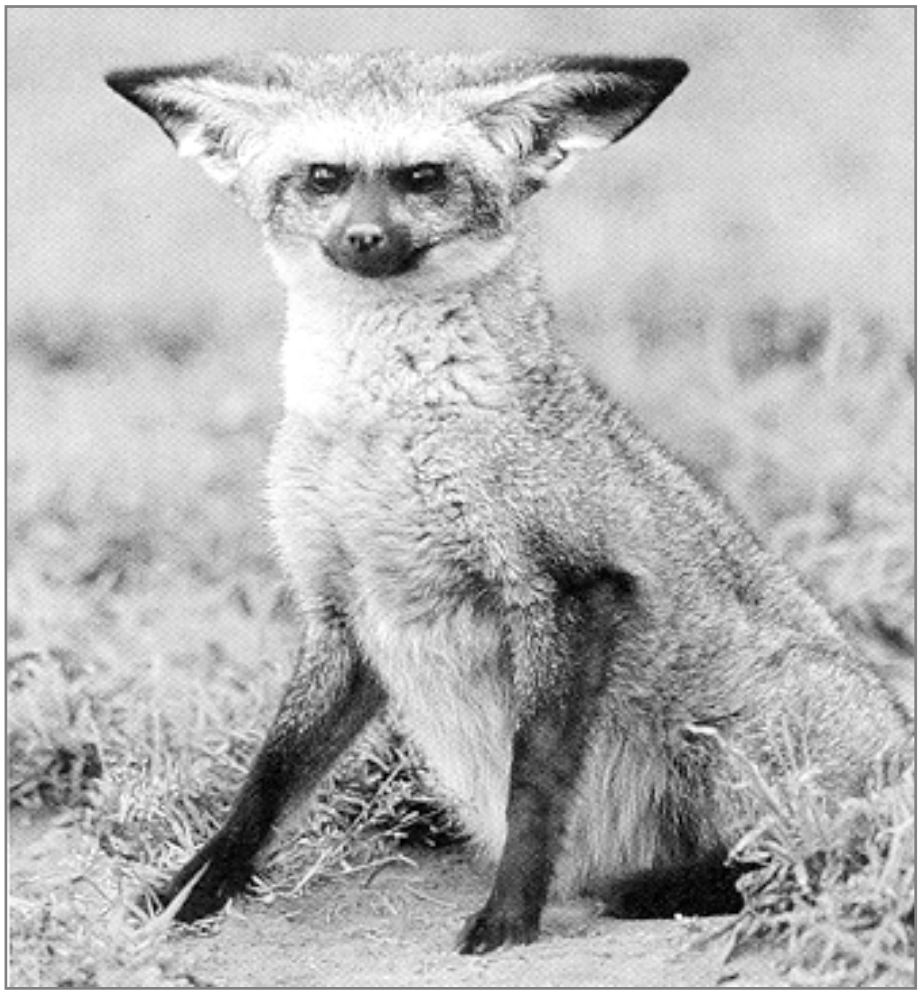
Halt! Whoa! Stop it right there!
Before you read any further there's
something you really should know.

You may have opened this little book expecting it
to be filled with answers, but (surprise, surprise!)
it’s actually a book about questions.



This may not be what you wanted to hear,
but, hey, that’s science for you!

# *Acknowledgements - Dankwoord*

"Some people believe in fate, others don't. I do, and I don't. It may seem at times as if invisible fingers move us like puppets on strings. But, for sure we are not born to be dragged along. We can grab the strings ourselves and adjust our course at every crossroad, or take off at any little trail into the unknown." (T. Heyerdahl)

Er zijn weinig momenten in het leven waar men de tijd neemt om terug te kijken en eventjes te overlopen wat men zoal heeft uitgespookt. Op het einde van deze vier jaar blijf ik met de vraag zitten hoe ik er uiteindelijk ben in geslaagd om dit werkje neer te leggen. Een mens maakt vele keuzes in het leven; dactylo-lessen in de lagere school, een wiskundige richting in de humaniora, één jaartje USA op mijn 17de, bio-ingenieur aan den unief, een passie voor alles wat met water te maken heeft …allemaal zaken die in de praktische zin uiteindelijk hebben bijgedragen tot dit werk. Maar zoals een groot meneer ooit zei, een doctoraat is 25% inspiratie en 75% transpiratie. Hij vergat hierbij echter te vertellen dat een extra 100% stimulatie noodzakelijk zijn! Vandaar dus onderstaande woorden van eeuwige dank aan iedereen die van ver of van dichtbij tot dit doctoraat heeft bijgedragen.

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## *Table of contents*

Acknowledgements/Dankwoord ..... I
Table of contents ..... III
List of abbreviations ..... V

**Chapter 1**

General introduction and conceptual framework ..... 1

**Chapter 2**

Mysid crustaceans as potential test organisms for the evaluation of environmental endocrine disruption ..... 13

**Chapter 3**

Energy metabolism of *Neomysis integer* in relation to a variable environment ..... 35

**Chapter 4**

Energy metabolism of *Neomysis integer* following exposure to tributyltin ..... 49

**Chapter 5**

Validation of the cellular energy allocation assay with the scope for growth assay in *Neomysis integer* ..... 63

**Chapter 6**

Testosterone metabolism of *Neomysis integer* as a biomarker for endocrine disruption: identification and quantification of *in vivo* produced testosterone metabolites and endogenous vertebrate-type steroids ..... 79

**Chapter 7**

Testosterone metabolism of *Neomysis integer* following exposure to tributyltin..... 91

**Chapter 8**

Testosterone and energy metabolism of *Neomysis integer* following exposure to endocrine disruptors ..... 105

**Chapter 9**

Exposure study in the Scheldt estuary: flame retardants, surfactants and organotins in sediment and mysid shrimp ..... 123

**Chapter 10**

Energy metabolism in field populations of *Neomysis integer* of the Scheldt estuary 139

**Chapter 11**

Testosterone metabolism in field populations of *Neomysis integer* of the Scheldt estuary ..... 159

**Chapter 12**

General conclusions and research perspectives ..... 173

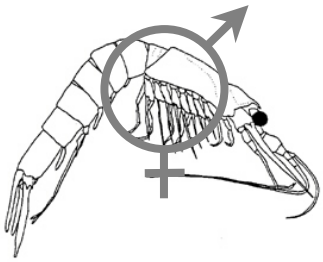
Summary ..... 179
Samenvatting ..... 183
References ..... 189
Curriculum vitae ..... 217

## *List of abbreviations*

| | |
|---|---|
| A | energy absorbed |
| AED | androstenedione |
| AED | atomic emission detection |
| ANOVA | analysis of variance |
| APCI | atmospheric pressure chemical ionization |
| APE | alkylphenol ethoxylates |
| ASTM | American Society for Testing of Materials |
| ATP | adenosine triphosphate |
| BCF | bioconcentration factor |
| BDE | brominated diphenyl ether |
| C:N | carbon:nitrogen |
| CAS | Chemical Abstract Service Registry |
| CEA | cellular energy allocation |
| CHH | crustacean hyperglycemic hormone |
| CSTEE | Scientific Committee on Toxicity, Ecotoxicity and the Environment |
| CYP | cytochrome P450 |
| DBT | dibutyltin |
| DDE | dichlorodiphenyldichloroethylene |
| DDT | dichlorodiphenyltrichloroethane |
| DEF | dihydrotestosterone equivalency factor |
| DHT | dihydrotestosterone |
| DO | dissolved oxygen |
| DPT | diphenyltin |
| dw | dry weight |
| E2 | 17β-estradiol |
| $E_a$ | energy available |
| $E_c$ | energy consumed |
| EC50 | median effect concentration (with denotation of exposure concentration) |
| EcR | ecdysteroid receptor |
| EDSTAC | Endocrine Disruptor Screening and Testing Committee |
| EEF | estradiol equivalency factor |
| EEQ | estradiol equivalents |
| ELISA | enzyme-linked immunosorbent assay |
| ER-CALUX | estrogen receptor-mediated luciferase reporter gene assay |

| | |
|---|---|
| EtOH | ethanol |
| ETS | electron-transport system |
| GC | gas chromatography |
| ICP | inductively coupled plasma mass spectrometry |
| ISO | International Organization for Standardization |
| HBCD | hexabromocyclododecane |
| HPLC | high-performance liquid chromatograpy |
| HSD | Tukey's Honestly Significant Difference test |
| IGR | Insect Growth Regulator |
| Kow | octanol-water partitioning coefficient |
| Kp | distribution coefficient |
| LC50 | median lethal concentration (with denotation of exposure concentration) |
| LC | liquid chromatography |
| LOEC | lowest observed effect concentration |
| LOI | loss on ignition |
| MAR | metabolic androgenization ratio |
| MBT | monobutyltin |
| MFO | mixed function oxygenase system |
| MIH | molt-inhibiting hormone |
| MPT | monophenyltin |
| MSPD | Matrix Solid Phase Dispersion |
| NA | not available |
| NaBEt4 | sodiumtetraethylborate |
| NADH | nicotinamide-adenine dinucleotide (reduced) |
| NADPH | nicotinamide-adenine dinucleotide phosphate (reduced) |
| NCI-MS | negative chemical ionization mass spectrometry |
| ND | not detected/not determined |
| NOEC | no observed effect concentration |
| NP | nonylphenol |
| NPE | nonylphenol ethoxylates |
| NPEC | nonylphenol ether carboxylates |
| OC | organic carbon |
| OECD | Organization for Economic Cooperation and Development |
| OP | octylphenol |
| OSPAR | Oslo and Paris Commission |
| PBB | polybrominated biphenyls |
| PBDE | polybrominated diphenyl ethers |

| | |
|---|---|
| PCB | polychlorinated biphenyl |
| PDMS | polydimethylsiloxane |
| R | energy respired |
| RT-PCR | reverse transcription polymerase chain reaction |
| S | salinity |
| SETAC | Society of Environmental Toxicology and Chemistry |
| SFG | scope for growth |
| SPM | suspended particulate matter |
| SPME | solid phase microextraction |
| SVTF | Endocrine Disruptor Screening and Testing Standardization and Validation Task Force (USEPA) |
| T | temperature |
| TBBPA | tetrabromobisphenol A |
| TBT(Cl) | tributyltin(chloride) |
| TLC | thin layer chromatography |
| TMAH | tetramethylammoniumhydroxide |
| TPT | triphenyltin |
| TPrT(Cl) | tripropyltin(chloride) |
| TST | testosterone |
| U | energy excreted |
| USEPA | United States Environmental Potection Agency |
| ww | wet weight |
| YAS | yeast androgen screen |
| YES | yeast estrogen screen |



# CHAPTER 1

# -

# General introduction and conceptual framework

# Chapter 1

## *General introduction and conceptual framework of the study*

### 1.1. Evolution and the environmental endocrine hypothesis

Before Darwin, how species originated remained the 'mystery of mysteries' for a very simple reason. Over the time span of recorded human history, let alone the life of a single human being, no new species had been recognized (although undoubtedly many new species had appeared). Indeed, it is quite difficult to determine how something happens if no one has ever observed it happening. One problem that many people have with Darwin's theory of evolution is that they think it all happened in the distant past.

In a great burst of evolution, a wide variety of invertebrate animals appeared in the sea near the beginning of the Cambrian period, about 600 million years ago. About 200 thousand years ago, our own species, *Homo sapiens*, first appeared on this planet. It was, however, only 200 years ago that increasing industrial pollution began with burning of huge quantities of coal for fuel and that man was first exposed to anthropogenic chemicals (Audesirk and Audesirk, 1997). Finally, only 60 years ago, the first organic pesticides were produced. Today, we are potentially exposed to more than 22,000,000 chemicals and about 4,000 new substances are registered every day (CAS, 2003). As such, one good example of present-day evolution has been caused by the recent outburst in environmental pollution. Contrary to many lower species, man has only been exposed to these new organic chemicals for one generation, therefore, the potential transgenerational effects of these compounds will only become apparent in the following generations.

For nearly half a century, the fear of toxic chemicals has been largely synonymous with the fear of cancer. The world's obsession with cancer - the dread disease - has been the dominant lens through which the study of toxic chemicals has been viewed. Cancer is certainly not the only adverse health effect of industrial chemicals, but it has largely eclipsed other diseases and reproductive effects as an object of public concern and scientific research. However, in the last few years a new theory of environmental disease has emerged that explores a variety of human and animal abnormalities that are not explained by or investigated with this dominant cancer paradigm. The guiding concept of this new

paradigm is that some chemicals can interfere with the body's natural hormones. Mostly synthetic organic chemicals, these compounds have been implicated in an extensive number of human and animal disorders, including reproductive and developmental abnormalities, immune dysfunction, cognitive and behavioral pathologies, and cancer. Some of the postulated effects of endocrine-disrupting chemicals (or endocrine disruptors) have been correlated in wildlife and laboratory studies. If this hypothesis is confirmed for human effects, science will have discovered an important new etiology of environmental disease. From the point of view of human pathology, the environmental endocrine hypothesis could turn out to be the most significant environmental health hypothesis since the discovery of chemical mutagenesis leading to cancer. It has the potential to change radically the way we think about the contribution of environmental factors to disease by shifting the focus from cancer and acute toxicity to the reproductive, neurophysiological, and developmental effects of chemicals. Although certain components of the environmental endocrine hypothesis are still debated among scientists, industrial nations have already begun considering how to regulate a class of chemicals that is currently ill-defined yet ubiquitous. Governments will be faced with the challenge of assessing the health and environmental effects of numerous individual chemicals as well as the cumulative effects of multiple exposures to many kinds of chemicals (Colborn et al., 1996; Krimsky, 2000).

### 1.2. Endocrine-disrupting chemicals

In developing management strategies to address the potential problem of endocrine disruption, there has been a tendency for responsible bodies (e.g. environmental agencies, environmental legislators) to attempt to identify chemicals that might interact with hormone receptors, then measure the concentrations and distributions of each chemical in the environment. Such an approach seems doomed to failure because of the enormous number of chemicals that have endocrine-disrupting potential. Many have little in common structurally or in terms of their chemical properties. Furthermore, they exert their effects by different mechanisms. A general definition for an endocrine disruptor was given by Holmes et al. (1997) as "a substance which causes adverse effects in an intact organism, or its progeny, subsequent to changes in endocrine function". Consequently, an extensive list of chemicals thought to be capable of disrupting the reproductive endocrine system of animals has been assembled (Depledge and Billinghurst, 1999). They can be categorized as follows (modified from Gray et al., 1996):

- Environmental estrogens (estrogen receptor mediated): methoxychlor, bisphenolic compounds, ethinylestradiol

- Environmental anti-estrogens: dioxins, endosulphan
- Environmental anti-androgens: vinclozolin, DDE, kraft mill effluent
- Toxicants that reduce steroid hormone levels: fenarimol and other fungicides, endosulfan
- Toxicant that affect reproduction primarily through effects on the central nervous system: dithiocarbamate pesticides, methanol
- Other toxicants that affect hormonal status: cadmium, benzidine-based dyes

At present chemicals receiving the most attention are the environmental estrogens and anti-estrogens since they have been shown to produce effects in vertebrates. However, whether other categories are of greater significance to invertebrates remains to be determined (Depledge and Billinghurst, 1999). Specific chemicals which have been shown to disrupt endocrine function in invertebrates are listed in Table 1.1.

**Table 1.1.** Chemicals which have been implicated in endocrine disruption in invertebrates (Depledge and Billinghurst, 1999)

| Group | Examples |
|---|---|
| Herbicides | Diquat dibromide, Atrazine, Simazine, Diuoron |
| Metals | Cadmium, Selenium, Zinc, Mercury, Lead, Tributyltin |
| PCBs | Clophen A50, Aroclor 1242 |
| Alkylphenols | Nonylphenol, Pentylphenol |
| Natural and synthetic vertebrate steroids | Diethylstilbestrol, Testosterone |
| Insecticides | Pyriproxyfen, DDT, MCPA, Endrin, Toxaphene, Piperonyl butoxide, Methoprene, Endosulfan, Pentachlorophenol, Diflubenzuron, Kelthane |
| Mixture | Tannery effluent, Paper and pulp mill effluent, Crude oil derivatives, Sewage effluents |

### 1.3. Endocrine disruption in invertebrates

Today, invertebrates dominate the animal kingdom constituting over 95% of the known animal species (Barnes, 1980). Those groups of invertebrates that have received the greatest attention with respect to toxicology (i.e. Crustacea, Insecta, Annelida, Mollusca) are the product of an evolutionary lineage distinct from that of the vertebrates (LeBlanc, 1998). Accordingly, the endocrine systems of most vertebrate groups share little with invertebrates (IPCS, 2002). The potential for endocrine-disrupting toxicity of chemicals has been relatively well documented in vertebrates including mammals, fish, birds, and reptiles which largely share the same basic neuro-endocrine and developmental cascades (Ankley et al., 1998).

Less well understood, is whether invertebrates are similarly susceptible to endocrine-disrupting toxicity (LeBlanc, 1999).

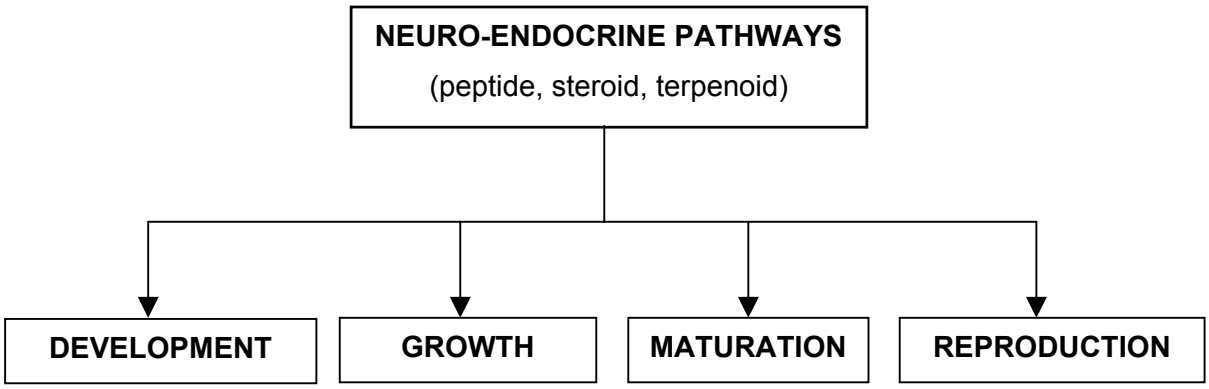
Among the invertebrates one well-documented example of environmental endocrine disruption exists. Reports of pseudohermaphroditic conditions in certain marine gastropod species surfaced in the scientific literature nearly three decades ago (Blaber, 1970). This condition, characterized by the imposition of male sex characteristics (i.e. penis, vas deferens) onto females, has become known as imposex (Smith, 1971). Imposex has been associated with skewed sex ratios, reduced fecundity, population declines, and local extinctions of affected gastropod populations (Gibbs and Bryan, 1986). There are remarkably few other case histories that document endocrine disruption in invertebrate field populations. Unfortunately, this reflects the state of knowledge at the present time (DeFur et al., 1999).

Although evidence of endocrine disruption in marine and estuarine invertebrates can be found in laboratory studies (e.g. Bodar et al., 1990; Fingerman et al., 1996; Schultz et al., 1980, Voogt et al., 1989), extremely limited field data are available. High prevalence of intersex in harpacticoid copepods has been reported in the vicinity of a sewage outfall in the Firth of Forth, Scotland (Moore and Stevenson, 1991; 1994). However, no conclusive causal relationship between the sewage outfall and the levels of intersex was established. Similarly, the lobster *Homarus americanus* has been observed with ovotestes in Nova Scotia, but it was not concluded whether this was a natural or site-related phenomenon (Sangalang and Jones, 1997). Minor effects have been reported on the microsomal cytochrome P450 monooxygenase system in sea stars collected from polluted sites in the influence rivers of the North Sea, like the Elbe (Germany), the Rhine (The Netherlands), the Scheldt estuary (The Netherlands), and the Humber (UK) (Den Besten et al., 1996; Postma and Valk, 1996; 1997). These and other observations indicate that endocrine disruption may be occurring in invertebrate populations, but as yet, with the exception of organotin effects in gastropods, data from systematic field investigations are unavailable (Depledge and Billinghurst, 1999; Oberdörster and Cheek, 2000).

## 1.4. The endocrine system of invertebrates with special reference to crustacean endocrinology

Relatively little is known about the endocrine systems of invertebrates and their susceptibility to environmental endocrine disruption. Yet, utilization of hormones to control and coordinate biochemical, physiological and behavioral processes is common to all invertebrate taxa. The majority of invertebrate hormones identified to date are peptide hormones, but non-peptide endocrine messengers such as ecdysteroids and juvenile hormones (juvenoids) are important in many groups, especially insects and crustaceans

(Pinder and Pottinger, 1998). As in vertebrates, these nonpeptide hormones may be more susceptible to endocrine toxicants because exogenous chemicals are more likely to interact with the receptors of these hormones (IPCS, 2002). Ecdysteroids and juvenoids in arthropods (i.e. crustaceans, insects, and some minor phyla) share significant structural and functional homology with the sex steroids and retinoids of vertebrates. Ecdysteroids are primarily recognized for their regulation of molting, embryo development, diapause, cuticle formation, ovulation, and spermatogenesis (Hagedorn, 1985; Koolman, 1989). Terpenoids (specifically the juvenoids) have been recognized primarily in insects for their role in promoting juvenile to adult metamorphosis (LeBlanc et al., 1999). Terpenoids are now recognized to function in concert with, and perhaps independently of, ecdysteroids to regulate a variety of functions including reproduction, caste determination, behavior, diapause, and metabolism (Nijhout, 1998). Methyl farnesoate, the unepoxidated form of Juvenile Hormone III, functions as a gonadotropin in crustaceans and may be functionally homologous to the juvenile hormone in insects (Homola and Chang, 1997; Laufer et al., 1993). Figure 1.1 gives an overview of endocrine-regulated processes in crustaceans that can be used as indicators of endocrine disruption in toxicity testing.




**ENDPOINTS (BIOMARKERS)**

| | | | |
|---|---|---|---|
| Developmental retardation | Total length | Sex characteristics | Offspring production |
| Vitellogenin production | Molt frequency | Time to first brood | Brood production |
| Lipid profiles | Energy metabolism | Energy metabolism | Offspring viability |
| Hormone metabolism | Hormone metabolism | Hormone metabolism | Reproductive behavior |
| | | | Fertilized egg production |
| | | | Fertilized hatch hatchibility |
| | | | Hormone metabolism |

**Figure 1.1.** Endocrine-regulated parameters in crustaceans that can be used as indicators of endocrine disruption in toxicity testing (redrafted after LeBlanc, 1999).

Most of what is known about crustacean endocrinology has been obtained through studies with larger decapods (crabs, lobsters, crayfish and shrimp). Some other groups of crustaceans, however, have proven useful as models for the elucidation of some hormonal processes (most notable, the androgenic hormone of amphipods). The effects of organic and inorganic contaminants on functions regulated by hormones in crustaceans are being investigated with increasing frequency because several of these phenomena show promise of being useful biomarkers of environmental contamination and endocrine disruption (DeFur et al., 1999; Fingerman et al., 1996, 1998; Medesani et al., 2001).

Unfortunately, relatively little data is available on the hormonal control of biological processes in mysid shrimp, the organisms used in this doctoral study. As such, the current knowledge in mysids is mainly derived from studies with other crustaceans. While the present doctoral research primarily focuses on the use of energy and steroid metabolism of *Neomysis integer*, other hormone-regulated processes such as vitellogenesis and ecdysteroid metabolism in mysids might prove to be valuable endpoints for studying chemical-induced endocrine disruption. In this perspective, ecdysteroid metabolism and vitellogenesis in *N. integer*, is the subject of present and future research activities in our laboratory. For a comprehensive overview on the endocrinology and promising hormone-regulated endpoints in mysids, we refer to chapter 2 of this doctoral thesis.

### 1.5. Selection of suitable test species for evaluating endocrine disruption

Selection criteria for test species for evaluating endocrine disruption are given in DeFur et al. (1999) and Depledge and Billinghurst (1999):

- Species should be chosen as representative of several different invertebrate phyla
- Primary mode reproduction should be known
- Ease of culture (organisms readily available and adapted to laboratory conditions)
- Generation time should not be too long (i.e. transgenerational exposures)
- Size (i.e. measurable tissue concentrations of hormones)
- Knowledge of endocrinology
- Standard methods (i.e. ASTM, OECD, USEPA, ISO)
- Relative sensitivity
- Ecological importance
- Ability for *in situ* testing

Clearly, mysids fulfill almost all of the above criteria. Specimens are available in high densities, generally throughout the year, and are easily collected. Collection is unlikely to deplete field populations since mysids occur in such large numbers. Mysids are easily

maintained in the laboratory, and readily feed on brine shrimp (*Artemia*) nauplii. In addition, mysids have a relatively short life cycle, reproduce sexually, and ovigerous females carry their developing embryos in a ventral brood pouch (which is why they are also called opossum shrimp), allowing various aspects of their reproductive biology to be studied. Their size allows individual measurement of hormones and other biochemical fractions. The role of mysids in marine and estuarine food webs clearly demonstrates their ecological importance (Hostens, 2003; Roast et al., 1998a). Mysids are standard test organisms (ASTM, USEPA) which have been used in laboratory toxicity testing since the early 1970s. They have been demonstrated to be more sensitive than many other marine species. Furthermore, mysid shrimp were put forward as a representative of an important and diverse group within the crustacean class, which may serve as a viable surrogate for many crustaceans. In this perspective, they have been put forward as suitable test organisms for the evaluation of endocrine disruption by several regulatory bodies (CSTEE, 1999; DeFur et al., 1999; LeBlanc, 1999). Finally mysids have been used in cage-experiments in the field. Unfortunately, basic information on their endocrinology is limited but relatively high compared to many other invertebrates since they are crustaceans (refer to Chapter 2 for detailed info).

*Neomysis integer* (Figure 1.2), the mysid used in this doctoral study, has the additional advantages that it is a key species in the hyperbenthic community of many North-European estuaries. Furthermore, it has a strong tolerance for temperature and salinity changes, allowing its use in coldwater and estuarine testing, which is not possible with the standard American test species *Americamysis bahia*. The biology and ecology of this species in the Scheldt estuary, which was chosen as the site for the field-study in this doctoral thesis, have been extensively studied (Fockedey and Mees, 1999; Mees and Hamerlynck, 1992; Mees et al., 1993a,b; 1994;1995a,b). In addition, Roast and co-workers (1998a,b; 1999a,b,c; 2000a, b,c; 2001a,b; 2002) have demonstrated the successful use of this species in sub-lethal toxicity testing. Finally, this species has previously been used for toxicity testing and cultured with success in our laboratory (Heijerick, 1994).

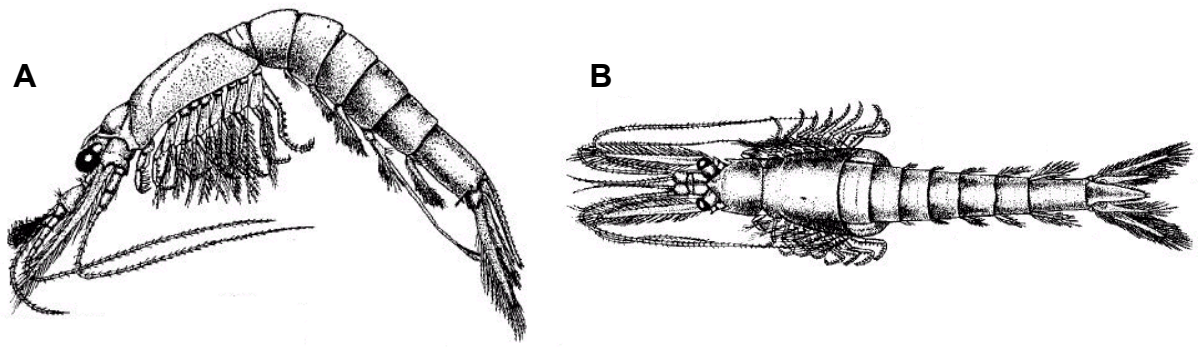



**Figure 1.2.** *Neomysis integer* (Crustacea: Mysidacea). A, adult male; B, adult female (Tatersall and Tatersall, 1951).

### 1.6. The biomarker approach

Detecting endocrine disruption *in situ*, and provision of early warning of potentially ecologically relevant effects might be achieved using biomarkers (Depledge and Billinghurst, 1999). Biomarkers are defined as biochemical, physiological and histological changes as well as aberrations in organisms to estimate either exposure to or adverse effects of anthropogenic chemicals (Huggett et al., 1992). In the context of ecotoxicology, it is widely accepted that the primary concern should be to ensure that environmental contamination does not significantly disturb population dynamics, community structure and ecosystem processes (Depledge, 1996). At present, biomarker applications in ecotoxicology suffer from the following disadvantages. As little information is available on the basic biochemistry and physiology of the commonly used test organisms, it is difficult to estimate the 'normal' ranges of effects which may be encountered (Mayer, 1983; Mehrle and Mayer, 1980). Additionally, biomarker endpoints suffer from various sources of variability (e.g. organismal, environmental and methodological). As most of the efforts of biomarker research are currently put into the development of new test systems rather than on the evaluation of existing methodologies under realistic field conditions, in general, little is known about their use in field situations (Huggett et al., 1992).

Biomarkers which signal endocrine disruption have already been developed and there appears to be considerable potential for the extension of this approach. A good example of this is vitellogenin induction in fish as a biomarker for exposure to environmental estrogens (Allen et al., 1999; Matthiessen, 1998; Purdom et al., 1994; Sumpter and Jobling, 1995). If potential effects of endocrine-disrupting chemicals are to be properly assessed, then it will be necessary to develop test systems which are ecologically relevant, provide some means of gaining early warning of ensuing adverse effects, and link cause and effect. No single test system is likely to be capable of fulfilling all of these requirements. It is much more likely that a multi-faceted approach will be most successful (Depledge and Billinghurst, 1999).

One of the most successful types of biomarkers are those linked with the energy metabolism. Physiological energetics offer the advantage that this type of research provides information on key processes in the organism's energy acquisition and expenditure, possibly also elucidating the mode of action of the toxicant (De Coen, 1999). Crustaceans have provided an important and valuable model for investigation of physiological regulation and integration and there is a large body of information available on the neuroendocrine pathways of physiological regulation in crustaceans. Since energetic processes are hormone-regulated they are, by definition, sensitive to hormone disruption. Several measurements of energy reserves and consumption may serve as useful biomarkers of endocrine-disrupting substances in crustaceans (McKenney, 1999). Furthermore, alterations

in the energy metabolism of mysids have been successfully used as indicators of stress to toxicant exposure in *Americamysis bahia* (McKenney, 1982, 1985, 1998; McKenney and Matthews, 1990; McKenney et al., 1991), *Praunus flexuosus* (Garnacho et al., 2001), *Neomysis awatschensis* (Chin et al., 1998a,b; Shin et al., 2000), and *Neomysis integer* (Roast et al., 1999c, 2000b). In light of the biomarker disadvantages mentioned earlier, we selected a validated biomarker of physiological effects, namely the cellular energy allocation (CEA) assay which was successfully used in daphnids by De Coen (1999) to detect toxicant-induced effects. These effects could be correlated with effects at higher levels of biological organization. Furthermore, it was chosen to investigate CEA variability in relation to a variable environment in the laboratory. Finally, we wanted to validate the CEA with other physiological biomarkers, such as the long-established scope for growth assay, and evaluate its use in the field (i.e. Scheldt estuary).

Pollutants may exert their adverse effects via interference with the hormonal reproduction by steroids. Baldwin and co-workers have published several papers proposing that changes in steroid metabolism in crustaceans could provide an early indication of potential reproductive toxicity after sublethal exposure to suspected endocrine disruptors (Baldwin et al., 1995, 1997, 1998; LeBlanc and McLachlan, 2000). However, these mechanisms were never investigated in mysid species. Consequently, steroid metabolism of *N. integer* was chosen as a second hormone-regulated process in this doctoral study.

### 1.7. Research needs in endocrine disruption testing

An international SETAC (Society of Environmental Toxicology and Chemistry) workshop on endocrine disruption in invertebrates was held in The Netherlands in 1998. The most important research needs within the field of endocrine disruption in invertebrates were summarized in the proceedings of this workshop (DeFur et al., 1999):

- Basic research on the endocrinology of invertebrates is urgently needed to remedy our ignorance of mechanisms of action, physiological control, and hormone structure and function.
- Research is needed to test known endocrine disruptors with a variety of invertebrate bioassays. More specific, testing is required with specified reference compounds to determine potential effects, especially of nonvertebrate-type endocrine disruptors in invertebrates.
- Field assessments and surveys need to be able to identify endocrine-disrupting effects. These efforts will have to be informed by validated biomarkers and other indicators of exposure and/or effects of endocrine disruptors.

- Spending is likely to be most cost-effective if it supports research on those groups of invertebrates that are well known, abundant, ecologically important, or economically significant.
- Ecotoxicological approaches in current use should be adapted so that they can detect endocrine disruptors.
- The invertebrates potentially offer a wealth of knowledge in understanding comparative and ecological aspects of endocrine disruption. For these reasons, invertebrate systems should be a high priority for research, screening and testing, and methods development.

### 1.8. Conceptual framework of the study

Addressing the research needs mentioned above, the overall aim of the present study is to investigate endocrine disruption in an ecologically relevant invertebrate test species through laboratory and field research. More specifically the research goals of this study are:

- A fundamental study of the hormone-regulated processes of energy and steroid metabolism in the mysid *Neomysis integer*
- Evaluation and validation of the use of these processes as endpoints to evaluate environmental endocrine toxicity in the laboratory
- Evaluation of the exposure and effects of endocrine disruptors in the Scheldt estuary on the energy and steroid metabolism in the resident *N. integer* population

This dissertation is made up of ten research chapters followed by a general conclusion. All ten chapters were published or submitted in one or more separate research papers (12 peer-reviewed papers in total). To avoid excessive repetition, the introduction and material and methods sections were adjusted in each chapter. An overview of the different chapters and their content is given below:

In **chapter 2**, a review on the use of mysid shrimp as potential test organisms for the evaluation of environmental endocrine disruption is given. This chapter should be considered as an extension to this introductory chapter as it describes the biology, ecology, endocrinology and relevance of mysid species in endocrine-disruption and general toxicity testing and, thus, describes in detail the rationale behind the use of mysids in endocrine-disrupting testing.

In **chapter 3**, the energy metabolism in *N. integer* is investigated in relation to a variable environment. The cellular energy allocation (CEA) assay is evaluated and adapted for use with *N. integer* and the response of this biomarker is investigated following exposure

to environmentally (Scheldt estuary) relevant combinations of temperature, salinity and dissolved oxygen.

In **chapter 4**, the CEA assay, a biomarker to evaluate effects on energy metabolism in *N. integer*, is used to determine the effects of acute exposure to the known endocrine disruptor tributyltin. In addition, the uptake of this compound is investigated.

In **chapter 5**, the physiological responses of *N. integer*, following exposure to the organophosphate pesticide chlorpyrifos, were compared using the CEA and scope for growth assay via an interlaboratory validation study.

In **chapter 6**, the steroid metabolism and metabolic capacity of *N. integer* are investigated. Protocols are developed to quantify the testosterone elimination profile of *N. integer* via thin-layer chromatography and liquid chromatography coupled with multiple mass spectrometry. In addition, the endogenous presence of a number of vertebrate-type steroids is described for the first time in mysids.

In **chapter 7**, the effects of a known disruptor of invertebrate steroid metabolism, tributyltin, on testosterone metabolism in *N. integer* are described.

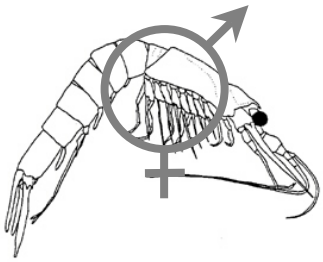
In **chapter 8**, the steroid and energy metabolism of *N. integer* are validated via laboratory exposures to a number of 'reference' endocrine disruptors. In addition, acute 96-h toxicity of these chemicals to *N. integer* is assessed.

In **chapter 9**, sediment and mysids (*N. integer*) are collected from the Scheldt estuary for an *in situ* exposure study. Concentrations of flame retardants, organotins and surfactants are reported in mysids for the first time and discussed in relation to sediment concentrations of these compounds.

In **chapter 10**, the use of the CEA assay in *N. integer* is evaluated in the field through several sampling campaigns in the Scheldt estuary. Seasonal and spatial patterns in cellular energy allocation of the resident *N. integer* are described.

In **chapter 11**, the use of the testosterone metabolism assay in *N. integer* is evaluated in the Scheldt estuary. Spatial patterns are investigated and discussed in the context of *in situ* exposure to endocrine disruptors.

In **chapter 12**, general conclusions are drawn and future research needs are formulated.



# CHAPTER 2

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# Mysid crustaceans as potential test organisms for the evaluation of environmental endocrine disruption: a review

**Redrafted after :**

Verslycke T, Fockedey N, McKenney CL Jr, Roast SD, Jones MB, Mees J, Janssen CR (accepted) Mysid crustaceans as potential test organisms for the evaluation of environmental endocrine disruption: a review. *Environmental Toxicology and Chemistry.*

# Chapter 2

## *Mysid crustaceans as potential test organisms for the evaluation of environmental endocrine disruption*

**Abstract**

Anthropogenic chemicals which disrupt the hormonal systems (endocrine disruptors) of wildlife species have recently become a widely investigated and politically charged issue. Invertebrates account for roughly 95% of all animals, yet surprisingly little effort has been made to understand their value in signaling potential environmental endocrine disruption. This omission can be attributed largely to the high diversity of invertebrates and the shortage of fundamental knowledge of their endocrine systems. Insects and crustaceans are exceptions and, as such, appear excellent candidates for evaluating the environmental consequences of chemically-induced endocrine disruption. Mysid shrimp (Crustacea: Mysidacea) may serve as a viable surrogate for many crustaceans and have been put forward as suitable test organisms for the evaluation of endocrine disruption by several researchers and regulatory bodies (e.g. USEPA). Despite the long-standing use of mysids in toxicity testing, little information exists on their endocrinology and few studies have focused on the potential of these animals for evaluating the effects of hormone-disrupting compounds. Therefore, the question remains as to whether the current standardized mysid endpoints can be used or adapted to detect endocrine disruption, or if new procedures need to be developed, specifically directed at evaluating hormone-regulated endpoints in these animals. This review summarizes the ecological importance of mysids in estuarine and marine ecosystems, their use in toxicity testing and environmental monitoring, and their endocrinology and important hormone-regulated processes to highlight their potential use in assessing environmental endocrine disruption.

### 2.1. Introduction

Anthropogenic chemicals that disrupt the hormonal systems (endocrine disruptors) of wildlife species have recently become a widely investigated and politically charged issue (Colborn et al., 1996; Depledge and Billinghurst, 1999; Krimsky, 2000). 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 (Billinghurst et al., 2000; Crisp et al., 1997; DeFur et al., 1999; Fingerman et al., 1998; Leblanc, 1999; Leblanc and Bain, 1997; McKenney, 1999; Oberdörster and Cheek, 2000). Although growth, reproduction, development, and other aspects of invertebrate physiology are known to be under hormonal control, the endocrine systems and hormones produced and used in invertebrates are not directly analogous to those of vertebrates (USEPA, 2002a). In invertebrates, the selection of suitable test methods and species for evaluating endocrine disruption is confounded by diversity. The use of a limited number of species as representative of this diversity is a naive approach destined to failure in the absence of suitable safeguards (Depledge and Billinghurst, 1999; LeBlanc, 1999). Hence, the key challenge for environmental assessment is to find invertebrate species, selected from multiple levels of ecosystem function, to efficiently monitor and evaluate the complexity of potential environmental effects of endocrine-disrupting chemicals at a reasonable financial cost (Dale and Beyeler, 2001).

Many anthropogenic pollutants have the world's oceans and seas as a final sink, carried there through riverine and estuarine conduits (Nimmo and Hamaker, 1982; Oberdörster and Cheek, 2000). Estuaries, intrinsically and commercially important ecosystems, are amongst the first recipients of endocrine disruptors in their seaward transport. Of the estuarine organisms that could be adversely affected by these compounds, crustaceans are good candidates for the study of potential impacts. Crustaceans are common in freshwater, estuaries and shallow coastal waters and form vital links in aquatic food webs (Cattrijsse et al., 1997; Fossi et al., 2001; Havens, 1991; Hostens and Mees, 1999; Mees et al., 1994; Moreira et al., 1992). In addition, crustaceans are susceptible to the effects of endocrine disruptors (USEPA, 2002a). 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 chemically-induced endocrine disruption by virtue of the 'wealth' of information available on their endocrinology compared with other invertebrates (Chang, 1993; Downer and Laufer, 1983; Laufer and Downer, 1988; LeBlanc, 1999; Oberdörster and Cheek, 2000).

Of the crustaceans, mysid shrimp have been put forward as suitable test organisms for the evaluation of endocrine disruption (CSTEE, 1999; DeFur et al., 1999; LeBlanc, 1999). The United States Environmental Protection Agency (USEPA) established the Endocrine Disruptor Screening and Testing Standardization and Validation Task Force (SVTF) to coordinate and conduct the scientific and technical work necessary to validate the screens and tests recommended by the Endocrine Disruptor Screening and Testing Committee (EDSTAC). The STVF recommended a two-tiered approach for determining whether a

chemical is an endocrine disruptor, and mysids were proposed as a suitable invertebrate assay in the Tier 2 testing (*in vivo* testing) (http://www.epa.gov/scipoly/oscpendo) for a two-generation reproductive/developmental toxicity test. Recently, a draft review paper was compiled on mysid life-cycle toxicity testing (USEPA, 2002a) and the two-generation mysid life-cycle assay was proposed to the Organization for Economic Cooperation and Development (OECD) as a new OECD test guideline. Despite the long-standing use of mysids in toxicity testing, little information on their endocrinology has been published and few studies have focused on the potential of these animals for evaluating the effects of hormone-disrupting compounds. Therefore, the question remains as to whether the current standardized mysid endpoints can be used or adapted to detect endocrine disruption, or if new procedures need to be developed, specifically directed at evaluating hormone-regulated endpoints in these animals.

This present review provides an overview of the available information on mysids relevant to the issue of endocrine disruption, including their ecological role in marine and estuarine ecosystems, their use in toxicity testing and environmental monitoring, and their endocrinology. A case is made for their potential use in assessing the environment consequences of endocrine-disrupting chemicals.

### 2.2. Mysid biology and ecology

Mysids (Malacostraca: Peracarida: Mysidacea) are relatively small (the majority of species being between 5 mm and 25 mm in length), shrimp-like crustaceans, often referred to as 'opossum shrimp' due to the oostegites forming a ventral female marsupium for carrying the developing embryos. The latter feature distinguishes mysids from other shrimp-like crustaceans. Mysids are identified from other peracarids (Amphipoda, Isopoda, Cumacea, Tanaidacea) by the presence of a statocyst (containing large endogenous statoliths, the primary equilibrium organs for mysids) on the proximal part of the uropodal endopod. Mysids are distributed from 80°N to 80°S and occur in various aquatic environments, including freshwater, groundwater, brackish, estuarine, coastal and oceanic habitats (Mauchline, 1980; Tattersall and Tattersall, 1951). Mauchline and Murano (1977) published a world list of mysids (765 species distributed between approximately 120 genera), however, this number is ever increasing through improved sampling techniques and exploration of new habitats. The present count is more than 1000 species belonging to approximately 160 genera (Meland, 2002 onwards). A comprehensive database on the world mysid fauna ('Nemys', http://intramar.ugent.be/nemys), containing links to relevant information (i.e. taxonomical, morphological, ecological, biogeographic, literature, pictorial and molecular info) on the species level, is presently being constructed at the Section Marine

Biology (Ghent University, Belgium) (Tim Deprez, unpublished data).

In general, mysids are regarded as omnivores, feeding on phytoplankton, zooplankton and organic detritus (Fockedey and Mees, 1999; Jerling and Wooldridge, 1994; Mauchline, 1980; Odum and Heald, 1972; Tattersall and Tattersall, 1951). Pelagic forms filter particles during swimming while benthic species have been observed actively hunting and grabbing small particles (Tattersall and Tattersall, 1951). Mysids form important links in the food webs of aquatic ecosystems and often feed selectively for size and/or species of prey (Mauchline, 1980; Mees and Jones, 1997). Consequently, they have the potential for structuring zooplankton communities (Fulton, 1982; Rudstam et al., 1989) and influencing the structure of phytoplankton, tychoplankton and meiofaunal communities (Grossnickle, 1982; Johnston and Lasenby, 1982; Kost and Knight, 1975; Mees and Jones, 1997; Siegfried and Kopache, 1980; Viherluoto and Viitasalo, 2001a; Web et al., 1987; Wooldridge, 1989). Most mysids utilize organic detritus to a considerable extent and are capable of remineralizing a substantial portion of the non-refractory detritus suspended in the water column or buried in the surface sediments (Fockedey and Mees, 1999; Jansen, 1985; Kost and Knight, 1975; Roast et al., 1998a). Mysid size is intermediate between mesozooplanktonic (µm) and endo- or epibenthic (cm) prey items, and mysids often progressively replace copepods in the diet of many post-larval and juvenile commercial fish species (Hostens and Mees, 1999; Markle and Grant, 1970; Mauchline, 1980; Stickney et al., 1974). In addition, mysids may serve as prey for larger crustaceans, marine mammals or wading birds (Lussier et al., 1988; Mauchline, 1980; Mees and Jones, 1997; Moffat, 1996; Ogle and Pierce, 1976; Tattersall and Tattersall, 1951).

Estuarine mysids have a flexible physiology which responds to a host of dynamically changing environmental variables, characteristic of the complex chemistry of estuaries. Temperature and salinity are the dominant ecological variables, and may act either singly or in combination to modify the physiological and ecological properties of estuarine organisms as well as responses to xenobiotic exposure. Empirical determination of the optimal salinity-temperature conditions of estuarine mysids is, therefore, essential for the development of optimum laboratory culture of these organisms and their use in toxicity and hazard assessment. For example, the optimal salinity-temperature conditions for growth of *Americamysis bahia* through its entire life cycle (McKenney and Celestial, 1995) are correlated with resistance patterns to these dominant environmental variables (McKenney, 1994b) and its distribution in estuaries. Moreover, temperature and salinity interact to modify the reproductive capacity of this species (McKenney, 1996).

## 2.3. Mysids and toxicology

Mysids are sensitive to chemical contaminants at environmentally relevant concentrations and have been used in regulatory toxicity testing for more than 20 years (Brandt et al., 1993; Emson and Crane, 1994; Gaudy et al., 1991; Harmon and Langdon, 1996; Hunt et al., 1997; Jacobs and Grant, 1974; Khan et al., 1992; Langdon et al., 1996; Lussier et al., 1985, 1999; Martin et al., 1989; McKenney, 1998; Nimmo and Hamaker, 1982; Roast et al., 1998a, 1999a, 2001a; Wildgust and Jones, 1998). USEPA and ASTM (American Society for Testing of Materials) have adopted the sub-tropical *Americamysis* (formerly *Mysidopsis*) *bahia* as a key testing species for coastal and estuarine monitoring, and standard guides for conducting life-cycle toxicity tests with this species have been developed (ASTM, 1998, 1999, 2002; USEPA, 1995a,b, 1997, 2002a). While there is a relatively large amount of published toxicity data for *Americamysis* species, there are relatively limited data on the sensitivity of other mysid species to toxicants (Roast et al., 1999a). The available evidence, however, suggests that mysids are generally more sensitive to toxic substances than many other test species (Hunt et al., 2002; Morton et al., 1997; Roast et al., 1998a; Verslycke et al., 2003b). Toxicity test procedures have been published for *Neomysis mercedis* (Brandt et al., 1993), *Mysidopsis intii* (Langdon et al., 1996), *Holmesimysis costata* (Martin et al., 1989), *Americamysis bigelowi* (Gentile et al., 1982), *Neomysis integer* (Roast et al., 1999a; Heijerick, 1994; Verslycke et al., 2003b), *Tenagomysis novae-zealandiae* (Nipper and Williams, 1997) and *Neomysis awatschensis* (Kang et al., 1997) (Table 2.1). In addition, methods for maintaining viable populations of different mysid species under laboratory conditions have been described by several researchers (Domingues et al., 1999; Langdon et al., 1996; Lussier et al., 1988; McKenney, 1987; Nimmo et al., 1991; Nipper and Williams, 1997; Reitsema and Neff, 1980; Verslycke et al., 2003b; Ward, 1984). Recently, a strong correlation between the toxic response of daphnids and mysids (which were generally more sensitive) for pesticides and organics was reported, emphasizing the use of mysids in future toxicity testing (Robinson, 1999).

Mysids have been used successfully to measure various sublethal toxicant effects, such as growth, swimming capability, feeding behavior, molting, energy budget, reproduction, sexual maturity and vitellogenesis (described in detail in the following paragraphs and summarized in Table 2.2). Also, field studies and caging experiments with mysids have been published (Clark et al., 1986; Fossi et al, 2001; McKenney et al., 1985; Rand and Clark, 2000; Verslycke et al., submitted-Chapter 10,11).

From their ecological importance, wide geographic distribution, year-round availability in the field and easy transportation, ability to be cultured in the laboratory, and sensitivity to contaminants, it is concluded that mysids are appropriate toxicity test organisms.

**Table 2.1.** Candidate mysid test species for toxicity testing with details on their natural habitat and culturing

| Species name | Distribution | Habitat description | Commercial culture | Culture protocol |
|---|---|---|---|---|
| *Americamysis bahia* (= *Mysidopsis bahia*) | Coastal estuaries and embayments ranging from the Gulf of Mexico to Narragansett (RI, USA)[a] | Marine (>15‰), <20-34°C[b] | yes | Lussier et al., 1988<br>Ward, 1984, 1991 |
| *Americamysis bigelowi* (= *Mysidopsis bigelowi*) | Eastern coast of the USA from MA (Georges Bank) to FL, often together with *A. bahia*[a] | Marine (30-35‰), 2-30°C | no | Lussier et al., 1988 |
| *Americamysis almyra* (= *Mysidopsis almyra*) | Eastern coast of the USA, inshore waters along the entire coast of Gulf of Mexico and northward along Atlantic coast to Patapsco River (MD)[a] | Marine (10-20‰), >20°C | yes | Reitsema and Neff, 1980<br>Domingues et al., 1998, 1999 |
| *Holmesimysis costata* (= *Acanthomysis sculpta*) | Principal species of the genus, from Southern California (USA) to British Columbia (Canada)[c] | Marine, planktonic, lives within surface canopy of kelp | no, field-collected animals available[c] | USEPA, 2002b<br>Turpen et al., 1994 |
| *Mysidopsis intii* | Eastern pacific from South-America to the southern California coast of the USA[a,d] | Marine, epibenthic, optimal temperature 20-22°C, optimal salinity 28-35‰ | no | Langdon et al., 1996 |
| *Mysis mixta* | Eastern (from White Sea to Iceland) and Western (Greenland coastal waters down to Cape Cod, USA) Atlantic regions[e] | Brackish, low salinity, coldwater | no | Gorokhova and Hansson, 2000 |
| *Neomysis awatschensis* | Pacific coast of Japan, Korea and USA[f] | Marine, estuarine | no | Kim and Chin, 1991 |
| *Neomysis mercedis* | North-Eastern pacific coast (southern Alaska to Goviota Bay, CA, USA)[g] | Freshwater, estuaries and coastal lakes, planktonic/epibenthic, euryhaline (<0.5 to >25‰), 6-22°C | no | Brandt et al., 1993 |
| *Neomysis integer* | North-European estuaries and coastal waters, oligohaline and freshwater lakes[h] | Marine, estuarine, freshwater, hyperbenthic, euryhaline (<0.5 to >25‰), coldwater (< 20°C)[i] | no | Heijerick, 1994<br>Verslycke et al., 2003b |
| *Praunus flexuosus* | North-European coastal waters | Hyperbenthic/planktonic, euryhaline, eurytherm[j] | no | Winkler and Greve, 2002 |

[a] Price et al., 1994; [b] McKenney, 1994b, 1996; McKenney and Celestial, 1995; [c] USEPA, 2002b, Turpen et al., 1994; [d] Langdon et al., 1996; [e] Mauchline, 1980; [f] Kim and Chin, 1991; [g] Brandt et al., 1993; [h] Mees et al., 1994, Roast et al., 1998a; [i] Kuhlmann, 1984; [j] McLusky, 1979, McLusky and Heard, 1971.

### 2.4. Candidate mysid test species for endocrine disruption research

General selection criteria for the most appropriate mysid species for toxicological testing are given by Nimmo and Hamaker (1982) and Roast et al. (1998a). In addition, the important characteristics for the selection of a suitable test species for identifying the effects of endocrine disruption in the environment are given by DeFur et al. (1999). Some attributes described in the latter publication (for example, mode of reproduction or knowledge of endocrinology) do not allow for discrimination among candidate mysid species. A very useful document in this context is a draft review paper on life-cycle toxicity testing with mysids in which several species (*Americamysis bahia*, *Americamysis almyra*, *Americamysis bigelowi*, *Holmesimysis costata*, *Mysidopsis intii*, *Neomysis mercedis* and *Neomysis integer*) are considered for their potential utility in endocrine disruption testing (USEPA, 2002a). From this review, it may be concluded that, although *A. bahia* has many strengths, limited ecological relevance for high latitude and low saline systems preclude its general utility. However, given the high degree of standardization in *A. bahia*, progress in development of standardized test protocols for endocrine disruption testing should be fastest in this species. Table 2.1 summarizes the distribution, habitat description and available culture protocols for other candidate mysid test species.

### 2.5. Mysid endocrinology and hormone-regulated endpoints

The use of hormones to regulate biological processes is a strategy common to vertebrates and invertebrates. Although the endocrine systems of invertebrates regulate many of the same processes in vertebrates (development, growth and reproduction), some endocrine-regulated processes are unique to specific groups of invertebrates. For example, molting, diapause and limb regeneration are endocrine-regulated processes associated with some invertebrate groups that are rare or absent among vertebrates (DeFur et al., 1999).

Most of the current knowledge of crustacean endocrinology is based upon studies with decapods such as crabs, lobsters, crayfish and shrimp and has been reviewed previously (Carlisle and Knowles, 1959; Chang, 1997; Charmantier et al., 1997; DeFur et al., 1999; Fingermann, 1987; Lafont, 2000; Quackenbush, 1986). The main biological processes, such as growth, molting and reproduction, are cyclic and fairly well understood in benthic and terrestrial malacostracans such as decapods, isopods and amphipods (Chang, 1993; Cuzin-Roudy and Saleuddin, 1989; Spindler et al., 1984). These biological processes are regulated by a complex endocrine system (Charmantier et al., 1997; Cuzin-Roudy and Saleuddin, 1989). Basically, inputs from the environment are integrated by the central nervous system; neurotransmitters and neuromodulators govern the release of neuropeptides which control

the production of hormones by the endocrine glands (Huberman, 2000). The main crustacean endocrine centers include the Y-organ, mandibular organ, androgenic gland, X-organ and sinus gland (Charmantier et al., 1997; DeFur et al., 1999).

The effects of organic and inorganic contaminants on crustacean functions regulated by hormones are being investigated with increasing frequency as several show promise as biomarkers of environmental contamination and endocrine disruption (DeFur et al., 1999; Fingerman et al., 1996, 1998; Medesani et al., 2001). Unfortunately, relatively little data are available on the hormonal control of biological processes in mysids. Having said that, certain endpoints relevant to the testing of suspected endocrine disruptors, such as survival, fecundity, sexual maturation and biomass increase, are already standardized procedures for some mysids such as *Americamysis bahia*, *Americamysis bigelowi* (partly), *Americamysis almyra*, *Holmesimysis costata* and *Neomysis mercedis*, and many other endpoints or species are promising (DeFur et al., 1999). The use of potential mysid hormone-regulated endpoints as biomarkers of exposure or effects of endocrine disruptors are discussed in detail in the following paragraphs and summarized in Table 2.2. Although many, if not all, of these endpoints may indicate a response to an endocrine disruptor, most also vary in response to exposure to other stressors and this is further confounded by the interrelatedness of some of these endpoints (USEPA, 2002a). The key to the interpretation of these endpoints as indicators of endocrine disruption will be to create a large database on the 'normal' response of organisms and/or good reference sites when working under field conditions.

### *2.5.1. Growth and molting*

Most commonly, growth is measured either by increases of dry weight or body length per time interval (Cleveland et al., 2000; Mauchline, 1985; McKenney, 1986; Langdon et al., 1996) and, for crustaceans, is often expressed in terms of intermolt period and growth factor (Mauchline, 1980). Growth curves (such as the von Bertalanffy equation) can be fitted to the growth data (Cuzin-Roudy et al., 1981; Ikeda, 1992; Mees et al., 1994), allowing comparison of the different growth parameters between treatments. While several studies have focused on growth and molting in mysids under natural conditions (Astthorsson and Ralph, 1984; Gorokhova and Hansson, 2000; Kim and Chin, 1991; Kuhlmann, 1984; Mauchline, 1973, 1985; McKenney and Celestial, 1995; Turpen et al., 1994; Winkler and Greve, 2002), exposure experiments have also confirmed the sensitivity of these endpoints in toxicology (Brandt et al., 1993; Cleveland et al., 2000; Gorokhova, 1998; Hunt et al., 1997; Kang et al., 1997; Lussier et al., 1999; McKenney, 1982, 1985, 1986, 1994a; McKenney and Celestial, 1996; McKenney and Matthews, 1990; McKenney et al., 1985, 1991; Voyer and McGovern, 1991). For mysids, reduced growth is the most common sublethal response to toxicant

exposure and this has important implications for reproductive success since fecundity is related directly to female body size (Kang et al., 1997; Mauchline, 1973; Mees et al., 1994; Winkler and Greve, 2002). In crustaceans, significant growth occurs only as a result of molting, therefore, disruption of molting may result in alterations in growth (Toda et al., 1984; USEPA, 2002a).

Ecdysteroids (the molting hormones in crustaceans) also function in the control of reproduction and embryogenesis (Charmantier et al., 1997; Subramoniam, 2000), therefore, the crustacean molt cycle has profound effects on many aspects of organismal function, including physiology, behavior and changes in biochemical composition (Chang, 1995; Oberdörster et al., 1999). Molting is regulated by a multihormonal system but is under the immediate control of molt-promoting steroid hormones (ecdysteroids) secreted by an ecdysial gland, called the Y-organ (the homologue of the prothoracic gland in insects) (Huberman, 2000; Zou and Fingerman, 1997). The Y-organ secretes ecdysone which, on release in the hemolymph, is converted into active 20-hydroxyecdysone (synonyms: crustecdysone, ecdysterone). Circulating titers of 20-hydroxyecdysone vary impressively during the molt cycle (Chang, 1995; Huberman, 2000). The Y-organ produces two other ecdysteroids, 3-dehydroxyecdysone and 25-deoxyecdysone, the latter forming the immediate precursor to the active ponasterone A (Subramoniam, 2000). More studies have been done on the effects of contaminants on molting and limb generation than on any other hormone-mediated process in crustaceans (Fingerman et al., 1998; Oberdörster et al., 1999, 2001).

Molt staging, based on changes in the integument, has been developed for various crustaceans and is generally divided into four major periods: postmolt, intermolt, premolt and molt (ecdysis). Mysid molt stages have been described for *Siriella armata* (Cuzin-Roudy and Tchernigovtzeff, 1985; Cuzin-Roudy et al., 1989), *Mysis mixta* (Gorokhova, 2002) and *Neomysis integer* (Gorokhova, 2002). In mysids, ecdysis is instantaneous with the entire carapace lifting up and the mysid sliding out of the old cuticle while swimming. For female mysids, integumental development during molt preparation, marsupial brood development and development of new eggs in the ovary are synchronized, facilitating molt staging (Cuzin-Roudy and Saleuddin, 1989). To date, only one study has quantified ecdysteroid titres during the mysid molt cycle and this study was with *Siriella armata* (Cuzin-Roudy et al., 1989). However, both ecdysone and 20-OH ecdysone have been identified in *N. integer* (Ghekiere et al., unpublished data) and *Americamysis bahia* (Tuberty and McKenney, unpublished data).

As mentioned previously, molting is controlled by ecdysteroids (Zou and Fingerman, 1997). Ecdysteroids, like other steroid hormones, control the activity of specific genes at the transcriptional level by interacting with the intracellular ecdysteroid receptor (EcR) (Huberman, 2000; Oberdörster et al., 1999; Segraves, 1991). In arthropods, the ecdysone

receptor is in the same gene family as the vertebrate thyroid receptor but, interestingly, steroidal estrogens do not agonize or antagonize the EcR (Sarker et al., 1999). There is evidence (Sarker et al., 1999) that some non-steroidal environmental estrogens are ecdysteroid antagonists (e.g. lindane, bisphenol A, diethylphthalate and p,p'-DDT). In addition, several classes of phytochemicals antagonize ecdysone activity (Oberdörster et al., 2001). The apparent ubiquity of the anti-ecdysteroidal activity of environmental chemicals necessitates investigation into their potential effects on crustaceans (Mu and LeBlanc, 2002a,b). Many pesticides, generally classed as Insect Growth Regulators (IGRs), function as ecdysone agonists (DeFur et al., 1999; McKenney, 1999). As suggested by Zou and Fingerman (1997), future investigations of molting in crustaceans should have two emphases, one examining interactions between potential endocrine disruptors and the ecdysone receptor, and one focusing on the possible impairment of ecdysteroidogenesis by these agents. *In vitro* assays can determine quickly whether a chemical has (anti-) ecdysteroidal activity (Dinan et al., 2001; Smagghe et al., 2002). Given the current methods for quantifying ecdysteroids using radioimmunoassay (e.g., Cuzin-Roudy et al., 1989, Mu and LeBlanc, 2002b) and the available methods for molt staging (Cuzin-Roudy and Tchernigovtzeff, 1985; Gorokhova, 2002), it should be possible to evaluate the potential interaction of these chemicals with the process of molting in mysids.

As ecdysteroids are used as major endocrine signaling molecules in crustaceans (DeFur et al., 1999), and little is known of their other functions, it may be expected that a chemical with (anti-)ecdysteroidal activity will also affect other hormone-regulated processes in crustaceans. Support for this hypothesis is provided by Mu and LeBlanc (2002b) who demonstrated that the fungicide fenarimol altered embryo development in daphnids by interfering with the ecdysteroid metabolism. However, one major advantage of using ecdysteroid metabolism as an endpoint is that it provides a means of evaluating the impact of environmental chemicals on crustaceans (and potentially other arthropods), while not necessarily affecting vertebrates. Since (anti-)ecdysteroidal activity has been proven *in vitro* for certain chemicals, and disruption of molting has been observed as a result of chemical exposure, these chemicals should be tested in exposures with mysids. In these exposures, endpoints such as intermolt period, growth, morphological aberrations, ecdysone titers, protein concentrations, integument development, as well as related endpoints, should detect *in vivo* effects of chemicals on ecdysteroid metabolism and molting in mysids. *In vitro* assays should aid the mechanistic understanding of chemical action to better allow distinction between endocrine-specific and pharmacological effects.

*2.5.2. Energy metabolism*

Biomarkers linked with physiological energetics provide information on key processes in the organism's energy acquisition and expenditure, possibly also elucidating the mode of action of the toxicant. Under normal conditions, specific amounts of energy are allocated to basal metabolism, growth and reproduction, and therefore, theoretically, changes in metabolic turnover and specific allocations should be linked to effects at higher levels of ecological organization (De Coen, 1999). There is a large body of information available on the neuroendocrine pathways of physiological regulation in crustaceans. While typical and well-studied challenges to the endogenous energy metabolism include environmental hypoxia, functional (internal) hypoxia, changing energetic requirements, disturbance to water balance/ion-homeostasis and changes in temperature (for review, see Morris and Airriess, 1998), exposure to toxicants will also result in an energetic challenge. Since energetic processes are hormone-regulated, they are, by definition, sensitive to hormone disruption and several measurements of energy reserves and consumption may serve as useful biomarkers of endocrine-disrupting substances in crustaceans (McKenney, 1999).

Alterations to the energy metabolism of mysids have been used successfully as an indicator of stress to toxicant exposure in *Americamysis bahia* (McKenney, 1982, 1985, 1998; McKenney and Matthews, 1990; McKenney et al., 1991), *Praunus flexuosus* (Garnacho et al., 2001), *Neomysis awatschensis* (Chin et al., 1998a,b; Shin et al., 2000) and *Neomysis integer* (Roast et al., 1999c, 2000b; Verslycke and Janssen, 2002; Verslycke et al., 2003c). In *A. bahia*, *P. flexuosus* and *N. awatschensis*, weight-specific respiration, ammonia excretion rates and O:N ratios were measured following toxicant exposure. In *N. integer*, Roast et al. (1999c) used Scope for Growth (Widdows and Page, 1993), while Verslycke and Janssen (2002) and Verslycke et al. (2003c) used the Cellular Energy Allocation assay (De Coen and Janssen, 1997). Both methods are promising and were recently validated in *N. integer* following exposure to the pesticide chlorpyrifos (Verslycke et al., submitted-Chapter 5). The Cellular Energy Allocation assay was also validated in the field (Verslycke et al., submitted-Chapter 10). The ecological relevance and utility of short-term bioindicators of metabolic processes in *A. bahia* have been demonstrated following chronic exposure to pesticides (McKenney, 1982, 1985, 1998; McKenney and Matthews, 1990; McKenney et al., 1991). In these studies, pesticide-exposed juvenile mysids had a greater reliance on the more energy-rich lipid substrates during maturation to support elevated metabolic demands, resulting in less lipid material available for gamete production and reduced reproductive success. Unexposed mysids shift toward more proteinaceous substrates during maturation, as demonstrated for *A. bahia* (McKenney, 1998) and *N. integer* (Raymont et al., 1968; Verslycke and Janssen, 2002). These changes in metabolic substrate

usage can be measured by monitoring the oxygen/nitrogen ratio (O:N) (McKenney, 1982; Laughlin and Lindén, 1983), the lipid and protein content (Verslycke and Janssen 2002) or the carbon/nitrogen (C:N) ratio of the test organism (Gorokhova and Hansson, 2000). On the other hand, hyperglycemia is a common response to environmental or functional hypoxia and contaminant exposure in numerous decapods, and it is thought to be triggered by the action of crustacean hyperglycemic hormone (CHH) on various target tissues (Fingerman et al., 1998; Morris and Airries, 1998). The amino acid sequence of CHH is highly homologous with the molt-inhibiting hormone (MIH), another product of the sinus glands in crustaceans, indicating possible involvement in the control of molting and reproduction (Morris and Airries, 1998). Several investigators have examined the effects of metals and organic contaminants on blood glucose concentrations and CHH titers in crustaceans (Fingerman et al., 1998). Changes in blood glucose levels in mysids exposed to potential endocrine disruptors may indicate disruption of hormonal activity other than that associated with molting or reproduction (USEPA, 2002a).

The methods described above are transferred easily to other mysid species. Endpoints related to energetic processes are relatively easy to measure, but a better and holistic understanding of the role of the different hormones involved in energy metabolism, such as CHH, is needed to evaluate the potential impact of hormone-mimicking substances on mysids. In this context, new immunoassays for determination of circulating hormones in the hemolymph, such as CHH, are promising (Chang et al., 1998).

Other endpoints related to metabolism in mysids have been studied. High acetylcholinesterase activity in *Siriella clausi*, indicating a high metabolic rate, identified this mysid as particularly suited for research based on biomarkers in the marine environment (Fossi et al., 2001). In addition, *N. integer* has been used as a viable alternative model for the partial replacement of vertebrate animals in metabolic studies with illegal growth promoters and veterinary drugs (De Wasch et al., 2002). Finally, respiratory responses have been studied in mysids in relation to a variable environment and toxic exposure (Garnacho et al., 2001; Kang et al., 1997; Kim and Chin, 1991; Laughlin and Lindén, 1983; Marshall et al., 2003; McKenney, 1982, 1998; Roast et al., 1999b; Weisse and Rudstam, 1989).

While undoubtedly having environmental relevance and being fairly easily extrapolated to higher levels of biological organization, the major disadvantage of endpoints related to energy metabolism is their difficulty in mechanistically explaining hormone-regulated responses as can be expected from exposure to endocrine disruptors. Many abiotic and toxic stressors affect the energy metabolic processes of organisms (i.e. Gorokhova and Hansson, 2000; McKenney, 1994a; Roast et al., 1999b,c, 2000b; Shin et al., 2000; Verslycke and Janssen, 2002; Verslycke et al., 2003c), while not necessarily being related to disruption in normal hormonal regulation. The successful use of biomarkers for the evaluation of

endocrine disruption will, therefore, be limited by the amount of background data on natural variation and 'normal' levels of the endpoints in question, and also by the fundamental understanding of the toxicant action at the (sub)cellular level.

*2.5.3. Steroid metabolism and cytochrome P450*

The presence of sex hormones has been suggested in many, if not all, arthropods (De Loof and Huybrechts, 1998). Vertebrate-type steroids (such as 17β-oestradiol, testosterone, and progesterone) have been measured in several malacostracan crustaceans (DeFur et al., 1999; James and Boyle, 1998). Although the lack of a role for vertebrate sex steroid hormones in arthropods has been highlighted (DeFur et al., 1999; De Loof and Huybrechts, 1998), fragmented evidence suggests that some of these compounds may function as hormones in crustaceans (DeFur et al., 1999; Mu and LeBlanc, 2002a; Verslycke et al., 2002).

Pollutants may exert reproductive effects through interference with the normal steroid metabolism. In daphnids, changes in steroid metabolism could provide an early indication of potential reproductive toxicity after sublethal exposure to suspected endocrine disruptors (Baldwin et al., 1995, 1997, 1998; LeBlanc and McLachlan, 2000). These chemicals often interfere with the microsomal P-450 monooxygenase system, also called the mixed-function oxygenase (MFO) system. The MFO system is involved not only in the metabolism of organic toxicants but also in steroid metabolism; consequently, induction or inhibition of the MFO system may also have repercussions for the hormonal control of reproduction. A relationship between reproductive failure and the effects of pollutants on endocrine function was found in teleost fish (Singh and Singh, 1980; Thomas, 1989). For invertebrates, several studies have focused on pollutant-induced steroid metabolism alterations. In sea stars, a linkage was demonstrated between impaired reproductive success, pollution-modulated endocrine function and induction of the MFO system (Den Besten et al., 1991). In gastropods, much work on steroid metabolism has been initiated by the observation of tributyltin-induced imposex, a state of pseudohermaphrodism in which females exhibit functional secondary male characteristics. Although the underlying mechanism by which tributyltin causes imposex in gastropods has not been elucidated conclusively, the weight of evidence is in favor of the cytochrome P450-dependent aromatase inhibition hypothesis (Gooding and LeBlanc, 2001; Matthiessen and Gibbs, 1998; Oberdörster et al., 1998a,b; Oehlmann and Bettin, 1996). In crustaceans, alterations in steroid metabolism have been studied in *Daphnia magna* (Baldwin and LeBlanc, 1994a,b; Baldwin et al., 1998; LeBlanc and McLachlan, 2000; Oberdörster et al., 1998a) and in the blue crab *Callinectes sapidus* (Oberdörster et al., 1998b). Verslycke et al. (2002) reported the testosterone metabolism and the presence of

vertebrate-type steroids in *Neomysis integer*, and demonstrated the presence of a complex steroid hydroxylase system consisting of different P450 isozymes. The remarkable diversity of testosterone hydroxylation exhibited should stimulate further studies on the induction, stereo-specificity and regulation of the enzyme systems of *N. integer* and other mysids. More recently, alterations in the phase I and II testosterone metabolism in *N. integer* following acute exposure to tributyltin have been demonstrated (Verslycke et al., 2003a). In addition, the assay used in these studies has been used recently in exposure experiments with other chemicals, such as nonylphenol and methoprene, and also in the field (Verslycke et al., submitted-Chapter 8,11).

Endogenous androgens may be the precursors for other hormones, therefore, exposure to exogenously added androgens could elicit activity through receptors other than the androgen receptor. Although this has not been determined in crustaceans, Verslycke et al. (2002) found evidence of a sex-specific production of androgens, such as testosterone and androstenedione, in *N. integer*. Similarly, LeBlanc and McLachlan (1999) reported various rates of testosterone conversion to androstenedione in daphnids. Future studies are needed to reveal if these conversions are affected by age, gender, reproductive state, or changes in the abiotic environment. It should be noted that an androgen receptor has not been found or cloned in crustaceans. Therefore, its identification and characterization should be a priority for research to explore the usefulness of sex steroids for evaluating endocrine disruption in crustaceans and other invertebrates.

Studies over the last 30 years have established the important role of cytochrome P450 in the biotransformation of xenobiotics and endogenous compounds (such as ecdysteroids) in crustaceans (for a review on crustacean P450, refer to James and Boyle, 1998). Although no structural information on cytochromes P450 in crustaceans is available, it is clear that they are involved in several steps in the biosynthesis of ecdysteroids and other physiologically important substrates in crustaceans (Lachaise and Sommé, 1998). More studies are needed to understand the effects, if any, of various classes of environmental and other chemicals that are known modulators of cytochrome P450 expression or activity. New molecular tools, such as primer-based RT-PCR procedures and expression of P450s in heterologous systems, should result in better insights into the function and expression of P450s in the context of endocrine disruption. In addition, *in vivo* metabolic studies with different substrates (testosterone, ecdysone) will provide valuable tools for evaluating the effects of toxicant exposure, particularly when linked with effects on higher levels of biological organization. Though information on the identity of P450s and their functional role in mysids is, to our knowledge, non-existent, mysids should be a good model to study these mechanisms. From the preliminary studies with *N. integer* by Verslycke et al. (2002, 2003a, submitted-Chapter 8), there is sufficient information to suggest that mysids have an

enzymatic biotransformation system which rivals that of other invertebrates and vertebrates. Metabolic studies with physiologically relevant substrates, that also measure hormone-regulated effects at a higher level of biological organization (i.e. reproductive success), will be valuable.

*2.5.4. Reproduction and vitellogenesis*

Although the main neurosecretory centers and the sinus gland in mysids resemble these from decapods, sexual differentiation in juveniles and mysid reproduction is more like that of amphipods and isopods, and is strictly linked to the molt cycle (Cuzin-Roudy and Saleuddin, 1989). In mysids, embryonic and post-embryonic development occurs in the female marsupium and include five consecutive stages from oviposition to the juvenile stage (Mauchline, 1980; Wittmann, 1981a,b; Wortham and Price, 2002). Juveniles are liberated 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. Secondary vitellogenesis is not only cyclical, as in other crustaceans (Charniaux-Cotton, 1985), but also strictly linked to the molt cycle, offering an example of the type-2 pattern (e.g. Amphipoda, Isopoda, Decapoda) for the regulation of simultaneous gonadal and somatic growth in crustaceans (Adiyodi and Subramoniam, 1983; Charniaux-Cotton, 1985). Cuzin-Roudy and Saleuddin (1989) published an excellent review on the use of the mysid *Siriella armata* as a biological model for the study of hormonal control of molt and reproduction, which should be extended for other mysid species. In addition, Wortham and Price (2002) and Greenwood et al. (1989) published studies on the *in vitro* culture of mysid marsupial developmental stages at different temperatures. These assays should be evaluated further as a means of detecting effects of contaminants on marsupial development in mysids.

In general, there are few studies of the effects of contaminants on gonadal maturation of crustaceans (Fingerman et al., 1998), however, there has been much recent attention on vitellogenin, the precursor to the yolk protein vitellin in egg-laying invertebrates and vertebrates as indicators of exposure to estrogenic xenobiotics (Billinghurst et al., 2000; Lee and Chang, 1997; Lee and Watson, 1994, 1995; Lee et al., 1996; Oberdörster et al., 2000a,b; Tuberty et al., 2002; Warrier et al., 2001). Control of vitellogenesis is being studied intensively because yolk is an excellent model for studying mechanisms of hormonal control at the cellular and molecular levels (Billinghurst et al., 2000; Tuberty et al., 2002). To assess the potential adverse effects of xenobiotics on crustacean reproduction, it is important to measure accurately vitellogenin and vitellin in crustacean models (an overview of crustacean species from which vitellin, vitellogenin or lipovitellin has been isolated or partially

characterized is given in Tuberty et al. (2002). Recently, a quantitative enzyme-linked immunosorbent assay (ELISA) was developed for the mysid *Americamysis bahia* using polyclonal antisera (Tuberty et al., 2002). In addition, studies are under way to characterize and purify vitellin of the mysid *Neomysis integer* (Ghekiere et al., unpublished data). Future laboratory and field studies with mysids are needed to evaluate the use of these immunoassays for investigating effects of xenobiotics on crustacean vitellogenesis. A good example of this is given by Oberdörster et al. (2000a,b) who reported the effects of chronic pyrene exposure on molting and reproduction assessed in the grass shrimp *Palaemonetes pugio* using a monoclonal ELISA for vitellin. Other studies have also found an impact of xeno-estrogens on the production of crustacean proteins (e.g. vitellin, cypris major protein) which are thought to be under estrogen control (Billinghurst et al., 2000; Okumura et al., 1992). Future work on sequence determination of vitellogenic genes and their hormonal activity will provide interesting insight into the vitellogenic process in mysids. Genomic and non-genomic effects of ecdysteroids on ovarian maturation is another potential area of work. Synergistic and antagonistic actions of the different neuropeptides, and the mandibular organ control over molting and reproduction, are other areas requiring further study as a basis for use of crustaceans for endocrine disruption testing in the future (USEPA, 2002a).

### *2.5.5. Life-cycle testing, population and field studies*

Despite superficial resemblance to decapod shrimps, mysids are more closely related to amphipods and isopods, and are grouped together in the superorder Peracarida. All three orders are good candidates for toxicological testing, and amphipods and mysids are used routinely. However, for endocrine disruption testing, especially for life-cycle tests, mysids offer clear advantages over amphipods. Most marine amphipods used in toxicological testing must be collected from their natural habitats prior to use in tests. Although they can be held for a few weeks prior to testing, they generally are not cultured for tests. Conversely, several mysid species have been cultured in the laboratory and used in life cycle tests (USEPA, 2002a). There are several measures of reproductive performance that can be used to assess sublethal response in life-cycle testing, including sexual maturity, the time to first brood release, the time required for egg development (and its separate phases), fecundity, brood success and alterations in reproductive characteristics in populations (Gentile et al., 1983; Khan et al., 1992; Lussier et al., 1985; McKenney, 1982, 1985, 1986, 1998, 1999; McKenney and Celestial, 1996; McKenney et al., 1991, 1999) (Table 2.2). Inhibited reproduction is the most sensitive sublethal, population response of *Americamysis bahia* chronically exposed to pesticides (McKenney, 1998). Numerous studies have described the use of reproductive endpoints in mysids following toxic exposure and changes in the abiotic environment (Nimmo

and Hamaker, 1982; Van Sprang et al., 1991; Voyer and McGovern, 1991; Winkler and Greve, 2002). Although standard chronic assays, including reproductive endpoints, are described for *A. bahia*, these should be applicable to other mysids, though the longer life cycle in other species may restrict their use in routine testing.

The life history of *A. bahia* is very amenable to demographic modeling because of rapid growth, early sexual differentiation (at 14 days) and reproduction (commencing around 17 days), and frequency of brood production (average of five to seven per female) over the full life span of 90 days (Kuhn et al., 2000, 2001). These endpoints provide useful information for predicting population-level effects of reproductive toxicants. However, further validation is needed in multi-generational laboratory studies as well as incorporation of other population growth parameters such as density dependence, predation, migration and competition, before conclusions can be formulated which are relevant for natural environmental conditions. Preliminary transgenerational responses of *A. bahia* to a pesticide acting as a juvenile hormone agonist have been reported (McKenney et al., 1999). Survival, growth, development and reproduction of this estuarine mysid were monitored through an entire life-cycle exposure to fenoxycarb and during the second generation without additional exposure. Juvenile mysid growth, and carbon and nitrogen accumulation, as well as mysid survival through the first brood production, were significantly affected by fenoxycarb. On the other hand, neither maturation time, sex determination nor young production were significantly altered during the life-cycle exposure. However, second generation adults, exposed to fenoxycarb only as developing embryos and juveniles, produced fewer young and contained significantly less males. These results demonstrate clearly the need for transgenerational studies with mysids to fully understand the potential chronic impact of endocrine disruptors.

Detailed information and the short life cycle of *A. bahia* clearly favor the use of this species in the initial development and further validation of population models based on reproductive endpoints. A concise draft of a detailed review paper has been produced by USEPA (2002a) on a recommended protocol and additional data needs for a two-generation life-cycle test with *A. bahia* in the context of endocrine disruptors. In this review, the following endpoints and their preferred methods for quantification are given: survival, molting frequency, reproduction (sexual maturity, time to first brood release, brood size, offspring produced), metabolic disruption, disruption in steroid metabolism, vitellogenin induction, cytochrome P450 levels and blood glucose levels. Table 2.2 summarizes the potential endpoints for evaluating environmental endocrine disruption in mysids.

**Table 2.2.** Potential endpoints for evaluating endocrine disruption in mysids

| Endpoint | Use in mysids[a] | Reference[b] |
|---|---|---|
| Survival (acute) | *Americamysis bahia* (S) | ASTM, 2002; USEPA, 2002b |
| | *Americamysis bigelowi* (S) | ASTM, 2002 |
| | *Americamysis almyra* (S) | ASTM, 2002 |
| | *Holmesimysis costata* (S) | ASTM, 1998; Martin et al., 1989; USEPA, 2002b |
| | *Neomysis mercedis* (S) | ASTM, 1998; Brandt et al., 1993 |
| | *Mysidopsis intii* (L) | Harmon and Langdon, 1996; Langdon et al., 1996 |
| | *Neomysis integer* (L) | Emson and Crane, 1994; Roast et al., 1999a; Verslycke et al., 2003b |
| | other species (L) | Kang et al., 1997; Nipper and Williams, 1997 |
| Life-cycle testing | *A. bahia* (S) | ASTM, 1999; USEPA, 1995a, 1996, 2002b |
| | *A. bigelowi* (S), *A. almyra* (S) | ASTM, 1999 |
| | *H. costata* (S) | ASTM, 1999; Hunt et al., 1997; USEPA, 1995b |
| | *M. intii* (L) | Harmon and Langdon, 1996; Langdon et al., 1996 |
| | *N. integer* (L) | Laughlin and Lindén, 1983 |
| Two-generation testing | *A. bahia* , *A. bigelowi, A. almyra* (S in prep, L) | McKenney et al., 1999; Nimmo et al., 1980; USEPA, 2002a |
| | *M. intii* | method should be developed |
| | *H. costata*, *N. mercedis*, *N. integer* | probably impractical due to long generation time |
| Fecundity (brood size) | *A. bahia, A. bigelowi*, *A. almyra* (S) | ASTM, 1999; USEPA, 1995a |
| | *H. costata* (S) | Turpen et al., 1994; USEPA, 1995b |
| | *N. mercedis* (L) | Murtaugh, 1989 |
| | *M. intii* (L) | Harmon and Langdon, 1996; Langdon et al., 1996 |
| | *N. integer*, *Praunus flexuosus* (L) | Irvine et al., 1995; Winkler and Greve, 2002 |
| | *Mysis mixta* (L) | Gorokhova and Hansson, 2000 |
| | *N. awatschensis* (L) | Kim and Chin, 1991 |
| Embryonic development | *A. bahia* (L) | Wortham and Price, 2002 |
| | *Mesopodopsis slabberi* (L) | Greenwood et al., 1989 |
| Sexual maturatity | *A. bahia* (S/L) | Gentile et al., 1983; Khan et al., 1992; Lussier et al., 1985; McKenney, 1996 |
| Time to first brood release | *H. costata* (L) | Turpen et al., 1994 |
| | *N. integer*, *P. flexuosus* (L) | Winkler and Greve, 2002 |
| Egg development time | *M. mixta* (L) | Gorokhova and Hansson, 2000 |
| | *M. intii* | Harmon and Langdon, 1996; Langdon et al., 1996 |
| Sex ratio and intersexuality | *A. bahia* (S/L) | ASTM, 1999; McKenney et al., 1999; USEPA, 2002b |
| | *N. integer* (L) | Chojnacki and Ciupinski, 1986; Hough et al., 1992; Mees et al., 1995 |
| Molt time and success | *A. bahia* (L) | De Lisle and Roberts, 1994 |
| | *N. integer* (L) | Astthorsson and Ralph, 1984; Gorokhova, 2000 |
| | *M. mixta* (L) | Gorokhova, 2000 |
| | *Siriella armata* (L) | Cuzin-Roudy and Tchernigovtzeff, 1985 |
| | *N. awatschensis* (L) | Kim and Chin, 1991 |

| Endpoint | Use in mysids[a] | Reference[b] |
|---|---|---|
| Growth, Biomass | *A. bahia, A. bigelowi*, *A. almyra* (S) | ASTM, 1999; USEPA, 1995a |
| | *H. costata* (S/L) | Hunt et al., 1997; Turpen et al., 1994; USEPA, 1995b |
| | *N. mercedis* (L) | Brandt et al., 1993 |
| | *M. intii* (L) | Harmon and Langdon, 1996; Langdon et al., 1996 |
| | *M. mixta* (L) | Gorkhova, 1998 |
| | *N. integer* (L) | Kuhlman, 1984; Mauchline, 1985 |
| | *N. awatschensis* (L) | Chin et al., 1998a,b; Kang et al., 1997; Kim and Chin, 1991 |
| | *P. flexuosus* (L) | Gorokhova and Hansson, 2002 |
| | *Tenagomysis novae-zealandiae* (L) | Nipper and Williams, 1997 |
| Energy metabolism | *A. bahia* (L) | McKenney, 1998 |
| O:N ratio, C:N ratio | *N. mercedis* (L) | Chigbu and Sibley, 1996 |
| Respiration | *N. integer* (L) | Roast et al., 1999c; Verslycke et al., 2003c |
| | *M. mixta* (L) | Gorokhova and Hansson, 2000 |
| | *N. awatschenchis* (L) | Chin et al., 1998a,b; Shin et al., 2000 |
| | *P. flexuosus* (L) | Garnacho et al., 2001 |
| | *Leptomysis lingvura* (L) | Gaudy et al., 1991 |
| | *Mysis relicta* (L) | Adare and Lasenby, 1994; Chess and Stanford, 1998 |
| | *Gastrosaccus brevifissura* (L) | Marshall et al., 2003 |
| Ecdysteroid metabolism | *A. bahia* (U) | Tuberty and McKenney |
| | *N. integer* (U) | Ghekiere et al. |
| | *S. armata* (L) | Cuzin-Roudy et al., 1989 |
| Steroid metabolism | *N. integer* (L) | De Wasch et al., 2002; Verslycke et al., 2002, 2003a |
| P450 enzymes | *N. integer* (L) | Verslycke et al., 2002, 2003a |
| Vitellogenesis | *A. bahia* (L) | Tuberty et al., 2002 |
| | *S. armata* (L) | Cuzin-Roudy et al., 1989 |
| | *N. integer* (U) | Ghekiere et al. |
| Osmoregulation | *A. bahia* (L) | De Lisle and Roberts, 1986, 1987, 1994 |
| | *N. integer* (L) | Bobovich, 1976 |
| | *P. flexuosus* (L) | McLusky et al., 1982 |
| | other species | Webb et al., 1997 |
| Morphology, histology | *A. bahia, A. bigelowi* (L) | Gentile et al., 1982 |
| | *N. integer* (L) | Mees et al., 1995 |
| Swimming behavior | *N. integer* (L) | Buskey, 1998; Roast et al., 1998a, 2000a,c, 2001b |
| Feeding behavior | *A. bahia* (L) | Nimmo et al., 1981; Cripe et al., 1981 |
| | *M. mixta* (L) | Engstrom et al., 2001; Viherluoto and Viitasalo, 2001a |
| Other behavioral endpoints | mating, grooming, swarming, burrowing ability, predator/prey dynamics | Acosta and Poirrier, 1992, Buskey, 2000; Modlin, 1990; Nel et al., 1999; Rademacher and Kils, 1996, Ritz et al., 1997; Roast et al., 2002 |

[a] S: standard; L: published in literature; U: unpublished data.
[b] Selection of references is given, refer to reference list for additional references.

The use of mysids in field studies has been extremely limited. McKenney et al. (1985) and Clark et al. (1986) performed experiments with caged mysids to evaluate the lethal and sublethal responses of *A. bahia* during field applications of fenthion, an organophosphate insecticide. To our knowledge, these are the only published studies on *in situ* exposures with caged mysids. In addition, studies which have investigated biomarker responses in field-exposed mysids is also very limited (Fossi et al. 2001; Verslycke et al., submitted-Chapter 10,11). Clearly, field validation of the biomarkers described in this review, is a strong research need for the future.

*2.5.6. Morphology and histology*

Morphological changes resulting from exposure to contaminants have been documented for many taxa, including arthropods, but have not been considered widely in mysid toxicological studies as a measurable endpoint (USEPA, 2002a). Gentile et al. (1982) reported morphological aberrations at the onset of sexual maturity in *Americamysis bahia* and *Americamysis bigelowi* exposed to cadmium in the laboratory. In addition, field observations of intersexuality and variable telson morphology were reported in *Neomysis integer* from different European estuaries and the Baltic (Chojnacki and Ciupinski, 1986; Hough et al., 1992; Mees et al., 1995). Most of the telson differences may be explained by regeneration of parts damaged by predation and cannot be related directly to physiological perturbations during molting. Still, a genetic or epigenetic basis cannot be ruled out completely (Mees et al., 1995). The degree of fluctuating asymmetry in mysids has been proposed as a quantifiable measure of morphological aberrations and is thought to arise from environmental or genetic stress during development (USEPA, 2002a). Since the results from earlier studies on morphological aberrations could not give a clear mechanistic explanation for the observed effects, preliminary studies examining different potential characteristics would first have to be performed in mysids, before further considering this endpoint.

2.5.7. Behavioral and other endpoints

Disruption of mysid swimming and position maintenance behavior has been investigated in laboratory studies with *Neomysis integer* exposed to sublethal concentrations of chlorpyrifos (an organophosporous pesticide) and cadmium (Buskey, 1998; Roast et al., 1998a, 2000a,c, 2001b, 2002). Although the mode of action of the toxicant on swimming remains unknown, the authors speculated that the disruption in chlorpyrifos-exposed mysids was probably due to the inhibitory action on acetylcholinesterase. In addition, Cripe et al. (1981) reported a reduction in the maximum sustained swimming speed of *Americamysis*

*bahia* following exposure to sublethal levels of two pesticides. Other authors have investigated the swarming behavior of mysids either in laboratory or field studies (Modlin, 1990; Ritz et al., 1997). For mysids, disruption of swimming/swarming behavior may lead to increased predation or displacement from optimum sites in the estuary (Roast et al., 1998a).

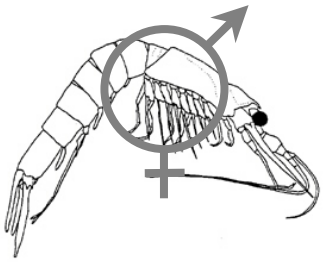
Other behavioral responses that have been measured in mysids include feeding activity (Engstrom et al., 2001; Jerling and Wooldridge, 1995; Nimmo et al., 1981; Viherluoto and Viitasalo, 2001a,b), swarming behavior (Buskey, 2000), grooming behavior (Acosta and Poirrier, 1992), burrowing ability (Nel et al., 1999) and predator/prey dynamics (Rademacher and Kils, 1996). The applicability of using behavioral responses as a monitoring tool, however, has little utility unless behavioral changes are understood within an ecological context, i.e. how well the patterns are understood within the context of an animal's natural life habits and ecological requirements (Olla et al., 1980a) and if the changes can be related clearly to internal residue levels or environmental levels of specific contaminants (Olla et al., 1980b).

Several studies have been published on osmotic regulation in mysids (Webb et al., 1997 and references therein) and the interaction between osmoregulation and chemical exposure (Kline and Stekoll, 2000; Wildgust and Jones, 1998). Other hormonal responses and disturbances in crustaceans, such as color changes (one of the earliest studied phenomenon that provided definite proof of a hormone-mediated process in a crustacean), retinal pigments and limb regeneration are discussed in a review by Fingerman et al. (1998). However, the use of these endpoints in mysids awaits further study.

### 2.6. Conclusions

This review demonstrates clearly the ecological relevance and the potential use of mysid shrimps as a test species for the evaluation of environmental endocrine disruption and as a potential surrogate for many other crustaceans. The highly standardized use of mysids in toxicity testing is an important advantage and research should be directed at evaluating the current standardized endpoints, such as survival, growth, and reproduction preferably through an entire life cycle, with a number of endocrine disruptors. In this context, a number of reference chemicals, chosen for their possible mode of action (i.e. ecdysone agonist, estrogen antagonist, juvenile hormone agonists, etc.) was proposed in DeFur et al. (1999) for evaluating relative endpoint sensitivity to potential endocrine-disrupting compounds. In addition, evidence of transgenerational effects has been published and presently a two-generation life-cycle protocol is being investigated with the standard species *Americamysis bahia*. However, an extensive list of non-standardized endpoints has been published and should be investigated further. Some of these endpoints, such as disruption of the

ecdysteroid metabolism and embryonic development, might differentiate for invertebrate-specific effects of chemicals. The selection of which mysid species to use will be a balance of its ecological relevance and its ease of use for measuring the selected endpoints. Clearly, the amount of available information and the relatively short life cycle of *A. bahia*, favor the use of this species, but its narrow salinity and temperature range limit its use in colder water or low-salinity testing. Various other mysid species are proposed in this review, together with a list of potential endpoints to evaluate the effects of endocrine disruptors in these animals. These should stimulate the scientific community to explore further the use of mysid shrimp as an invertebrate model for the evaluation of environmental endocrine disruption.



# CHAPTER 3

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# Energy metabolism of *Neomysis integer* in relation to a variable environment

**Redrafted after :**

Verslycke T, Janssen CR (2002) Effects of a changing abiotic on the energy metabolism in the estuarine mysid shrimp *Neomysis integer* (Crustacea: Mysidacea). *Journal of Experimental Marine Biology and Ecology* 279:61-72.

# Chapter 3

## *The energy metabolism of Neomysis integer in relation to a variable environment*

**Abstract**

Adaptations to life in an estuary include a wide salinity tolerance, an extremely efficient osmoregulatory and respiratory physiology. These adaptive mechanisms are energy-consuming and relatively little data is available on the combined effects of abiotic stress factors on the energy metabolism of mysid shrimp. A new methodology (cellular energy allocation, CEA) to assess the energy budget was adopted for the estuarine crustacean *Neomysis integer* (Crustacea: Mysidacea). The biochemical composition of *N. integer* was determined: protein (7.39 ± 1.81% wet weight), lipid (3.99 ± 1.05% ww) and sugar (0.42 ± 0.18% ww). To assess the effect of natural variability on the energy metabolic processes in *N. integer*, a fractional factorial test design was set up with different naturally (Scheldt estuary, The Netherlands) occurring combinations of temperature, salinity and dissolved oxygen. The different abiotic factors had no significant effect on the CEA of *N. integer* within the tested range, although significant effects were observed on the energy reserves and energy expenditure. Temperature and dissolved oxygen, in general, had the strongest effect on the energy allocation in *N. integer*. The present study demonstrates that *N. integer* efficiently regulates its energy metabolism in response to a variable environment to minimize changes in the CEA. By modeling the influence of these abiotic stresses on the energy metabolism (CEA) of *N. integer*, it will be possible to use the CEA as an ecologically relevant biomarker of exposure to pollutants in estuaries.

### 3.1. Introduction

Few environments expose their inhabitants to such a variety of abiotic variables and of such large magnitude, as does an estuary. The mysid *Neomysis integer* (Crustacea: Mysidacea) dominates the hyperbenthic fauna of the low-salinity regions of western European estuaries (Mees et al., 1995b; Mees and Jones, 1997). In these upper estuarine regions, this species is exposed to large tidal and seasonal fluctuations in temperature, salinity and dissolved oxygen (Moffat and Jones, 1992). For example, *N. integer* maintains a relatively permanent position in the East Looe River Estuary (Cornwall, UK) and the Scheldt

estuary (The Netherlands), despite being exposed to large daily fluctuations in its abiotic environment (Mees et al., 1994; Roast et al., 1998b). Adaptations to life in such an environment include a wide salinity tolerance (Mauchline, 1971), an extremely efficient osmoregulatory and respiratory physiology (hyper–hypo-osmoregulator) (McLusky and Heard, 1971; Roast et al., 1999b). These adaptive mechanisms are energy-consuming and relatively little data is available on the combined effects of abiotic stress factors on the energy metabolism of mysid shrimp. Changes in the energy metabolism, in general, will ultimately influence future life characteristics such as growth and reproduction.

In this context, a new methodology (cellular energy allocation, CEA) to assess the energy budget was adopted for the estuarine crustacean *N. integer* (De Coen and Janssen, 1997). Available energy reserves (total sugar, lipid and protein contents) and energy consumption (as derived from the electron transport activity) were quantified biochemically and integrated into a general stress indicator. To assess the natural variability of the energy metabolic processes in *N. integer*, a fractional factorial test design was set up with different naturally (Scheldt estuary) occurring combinations of temperature, salinity and dissolved oxygen. The influence of these natural variables on the energy metabolism in *N. integer* is described.

## 3.2. Material and Methods

### *3.2.1. Animal collection and maintenance*

Initial *Neomysis integer* populations were collected from the shore by hand net in the Galgenweel (a brackish water with a salinity of 3–5‰ near the river Scheldt, Antwerp, Belgium). After a 24-h 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‰. A 14-h light:10-h dark photoperiod was used during culturing and water temperature was maintained at 15 ± 1°C. Cultures were fed daily with 24–48-h-old *Artemia* nauplii *ad libitum* to prevent adult mysids from cannibalizing their young. Hatching of the *Artemia* cysts was performed in 1-l conical vessels under vigorous aeration and continuous illumination at 25°C.

### *3.2.2. Exposure to different abiotic variables*

The range of the abiotic variables was chosen from field data for the Scheldt estuary. The Scheldt estuary is the lower part of the river Scheldt (see Fig. 9.1 in Chapter 9). It is the last remaining true estuary of the Delta area and it is characterized by a marked salinity

gradient. The estuarine zone of the tidal zone extends from the North Sea (Vlissingen) to Antwerp, 80-km inland. The abiotic environment is further described in Chapter 9 and 10.

All exposures were done in a temperature-controlled chamber (Liebher®, Laborimpex, Brussels, Belgium). Temperature was set at 5 or 20°C (± 1°C) and dissolved oxygen was maintained at 110% or 70% saturation by aerating with respectively atmospheric air or reduced-oxygen air (14% $O_2$, Air Liquide, Belgium). The required salinity (5‰ or 25‰) was obtained by diluting artificial sea water (Instant Ocean®) with carbon-filtered deionized tap water. Final salinity was confirmed with a portable refractometer (Digit 032, CETI, Belgium). Dissolved oxygen and temperature were measured at least two times a day (Oxi 191, WTW, Germany) and were within 5% of the desired value.

Test organisms of about equal size (average wet weight of all animals used: 11.9 ± 4.6 mg) were collected from the cultures and randomly distributed in 1-l glass beakers (10 organisms/beaker). Test animals were allowed a 24-h acclimation period to the test conditions, after which 20 organisms were collected (animals day 0), shock-frozen in liquid nitrogen and kept at -80°C until analysis. The remaining organisms were further exposed to a selected temperature–salinity–dissolved oxygen combination and were collected in the same way after 120 h (animals day 4). It was decided not to feed the animals during the exposure period, to avoid variance due to individual differences in feeding.

### *3.2.3. Factorial design*

The influence of the different abiotic parameters on the energy metabolism of *Neomysis integer* was evaluated in a factorial design (Statsoft, 1994). In such a design, factors are tested at two levels. This implies that only linear relationships can be detected because only two levels are tested per factor and curvature cannot be detected from that portion alone. The most intuitive approach to study the influence of multiple factors is to vary the factors of interest in a full factorial design to try all possible combinations of the settings. In this way, the number of necessary runs in the experiment increases geometrically. In this study, temperature, salinity and dissolved oxygen were varied according to a $2^3$ full factorial design (eight designs were tested) and, to avoid systematic errors, the order of the runs was randomized (Table 3.1). Temperature, salinity and dissolved oxygen were varied within a naturally occurring range, representative of the physiological limits of *N. integer*.

### *3.2.4. CEA measurement*

The CEA was measured according to De Coen and Janssen (1997) with major modifications. The different energy reserve fractions (lipids, protein, sugar) were determined

**Table 3.1.** Full factorial design to assess the effects of the abiotic variables temperature (T), salinity (S) and dissolved oxygen (DO) on the energy metabolism of the mysid *N. integer*

| Design no. | Water parameters | | | Cellular energy allocation | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | T (°C) | S (‰) | DO (%) | ΔSugar reserve[a] (mJ/mg ww) | ΔProtein reserve[a] (mJ/ mg ww) | ΔLipid reserve[a] (mJ/ mg ww) | $\Delta E_a$ (mJ/mg ww) | 4d-$E_c$ (mJ/ mg ww) | CEA |
| 1 | 5 | 5 | 110 | 20.8 ± 27.2 | -149.8 ± 651.4 | -695.4 ± 667.3[b] | -824 ± 933[b] | 6973 ± 1629[b] | -0.118 ± 0.137 |
| 2 | 20 | 25 | 110 | 3.4 ± 13.0 | 448.1 ± 886.3 | 163.2 ± 743.8 | 614 ± 1157 | 5633 ± 1595 | 0.109 ± 0.208 |
| 3 | 20 | 5 | 70 | 13.5 ± 10.8[b] | 1067.5 ± 437.4[b] | 570.5 ± 422.7[b] | 1651 ± 608[b] | 4498 ± 2190 | 0.367 ± 0.224 |
| 4 | 5 | 25 | 70 | 24.9 ± 13.0[b] | 183.5 ± 563.3 | 269.2 ± 269.1 | 478 ± 624 | 6393 ± 1735 | 0.075 ± 0.100 |
| 5 | 5 | 5 | 70 | -4.0 ± 14.0 | 37.3 ± 245.9 | -19.8 ± 222.0 | 13 ± 332 | 4724 ± 753 | 0.003 ± 0.070 |
| 6 | 20 | 25 | 70 | 17.7 ± 6.0[b] | 68.6 ± 286.1 | -11.7 ± 270.1 | 75 ± 393 | 3359 ± 643[b] | 0.022 ± 0.117 |
| 7 | 20 | 5 | 110 | 1.0 ± 19.3 | -39.8 ± 133.5 | -145.4 ± 373.7 | -184 ± 397 | 5091 ± 1443 | -0.036 ± 0.079 |
| 8 | 5 | 25 | 110 | 40.9 ± 29.6[b] | -588.2 ± 134.5[b] | -141.3 ± 455.8 | -689 ± 476[b] | 4769 ± 1090 | -0.144 ± 0.105 |

[a] Energy reserve day 0 – energy reserve day 4.
[b] Significant change in energy reserve, energy consumption or CEA between day 0 and day 4 (ANOVA, $p < 0.05$).

spectrophotometrically and transformed into energetic equivalents using their respective energy of combustion (17,500 mJ/mg glycogen, 24,000 mJ/mg protein, 39,500 mJ/mg lipid) (Gnaiger, 1983). The energy consumed ($E_c$) was estimated by measuring the electron transport activity according to Owens and King (1975). The quantity of oxygen consumed per mysid, as derived from the ETS data, was transformed into energetic equivalents using the oxyenthalpic equivalents for an average lipid, protein and sugar mixture (484 kJ/mol $O_2$) (Gnaiger, 1983).

The $E_a$, $E_c$ and CEA values were calculated as follows:

$\Delta E_a$ (difference in available energy, $\Delta E_a$) = $E_{a,\ day\ 0} - E_{a,\ day\ 4}$

$E_c$ (average energy consumption, 4d-$E_c$) = 1/2*($E_{c,\ day\ 0}$ + $E_{c,\ day\ 4}$)

CEA (cellular energy allocation) = $\Delta E_a/E_c$

Alternatively, since all animals were collected from the same laboratory culture, the variation in the initial energy status of the animals used for the different experiments can be considered insignificant in comparison to the variation induced by the exposure. As such, the energetic status (lipid, sugar and protein content at day 4, $E_{a,\ day\ 4}$, $E_{c,\ day\ 4}$, and $E_a/E_c$) of the animals at the end of the 96-h exposure period is also indicative of the exposure effect.

*3.2.5. Statistical analysis*

All data were checked for normality and homogeneity of variance using Kolmogorov–Smirnov and Levene's test, respectively with an $\alpha$ = 0.05. The factorial design was analyzed using the experimental design module in the software package Statistica™. The effect of the treatment was tested for significance using a one-way analysis of variance (Tukey's Honestly Significant Difference test, Statistica™). In addition, a factorial ANOVA was performed on the data at day 4 (lipid, sugar, protein, $E_{a,\ day\ 4}$, $E_{c,\ day\ 4}$, and $E_a/E_c$). The effect of temperature, salinity and dissolved oxygen were tested individually and in combination. Significant differences were detected via Tukey's Honestly Significant Difference test .

**3.3. Results**

The biochemical composition of *Neomysis integer* was as follows: protein (7.39 ± 1.81% wet weight), lipid (3.99 ± 1.05% ww) and sugar (0.42 ± 0.18% ww). The changes in energy reserves during the 4-day exposure period are summarized in Table 3.1. Sugar reserves were mainly used as an energy source and higher salinities were correlated with a higher sugar consumption. The protein fraction was quantitatively the most important energy fraction and exhibited large variation (1714.6 ± 467.5 mJ/mg ww). Similar large variations

were found in the lipid content (1553.8 ± 493.6 mJ/mg ww). The energy consumption as measured by the electron transport activity was relatively unaffected after the 4-day exposure, except for a significant reduction in design 1 (5°C – 5‰ – 110% DO) and design 6 (20°C – 25‰ – 70% DO).

To determine individual effects of the tested abiotic factors, an analysis of variance was performed on the complete data set (Table 3.2). The different abiotic factors did not result in a significant effect on the energy metabolism of *N. integer* within the tested range. The small variance in energy consumption could not be explained by a single abiotic factor. Temperature explained some of the variation in lipid and protein content, the change in total energy reserves ($\Delta E_a$) and the CEA. Salinity was the most important factor explaining the variations in sugar reserves, but both temperature and salinity effects were not significant.

However, when the different energy parameters at day 4 (lipid, sugar, protein, $E_{a,\ day\ 4}$, $E_{c,\ day\ 4}$, and $E_a/E_c$) were analyzed in a multifactorial ANOVA, significant effects were observed for all parameters with temperature as an independent variable (i.e., all parameters were higher at 5°C in comparison with these at 20°C). In addition, dissolved oxygen had a significant effect on $E_{a,\ day\ 4}$ and $E_{c,\ day\ 4}$ (Fig. 3.1). Significant interaction effects were observed between temperature and dissolved oxygen on $E_a/E_c$. Since an increase in energy consumption also led to an increase in the available amount of total energy, the net effect on the cellular energy allocation was almost zero. From this it can be concluded that, within a variable environment, mysids efficiently adapt their energy metabolism to cope with an increase in energy demand and to preserve normal metabolic function.

**Table 3.2.** The influence of temperature (T), salinity (S) and dissolved oxygen (DO) on the energy metabolism of *N. integer*

| Energetic fraction | Abiotic factor[a] | | |
|---|---|---|---|
| ΔSugar | S (p = 0.24, ↓) | T (p = 0.31, ↑) | DO (p = 0.75, ↓) |
| ΔProtein | T (p = 0.15, ↓) | DO (p = 0.21, ↑) | S (p = 0.52, ↑) |
| ΔLipid | DO (p = 0.15, ↑) | T (p = 0.27, ↓) | S (p = 0.56, ↓) |
| $\Delta E_a$ | DO (p = 0.15, ↑) | T (p = 0.16, ↓) | S (p = 0.93, ↑) |
| 4d-$E_c$ | DO (p = 0.90, ↑) | T (p = 0.95, ↑) | S (p = 0.96, ↑) |
| CEA | DO (p = 0.15, ↑) | T (p = 0.16, ↓) | S (p = 0.70, ↑) |

[a] The abiotic factors are ranked according to their importance in explaining the variance of the tested variable (full factorial design, ANOVA, Statistica™). The level of significance and the nature of the effect are shown (↑ positive; ↓ negative).

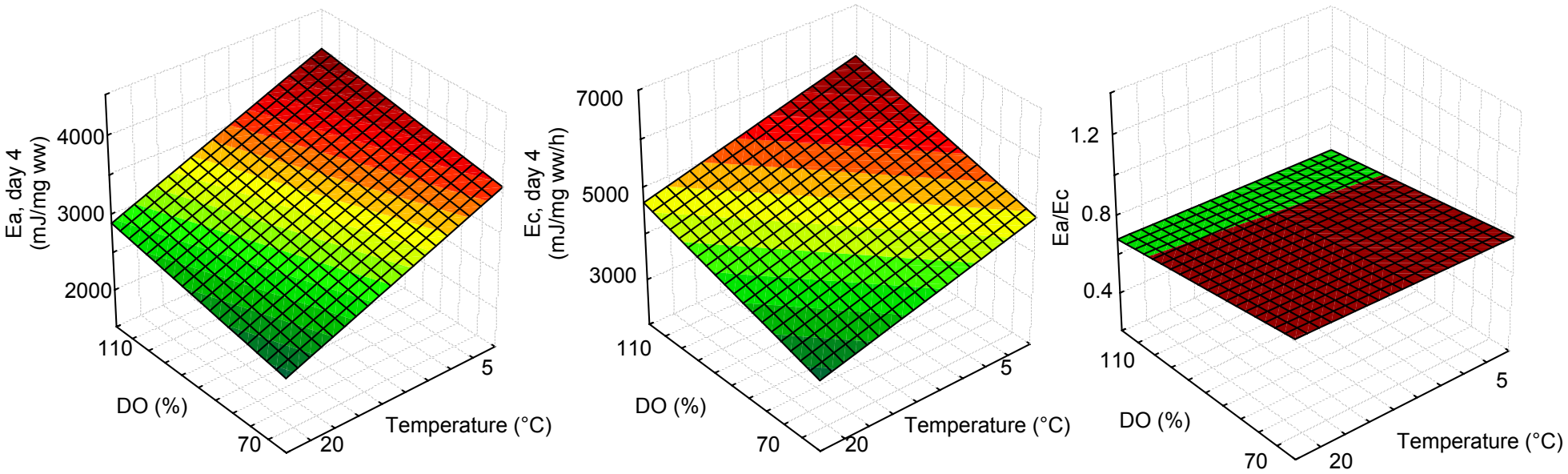



**Figure. 3.1.** Surface-response plots of the available energy $E_{a,\ day\ 4}$, the energy consumption $E_{c,\ day\ 4}$ and the cellular energy allocation $E_a/E_c$ following a 96-h exposure period in *N.* integer as a function of dissolved oxygen (DO) and temperature (T).

Interaction effects of the different abiotic factors on the CEA (as given in Table 3.1) were also tested (Fig. 3.2). Although interaction effects were apparent (deviation from 0-effect on CEA in the abscissa), no significant effect could be found on the energy budget by the interaction of the three tested abiotic factors; temperature, in general, had the most adverse effect on the CEA. Contrary to this, significant interactions between dissolved oxygen and temperature on the CEA (calculated as $E_a/E_c$ ) were found when analyzing these parameters at day 4 (Fig. 3.1). Most probably, these effects were not found to be significant when calculating with the energy difference between day 0 and day 4 (Table 3.1, 3.2 and Fig. 3.2), because animals had already partly adapted their energy metabolism over the acclimation period of 24 h.

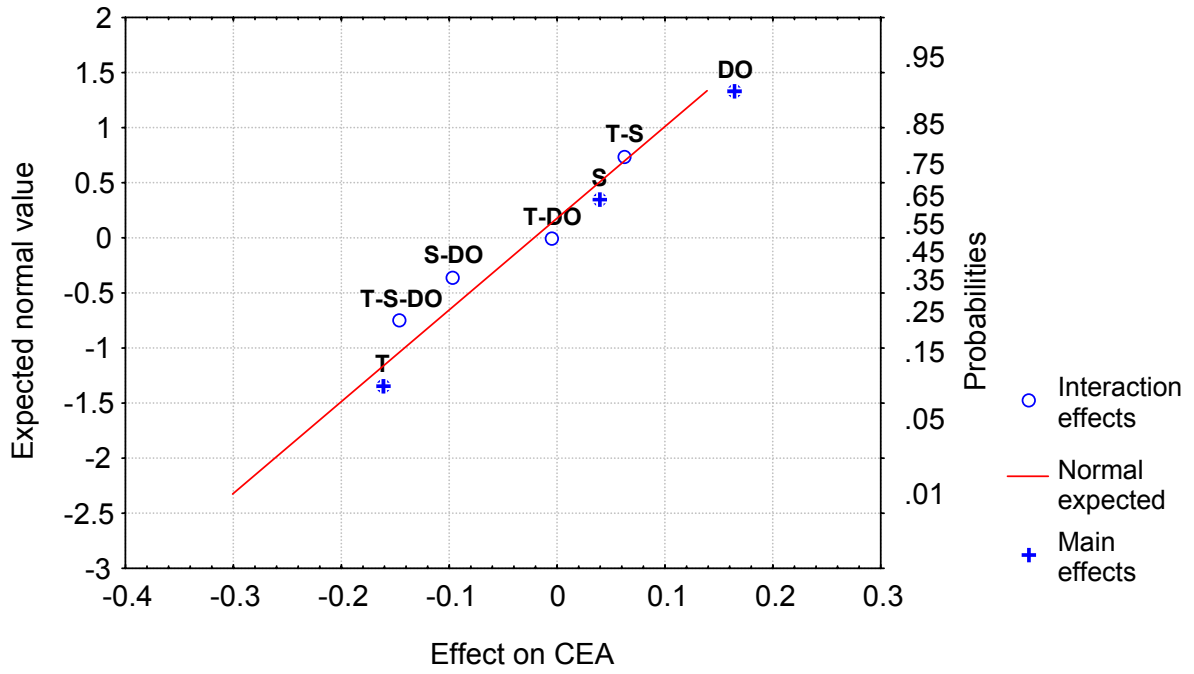



**Figure. 3.2.** Normal probability plot of the individual and interaction effects of the selected abiotic factors (T: temperature; S: salinity; DO: dissolved oxygen) on the energy metabolism of *N. integer*. True effects are seen as outliers on the graph (Statistica™).

## 3.4. Discussion

### *3.4.1. Biochemical composition of N. integer*

Knowledge of the biochemical composition of a species is essential to the understanding of its metabolism. The different biochemical fractions of *Neomysis integer* were determined simultaneously. Although lower than reported by Raymont et al. (1964), the protein content (7.39 ± 1.81% ww) of *N. integer* was found to be high and quantitatively the

most important fraction. The low levels of sugar (0.42 ± 0.18% ww) and lipid (3.99 ± 1.05% ww) confirm earlier findings (Bhat and Wagh, 1992; Raymont et al., 1964). Differences in energy content reported in our study in comparison to other studies might be caused by the artificial feeding of *N. integer* with lipid-enriched *Artemia* (*Artemia* were enriched with lipid-containing SuperSelco-INVE, Aquaculture NV, Belgium), or as a result of the different (colorimetric) methods used in our study. Mysids are known to be omnivores and base their metabolism on food, which is more or less immediately available, and food storage may be less significant (Fockedey and Mees, 1999; Mauchline, 1971; Tattersall and Tattersall, 1951). The artificial feeding of these animals in the laboratory may thus explain the different biochemical composition compared to that of field-collected mysids. Since the main objective of this study was to investigate changes in the energy metabolism during a relatively short period under different environmental conditions, feeding was stopped during the exposures. In this way, the metabolic use of energy reserves were quantified, without having interference of effects on feeding behavior and energy intake (energy was only lost during the experiment).

### *3.4.2. Effects on energy reserves*

The effects of different combinations of temperature, salinity and dissolved oxygen on the energy status of *Neomysis integer* were tested to gain insight into the natural variability of the energy metabolism under field-based conditions.

As the animals were not fed during the exposure period, no significant increase of any of the biochemical fractions was expected. The significant increase in some biochemical fractions are attributed to cannibalism (personal observation) and were taken into account when interpreting the results (only negative effects were seen as true effects).

With exception of design 5 (5°C – 5‰ – 70% DO), all tested designs resulted in a reduction of the sugar content (Table 3.1). Sugar depletion could be correlated, although not significantly ($p = 0.24$), with changes in salinity (Table 3.2). *N. integer* is a hyper- and hypo-osmoregulator with its iso-osmotic point situated around 18‰, and a high tolerance for salinities between 1‰ and 40‰ (McLusky and Heard, 1971; Roast et al., 2001a). Thus, at salinities of 5‰ and 25‰, *N. integer* actively maintains its hemolymph respectively hyper- or hypo-osmotic to the external environment. The actual osmoregulatory mechanisms of mysids have not been demonstrated, and may be different from those of decapods (Tattersall and Tattersall, 1951). It is, however, obvious that when placed in a medium of higher or lower osmotic pressure, energy-consuming processes are required to maintain the internal osmotic pressure constant. Glucose delivers fast energy in the form of ATP via the process of glycolysis and oxidative phosphorylation and is the major circulating carbohydrate in

crustaceans (Morris and Airriess, 1998). Although lipid and protein utilization is variable in crustaceans, generally, carbohydrate is used before lipid and protein as the preferred fuel for metabolic processes (Garret and Grisham, 1995; Morris and Airriess, 1998). The mysids in this study were acclimated to a salinity of 5‰ and transferred into a test medium of either the same (5‰) or higher salinity (25‰). Transfer to a higher salinity requires the organism to shift its osmoregulation from hypo- to hyperosmotic, which may explain the significantly higher use of sugar reserves (in designs 4-6-8) as a fast energy source to fuel these processes.

The effects of the abiotic factors on the lipid reserves were not clear. Higher temperatures, higher salinities and lower dissolved oxygen concentrations resulted in increased lipid use. Linford (1965) found that *N. integer* starved for 96 h showed little decrease in lipid content. Contrary to this, Morris (1971) found significant changes in the lipid content (25% lipid loss after 7 days) of *N. integer* in a series of experiments to assess the effects of temperature and salinity. Morris (1971) also suggested that the lipid fraction of *N. integer* contains a very conservative (in its fatty acid composition) element, which plays an important role in the metabolism of the animal. Our findings support the views of Morris that relatively large variations in lipid reserves are found in *N. integer*. Organisms were not sexed and consequently male and female organisms were randomly used for each experiment. The eggs of mysids contain high amounts of lipids and the lipid metabolism of ovigerous females can be assumed to be different from males (Linford, 1965). The large variations in lipid metabolism observed in our study might be partially caused by a sex-specific lipid metabolism.

The biochemical composition of the estuarine mysid *N. integer* contrasts sharply with the typical mammal in its low carbohydrate reserves and fat depots. The large amount of protein observed in our study, might suggest that *N. integer* actively deaminates its body proteins, especially should food shortage occur. In this event, the mysid, a relatively small aquatic animal should experience no great difficulty in ridding its body of ammonia arising from deamination (Raymont et al., 1968). The potential use of protein as a metabolic reserve has also been suggested by Bhat and Wagh (1992) for marine zooplankton. The decline in protein reserves in our study after a 4-day exposure supports the hypothesis that *N. integer* can actively use protein as an energy source under starvation or stress conditions. Longer exposures will be performed to confirm these results. In these experiments, the effect of ecdysis on the protein metabolism and the general energy metabolism will also be assessed. The exoskeleton of *N. integer* contains large amounts of protein and ecdysis occurs in a temperature and age dependent way. In juvenile *N. integer*, the intermolt period varies from 2–3 up to 10 days at 9°C and 16°C, respectively (Asthorsson and Ralph, 1984; Mauchline, 1985). Although no exoskeletons were observed in the exposure beakers (the exoskeleton is

however rapidly consumed by other animals), large variations (as in design 3) could be attributed to ecdysis.

*3.4.3. Effects on energy consumption*

The oxygen consumption of *Neomysis integer* is dependent on several abiotic (e.g. temperature, salinity) and biotic (age, weight, gender, reproductive status) parameters (Burggren and Roberts, 1991; Kinne, 1970, 1971; Newell and Branch, 1980; Roast et al., 1999b; Schmidt-Nielsen, 1997). Each factor may have an independent effect, or all factors may interact and influence the respiratory metabolism, making it a difficult parameter to interpret. The method described in this study to measure respiration was adopted from Owens and King (1975). The method for measuring the activity of the electron transport system provides an estimate of the potential whole organism respiration rate and has been validated in our laboratory for *N. integer* against real-time respiration measurements with a respirometer (RC650 Strathkelvin Instruments). Both measurements were highly correlated ($R^2 = 0.94$; $p < 0.01$, $n = 6$; data not shown).

From the analysis of variance on the average energy consumption (4d-$E_c$), it can be concluded that none of the three tested abiotic parameters had a significant effect on the measured ETS activity (Table 3.2). Biotic variables such as age, weight and gender probably have a larger influence on respiration rates. Furthermore, the ETS assay measures the maximum ETS activity under saturated substrate (NADH, NADPH) conditions and observed changes in activity must therefore be realized through changes in the amount of enzymes produced by the organism. Consequently, ETS activity responds much slower to changes in the environment than the respiration rate (Båmstedt, 1980; Mayzaud, 1986; Skjoldal et al., 1984). Båmstedt (2000) has recently published an alternative to the Owens and King (1975) method based on natural levels of substrate in an attempt to achieve a better correlation between the ETS assay and ambient respiration rates. This method looks promising but needs further validation.

Taking into account the inherent properties of the ETS assay, the energy consumption reported in our study is an overestimation of the ambient respiration. This is clear when considering the energetic content calculations for the organisms. The average calculated energetic content ($E_{a,\ day\ 0}$) on day 0 was 3405 mJ/mg ww, the average energy consumption (4d-$E_c$) for the 4-day period derived from the ETS assay was 5180 mJ/mg ww. From these calculations *N. integer* would use up all of its energy resources in less than 3 days provided there is no energy intake. As mentioned before, the ambient respiration is a lot lower than calculated by the ETS assay, and mysids consumed considerably less energy during the exposure period (max. 1651 mJ/mg ww; $\Delta E_a$, Table 3.1). Although energy consumption

calculated by ETS activity is thus an overestimation of actual respiration and can also be derived more correctly from the decline in energy reserves, there are advantages associated with ETS measurements. Especially with reference to exposures with toxic compounds, the ETS assay can mechanistically explain alterations in the energy consumption through specific interaction of the toxicant with the electron transport system (Oberdörster et al., 1998c; Spicer and Weber, 1991). It should, however, be mentioned that, although effects on average ETS activities between day 0 and day 4 (4d-$E_c$) were not found significant by the factorial design, ETS activities at day 4 were significantly affected by temperature and dissolved oxygen (Fig. 3.1).

*3.4.4. Interaction effects on the cellular energy allocation (CEA)*

Although apparent interaction between temperature, salinity and dissolved oxygen can be derived from the normal-probability plot shown in Fig. 3.2, none of these effects was significant. Thus, the energy metabolic processes of *Neomysis integer* seem to be relatively unaffected by changes in the abiotic environment. Interestingly, significant effects of temperature and dissolved oxygen were found on available energy and energy consumption when considering the animals at day 4. However, the joint effect of these factors on the energy allocation was almost zero (Fig. 3.1). This may be explained by an efficient physiological adaptation mechanism in *N. integer* which responds to an increase in energy demand, by a more efficient metabolism, resulting in more available energy and consequently only a minor change in overall energy allocation.

Overall, temperature and dissolved oxygen had the strongest effect on the CEA (Table 3.2; Fig. 3.1). The relatively higher sensitivity towards changes in temperature might be explained by the fact that this parameter is also the least variable over a small time period in the natural environment. Where salinity (and to a smaller extent, dissolved oxygen) show high fluctuations linked to tidal influences in an estuary, temperature changes more slowly with a seasonal variability. Furthermore, there is a direct physico-chemical link between temperature and dissolved oxygen, which would explain observed correlations in physiological response to these two factors. This conclusion is supported by Roast et al. (2000b) examining the effect of fluctuating abiotic factors (temperature, salinity) on the egestion rate of *N. integer*. In this study, temperature was the most important factor causing increased egestion rates in *N. integer*. These would correspond with a change in CEA in our study. Weisse and Rudstam (1989) also found temperature to be the most important abiotic factor explaining variance in excretion and respiration rates in *N. integer*. We are currently performing an inter-laboratory validation of our CEA method with the Scope for Growth approach based assay of Roast et al. (1999c), to further mechanistically explain our common

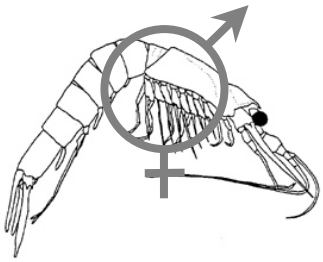
results and examine the future use of CEA as a biomarker for exposure (Verslycke et al., submitted-Chapter 5).

The CEA methodology described in this study not only provides an integrated quantification of the organism's energy budget but also helps to elucidate different modes of action upon exposure to a varying abiotic environment and could potentially explain the different modes of action of toxicants. Furthermore the CEA methodology has been used with success with other organisms, e.g. the crustacean *Daphnia magna* (De Coen and Janssen, 1997) and catfish *Clarias gariepinus* where Nguyen (1997) demonstrated that the CEA criterion was predictive of long-term effects on growth. This illustrates the potential use of the cellular energy allocation as a methodology to assess the energy budget of organisms using simple colorimetric measurements. Due to the observed relationships with long-term population effects, as described by De Coen and Janssen (1997) for *D. magna*, this biomarker might also be useful in field monitoring programs.

### 3.5. Conclusion

In addition to its potential as a biomarker of exposure to environmental toxicants, the CEA methodology can be very useful as an applied physiological research tool to study the energy metabolism of an organism, as shown in this study. In contrast to the Scope for Growth assay (Warren and Davies, 1967), the CEA methodology offers the possibility to assess (separately) both energy conversion and energy allocation. Although no significant effects of the tested abiotic variables were apparent during the 4-day exposure period, preliminary conclusions on the involved mechanisms and potential effects of natural variability in *Neomysis integer*'s natural environment, are possible. Generally, these results were in accordance with previous studies on the energy metabolism of *N. integer.* Studies like this allow a more integrative approach when studying field effects in laboratory experiments. In this respect, the used factorial design approach has great potential when a large number of parameters need to be tested.

Future research will focus on the validation of the CEA methodology with other described methodologies and the use of the CEA as biomarker of exposure in laboratory and field. The use of the CEA assay is, in this context, also being tested *in situ* on resident mysid populations in the Scheldt estuary.



# CHAPTER 4

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# Energy metabolism of *Neomysis integer* following exposure to tributyltin

**Redrafted after :**

Verslycke T, Vercauteren J, Devos C, Moens L, Sandra P and Janssen CR (2003) Cellular energy allocation in the estuarine mysid shrimp *Neomysis integer* (Crustacea: Mysidacea) following tributyltin exposure. *Journal of Experimental Marine Biology and Ecology* 288:167-179.

**and**

Verslycke T, Vercauteren J, Moens L, Sandra P and Janssen CR (submitted) Uptake and effects of tributyltin in the estuarine mysid shrimp *Neomysis integer* (Crustacea: Mysidacea). *Marine Environmental Research*.

# Chapter 4

## *Energy metabolism of Neomysis integer following exposure to tributyltin*

**Abstract**

Recently, we described the cellular energy allocation (CEA) methodology to asses the effects of abiotic stress on the energy metabolism of the estuarine crustacean *Neomysis integer* (Crustacea: Mysidacea) (Verslycke and Janssen, 2002). This short-term assay is based on the biochemical assessment of changes in the energy reserves (total carbohydrate, protein and lipid content) and the energy consumption (electron transport activity) and has been shown to be predictive of effects at the population level in daphnids (De Coen and Janssen, 1997). In the present study, the CEA methodology was evaluated using adult *N. integer* exposed for 96 h to the antifoulant tributyltinchloride (TBTCl). From a range-finding experiment with juvenile *N. integer*, a 96-h LC50 of 164 ng TBTCl/l was calculated. The energy metabolism of *N. integer*, as summarized by the CEA, was significantly altered by TBTCl exposure. Mysids exposed to 10, 100 and 1000 ng TBTCl/l contained less energy and had lower respiration rates (in 10 and 1000 ng TBTCl/l treatment) than the control, resulting in a lower CEA. These changes at the cellular level occurred at environmentally relevant concentrations of the toxicant TBTCl which were an order of magnitude lower than reported effect concentrations for scope for growth in other marine invertebrates. The high sensitivity of mysids towards the sublethal and lethal effects of TBT may be a result of the low *in vivo* metabolism of this compound in mysids leading to high TBT body burdens.

### 4.1. Introduction

Tributyltin (TBT) is a highly toxic and widespread contaminant in aquatic environments and has caused worldwide imposex (a pseudohermaphroditic condition, characterized by the development of a penis, vas deferens, and seminiferous tubules in females) in marine gastropods (DeFur et al., 1999). The main inputs of TBT in the marine environment are from antifouling paints used on boats. In 1985, the world production of triorganotins with biocide properties was in the range of 8-10,000 tons annually (de Mora, 1996). Over recent decades, the use and the production of TBT-containing paints has been restricted, resulting in

significant reductions in environmental concentrations (Alzieu, 1998). Despite these restrictive regulations, coastal TBT contamination can still reach up to 200 ng TBT/l (Michel et al., 2001).

In order to evaluate effects of pollutants, a number of assays have been developed linking effects at the (sub)organismal level with population level effects (Kooijman and Metz, 1984; Nisbet et al., 1989). The 'metabolic cost' hypothesis suggests that toxic stress induces metabolic changes in an organism which might lead to a depletion of its energy reserves resulting in adverse effects on growth and reproduction (Calow and Sibly, 1990). Probably the most successful application of this principle has been the 'scope for growth' concept, combining individual responses, such as respiration, excretion rate and assimilation efficiency into a single, integrated bioassay (Widdows and Salkeld, 1993b). Scope for growth provides a measurement of the energy status of an organism, but remains labor-intensive, often results in a highly variable response (Bühringer and Danischewski, 2001) and is not easily employed in routine environmental impact assessments (De Coen and Janssen, 1997). Recently, we described an alternative methodology (cellular energy allocation, CEA) to assess the energy budget for the estuarine crustacean *Neomysis integer* (Verslycke and Janssen, 2002). The CEA methodology not only provides an integrated quantification of the organism's energy budget but also helps to elucidate different modes of action upon exposure to a varying abiotic environment and could potentially explain the different modes of action of toxicants.

In this paper, the CEA methodology was used to assess the effect of tributyltinchloride (TBTCl) on the energy allocation in the mysid *N. integer*. To evaluate acute toxicity of TBTCl to *N. integer*, an initial toxicity test was set up with juvenile mysids. In a subsequent exposure experiment, the effects of TBTCl on the energy metabolism of *N. integer* were evaluated. Finally, the uptake of TBTCl by *N. integer* was evaluated in an additional experiment.

Mysids are used routinely in laboratory toxicity tests and standard guides for conducting life-cycle toxicity tests with saltwater mysids have been developed using the sub-tropical American species, *Americamysis* (formerly *Mysidopsis*) *bahia* as a model (ASTM, 1999; USEPA, 1995a, 1997). While specifically derived from work with *A. bahia*, these standard methods can be modified for testing with other mysid species (Roast et al., 1998a; Verslycke et al., submitted-Chapter 2). Since *A. bahia* has limited tolerance for lower salinities and is not representative of European waters, *N. integer* has been proposed as a test species for pollution monitoring in European estuaries (Emson and Crane, 1994; Roast et al., 1998a; Roast et al., 1998b; Roast et al., 1999b; Verslycke et al., submitted-Chapter 2). *N. integer* is the dominant hyperbenthic mysid in European estuaries (Mees et al., 1995; Mees and Jones, 1997) and is sensitive to many toxicants at environmental concentrations (Roast et al., 1998a; Verslycke et al., 2003b; Wildgust and Jones, 1998). Furthermore, the

extensive works of McKenney (1986), McKenney and Celestial (1996), McKenney and Matthews (1990) and McKenney et al. (1991) with *A. bahia* have resulted in several test procedures evaluating alterations in growth and reproductive responses upon toxicant exposure. In addition, transgenerational exposures are possible, gross morphological and histopathological changes may be observed, feeding and mating behavior and osmoregulatory capacities may be quantified and cage experiments with mysids have been successfully performed in the field (for a review refer to Verslycke et al., submitted-Chapter 2).

## 4.2. Material and Methods

### *4.2.1. Animal collection and maintenance*

Initial *Neomysis integer* populations were collected from the Galgenweel (Antwerp, Belgium) and cultured in the lab as described in Chapter 3 (§ 3.2.1).

### *4.2.2. Acute toxicity of TBTCl*

Juvenile mysids were randomly distributed in 1.5-l glass beakers (10 per concentration, two replicates), with each containing 1 l of the required TBTCl concentration (control, 10, 100, 1000, 10,000, or 100,000 ng/l) in water with a salinity of 5‰ (diluted from artificial sea water, Instant Ocean®; Aquarium Systems, Sarrebourg, France). The TBT was delivered to the exposure solutions in absolute ethanol. The ethanol concentration in the solvent control was 0.01%. Exposure temperature was 15 ± 1°C, and test solutions were renewed after 48 h. Animals were fed twice daily with 24- to 48-h-old *Artemia* nauplii (about 75 *Artemia*/mysid). Mortality was recorded daily.

### *4.2.3. Effects of TBTCl on the CEA of N. integer*

Duplicate groups of *Neomysis integer* were exposed to 10, 100 and 1000 ng TBTCl/l and a control for 96 h. Animals were exposed in duplicate glass aquaria (20 × 15 × 15 cm) divided into six chambers by nylon screen (each chamber containing two individuals) resulting in 24 mysids per test concentration. Test organisms of about equal size (average wet weight of all animals used: 10.9 ± 3.3 mg) were collected from the cultures and either directly shock-frozen in liquid nitrogen and kept at -80°C until analysis (day 0 animals) or randomly distributed into the test chambers. After 96 h, the mysids were removed from the exposure, shock-frozen in liquid nitrogen and kept at -80°C until analysis (day 4 animals). It was

decided not to feed the animals during the exposure period, to avoid variance due to individual differences in feeding. In case of partial mortality at the end of the 96-h exposure period, surviving animals were divided over the different biochemical analyses (sugar, lipid, protein and electron transport activity) in a way that at least three replicate measurements were performed for each parameter in each treatment.

All exposures were done in a temperature-controlled chamber (Liebher®, Laborimpex, Brussels, Belgium). Temperature was set at 15°C (± 1°C) and dissolved oxygen was maintained at saturation levels by continous aeration. The required salinity (5‰) was obtained by diluting artificial sea water (Instant Ocean®) with carbon-filtered deionized tap water. Final salinity was confirmed with a portable refractometer (Digit 032, CETI, Belgium). Dissolved oxygen and temperature were measured twice daily (Oxi 191, WTW, Germany) and were within 5% of the desired value. The tributyltin was delivered to the exposure solutions in absolute ethanol and concentrations were checked with GC/ICP-MS. The concentration of ethanol in the solvent control was 0.01%. Test concentrations were renewed after 48 h.

*4.2.4. Uptake of TBTCl by N. integer*

In an additional experiment the uptake of TBTCl was investigated in *Neomysis integer* following a 96-h exposure period to the same concentrations as in the CEA experiment. Juvenile mysids were randomly distributed to 1.5-l glass beakers (15 per concentration, two replicate beakers per concentration), each containing 1 l of the required TBTCl concentration (10, 100 and 1000 ng TBTCl/l) in water with a salinity of 5‰ (diluted from artificial sea water, Instant Ocean®, with carbon-filtered deionized tap water). The tributyltin was delivered to the exposure solutions in absolute ethanol. The concentration of ethanol in the solvent control was 0.1%. Exposure temperature was 15 ± 1°C and exposure solutions were renewed after 48 h. Animals were fed twice daily with 24h- to 48-h-old *Artemia* nauplii (about 75 *Artemia/*mysid). At the end of the 96-h exposure period, the animals were removed from the exposure medium, blotted dry on paper and randomized aliquots of 50 ± 1 mg wet mysid biomass (about 2-3 mysids) were weighed on an analytical balance and placed in separate 1.5 ml cryogen vials. Ten of these aliquots were prepared per exposure concentration. These samples were then homogenized with a motor-driven teflon plotter in 100 µl double-deionized carbon-filtered water, shock-frozen in liquid-nitrogen and kept at -20°C until TBT analysis. Three replicate water samples per concentration, both fresh and 48-h old solutions, were transferred into 100 ml dark bottles and kept at less than 4°C until determination of the TBT/DBT/MBT-concentrations by GC/ICP-MS within a week.

*4.2.5. TBTCl analysis in mysid and water*

To allow accurate quantification of TBTCl concentrations in mysids, both an internal standard (tripropyltinchloride, TPrTCl) and standard addition (TBTCl) were used. Five replicate measurements were performed with a TBT spike and five without this spike. Standard addition allows a calculation of the signal/response ratio in the sample matrix. Internal standardization with TPrT corrects for small differences in derivatization efficiency, extraction efficiency, ICP-MS sensitivity, etc. Mysids were destructed to release organotins using a tetramethylammonium-hydroxide (TMAH) solution. Next, an internal standard (5 ng TPrTCl) and a tributyltinchloride spike (5 ng TBTCl), in case of standard addition, were added: 40 ml vial + 50 mg mysids + 50 µl TPrTCl (100 µg/l in EtOH) + [50 µl TPrTCl (100 µg/l in EtOH)] + 750 µl TMAH (25% in MQ-water) + Teflon® stir bar; stir for 60 min at 60°C.

Since most organotins are not sufficiently volatile and too polar to be separated by gas chromatography (GC), they were ethylated in a 500 µl sodiumtetraethylborate ($NaBEt_4$) solution (1% in MQ-water). This reaction is optimal in an acetate buffer with a pH of 5.0 (0.2 M NaOAc + HOAc).

The third step is the headspace solid phase microextraction (SPME) in which the organotins are extracted with a polydimethylsiloxane (PDMS)-coated fiber (100 µm PDMS, Supelco). The needle of the SPME device was pierced through the septum of the sample vial and the PDMS fiber was exposed to the sample headspace for 15 min at 50°C. Subsequently, the fiber was withdrawn from the sample and inserted into the GC liner for thermal desorption (2 min at 250°C) of the organotin compounds and GC/ICP-MS analysis.

Following desorption, the organotin compounds are separated on a Perkin Elmer autosystem GC using a 30-m column with a DB-1 phase (100% PDMS). The end of this column is connected to a fused silica capillary which transfers (at 260°C) the compounds to the detector. Organotins were detected with a Perkin Elmer 5000 inductively coupled plasma mass spectrometer (ICP-MS). Note that, dibutyltin (DBT) and monobutyltin (MBT) were not quantified in mysids since they were present in high background concentrations, probably via the water and/or the derivatization solution.

The analysis of TBT in the water was similar to the analysis in mysids as described above. Digestion was not needed and 5 ml acetate buffer was added before addition of the $NaBEt_4$-solution. Because of the higher sensitivity, due to the absence of organic material, 10 min headspace SPME at room temperature was sufficient.

*4.2.6. CEA measurement*

The $E_a$, $E_c$ and CEA values were calculated as described in Chapter 3 (§ 3.2.4).

*4.2.7. Statistical analysis*

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$^{TM}$). The 96-h-LC50 values were calculated using the moving-average method (Stephan, 1977).

## 4.3. Results

*4.3.1. Acute toxicity of TBTCl to N. integer*

*Neomysis integer* were exposed to different concentrations of TBTCl in the water (10, 100, 1000, 10,000 and 100,000 ng TBTCl/l) for 96 h and mortality was recorded (Fig. 4.1). Experimental TBTCl concentrations in freshly prepared solutions and after 48 h were determined with GC/ICP-MS (Table 4.1). The experimental TBTCl concentrations in the freshly prepared solutions correlated well with the nominal values, except in the lower concentrations (10 and 100 ng TBTCl/l). From the mortality data, a 96-h LC50 of 114 ng TBTCl/l could be calculated (95% confidence limits: 33-436 ng TBTCl/l) based on the nominal concentrations. Based on the measured average concentrations (average between 0- and 48-h-old concentrations), the 96-h LC50 was slightly higher: 164 ng TBTCl/l (95% confidence limits: 52-373 ng TBTCl/l).

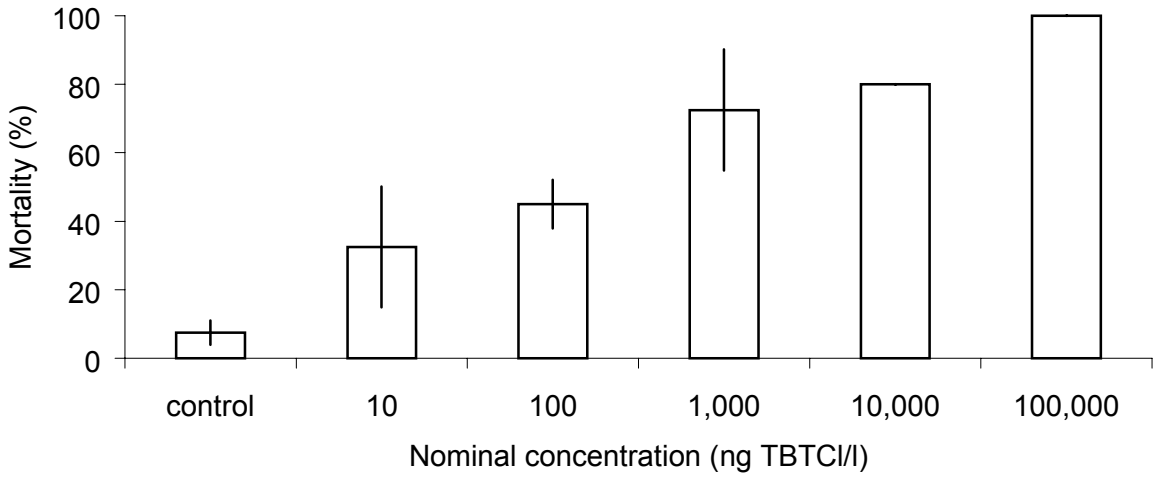



**Figure 4.1.** Acute 96-h toxicity of tributyltinchloride (TBTCl) to the mysid *N. integer*. Error bars represent standard deviation on the mean of two replicates; n = 10 in each replicate treatment.

**Table 4.1.** Aqueous tributyltinchloride (TBTCl) concentrations (ng/l) in the 96-h toxicity experiment with *N. integer* as determined by GC/ICP-MS

| Nominal TBTCl concentrations | Experimental TBTCl concentrations | |
|---|---|---|
| | Fresh solution | After 48 h |
| control | 8 | 9 |
| 10 | 32 | 28 |
| 100 | 150 | 86 |
| 1000 | 1050 | 1210 |
| 10,000 | 8540 | 2630 |
| 100,000 | ND | ND |

ND, not determined.

*4.3.2. Cellular energy allocation*

The effects of the 96-h exposure to tributyltinchloride on the different CEA parameters and the CEA of *Neomysis integer* are shown in Fig. 4.2 and Table 4.2, respectively.

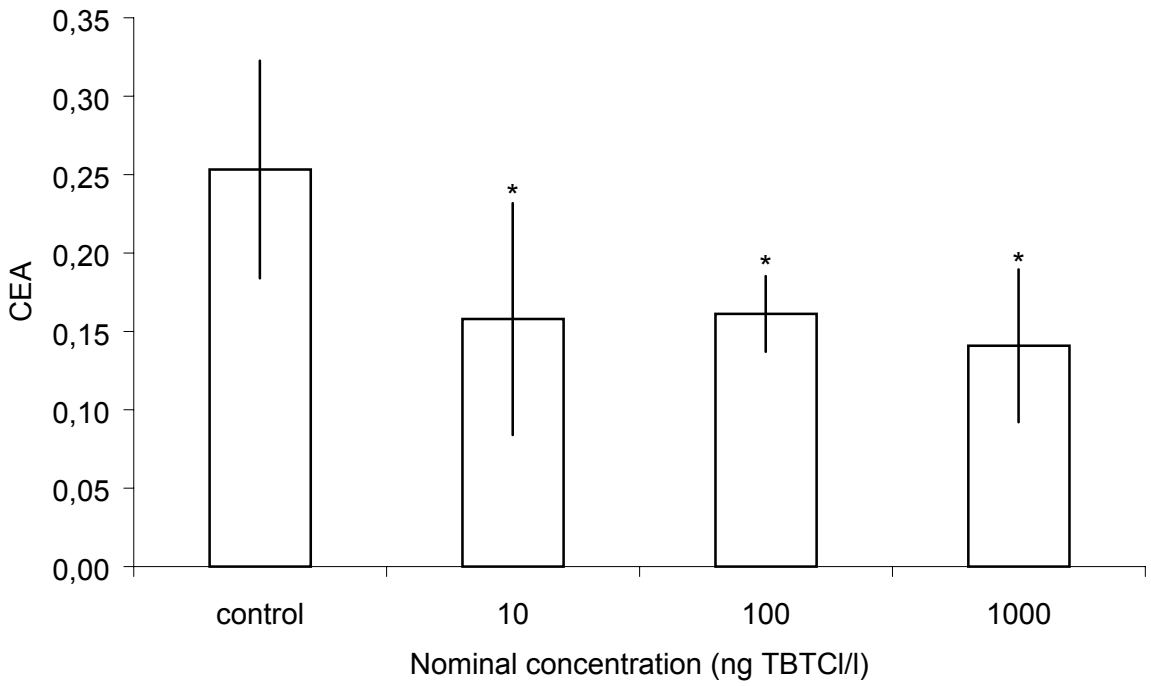



**Figure 4.2.** Effect of 96-h exposure to tributyltinchloride (TBTCl) on the cellular energy allocation (CEA) in *N. integer*. Error bars represent standard deviation on the mean, with n = 5; * significantly different from control (Dunnett's; $p < 0.05$).

**Table 4.2.** Effects of 96-h exposure to tributyltinchloride (TBTCl) on the cellular energy allocation of the mysid *N. integer* (data are shown as mean ± standard deviation)

| Nominal concentration (ng TBTCl/l) | Cellular energy allocation | | | | |
|---|---|---|---|---|---|
| | ΔSugar reserve[a] (mJ/mg ww) | ΔProtein reserve[a] (mJ/ mg ww) | ΔLipid reserve[a] (mJ/ mg ww) | $\Delta E_a$[a] (mJ/mg ww) | 4d-$E_c$ (mJ/ mg ww) |
| Control | 66.6 ± 49.1 | 449 ± 364 | 1037 ± 423 | 1553 ± 688 | 5904 ± 1194 |
| 10 | 34.4 ± 87.9 | 21.1 ± 154.0 | 790 ± 351 | 845 ± 203 | 5335 ± 862 |
| 100 | 63.6 ± 38.4 | 73.9 ± 521.3 | 921 ± 467 | 1058 ± 345 | 6663 ± 1124 |
| 1000 | 35.3 ± 65.6 | -300 ± 639 | 975 ± 380 | 711 ± 290[b] | 4991 ± 232 |

[a] Energy reserve day 0 – energy reserve day 4.
[b] Significantly different from control (Dunnett's; $p < 0.05$).

**Table 4.3.** Uptake of tributyltinchloride (TBTCl) by the mysid *N. integer* following a 96-h exposure period

| Nominal concentration (ng TBTCl/l) | Test solutions (ng/l)[a] | | | | | | | Mysids (ng/g ww)[a] | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 h | | | 48 h | | | average | 96 h | |
| | TBT | DBT | MBT | TBT | DBT | MBT | TBT | TBT | BCF |
| Control | 0.7 ± 0.4 | 97 ± 74 | 29 ± 25 | 2.6 ± 1.0 | 31 ± 19 | 35 ± 27 | 1.7 ± 1.3 | 12 ± 5 | 7015 |
| 10 | 8.7 ± 1.3 | 34 ± 16 | 17 ± 14 | 6.9 ± 0.5 | 21 ± 11 | 21 ± 9 | 7.8 ± 1.3 | 17 ± 6 | 2248 |
| 100 | 72 ± 4 | 53 ± 29 | 42 ± 25 | 54 ± 6 | 65 ± 47 | 49 ± 30 | 63 ± 11 | 70 ± 24 | 1115 |
| 1000 | 760 ± 15 | 62 ± 11 | 31 ± 5 | 627 ± 45 | 105 ± 14 | 46 ± 11 | 693 ± 82 | 1127 ± 154 | 1625 |

[a] Mean ± standard deviation of 3 replicate measurements (test solutions) or 5 replicate measurements (mysids).

Compared with mysids in the control, metabolic use of lipid, protein and sugar reserves was reduced (although not significantly) in all TBTCl-exposed mysids. Energy consumption after 96-h exposure to TBTCl ($E_{c,\ day\ 4}$), as derived from the electron transport activity, was higher in the 100 ng/l treatment compared to the control, but this was not statistically significant (data not shown). The average 96-h energy consumption (4d-$E_c$) in mysids exposed to 10 and 1000 ng TBTCl/l was reduced, but this was not significant when compared with the control ($p = 0.4$ and $p = 0.2$, respectively). The ratio of the observed changes in energy reserves over the average energy consumption, defined as the cellular energy allocation (CEA), was significantly lower in all TBTCl-exposed mysids.

*4.3.3. Uptake of TBT by N. integer*

Fresh and 48-h old test solutions were sampled and tri-, di- and monobutyltin concentrations were determined (Table 4.3). The measured TBT concentrations in the test solutions, averaged between fresh and 48-h old solutions, were between 22 and 37% lower than the nominal concentrations. Blank test solutions contained measurable TBT concentrations (1.7 ± 1.3 ng/l). Loss of TBT after 48 h was 21 ± 4%. In addition, mysids were sampled at the end of 96-h exposure period, TBT body burdens were determined and the bioconcentration factors (BCF) were calculated (Table 4.3). The BCF factor in TBT-exposed mysids varied between 1115 and 2248. A high BCF of 7015 was found in control mysids.

**4.4. Discussion**

Although the direct toxic effects and alterations in steroid metabolism leading to imposex following TBT exposure have been extensively described, relatively few data are available on the interaction of this toxicant with the processes that regulate energy metabolism (for review refer to Fent, 1996). The imposex and intersex phenomena have demonstrated that it is important that toxicity and effects of environmental chemicals are studied on their basic modes of action at the subcellular level. Still, in the case of TBT-induced imposex, these underlying mechanisms have not been conclusively unraveled (DeFur et al., 1999).

Organotins exert a number of important cellular, biochemical, and molecular effects. For instance, elevation of intracellular calcium concentration appears to be responsible for the thymocyte killing and stimulation of apoptosis and thus cytotoxic action of organotins (Aw et al., 1990). In reference to the energy metabolism, organotins are inhibitors of oxygen uptake into tissues and mitochondria of cells, and are potent inhibitors of ATP synthesis (Matsuno and Hatefi, 1993). The ability of trisubstituted organotins to uncouple or directly

inhibit oxidative phosphorylation in mitochondria has been attributed to three basic modes of action: direct inhibition of the ATPase complex (Aldridge and Street, 1964), inhibition of proton flow through the inner mitochondrial membrane (Selwyn, 1976), and gross swelling of mitochondria leading to the loss of most energetic functions (Aldridge and Rose, 1969).

Consequently, it can be hypothesized that changes in the energetic processes of *Neomysis integer* following acute TBT exposure, could be good indicators of the sublethal effects of this toxicant. In this perspective, we used the recently described cellular energy allocation technique to investigate the effects of TBT on the energy metabolism in the mysid *N. integer* (Verslycke and Janssen, 2002).

First, the acute toxicity of TBTCl to juvenile *N. integer* was assessed with a range-finding test. The acute LC50 for juvenile *N. integer* of 164 ng TBTCl/l (95% confidence limits: 52-373 ng TBTCl/l) found in our study is lower than those reported by Goodman et al. (1988) for juvenile *Americamysis bahia* (1100 ng TBTCl/l) and Salazar and Salazar (1989) for juvenile *Metamysidopsis elongata* (973 ng TBTCl/l). Davidson et al. (1986) and Valkirs et al. (1987) observed acute TBT toxicity within the same range (420-610 ng TBT/l) for mysid shrimp (for reviews refer to Fent, 1996 and USEPA, 2002c). TBT compounds are thus highly toxic to juvenile mysid shrimp. A greater sensitivity of the younger stages of mysids was previously found by Cripe (1994) from a review of toxicity data for *A. bahia*. The 60% mortality in the 1000 ng TBTCl/l treatment with adult mysids for the CEA experiments, illustrates that adult mysids differ from juveniles in their sensitivity to TBTCl (96-h-LC50 of TBTCl for adult *N. integer* in the µg/l range, data not shown). It can be speculated from these toxicity results that coastal TBT contamination, which can still reach concentrations of 200 ng TBT/l despite restrictive regulations (Michel et al., 2001), may be a potential threat to resident mysid populations.

Second, the effects of TBTCl exposure on the cellular energy allocation in adult mysids were investigated. Protein, lipid and sugar allocation over the 96-h exposure period, was different in all TBT-exposed mysids compared with the control (although not statistically significant), and this effect was strongest on the protein allocation in the 1000 ng TBTCl/l treatment ($p = 0.05$, compared with control). The change in available energy ($\Delta E_a$) over the 96-h exposure period to TBTCl was lower in the 10 ($p = 0.07$), 100 ($p = 0.14$) and 1000 ng/l ($p = 0.04$) treatment compared with the control. These results indicate that TBT interferes with energy metabolism in *N. integer*, by disrupting energy-producing processes of the catabolism. Since animals were not fed during the exposure, the intermediary energy-producing metabolism relies on the substrates available at day 0 (protein, sugar and lipid). Deleterious effects of organotins on the mitochondria, which are the sites of electron transport, oxidative phophorylation and fatty acid oxidation, have been described in literature (Fent, 1996). Alterations in the mitochondrial function would lead to a disruption of the

catalytic processes and thus to a reduced breakdown of cellular energy reserves, as observed in the TBT-exposed mysids in this study. Obviously, these catalytic processes do not solely depend on mitochondrial integrity. The first steps, not only in the oxidation of fatty acids but also in the protein degradation and glycolysis, occur in the cytosol, independent of mitochondrial function. This could explain why significant breakdown of protein, sugar and lipid reserves still occurs in TBT-exposed mysids.

The differential effect on sugar, lipid and protein reserves is more difficult to explain. Protein allocation was more affected than lipid and sugar allocation. The biochemical composition of the estuarine mysid *N. integer* contrasts sharply with that of a typical mammal, i.e. the former has low carbohydrate reserves and fat depots. The potential use of protein as a metabolic reserve has previously been suggested by Bhat and Wagh (1992) for marine zooplankton and was also found in our previous studies with *N. integer* (Verslycke and Janssen, 2002). Still, we found that protein reserves exhibit high variability, which is probably partly related to ecdysis in these organisms. Further investigation and longer exposures should provide a better insight into the differential effect of TBT on protein, sugar and lipid metabolism.

The electron transport system (ETS) is found in a cell's mitochondria and its microsomes, and consists of a complex chain of cytochromes, flavoproteins, and metallic ions that transport electrons from catabolized foodstuffs (lipid, sugar, protein as fatty acids, glucose and amino acids) to oxygen (Packard, 1971). Owens and King (1975) demonstrated that the use of INT (*p*-iodonitrotetrazolium violet) as an artificial electron acceptor in the ETS with the suitable substrates provides a measure of planktonic potential oxygen consumption. We previously reported a high correlation between potential whole organism respiration rates (as derived from the ETS assay) and real-time respiration measurements with a respirometer in *N. integer* (Verslycke and Janssen, 2002). TBT has a selective and species-specific effect on different enzymes of the microsomal ETS in freshwater fish, leading to destruction of native enzymes and inhibition of enzyme activity (Fent et al., 1998). It has been suggested that damage to mitochondria (and so decreasing respiration) is one of the most important mechanisms of TBT toxicity (Huang and Wang, 1995). We only observed a modest non-significant decrease in energy consumption in the 10 and 1000 ng TBTCl/l treatment and a non-significant increase in ETS activity in the intermediate exposure treatment. Either actual respiration rates were indeed affected but these effects were masked because potential *in vitro* respiration rates were measured, or the possible differential effects of TBT on the mitochondrial or microsomal ETS were not detected by the ETS assay. Selective administration of NADH (electron donor for the mitochondrial ETS; Garret and Grisham, 1995) or NADPH (electron donor for the microsomal ETS; Lemberg and Barrett, 1973) could provide more information on potential selective effects of TBT on the microsomal and/or

mitochondrial ETS. Actual mysid respiration rates should be monitored in future exposures to confirm if the respiration of TBT-exposed animals was actually reduced. Based on the present study, it is difficult to assign the observed decline in CEA to a single effect of TBT on either a decrease in catabolic activity, a decrease in actual respiration rates, an inhibition of the ETS, or an effect on the mitochondrial integrity. Likely, a combination of the above led to the observed decrease in CEA, even at the lowest exposure concentration.

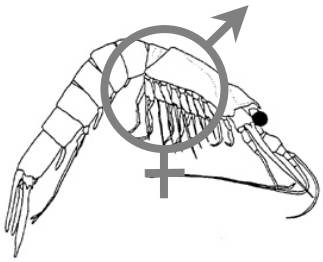
Generally, imposex in gastropod mollusks is believed to be the most dramatic effect of TBT exposure, occurring at levels of 2 ng TBT/l and above (Alzieu et al., 1986; Bryan et al., 1986; Curtis and Barse, 1990; Smith, 1980). The effects of TBT on gastropods are the most complete example of endocrine disruption in marine invertebrates (DeFur et al., 1999). However, TBT has also been reported to exert some endocrine disruptive effects in crustaceans. The following observations have been described in literature; effects on testosterone metabolism in daphnids (Leblanc and McLachlan, 2000; Oberdörster et al., 1998a) and mysids (Verslycke et al., 2003a, submitted-Chapter 8); effects on regenerative limb growth (Reddy et al., 1991; Weis et al., 1987); effects on molting (Reddy et al., 1992) and on calcium resorption from the exoskeleton (Nagabushanam et al., 1990). These effects typically occur at levels in the µg or mg/l range, levels which are acutely toxic to *N. integer*. We previously found significant interference of TBT with the testosterone phase I and II metabolism in *N. integer* (Verslycke et al., 2003a). Phase I reductase and hydroxylation activity and metabolic androgenization were induced in the 10 ng TBTCl/l treatment, whereas higher concentrations (100 and 1000 ng TBTCl/l) resulted in a reduction of phase II sulfate conjugation. In the present study, we found a significant alteration of the cellular energy allocation at the lowest exposure concentration (10 ng TBTCl/l). The general definition of an endocrine disruptor, i.e. an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function, would allow the observed effects on cellular energy allocation to be cataloged as endocrine disruptive, since effects on CEA have been linked to effects on reproduction at the population level (De Coen and Janssen, 1997). On the other hand, it is easy to argue the contrary, since no direct disruption in endocrine function was observed, only the secondary effects. In conclusion, although a discussion on ‘actual’ endocrine disruption is valid and a hot topic in current environmental science, the outcome of this discussion and so the actual underlying mechanism leading to individual and population effects is irrelevant for the organism in question. The observed effects on CEA and testosterone metabolism in *N. integer* occur at environmentally relevant concentrations of TBT, and research should be directed to the extrapolation of these data to field situations.

An important step in laboratory to field extrapolation of TBT effects is the understanding of the kinetics of this toxicant in mysids. High differences in the toxicity of organotins and the

metabolic capacity of aquatic organisms have been reported (Ohji et al., 2002). TBT is metabolized *in vivo* by a detoxifying system involving two phases. The phase I reactions involve the cytochrome P-450 dependent monooxygenase system (MFO) which hydroxylates TBT to alpha-, beta-, gamma-, and delta-hydroxydibutyltin derivatives (Fish et al., 1976). The phase II reactions conjugate sugars or sulphate to hydroxydibutyltin, and these polar conjugates are then rapidly eliminated from the organism. The MFO system of vertebrates and invertebrates is associated with the endoplasmatic reticulum of the cell and is a multicomponent enzyme system composed of phospholipid, cytochrome P-450, and NADPH cytochrome P-450 reductase (Lee, 1981; Lu, 1976; Stegeman, 1981). Thus, metabolism of a compound generally reduces persistence, increases elimination, and reduces toxicity (Lee, 1996). Mollusks have lower cytochrome P-450 content and mixed function oxygenase activity than crustaceans (Anderson, 1985; Lee, 1981; Livingstone, 1991; Livingstone and Farrar, 1985). This may be one reason why crustaceans are not as sensitive as mollusks to TBT exposure (Oberdörster et al., 1998c). In addition, it is also considered that differences between organisms in terms of metabolic capacity occur due to the inhibition of the cytochrome system by TBT. The binding of TBT to glutathione S-transferase and cytochrome P-450 results in the inhibition of these two detoxifying enzyme systems (Henry and Byington, 1976; Rosenberg and Drummons, 1983). We previously reported TBT-induced effects on the phase I and II biotransformation system of *N. integer* (Verslycke et al., 2003a). These effects of TBT on the metabolic capacity of mysids may explain their high sensitivity to the toxic effects of TBT. Unfortunately, no data are available regarding the uptake and metabolism of TBT in mysids. Data on TBT metabolism in crustaceans, in general, are scarce (USEPA, 2002c). The present study demonstrates that TBT is readily taken up by *N. integer* following a 96-h exposure period in all treatments. No TBT elimination or TBT conversion to DBT/MBT were monitored in the present study, so it was not possible to determine if steady-state levels of TBT were reached following this short exposure period. However, we previously reported high body burdens of TBT in *N. integer* of the Scheldt estuary, ranging from 927 to 1209 ng TBT/g dw (Verslycke et al., submitted-Chapter 9). Remarkably, The TBT/total butyltin ratio in these field-exposed mysids was very high (0.98 ± 0.01), which is significantly higher than previously reported values of 0.5-0.8 by Morcillo et al. (1999), 0.8 by Regoli et al. (2001) and 0.7 by Bouma et al. (2000) for mussels, indicating a very slow TBT metabolism. TBT body burdens in mysids from the present laboratory study were in the same range, i.e. 101 ng TBT/g dw in the 10 ng/l treatment to 6545 ng TBT/g dw in the 1000 ng/l treatment (calculated using a dw/ww ratio of 0.154; Verslycke et al., submitted-Chapter 9). So, although it was unknown if TBT concentrations in mysids of the laboratory experiment had reached a steady-state, absolute concentrations were comparable to levels in field-exposed mysids.

## 4.5. Conclusions

This study shows that the CEA assay can be used to detect sublethal interactions of environmental pollutants with the energetic processes of an organism. These changes at the cellular level occurred at environmentally relevant concentrations of the toxicant TBTCl and were an order of magnitude more sensitive than reported TBT effects on scope for growth in other marine invertebrates (Bühringer and Danischewski, 2001; Widdows and Page, 1993). In addition, the CEA assay allows an evaluation of specific interactions with subcellular mechanisms linked with the energy metabolism, such as electron transport activity and lipid, protein and sugar allocation. Finally, the apparent low metabolic capacity for TBT in field-exposed mysids, the effects of TBT on the metabolic capacity of laboratory-exposed mysids, and the high TBT accumulation potential in these animals, may explain the high sensitivity of mysids towards the lethal and sublethal effects of TBT. Consequently, the potential effects of chronic TBT exposure should be further investigated in the laboratory and linked with biomarker responses in field-exposed mysids.



# CHAPTER 5
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# Validation of the cellular energy allocation assay with the scope for growth assay in *Neomysis integer*

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# Chapter 5

## *Validation of the cellular energy allocation assay with the scope for growth assay in Neomysis integer*

**Abstract**

Mysids (Crustacea: Mysidacea) are used routinely in acute toxicity testing to evaluate the comparative toxicity of chemicals to aquatic organisms. The need for sub-lethal endpoints that provide more comprehensive understanding of the potential impacts of toxicants to natural populations has resulted in examination of several physiological responses in mysid shrimp, including scope for growth (SFG) and cellular energy allocation (CEA). Both assays, based on the concept that energy in excess of that required for normal maintenance will be available for growth and reproduction, have been reported independently for the mysid *Neomysis integer*. The present study compares the responses of *N. integer* following exposure to environmentally realistic concentrations of the organophosphate pesticide chlorpyrifos using both assays. Oxygen consumption in the SFG assay was significantly correlated with cellular respiration rate in the CEA assay, and both were significantly increased by chlorpyrifos exposure. In addition, the protein, sugar, lipid and total energy content in the CEA assay and the egestion rate in the SFG assay were significantly different in chlorpyrifos-exposed mysids compared with control mysids. In contrast, absorption efficiency in the SFG assay was unaffected by pesticide exposure. Significant effects in the SFG and CEA assays were more pronounced following short (i.e. 48 h) compared with longer exposure periods (e.g. 168 h). SFG was significantly reduced at near-lethal concentrations (72 and 100 ng chlorpyrifos/l), whereas CEA was reduced in all chlorpyrifos-exposed mysids (38, 56, 72 and 100 ng chlorpyrifos/l). Differences in sensitivity between these assays may be a reflection of the effects of chlorpyrifos at different levels of biological organization (e.g. CEA, cellular and SFG, organismal). The present study, however, does not permit a conclusive statement as to whether one assay is better than the other, as both assays have their own strengths and weaknesses.

### 5.1. Introduction

To evaluate effects of pollutants on animal populations, communities and ecosystems, various methods have been developed ranging from the (sub)cellular to the ecosystem level of

biological response. However, the predictive ability of measurements at higher levels of biological organization is limited because ecologically important effects (e.g. death or impaired organismal function) have already occurred before they can be detected at population and community levels. Over the last decades, biomarkers at the suborganismal levels of organization (biochemical, physiological, and histological) have been considered to be viable measures of responses to stressors (Huggett et al., 1992). Some of the most successful types of biomarkers are those linked to metabolism and energetics. Crustaceans have provived an important and valuable study model for investigation of regulation and integration in physiology and there is a large body of information available on the neuroendocrine pathways of physiological regulation. While typical and well-studied challenges to the endogenous energy metabolism include environmental hypoxia, functional (internal) hypoxia, changing energetic requirements, disturbance to water balance/ion-homeostasis and changes in temperature (for a review refer to Morris and Airriess, 1998), exposure to toxicants will also result in an energetic challenge.

Several physiological responses have been used to quantify the sub-lethal effects of contaminants in mysid shrimp, including respiration rates, feeding and excretion (Engstrom et al., 2001; Gaudy et al., 1991; McKenney, 1986, 1998; McKenney and Celestial, 1996; McKenney and Matthews, 1990; Roast et al., 1999b, 2000b; Voyer and McGovern, 1991). Physiological energetics provide information on key processes in the organism's energy acquisition and expenditure, possibly also elucidating the mode of action of the toxicant. The allocation of specific amounts of energy to basal metabolism, growth and reproduction, will vary in response to changing environmental conditions, and theoretically exposure to a pollutant will disturb this allocation. In addition, changes in metabolic turnover and specific allocations will be linked to effects at higher levels of ecological organization (De Coen, 1999; McKenney, 1998). Based upon this concept, several single integrated bioassays, such as 'scope for growth' (SFG) and 'cellular energy allocation' (CEA), which both provide rapid, instantaneous measurements of the energy status of an organism, were developed (De Coen and Janssen, 1997; Widdows and Donkin, 1992). To date, there have been few attempts to compare different physiological responses of the same species to determine which is the most sensitive, or most appropriate, for environmental monitoring (e.g. Bamber and Depledge, 1997). This paper addresses the question of the relative value of SFG and CEA as biomarkers of environmental contamination.

Recently, the effects of the pesticide chlorpyrifos (*O*, *O*,-diethyl *O*-3,5,6-trichloro-2-pyridylphosphorothioate), a non-systemic organophosporothioate insecticide (Whitehead, 1997), on the respiration, feeding rate, absorption efficiency and SFG of the estuarine mysid *Neomysis integer* (Leach) were reported (Roast et al., 1999c). Also, the CEA assay has been used with *N. integer* to detect subtle effects of exposure to the antifoulant biocide tributyltinchloride (Verslycke et al., 2003c). The present study set out to compare and evaluate

both assays through an identical test set-up with the same species, *N. integer*, and the same toxicant, chlorpyrifos. The potential of each individual CEA and SFG parameter as toxicity test endpoints are assessed, and the response of both CEA and SFG are compared.

Mysids (Crustacea: Peracarida) have been used in regulatory (and other) toxicity testing for more than 20 years. The sub-tropical American species *Americamysis* (formerly *Mysidopsis*) *bahia* is a standard test organism adopted by the United States Environmental Protection Agency (USEPA) for marine and estuarine testing (USEPA, 1995a; 1997) and standard test protocols have been developed for other mysid species, such as *Neomysis mercedis* (Brandt et al., 1993), *Mysidopsis intii* (Langdon et al., 1996) and *Holmesimysis costata* (Martin et al., 1989). *A. bahia* has many strengths and few weaknesses as a candidate test species. Its primary advantages include widespread availability and ease of culture. The most important criticism of the widespread use of *A. bahia* in toxicological testing is its limited tolerance to dilute salinities and colder waters, casting doubt on its value as a test organism for estuarine studies (Pillard et al., 1999). More importantly, *A. bahia* is not representative of European estuaries and there is a clear need to identify indigenous estuarine species for toxicity testing in Europe (e.g. Roast et al., 1998a). *N. integer*, the dominant hyperbenthic mysid of the upper reaches of European estuaries (Mees et al., 1995b; Mees and Jones, 1997) is sensitive to many toxicants at environmentally relevant concentrations (Emson and Crane, 1994; Roast et al., 1999a, 2001a; Verslycke et al., 2003b; Wildgust and Jones, 1994). *N. integer* has been proposed as a test species for monitoring European estuaries (Emson and Crane, 1994; Roast et al., 1998a; Verslycke et al., 2003b) and the present paper contributes further to this suggestion.

## 5.2. Material and Methods

### *5.2.1. Animal collection and maintenance*

For the CEA assays, *Neomysis integer* was taken from a laboratory culture in the Laboratory for Environmental Toxicology and Aquatic Ecology (Ghent University, Belgium); mysids were collected initially from the Galgenweel (a brackish-water inlet with a salinity of 4 ± 1‰ near the river Scheldt, Antwerp, Belgium) as described in Chapter 3 (§ 3.2.1). Culture medium was artificial seawater (Instant Ocean®, Aquarium Systems, France), diluted with aerated deionized tap water to a final salinity of 5‰. A 14-h light:10-h dark photoperiod was used during culturing and water temperature was maintained at 15 ± 1°C. Cultures were fed daily *ad libitum* with 24–48-h-old *Artemia* nauplii to prevent adult mysids from cannibalizing their young. Hatching of the *Artemia* cysts was performed in 1-l conical vessels under vigorous aeration and continuous illumination at 25°C.

For the SFG experiments, *N. integer* was initially collected from the southern side of

the Terras Bridge, East Looe River estuary (Cornwall, UK) as described by Roast et al. (1998b). Animals were returned to the laboratory and placed in a shallow 15-l holding tank at a salinity of 10 ± 1‰ (made by combining filtered seawater and double-distilled, deionized water) in a constant-temperature room (15 ± 1°C). Lighting was provided at ambient laboratory levels by overhead fluorescent lights; a time switch provided a 16-h light:8-h dark photoperiod. Mysids were fed *ad libitum* with 24–48-h-old *Artemia* nauplii hatched in the laboratory from cysts.

### *5.2.2. Chlorpyrifos exposure*

In the SFG experiment, mysids were exposed to chlorpyrifos concentrations of 0.038, 0.056, 0.072 and 0.100 µg/l for 3 time periods (48, 96 and 168 h) as described by Roast et al. (1999c). In the CEA experiment, exposure concentrations and conditions were identical to these of the SFG experiment, except that only 2 time periods (48 and 168 h) were evaluated and animals were acclimated gradually for 72 h to the test salinity of 10‰. In both CEA and SFG experiments, a solvent control (100 µl acetone/l) was used to assess any effects of acetone on mysid physiology. As oxygen consumption by *Neomysis integer* is gender specific (Roast et al., 1999b), only males were used in these experiments. In the SFG experiment, 10 mysids of equal length (12 ± 1 mm from the anterior margin of the rostrum to the tip of the telson; average wet weight was 7.5 mg) were placed together in 2-l tall-form glass beakers containing 1500 ml of exposure water. In the CEA experiment, 50 mysids of equal length (visual selection of animals with a length of about 10 mm; average wet weight was 7.3 mg) were placed together in 10-l solid-glass aquaria containing 5000 ml of exposure water. Two replicate aquaria were used for each concentration in the CEA experiment. Due to the unstable nature of chlorpyrifos (ca. 55% loss within 24 h; Roast et al., 1999a), exposure solutions were replaced every 24 h with a freshly-made solution (chlorpyrifos concentrations were not measured in the CEA experiment, but considered similar to those in the SFG experiment). At this time, dead or moribund mysids were removed from the test vessels (mortality was concentration-dependent and, in both test set-ups, more than 50% had died after 7 days exposure to 0.1 µg chlorpyrifos/l). Toxicant concentrations were prepared from an initial stock of 1 g chlorpyrifos/l acetone by dilution in 10‰ water. Mysids were fed twice daily with equal amounts of 36 ± 12-h-old *Artemia* nauplii (100 *Artemia*/mysid/day), resulting in a continuous availability of food.

### *5.2.3. Scope for Growth assay*

Oxygen consumption, feeding rate and absorption efficiency by *Neomysis integer*, and subsequent calculation of SFG, was measured by Roast et al. (1999c). In summary, 9 replicate

respiration measurements were performed in closed respiration chambers (Strathkelvin Instruments Ltd.) at 15 ± 1°C and a salinity of 10 ± 1‰, using freshly-made exposure solutions of the same chlorpyrifos concentration to which the mysids had been exposed. Mysid egestion rate (measured by faecal production) correlates well with ingestion rate, and the former is a valid, indirect measurement of feeding rate (Murtaugh, 1984). Faecal production by *N. integer*, measured according to the method described by Roast et al. (2000b), was measured from 10 replicates for each pesticide concentration and exposure period. Finally, mysid absorption efficiency was estimated by the ratio method (Conover, 1966), using pooled faecal material from several mysids. SFG was calculated by converting oxygen consumption and feeding rates into energy equivalents (J/h), and calculating the net energy gain/loss through physiological processes using the equation:

$$SFG = A - (R + U) \quad \text{(J/mg mysid dry weight/h)}$$

where: $A$ = energy absorbed, $R$ = energy respired and $U$ = energy excreted (Widdows and Salkeld, 1993). The rate of ammonia excretion is usually closely related with respiration rate and, contributing usually <5% of metabolic energy expenditure, is usually omitted from the SFG calculation (Widdows and Salkeld, 1993). Ammonia excretion was not measured in the present study, thus SFG was calculated from the equation $P = A - R$ (units as defined above) (Roast et al., 1999c).

### *5.2.4. Cellular Energy Allocation assay*

Mysids were shock-frozen in liquid nitrogen and kept at -80°C until analysis. CEA was measured according to Verslycke and Janssen (2002) with minor modifications. The different energy reserve fractions $E_a$ (lipid, protein, sugar) were determined spectrophotometrically and transformed into energetic equivalents using their respective energy of combustion (39500 mJ/mg lipid, 24000 mJ/mg protein, 17500 mJ/mg glycogen) (Gnaiger, 1983). The energy consumed ($E_c$) was estimated by measuring the electron transport activity (ETS) according to Owens and King (1975). The quantity of oxygen consumed per mysid, as derived from the ETS data, was transformed into energetic equivalents using the oxyenthalpic equivalents for an average lipid, protein and sugar mixture (484 kJ/mol $O_2$) (Gnaiger, 1983).

The $E_a$, $E_c$ and CEA value were calculated as follows:

$E_a$ (available energy $E_a$) = sugar + lipid + protein (mJ/mg mysid wet weight)

$E_c$ (energy consumption $E_c$) = ETS activity (mJ/mg mysid wet weight/h)

CEA (cellular energy allocation) = $E_a/E_c$

From this calculation, it can be deduced that a decline in CEA indicates either a reduction in available energy or a higher energy expenditure, both resulting in a lower amount of energy available for growth or reproduction. Ten replicate measurements of lipid, sugar and protein content and ETS activity were performed for each pesticide concentration and exposure period, except in the highest test concentration after 7 d exposure (n = 7) due to high mortality. All data are expressed as mJ per mg wet weight of the animal. For wet weight measurements, mysids were blotted dry and weighed with an analytical balance (± 0.01 mg). To express results on a dry-weight base, a mysid dry wt/wet wt ration of 20% can be used (Mauchline, 1980).

### *5.2.5. Statistical analysis*

All data were checked for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene's test, respectively, with an $\alpha$ = 0.05. The effects of exposure concentration and exposure duration were examined statistically by 1- and 2-way analysis of variance (ANOVA). SFG data are presented as means of the SFG calculated for the 48, 96 and 168-h exposure periods. Similar, CEA data are presented as means of the CEA calculated for the 48 and 168-h exposure periods. Where significant *F*-ratios were calculated by ANOVA, Tukey's Honestly Significant Difference (HSD) test or Dunnett's Test were applied to identify which datasets were different.

## 5.3. Results

### *5.3.1. Oxygen consumption rate*

Respiration by *Neomysis integer*, measured *in vivo*, was affected by exposure concentration (ANOVA, *F*-ratio = 718, df = 4, $p < 0.001$) and duration (ANOVA, *F*-ratio = 109, df = 4, $p < 0.001$). All chlorpyrifos concentrations caused increased oxygen consumption with a clear concentration response ($p < 0.01$; Fig. 5.1). Mysids exposed to chlorpyrifos for 48 h showed a higher rate of oxygen consumption than those exposed for 96 or 168 h ($p < 0.01$). Although there was a significant interaction between pesticide concentration and exposure period (2-way ANOVA, *F*-ratio = 4.65, df = 6, $p < 0.01$), the effect was not obvious, and the individual effects of concentration and exposure are considered more important.

Cellular respiration was measured in mysids following chlorpyrifos exposure for 48 and 168 h via the *in vitro* electron transport system (ETS) activity. ETS activities were significantly affected by exposure concentration (ANOVA, *F*-ratio = 5.90, df = 4, $p < 0.001$; Table 5.1), but not by exposure duration (ANOVA, *F*-ratio = 3.08, df = 2, $p > 0.05$). At the three highest exposure concentrations, mysids had higher respiration rates ($p < 0.05$) than control mysids

after 48 and 168 h (not significant in the 0.072 µg chlorpyrifos/l treatment after 168 h). In addition, a significant correlation was found between the real-time respiration rates in mysids from the SFG experiment and ETS activities in mysids from the CEA experiment via linear regression analysis ($R^2 = 0.65$, $p < 0.01$; Fig. 5.2)

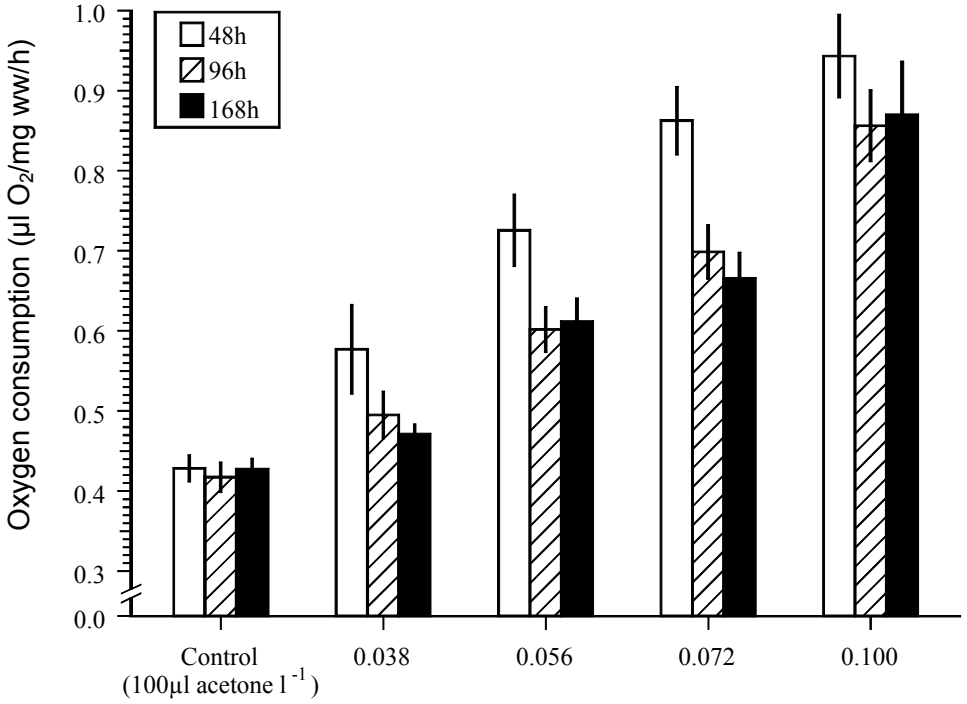



**Figure 5.1.** Oxygen consumption by *N. integer* following exposure to chlorpyrifos. n=9 for each exposure concentration/period. Error bars correspond to standard deviations on the mean (all chlorpyrifos-exposed mysids had a significantly higher oxygen consumption rate than control mysids, $p < 0.01$) .

*5.3.2. Egestion rates and absorption efficiency*

Egestion rates of *Neomysis integer* were significantly affected by chlorpyrifos concentration (ANOVA, *F*-ratio = 27.59, df = 4, $p < 0.001$) but not by exposure duration (ANOVA, *F*-ratio = 0.72, df = 2, $p > 0.05$). Egestion rates were significantly different from control mysids following each of the 3 exposure periods at 0.1 µg chlorpyrifos/l ($p < 0.01$), and after 48 h at 0.072 µg chlorpyrifos/l ($p < 0.05$) (Fig. 5.3).

Irrespective of exposure concentration and duration, the absorption efficiency of *N. integer* was unaffected by chlorpyrifos (data presented in Roast et al., 1999c). The small variations recorded in absorption efficiency (absorption efficiencies of all pesticide-exposed mysids were within 1% of control mysid values) are assumed to be due to experimental variation and not to pesticide exposure.

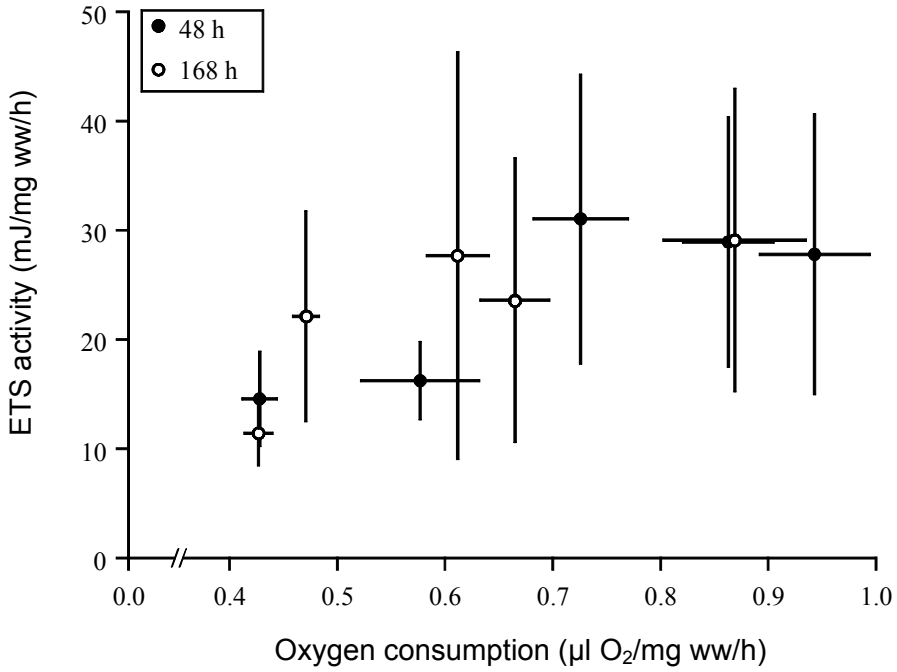



**Figure 5.2.** Correlation between *in vitro* electron transport system (ETS) activities and whole organism respiration rates in *N. integer* exposed to chlorpyrifos. n=9 for each exposure concentration/period. Error bars correspond to standard deviations on the mean.

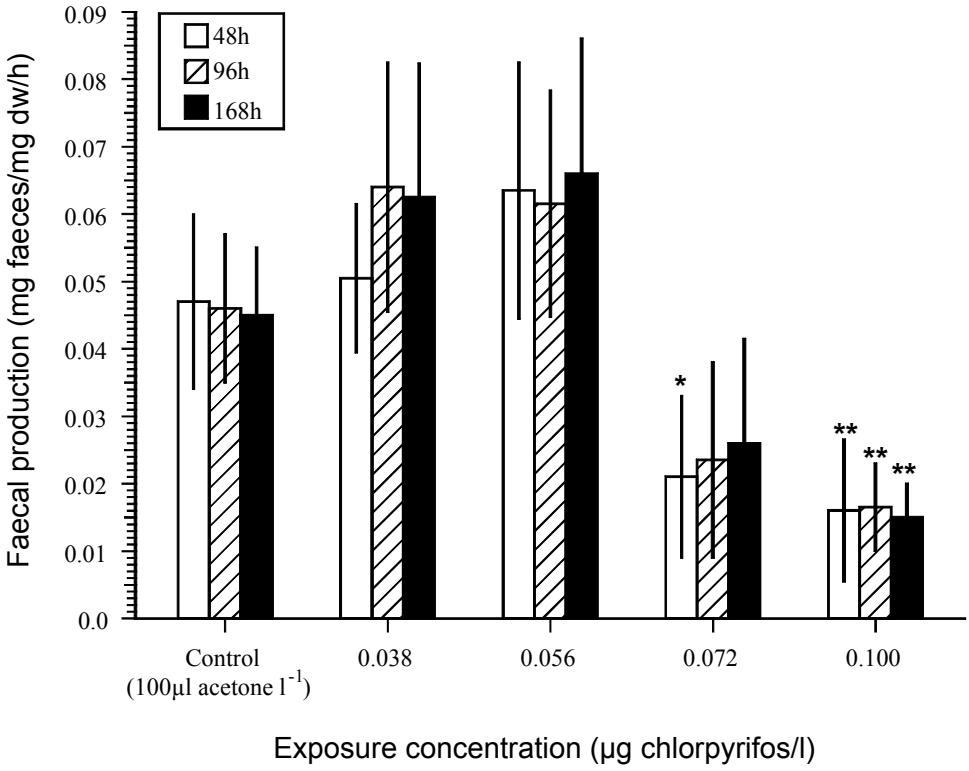



**Figure 5.3.** Egestion by *N. integer* following exposure to chlorpyrifos. n=10 for each exposure concentration/period. Error bars correspond to standard deviations on the mean. * significantly different from control of the same exposure duration (* $p < 0.05$, ** $p < 0.01$).

**Table 5.1.** Effects of chlorpyrifos exposure on the cellular energy allocation (CEA) in *N. integer* (data are shown as mean ± standard deviation)

| Nominal concentration (µg chlorpyrifos/l) | Exposure duration | Energy allocation | | | | |
|---|---|---|---|---|---|---|
| | | Sugar reserve (mJ/mg ww) | Protein reserve (mJ/mg ww) | Lipid reserve (mJ/mg ww) | $E_a$[a] (mJ/mg ww) | $E_c$[b] (mJ/mg ww/h) |
| | 0h | 22.45 ± 11.45 | 508.09 ± 202.19 | 1283.59 ± 455.15 | 1814.14 ± 498.18 | 13.74 ± 2.94 |
| Control (n = 10) | 48h | 32.71 ± 13.39 | 517.14 ± 87.80 | 1667.13 ± 458.42 | 2216.98 ± 466.96 | 14.55 ± 4.31 |
| | 168h | 16.75 ± 6.35 | 1186.25 ± 407.45 | 1197.30 ± 317.91 | 2389.29 ± 516.99 | 11.41 ± 2.96 |
| 0.038 (n = 10) | 48h | 36.97 ± 25.97 | 941.01 ± 358.01** | 1752.08 ± 360.16 | 2730.06 ± 508.50 | 16.22 ± 3.53 |
| | 168h | 20.98 ± 9.33 | 898.5 ± 326.89 | 1364.81 ± 593.70 | 2284.28 ± 677.96 | 22.11 ± 9.60 |
| 0.056 (n = 10) | 48h | 39.49 ± 12.87 | 1053.66 ± 414.00*** | 1587.87 ± 755.85 | 2681.09 ± 862.01 | 31.04 ± 13.22*** |
| | 168h | 33.88 ± 12.44*** | 1079.98 ± 292.96 | 1533.521 ± 459.83 | 2647.37 ± 545.68 | 27.67 ± 18.62* |
| 0.072 (n = 10) | 48h | 45.50 ± 15.72 | 1178.55 ± 176.17*** | 1728.23 ± 612.31 | 2952.29 ± 637.45* | 28.93 ± 11.44*** |
| | 168h | 25.86 ± 10.65 | 796.11 ± 235.86* | 1875.51 ± 804.63* | 2697.94 ± 838.66 | 23.60 ± 12.97 |
| 0.100 (n = 7) | 48h | 32.36 ± 13.96 | 1080.20 ± 266.49*** | 2135.42 ± 962.47 | 3247.98 ± 998.86** | 27.79 ± 12.81*** |
| | 168h | 16.19 ± 8.49 | 851.97 ± 330.12 | 2015.43 ± 1005.70* | 2883.59 ± 885.18 | 29.09 ± 13.86* |

[a] $E_a$ = Energy available (sum of sugar, protein and lipid).
[b] $E_c$ = Energy consumption (as derived from electron transport activity).
* significantly different from control of the same exposure duration (ANOVA, Dunnett; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

*5.3.3. Sugar, protein, lipid and total energy content*

The effects of chlorpyrifos on the different CEA component values (sugar, lipid and protein) and the total energy content of *Neomysis integer* ($E_a$) are shown in Table 5.1. Sugar content of *N. integer* was affected significantly by chlorpyrifos concentration (ANOVA, *F*-ratio = 2.84, df = 4, $p < 0.05$) and exposure duration (ANOVA, *F*-ratio = 19.31, df = 2, $p < 0.001$). No significant interaction effects between exposure concentration and duration on sugar content were observed (2-way ANOVA, *F*-ratio = 0.71, df = 4, $p > 0.05$). All chlorpyrifos-exposed mysids had a higher sugar content than control mysids, except those in the highest exposure concentration. Sugar content was significantly higher in mysids exposed to 56 µg chlorpyrifos/l for 168 h than at other exposure concentrations ($p < 0.001$).

The mean protein content of *N. integer* exposed for 48 and 168 h was significantly affected by exposure duration (ANOVA, *F*-ratio = 35.07, df = 2, $p < 0.001$), but not by chlorpyrifos concentration (ANOVA, *F*-ratio = 1.04, df = 4, $p > 0.05$), although significant exposure effects were observed when considering 48 h and 168 h-exposed mysids separately. Significant interaction effects between exposure concentration and duration on protein content were observed (2-way ANOVA, *F*-ratio = 8.27, df = 4, $p < 0.001$). Following 48 h exposure to chlorpyrifos, all mysids had significantly higher protein contents than control mysids (HSD, $p < 0.01$). However, the opposite was observed in the 168 h-exposed mysids, where protein concentrations in chlorpyrifos-exposed mysids were lower than these of control mysids, although this was not statistically significant ($p > 0.05$).

Finally, lipid content of *N. integer* was significantly affected by exposure duration (ANOVA, *F*-ratio = 7.54, df = 2, $p < 0.001$) and concentration (ANOVA, *F*-ratio = 2.57, df = 4, $p < 0.05$), but no significant interaction effects between exposure concentration and duration were observed (2-way ANOVA, *F*-ratio = 0.74, df = 4, $p > 0.05$). All chlorpyrifos-exposed mysids had a higher lipid content than control mysids, but this was only significant in the two highest test concentrations following 168 h exposure ($p < 0.05$).

Total energy available ($E_a$) was calculated as the sum of the individual energy components sugar, lipid and protein. The $E_a$ of *N. integer* was significantly affected by exposure duration (ANOVA, *F*-ratio = 26.62 df = 2, $p < 0.001$) and concentration (ANOVA, *F*-ratio = 3.194, df = 4, $p < 0.05$), but no significant interaction effects between exposure concentration and duration were observed (2-way ANOVA, *F*-ratio = 0.58, df = 4, $p > 0.05$). The total energy content of chlorpyrifos-exposed mysids was higher than the $E_a$ in control mysids. This effect was significant in 48-h exposed mysids in the two highest test concentrations ($p < 0.05$).

*5.3.4. Scope for Growth*

Since different mysids were used to measure the individual components of the SFG calculations (i.e. oxygen consumption, feeding rate and absorption efficiency), SFG was estimated by using the mean component values. True replicates were, therefore, not possible and a single SFG estimate was calculated for pesticide concentration and exposure period. However, with the exception of mysids exposed for 48 h, there was no effect of exposure duration on the oxygen consumption or egestion rate of *N. integer*, therefore, mean SFG values at each pesticide concentration were calculated by averaging SFG values at 48, 96 and 168 h, allowing comparison of concentration effects. Exposure to chlorpyrifos had a significant effect on SFG of *Neomysis integer* (ANOVA, *F*-ratio = 61, df = 4, $p < 0.01$) (Fig. 5.4). At the 2 higher pesticide concentrations, mysids had significantly reduced SFG compared with controls ($p < 0.01$). The mean SFG for mysids exposed to 0.100 µg chlorpyrifos/l was close to zero, with negative values for the lower 95% confidence interval, indicating some mysids may have been utilizing more energy than they were assimilating.

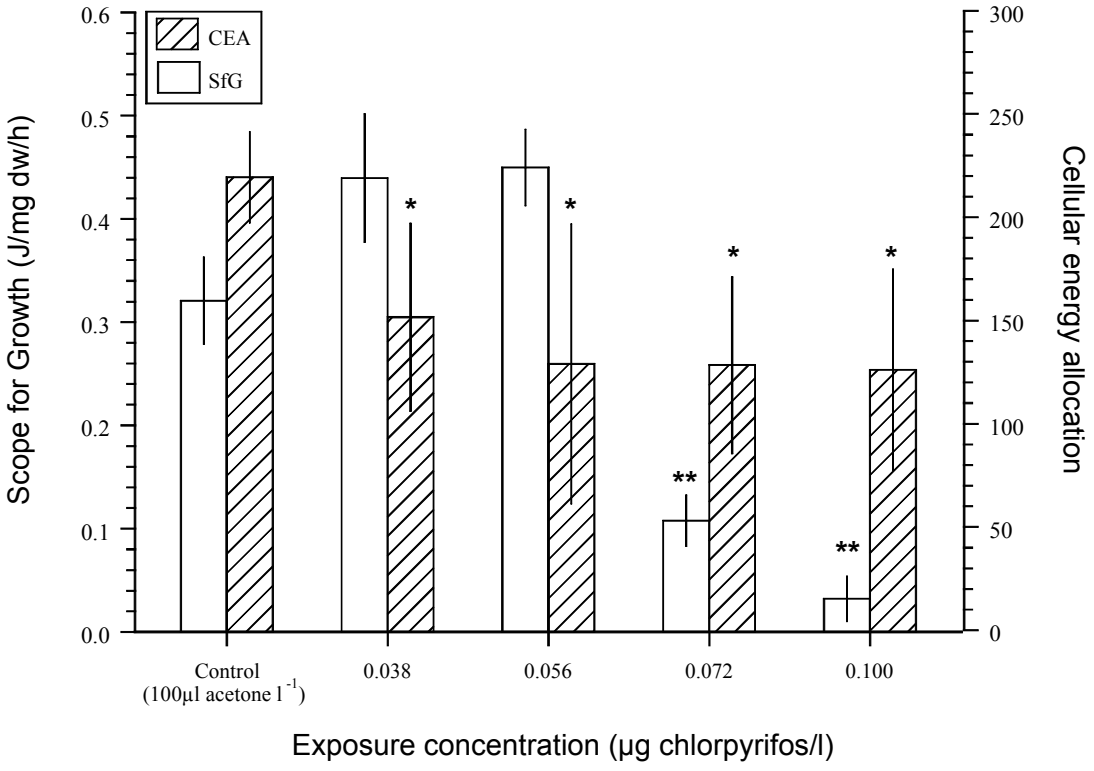



**Figure 5.4.** Scope for growth (SFG) and Cellular energy allocation (CEA) of *N. integer* following exposure to chlorpyrifos. Data pooled from SFG calculated after 48, 96 and 168 h and CEA calculated after 48 and 168 h. Error bars correspond to standard deviations on the mean. * significantly different from control (* $p < 0.05$, ** $p < 0.01$).

*5.3.5. Cellular energy allocation*

Cellular energy allocation was calculated as the ratio of the available energy $E_a$ (sum of protein, sugar and lipid reserve) to the energy consumption $E_c$ (as derived from the ETS activity) (Fig. 5.4). Thus, a decline in CEA, indicates either a reduction in available energy or a higher energy expenditure, both resulting in a lower amount of energy available for growth or reproduction. Although the exposure period had an effect on all CEA components (except energy consumption), a single CEA estimate was calculated by averaging CEA values at 48 h and 168 h, allowing comparison of concentration effects and allowing a comparison with the SFG results. From this calculation, it was derived that all chlorpyrifos-exposed mysids had a reduced CEA compared with control mysids ($p < 0.05$).

## 5.4. Discussion

In the present study, chlorpyrifos was found to alter energy allocation and scope for growth in the mysid *Neomysis integer*. Mysids exposed to the highest chlorpyrifos concentration had cellular respiration rates (measured as ETS activity) which were twice the rate of control mysids. In addition, a significant correlation ($R^2 = 0.65$, $p < 0.01$) was found between cellular respiration rate and whole animal respiration rate, the latter also being twice as high in the highest exposure concentration compared with control mysids. This correlation is apparent, despite measurements being carried out on different and separately exposed animals. Previously, we reported a high correlation ($R^2 = 0.94$; $p < 0.01$) between ETS activities *in vitro* and real-time *in vivo* animal respiration in *N. integer*, but these were measured on the same animals (Verslycke and Janssen, 2002). Thus, ETS activities appear to be a valid alternative to whole animal respiration measurements. Increased rates of oxygen consumption have been reported previously for mysids exposed to toxicants, including *Neomysis americana* exposed to naphthalene (Smith and Hargreaves, 1984), *Americamysis bahia* exposed to the pesticides thiobencarb, endrin, fenthion and DEF (McKenney, 1998), and *N. integer* exposed to methoprene and nonylphenol (Verslycke et al., submitted-Chapter 8). In addition to the concentration response, there was also an exposure period effect on whole animal respiration, where mysids exposed to chlorpyrifos for 48 h consumed oxygen at a greater rate than mysids exposed for 96 or 168 h. However, this effect was not observed in the ETS activities of chlorpyrifos-exposed mysids in the CEA experiment. The ETS assay measures the maximum ETS activity under saturated substrate (NADH, NADPH) conditions and observed changes in activity must therefore be realized through changes in the amount of enzymes produced by the organism. Consequently, it is assumed that ETS activity is an overestimation of the ambient respiration and responds much slower to changes in the environment than the respiration rate

(Båmstedt, 1980; Mayzaud, 1986; Skjoldal et al., 1984). Nevertheless, the concentration response in ETS activities was significant after 48 h of exposure and was not significantly different from the response after 7 d of chlorpyrifos exposure. It could, therefore, be hypothesized that mysids suffered from acute physiological stress following 48 h exposure, whereas at 168 h, although the cellular effects were still present, other mechanisms were operating, thus resulting in lower whole animal respiration rates. Monooxygenase systems are important in the metabolism of foreign organic compounds and are believed to play a role in the metabolism of organophosphate pesticides (WHO, 1986). We reported a very complex biotransformation system in *N. integer* (Verslycke et al., 2002), which can be modulated by toxicant exposure (Verslycke et al., 2003a). Consequently, alterations in the detoxification mechanism of *N. integer* could explain the observed exposure duration effects on respiration.

Crustacean feeding rates are frequently suppressed following exposure to toxicants (e.g. Crane and Maltby, 1991; Crane et al., 1995; Guerin and Stickle, 1995). Although feeding rates have been studied in mysids (Engstrom et al., 2001; Nimmo et al., 1981; Jerling and Wooldridge, 1995; Roast et al., 2000b; Viherluoto and Viitasalo, 2001a,b), few studies have investigated the effects of toxicants on mysid feeding rates. One study demonstrated a 50% decrease in cadmium-exposed mysid feeding rates, compared with control mysids (Gaudy et al., 1991). In the present study, egestion rate of *N. integer* (and, therefore, feeding rate) following exposure to chlorpyrifos was significantly affected, and mysids in the two highest test concentrations had decreased egestion rates compared with control mysids. On the other hand, absorption efficiencies were unaffected. In general, absorption efficiency is less sensitive to toxicant exposure than actual feeding rates and ingestion rate is considered the most important process in energy acquisition (e.g. Crane and Maltby, 1991; Roast et al., 2000b). It may be anticipated that feeding rate is closely related to the energy content of the mysids. Both $E_a$ and egestion rates were indeed affected most strongly at the two highest exposure concentrations (Fig. 5.2, Table 5.1), however, the observed effects were contradictory. Since it is assumed that mysid egestion rates are a good estimate of feeding rate (Murtaugh, 1983), lower egestion rates are expected to decrease the energy uptake and consequently the $E_a$. Unexpectedly, mysids from the two highest exposure concentrations had a higher energy content (significant after 168 h) than mysids from the control. The reason behind this observation remains unclear. Perhaps, the correlation between feeding and egestion could be altered by toxicant exposure, although no chlorpyrifos-induced effects on absorption efficiency were apparent.

Clearly, present results indicate that chlorpyrifos exposure has significant effects on the energy expenditure/acquisition of *N. integer*. The increased oxygen uptake by *N. integer* reflects increased energy expenditure. Similarly, given that absorption efficiency and total energy content were relatively unaffected (there was a small concentration dependent

increase in $E_a$) by exposure to chlorpyrifos, increased energy expenditure will ultimately lead to reduced energy acquisition. In the CEA assay, the increase in energy consumption in chlorpyrifos-exposed mysids was the determining factor for the observed decline in CEA. In the SFG assay, on the other hand, feeding rate was reflected most strongly in the mysid SFG value. Both SFG and CEA incorporate the various components of an organism's energy budget by transformation of these components into energy equivalents (i.e. J/h), thus identifying the overall physiological effect of chlorpyrifos on *N. integer*. In this study, exposure to 0.072 and 0.100 µg chlorpyrifos/l led to a marked and concentration dependent reduction in SFG compared with control mysids (Fig. 5.4). Significant effects of chlorpyrifos on CEA were detected above 0.038 µg chlorpyrifos/l, but there was no evidence of a concentration-response relationship (Fig. 5.4). These effect on energy allocation would ultimately result in the mysid utilizing its energy reserves, and thus a decrease in $E_a$. Indeed, mysids exposed to 0.100 µg chlorpyrifos/l had a mean SFG close to zero and the standard deviation approaches zero, indicating that some individuals were potentially mobilizing their energy reserves. However, the $E_a$ was not significantly lower in these animals in the present study and such an effect might only become apparent following longer exposures, which are likely to result in complete mortality, given the 168-h LC50 of 0.084 µg chlorpyrifos/l.

One final consideration is to assess which of the physiological responses measured in the present study offers the best potential for regulatory or environmental assessment purposes. Oxygen consumption was the most sensitive response and increased following exposure to the lowest pesticide concentration (0.038 µg chlorpyrifos/l) in the SFG experiment. Although ETS activities in the CEA experiment were clearly induced in mysids exposed to the same concentration, this effect was only significant at 0.056 µg/l. Advantages of the ETS measurement are the fact that no respirometer is required and that it can be performed on homogenized animals at any time, which is imperative for field studies. On the other hand, given the initial cost of a respirometer, *in vivo* respiration measurements are very cheap and don't require the sacrifice of the animal. The ETS assay can mechanistically explain alterations in the energy consumption through specific interaction of the toxicant with the electron transport system (Oberdörster et al., 1998c; Spicer and Weber, 1991). However, ETS measurements had a greater variance in the present study (Fig 5.2) and did not show a linear concentration-relationship, making it more difficult to interpret. Both the ETS assay and whole animal oxygen consumption have the disadvantage that they are difficult to interpret as an isolated physiological response, especially since respiratory responses are also highly dependent on several abiotic and biotic factors (Roast et al., 1999b).

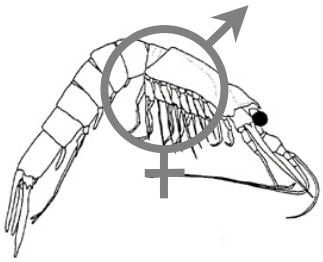
Scope for growth and cellular energy allocation are clearly the most appropriate responses to measure from a physiological point of view. However, SFG was disrupted only at concentrations approaching those causing lethal effects, especially for the longer

exposure periods used in the present study. Egestion rates were significantly affected at 0.072 µg chlorpyrifos/l in the SFG experiment, which is just below the 168-h LC50 of 0.084 µg chlorpyrifos/l for *N. integer*. This narrow range between sub-lethal and lethal concentrations may be a feature of active (motile) crustaceans in contrast to sessile bivalves where SFG is affected at much lower toxicant concentrations than causing mortality (Widdows and Donkin, 1992). The cellular responses of the CEA were more sensitive (lowest observed effect concentration (LOEC) of 0.038 µg chlorpyrifos/l) than the organismal responses of the SFG (LOEC of 0.072 µg chlorpyrifos/l), which corroborates the theory of higher sensitivities of endpoints at a lower level of biological organization (e.g. biochemical vs. physiological). We found a similar sensitivity of the CEA in *N. integer* exposed to the biocide tributyltinchloride, where CEA was significantly affected at 0.01 µg tributyltinchloride/l, which was an order of magnitude lower than the 96-h LC50 of 0.164 µg tributyltinchloride/l (Verslycke et al., 2003c). A disadvantage of the CEA response in the present study was the lack of a linear concentration-dependent response in the range between low-sublethal and high sub-lethal effects of chlorpyrifos.

Although the interpretation of the individual components of the CEA (lipid, sugar and protein content) requires extensive background and control data, especially when used in field-collected organisms (Verslycke et al., submitted-Chapter 10), the incorporation of energy consumption makes the CEA a relevant biomarker with a similar concept as the SFG. In addition, the CEA offers the possibility to assess (separately) the toxicant-induced effects on the major metabolic pathways, e.g. energy conversion towards growth (protein metabolism) might be separated from reserve depletion (lipids and sugar) to meet metabolic demands (De Coen, 1999). This differential effect on metabolic pathways has been used successfully as a sensitive endpoint by McKenney (1998) in mysids. This author demonstrated a significant effect of pesticide exposure on O:N ratios, which are indicative of differences in carbohydrate and/or lipid versus protein substrate utilization, and which resulted in reduced reproductive effects in the mysid *Americamysis bahia*. Similarly, the biochemical responses of the CEA assay have been correlated significantly with population-level effects in toxicant-exposed daphnids, such as the intrinsic rate of natural increase and the mean total offspring per female (De Coen and Janssen, 1997). In the present study, proteins were the fraction that was most strongly affected in chlorpyrifos-exposed mysids (Table 5.1). Several investigators have demonstrated that mysid metabolism is protein-based (Chin, 1974; Fergusson, 1973; Gaudy et al., 1980) and, therefore, protein metabolism might be an important endpoint to measure in toxicant-exposed mysids.

## 5.5. Conclusions

In summary, exposure to chlorpyrifos caused significant effects on the physiology of *Neomysis integer* which were detected by both the SFG and CEA assay. While some of the individual SFG and CEA components, such as oxygen consumption and protein content, were sensitive endpoints, it is unknown whether these are a consistent response to a wide range of chemicals. Furthermore, both SFG and CEA are integrative and provide more information concerning the effect of contaminants on the overall physiology of the animal, and potential effects at the population level. From the present study, CEA would appear to be a more sensitive biomarker than SFG [perhaps because it measures effects at a lower level of biological organization (cellular) than SFG (sub-organismal)], but in contrast to SFG does not show a concentration-dependent response in the sub-lethal to lethal range. However, this is only the first publication in which CEA and SFG responses are compared in a similar test set-up. Furthermore, a considerable amount of work has been conducted on the influence of extrinsic and intrinsic factors on SFG, and of all stress indicators, SFG is probably the most developed and field-validated bioassay through several ecosystem monitoring programs (Huggett et al., 1992). The present study should, however, stimulate the use of CEA as an alternative or complementary approach to measure physiological aberrations in animals. It has been used in daphnids (De Coen and Janssen, 1997), fish (Nguyen, 1997; Smolders et al., 2003) and insects (Lock et al., submitted), and is easily adopted for use in other vertebrate or invertebrate species. Future studies should focus on improving the interpretation of CEA and on correlating both SFG and CEA responses in mysids from short-term exposures with higher level (i.e. reproduction, population) chronic effects. From the extensive work by McKenney and co-workers (1986, 1990, 1996, 1998) on physiological dysfunction in *Americamysis bahia* following chronic pesticide exposure, it can be concluded that metabolic alterations in mysids may provide sensitive assays to detect population-level effects. In conclusion, the present study does not permit a conclusive statement as to whether one assay is better than the other, as both assays had inherent strenghts and weakenesses.



# CHAPTER 6

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# Testosterone metabolism of *Neomysis integer* as a biomarker for endocrine disruption: Identification and quantification of *in vivo* produced testosterone metabolites and endogenous vertebrate-type steroids

**Redrafted after :**

Verslycke T, De Wasch K, De Brabander HF, Janssen CR (2002) Testosterone metabolism in the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea): Identification of testosterone metabolites and endogenous vertebrate-type steroids. *General and Comparative Endocrinology* 126:190-199.

**and**

De Wasch K, Poelmans S, Verslycke T, Janssen C, Van Hoof N, De Brabander HF (2002) Alternative to vertebrate animal experiments in the study of metabolism of illegal growth promotors and veterinary drugs. *Analytica Chimica Acta* 473:59-69.

# Chapter 6

## *Testosterone metabolism of Neomysis integer as a biomarker of endocrine disruption: Identification and quantification of in vivo produced testosterone metabolites and endogenous vertebrate-type steroids*

**Abstract**

Testosterone metabolism by *Neomysis integer* (Crustacea: Mysidacea) was assessed to obtain initial data on its metabolic capacity. *N. integer* were exposed to both testosterone and [$^{14}$C]testosterone. Identification of *in vivo* produced testosterone metabolites and endogenous vertebrate-type steroids was performed using thin-layer chromatography (TLC) and liquid chromatography with multiple mass spectrometry (LC-MS$^n$). Endogenous production of testosterone in mysids was detected for the first time. *N. integer* were exposed to testosterone and metabolized administered testosterone extensively. At least 11 polar testosterone metabolites ($R_{f,\text{metabolite}} < R_{f,\text{testosterone}}$), androstenedione, dihydro-testosterone, and testosterone were produced *in vivo* by *N. integer*. A sex-specific testosterone metabolism was also observed in mysids, although this observation requires further confirmation. The anabolic steroid β-boldenone was identified for the first time in invertebrates. The metabolic pathway leading to the formation of β-boldenone remains unknown, since the steroidal precursor androstadienedione could not be detected. These results reveal interesting similarities in enzyme systems in invertebrate and vertebrate species. Alterations in steroid hormone metabolism may be used as a new biomarker for the effects of endocrine disruptors in invertebrates.

### 6.1. Introduction

Changes of the hormone system due to environmental contaminants (endocrine disruptors) has recently become a widely investigated and politically charged issue (Colborn et al., 1996; Krimsky, 2000). Invertebrates account for 95% of all animals (Barnes, 1980), yet surprisingly little effort has been made to understand their value in signaling potential environmental endocrine disruption (Alvarez and Ellis, 1990; Crisp et al., 1997; DeFur et al., 1999; Depledge and Billinghurst, 1999; LeBlanc, 1999; LeBlanc and Bain, 1997; Oberdörster and Cheek, 2000).

The use of hormones to regulate biological processes is a strategy common to both vertebrates and invertebrates. Endocrine control strategies and basic hormonal mechanisms to regulate biological processes have been widely conserved among animal phyla (DeFur et al., 1999). Vertebrate-type steroids such as 17β-estradiol (De Loof and De Clerk, 1986), testosterone (Burns et al., 1984; Fairs et al., 1989) and progesterone (Kanawaza and Teshima, 1971; Yano, 1985) have been detected in malacostracan crustaceans. Fragmented evidence even suggests a functional role for some of these compounds in crustaceans (Nagabhushanam and Kulkarni, 1981; Sarojini, 1963). There are, however, no data on the occurrence and metabolic pathways of vertebrate-type steroids in mysid shrimp. Very little information exists in literature on the endocrinology of mysids and crustaceans in general (DeFur et al., 1999).

Recent studies have used steroid metabolism as a new biomarker for identifying endocrine-disrupting effects in invertebrates (Baldwin and Leblanc, 1994a,b; Baldwin et al., 1997, 1998; Morcillo et al., 1998; Oberdörster et al., 1998a,b,c; Ronis and Mason, 1996). Alterations in steroid hormone metabolism often correlate with effects on steroid hormone-dependent processes such as growth and reproduction (Parks and LeBlanc, 1996).

Vertebrate-type steroids have been used as substrates to study the various P450 enzymes of the oxidative metabolism in invertebrates. These enzymes are also responsible for the biotransformation and elimination of lipophilic environmental pollutants (Hahn and Stegeman, 1994). Results from these studies provide information on the complexity of the P450 systems in invertebrates and its degree of similarity to P450s in vertebrates. It has been suggested that modulation of the P450 activity has potential as a biomarker of toxicant exposure in invertebrates (Baldwin and LeBlanc, 1994a,b). In addition, these studies could provide alternative models for the partial replacement of vertebrate animals in metabolic studies with, for instance, illegal growth promoters and veterinary drugs.

The subtropical mysid *Americamysis* (=*Mysidopsis*) *bahia* is a standard organism used in laboratory toxicity testing (ASTM, 1999; USEPA, 1995a, 2002b). The available toxicity data suggest that mysids *sensu lato* are very sensitive to toxic chemicals (Roast et al., 1998a). Mysids have also been proposed as standard test organisms for evaluating the effects of endocrine disruptors (EDSTAC, 1998). There is, however, growing interest in using indigenous mysids in toxicity testing and environmental monitoring (Langdon et al., 1996). Consequently, *Neomysis integer* has been identified as a possible European alternative to the standard American species (Emson and Crane, 1994; Roast et al., 1999a; Wildgust and Jones, 1998). *N. integer* has been reported to be present in all European coastal waters of the Atlantic Ocean (Tattersall and Tattersall, 1951), where it dominates the hyperbenthic fauna of the low-salinity regions of estuaries (Mees and Hamerlynck, 1992; Mees et al., 1995b).

In this study, testosterone metabolism by *N. integer* was assessed to obtain initial data

on its metabolic capacity. Testosterone metabolites and endogenous vertebrate-type steroids were identified. Furthermore, results from this study provide information on the complexity of the P450 system in *N.* integer and its degree of similarity to P450s in vertebrates. Finally, these results are of value in future exposure studies to evaluate modulations of the testosterone metabolism profile by specific xenobiotics.

## 6.2. Material and Methods

### *6.2.1. Animal collection and maintenance*

Initial *Neomysis integer* populations were collected from the Galgenweel (Antwerp, Belgium) and cultured in the lab as described in Chapter 3 (§ 3.2.1).

### *6.2.2. In vivo exposure to testosterone*

Adult male and female *Neomysis integer* were taken from the 200-l aquaria and individually placed into 5-ml glass recipients containing 2 ml culture medium. Two micrograms of testosterone (Sigma-Aldrich, Bornem, Belgium) dissolved in 10 µl methanol was added to the recipients and a 16-h exposure period was applied. For the radioactive experiments 10 µl [4-$^{14}$C]testosterone (specific activity: 53.6 mCi/mmol; packaging: 0.04mCi/ml ethanol) (NEN, Boston, MA, USA) was added to the test medium and metabolized for 16h. These test conditions were modified for *N. integer* from similar experiments with the Cladoceran *Daphnia magna* (Baldwin and LeBlanc, 1994b). Replication was used for these experiments and blanks were run to account for breakdown or microbial transformation of testosterone in the absence of mysids.

### *6.2.3. Thin-layer chromatography analysis of testosterone and [$^{14}$C]testosterone metabolites*

Extraction and thin-layer chromatography (TLC) of testosterone metabolites was adopted from Baldwin and LeBlanc (1994a,b) with minor modifications. *Neomysis integer* were isolated from the exposure medium, shock-frozen in liquid nitrogen, and homogenized on ice in 100 µl deionized water using a motor-driven teflon pestle. Testosterone metabolites were extracted using 4 ml ethyl acetate (2 x 2 ml) and phase-separated using centrifugation. The ethyl acetate fractions were pooled and evaporated under a stream of nitrogen. The residue was redissolved in 40 µl ethyl acetate and spotted onto an aluminum-backed silica gel TLC plate (Merck; 20 cm x 20 cm x 0.25 mm). The metabolites were separated with a double-solvent system consisting of methylene chloride:acetone (4:1, v/v) and chloroform:ethyl acetate:ethanol (4:1:0.7, v/v/v). All of these solvents were of HPLC-grade

(Sigma-Aldrich, Belgium). The [$^{14}$C]testosterone metabolites were visualized using autoradiography film (Kodak X-OMAT AR). For visualization of the nonradioactive testosterone metabolites and authentic standards, the plates were sprayed with a $H_2SO_4$/Acetone (1:1, v/v) mixture and charred in the oven at 160°C for 15 min. The different metabolites were identified on the basis of their cochromatography with the authentic standards (Table 6.1).

**Table 6.1.** Retention of authentic steroid standards following TLC and LC-MS$^n$. Identification of testosterone metabolites and vertebrate-type steroids after *in vivo* exposure of *N. integer* to 2 µg testosterone for 16h

| Standards | Chromatography | | Identified | |
|---|---|---|---|---|
| | $R_{f,rel}$ TLC | $T_{r,rel}$ LC-MS$^n$ | Organism | Medium |
| Androstenedione[a] | 1.18 | 0.90 | ●■ | ●■ |
| Androstenolone[a] | 1.17 | 1.05 | - | - |
| Androstadienedione[a] | 1.12 | 0.74 | - | - |
| Dihydrotestosterone[a] | 1.08 | 1.16 | ●■ | ●■ |
| Androsterone[a] | 1.07 | 1.26 | - | - |
| 17β-Estradiol[a] | 1.02 | 0.96 | - | - |
| Testosterone[b] | 1.00 | 1.00 | ●■ | ●■ |
| β-Boldenone[a] | 0.89 | 0.87 | ● | ●■ |
| 2α-OH[a] | 0.82 | 0.76 | ● | ●■ |
| 16β-OH[a] | 0.77 | 0.72 | ● | ● |
| 6β-OH[a] | 0.71 | 0.55 | ● | ● |
| 6α-OH[a] | 0.63 | 0.41 | ● | ●■ |
| 11β-OH[a] | 0.53 | 0.79 | ●■ | ●■ |
| 7α-OH[a] | 0.49 | 0.55 | - | ●■ |
| 16α-OH[a] | 0.44 | 0.64 | ● | ●■ |
| 11α-OH[a] | 0.41 | 0.72 | - | ● |
| 15α-OH[a] | 0.36 | 0.53 | - | ●■ |

[a] Purchased from Steraloids Inc. (UK).
[b] Purchased from Sigma-Aldrich (Belgium).
●, identified by TLC.
■, identified by LC-MS$^n$.

*6.2.4. Liquid chromatography with multiple mass spectrometry analysis of testosterone metabolites and endogenous vertebrate-type steroids*

Exposure and extraction procedures for liquid chromatography with multiple mass spectrometry (LC-$MS^n$) analysis of the metabolites were identical to those for the TLC analyses. The HPLC apparatus was composed of an Agilent 1100 series pump, a model AS3000 autosampler (TSP, San Jose, CA, USA) and vacuum degasser (Agilent, Palo Alto, USA). Separation was performed on a Symmetry $C_{18}$ column (5 µm, 150 mm x 2.1 mm, Waters, Milford, USA). The flow rate was 0.3 ml/min. Testosterone and metabolites were ionized using atmospheric pressure chemical ionization (APCI) and chromatographed using gradient elution. Analysis was carried out using an LCQdeca Ion Trap Mass Analyzer (ThermoQuest, San Jose, USA), with an APCI interface and XCalibur 1.2. software. The metabolites were detected in MS-MS-full scan positive ion mode. The solvents for preparation of the mobile phase were HPLC-grade obtained from Merck (Darmstadt, Germany). To separate the different compounds, a linear gradient consisting of a mixture of 60% 0.02M formic acid and 40% methanol was used. The methanol percentage increased from 40% to 80% in 25 min. The column was allowed to equilibrate at initial conditions for 8 min. The different testosterone metabolites were identified on the basis of their relative retention times (calculated as a ratio of the retention time of testosterone) as compared with the retention times of authentic standards (Table 6.1).

## 6.3. Results

*6.3.1. TLC analysis of testosterone metabolites*

Female and male *Neomysis integer* produced at least 11 polar metabolites ($R_{f,\text{metabolite}} < R_{f,\text{testosterone}}$) and a number of nonpolar metabolites ($R_{f,\text{metabolite}} > R_{f,\text{testosterone}}$) during *in vivo* exposure to [$^{14}$C]testosterone (Fig. 6.1). The metabolites were identified on the basis of their comigration with authentic standards, using the solvent system described under Material & Methods (Table 6.1).

The nonpolar metabolites were poorly resolved from one another in the TLC solvent system used, and only androstenedione and dihydrotestosterone could be identified and confirmed by LC-$MS^n$.

Of the 11 polar metabolites, β-boldenone, 9 monohydroxy metabolites (2α-, 16β-, 6β-, 6α-, 11β-, 7α-, 16α-, 15α-, and 11α-hydroxytestosterone), and an unknown metabolite were identified by their relative retention in the solvent system used. Of these polar metabolites, β-boldenone and 6 monohydroxymetabolites (2α-, 6α-, 11β-, 7α-, 16α-, and 15α-

hydroxytestosterone) could be confirmed with LC-MS$^n$.

From the autoradiogram depicted in Fig. 6.1, it can be concluded that the nonpolar lipophilic metabolites were preferentially retained by *N. integer*, while polar hydrophilic metabolites were most abundant in the test medium.

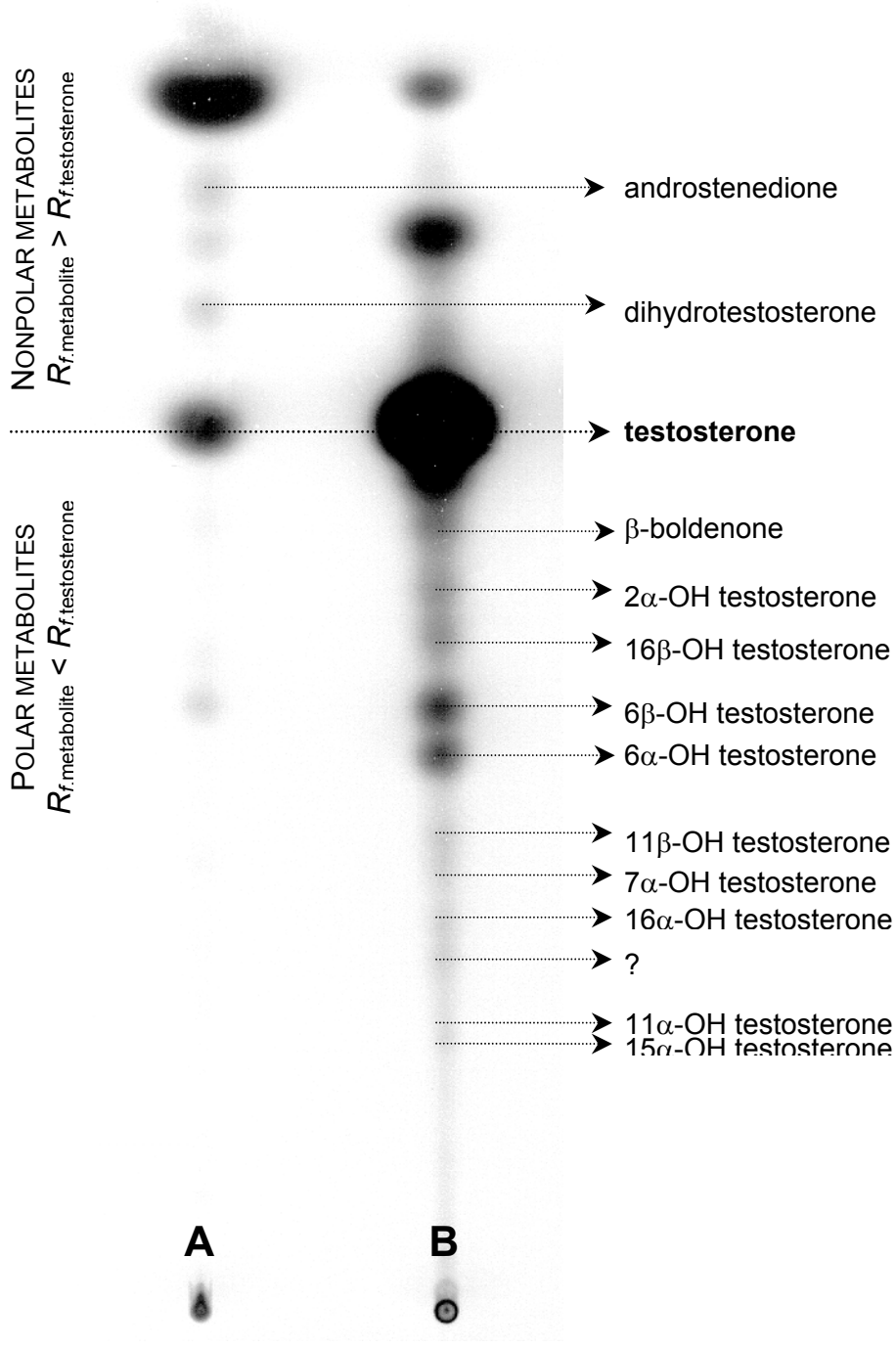



**Figure 6.1.** Autoradiography (96 h) of [$^{14}$C]testosterone metabolites in homogenates (A) and in the test medium (B) of exposed *N. integer* following TLC. The different metabolites are identified on the basis of their cochromatography with authentic standards.

### *6.3.2. LC-MS$^n$ analysis of testosterone metabolites*

The metabolites in *Neomysis integer* and the test medium after *in vivo* exposure to testosterone were identified using LC-MS$^n$ (Fig. 6.2). A protocol was adopted to separate 9 different monohydroxy standards and vertebrate-type steroids in one run. The relative retention times (calculated as a ratio of the retention time of testosterone) of the different steroids on the LC-MS$^n$ are shown in Table 6.1 as compared with the relative retentions in the TLC solvent system. Six monohydroxy testosterone metabolites and two unknown polar testosterone metabolites were detected in the test medium by their chromatographic retention and mass spectrum (MS$^2$). Only 11β-hydroxytestosterone could be identified in extracts of *N. integer* exposed to testosterone.

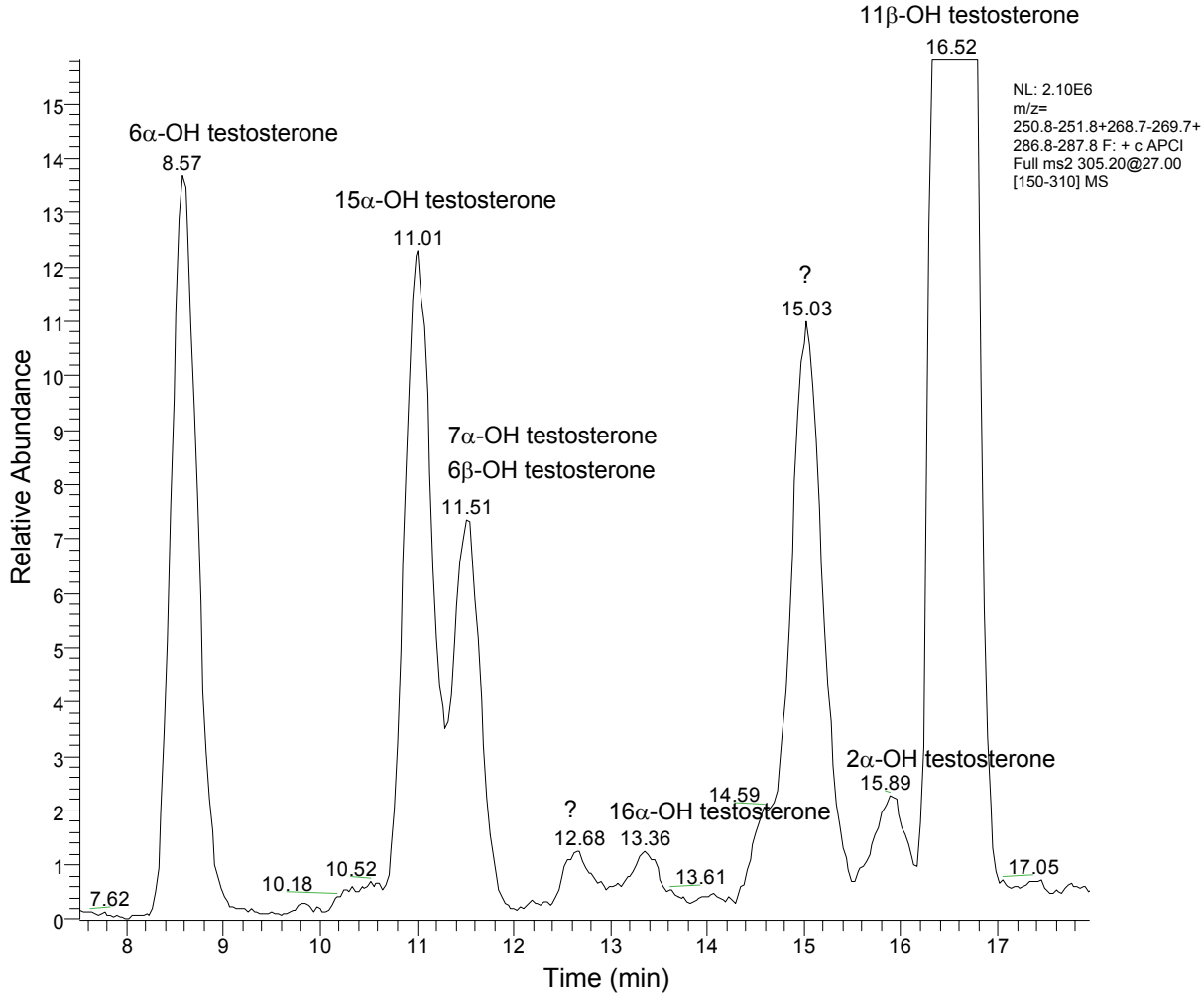



**Figure 6.2.** Detection of monohydroxy testosterone metabolites with LC-MS$^n$ after *in vivo* testosterone biotransformation in *N. integer*.

### *6.3.3. Detection of vertebrate-type steroids by LC-MS$^n$ in testosterone-exposed and unexposed N. integer*

Testosterone-exposed and unexposed *Neomysis integer* were tested for different vertebrate-type steroids (Table 6.1). In both male and female unexposed *N. integer*, endogenous testosterone was identified (Fig. 6.3). Preliminary quantification results indicate significantly higher (about 5 times higher in male; data not shown) endogenous testosterone levels in male mysid compared to those in females. Androstenedione was endogenously produced by male mysids, but could not be detected in female organisms. Further confirmation is needed to investigate the possibility of a sex-specific testosterone metabolism in *N. integer*.

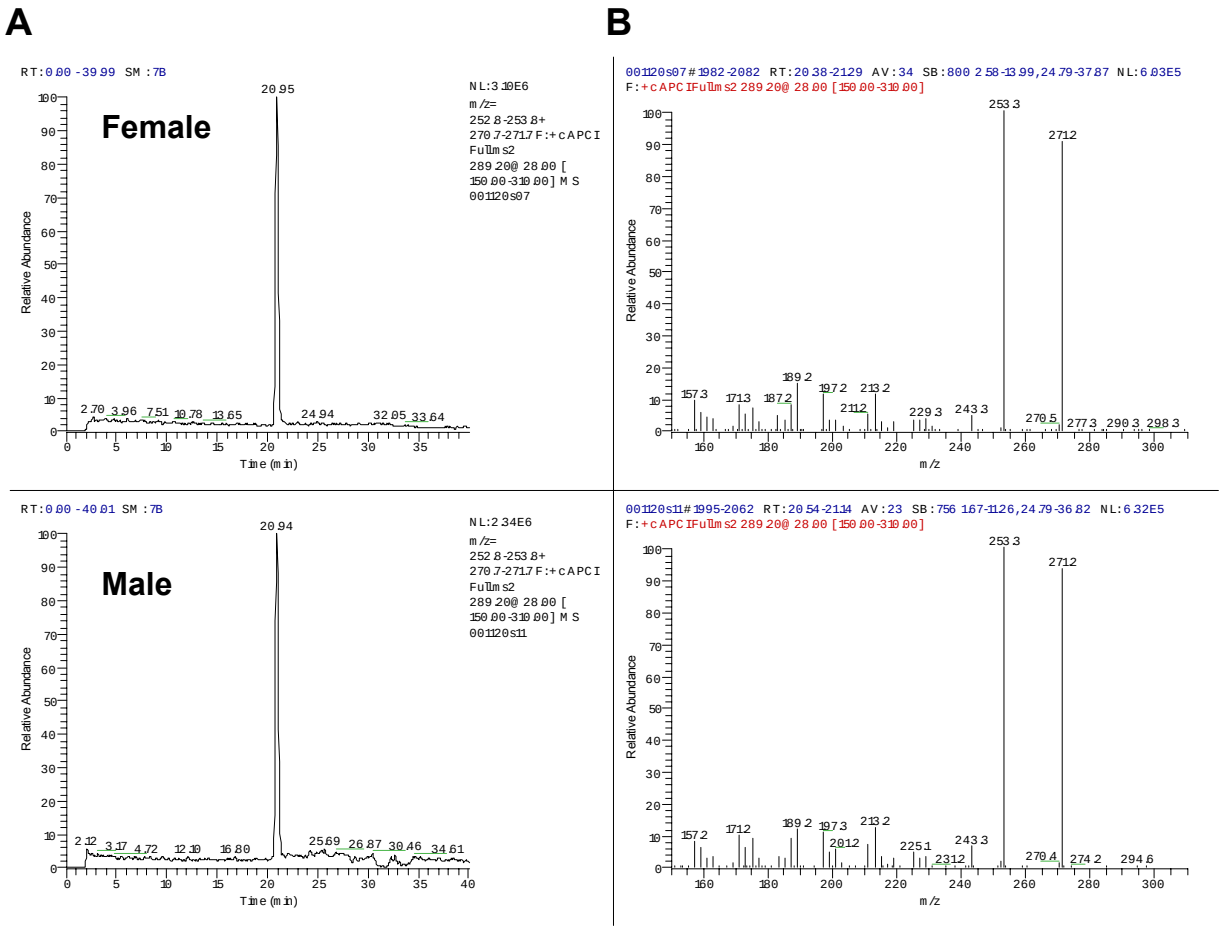



**Figure 6.3.** Homogenates of *N. integer* were extracted and analyzed on LC-MS$^n$. The chromatogram shows evidence of endogenous testosterone production in male and female *N. integer*. (A) Retention time for testosterone; (B) mass spectrum for testosterone.

β-Boldenone, an anabolic steroid, was detected in the test medium of exposed male and female organisms, but the steroid precursor androstadienedione could not be detected. Dihydrotestosterone and androstenedione were also found in the test medium of *N. integer* exposed to testosterone (Fig. 6.4).

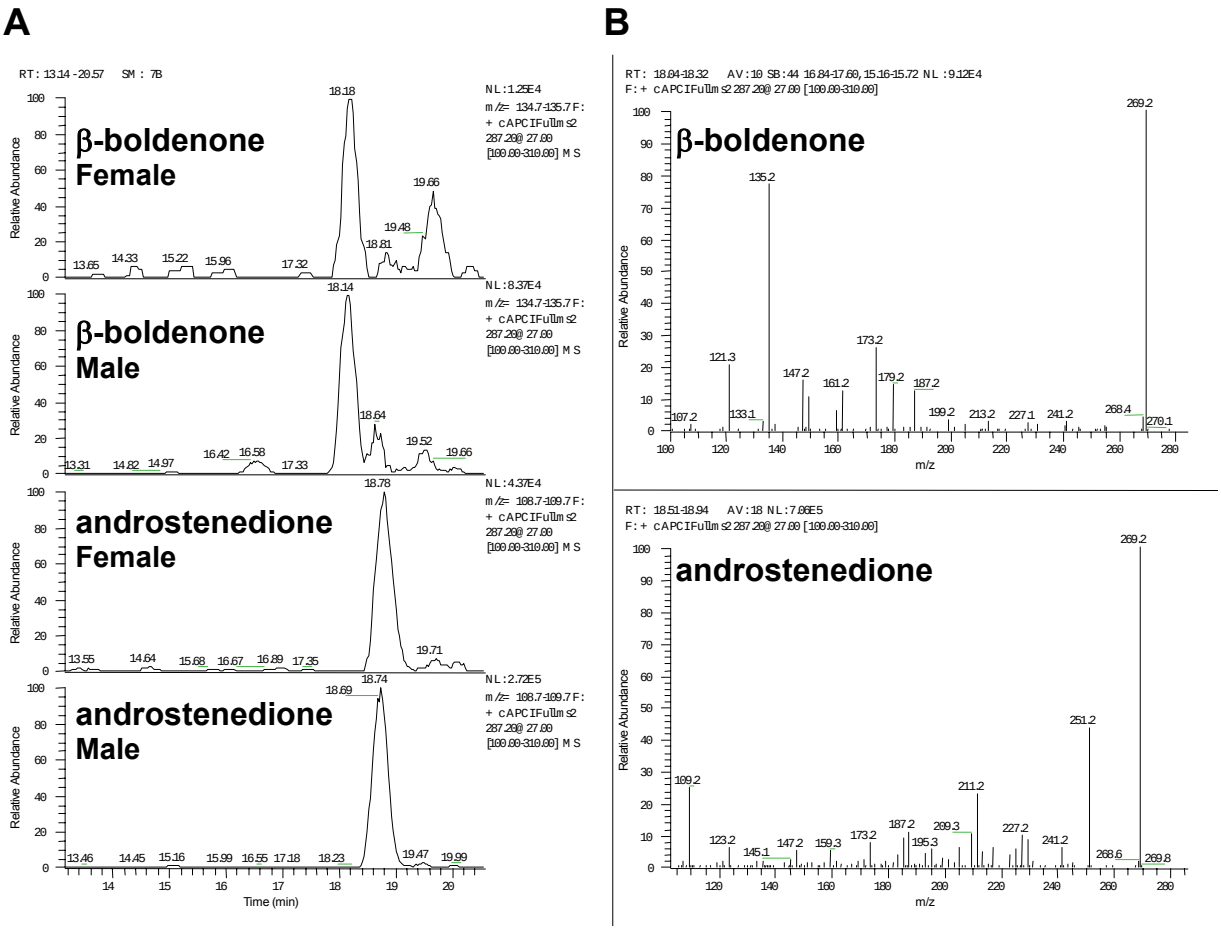



**Figure 6.4.** Detection of the anabolic steroid β-boldenone and the vertebrate-type steroid androstenedione by LC-MS$^n$ in male and female *N. integer* exposed to 2 µg testosterone. (A) Retention time, (B) mass spectrum.

### 6.4. Discussion

*Neomysis integer* produced all the monohydroxy testosterone metabolites (2α-, 16β-, 6β-, 6α-, 7α-, 15α-) found in a similar study with the crustacean *Daphnia magna* (Baldwin and Leblanc, 1994a), but also three new monohydroxy metabolites (11β-, 16α-, 11α-) which were not identified in the daphnid study. This could be a result of the greater identification capability of the combined chromatography methods (TLC and LC-MS$^n$) used in the present study.

Of the nonpolar metabolites, only androstenedione and dihydrotestosterone could be identified. Since the nonpolar metabolites were very poorly resolved by TLC, a more detailed analysis for vertebrate-type steroids was carried out on extracts of exposed and unexposed *N. integer* with LC-MS$^n$. This led to the identification of β-boldenone (1,4-androstadiene-17β-ol-3-one), a close derivative of testosterone (4-androstene-17β-ol-3-one) differing only by the addition of a second double bond in the A ring of the structure. The direct precursor of boldenone is androstadienedione, which differs from testosterone's direct precursor only by the same alteration (Fig. 6.5).

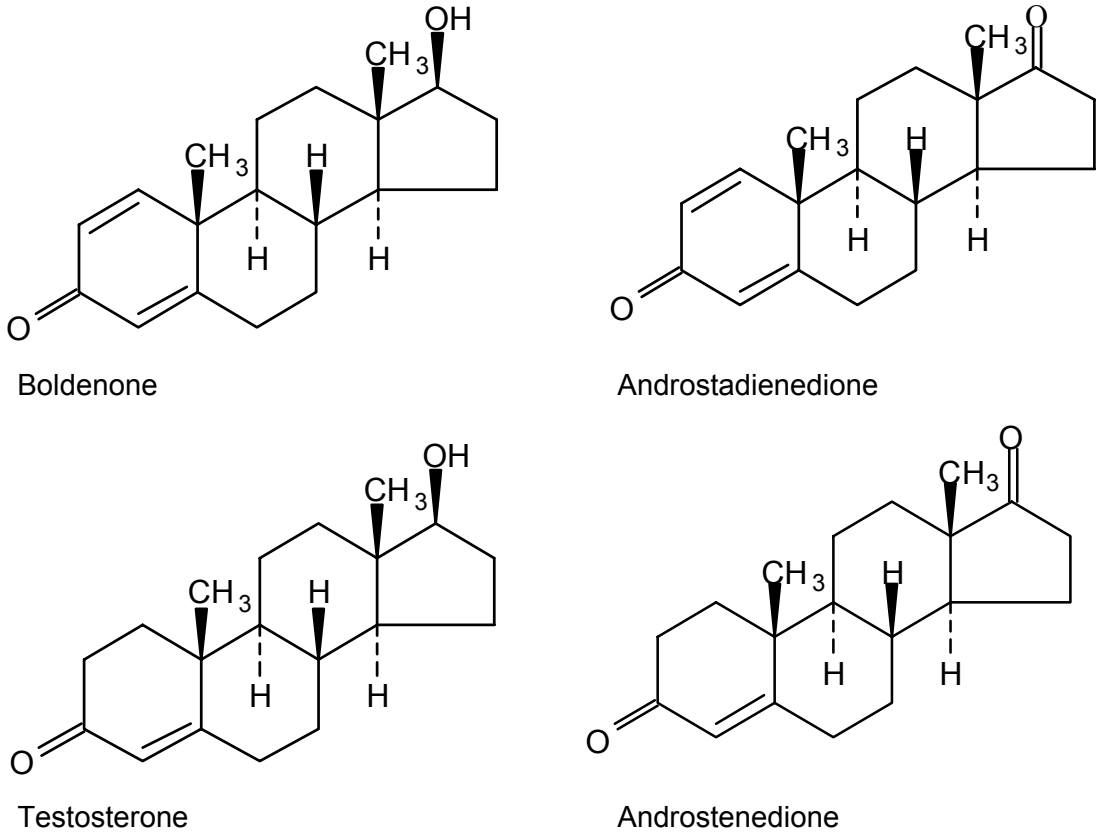



**Figure 6.5.** Structure of testosterone, the anabolic steroid boldenone, and their respective precursors androstenedione and androstadienedione.

To our knowledge, boldenone has never been identified in invertebrates. As it was not detected in unexposed organisms, it can be hypothesized that it is a biotransformation product of testosterone (by dehydrogenation of testosterone in position 1,2). The metabolism of this compound is not clearly understood in vertebrates, where it is mostly used as a performance enhancer in the form of boldenone-17-undecylenate. Androstenedione was produced endogenously by male mysid and was also found in male and female *N. integer* exposed to testosterone, which illustrates the presence of the widely distributed body enzyme 17β-hydroxysteroid dehydrogenase (which interconverts testosterone-

androstenedione and boldenone-androstadienedione between inactive 17-keto and active 17-beta hydroxy form) in *N. integer*. Unexpectedly, androstadienedione could not be detected. Further research is required to elucidate the possible biotransformation pathways of boldenone and its metabolites. Important to mention is that the reproducibility of the experiments in the case of boldenone was a problem. During a certain period all exposed samples were clearly positive for β-boldenone. Later, we had only a few positive responses and sometimes none showed a response for boldenone. Furthermore, only after exposure to a high concentration of an anabolic steroid (testosterone and also stanozolol, data not shown) was boldenone formed and detected in the medium (highest response) and organism (lowest response). The reason for this observation is, as yet, unknown.

One step in identifying testosterone and its metabolites with LC-$MS^n$ was to look for endogenous testosterone production in *N. integer*. Baldwin and Leblanc (1994b) found no indication of testosterone production by daphnids. The results of our study clearly show the presence of endogenous testosterone in unexposed male and female *N. integer*. To our knowledge, evidence for testosterone production has only been described for some decapod crustaceans (Burns et al., 1984; Fairs et al., 1989), but never for mysid shrimp. The limited available data on vertebrate-type steroids in crustaceans are insufficient to conclude that these steroids function as hormones (DeFur et al., 1999). Future studies will address the possible functional role for testosterone or its metabolites in *N. integer*.

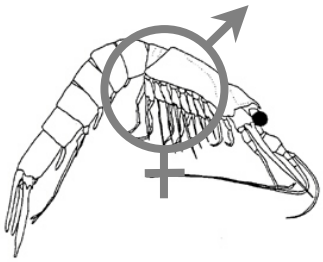
The main vertebrate estrogen 17β-estradiol could not be detected in *N. integer*. Although previous studies have identified this compound in malacostracan crustaceans (De Loof and De Clerk, 1986), the functional role of estrogens has not been established in arthropods. Androsterone, a 17-keto steroid derived from testosterone in humans and androstenolone (dehydroepiandrosterone), a precursor of androstenedione, also were not detected in our study.

The results from the *in vivo* biotransformation experiments with *N. integer* demonstrate the presence of a complex steroid hydroxylase system consisting of different P450 isozymes. The remarkable diversity of testosterone hydroxylation exhibited should stimulate further studies on the induction, stereo-specificity, and regulation of the enzyme systems of *N. integer*. These results are also in line with previous observations that have indicated that testosterone is a good steroid substrate to study enzyme systems in crustaceans (James, 1989; James and Boyle, 1998).

Steroid hydroxylation reactions are catalyzed by most P450 isozymes found in mammalian liver. Theoretically, testosterone can be hydroxylated at 20 different sites by different isozymes. Since each P450 isozyme has a distinct hydroxylation pattern, this pattern can be used to determine the relative concentration of these enzymes (Correia, 1995). These hydroxylation patterns can be affected by exposure to xenobiotics through a

number of different mechanisms (e.g., transcriptional inhibition or induction of P450 enzymes, competition of inducible or constitutive enzymes for limited cellular heme pools, perturbation of the hormonal regulation of P450 enzyme expression, etc.) (Waxman, 1988; Yeowell et al., 1987). Endocrine-disrupting compounds also have the potency to affect hydroxylation patterns in invertebrates (Baldwin et al., 1995; Oberdörster et al., 1998c). As a result, modulation of the P450 activity in *N. integer* can potentially be used as a predictive biomarker for exposure to endocrine disruptors.

The LC-$MS^n$ method described here has the advantage over the TLC method that results are obtained rapidly and without the use of radioactive substrates. Difficulties due to interference with endogenous steroid concentrations, which is not a problem when working with labeled compounds, may be encountered. Another advantage of working with labeled compounds is the possibility of detecting very small quantities of a metabolite by increasing the autoradiography exposure period. An autoradiography period of 96 h was applied for the identification of the different metabolites in the homogenates of exposed *N. integer*. These metabolites were below the detection limit of the LC-$MS^n$ method. The LC-$MS^n$ method is, however, convenient for use in routine biomonitoring, and additional studies are underway to further identify and quantify metabolites by LC-$MS^n$ and validate these results as a biomarker for endocrine disruption in *N. integer*.



# CHAPTER 7

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# Testosterone metabolism of *Neomysis integer* following exposure to tributyltin

**Redrafted after :**

Verslycke T, Poelmans S, De Wasch K, Vercauteren J, Devos C, Moens L, Sandra P, De Brabander HF, Janssen CR (2003) Testosterone metabolism in the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) following tributyltin exposure. *Environmental Toxicology and Chemistry* 22: 2030-2036.

# Chapter 7

## *Testosterone metabolism of Neomysis integer following exposure to tributyltin*

**Abstract**

Current evidence suggests that the biocide tributyltin (TBT) causes the development of imposex, a state of pseudohermaphrodism in which females exhibit functional secondary male characteristics, by altering the biotransformation or elimination of testosterone. Imposex in gastropods following TBT exposure is the most complete example of the effects of an endocrine disruptor on marine invertebrates. Previous studies have demonstrated that the estuarine mysid *Neomysis integer* converts testosterone into multiple polar and nonpolar metabolites resulting from both phase I and phase II biotransformations (Chapter 6). In the present study, the effects of tributyltinchloride (TBTCl) on the phase I and phase II testosterone metabolism of *N. integer* were evaluated. TBTCl was highly toxic to *N. integer* (96h-median lethal concentration of 164 ng/l). To assess the effects on testosterone metabolism, mysids were exposed for 96 h to different concentrations of TBTCl (control, 10, 100 and 1000 ng/l), and testosterone elimination as polar hydroxylated, nonpolar oxido-reduced, and glucose- and sulfate-conjugated metabolites was examined. TBTCl differentially affected testosterone metabolism. The effect of TBTCl on phase I metabolism was unclear and has been shown to vary among species, likely depending on the inducibility or presence of certain P450 isozyme families. Reductase activity and metabolic androgenization were induced in the 10 ng/l treatment, whereas higher concentrations resulted in a reduction of sulfate conjugation. The exact mechanisms underlying TBT-induced imposex and alterations in the steroid metabolism need to be further elucidated.

### 7.1. Introduction

Anthropogenic chemicals that can disrupt the hormonal systems (endocrine disruptors) of wildlife species have recently become a widely investigated and politically charged issue (Colborn et al., 1996; Depledge and Billinghurst, 2000; Krimsky, 2000). Invertebrates account for 95% of all animals (Barnes, 1980), yet surprisingly little effort has been made to understand their value in signaling potential environmental endocrine disruption (Alvarez and Ellis, 1990;

Crisp et al., 1997; DeFur et al., 1999; Depledge and Billinghurst, 1999; LeBlanc, 1999; LeBlanc and Bain, 1997; Oberdörster and Cheek, 2000). Presently, very few clear examples of endocrine disruption in invertebrates have been reported, perhaps because their hormonal systems have not been documented comprehensively (DeFur et al., 1999).

Imposex in marine neogastropods following exposure to tributyltin (TBT), a marine biocide used in antifouling paints, is the most complete example of the effects of an endocrine disruptor on marine invertebrates (DeFur et al., 1999). This pseudohermaphroditic condition occurs at environmentally relevant concentrations (LeBlanc and Bain, 1997) of TBT, which has been associated with population-level effects on marine neogastropods (Bryan et al., 1986; Matthiessen et al., 1995). Although to our knowledge the underlying mechanism by which TBT causes imposex in gastropods has not been conclusively elucidated, the weight of evidence favors the aromatase-inhibition hypothesis (Matthiessen and Gibbs, 1998). This hypothesis states that higher levels of TBT in the presence of natural levels of testosterone probably inhibit competitively cytochrome P450-dependent aromatase (CYP19), thereby preventing the conversion of testosterone to 17β-oestradiol (Bettin et al., 1996; Spooner et al., 1991; Stroben et al., 1991). This causes an increase in testosterone levels, leading to imposex induction. Alternatively, Ronis and Mason (1996) suggested that TBT causes imposex in the periwinkle *Littorina littorea* by blocking phase II sulfate conjugation (and, hence, excretion) of testosterone and its potent metabolites (e.g. androstenedione, dihydroandrostenedione, dihydrotestosterone, and dihydrotestosterone-diols). However, this hypothesis could not be confirmed by Oberdörster et al. (1998b), who found no overall change in the elimination of testosterone as polar conjugates in TBT-exposed normal and imposex mud snails *Ilyanassa obsoleta*. Similarly, Gooding and LeBlanc (2001) concluded that mud snails do not readily eliminate testosterone as polar derivatives. Finally, TBT can also interfere directly with the neurohormonal system of mollusks and, consequently, lead to changes in steroid titers only as a secondary effect (Féral and Legall, 1983; Oberdörster and McClellan-Green, 2000). Although TBT obviously interacts with some part of the endocrine system of mollusks (Oberdörster and Cheek, 2000), further investigations are needed to reveal the underlying mechanisms of imposex in these animals as well as to examine the interactions of this chemical with the hormone system of other invertebrates.

Most of the knowledge regarding crustacean endocrinology is derived from studies with larger decapods, such as crabs, lobsters, crayfish, and shrimp. Although some examples of laboratory experiments demonstrating the effects of endocrine disruptors in these animals have been reported, the conclusions are often ambiguous. Crustaceans may serve as good indicator species of endocrine disruption because of their economic importance, ecological significance, and extensive use as model invertebrates in laboratory toxicity testing (DeFur et al., 1999). In this context, we are investigating the potential of the estuarine mysid *Neomysis integer* as an

indicator species for the potential effects of endocrine disruptors. Standard guidelines for conducting life-cycle toxicity tests with saltwater mysids have been developed using *Americamysis bahia* as a model species (ASTM, 1999; USEPA 1995a, 1997). These standard methods, however, can be modified for testing with other mysid species (Verslycke et al., submitted-Chapter 2). The mysid *N. integer* dominates the hyperbenthic fauna of the low-salinity regions of western European estuaries (Mees et al., 1995b; Mees and Jones, 1997), and it has been proposed as a European alternative to the standard test species *A. bahia* (Emson and Crane, 1994; Roast et al. 1998a; Roast et al. 1999b; Verslycke and Janssen, 2002). The works of Roast et al. (1998a,b; 1999a,b,c; 2000a,b,c; 2001a,b) and Verslycke et al. (2002, 2003a,b,c) demonstrate the successful use of this species for ecotoxicological work. Unfortunately, little information is available in literature regarding the endocrinology of mysids.

Generally, most studies regarding the effects of endocrine disruptors in crustaceans have documented the interference of juvenile hormone analogs and other insecticides in the molting and metamorphosis of these animals (Oberdörster and Cheek, 2000). A number of studies have been published on the steroid metabolism in crustaceans as a biomarker for the endocrine-disruptive effects of TBT. Several studies, for example, investigated changes in the testosterone metabolism of daphnids following exposure to various xenobiotics, including TBT (Baldwin and LeBlanc, 1994a,b; Baldwin et al., 1995, 1997, 1998; Parks and LeBlanc, 1996; LeBlanc and Bain, 1997; LeBlanc and McLachlan, 2000). Other studies (Oberdörster et al., 1998a,c) have examined changes in the testosterone metabolism in daphnids (*Daphnia magna*) and the blue crab (*Callinectes sapidus*) following TBT exposure. In addition, we have recently reported on the testosterone metabolism of *N. integer* (Verslycke et al., 2002).

To our knowledge, however, no studies regarding the effects of TBT on the steroid metabolism of mysids have been published. Hence, the purpose of this study was to examine the effects of TBTCl on the phase I and II testosterone metabolism of *N. integer*.

## 7.2. Material and Methods

### *7.2.1. Chemicals*

Testosterone (4-androsten-17β-ol-3-one) and methyltestosterone (4-androsten-17α-methyl-17β-ol-3-one) were obtained from Sigma-Aldrich (Bornem, Belgium). Androstenedione (4-androsten-3,17-dione), dihydrotestosterone (5α-androstan-17β-ol-3-one), boldenone (1,4-androstadien-17β-ol-3-one), and the different testosterone metabolites (4-androsten-[2α-, 6α-, 6β-, 7α-, 11α-, 11β-, 15α-, 16α-, 16β-], 17β-diol-3-one) were purchased from Steraloids (Newport, RI, USA). Tributyltinchloride (96% purity) was obtained from Janssen Chimica (Berchem, Belgium). Other solvents and reagents were analytical

grade and purchased from Merck Eurolab (Leuven, Belgium).

### *7.2.2. Animal collection and maintenance*

Initial *Neomysis integer* populations were collected from the Galgenweel (Antwerp, Belgium) and cultured in the lab as described in Chapter 3 (§ 3.2.1).

### *7.2.3. TBTCl exposure*

Juvenile mysids were randomly distributed in 1.5-l glass beakers (10 per concentration, two replicates), with each containing 1 l of the required TBTCl concentration (control, 10, 100, 1000, 10,000, or 100,000 ng/l for the toxicity experiment and control, 10, 100, or 1000 ng/l for the testosterone metabolism experiment) in water with a salinity of 5‰ (diluted from artificial sea water, Instant Ocean®). The TBT was delivered to the exposure solutions in absolute ethanol. The ethanol concentration in the solvent control was 0.01%. Exposure temperature was 15°C, and test solutions were renewed after 48 h. Animals were fed twice daily with 24- to 48-h-old *Artemia* nauplii (about 75 *Artemia*/mysid). Mortality was recorded daily.

### *7.2.4. TBTCl analysis*

The pH of the medium was adjusted to 5.3 with a sodium acetate/acetic acid buffer before extraction. After ethylation in an aqueous solution containing 1% of tetraethylborate, organotins were extracted with hexane, separated with a Perkin-Elmer Autosystem (Norwalk, CT, USA) gas chromatograph (GC), and measured with a Perkin-Elmer Sciex Elan 5000 inductive-coupled plasma mass spectrometer (ICP-MS). Tripropyltin was used as internal standard. Details on the GC/ICP-MS operating conditions are given by De Smaele et al. (2001). More detailed information on the organotin analysis is also given in Chapter 4 (§ 4.2.5).

### *7.2.5. Testosterone metabolism*

Juvenile mysids were exposed to 10, 100, and 1000 ng/l of TBTCl and to a solvent control as described above. Following the 96-h exposure period, mysids were individually placed into 5-ml glass tubes containing 2 ml of the same TBTCl concentration. Two micrograms of testosterone dissolved in 10 µl of methanol was added to the tubes, and a 6-h exposure period was applied. Exposure temperature was 15°C. Blanks were run to account for breakdown or microbial transformation of testosterone in the absence of mysids.

*Neomysis integer* were subsequently isolated from the exposure medium, dried on a paper towel, weighed on an analytical balance, shock-frozen in liquid nitrogen, and homogenized on ice in 100 µl deionized water using a motor-driven Teflon® pestle. Methyltestosterone (50 ng in 50 µl of methanol) was always added before extraction as an internal standard. Testosterone metabolites were extracted from the homogenized organisms using 2 ml ethyl acetate (2 × 1 ml) and phase-separated using centrifugation (5 min at 14000 *g*). The two ethyl acetate fractions were pooled for analysis. Testosterone metabolites were extracted from the medium in the same way using 4 ml ethyl acetate (2 × 2 ml).

Following ethyl acetate extraction, the remaining polar phase II metabolites were hydrolyzed according to Baldwin and Leblanc (1994b). In short, the assay medium is evaporated, and subsequently, the testosterone conjugates are hydrolyzed for β-glucose, sulfate and α-glucose-conjugated metabolites and extracted with 4 ml ethyl acetate (2 × 2 ml).

### *7.2.6. Liquid Chromatography with Multiple Mass Spectrometry analysis of testosterone metabolites*

The high-performance liquid chromatography (HPLC) apparatus was comprised of an Agilent 1100 series pump, autosampler, and vacuum degasser (Agilent, Palo Alto, CA, USA). Chromatographic separation was achieved using a Symmetry $C_{18}$ column (5 µm, 150 × 2.1 mm; Waters, Milford, USA). The flow rate was 0.3 ml/min. Analysis was carried out using an LCQ$^{DECA}$ ion-trap mass analyzer (ThermoQuest, San Jose, CA, USA) with an atmospheric pressure chemical ionization (APCI) interface and XCalibur 1.2. software (Batavia, NY, USA). The metabolites were detected in MS-MS-full scan positive-ion mode. The solvents for preparation of the mobile phase were HPLC-grade obtained from Merck Eurolab (Leuven, Belgium). To separate and quantify the different compounds, samples were vacuum evaporated to dryness (Speedvac SC210A, Farmingdale, NY, USA) and reconstituted in 30 µl MeOH and 90 µl 0.02 M HCOOH. Sixty microliters were injected on column. A gradient elution was used (0.02 M HCOOH:MeOH, from 60:40 to 20:80 in 25 min, hold for 5 min). The different testosterone metabolites and endogenous steroids were identified on the basis of their relative retention times (calculated as a ratio of the retention time of testosterone) compared with the retention times of authentic standards. For more details, refer to Chapter 6 (§ 6.2.4). All metabolite concentrations were normalized for the wet weight (ww) of the animals. The weights of the TBTCl-exposed mysids were not significantly different from the weights in the control (Dunnett's test, $p > 0.05$).

*7.2.7. Statistics*

The 96-h median lethal concentrations (LC50) values were calculated using the moving-average method (Stephan, 1977). 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. Acute toxicity of TBTCl to N. integer*

*Neomysis integer* were exposed to 10, 100, 1000, 10,000 and 100,000 ng/l of TBTCl for 96 h, and mortality was recorded (Chapter 4; Fig 4.1). Experimental TBTCl concentrations in freshly prepared test solutions and after 48h were determined with GC/ICP-MS (Chapter 4; Table 4.1). The experimental TBTCl concentrations in the freshly prepared solutions correlated well with the nominal values, except in the lower concentrations (10 and 100 ng TBTCl/l). From the mortality data, a 96-h LC50 of 114 ng TBTCl/l (95% confidence limits, 33-436 ng TBTCl/l) could be calculated based on the nominal concentrations. Based on the measured average concentrations (average between concentrations in fresh solution and after 48 h), the 96-h LC50 was 164 ng TBTCl/l (95% confidence limits, 52-373 ng TBTCl/l).

*7.3.2. Metabolic elimination of testosterone following TBTCl exposure*

As previously described, *Neomysis integer* produces a range of testosterone metabolites that can be divided into oxido-reduced/hydroxylated (phase I biotransformation) derivatives and conjugated (phase II biotransformation) derivatives (Verslycke et al., 2002). Following 96-h exposure to TBTCl, the ability of the mysids to eliminate testosterone as various metabolic derivatives was evaluated (Table 7.1). The major metabolites detected in the medium were the nonpolar testosterone derivatives androstenedione and dihydrotestosterone. Dihydrotestosterone (not statistically significant) and androstenedione production was higher in the 10 ng TBTCl/l treatment than in the control. Exposure of *N. integer* to 1000 ng TBTCl/l resulted in a reduced production of nonpolar metabolites.

**Table 7.1.** Metabolic elimination of testosterone by *N. integer* following 96-h exposure to tributyltinchloride (TBTCl)

| Concentration (ng/l) | Polar metabolites[a] | | | | | | | Nonpolar metabolites[a] | | | TST[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | boldenone | 2α-OH[b] | 6α-OH | 7α/15α-OH | 11α-OH | 11β-OH | Total | AED[b] | DHT[b] | Total | |
| **Phase I** | | | | | | | | | | | |
| Control (n = 10) | 24.9 ± 12.3 | — | 18.8 ± 8.5 | 6.3 ± 2.3 | — | 20.1 ± 11.3 | 70.0 ± 28.4 | 2005 ± 1447 | 358 ± 453 | 2363 ± 1471 | |
| 10 (n = 9) | 40.0 ± 17.6[c] | — | 19.1 ± 7.5 | 5.0 ± 2.1 | — | 29.1 ± 16.0 | 93.2 ± 27.5[c] | 3941 ± 1912[d] | 583 ± 482 | 4524 ± 1855[d] | |
| 100 (n = 8) | 16.6 ± 5.2 | — | 13.4 ± 6.2 | 3.1 ± 1.0[d] | x[e] | 15.0 ± 3.4 | 48.1 ± 10.6 | 2025 ± 1119 | 293 ± 106 | 2318 ± 1152 | |
| 1000 (n = 8) | 18.4 ± 6.2 | — | 12.3 ± 3.7 | 3.5 ± 1.6[d] | x | 14.5 ± 6.2 | 48.6 ± 14.8 | 1721 ± 1011 | 189 ± 153 | 1911 ± 1049 | |
| **Phase II glycosylation** | | | | | | | | | | | |
| Control | — | 1.5 ± 1.1 | — | — | 2.9 ± 1.2 | 23.3 ± 15.5 | 27.7 ± 16.0 | — | 131 ± 44 | 131 ± 44 | 3542 ± 1610 |
| 10 | — | 1.2 ± 0.7 | — | — | 2.7 ± 1.8 | 25.0 ± 16.0 | 28.9 ± 17.8 | — | 190 ± 162 | 190 ± 162 | 3440 ± 1237 |
| 100 | — | 0.9 ± 0.4 | — | — | 4.0 ± 2.8 | 16.0 ± 8.9 | 20.8 ± 10.2 | — | 250 ± 110[c] | 250 ± 110[c] | 2389 ± 1161 |
| 1000 | — | 0.8 ± 0.7 | — | — | 4.2 ± 1.4 | 18.0 ± 6.0 | 22.9 ± 6.4 | — | 151 ± 60 | 151 ± 60 | 3103 ± 984 |
| **Phase II sulfation** | | | | | | | | | | | |
| Control | — | 3.7 ± 3.0 | — | 3.0 ± 2.5 | 4.1 ± 2.3 | 34.0 ± 20.2 | 44.8 ± 23.6 | — | 278 ± 155 | 278 ± 155 | 5319 ± 3563 |
| 10 | — | 3.0 ± 0.5 | — | 2.7 ± 1.3 | 3.7 ± 1.4 | 36.2 ± 16.9 | 45.7 ± 18.3 | — | 268 ± 148 | 268 ± 148 | 5018 ± 1692 |
| 100 | — | 3.0 ± 1.1 | — | 1.8 ± 1.3 | 1.8 ± 0.8[d] | 17.3 ± 10.6[c] | 23.9 ± 11.9[c] | — | 246 ± 148 | 246 ± 148 | 2432 ± 838[c] |
| 1000 | — | 2.3 ± 0.9 | — | 1.1 ± 0.3[c] | 3.0 ± 1.5 | 17.1 ± 6.0* | 22.9 ± 6.8[c] | — | 155 ± 67 | 155 ± 67 | 2826 ± 991[c] |

[a] Values obtained from LC-MS$^n$ analysis. Data are presented as mean ± standard deviation in ng/g wet weight.
[b] OH: hydroxytestosterone; DHT: dihydrotestosterone; AED: androstenedione; TST: testosterone.
[c] Significantly higher/lower than the control at p = 0.05.
[d] Significantly higher/lower than the control at p = 0.01.
[e] x: metabolite was only detected in one replicate sample.

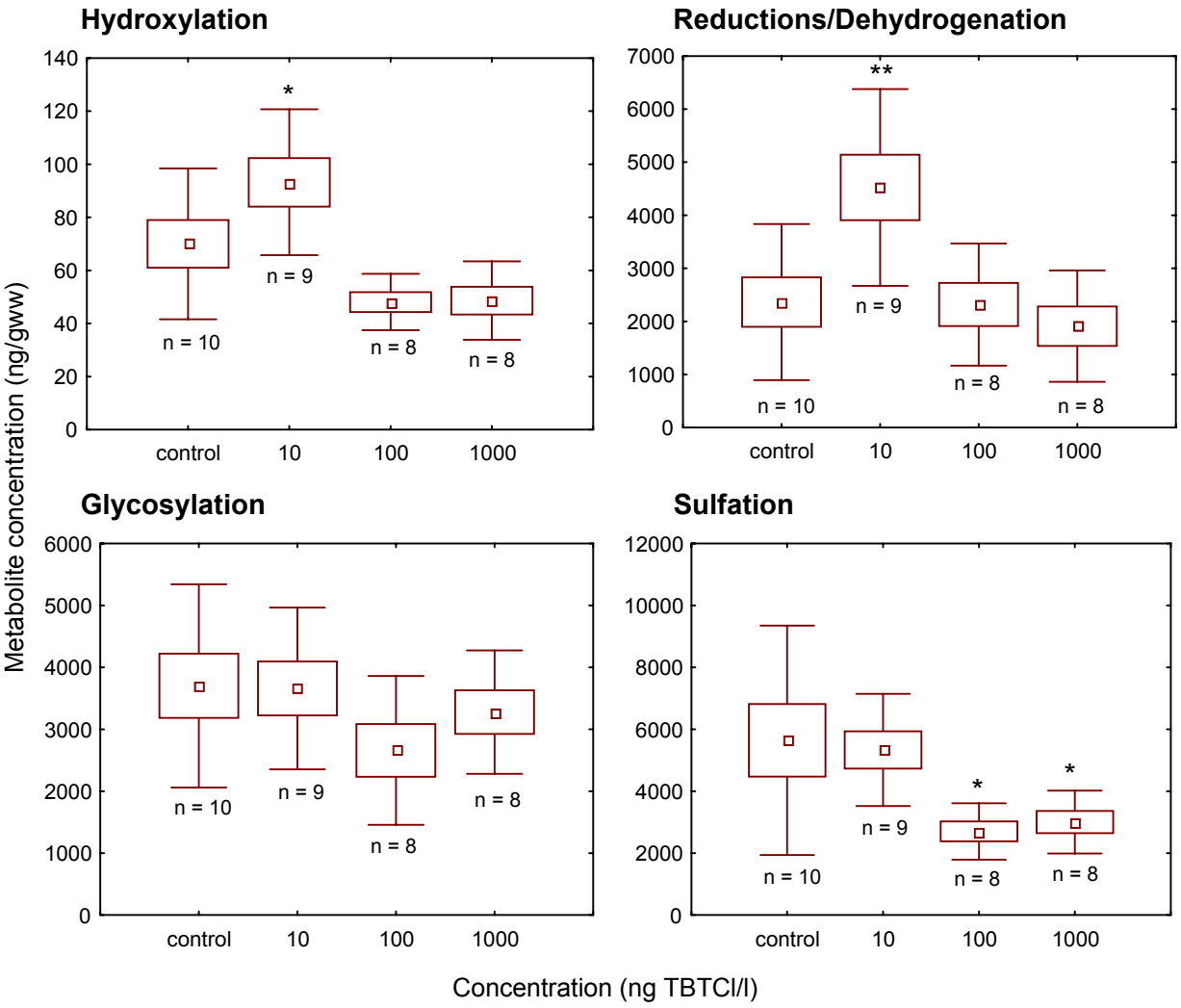



**Figure 7.1.** Metabolic elimination of testosterone by *N. integer* following 96-h exposure to tributyltinchloride (TBTCl) as hydroxylated, oxido-reduced, glucose-conjugated, and sulfate-conjugated testosterone derivatives. Number below the whisker indicates the number of observations. (Dunnett's: * $p < 0.05$; ** $p < 0.01$, significance compared to control).

Minor metabolites in the medium were the polar compounds boldenone, 6α-hydroxytestosterone, 11β-hydroxytestosterone and 7α+15α-hydroxytestosterone. In some samples, 11α-hydroxytestosterone was also randomly detected in low concentrations.

Mysids exposed to 100 and 1000 ng TBTCl/l produced lower amounts of polar monohydroxy metabolites as compared to mysids in the control. This reduction was significant for the metabolite 7α+15α-hydroxytestosterone. Similar to the observed induction for the nonpolar metabolites, the concentration of some polar metabolites (boldenone and 11β-hydroxytestosterone) in the medium was significantly higher in the 10 ng TBTCl/l treatment compared to the control.

The medium was also analyzed for phase II testosterone conjugates. The addition of testosterone and its metabolites to α-glucose was negligible in mysids. The elimination rates of β-glucose-conjugated, sulfate-conjugated, oxido-reduced, and hydroxylated testosterone and its derivatives were differentially affected by TBTCl exposure (Fig. 7.1). Sulfation and glycosylation were reduced after exposure to increasing TBTCl concentrations (significant reduction of sulfation in the 100 and 1000 ng TBTCl/l treatments compared to the control).

Hydroxylation and conjugation of testosterone leads to inactivation and preferential elimination, whereas oxido-reduced metabolites are preferentially retained and many serve as androgens or androgen precursors (Baldwin and LeBlanc, 1994b). Similar to Baldwin et al. (1997), we used the ratio of the concentrations of the eliminated oxido-reduced products and the polar products (hydroxylated plus conjugated) to derive a metabolic androgenization ratio. This value has been used as a numerical interpretation for the total effect of a chemical on androgen metabolism in daphnids. Mysids exposed to 10 and 100 ng/l of TBTCl had higher metabolic androgenization ratios, but these were not significantly different from that of the control (Dunnett's test; p = 0.1 for 10 ng TBTCl/l and 0.4 for 100 ng TBTCl/l) (Fig. 7.2).

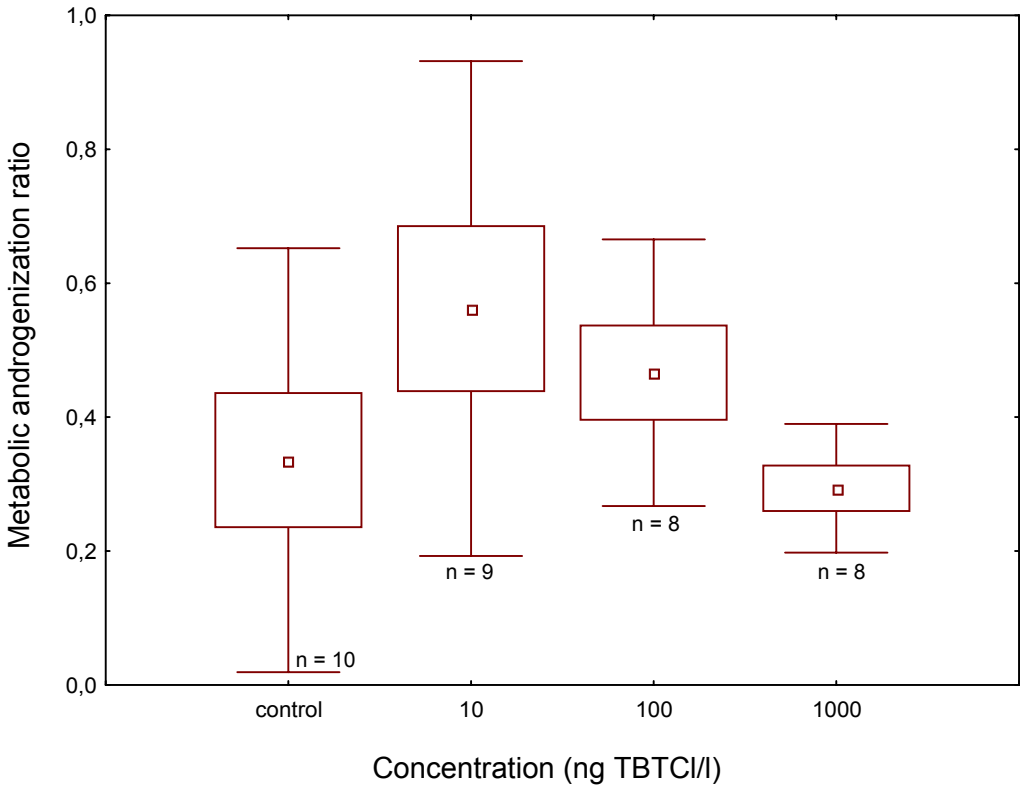



**Figure 7.2.** Metabolic androgenization of tributyltinchloride (TBTCl)-exposed *N. integer*, calculated as the ratio of oxido-reduced to glucosylated/sulfated/hydroxylated metabolites of testosterone (Baldwin et al., 1997). Number below the whisker indicates the number of observations.

## 7.4. Discussion

### *7.4.1. Acute toxicity of TBTCl to N. integer*

Literature regarding toxicity testing with the standard mysid species *Americamysis bahia* demonstrates the high sensitivity of mysids, in many cases at levels that are likely to occur in the environment (McKenney, 1998; Nimmo and Hamaker, 1982; Verslycke et al., submitted-Chapter 2). The acute LC50 for juvenile *Neomysis integer* of 164 ng TBTCl/l found in this study is lower than that reported by Goodman et al. (1988) for *A. bahia* (1100 ng TBT/l). Davidson et al. (1986) and Valkirs et al. (1987) also observed acute TBT toxicity within the same range (420-610 ng TBT/l) for mysid shrimp (for review, refer to Fent, 1996). Tributyltin compounds thus are highly toxic to juvenile mysid shrimp. It can be speculated from these results that coastal TBT contamination, which can still reach concentrations of 200 ng TBT/l despite restrictive regulations (Michel et al., 2001), may be a potential threat to resident mysid populations (see detailed discussion in Chapter 4).

### *7.4.2. Metabolic elimination of testosterone*

Exposure to TBTCl differentially altered metabolic elimination of testosterone in *Neomysis integer* as phase I metabolites. An induction of both nonpolar and polar metabolites was observed in the lowest exposure (10 ng TBTCl/l), but higher concentrations resulted in either no effect or a lower elimination rate.

The metabolism of boldenone (1,4-androstadiene-17β-ol-3-one) is poorly understood, but boldenone probably requires testosterone or androstenedione (AED) as a precursor (Verslycke et al, 2002). Therefore, changes in boldenone production may reflect changes in the availability of these substrates. The production of both boldenone and AED were significantly induced in the 10 ng TBTCl/l treatment. Interestingly, we also observed a similar pattern for endogenous AED body burdens in the mysids exposed during this experiment (Table 7.2). Elimination of the nonpolar metabolite dihydrotestosterone was affected in the same way. Oberdörster et al. (1998b) described a similar maximum in the production of AED at a concentration of 10 ng TBTCl/l in the mud snail *Ilyanassa obselata*, which was lowered at the highest tested concentrations (20 and 200 ng TBTCl/l).

None of the detected hydroxytestosterone metabolites were induced by TBTCl. It has been demonstrated that TBTCl induces the expression of CYP3A-like proteins in crab hepatopancreas (Oberdörster et al., 1998c). Similarly, Leblanc and McLachlan (2000) observed an increased production of the CYP3A-dependent metabolite 6β-hydroxy-testosterone in *Daphnia magna* exposed to TBTCl. Other studies were unable to

demonstrate changes in the phase I metabolism of TBTCl-exposed organisms (Oberdörster et al., 1998a). The CYP2 and CYP3 family are largely responsible for hydroxylation of hormones and drugs. We have previously demonstrated the ability of *N. integer* to metabolize testosterone to different monohydroxymetabolites, such as 2α-, 6α-, 11α-, 11β-, 7α-, 16α-, and 15α-hydroxytestosterone (Verslycke et al., 2002). All these metabolites are linked to the CYP2 family. No monohydroxymetabolites of testosterone linked to the CYP3A family (6β-, 2β-, 15β-, 18β-hydroxytestosterone) were ever identified in our studies, which explains the present results. Apparently, TBTCl upregulates the expression of certain P450 isozyme families that are not ubiquitous in all invertebrates, resulting in a variable response among species (for a review of crustacean P450, refer to James and Boyle, 1998).

**Table 7.2.** Retention of testosterone (TST) and androstenedione (AED) by *N. integer* following 96-h exposure to tributyltinchloride (TBTCl)

| Concentration (ng/l) | Metabolite in organism (ng/g ww)[a] | |
|---|---|---|
| | TST | AED |
| Control (n = 10) | 285.7 ± 113.9 | 95.6 ± 58.5 |
| 10 (n = 9) | 259.5 ± 114.8 | 154.9 ± 79.7[b] |
| 100 (n = 8) | 157.8 ± 72.1[c] | 95.7 ± 69.1 |
| 1000 (n = 8) | 148.2 ± 43.8[c] | 55.1 ± 28.0 |

[a] Data are presented as mean ± standard deviation.
[b] Significantly higher than the control at p = 0.05.
[c] Significantly lower than the control at p = 0.01.

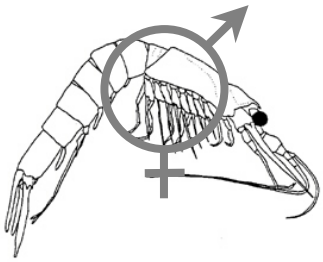
Except for a small induction in the amount of glucose-conjugated dihydrotestosterone, glucose conjugation of testosterone metabolites in *N. integer* was unaffected by TBTCl exposure. Elimination of testosterone as a glucose conjugate was not significantly affected by TBTCl, but was lower in the two highest treatments compared to the control. Sulfate conjugation was significantly lowered, in a concentration-dependent manner, for both polar and nonpolar metabolites and testosterone. Ronis and Mason (1996) hypothesized that TBT inhibits sulfur conjugation of testosterone as well as its metabolites and their excretion, resulting in a build-up of pharmacologically active androgens in the tissues. Our study corroborates, in part, the findings of Ronis and Mason, because the major biochemical targets of TBT appeared to be sulfate conjugation. However, we could not demonstrate that the build-up of active androgens in whole-body homogenates of the exposed mysids coincided with a reduced sulfation in the 100 and 1000 ng TBTCl/l treatments (Table 7.2). On the contrary, testosterone concentrations (as derived from whole-body concentrations) were significantly lower at the two highest test concentrations. This could not be related,

however, to an induced metabolic elimination of testosterone metabolites in the medium. These lower testosterone concentrations may have resulted from a reduced uptake of testosterone, although Ronis and Mason (1996) found no indication of changes in testosterone uptake. Similarly, we found no differences in testosterone concentrations in the medium, indicating that testosterone uptake from the medium was similar in all treatments (data not shown). Considering that conjugation of testosterone decreased instead of increased at higher TBT concentrations, the processes behind the observed decline in endogenous testosterone remain unclear. Hypothetically, testosterone could have been partly eliminated as metabolites which were not identified by the described LC-MS method (extracts were only scanned for metabolites mentioned in Materials and Methods). These unidentified metabolites could, for instance, be androstanediols or androstenediols as observed in testosterone elimination experiments with the daphnid *D. magna*, and the mollusks *I. obselata* and *Littorina littorea* (LeBlanc and McLachlan, 2000; Oberdörster et al., 1998b; Ronis and Mason, 1996).

The overall effect of TBTCl on the metabolic elimination of testosterone can be summarized by the ratio of the eliminated oxido-reduced products and the polar products (hydroxylated plus conjugated), that is, the metabolic androgenization ratio (Fig. 7.2). Exposure of *N. integer* for 96 h to 10 and 100 ng TBTCl/l resulted in an increased metabolic androgenization ratio (although not statistically significant from that of the control). This effect can be attributed to an induction of the reductase activity, resulting in higher productions of the nonpolar metabolites androstenedione and dihydrotestorone in the 10 ng TBTCl/l treatment. This induction corresponds with an increased build-up of the pharmacologically active androgen androstenedione in the tissues of *N. integer* (Table 7.2). The 1000 ng TBTCl/l treatment had no effect on the metabolic androgenization ratio, although a significant decrease in sulfate addition was observed, indicating that this mechanism might be important in explaining imposex phenomena. As proposed by LeBlanc and McLachlan (2000), the differential effect on phase II conjugation may be related to the position in the cell of the involved enzymes. Both testosterone hydroxylase and glycosyltransferase enzymes are located in the endoplasmatic reticulum, whereas sulfotransferase is a water soluble protein in the cytosol. The effect of TBTCl on phase I metabolism was unclear and has been shown to vary among species, probably depending on the inducibility of certain P450 isozyme families. Future research should focus on the fundamental understanding of the mechanism behind TBT toxicity and the cellular effects of this compound on the enzymes involved in steroid regulation.

### 7.5. Conclusions

Tributyltin is highly toxic to mysids, and at present environmental concentrations, acute or chronic toxic effects could result in mortality and a decline in coastal or estuarine mysid populations. The sublethal effects of TBT on steroid metabolism remain unclear. Although we could demonstrate significant alterations in the testosterone metabolism (especially reductase induction and reduction in sulfate conjugation) of *Neomysis integer* following acute exposure to sublethal concentrations of TBTCl, the mechanisms involved need further elucidation. In conclusion, testosterone metabolism of *N. integer* provides a sensitive research tool to investigate the sublethal effects of tributyltin. The applicability and sensitivity of these techniques should, therefore, be evaluated following exposure to other endocrine disruptors.



# CHAPTER 8

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# Testosterone and energy metabolism of *Neomysis integer* following exposure to endocrine disruptors

**Redrafted after :**

Verslycke T, Poelmans S, De Wasch K, De Brabander HF, Janssen CR (submitted) Testosterone and energy metabolism in the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) following exposure to endocrine disruptors. *Environmental Toxicology and Chemistry*.

# Chapter 8

## *Testosterone and energy metabolism of Neomysis integer following exposure to endocrine disruptors*

**Abstract**

Recently, we described the cellular energy allocation (CEA) methodology to asses the effects of abiotic and toxic stress on the energy metabolism of the estuarine crustacean *Neomysis integer* (Crustacea: Mysidacea) (Verslycke and Janssen, 2002; Verslycke et al., 2003c). In addition, the testosterone metabolism of *N. integer* proved to be a valuable endpoint in evaluating the potential effects of tributyltinchloride, a marine biocide which is generally considered responsible for causing imposex in female gastropods (Verslycke et al., 2003a). In the present study, a diverse set of reference compounds suspected of having an endocrine-disrupting mode of action were tested for acute toxicity, i.e. testosterone, flutamide, ethinylestradiol, precocene, nonylphenol, fenoxycarb and methoprene. *N. integer* was very sensitive to all tested compounds, with 96-h median lethal concentrations in a narrow range between 0.32 and 1.95 mg/l. The pesticides methoprene and fenoxycarb, both synthetic insect juvenile hormone analogs, were most toxic to *N. integer*. In addition, the short-term sublethal effects of methoprene and nonylphenol (an estrogen agonist) on the energy and steroid metabolism of *N. integer* were evaluated. Both compounds significantly affected energy and testosterone metabolism of *N. integer* at concentrations below acute toxicity levels. Energy consumption in methoprene and nonylphenol-exposed mysids was significantly induced at 100 µg/l, resulting in a lower CEA in these animals. Testosterone phase I metabolism was affected at 10 µg/l, whereas glycosylation was the most important phase II pathway affected in mysids exposed to 100 µg/l of both compounds. Methoprene exposure resulted in a concentration-dependent increase in the metabolic androgenization ratio (MAR: ratio of nonpolar and polar testosterone derivatives). Mysids exposed to 10 µg/l nonylphenol had a significantly higher MAR. The present study indicates that energy and testosterone metabolism of mysids, as endpoints, are able to detect endocrine disruptive activity of chemicals following short-term exposure to environmental realistic levels of endocrine disruptors.

## 8.1. Introduction

Invertebrates account for roughly 95% of all known species of animals on our planet, yet our knowledge of their basic endocrinology is limited, and only recently have we begun to understand their value in signaling environmental endocrine disruption (DeFur et al., 1999). The majority of evidence for chemically-induced endocrine disruption in invertebrates stems from exposures to pesticides specifically designed to disrupt endocrine-regulated processes such as growth, metamorphosis and molting (Fingerman et al., 1998). Historically, most assays used to evaluate endocrine function in invertebrates have involved the use of general endpoints which did not reveal specific mechanisms. Fortunately, new and more sophisticated analytical techniques allow analyses of specific pathways and use microquantities of biological material (LeBlanc, 1999). In this perspective, a number of new hormone-regulated endpoints have been proposed as promising biomarkers to detect endocrine disruption in invertebrates (DeFur et al., 1999).

Chronic toxicity tests with invertebrates such as daphnids (*Daphnia* and *Ceriodaphnia*) and mysid shrimp (*Americamysis*) have been required for decades for the evaluation of the toxicity of environmental pollutants. The short life cycle of these organisms allows for the measurement of endocrine-regulated endpoints in a time frame sufficiently short to qualify as screening tests (DeFur et al., 1999; LeBlanc, 1999). The endpoints currently used in acute and chronic toxicity testing are intended to define organism or population status (e.g. survival, growth). These endpoints may not be the most suitable endpoints for detecting chemicals that have an endocrine-disrupting mode of action. Presently, the most appropriate evaluation criteria cannot be identified because there is a lack of data to select the most discriminating endpoint. In absence of data which can suggest that existing tests and effect assessment procedures adequately protect invertebrate communities, the number of new endpoints or new test species might increase.

Mysid shrimp may serve as a viable surrogate for many crustaceans and have been put forward as suitable test organisms for the evaluation of endocrine disruption (Verslycke et al., submitted-Chapter 2). In addition, we have recently demonstrated that alterations in the energy and steroid metabolism are useful biomarkers to evaluate the effects of chemicals on mysids (Verslycke et al., 2003a,c). To validate the use of these assays as sensitive endpoints to detect the potential effects of chemicals with endocrine-disrupting properties in mysids, we are presently evaluating relative endpoint responses via exposure experiments with a selection of reference chemicals. A list of reference chemicals (based on their possible mode of action) for evaluating endpoint and/or species sensitivity to potential endocrine-disrupting compounds was suggested at a workshop on endocrine disruption in invertebrates held in The Netherlands in 1998 (DeFur et al., 1999). This list includes methoprene (juvenile hormone agonist), precocene

(juvenile hormone antagonist), 20-OH ecdysone (ecdysone agonist), homobrassinolide and luteolin (ecdysone antagonist), fadrozole (aromatase inhibitor), methyl testosterone (androgen agonist), flutamide (androgen antagonist), 4-tert-pentylphenol (weak estrogen agonist), ethinylestradiol (strong estrogen agonist) and ZM-189,154 (estrogen antagonist).

The present study investigates the acute toxicity of a number of these reference chemicals to the mysid *Neomysis integer*, i.e. testosterone, flutamide, ethinylestradiol, methoprene and precocene. In addition, another juvenile hormone agonist was included, the pesticide fenoxycarb, which has been reported to affect energy metabolism of juvenile mud crabs (Nates and McKenney, 2000). As an estrogen agonist, nonylphenol instead of 4-tert-pentylphenol was used, since it has been demonstrated that this chemical alters testosterone metabolism in *Daphnia magna* (Baldwin et al., 1997). Furthermore, *N. integer* is exposed to high concentrations of this chemical in the Scheldt estuary (Verslycke et al., submitted-Chapter 9).

Next to mortality, the effects of nonylphenol and methoprene on the testosterone and energy metabolism of *N. integer* were evaluated following short-term exposures. It has been reported that methoprene significantly affects energy metabolism in the mysid shrimp *Americamysis bahia* (McKenney and Celestial, 1996) and that the development of secondary sex characteristics in daphnids can be altered by this compound (Olmstead and LeBlanc, 2001). Consequently, both nonylphenol and methoprene are suitable reference compounds to evaluate the steroid and energy metabolic processes in *N. integer* as biomarkers for endocrine disruption.

## 8.2. Material and Methods

### *8.2.1. Animal collection and maintenance*

Initial *N. integer* populations were collected from the Galgenweel (Antwerp, Belgium) and cultured in the laboratory as described in Chapter 3 (§ 3.2.1).

### *8.2.2. Acute toxicity test*

Juvenile mysids of equal size (visual selection of animals 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‰ (diluted from artificial sea water [Instant Ocean®] with deionized, carbon-filtered tap water). For each test concentration, 2 replicate beakers with 5 mysids were used. Mysids were exposed for 96 h to increasing concentrations of the test compounds testosterone, flutamide, ethinylestradiol, nonylphenol,

methoprene, fenoxycarb and precocene (Table 8.1). All compounds were delivered to the exposure solutions in absolute ethanol. The concentration of ethanol in the solvent control was 0.1%. Exposure temperature was 15 ± 1°C and exposure solutions were renewed after 48 h. Animals were fed twice daily with 24h- to 48-h-old *Artemia* nauplii (30-50 *Artemia*/mysid) and mortality was noted daily. At the end of the 96-h exposure period, the median lethal concentrations were calculated.

**Table 8.1.** Reference compounds selected for endpoint evalulation in *N. integer*

| Compound | CAS no. | Log $K_{ow}$ | Tested range (mg/l) | Possible mode of action |
|---|---|---|---|---|
| Testosterone | 58-22-0 | 3.32[a] | control-0.01-0.1-1-10-50-100 | natural vertebrate hormone, androgen agonist |
| Flutamide | 13311-84-7 | 3.35[b] | control-0.01-0.1-1-10-50-100 | androgen antagonist |
| 17α-Ethinylestradiol | 57-63-6 | 3.67[c] | control-0.01-0.1-1-10-50 | xenoestrogen |
| Nonylphenol | 25154-52-3 | 4.5[d] | control-1E-5-0.001-0.01-0.1-1-10-50 | environmentally relevant xenoestrogen |
| Methoprene | 40596-69-8 | NA[e] | control-1E-5-1E-4-0.001-0.01-0.1-1-10-100 | pesticide, juvenile hormone agonist |
| Fenoxycarb | 79127-80-3 | 4.3[f] | control-0.01-0.1-1-10-50 | pesticide, juvenile hormone agonist |
| Precocene | 644-06-4 | NA[e] | control-0.01-0.1-1-10-50 | juvenile hormone antagonist |

[a] Hansch and Leo, 1985.
[b] Morris et al., 1991.
[c] Hansch et al., 1995.
[d] http://hazard.com/msds.
[e] NA = not available.
[f] http://www.msdssearch.com.

*8.2.3. 96-h exposures to sublethal concentrations of methoprene and nonylphenol*

Juvenile mysids of equal size (average wet weight 2.9 ± 1.5 mg and 2.8 ± 1.4 mg in methoprene and nonylphenol experiments, respectively) were taken from the culture and randomly distributed to 400-ml glass beakers containing 200 ml of the desired test concentration in water with a salinity of 5‰. For each test concentration, 50 mysids were exposed for 96 h to sublethal concentrations of methoprene (control-0.01-1-100 µg/l) and nonylphenol (control-0.01-1-100 µg/l). Again, maximum 5 mysids were used per beaker. All compounds were delivered to the exposure solutions in absolute ethanol. The concentration of ethanol in the solvent control was 0.1%. Exposure temperature was 15 ± 1°C and exposure solutions were

renewed after 48 h. Animals were fed twice daily with 24h- to 48-h-old *Artemia* nauplii (30-50 *Artemia*/mysid). At the end of the exposure period, 10 mysids were removed per concentration, and exposed for another 6 h to testosterone in 5-ml glass tubes containing 2 ml of the same toxicant test concentration to which 2 µg of testosterone (in 10 µl methanol) was added. This additional exposure was used to evaluate the ability of mysids to eliminate testosterone as polar and nonpolar derivatives. The other mysids were directly shock-frozen in liquid nitrogen and kept at -80°C until analysis of the cellular energy allocation.

### *8.2.4. Testosterone metabolism and cellular energy allocation assay*

The testosterone metabolism assay and the liquid chromatography with multiple mass spectrometry (LC-$MS^n$) analyses were performed as described in Chapter 6 (§ 6.2). The CEA was performed as described in Chapter 5 (§ 5.2.4). Both testosterone metabolism and CEA data were normalized for the wet weight (ww) of the animals.

A number of the testosterone derivatives, especially the polar monohydroxy metabolites, were only detected in low concentrations in some samples and were below the LC-$MS^n$'s limit of detection in other samples from the same treatment. Metabolites which were below detection limit were set equal to zero to allow calculation of averages and statistically compare averages between treatments. Alternatively, the value for an undetected metabolite could be replaced by the lowest detected concentration in all samples. Since this value would, therefore, be variable between experiments, it was considered appropriate to replace all undetected values by zero. Thus, the calculated averages for the production of testosterone derivatives are potentially higher.

### *8.2.5. Statistical analysis*

The 96-h median lethal concentration (LC50) values were calculated using the moving-average method (Stephan, 1977). All analyses were done with the software package Statistica™ (Statsoft, Tulsa, OK, USA). Data were checked for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene's test, respectively, with an $\alpha$ of 0.05. Data were log-transformed if they did not meet with the above criteria. The effect of the treatment was tested for significance using a one-way analysis of variance (Dunnett's test). 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. Acute toxicity of the tested compounds*

The calculated lethal concentrations are summarized in Table 8.2. The LC50s obtained for the tested compounds ranged from 1.95 mg/l for testosterone to 0.32 mg/l for methoprene. The highest test concentrations resulted in complete mortality for all compounds, indicating that a sufficiently broad concentration range was tested. Mysid control survival in all 96-h tests ranged from 80 to 100 %.

**Table 8.2.** 96-h toxicity of a number of endocrine disruptors to juvenile *N. integer*. The 95% confidence interval is given in parentheses

| Compound | 96-h LC50 (mg/l) | Literature LC50 (mg/l) |
|---|---|---|
| Testosterone | 1.95 (0.55 – 9.08) | 5.6[a] (4.7 – 6.6) |
| Flutamide | 1.38 (0.49 – 4.12) | 5.4[b] (4.2 – 7.0) |
| 17$\alpha$-ethinylestradiol | 1.20 (0.39 – 3.78) | 1.1[c] (NA[d]) |
| Precocene | 0.77 (0.24 – 2.26) | NA |
| Nonylphenol | 0.59 (0.15 – 3.85) | 0.1 – 0.5[e] |
| Fenoxycarb | 0.53 (0.16 – 1.51) | 0.92[f] (NA) |
| Methoprene | 0.32 (0.10 – 1.00) | 0.125[g] (NA) |

[a] 48u-LC50 for *Acartia tonsa* (Crustacea: Copepoda) (Andersen et al., 2001).
[b] 48u-LC50 for *A. tonsa* (Crustacea: Copepoda) (Andersen et al., 2001).
[c] 48u-LC50 for *A. tonsa* (Crustacea: Copepoda) (Andersen et al., 2001).
[d] NA: not available.
[e] 96u-LC50 for aquatic organisms (Baldwin et al., 1997).
[f] 96u-LC50 for *Palaemonetes pugio* (Crustacea: Decapoda) (Key and Scott, 1994).
[g] 96u-LC50 for *Americamysis bahia* (Crustacea: Mysidacea) (McKenney and Celestial, 1996).

### *8.3.2. Sublethal effects of methoprene on testosterone and energy metabolism of N. integer*

The effects of 96-h exposure to sublethal concentrations of methoprene (control, 0.01, 1 and 100 µg/l) on the testosterone and energy metabolism of *Neomysis integer* are summarized in Table 8.3 and Figures 8.1 and 8.2.

**Table 8.3.** Metabolic elimination of testosterone by *N. integer* following 96-h exposure to methoprene

| Concentration (µg/l) | Polar metabolites[a] | | | | | | | | Nonpolar metabolites[a] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | boldenone | 2α-OH[b] | 6α-OH | 7α/15α-OH | 11α-OH | 11β-OH | 16α-OH | Total | AED[b] | DHT[b] | TST[b] | Total |
| **Phase I** | | | | | | | | | | | | |
| Control | x[c] | — | 39 ± 14 | — | — | — | — | 39 ± 14 | 1137 ± 933 | — | | 1137 ± 933 |
| 0.01 | x | x | 34 ± 12 | 10 ± 7.1 | — | 10 ± 18 | — | 56 ± 20* | 405 ± 811 | — | | 405 ± 811 |
| 1 | x | — | 25 ± 6.4* | 7.6 ± 6.3 | — | 5.0 ± 6.2 | — | 34 ± 10 | 725 ± 868 | — | | 725 ± 868 |
| 100 | x | x | 26 ± 13* | 4.3 ± 4.3 | — | 5.0 ± 5.0 | — | 36 ± 12 | 783 ± 443 | — | | 783 ± 443 |
| **Phase II glycosylation** | | | | | | | | | | | | |
| Control | — | x | — | — | — | 22 ± 34 | — | 22 ± 34 | — | — | 17123 ± 12938 | 17123 ± 12938 |
| 0.01 | — | x | — | — | — | 30 ± 23 | — | 32 ± 21 | 89 ± 178 | — | 15690 ± 11758 | 15768 ± 11665 |
| 1 | — | 8.1 ± 19 | — | — | — | 11 ± 18 | — | 19 ± 24 | — | — | 11175 ± 8282 | 11175 ± 8282 |
| 100 | — | — | — | — | — | — | — | — | — | — | 3391 ± 1036** | 3391 ± 1036** |
| **Phase II sulfation** | | | | | | | | | | | | |
| Control | — | — | — | — | — | — | — | — | — | — | 13732 ± 8248 | 13732 ± 8248 |
| 0.01 | — | — | — | — | — | 11 ± 20 | — | 11 ± 20 | — | — | 12410 ± 5457 | 12410 ± 5457 |
| 1 | — | — | x | 3.2 ± 6.3 | — | 27 ± 30 | — | 31 ± 35 | — | — | 15239 ± 6226 | 15239 ± 6226 |
| 100 | — | — | — | — | — | 14 ± 22 | — | 14 ± 22 | — | — | 8703 ± 3358 | 8703 ± 3358 |

[a] Values obtained from LC-MS$^n$ analysis. Data are presented as mean ± standard deviation in ng/g wet weight.
[b] OH: hydroxytestosterone; DHT: dihydrotestosterone; AED: androstenedione; TST: testosterone.
[c] x: metabolite was only detected in one replicate sample.
* Significantly different from control (ANOVA, Dunnett; * $p < 0.05$; ** $p < 0.01$).

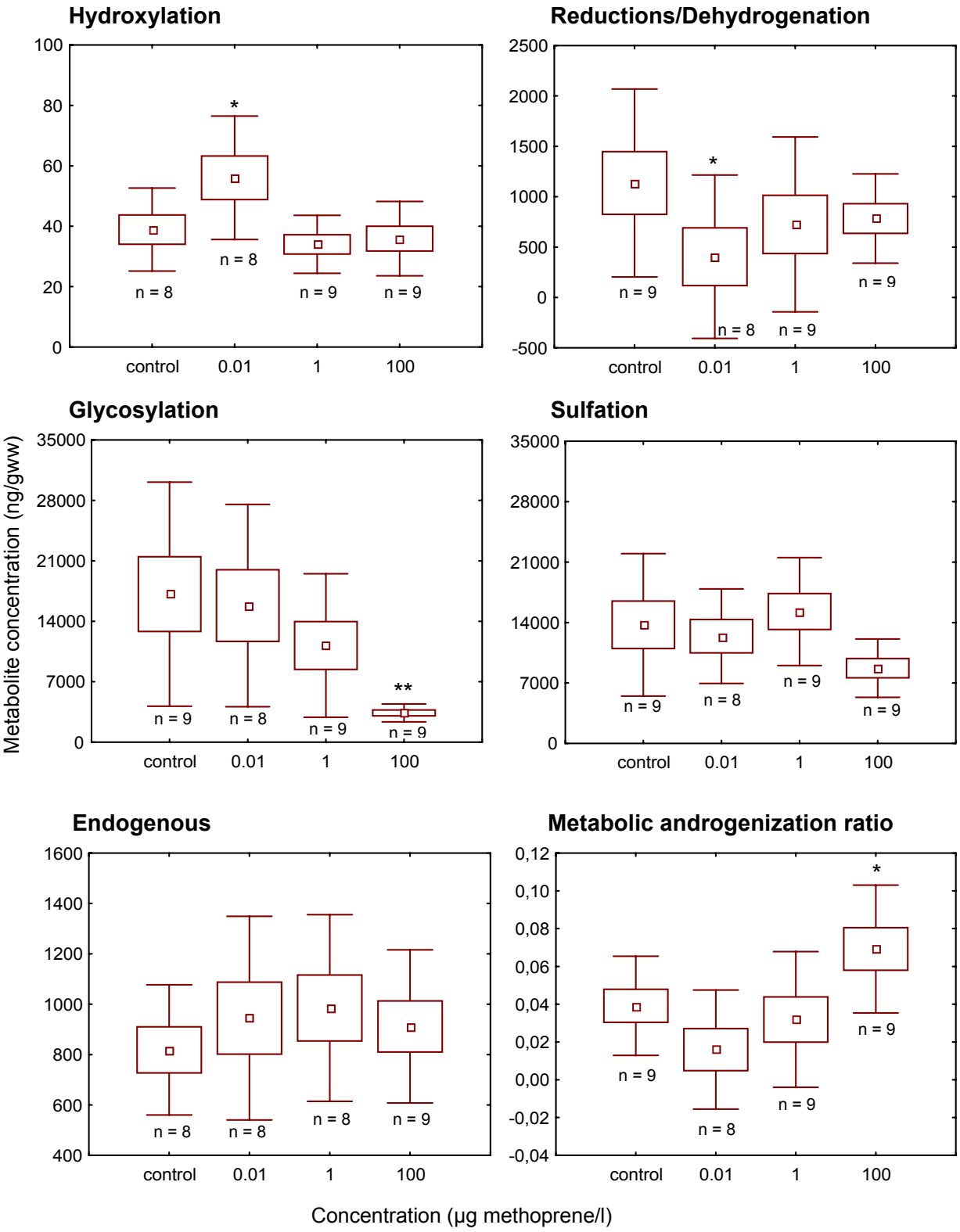



**Figure 8.1.** Metabolic elimination of testosterone by *N. integer* following 96-h exposure to sublethal concentrations of methoprene (ANOVA, Dunnett; * $p < 0.05$; ** $p < 0.01$, significance from control). Number below the whisker indicates the number of observations.

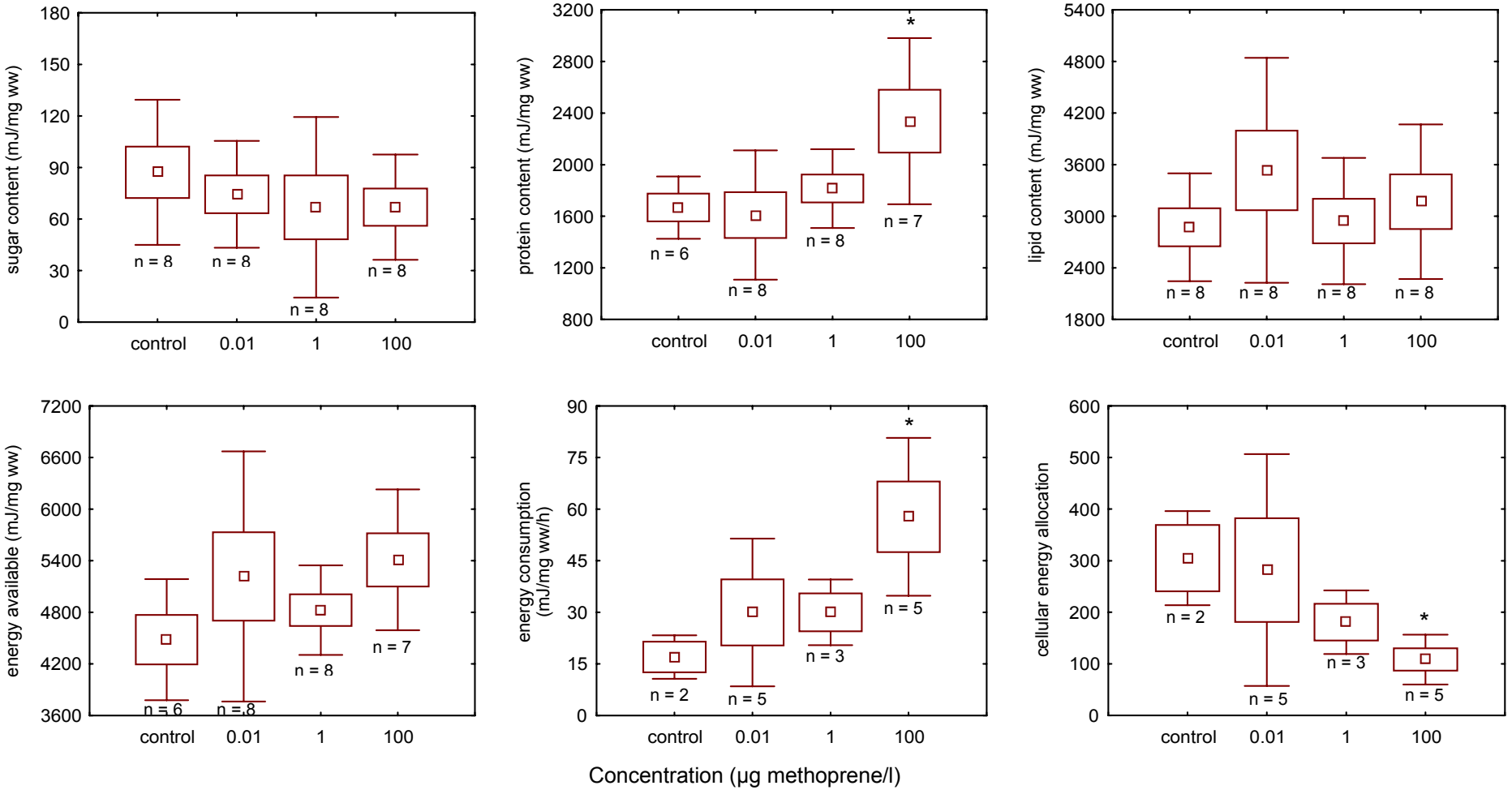



**Figure 8.2.** Cellular energy allocation in *N. integer* following 96-h exposure to sublethal concentrations of methoprene (ANOVA, Dunnett; * $p < 0.05$, significance from control). Number below the whisker indicates the number of observations.

Methoprene had significant effects on the different pathways of energy allocation in *Neomysis integer*. Of the individual energy reserve fractions, only proteins were significantly higher in the highest exposure concentration, compared with the control. The overall available energy (sum of protein, sugar and lipid) was higher in methoprene-exposed mysids, although this was not statistically significant. Energy consumption, however, was significantly induced by methoprene exposure and was almost four times higher in the 100 µg methoprene/l treatment compared with control mysids. This increased energy consumption resulted in significant alterations in the energy allocation of methoprene-exposed mysids which had a lower cellular energy allocation (CEA) value, indicating that they were allocating more energy to energy-demanding processes to cope with chemical exposure.

The effects on energy allocation were also reflected in the metabolic capacity of *N. integer* to eliminate testosterone as polar and nonpolar derivatives. Phase I testosterone metabolism (hydroxylation and reductions/dehydrogenation) was significantly different in the lowest methoprene exposure concentration (10 ng/l), compared to control mysids. Elimination of testosterone as polar phase II conjugates was lower in methoprene-exposed mysids and this was significant for glycosylation in the highest exposure concentration. This reduction in the elimination of testosterone as polar metabolites, results in a build-up of various nonpolar androgen-active metabolites such as androstenedione, dihydrotestosterone and testosterone in the tissues of methoprene-exposed mysids. The overall impact of a chemical on the metabolic elimination of testosterone can be calculated by the metabolic androgenization ratio (MAR), which is the ratio of the rates of production of the eliminated reduced/dehydrogenated products and the polar products (hydroxylated plus conjugated). This ratio was significantly higher in the 100 µg/l treatment compared to the control.

### *8.3.3. Sublethal effects of nonylphenol on testosterone and energy metabolism of N. integer*

The effects of 96-h exposure to sublethal concentrations (control, 0.01, 1 and 100 µg/l) of nonylphenol on the testosterone and energy metabolism of *Neomysis integer* are summarized in Table 8.4 and Figures 8.3 and 8.4.

**Table 8.4.** Metabolic elimination of testosterone by *N. integer* following 96-h exposure to nonylphenol

| Concentration (µg/l) | Polar metabolites[a] | | | | | | | | Nonpolar metabolites[a] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | boldenone | 2α-OH[b] | 6α-OH | 7α/15α-OH | 11α-OH | 11β-OH | 16α-OH | Total | AED[b] | DHT[b] | TST[b] | Total |
| **Phase I** | | | | | | | | | | | | |
| Control | x[c] | — | 17 ± 10 | 8.2 ± 4.9 | — | 25 ± 22 | — | 51 ± 23 | 1027 ± 298 | — | | 1027 ± 298 |
| 0.01 | x | — | 61 ± 40 | 5.7 ± 11 | — | 8.4 ± 15 | 24 ± 36 | 97 ± 45* | 2062 ± 1187* | — | | 2062 ± 1187* |
| 1 | x | 6.7 ± 7.8 | 35 ± 20 | 9.4 ± 11 | — | 9.6 ± 15 | 7.2 ± 11 | 68 ± 28 | 1138 ± 435 | — | | 1138 ± 435 |
| 100 | x | — | 27 ± 19 | 4.6 ± 8.1 | x | 17 ± 21 | x | 52 ± 20 | 1768 ± 945 | — | | 1768 ± 945 |
| **Phase II glycosylation** | | | | | | | | | | | | |
| Control | 6.5 ± 9.2 | — | x | x | — | 31 ± 9.6 | x | 39 ± 15 | x | — | 2554 ± 1066 | 2558 ± 1073 |
| 0.01 | x | — | — | x | — | 12 ± 21* | — | 12 ± 21** | 15 ± 22 | — | 1417 ± 859 | 1432 ± 874 |
| 1 | 6.4 ± 9.9 | — | — | x | — | 25 ± 15 | — | 31 ± 18 | 10 ± 17 | — | 2292 ± 938 | 2302 ± 943 |
| 100 | 5.6 ± 11 | x | x | 0.9 ± 1.9 | — | 27 ± 10 | — | 35 ± 16 | 2.9 ± 8.2 | — | 3820 ± 1879 | 3823 ± 1884 |
| **Phase II sulfation** | | | | | | | | | | | | |
| Control | 4.6 ± 9.1 | — | x | — | 1.0 ± 1.8 | 36 ± 26 | x | 42 ± 21 | 4.5 ± 6.9 | — | 4326 ± 2669 | 4372 ± 2683 |
| 0.01 | — | x | x | — | — | 24 ± 28 | x | 34 ± 34 | 18 ± 33 | — | 6617 ± 4695 | 6666 ± 4752 |
| 1 | x | — | — | — | — | 17 ± 15 | — | 23 ± 25 | x | — | 2960 ± 2334 | 2984 ± 2348 |
| 100 | 4.7 ± 8.9 | 0.8 ± 1.5 | — | 1.4 ± 1.6 | x | 15 ± 9.7* | — | 23 ± 15 | 9.7 ± 15 | — | 3042 ± 1839 | 3075 ± 1842 |

[a] Values obtained from LC-MS$^n$ analysis. Data are presented as mean ± standard deviation in ng/g wet weight.
[b] OH: hydroxytestosterone; DHT: dihydrotestosterone; AED: androstenedione; TST: testosterone.
[c] x: metabolite was only detected in one replicate sample.
* Significantly different from control (ANOVA, Dunnett; * $p < 0.05$; ** $p < 0.01$).

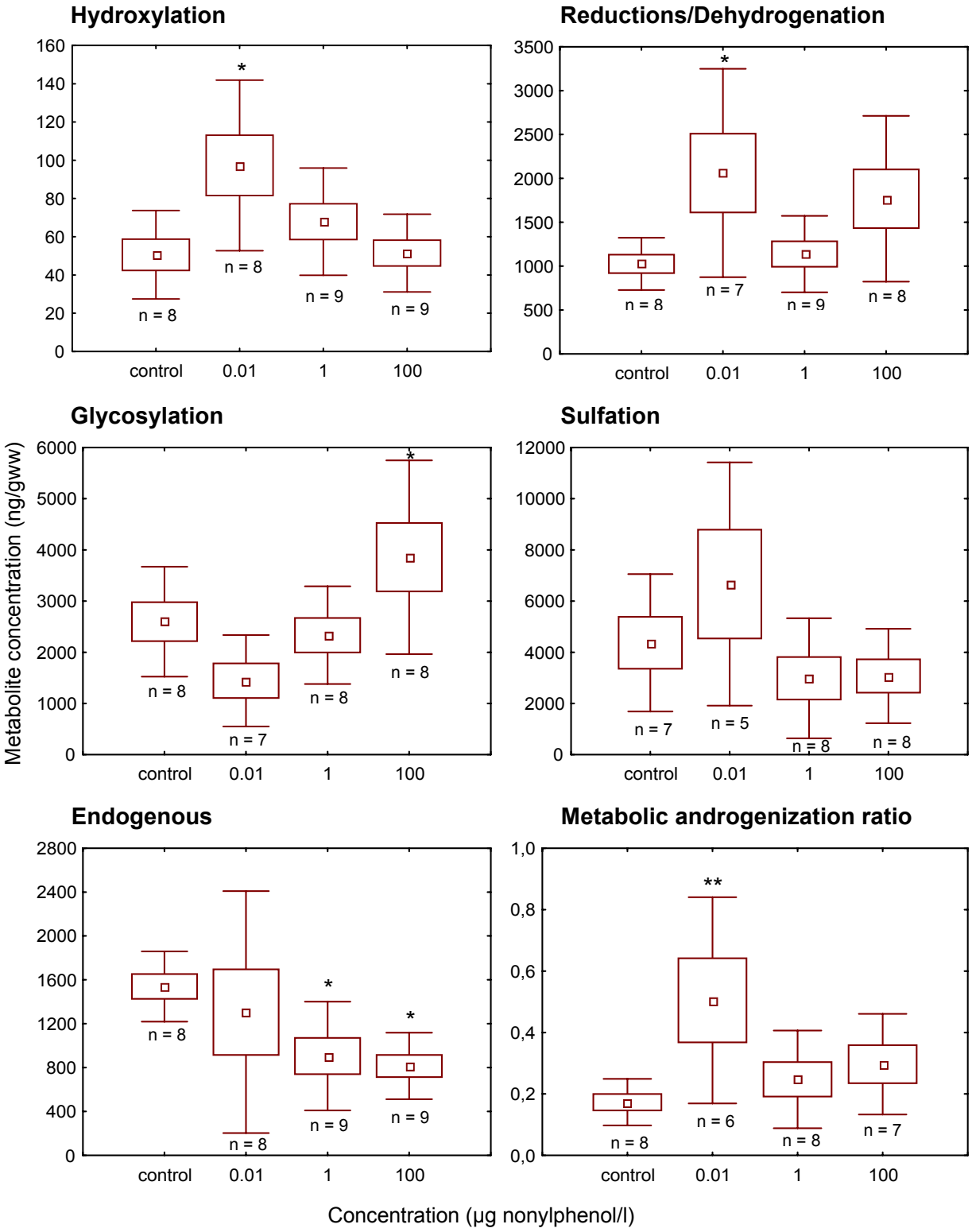



**Figure 8.3.** Metabolic elimination of testosterone by *N. integer* following 96-h exposure to sublethal concentrations of nonylphenol (ANOVA, Dunnett; * $p < 0.05$; ** $p < 0.01$, significance from control). Number below the whisker indicates the number of observations.

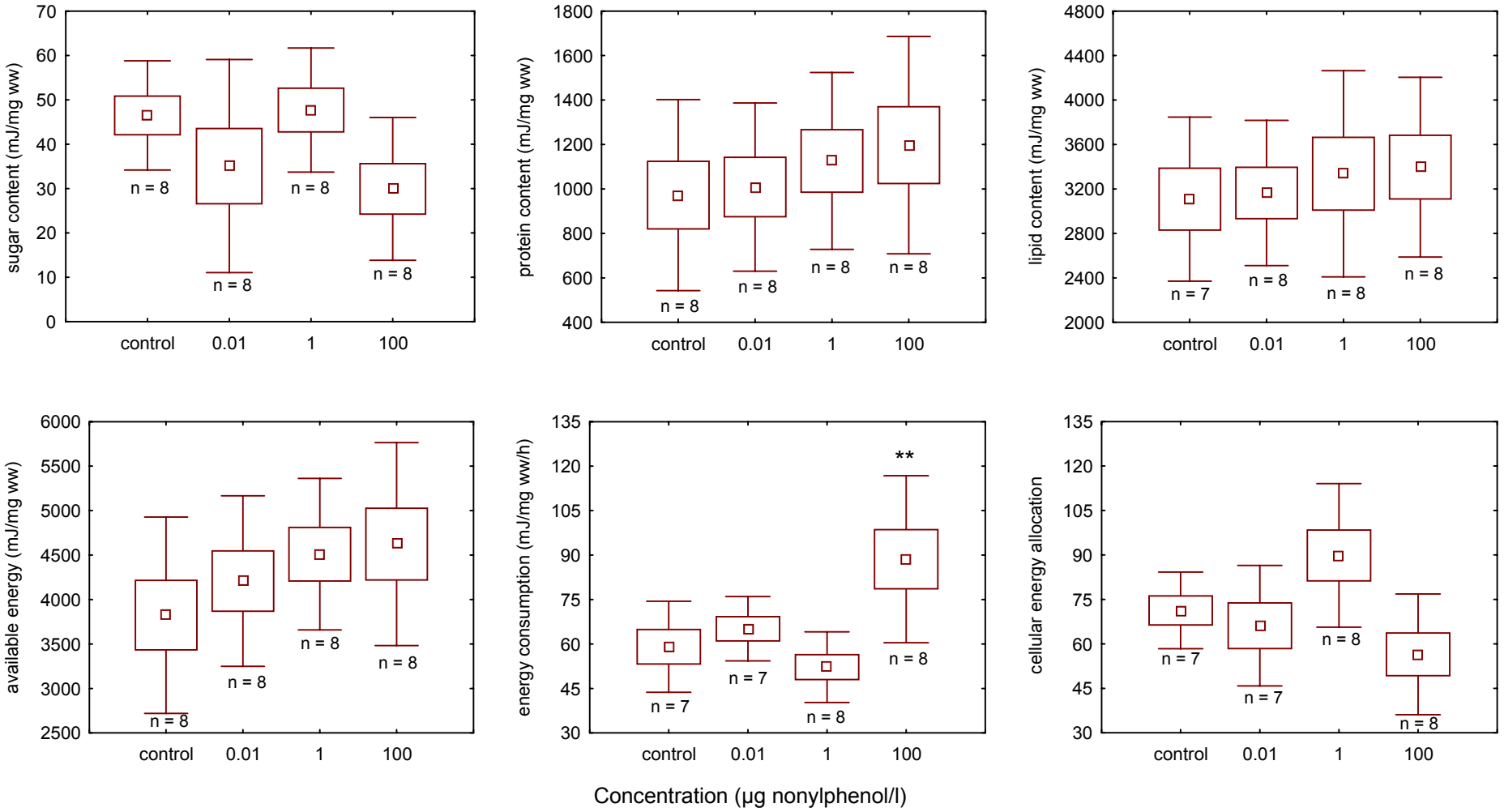



**Figure 8.4.** Cellular energy allocation in *N. integer* following 96-h exposure to sublethal concentrations of nonylphenol (ANOVA, Dunnett; * $p < 0.05$; * $p < 0.01$, significance from control). Number below the whisker indicates the number of observations.

In general, the effects of nonylphenol on energy metabolism were similar to what was observed for methoprene-exposed mysids, i.e. sugar content decreased, protein content increased and lipid content was relatively unaffected. The overall effect of nonylphenol on the individual energy reserve fractions of *N. integer* results in an concentration-dependent increase in the total available energy. Similar to what was observed in the methoprene experiment, this increase in total available energy was not significant from the control treatment. In addition, a similar significant increase in energy consumption as observed in methoprene-exposed mysids, was found in the highest nonylphenol concentration. This increased energy demand resulted in a decrease in cellular energy allocation in the highest exposure treatment which was, however, not significant.

The effects of nonylphenol on testosterone metabolism were very different from what was observed for methoprene. Although a similar induction in the oxidative metabolism was apparent in the lowest exposure concentration, the effects on phase I reductive metabolism and phase II conjugation were different. Glycosylation was significantly increased in the highest nonylphenol exposure concentration, whereas a significant reduction was observed in this treatment for methoprene. However, in both experiments glycosylation appeared to be the major biochemical target of the tested compounds. Similar to what was observed with methoprene, phase II conjugation was strongly correlated with endogenous concentrations of vertebrate-type androgens, although the effects were opposite. The metabolic androgenization ratio was higher in all nonylphenol-exposed mysids, but this was only significant for the lowest nonylphenol exposure concentration (10 ng/l).

## 8.4. Discussion

### *8.4.1. Acute toxicity of the tested compounds*

As mentioned in the introduction, the incentive for investigating whether the steroid and energy metabolism are sensitive to endocrine-disrupting compounds was the finding that tributyltin affects these processes at lower concentrations than those found to be acutely toxic. In this perspective, acute toxicity to *Neomysis integer* was determined for a range of reference compounds as shown in Table 8.2. From these experiments it can be concluded that *N. integer* is at least as sensitive to these compounds than what has been previously determined for other aquatic invertebrates. This corroborates earlier toxicity work with mysids which demonstrated that mysids are among the most sensitive aquatic species to a wide range of chemicals (DeFur et al., 1999; Roast et al., 1998a, 1999a, 2000c; USEPA, 2002c; Verslycke et al., 2003b).

*8.4.2. Sublethal effects of methoprene and nonylphenol on the energy metabolism of N. integer*

The effects of chemicals on the energy allocation of *Neomysis integer* are consistent between the different exposure experiments. We previously investigated the effects of tributyltinchloride and chlorpyrifos on the cellular energy allocation in this species following short-term exposure (refer to Chapters 4 and 5). Both 48-h exposure to chlorpyrifos and 96-h exposure to tributyltinchloride (TBTCl) resulted in a significant increase in proteins, a decrease in sugars and a relatively unaffected lipid fraction. The concentration-dependent increase in energy consumption, as derived from the electron transport activity, is believed to cause a more efficient metabolism in mysids to cope with this increased energy demand. Consequently, exposed mysids have a higher amount of energy available, but this is insufficient to cope with the elevated energy demand in the higher test concentrations, resulting in a lower CEA. This general scheme was confirmed with the experiments in this study, although these effects were stronger in methoprene-exposed mysids, compared to nonylphenol-exposed mysids. Olmstead and LeBlanc (2000) found that daphnids exposed to 50 and 100 µg methoprene/l for 3 and 6 days were significantly smaller. Mysids in our study exposed to 100 µg methoprene/l had a significantly lower CEA, which indicates that there was less energy available for growth and reproduction. This would corroborate the growth effects observed in daphnids. The wet weight of methoprene-exposed mysids was not significantly different from the wet weight of control mysids, thus, not confirming this assumption following short-term exposure. Long-term exposures are needed to further elucidate potential effects of methoprene on mysid growth.

The 96-h median lethal concentrations indicate that methoprene is almost twice as toxic as nonylphenol, and this was also reflected in the CEA response which was significantly reduced in the 100 µg/l treatment in methoprene-exposed mysids, but not in nonylphenol-exposed mysids. However, cellular respiration was significantly higher in the 100 µg/l treatment for both compounds, compared with control mysid respiration. As such, the NOEC (no observed effect concentration) for CEA in mysids following exposure to methoprene and nonylphenol was 1 and 100 µg/l, respectively. Previously, we found a 96-h LOEC (lowest observed effect concentration) for tributyltinchloride of 10 ng/l (tested concentrations: control-10-100-1000 ng TBTCl/l) and a 48-h LOEC of 38 ng/l for chlorpyrifos (tested concentrations: control-38-56-72-100 ng chlorpyrifos/l). The respective LC-50s for these compounds using the same exposure duration was 164 ng tributyltinchloride/l (Verslycke et al., 2003c) and 270 ng chlorpyrifos/l (Roast et al., 1999c). The acute toxicity of these chemicals is thus clearly reflected in the different sensitivities in the CEA assays. In conclusion, sublethal effects of the chemicals tested in the present study on the CEA of *N. integer* are observed at

concentrations which are three to ten times lower than the median lethal concentration.

It has previously been demonstrated that the biochemical endpoints of the CEA analysis are significantly related with population-level effects in daphnids, indicating the possibility of correlating energy-based sub-organismal effects with those emerging at the higher levels of biological organization (De Coen, 1999). Furthermore, in these studies, the CEA criterion correlated much better with ecologically relevant population-level test criteria (i.e. mean brood size, mean number of young per female, net productive rate and intrinsic rate of natural increase) than the individual energy fractions of the CEA, due to its integrated nature. The life history of the mysids is very amendable to demographic modeling (Kuhn et al., 2000, 2001). Therefore, the correlation of the individual biochemical endpoints, as well as the integrated CEA value, with population parameters in mysids should be an area of future research. However, the long life span of *N. integer* might limit the practical use of such assays. Clearly, the standard mysid test species *Americamysis bahia* has the advantage over *N. integer* in allowing demographic modeling in much shorter periods (Kuhn et al., 2000, 2001). In this perspective, chronic pesticide exposure studies with *A. bahia* have demonstrated the ecological relevance and utility of short-term bioindicators of metabolic processes (McKenney, 1982, 1985, 1998; McKenney and Matthews, 1990). However, the cellular energy allocation has not been evaluated in chronic assays with mysids so far.

### *8.4.3. Sublethal effects of methoprene and nonylphenol on testosterone metabolism in N. integer*

The effects of methoprene and nonylphenol on the phase I oxidative elimination of testosterone were similar, and corroborate the results of a previous study with TBTCl in which a comparable induction was observed in the lowest test concentration, but not at the higher concentrations. Steroid hydroxylation reactions are catalyzed by P450 isozymes and, consequently, testosterone metabolism is a tool to study the activity of P450 isozymes. Many xenobiotics, such as polycyclic aromatic hydrocarbons can induce the expression of P450s (Fent, 1996; Oberdörster et al., 1998c). Induced expression of P450s results in higher monooxygenase activity and the production of more polar derivatives of exogenous compounds, which are more easily excreted from the body. The induction of P450s only at low exposure concentrations could be due to several factors. First, the highest test concentrations may not have induced P450 enzymes. Secondly, the test compounds may block the activity of P450s at high concentrations, similar to what is observed for TBTCl (Oberdörster et al., 1998c; Verslycke et al., 2003a). Finally, phase II metabolism may be an important reducing factor in the formation of phase I hydroxylated metabolites (Smeets et al., 1999). We previously studied the inducibility of P450s in *Neomysis integer* exposed to

benzo[a]pyrene and found that 1 µg/l had a stronger effect than 50 µg/l, although P450 activities were very low at both exposure concentrations (Verslycke et al., unpublished data). Since we did not measure total P450 activity in this experiment, it can not be determined if the tested compounds induced these isozymes. Consequently, future studies are needed to evaluate P450 induction in mysids. We are not aware of studies which have demonstrated the binding and inactivation of P450 activity by nonylphenol or methoprene. It was, however, remarkable that a strong correlation was observed between phase II glycosylation and phase I hydroxylation in the nonylphenol experiment, which might indicate that this chemical increases the activity of reductions/dehydrogenation and hydroxylation metabolic pathways resulting in the suppressed activity of conjugative pathways due to lack of substrate (testosterone). Baldwin et al. (1997) observed a similar effect of nonylphenol on the elimination profile of testosterone in *Daphnia magna*. They concluded, however, that the unique response profile of the four metabolic processes (hydroxylation, reduction/ dehydrogenation, glycosylation and sulfation) suggests that nonylphenol directly and differentially altered each process. In our opinion, glycosylation was probably induced by nonylphenol exposure in mysids, resulting in less testosterone available for the phase I metabolism, and this was also reflected in a significant decrease in the endogenous concentrations of testosterone (Figure 8.3). Similar, the decrease in phase II glycosylation in mysids exposed to 100 µg methoprene/l was correlated with an increase in endogenous androgen concentrations, which resulted in a significantly higher MAR.

At this time, it remains unclear how nonylphenol and methoprene affect the different processes of the testosterone metabolism in *N. integer*. Although none of the above mechanisms can be excluded, we hypothesize that chemicals interact with the phase II metabolism of *N. integer* leading to the observed effects on metabolic build-up of potent androgens. This is consistent with the findings of studies by Ronis and Mason (1996) and Oberdörster et al. (1998a) who evaluated testosterone metabolism in TBT-exposed invertebrates. Apparently, glycosylation may be the most important pathway in mysids, similar to what is observed in daphnids (Baldwin et al., 1997; LeBlanc and McLachlan, 2000), although a previous study with TBTCl in *N. integer* found that sulfation was most strongly affected (Verslycke et al., 2003a). Future studies will have to determine which of these processes is most important in the detoxification of chemicals in mysids, or if these processes might be differentially affected depending on the chemical.

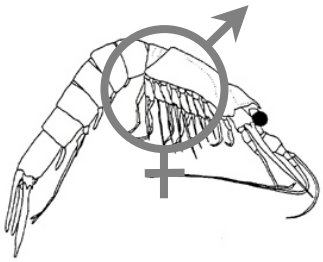
A final consideration is the observed metabolic androgenizing effect of methoprene observed in our study. Recently, Olmstead and Leblanc (2001) demonstrated that methoprene can modulate specific components of the sexual reproductive phase of daphnids. Exposure to 10 and 50 µg methoprene/l increased the percentage of male offspring produced in all broods in *D. magna*. The low concentrations at which methoprene

elicited these effects support the hypothesis that they are the consequence of this juvenile-hormone analog (juvenoid) acting at high affinity receptors to endogenous juvenoids, rather than some overt toxicological response. Our results corroborate these findings as effects on steroid metabolism were apparent in the same concentration range (LOEC of 100 µg/l). Consequently, the developmental abnormalities observed in daphnids might be a result of methoprene altering testosterone metabolism as was previously demonstrated for *D. magna* exposed to other compounds (LeBlanc, 1999; Mu and LeBlanc, 2002b). These observations suggest that steroidal androgens have a specific target site of toxicity in daphnids and mysids. Although a functional role for steroidal androgens has not been firmly established, testosterone has been reported to have androgen-like activity in some crustaceans (LeBlanc, 1999). Furthermore, it has been demonstrated that male daphnids are more susceptible to the toxicity of some chemicals than female (Olmstead and LeBlanc, 2000). We have reported endogenous concentrations of testosterone and other vertebrate-type androgens in *N. integer* and have also observed a sex-specific steroid metabolism in these animals (Verslycke et al., 2002). Thus, it can be hypothesized that several chemicals are likely to interact with testosterone metabolism in mysids which as a result could lead to functional abnormalities.

### 8.5. Conclusion

We previously reported that alterations in testosterone and energy metabolism are sensitive endpoints in mysids exposed to different xenobiotics (Verslycke et al., 2003a,c). Results from the present study corroborate this conclusion. While the effects of methoprene on testosterone metabolism occurred at the same concentration as the effects on CEA, nonylphenol-induced alterations in testosterone metabolism were apparent at the lowest tested concentration, whereas no significant effects were observed in the CEA assay. Similarly, we previously observed significant effects of tributyltin on testosterone metabolism in *N. integer* at concentrations ten times lower than those at which effects on energy metabolism were noted (Verslycke et al., 2003a,c). Clearly, these differences are a reflection of toxicant-specific interactions and need to be further examined using a more extensive list of compounds. The CEA assay has the advantage that it is, inherently, related with effects at higher levels of biological organization. This correlation should also be investigated for testosterone metabolism in mysids, following chronic exposures. Finally, the field applicability of these biomarkers should be validated in the future.

In conclusion, the present study indicates that energy and testosterone metabolism of mysids are sensitive endpoints that are able to detect endocrine-disruptive activity of chemicals following short-term exposure.



# CHAPTER 9

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# Exposure study in the Scheldt estuary: flame retardants, surfactants and organotins in sediment and mysid shrimp

**Redrafted after :**

Verslycke T, Vethaak AD, Arijs K, Janssen CR (submitted) Flame retardants, surfactants and organotins in sediment and mysid shrimp of the Scheldt estuary (The Netherlands). *Environmental Pollution*.

# Chapter 9

# *Exposure study in the Scheldt estuary: flame retardants, surfactants and organotins in sediment and mysid shrimp*

**Abstract**

Sediment and mysids from the Scheldt estuary, one of the largest and most polluted estuaries in Western Europe, were analyzed for a number of endocrine disruptors, i.e. organotins, polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA), nonylphenol ethoxylates (NPE) and transformation products nonylphenol (NP) and nonylphenol ether carboxylates (NPEC). In addition, *in vitro* estrogenic and androgenic potencies of water and sediment extracts were determined. Total organotin concentrations ranged from 84 to 348 ng/g dw in sediment and 1110 to 1370 ng/g dw in mysid. Total PBDE (excluding BDE-209) concentrations ranged from 14 to 22 ng/g dw in sediment and from 1765 to 2962 ng/g lipid in mysid. High concentrations of BDE-209 (240-1650 ng/g dw) were detected in sediment and mysid (269-600 ng/g lipid). Total HBCD concentrations in sediment and mysid were 14-71 ng/g dw and 562-727 ng/g lipid, respectively. Total NPE concentrations in sediment were 1422 ng/g dw, 1222 ng/g dw for NP and 80 ng/g dw for NPEC and ranged from 552 to 1119 ng/g dw for total NPE and from 206 to 435 ng/g dw for NP in mysid. Significant estrogenic potency, as analyzed using the yeast estrogen assay, was detected in sediment and water samples from the Scheldt estuary, but no androgenic activity was found. This study is the first to report high levels of endocrine disruptors in estuarine mysids.

## 9.1. Introduction

The presence of persistent anthropogenic chemicals in our environment is not a new problem. Since the 1960s, an increasing amount of environmental pollutants have been identified and their concentrations have been subject of continuous interest. During recent decades, reproductive and developmental problems in a wide range of wildlife species have been reported (Colborn et al., 1996; Krimsky, 2000; Vos et al., 2000). These disruptions are ascribed to the influence of particular compounds, so called endocrine disruptors, on the hormone systems of exposed animals and their offspring. Presently, no consensus list of

endocrine disruptors exists, although several regulatory bodies, such as the European Union (EU) and the Oslo and Paris Commission (OSPAR), have published indicative lists of potential hormone-disrupting chemicals. Environmental concentrations and the harmful effects of some of these chemicals, for instance PCBs, dioxins and organotins, have been extensively reviewed. On the other hand, there are significant amounts of other potential endocrine-disruptive chemicals in our environment, such as flame retardants and many surfactants, that we know much less about (Darnerud et al., 2001; Palm et al., 2002; Ying et al., 2002). While the use, production and regulation of compounds such as organotins, surface-active compounds and flame retardants is markedly different, they are frequently detected in high levels in the sediments and waters of estuarine environments worldwide (Darnerud et al., 2001; de Voogt et al., 2000; Fent, 1996; Ying et al., 2002). Unfortunately, little is known about the transfer of these chemicals to the hyperbenthic community and thus of the exposure levels in these organisms. Chemical exposure data on endocrine disruptors, in general, is very scarce for invertebrates, preventing an ecologically sound risk assessment for these compounds (DeFur et al., 1999). Organotins, flame retardants and surfactants were included into this study because strong indications of long-term effects on the endocrine system have been published in the literature (de Boer et al., 2003; Fent, 1996; Ying et al., 2002).

Mysid crustaceans are distributed from 80°N to 80°S and occur in most aquatic environments including freshwater, brackish, estuarine, coastal and oceanic (Mauchline, 1980; Tattersall and Tattersall, 1951). Mysids are also frequently used in toxicity testing (e.g. ASTM, 1998, 1999; McKenney, 1998; Nimmo and Hamaker, 1982; Roast et al., 1998a, 1999a; USEPA, 1995a, 1997; Verslycke et al., 2003b) and are sensitive to many toxicants at levels that are likely to occur in the environment (Roast et al., 1998a; Verslycke et al, 2003b). We have been using the hyperbenthic mysid *Neomysis integer* as a test organism for the evaluation of the endocrine-disruptive properties of chemicals in the laboratory and the Scheldt estuary (Verslycke et al., 2002, 2003a,c, submitted-Chapter 8, 10, 11).

A recent study on estrogenic contaminants in the aquatic environment of The Netherlands found high concentrations of flame retardants and surfactants in the Scheldt estuary (Vethaak et al., 2002). Furthermore, this estuary is apparently heavily contaminated with organotins, although only limited data are available (Bouma et al., 2000; OSPAR, 2002). While a few studies on environmental concentrations of organotins, flame retardants and surfactants in the Scheldt estuary are available, a survey of these potential endocrine disruptors in hyperbenthic invertebrates, such as mysids, has never been carried out. Hyperbenthic species play an important role in the coupling of benthic and pelagic food webs (Mees and Jones, 1997) and the ecological importance of *N. integer* in the Scheldt estuary has been thoroughly investigated (Fockedey and Mees, 1999; Mees et al.,1993b, 1994, 1995). These reasons necessitate a study on the exposure of these animals, which should provide important data for understanding

the transfer of these chemical pollutants in an estuary and is essential for understanding potential endocrine disruptive effects in (hyper-)benthic communities.

## 9.2. Material and Methods

### *9.2.1. Study area*

The river Scheldt takes its rise at Saint-Quentin in France about 350 km upstream of Vlissingen in The Netherlands where the river discharges into the North Sea (Fig. 9.1).

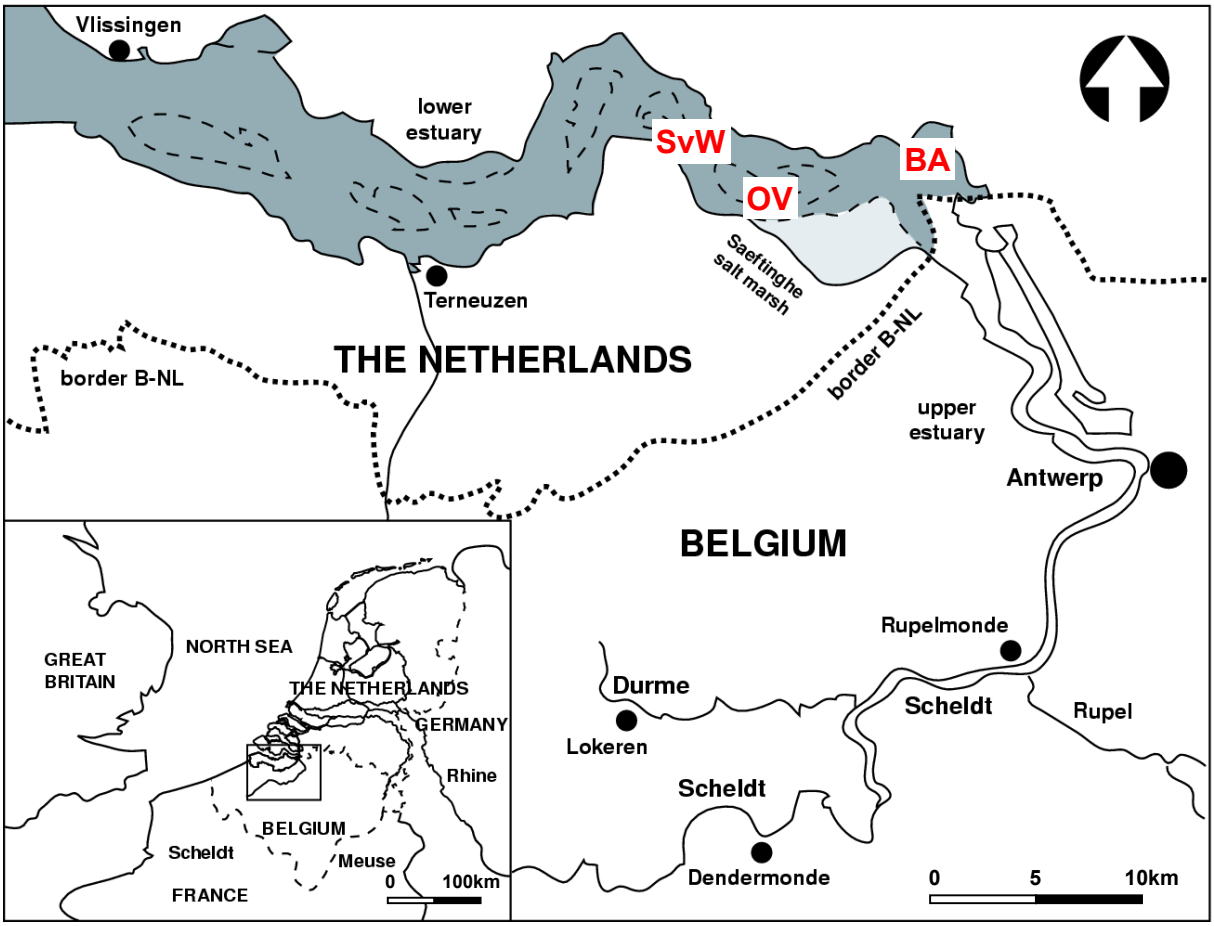



**Figure 9.1.** The Scheldt estuary, one of the largest and most polluted estuaries in Europe, was sampled for water, sediment and mysids at three sites (SvW, Schaar van Waarde; OV, Overloop van Valkenisse; BA, Bath) in november 2001, representative of the major distribution area of the estuarine mysid *N. integer*.

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. The Scheldt distinguishes itself from other estuaries by the fact that the relatively small average river discharge of 100 m³/s is strongly dominated by the large intertidal exchange volume of approximately 1 billion m³. The Scheldt

estuary is, therefore, characterized as a long and well mixed estuary with large intertidal areas. From an ecological point of view, the Scheldt estuary is one of the most important tidal river systems in Europe. It is an important passing, overwintering and feeding area for waterbirds and an important nursery for fish and shrimp. The Scheldt estuary was also ranked among the most polluted estuaries worldwide, based on contaminant concentrations in the dissolved as well as the particulate phase (Bayens et al., 1998). The physical, chemical and biological characteristics of the Scheldt estuary are further discussed in Bayens et al. (1998), Heip (1988, 1989), Herman et al. (1991), Van Eck et al. (1991) and Chapter 10 (§ 10.2.1).

### *9.2.2. Water, sediment and mysid sampling*

Water, sediment and mysid shrimp were collected from the Scheldt estuary (The Netherlands) at three different locations in November 2001 (see Fig. 9.1; Schaar van Waarde, Overloop van Valkenisse, and Bath) based on the sampling grid used by Mees et al. (1993b). The spatial spreading of these locations represents the major distribution zone of the estuarine mysid *Neomysis integer* (Mees et al., 1993b). Mysid samples were collected with a hyperbenthic sledge (Hamerlynck and Mees, 1991), consisting of a metal frame equipped with two mounted nets, one above the other as described in Chapter 10 (§ 10.2.2). *N. integer* specimens were sorted out on board and placed in hexane-rinsed aluminum foil packages and frozen at -20°C until analysis. Water was sampled with a Niskin-bottle about 1 m above the bottom, transferred into pre-rinsed dark glass 1-l bottles, and immediately extracted onboard. Sediments were sampled with a Van Veen grabber, collected in 250 ml glass recipients and maintained at 4°C until extraction. Salinity, dissolved oxygen concentration, and temperature were recorded at each location as secondary parameters (Chapter 10, Table 10.1).

### *9.2.3. Chemical analysis of organotins, surfactants and flame retardants*

All data were corrected for dry weight (or for lipid content in case of the flame retardant concentrations in mysids) of the sample. The water content of the sediment samples was determined gravimetrically after heating an aliquot of the sample to 105°C overnight. Loss on ignition (LOI) was determined gravimetrically after heating the dried sediment sample at 550°C for 2h. LOI at this temperature has been assumed to be due to volatilisation of organic matter. The carbon content in the sediment sample can roughly be estimated as the LOI divided by 2. The total lipid content of the mysid samples was determined by a chlorophorm/methanol extraction according to Bligh and Dyer (1959). All solvents used were

of analytical grade.

Organotin compounds in sediment and mysids were analyzed by *in situ* ethylation using gas chromatography linked with atomic emission detection (GC-AED). Sediments were sieved (<63 µm) and freeze-dried for analysis. Mysid samples were homogenized for analysis. Dry sediment or dry mysid homogenate were weighted and extracted with a mixture of methanol, hexane and glacial acid. Ethylation was carried out by adding sodiumtetraethylborate to the mixture at pH 5 (*in situ* ethylation and extraction). After ethylation, sodiumhydroxide was added to remove any boroxin (by-product of the ethylation). Clean-up of the hexane layer was carried out by eluting the hexane layer over a column filled with deactivated aluminiumoxide and C18 material. The concentrated extract was analyzed by GC-AED.

Extraction of mysids for the analysis of nonylphenol ethoxylates (NPE) and its transformation products nonylphenol ether carboxylates (NPEC) and nonylphenol (NP) was done by Matrix Solid Phase Dispersion (MSPD) as described by Zhao et al. (1999). The sediment samples were Soxhlet extracted with methanol and cleaned up with Solid Phase Extraction as described by de Voogt et al. (2000). LC-MS was used for metabolite identification. To that end an electrospray interface was employed. All samples were analyzed in duplo. Details of this method are published elsewhere (Jonkers et al., 2001).

Mysid samples were homogenized and mixed with anhydrous $Na_2SO_4$ for the analysis of flame retardants. Sediment samples were analyzed as such. Samples were Soxhlet extracted with a hexane/acetone mixture, followed by gel permeation chromatography and a chromatography over silica. The extracts were subsequently treated with concentrated sulfuric acid to remove any interfering substances. Final analysis was performed through capillary gas chromatography coupled with negative chemical ionization mass spectrometry (GC-NCI-MS). Details of this method are given by de Boer et al. (2000).

### *9.2.4. Yeast Estrogen Screen (YES) and Yeast Androgen Screen (YAS)*

One-liter water samples were extracted immediately after sampling on C18-packed solid-phase extraction disks (Bakerbond Speedisk™, J.T. Baker) with acetone and methanol according to the manufacturer's recommendations. The resulting extracts were evaporated and subsequently dissolved in 2 ml of ethanol for analysis in the YES and YAS. The YES was performed according to Routledge and Sumpter (1996) with the following modifications; absorbances (540/620 nm) were read after 8 days and EC50 values were calculated using the probit method (Stephan, 1977). The YAS was performed according to Sohoni and Sumpter (1998) with following modifications; test plates were incubated at 32°C for 24 hours

and were then placed at room temperature; absorbances (540/620 nm) were read after 8 days and EC50 values were calculated using the probit method (Stephan, 1977).

Sediment samples (10 g wet weight) were extracted via an automated soxhlet extraction system (Soxtec® system 1046, Foss, Belgium) using EPA method 3541 'Automated Soxhlet Extraction' (USEPA, 2003) with an acetone/hexane (1:1 v/v) mixture. Again, extracts were evaporated and dissolved in 2 ml ethanol for *in vitro* analysis in the YES and YAS. The estradiol equivalency factor (EEF) is calculated as the ratio of the EC50 of 17β-estradiol to the EC50 of the water or sediment sample. Similar, the dihydrotestosterone equivalency factor (DEF) is calculated as the ratio of the EC50 of dihydrotestosterone to the EC50 of the water or sediment sample.

## 9.3. Results and discussion

### *9.3.1. Analysis of organotins in sediment and mysids*

Total organotin concentrations, as shown in Table 9.1 (sum of tributyltin, TBT; dibutyltin, DBT; monobutyltin, MBT; triphenyltin, TPT; diphenyltin, DPT and monophenyltin, MPT) were in the µg/g dw range for mysid (1110-1370 ng/g dw) and a factor 5 lower in sediment (84-348 ng/g dw). The TBT/MBT and TBT/DBT-ratio were lower in the sediments sampled at the Bath site (most upstream sampling site, see Fig. 9.1). This lower TBT/DBT ratio indicates older contamination and a beginning of degradation, which can be expected to come from the harbor of Antwerp, the fourth largest harbor in the world (Basheer et al., 2002; Michel et al., 2001). A classification of TBT-contaminated sediments was proposed by Dowson et al. (1993), characterizing concentrations below 3 ng/g dw as uncontaminated, 3 to 20 ng/g dw as lightly contaminated, 20 to 100 ng/g dw as moderately contaminated, 100 to 500 ng/g dw as highly contaminated and above 500 ng/g dw as grossly contaminated. Using this scheme, the sampled surface sediments of the Scheldt estuary (45-156 ng TBT/g dw) can be classified as moderately to highly contaminated. All of the samples exceeded the ecotoxicological assessment criteria of 5 to 50 ng/g dw as proposed by the Oslo-Paris Commission (OSPAR, 2002). The concentrations of TBT in sediments of the Scheldt estuary sampled in this study are similar to those reported during the last decade for other harbors and contaminated coastal areas of the world as summarized in Brack (2002), but higher than these previously reported for the Scheldt estuary (3.6-46 ng/g dw) by OSPAR (2002). TBT concentrations in sediment from different harbors of the Scheldt estuary were summarized by Bouma et al. (2000) and ranged from 17.9 to 117 ng/g dw in the period 1996-2000.

**Table 9.1.** Concentrations of organotins, polybrominated diphenylethers (PBDE), hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA), nonylphenol ethoxylates (NPE), nonylphenol ether carboxylates (NPEC) and nonylphenol (NP) in sediment and mysid shrimp (*Neomysis integer*) from the Scheldt estuary (ng/g dry weight, except flame retardants in mysid in ng/g lipid weight)

| Compound | Sampling locations | | | | | |
|---|---|---|---|---|---|---|
| | Schaar van Waarde | | Overloop v. Valkenisse | | Bath | |
| | Mysid | Sediment | Mysid | Sediment | Mysid | Sediment |
| Dry weight (% ww) | 16.5 | 83.7 | 15.3 | 83.7 | 14.4 | 78.0 |
| Loss on ignition (% dw) | NA[a] | 0.4 | NA | 0.30 | NA | 1.6 |
| OC (% dw) | 15.6 | 0.2 | 15.6 | 0.15 | 15.6 | 0.8 |
| Lipid (% ww) | 1.3 | NA | 1.3 | NA | 1.1 | NA |
| Organotins | | | | | | |
| TBT | 927 | 155 | 1209 | 45 | 1199 | 156 |
| DBT | 23 | 30 | 21 | 16 | 25 | 123 |
| MBT | 12 | 22 | 8 | 16 | 9 | 36 |
| TPT | 148 | 9 | 56 | 7 | 137 | 22 |
| DPT | <3 | 3 | <3 | <3 | <3 | 11 |
| MPT | <3 | <3 | 22 | <3 | <3 | <3 |
| Total | **1110** | **219** | **1316** | **84** | **1370** | **348** |
| Flame retardants | | | | | | |
| BDE-28 | 31 | 0.2 | 46 | 0.2 | 46 | 0.7 |
| BDE-47 | 739 | 3.1 | 923 | 2.8 | 1182 | 4.4 |
| BDE-66 | 15 | 0.3 | 31 | 0.2 | 27 | 0.3 |
| BDE-71,75,77 | <8 | <0.1 | <8 | <0.1 | <9 | <0.1 |
| BDE-85 | 15 | 0.2 | 23 | 0.2 | 36 | 0.3 |
| BDE-99 | 646 | 3.0 | 677 | 2.6 | 1091 | 4 |
| BDE-100 | 215 | 1.0 | 262 | 0.9 | 364 | 1.7 |
| BDE-119 | <8 | <0.1 | 0.8 | <0.1 | <9 | <0.1 |
| BDE-138 | 3 | 0.5 | 5 | 14.0 | 7 | 0.1 |
| BDE-153 | 54 | 12.0 | 69 | 0.6 | 109 | 1.9 |
| BDE-154 | 46 | 1.8 | 62 | 0.5 | 100 | 1 |
| BDE-190 | <8 | <0.1 | <8 | <0.1 | <9 | <0.1 |
| BDE-209 | 331 | 250 | 269 | 240 | 600 | 1650 |
| ΣPBDE | **2095** | **272** | **2367** | **262** | **3562** | **1664** |
| TBBPA | <8 | <0.1 | 0.8 | < 0.1 | 0.9 | <0.1 |
| HBCD | 569 | 30.0 | 562 | 14.0 | 727 | 71.0 |
| NPE | | | | | | |
| NPE1 | 138 | ND[b] | 253 | ND | 136 | 51 |
| NPE2 | 981 | ND | 299 | ND | 220 | 221 |
| NPE3-16 | <52 | ND | <52 | ND | 74 | 1151 |
| ΣNPE | **1119** | ND | **552** | ND | **430** | **1422** |
| NP | 435 | ND | 332 | ND | 206 | 1222 |
| NPE1C | <46 | ND | <46 | ND | <46 | 28 |
| NPE2C | <39 | ND | <39 | ND | <39 | 52 |

[a]NA: not available; [b]ND: not detected. Refer to Fig. 9.1 for sampling locations.

The International Maritime Organization has decided to develop a binding international instrument to ban the use of organotin compounds in anti-fouling treatments on ships longer than 25 m. The target is to prohibit their application from 2003 and to require the removal of TBT from ships' hulls by the year 2008 (for an overview on TBT regulations see Champ, 2000). Despite these regulations, many areas still show legacy of historic TBT inputs, due to the persistence of organotins in sediment (Biselli et al., 2000; Stronkhorst et al., 1999). This is especially true for The Netherlands, who are on top of the TBT contributors (33%) list of the nine North Sea states, based on the annual percentage of merchant shipping (Davies et al., 1998). Within this context, the dogwhelk *Nucella lapillus* and the common whelk *Buccinum undatum* have locally dissapeared from the Scheldt estuary through the TBT-induced imposex phenomena. In addition, induced intersex was noticed in periwinkle *Littorina littorea* collected in the Scheldt estuary (De Wolf et al., 2001).

The concentrations of TPT in our study were in accordance with reported values for the Göta Älv estuary in Sweden and sediments from harbors in The Netherlands (Brack, 2002; de Boer et al., 2001) and generally lower than those reported for German North Sea and Baltic Sea marinas (Biselli et al., 2000).

Because of their filter-feeding behavior and high potential for bioaccumulation of contaminants, including organotins, bivalves have been widely used as sentinel organisms for monitoring contamination of aquatic systems. In addition, organotin concentrations in gastropods, fish and marine mammals, have been extensively investigated (for a review refer to Fent, 1996), but no data is available for hyperbenthic species such as mysids. In our study, TBT concentrations in mysids ranged from 927 to 1209 ng/g dw, which are similar to concentrations reported for bivalves and gastropods from contaminated sites, but higher than concentrations reported in fish, macroinvertebrates and macrophytes (Birchenough et al., 2002; Bouma et al., 2000; Fent, 1996). The TBT/total butyltin ratio in mysids for the three sites was 0.98 (± 0.01), which is significantly higher than previously reported values of 0.5-0.8 by Morcillo et al. (1999), 0.8 by Regoli et al. (2001) and 0.7 by Bouma et al. (2000) for mussels. This could indicate a very slow metabolism of TBT in mysids as compared to mussels, but could also result from a higher bioaccumulation for TBT as compared to DBT and MBT in mysids. Since the TBT/total butyltin ratio in sediment collected at the most inland site (0.49) was clearly lower when compared to the most marine/estuarine site (0.75), mysids apparently differentially bioaccumulate TBT and its degradation products to come to a steady-state independent of the ratio of these compounds in sediment. However, no data are available in literature on TBT uptake and metabolism in mysids to support this hypothesis. As organotins were not analyzed in the water column, no conclusions can be drawn from the exposure of mysids via this route.

We previously published a 96h-LC50 for TBTCl of 164 ng/l and also found that energy and steroid metabolism were significantly altered in *Neomysis integer* at 10 ng TBTCl/l (Verslycke et al., 2003c). Based on an estimated Kp value (ratio between TBT in the particulate and dissolved fractions) for TBT of 1 to 3 x $10^3$ l/kg as published by Fent (1996), water concentrations at the sampled sites can be estimated to range from 40 to 119 ng/l which corroborates the values reported for the Scheldt estuary in Bouma et al. (2000). This is higher than commonly reported concentrations of 1 to 50 ng/l for estuaries (Fent, 1996). These concentrations would be high enough to result in effects on resident mysids. Preliminary studies by our laboratory on the resident *N. integer* population in the Scheldt estuary, focusing on toxicant-induced biomarker responses and population effects, seem to confirm this. Indeed, we have found indications of alterations in the energy and testosterone metabolism of *N. integer* at the sampled sites (Verslycke et al., submitted-Chapter 10, 11).

### *9.3.2. Analysis of flame retardants in sediment and mysids*

Among the different groups of flame retardants, the most common are tetrabromobisphenol A (TBBPA), polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD) and polybrominated biphenyls (PBB). About one third of the production of brominated flame retardants is PBDEs, another third is TBBPA and the remainder is various other brominated compounds (Hyötyläinen and Hartonen, 2002). Flame retardants are extensively used as additives or reactives in paints, plastics, textiles, and electronics to inhibit or suppress the combustion process. They are used in large quantities worldwide (132,000 metric tons per year; Palm et al., 2002) and are persistent in the environment. Over the last decade, there have been indications of increased concentrations of these compounds in the environment and humans, although their levels are still lower than those of PCBs and DDT (Darnerud et al., 2001; Meerts et al., 2001). Recent data indicate that PBDEs may be more harmful than previously expected, although no complete toxicological evaluation is currently available on any of the commercially available PBDE mixtures or on any individual congener (Covaci et al., 2002; Darnerud et al., 2001; McDonald, 2002). PBDEs have been detected in several estuaries in Europe, among which those of the rivers Scheldt in The Netherlands and Mersey and Tees in the UK. In these rivers relatively high levels (up to µg/g dw) of BDE-209 were found (Bouma et al., 2000; de Boer et al., 2003).

Of the 15 congeners analyzed in our study, BDE-28, -47, -66, -85, -99, -100, -138, -153, -154, and -209 were found in all mysid and sediment samples (Table 9.1). Analysis of sediments from a number of European estuaries revealed high concentrations of BDE-209 (<0.5-1700 ng/g dw) in some rivers, e.g. the river Scheldt (200 ng/g dw), whereas most

samples showed levels below 20 ng/g dw (Sellström, 1999). The BDE-209 sediment concentrations in our study (240-1650 ng/g dw) corroborate earlier findings of high concentrations of this congener in the Scheldt estuary which are among the highest values found in European estuarine sediments and similar to concentrations found near point sources worldwide (Booij et al., 2002; Boon et al., 2002; Palm et al., 2002). BDE-209 is the most widely produced formulation, accounting for around 75% of the global production of PBDEs (Harner and Shoeib, 2002). The highest BDE-209 level detected in our study is about three times higher than recent published data for a nearby sampling place in the Scheldt estuary (up to 510 ng/g dw) by de Boer et al. (2003) and is among the highest reported until now. These authors concluded that the input from Antwerp appears to be more important than an assumed contribution from the bromine industry in Terneuzen. The PBDE concentrations in mysids and sediment from our study confirm this trend. Average levels of the other major congeners BDE-47 (3.43 ± 0.29 ng/g dw), BDE-99 (3.2 ± 0.72 ng/g dw), BDE-100 (1.2 ± 0.44 ng/g dw) were very similar to these reported by Sellström (1999) for European estuaries. In fact, total PBDE levels (excluding BDE-209) in sediment in our study ranged from 14 to 22 ng/g dw in sediment which corresponds well with previously published data (Palm et al., 2002).

While reports on PBDE exposure levels in vertebrates are exponentially increasing, the first data on exposure levels in marine invertebrates from the North Sea have only recently been published by Boon et al. (2002). The order of PBDE concentrations in mysids in our study is BDE-47, BDE-99 > BDE-100 > BDE-153, BDE-154 > BDE-28 > BDE-66, BDE-85 which is the same as found in other marine invertebrates such as sea star, hermit crab, mussels, whelk and shrimp (Boon et al., 2002; Bouma et al., 2000; de Boer et al., 2000). Other congeners (BDE-71, 75, 77, 119, 138, 190) were below the limit of detection. Similar to other studies we selected BDE-47 levels in mysid, the congener present in the highest concentration (36% of ΣPBDE in our study), for comparing with PBDE levels in animals from other areas. The BDE-47 levels in mysid ranged from 739 to 1182 ng/g lipid with a distinct upstream increase in concentrations. These concentrations are about 40 times higher than those found in North Sea invertebrates (10-38 ng/g lipid) and similar to the highest reported concentrations in fish and marine mammals worldwide (Manchester-Neesvig et al., 2001). From the data in Table 9.1, it can be derived that the bioaccumulation potential in mysids is highest for BDE-47, BDE-99 and BDE-100 and lowest for BDE-209. This corroborates the findings in bioaccumulation experiments with mussels (Gustafsson et al., 1999) and supports the available data which indicate that higher brominated compounds (heptaBDEs and above) do not bioaccumulate to a significant degree (Darnerud et al., 2001). A recent study by de Boer et al. (2003) found that suspended particulate matter (SPM) is an important carrier for higher brominated diphenylethers in the aquatic environment. They reported high BDE-209

concentrations in SPM (up to 4600 ng/g dw) and sediment (up to 510 ng/g dw) from the Scheldt estuary, most likely related to spills during the use of BDE-209 in the textile industries along the river Scheldt near Antwerp or further upstream. Bouma et al. (2000) also reported high concentrations of BDE-209 in SPM (297 ng/g dw) and sediment (107 ng/g dw) of the Scheldt estuary near the harbor of Terneuzen, which is situated downstream from the sampling locations in our study (Fig. 9.1). Mysids are known omnivores, feeding on detritus, algae and zooplankton (Fockedey and Mees, 1999; Mauchline, 1980). This feeding behavior would result in a high intake of SPM or sediment-associated toxicants such as PBDEs and could explain the high concentrations reported in this study as compared to concentrations found in other invertebrates. It should be noted that mysids were not depurated prior to analysis. This would be supported by the findings of Booij et al. (2002), who found that the larger part of the BDE-209 content in blue mussels was associated with ingested particles.

In addition, mysid and sediment samples from our study were also screened for hexabromocyclododecane (HBCD), used as a decaBDE substitute, and tetrabromobisphenol A (TBBPA). TBBPA was only detected in trace amounts in mysids from the two most inland sites (Overloop van Valkenisse and Bath) but was below detection limit in all sediment samples. Data on TBBPA concentrations in the aquatic environment are relatively limited, although they have previously been detected in a study near a Swedish plastics industry (34 to 270 ng/g dw). HBCD concentrations in sediments (38.3 ± 29.4 ng/g dw) and mysids (619.4 ± 93.5 ng/g lipid) from our study showed a similar upstream increase in concentrations towards the harbor of Antwerp, indicating that potential point sources are similar to these of PBDEs. HBCD has previously only been detected in environmental samples (fish and sediment) from Japan, Sweden and The Netherlands (Bouma et al., 2000; Sellström et al., 1998). HBCD concentrations in these studies ranged from below detection limit to 8000 ng HBCD/g lipid in fish and from below detection limit to 7000 ng/g ignition loss in sediment. Bouma et al. (2000) reported high HBCD concentrations associated with SPM (74 ng/g dw) and in sediment (25 ng/g dw) from the Scheldt estuary near Terneuzen, which resulted in high levels of this compound in fish (66-124 ng/g dw) and eggs of common tern *Sterna Hirundo* (533-844 ng/g dw). In the latter bird species, a decline in reproductive succes has been observed for several years in the Terneuzen population and these effects have been partly attributed to the high exposure of these animals to flame retardants (Bouma et al., 2000).

The general upstream increase in concentrations of flame retardants observed in our study can be discussed in the context of earlier work on PCB distribution in the Scheldt estuary. Like PCBs, PBDEs are very lipophilic (log Kow values 5 to 10; Rahman et al., 2001), have a high binding affinity for particles and a tendency to accumulate in sediments (de Wit, 2002). PBDE distributions in an estuary can be thought to be mainly influenced by tidal

hydrodynamics, suspended sediment transport and hydrophobic sorption. This would result in high accumulations of PBDEs in the zone of high turbidity at the head of the salt water intrusion, and little transport to the North Sea, as was modeled and validated by actual concentrations for PCBs in the Scheldt estuary by Vuksanovic et al. (1996). A zone of high turbidity in the Scheldt estuary is situated around the city of Antwerp, which would result in decreasing PBDE concentrations downstream of these locations as was observed in our study. In the Scheldt estuary, hyperbenthic life ceases shortly upstream of the Dutch-Belgian border due to oxygen depletion (oxygen saturation values of less than 40%), and the *N. integer* population is concentrated in a narrow zone of approximately 20 km (between Hansweert and the Dutch-Belgian border, in a salinity range of 25-8‰) throughout the year (Mees et al., 1993b). Within the hyperbenthic community of the Scheldt estuary, this distribution pattern places *N. integer* in the zone of highest pollution, due to the processes described above.

### *9.3.3. Analysis of surfactants in sediment and mysids*

Alkylphenol ethoxylates (APEs) are one of the most widely used classes of nonionic surfactants with an annual worldwide production of about 650,000 tons (Guenther et al., 2002). The most significant commercial APEs are octylphenol ethoxylates and nonylphenol ethoxylates (NPE). NPEs account for about 80% of the total APE use and 60% ends up in the aquatic environment. APEs can be biodegraded in the environment to form lower ethoxylate congeners, further transformation proceeds via oxidation of the ethoxylate chain, producing mainly alkylphenol ethoxy acetic acids (APEC1s), alkylphenoxy acetic acid (APE2C) and alkylphenols such as nonylphenol (NP) and octylphenol (OP) (Ying et al., 2002). NP and OP are known to be more toxic than their precursors and to mimic the effect of estrogens (Renner, 1997). Because of the formation of persistent metabolites and their estrogenic potential, APEs have been banned or restricted (Petrovic et al., 2002). In Western Europe and the USA, the APEs in household detergents have been completely replaced by alcohol ethoxylates, but because of their lower cost APEs are still being used in substantial amounts in institutional and industrial applications (de Voogt et al., 1997).

In our study, NPE were found in all mysid samples and total concentrations ranged from 430 to 1119 ng/g dw. The most important APE metabolite in mysid samples was nonylphenol diethoxylate (NPE2), followed by the mono (NPE1)- and higher (NPE3-16) ethoxylated metabolites in order of decreasing concentrations. However, only mysids sampled in Bath showed presence of these higher (NPE3-16) chainlength NPEs. NPE concentrations in invertebrates have rarely been reported. No detectable levels of APEs were found in blue mussel and zebra mussel in the Netherlands, whereas bream in this study

had similar NPE concentrations as mysids in our study (Vethaak et al., 2002).

Only one sediment sample (Bath) was analyzed for NPEs and contained long-chain NPEs, with a maximum for NPE3-4 and a total concentration of 1422 ng/g dw. This concentration is within the same range as reported total NPE concentrations in polluted marine sediments which are typically in the mg/kg dw range or lower (de Voogt et al., 1997; Petrovic et al., 2002; Ying et al., 2002). Other studies have identified high NPE concentrations in sediments from the Scheldt estuary near our sampling site (Vethaak et al., 2002) and in the channel Gent-Terneuzen which discharges into this estuary (de Voogt et al., 2000). The presence of long-chain NPEs in our study corroborates earlier findings by Shang et al. (1999) that little degradation occurs of NPEs once these compounds enter the sediment, with half-life estimates of more than 60 years. Sediment concentrations of nonylphenol ether carboxylates (NPEC), the major metabolites of NPE were found in a total concentration of 80 ng/g dw. However, these metabolites were below detection limit in all mysid samples. The higher concentrations of NPE2C (52 ng/g dw) as compared to NPE1C (28 ng/g dw) in sediment confirms earlier studies on aerobic biodegradation of APEs which consists of a rapid initiating step (ω-carboxylation) resulting in long carboxylated EO chains. Further biodegradation proceeds gradually into short-chain carboxylated EO with the most abundant species and recalcitrant species being NPE2C (de Voogt et al., 2000; Fenner et al., 2002; Ying et al., 2002). Furthermore, the presence of nonylphenol (NP) at a concentration of 1222 ng/g dw in the sediment sample indicates that sediments at the Bath site have aged long enough for degradation to play a significant role in the fate of the total contamination. This is similar to the findings for organotins in the present study. Earlier studies reported a NP sediment concentration of 3800 ng/g dw for a nearby upstream site in the Scheldt estuary (Vethaak et al., 2002). Blackburn et al. (1999) reported similar NP concentrations in estuarine sediments from the highly industrialized Tees estuary in the UK ranging from 1600 to 9050 ng/g dw. NP concentrations in mysid samples ranged from 206 to 435 ng/g dw. Almost no data are available on NP concentrations in invertebrates, although previous studies have reported bioconcentration factors of 100-280 in marine shrimp (Ekelund et al., 1990; McCleese et al., 1981) and relatively high NP levels (300-450 ng/g ww) were detected in zebra mussel (*Dreissena polymorpha*) taken from polluted freshwater rivers in The Netherlands (Vethaak et al., 2002). Considering a similar bioconcentration factor for mysid shrimp, estimated maximum NP concentrations of 4 µg/l can be expected in water at the sampled site in this study, which would correspond with earlier measurements at nearby sites in the Scheldt estuary (Vethaak et al., 2002) and NP concentrations in water of UK estuaries (Blackburn et al., 1999). Similar NP water concentrations could be extrapolated when using the reported distribution coefficient (Kp) of 6-700 l/kg by Johnson et al. (1998).

*9.3.4. In vitro estrogenicity and androgenicity of water and sediment*

A number of *in vitro* assays have been developed to screen substances for their estrogenic and/or androgenic activity. These bioassays offer an integrated measure of the potencies of environmental mixtures without knowing all relevant compounds beforehand. In addition, these assays have the advantage of being relatively inexpensive, rapid and not requiring large amounts of sample material (Murk et al., 2002). In our study, two reporter recombinant yeast assays based on estrogenic and androgenic response were used, the yeast estrogen screen (YES) and yeast androgen screen (YAS), respectively (Routledge and Sumpter, 1996; Sohoni and Sumpter, 1998). The sediment and water-associated estrogenicity and androgenicity as determined with the YES and YAS are given in Table 9.2.

**Table 9.2.** Estradiol (EEF) and dihydrotestosterone (DEF) equivalency factors in extracts of water and sediment collected from the Scheldt estuary (The Netherlands) measured in the yeast estrogen (YES) and androgen screen (YAS)

| Sampling location[a] | water | | sediment | |
|---|---|---|---|---|
| | EEF[b] (pmol E2/l) | DEF[c] (pmol DHT/l) | EEF (pmol E2/g dw) | DEF (pmol DHT/g dw) |
| Schaar van Waarde | 5.03 | < dl[d] | 5.64 | < dl |
| Overloop van Valkenisse | 7.07 | < dl | < dl | < dl |
| Bath | 5.82 | < dl | 7.67 | < dl |

[a] Refer to Fig. 9.1 for sampling locations.
[b] EC50(17β-estradiol)/EC50(sample).
[c] EC50(dihydrotestosterone)/EC50(sample).
[d] < dl: below detection limit, i.e. no positive estrogenic or androgenic response were detected.

Acetone/hexane-soluble extracts of the sediments had an estrogenic potency ranging from below detection limit to 7.7 pmol E2/g dw, but all sediments had an androgenic potency below the detection limit of the YAS. The estrogenic equivalency factor in the water samples ranged from 5 to 7 pmol E2/l. Previous studies in Belgium, The Netherlands and Spain have reported estrogenic potencies as measured with the YES assay in surface waters from below detection limit to 412 pmol EEQ/l (Garcia-Reyero et al., 2001; Tanghe et al., 1999; Vethaak et al., 2002). Studies on the *in vitro* estrogenic and androgenic potency of water samples collected from UK estuaries, demonstrated estrogenic potencies of below detection limit to 86 pmol EEQ/l and androgenic potencies of below detection limit to 31 pmol DHT/l (Thomas et al., 2001; 2002). Data on sediment-associated estrogenicity are relatively scarce, although a recent study on 12 marine sediments from the Netherlands found estrogenic potencies

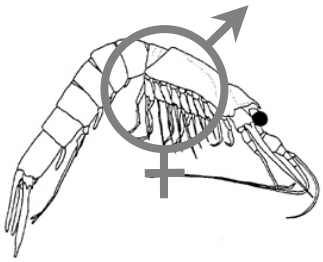
from 4.5–38.4 pmol EEQ/g dw as determined by a reporter gene assay (ER-CALUX) (Legler et al., 2002). However, Vethaak et al. (2002) did not identify high estrogenic activity in the Scheldt estuary, with an average of 0.06 pmol EEQ/l. High levels of androgenic activity were reported by Thomas et al. (2002) in 10 of 39 sediment samples from seven UK estuaries. Our study demonstrates the presence of chemicals with estrogenic potencies in water and sediment samples from the Scheldt estuary. Activities in water were relatively high compared to previously published data, whereas sediment-associated estrogenicity were in the same range. No detectable androgenic activity was observed in water and sediment of the Scheldt estuary, which is probably caused by the higher detection limit of the YAS assay as compared to the YES assay and/or the lower presence of androgen-active compounds in the aquatic environment. However, further research is needed to confirm these results. In general, caution must be exercised when comparing *in vitro* results from different studies using diverse sampling methods (e.g. unfiltered or filtered water) and different extraction or detection techniques (Vethaak et al., 2002).

Both the degradation products of APEs and PBDEs have shown estrogen agonism in the ER-CALUX assay (Legler et al., 2002; Meerts et al., 2001). The most potent of these chemicals in the ER-CALUX assay were BDE-100, NP and NPE (estrogenic equivalency factors from 3.8E-06 to 2.0E-05, relative to 17β-estradiol). Although the YES and ER-CALUX assay are clearly different in their sensitivity (Legler et al., 2002), the relatively high concentrations of BDE-100, NPEs and NP in sediments and mysids in our study could explain the observed estrogenic potencies in the YES. Similar, Thomas et al. (2001) suggested that industrially derived chemicals such as NP contribute to the *in vitro* estrogenic activity observed in industrialized UK estuaries. We are currently performing an in-depth study into the presence and distribution of a large number of known endocrine-disrupting chemicals in the Scheldt estuary (ENDIS-RISKS project, http://www.vliz.be/projects/endis) which will allow a better correlation between chemical analysis of water and sediment of the Scheldt estuary in relation to the observed estrogenic and androgenic potencies.

### 9.4. Conclusions

The present study reveals high concentrations of endocrine-disrupting chemicals such as flame retardants, organotins and surfactants in sediments of the Scheldt estuary and an important transfer of these target chemicals to the hyperbenthic mysid *Neomysis integer*. The presence of these compounds resulted in an elevated estrogenic potency in water and sediments. Current studies are focussing on measuring concentrations of a more comprehensive list of endocrine disruptors in mysids, water, sediment and suspended solids of the Scheldt estuary (ENDIS-RISKS project). The results of the present study demonstrate

warranted concern on potential population effects of endocrine-disrupting chemicals on the invertebrate population in the Scheldt estuary. While indications of changes in energy and steroid metabolism in *N. integer* of the Scheldt estuary have been observed (Verslycke et al., submitted-Chapter 10,11), we are currently investigating the long-term effects on the resident mysid populations. Overall, the present study indicates that more research is needed on the exposure of estuarine hyperbenthic invertebrates to endocrine disruptors which, due to their trophic position in these ecosystems, could give further insights into the presence, distribution, transfer and effects of these chemicals.



# CHAPTER 10

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# Energy metabolism in field populations of *Neomysis integer* of the Scheldt estuary

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Chapter

# 10

## *Energy metabolism in field populations of Neomysis integer of the Scheldt estuary*

**Abstract**

The seasonal and spatial patterns in cellular energy allocation of the estuarine mysid *Neomysis integer* (Leach, 1814) were investigated in the Scheldt estuary over a 2-year period. Using the recently developed cellular energy allocation (CEA) assay, energy reserves (protein, lipid and sugar) and energy consumption (as derived from the cellular respiration rate) were integrated into a general indicator of physiological stress. Total energy reserves were relatively unaffected by sampling season or location, whereas individual energy reserve fractions of *N. integer* were differentially influenced by sampling location and season. Seasonal effects were apparent for mysid weight and were related to the population biology, whereas spatial effects on the weight of *N. integer* may depend on pollution-induced effects on energy allocation in the two most upstream sites (Doel and Antwerp). These upstream sites coincide with the most polluted part of the sampled area and were characterized by a significant increase in energy consumption, resulting in a significantly lower CEA. Due to the recent amelioration in the oxygen concentration at these sites, it can be expected that *N. integer* will migrate further upstream, similar to what is observed in other European estuaries. It will, therefore, be important to assess the physiological consequences and potential population effects on mysids from these polluted areas in the Scheldt estuary. This study provides evidence that the CEA assay has potential under field conditions as an *in situ* biomarker of pollutant effects.

### 10.1. Introduction

Mysid crustaceans (Crustacea: Mysidacea) are shrimp-like animals which are distributed from 80°N to 80°S, and occur in most aquatic environments (Mauchline, 1980; Tattersall and Tattersall, 1951). Mysids are also frequently used in toxicity testing and there is growing interest in developing toxicity tests with mysids that are indigenous to local ecosystems (Roast et al., 1998a). In this context, *Neomysis integer* has been evaluated as a potential test organism for determining the effects of chemicals on northern European estuarine biota (Roast et al., 1998a, 1999a,b, 2000a,c, 2001a,b; Verslycke et al., 2002, 2003a,b,c, submitted-Chapter 5,8,9).

Baseline studies on the spatial and temporal patterns in the hyperbenthic component of the Scheldt estuary have identified the mysid *N. integer* as the dominant species in the brackish part of the Scheldt estuary (Mees and Hamerlynck, 1992; Mees et al., 1993a,b, 1994, 1995b). As a predator, *N. integer* can structure zooplankton populations and as a detrivore it can also affect the detrital chain (Fockedey and Mees, 1999; Mees et al., 1994). This species is also an important prey for demersal and pelagic fish and larger epibenthic crustaceans in the Scheldt estuary, and it is therefore believed to be a key species in the ecosystem of the brackish part of this estuary (Mauchline, 1980).

Estuaries and, in particular, the brackish part of these estuaries, are characterized by strong temporal and spatial fluctuations in the abiotic environment. In addition, many anthropogenic pollutants have the world's oceans and seas as a final sink, and are carried there through riverine and estuarine pipelines. As a result, many estuaries are heavily polluted with a variety of environmental contaminants. Animals which inhabit such extreme environments must be capable of maintaining normal metabolic function despite constant changes in their external environment (Oberdörster and Cheek, 2000). These adaptive mechanisms are energy-consuming and relatively little data is available on the combined effects of abiotic and pollutant stress factors on the energy metabolism of mysid shrimp. Growth, reproduction, and maintenance metabolism are the most important fractions of an organism's energy expenditure, so any increased maintenance requirement may result in reduced growth or reproduction (Huggett et al., 1992). Based upon this concept, several single integrated bioassays, such as 'scope for growth' (SFG) and 'cellular energy allocation' (CEA), which both provide rapid, instantaneous measurements of the energy status of an organism, have been developed (De Coen and Janssen, 1997; Verslycke and Janssen, 2002; Widdows and Salkeld, 1993). As with all energy-related biomarkers, these endpoints can be influenced by a large array of factors (e.g. diet, reproductive status, sex, age, location and season), hence, careful interpretation of energetics data is required. The key to the interpretation of field studies is to have a large database on the 'normal' condition of organisms and/or a good reference site for comparison.

The CEA assay, a recently developed bioassay of physiological status, has been used with *N. integer* to detect effects of changes in the abiotic environment (Verslycke and Janssen, 2002) and exposure to the antifoulant biocide tributyltinchloride under laboratory conditions (Verslycke et al., 2003c). The CEA assay has also recently been compared with the SFG assay in *N. integer* following laboratory exposure to the pesticide chlorpyrifos (Verslycke et al., submitted-Chapter 5). The present study, is a first attempt to investigate the potential *in situ* use of the CEA in indigenous *N. integer* of the Scheldt estuary. In this estuary, *N. integer* is exposed to a dynamic abiotic environment, but also to high concentrations of environmental pollutants, such as organotins, surfactants and flame retardants, resulting in high body burdens of these chemicals (Verslycke et al., submitted-Chapter 9). The potential interactive effects of these

environmental stresses on field populations have not been investigated in this species.

The goals of the present study were: (1) to investigate the potential use of the CEA assay as a research tool to study the interaction of toxic and abiotic stress factors in the Scheldt estuary, (2) to provide data relevant for the study of energy-flows in estuarine (benthic) food webs, and (3) to study the relation between population structure (related to season) and energy allocation in *N. integer*.

## 10.2. Material and Methods

### *10.2.1. Study area*

The river Scheldt takes its rise at Saint-Quentin in France about 350 km upstream of Vlissingen (The Netherlands) where the river discharges into the North Sea (Fig. 10.1).

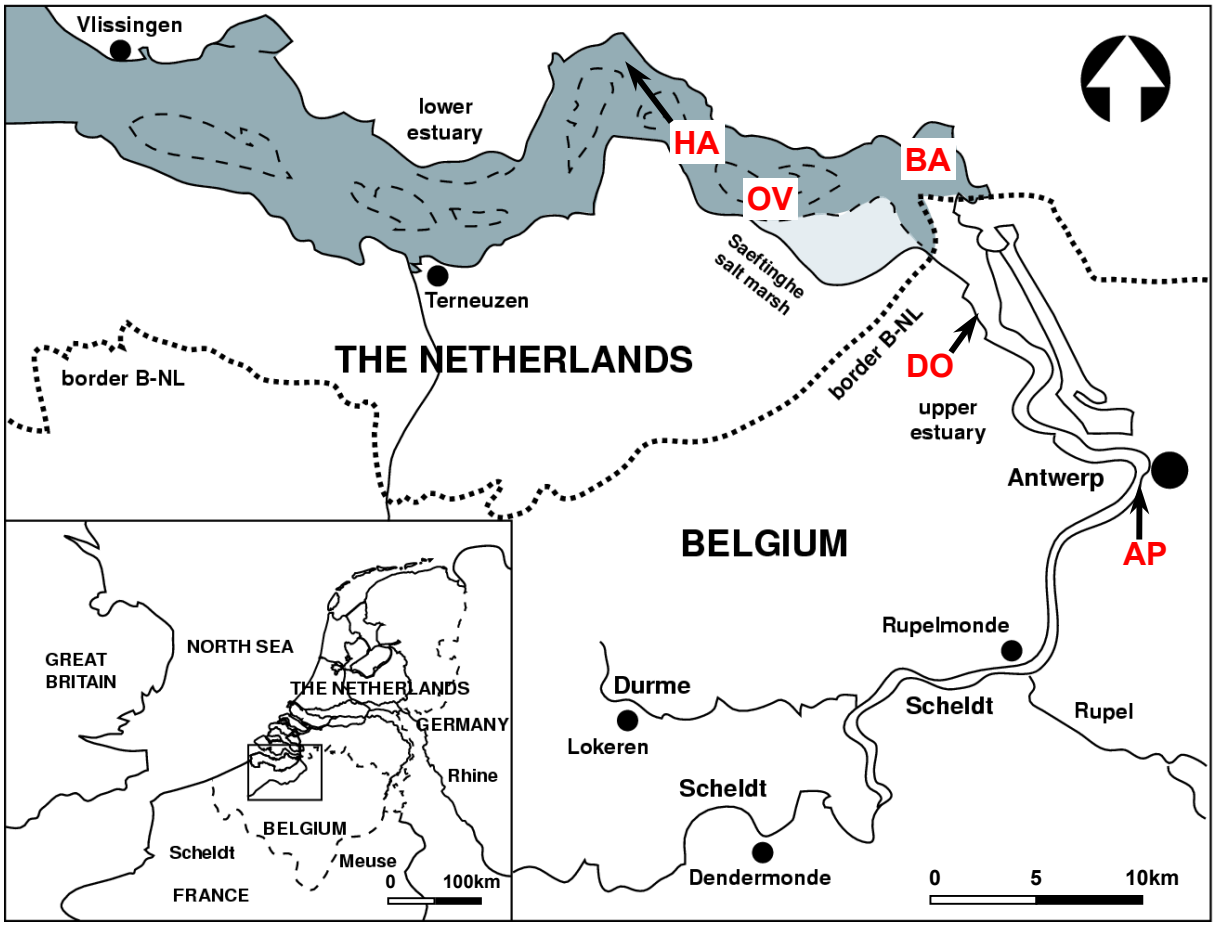



**Figure 10.1.** Map of the Scheldt estuary with location of the different sampling sites, representative of the major distribution area of the estuarine mysid *N. integer* (HA, Hansweert; OV, Overloop van Valkenisse; BA, Bath; DO, Doel; AP, Antwerp).

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. The Scheldt distinguishes itself from other estuaries by the fact that the relatively small average river discharge of 100 m³/s is strongly dominated by a large intertidal exchange volume of approximately 1 billion m³. The Scheldt estuary is, therefore, characterized as a long and well-mixed estuary with large intertidal areas and relatively stable salinity zones which are maintained in more or less the same position throughout a tidal cycle. Shifts in salinity zone distribution (over a few km) follow seasonal variations in freshwater inflow (Heip, 1989). River discharge is largely dependent on rainfall, and highest during winter (average 180 m³/s) and lowest in summer (60 m³/s) (Bayens et al., 1998). Maximum ebb and flood current velocities vary between 1 and 1.5 m/s at average neap and spring tides, respectively (Hostens, 2003). Dissolved oxygen concentration decreases sharply upstream of the Dutch-Belgian border and the riverine part is anoxic throughout most of the year (Herman et al., 1991). However, in the estuarine part of the Scheldt the water column is relatively well oxygenated, with concentrations of dissolved oxygen seasonally changing between 6 and 10 mg/l (Hostens, 2003). Turbidity is high, with $7.5 \times 10^5$ tons/year of fluvial fine sediments and $9 \times 10^4$ tons of marine suspended matter entering the system, which accumulate in the maximum turbidity zone, upstream of Antwerp. From an ecological point of view, the Scheldt estuary is one of the most important tidal river systems in Europe. It is an important passing, overwintering and feeding area for waterbirds and an important nursery for fish and shrimp. The Scheldt estuary was also ranked among the most polluted estuaries worldwide, based on contaminant concentrations in the dissolved as well as the particulate phase (Bayens et al., 1998; Duursma et al., 1988). The physical, chemical and biological characteristics of the Scheldt estuary are further discussed in Bayens et al. (1998), Heip (1988, 1989), Herman et al. (1991) and Van Eck et al. (1991).

### *10.2.2. Sampling*

Mysids were sampled during spring (March), summer (June) and winter (November-December) over a 2-year period (2001-2003), with a total of 7 campaigns. Sampling was done from the RV 'Zeeleeuw', the RV 'Scheldewacht', or the RV 'Belgica'. Each period, 3 stations were sampled (HA, OV and BA or DO). The samples from the BA site in the first sampling campaign were lost during handling (November 2001). During the last sampling campaign (March 2003), mysids were taken at one additional upstream site (AP) (Fig. 10.1). Mysid samples were collected with a hyperbenthic sledge (Hamerlynck and Mees, 1991), consisting of a metal frame equipped with two mounted nets, one above the other (Fig. 10.2). The nets were 4 m long and 1 m wide with a mesh size of 2 × 2 mm in the first 3 m and 1 × 1 mm in the last 1 m. The sledge was trawled over the bottom in front of the tidal current,

sampling the water column from 20 to 100 cm, over a distance of 1000 m (GPS readings from fixed points) at an average ship speed of approximately 2.3 m/s (4.5 knots). The total area sampled on each occasion was approximately 1000 m². The catch of the two nets were pooled for this study. All samples were taken during daytime when hyperbenthic animals are known to be concentrated near the bottom. Adult *Neomysis integer* specimens were sorted out on board, sex was not taken into account and no selection was made to avoid gravid females. The collected mysids were shock-frozen in liquid nitrogen, and kept at -80°C until analysis of the different CEA parameters. The two last sampling campaigns (December 2002, March 2003) were performed with a different hyperbenthic sledge of 301 cm long, 169 cm wide and 137 cm high (Fig. 10.2).

**Figure 10.2.** The hyperbenthic sledges used for the sampling of mysid shrimp in the Scheldt estuary. Left: the sledge operated from the RV 'Zeeleeuw' (inlay) during the first 5 campaigns (March-June-November 2001, March-June 2002). Right: the sledge operated from the RV 'Belgica' (inlay) during the last 2 campaigns (December 2002 and March 2003).

Two pairs of nets (71 cm wide, 3 m long) were mounted on the sledge next to each other. The mesh sizes of the two superimposed nets were 1 × 1 mm. Each net 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 to escape by swimming back or getting damaged by the strong flow. The collectors each have lateral openings covered with 0.5 mm gaze. An opening-closing mechanism automatically operates when touching the bottom, preventing contamination of the catch by upper water strata, and sampling the first 100 cm above the sea bottom. Salinity, dissolved oxygen concentrations and temperature were measured at all sites with a Sea-Bird SBE21 (Sea-Bird Electronics, Bellevue, WA, USA) thermosalinograph and a Sea-Bird SBE19 'SeaCat' CTD profiler (Table 10.1).

**Table 10.1.** Environmental variables, averaged per sampling season, during the period 2001-2003 for the different sampling sites (see Fig. 10.1) in the Scheldt estuary

| Parameter | Station | Spring[a] | Summer[a] | Winter[a] |
|---|---|---|---|---|
| Temperature (°C) | HA | 7.1 ± 0.4 | 16.9 ± 0.1 | 11.1 ± 3.0 |
| | OV | 7.8 ± 0.4 | 17.3 ± 0.2 | 11.3 ± 3.3 |
| | BA | 8.2 ± 0.3 | 18.3 ± 0.2 | 11.9 ± 2.9 |
| | DO | 8.9 ± 0.1 | 18.6 ± 0.1 | 12.4 ± 3.5 |
| | AP | 8.7 | NA[b] | 8.7 |
| Salinity (‰) | HA | 15.3 ± 0.4 | 20.3 | 18.4 |
| | OV | 8.0 ± 2.8 | 9.8 | 12.8 |
| | BA | 5.5 ± 0.7 | 8.6 ± 1.2 | NA |
| | DO | 3.2 | NA | 3.3 |
| | AP | 0.6 | NA | NA |
| Dissolved oxygen (mg/l) | HA | 10.2 ± 1.7 | 8.1 ± 1.2 | 8.7 ± 1.0 |
| | OV | 8.1 ± 2.3 | 7.4 ± 1.5 | 9.1 |
| | BA | 6.9 ± 1.0 | 6.5 ± 0.9 | 6.2 ± 0.3 |
| | DO | 6.6 | NA | 4.2 |
| | AP | 4.8 | NA | 2.6 |

[a] Data are shown as mean of the sampling season ± standard deviation (where possible).
[b] NA; not available.

*10.2.3. Cellular energy allocation assay*

The CEA was performed as described in Chapter 5 (§ 5.2.4). The $E_a$, $E_c$ and CEA value were calculated as follows:

$E_a$ (available energy) = sugar + lipid + protein (mJ/mg ww)

$E_c$ (energy consumption) = ETS activity (mJ/mg ww/h)

CEA (cellular energy allocation) = $E_a/E_c$

From this calculation, it can be deduced that a decline in CEA indicates either a reduction in available energy or a higher energy expenditure, both resulting in a lower amount of energy available for growth or reproduction. Ten replicate measurements of lipid, sugar and protein content and ETS activity were performed for each sampling site and period. For wet weight measurements, mysids were blotted dry and weighed with an analytical balance (± 0.1 mg). The dry weight of *N. integer* collected from the Scheldt estuary is typically around 14-17% of the wet weight (Verslycke et al., submitted-Chapter 9).

*10.2.4. Statistical analysis*

All analyses were done with the software package Statistica™ (Statsoft, Tulsa, OK, USA). Data were checked for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene's test, respectively, with an $\alpha$ of 0.05. Data were log-transformed if they did not meet with the above criteria. The effects of location and season on the energy allocation in mysids were examined statistically by 1- and 2-way analysis of variance (ANOVA) on the means of the different campaigns. Effects of location were also examined for each campaign individually. Where significant *F*-ratios were calculated by ANOVA, Tukey's Honestly Significant Difference (HSD) test was applied to identify which datasets were different. All box-plots were created with Statistica™ and show the mean (small square), standard error (box), and the standard deviation (whisker).

## 10.3. Results

*10.3.1. Weight and energy content*

The average wet weight (ww) of the collected *Neomysis integer* (n = 881) was 22.3 ± 11.0 mg ww. The weight of the collected animals was significantly dependent on the season (ANOVA, *F*-ratio = 159, df = 2, $p < 0.001$) and the sampling location (ANOVA, *F*-ratio = 6.1, df = 4, $p < 0.001$) (Fig. 10.3). Animals collected in spring (mean ww of 27.8 ± 11.1 mg) weighed significantly more than animals collected in summer (21.3 ± 8.4 mg) and winter (14.4 ± 6.5 mg). In addition, the weight of mysids collected at the most upstream site (AP) was significantly lower than the average seasonal weight of mysids collected at the other sites. When only considering the weight of the mysids sampled during the last campaign (March 2003), a significant weight reduction was observed for upstream collected mysids (significantly lower weights in DO and AP sites; data not shown).

The average total energy content ($E_a$) of *N. integer* of the Scheldt estuary was 3173 ± 888 mJ/mg ww (n = 220). The $E_a$ was significantly affected by location (ANOVA, *F*-ratio = 7.2, df = 4, $p < 0.001$), but not by season (ANOVA, *F*-ratio = 1.3, df = 2, $p = 0.28$), indicating spatial differences in total energy content of *N. integer* (Fig. 10.3). Mysids collected at the OV site had a significantly smaller $E_a$ than animals occuring at the HA and BA site.

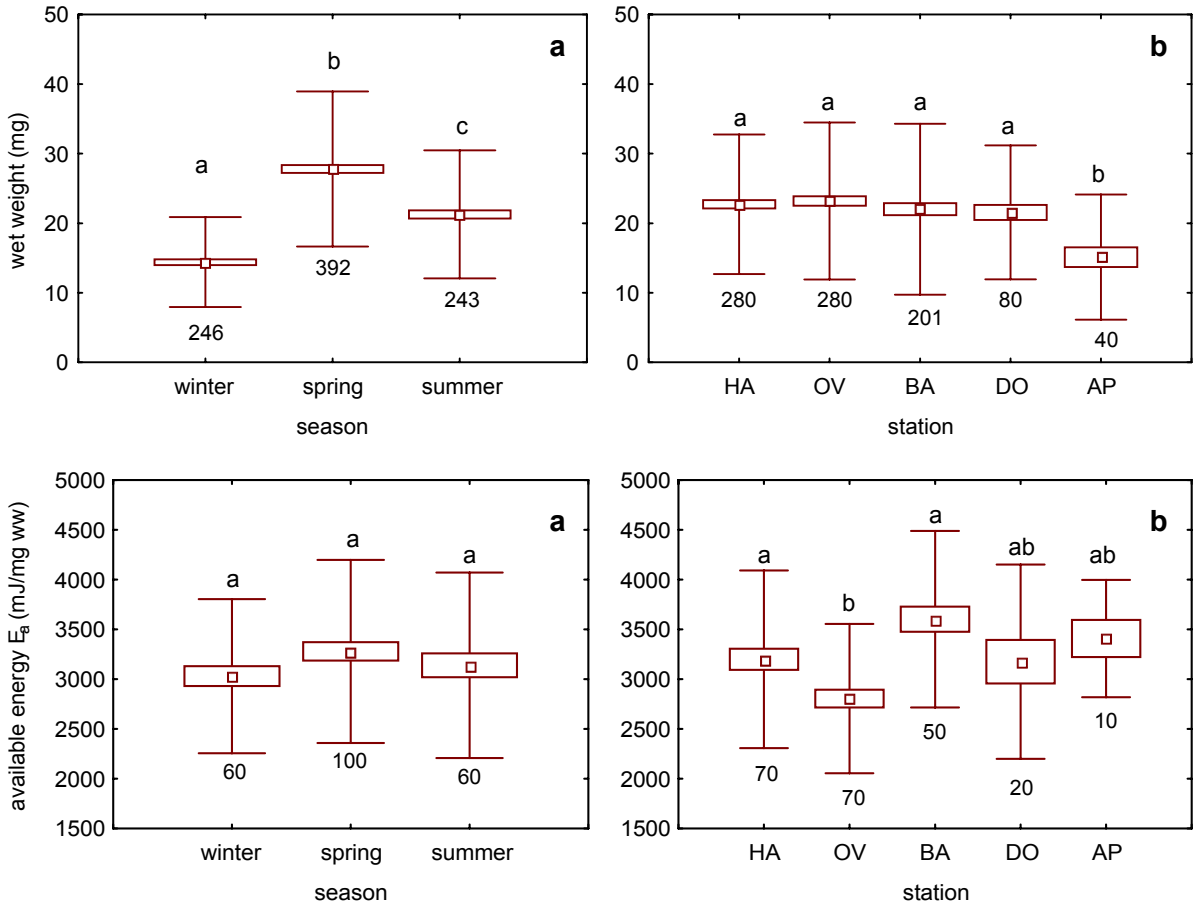



**Figure 10.3.** Seasonal (a) and spatial (b) patterns in wet weight (ww) and total energy content ($E_a$) of *N. integer* in the Scheldt estuary. Different letters indicate significant differences ($p < 0.05$). Number below the whisker indicates the number of observations. Refer to Fig. 10.1 for sampling locations.

### *10.3.2. Energy composition*

Sugar, lipid and protein content of *Neomysis integer* were determined (Table 10.2). Averaged over all sampled mysids, protein was the most important fraction (69.3 ± 23.4 µg protein/mg ww), followed by lipid (37.4 ± 18.4 µg lipid/mg ww), and sugar (1.9 ± 1.2 µg sugar/mg ww). By transforming these energy reserve fractions into energetic equivalents using their respective energy of combustion as described in Chapter 3 (§ 3.2.4), it can be derived that protein and lipid are quantitatively most important as energy sources (Fig. 10.4). Sugar, on the other hand, appears to be quantitatively less important as an energy source (1-2% of total energy content) in mysids.

The individual energy reserve fractions of *N. integer* were differentially affected by sampling location and season (Fig. 10.5). The average sugar content of *N. integer* in this

study was not significantly different between sampling locations (ANOVA, *F*-ratio = 0.9, df = 4, p = 0.44), but was significantly lower in winter compared to spring and summer (HSD, p < 0.05) (Fig. 10.5c). Average protein concentrations in mysids sampled at the OV site were significantly lower compared to these of mysids from the HA and BA site. Protein concentrations were not affected by season (ANOVA, *F*-ratio = 2.1, df = 2, p = 0.12) (Fig. 10.5a), whereas lipid concentrations were lower in summer compared to spring (HSD, p < 0.05). Interestingly, the average lipid content of mysids of all campaigns increased linearly when sampling from downstream to upstream locations (Fig. 10.5b).

In conclusion, although seasonal effects on the individual energy reserve fractions were obvious (Fig. 10.4-5), these did not result in a seasonal effect on total amount of energy available ($E_a$) in *N. integer* (Fig. 10.3a). On the other hand, significant spatial effects on $E_a$ were observed (Fig. 10.3b).

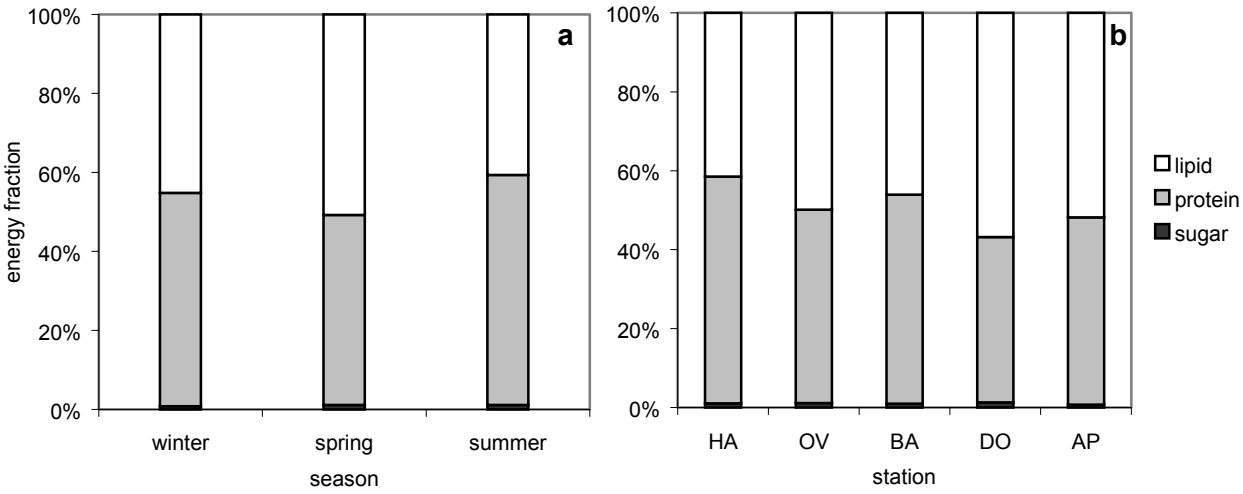



**Figure 10.4.** Seasonal (a) and spatial (b) patterns in energy composition of *N. integer* in the Scheldt estuary. Energy fractions are shown as percentage of the total energy content. Refer to Fig. 10.1 for sampling locations.

*10.3.3. Energy consumption*

Energy consumption ($E_c$) in mysids was calculated from the *in vitro* electron transport system (ETS) activity (measured as µl $O_2$/mg ww/h) and converted into energy equivalents as described in Chapter 3 (§ 3.2.4) (Table 10.2). The average $E_c$ in all sampled mysids was 30.9 ± 16.9 mJ/mg ww, and the $E_c$ was unaffected by season (ANOVA, *F*-ratio = 2.3, df = 2, p = 0.10) (Fig. 6a). However, the $E_c$ was significantly affected by location (ANOVA, *F*-ratio = 4.3, df = 4, p < 0.01) and increased in the more upstream sampling locations (significantly higher in the AP site) (Fig. 10.6b).

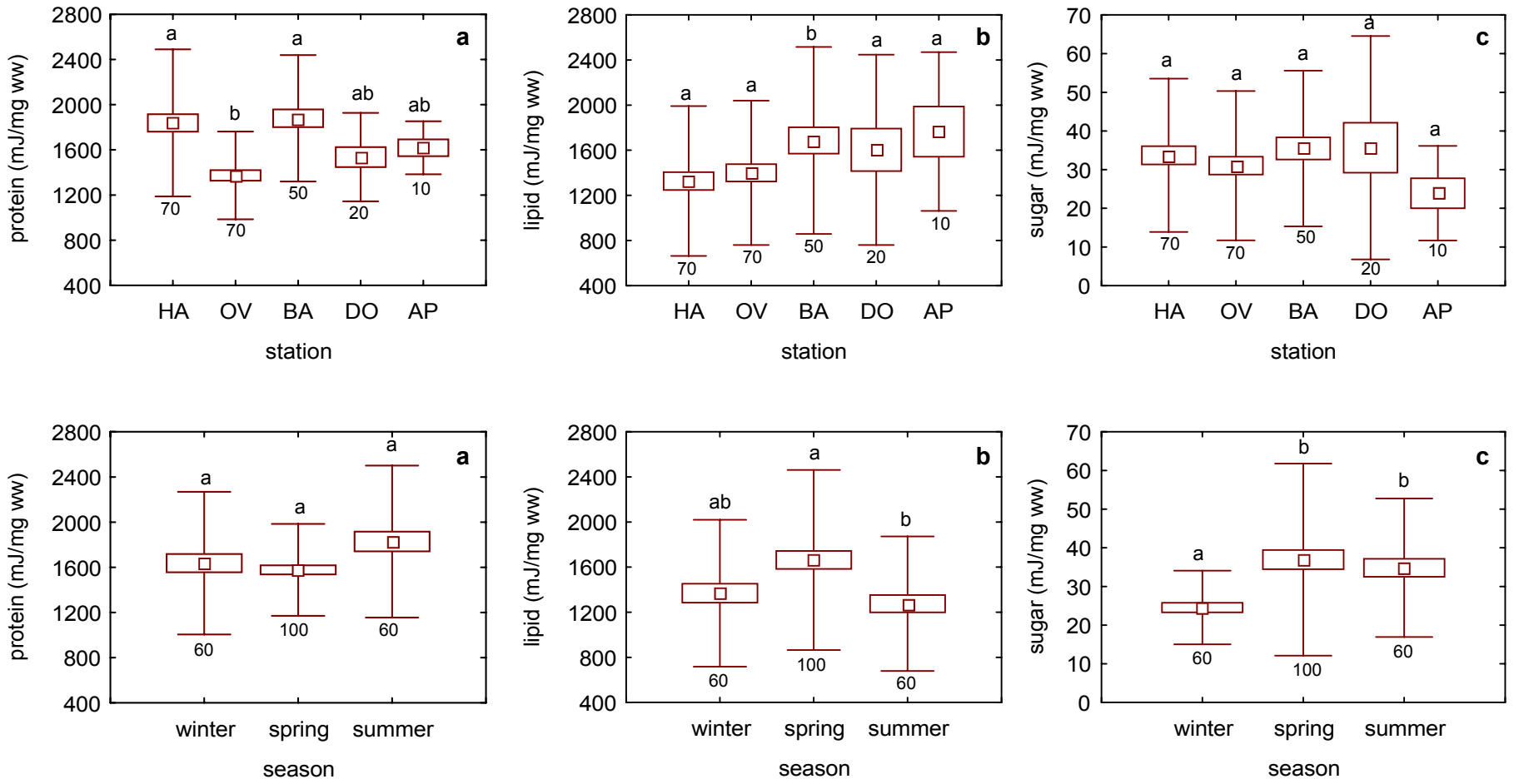



**Figure 10.5.** Seasonal (bottom) and spatial (top) patterns in protein (a), lipid (b) and sugar (c) content of *N. integer* in the Scheldt estuary. Different letters indicate significant differences ($p < 0.05$). Number below the whisker indicates the number of observations. Refer to Fig. 10.1 for sampling locations.

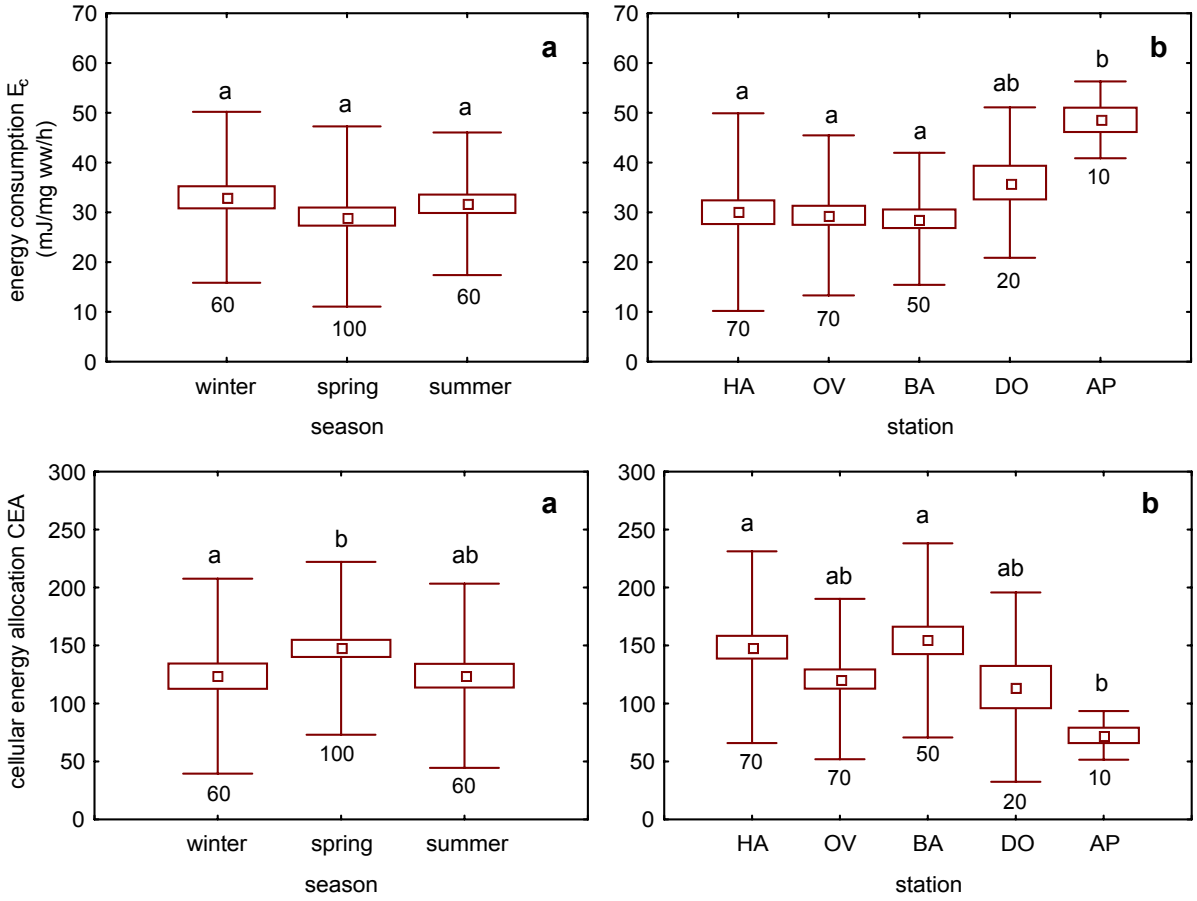



**Figure 10.6.** Seasonal (a) and spatial (b) patterns in energy consumption ($E_c$) and cellular energy allocation (CEA) of *N. integer* in the Scheldt estuary. Different letters indicate significant differences ($p < 0.05$). Number below the whisker indicates the number of observations. Refer to Fig. 10.1 for sampling locations.

### *10.3.4. Cellular energy allocation*

The cellular energy allocation (CEA), of *Neomysis integer* was calculated as the ratio of the amount of available energy ($E_a$) and the energy consumption ($E_c$) (Table 10.2). The CEA in *N. integer* was significantly affected by season, with mysids from the spring season allocating more energy to their energy reserves than these from the summer (HSD, $p = 0.08$) and winter season (HSD, $p < 0.05$) (Fig. 10.6a). However, the effects of sampling location on the variation in CEA were stronger than these resulting from differences in sampling season (2-way ANOVA), indicating important spatial differences in energy allocation. More energy was allocated towards energy consumption in the upstream sampling sites, especially in Doel (DO) and Antwerp (AP) (Fig. 10.6b). Consequently, the CEA at the AP site was significantly lower compared to the CEA of the HA and BA sites. If we considered HA (the most downstream and least polluted site) as the control site, than all sites except BA, had a significantly lower CEA (Dunnett's with HA as control, $p < 0.05$).

**Table 10.2.** Spatial (refer to Fig. 10.1 for locations ) and seasonal variation in cellular energy allocation in *N. integer* of the Scheldt estuary

| Date | Station | Cellular energy allocation | | | | | |
|---|---|---|---|---|---|---|---|
| | | Sugar reserve (mJ/mg ww) | Protein reserve (mJ/mg ww) | Lipid reserve (mJ/mg ww) | $E_a$ (mJ/mg ww) | $E_c$ (mJ/mg ww/h) | CEA |
| 15/03/01 | HA | 21.0 ± 4.4$^{a}$ | 1676.4 ± 361.0$^{a}$ | 898.5 ± 428.0$^{a}$ | 2595.9 ± 559.9$^{a}$ | 12.6 ± 1.4$^{a}$ | 205.9 ± 49.9$^{a}$ |
| | OV | 32.7 ± 14.8$^{b}$ | 1517.9 ± 337.9$^{a}$ | 1277.4 ± 632.3$^{a}$ | 2828.0 ± 717.1$^{a}$ | 13.7 ± 2.9$^{a}$ | 212.6 ± 70.1$^{a}$ |
| 14-15/6/01 | HA | 57.7 ± 24.5$^{a}$ | 2478.6 ± 666.9$^{a}$ | 1040.2 ± 178.9$^{a}$ | 3576.5 ± 690.9$^{a}$ | 47.2 ± 12.8$^{a}$ | 79.4 ± 26.5$^{a}$ |
| | OV | 37.0 ± 9.8$^{a}$ | 1692.6 ± 202.5$^{b}$ | 914.7 ± 321.0$^{a}$ | 2644.2 ± 379.7$^{b}$ | 23.7 ± 12.4$^{b}$ | 148.0 ± 80.4$^{a}$ |
| | BA | 34.4 ± 15.9$^{b}$ | 2462.7 ± 456.0$^{a}$ | 1700.0 ± 942.3$^{b}$ | 4197.1 ± 1047.0$^{a}$ | 31.2 ± 13.8$^{ab}$ | 171.4 ± 87.1$^{b}$ |
| 6-7/11/01 | HA | 24.8 ± 6.8$^{a}$ | 2325.0 ± 754.0$^{a}$ | 1025.9 ± 332.0$^{a}$ | 3375.8 ± 823.9$^{a}$ | 31.4 ± 25.3$^{a}$ | 161.8 ± 136.5$^{a}$ |
| | OV | 22.0 ± 8.4$^{a}$ | 1413.7 ± 519.6$^{ab}$ | 1466.6 ± 419.7$^{b}$ | 2902.3 ± 668.0$^{a}$ | 40.8 ± 11.7$^{a}$ | 73.9 ± 27.2$^{b}$ |
| | BA | 20.5 ± 9.3$^{a}$ | 2165.8 ± 447.8$^{a}$ | 1028.6 ± 112.2$^{a}$ | 3214.9 ± 461.7$^{a}$ | 41.7 ± 15.2$^{a}$ | 86.0 ± 33.6$^{a}$ |
| 4-5/3/02 | HA | 52.2 ± 16.3$^{a}$ | 2266.4 ± 460.9$^{a}$ | 1938.0 ± 652.4$^{a}$ | 4256.6 ± 798.9$^{a}$ | 50.9 ± 16.2$^{a}$ | 89.6 ± 33.1$^{a}$ |
| | OV | 65.6 ± 20.0$^{a}$ | 1598.4 ± 257.8$^{b}$ | 2026.6 ± 624.0$^{a}$ | 3690.6 ± 675.5$^{a}$ | 51.1 ± 21.1$^{a}$ | 83.9 ± 37.9$^{a}$ |
| | BA | 65.8 ± 13.8$^{a}$ | 1564.0 ± 348.6$^{b}$ | 2162.9 ± 471.3$^{a}$ | 3792.7 ± 586.4$^{a}$ | 23.5 ± 7.7$^{b}$ | 177.0 ± 64.4$^{b}$ |
| 5/6/02 | HA | 33.5 ± 12.3$^{a}$ | 1570.6 ± 284.6$^{a}$ | 1346.4 ± 554.3$^{a}$ | 2950.5 ± 623.2$^{a}$ | 37.1 ± 14.3$^{a}$ | 92.4 ± 40.5$^{ab}$ |
| | OV | 19.8 ± 5.3$^{b}$ | 903.0 ± 139.6$^{b}$ | 1224.0 ± 311.9$^{a}$ | 2146.8 ± 341.8$^{b}$ | 27.0 ± 8.3$^{ab}$ | 89.0 ± 30.7$^{a}$ |
| | BA | 26.7 ± 8.4$^{a}$ | 1864.2 ± 461.1$^{a}$ | 1429.3 ± 680.6$^{a}$ | 3320.3 ± 822.1$^{a}$ | 24.2 ± 10.5$^{b}$ | 164.0 ± 81.9$^{b}$ |
| 4/12/02 | HA | 33.5 ± 12.3$^{a}$ | 1397.7 ± 189.2$^{a}$ | 1972.5 ± 958.9$^{a}$ | 3403.6 ± 977.4$^{a}$ | 14.3 ± 4.0$^{a}$ | 249.9 ± 100.5$^{a}$ |
| | OV | 26.7 ± 8.4$^{a}$ | 1085.2 ± 180.4$^{a}$ | 1726.0 ± 724.1$^{a}$ | 2837.9 ± 769.9$^{a}$ | 27.8 ± 7.0$^{b}$ | 107.7 ± 37.2$^{b}$ |
| | DO | 19.8 ± 5.3$^{b}$ | 1436.7 ± 409.4$^{a}$ | 991.1 ± 244.3$^{b}$ | 2447.6 ± 476.8$^{b}$ | 42.1 ± 14.0$^{c}$ | 62.7 ± 24.1$^{c}$ |
| 18-19/3/03 | HA | 13.2 ± 5.6$^{a}$ | 1154.4 ± 103.7$^{a}$ | 1068.7 ± 242.2$^{a}$ | 2236.3 ± 263.6$^{a}$ | 16.9 ± 8.0$^{a}$ | 161.3 ± 78.5$^{a}$ |
| | OV | 13.4 ± 2.2$^{a}$ | 1406.2 ± 282.2$^{a}$ | 1165.4 ± 716.4$^{a}$ | 2585.0 ± 770.0$^{a}$ | 21.7 ± 8.0$^{ab}$ | 133.1 ± 63.1$^{a}$ |
| | BA | 30.0 ± 14.7$^{bc}$ | 1340.9 ± 236.2$^{a}$ | 2115.5 ± 1070.3$^{b}$ | 3486.4 ± 1096.1$^{b}$ | 23.0 ± 9.2$^{ab}$ | 174.1 ± 88.6$^{a}$ |
| | DO | 51.5 ± 34.2$^{bc}$ | 1635.5 ± 365.5$^{b}$ | 2216.8 ± 779.8$^{b}$ | 3903.8 ± 861.9$^{b}$ | 29.9 ± 14.3$^{b}$ | 165.6 ± 87.1$^{a}$ |
| | AP | 23.9 ± 12.2$^{ab}$ | 1618.6 ± 234.1$^{b}$ | 1766.1 ± 703.5$^{ab}$ | 3408.6 ± 741.6$^{b}$ | 48.6 ± 7.7$^{c}$ | 72.6 ± 19.5$^{b}$ |

Values within one sampling campaign not followed by the same letter are significantly different ($p < 0.05$). All data are given as mean ± standard deviation.

## 10.4. Discussion

### *10.4.1. Environmental variables and spatial community patterns*

The temperature in the water column was most strongly influenced by season (Table 10.1) and varied between 6.7°C and 18.6°C with lower average temperatures in spring (average over all stations: 8.0 ± 0.7°C), than in winter (11.3 ± 2.5°C) and summer (17.7 ± 0.8°C). Temperature always increased upstream, with a maximal difference of 2°C between the most upstream (AP) and downstream (HA) sites. A maximum temperature difference of 3°C between the mouth of the estuary and the 8‰ isohaline (around the DO site) was previously found in the Scheldt estuary (Mees et al., 1995). Salinity zones are relatively stable in the Scheldt estuary (Heip, 1989), but seasonal variations do occur due to seasonal differences in freshwater inflow, which are largely dependent on rainfall. On average, salinity was lower during the spring campaigns compared to summer and winter campaigns, which corroborates the findings of similar studies in the Scheldt estuary (Hostens, 2000; Mees et al., 1994). Dissolved oxygen concentration averaged 7.6 ± 1.9 mg/l but dropped well below saturation values upstream of the BA site, with very low values recorded at the AP site (2.6 mg/l or 22.7% saturation in December 2002). Better wastewater treatment have led to reductions in the nutrient load of the Scheldt estuary over the last 30 years resulting in significant higher dissolved oxygen concentrations in the more upstream sites, from around 2 mg/l in 1970 to values of more than 6 mg/l in recent years (values for the DO site as taken from http://www.waterbase.nl). It is expected that dissolved oxygen concentrations will continue to increase with new wastewater treatment plants treating the wastewater from the city of Brussels, which enters the Scheldt estuary via the river Rupel.

In the Scheldt estuary, *Neomysis integer* are always concentrated near the limit of viable oxygen concentration (about 40% of the saturation value) regardless of salinity (Mees et al., 1993a,b). Due to the low dissolved oxygen concentrations upstream of BA, *N. integer* was never found beyond this site in the period 1988-1991 (Mees et al., 1993a,b, 1994). In this period, the low salinity hyperbenthic community was completely absent in the Scheldt estuary, which was markedly different from other European estuaries such as the Eems (N. Netherlands) and the Gironde (S-W. France) (Mees et al., 1995b). Furthermore, *N. integer* occurred in much higher densities in the Scheldt estuary (242 ind/m² and 129 ind/m² at 19‰ and 10‰, respectively) than it did in these other estuaries (30 ind/m² and 33 ind/m², for Eems and Gironde at 4‰ and 2‰, respectively). This suggests a spatial compression of the population between critically low oxygen concentrations on the freshwater side (which have, since the time of the Mees et al. studies, significantly increased) and perhaps species better adapted to higher salinities on the seaward side (Mees et al., 1995b). The presence of *N. integer* at the DO and

AP site in the present study corroborates the fact that increased oxygen concentrations result in an upstream shift of the *N. integer* population. Since these sites are also more polluted (Verslycke et al., submitted-Chapter 9), these populations are at risk of potential adverse effects on growth and/or reproduction. We are presently looking at the population structure of *N. integer* in the scope of the ENDIS-RISKS project (http://www.vliz.be/projects/endis) to investigate potential changes in this population, as compared to the 1988-1991 situation.

### *10.4.2. Weight and energy content*

The *Neomysis integer* population of the Scheldt estuary consists of three annual cohorts (Mees et al., 1994). The overwintering generation lives from autumn until the following spring. The spring generation is born in early spring and lives for about 3 months, while the summer generation lives from summer until early winter. These 3 cohorts show a marked difference in their biology. The seasonal patterns in weight of the collected animals in our study correlate well with the growth curves of the 3 cohorts as described in Mees et al. (1994). Similar to their conclusions, we found the largest individuals in the spring cohort (27.8 ± 11.1 mg ww), and smaller individuals in summer (21.3 ± 8.4 mg ww) and winter (14.4 ± 6.5 mg ww). However, it should be noted that a non-randomized selection procedure was used for the collection of mysids in our study, i.e. only adult mysids were used, the sex was not taken into account and no selection was made to avoid gravid females. As such, the reported values are not representative for the entire population, but represent the average weight of the adult fraction of the population.

Since it has been demonstrated that incubation period, post-marsupial development time, and intermolt period are all highly temperature-dependent, temperature is most probably the driving factor behind the growth-based cohort separation as observed in different estuaries (Astthorsson & Ralph, 1984; Kuhlmann, 1984; Mauchline, 1985; Winkler and Greve, 2002). The fact that summer generation mysids will achieve sexual maturity faster than early or late season populations (through temperature-dependent induction in the molt frequency), will also have important consequences for the energy allocation in these different cohorts. For instance, reproduction is completely halted in winter, which would allow mysids to allocate more energy towards growth. Indeed, a higher total energy content was observed in mysids of the spring generation compared with summer and winter mysids, but this effect was not significant (Fig. 10.3a).

Significant spatial differences were observed in the total energy content ($E_a$) of mysids (Fig. 10.3b). Mysids from the OV site had a significantly lower $E_a$, mainly due to their low protein content (Fig. 10.5a). The reason for this effect remains unclear, but might be explained by the lateral seasonal migration of mysids at this site. Mysids at the OV site are

known to migrate onto the salt marsh of Saeftinghe in summer and autumn for reproduction, situated on the left bank north of the harbor of Antwerp (Mees et al., 1993a) (Fig. 10.1). The other sites in our study are typically representative of the subtidal channels, where lateral migration is not observed. It might, therefore, be hypothesized that the difference in energy composition (i.e. a lower protein content) between the OV and the other sites are a reflection of this tidal migration onto the Saeftinghe salt marsh during these periods. This would be supported by the protein data depicted in Table 10.2 for the OV site, which were most strongly decreased in the two summer and two autumn campaigns in comparison with the three spring campaigns. In fact, no significantly lower protein content was observed in mysids from the OV site when only considering the spring data. However, longer time-series are required to demonstrate whether this is a true effect and what the causal reasons might be. Generally, the $E_a$ of *N. integer* was remarkably stable, independent of season and location (except the lower $E_a$ in the OV site).

### *10.4.3. Energy composition*

Although the total energy content was relatively unaffected, distinct differences in energy composition were observed between mysids collected during different seasons (Fig. 10.4a). Generally, the relative lipid content was higher in the overwintering generation (measured in spring), which could be the result of the stop in reproduction in *Neomysis integer* (Mees et al., 1994), and thus a lower demand for lipids necessary for gamete production in mysids (Linford 1965). On the other hand, it has been demonstrated that adult mysids have a significantly lower O:N ratio compared to developing juveniles, suggesting a shift to more proteinaceous substrates during the maturation process (McKenney, 1998), which would also result in a higher relative lipid content. Other investigators have analyzed oxygen to nitrogen ratios and have described the metabolism of mysids as protein based (Chin, 1974; Fergusson, 1973; Gaudy et al., 1980). In our study, however, no significant seasonal effects were observed on protein allocation (Fig. 10.5a).

Sugar content, which was quantitatively the smallest fraction (1-2% of $E_a$), was significantly lower in winter (Fig. 10.5c). Glucose can deliver fast energy in the form of ATP, via the process of glycolysis and oxidative phosphorylation and is the major circulating carbohydrate in crustaceans (Morris and Airriess, 1998). The seasonal effects on sugar, might be a reflection of increased energy demands since energy consumption derived from ETS activity was indeed higher in winter (33.0 ± 17.2 mJ/mg ww/h), then in summer (31.7 ± 14.3 mJ/mg ww/h) and spring (29.2 ± 18.1 mJ/mg ww/h), however, this effect was not significant. Certainly other seasonally dependent environmental variables, such as food availability and food composition, could also affect energy metabolism. However, these effects could not be

assessed in the present study. Raymont et al. (1966) reported significant decreases in carbohydrate content (November, December and January) and lipid content (November, January, and March), but not in protein content and total energy content in *N. integer* collected from the River Test (Southampton, England). These authors hypothesized that the decrease in lipid content in March may be associated with the utilization of lipid reserves in the development of the eggs in the marsupium at time when phytoplankton food is not abundant. Since our data were not a continuous recording over an entire year, it is difficult to interpret seasonal changes, since processes related to reproduction in mysids, for instance, vary over a period of a few weeks, and may thus be missed when only sampling three times a year. It would, therefore, be interesting to investigate changes in protein, lipid and sugar allocation in *N. integer* over an entire life cycle, or indeed during an entire year. In such a study, it would be worthwhile to look at age- and sex-specific effects.

Different spatial patterns in protein and sugar content were observed (Fig. 10.5a,c). Lipid content in *N. integer* decreased towards the estuary mouth (Fig. 10.5b). Protein content was variable, which corroborates a previous laboratory study by Verslycke and Janssen (2002), investigating the effects of a variable environment on energy allocation in *N. integer* (Fig. 10.5a). Sugar content was lower in the most upstream site (AP), but this was not statistically significant (Fig. 10.5c). Many pollutants have an impact on carbohydrate metabolism, which generally results in an increase in glycolytic activity (De Coen et al., 2001). Hyperglycemic responses and subsequent mobilization of stored glycogen reserves have been observed in many invertebrates exposed to numerous pollutants, such as DDT, fenitrothion, pentachlorophenol, etc. (Huggett et al., 1992). In the sampled region of the Scheldt estuary in our study, the main input of environmental pollutants comes from the harbor of Antwerp and via the tributaries of the Scheldt river, upstream of Antwerp (Verslycke et al., submitted-Chapter 9). Potential effects on energy allocation in *N. integer*, are thus expected to be more severe in the most upstream locations, which are characterized by significantly higher chemical body burdens as compared with more downstream locations (Verslycke et al., submitted-Chapter 9). As suggested by Huggett et al. (1992), effects on glucose, protein or lipid can be theoretically predictive of higher level effects, but the influence of accessory factors, present under field conditions often hinders the interpretation of these biomarkers relative to toxicant-induced stress. It is, therefore, essential to establish baseline conditions for glucose, lipid and protein in *N. integer* under field conditions, if these are to be used in the future as biomarkers of exposure to toxicants.

### *10.4.4. Energy consumption*

Although small seasonal effects on energy consumption were observed, i.e. spring

mysids consumed less energy than summer and winter mysids, these effects were not statistically significant (Fig. 10.6a). The respiratory physiology of *Neomysis integer* is adapted to an environment which is subject to frequent and reciprocal fluctuations in temperature and salinity (Roast et al., 1999b). Roast et al. (1999b) predicted that oxygen consumption would increase in mysids of the East Looe River estuary (Cornwall, England) in summer when water temperature increases, or, alternatively, that *N. integer* becomes acclimated to constant temperatures, since such seasonal temperature changes occur over a long period, which would result in a minimal effect on oxygen consumption. Our study corroborates the latter hypothesis, since no significant seasonal effects on oxygen consumption were observed. This is in contrast with the findings of Garnacho et al. (2001) who found that the mysid *Praunus flexuosus* from the West Solent (S. England) showed different metabolic rates between seasons. Respiration rates were two to three times lower in winter at 5°C than in summer at 20°C. Similar results were reported for *N. integer* from the River Test (Southampton, England) by Raymont et al. (1966). Clearly, more research is needed to fully understand the potential adaptive respiratory physiology of mysids under field conditions.

A significant upstream increase in energy consumption was observed in our study (Fig. 10.6b). When compared with the average energy consumption ($E_c$) (averaged over the different campaigns) at the HA, OV, BA and DO sites, mysids from the AP location consumed significantly more energy. Since the AP site was only sampled during the last campaign (March 2003), we also analyzed the variance of energy consumption within this last campaign (Table 10.2). Effects were more obvious, and a strong increase was observed in $E_c$, which was significantly higher in both the DO and AP site compared to the other sites. Metabolic rate (respiration and excretion) reflects energetic demands and the pattern of substrate metabolism of an organism. Alterations of those patterns will affect the survival and productivity of a population (Garnacho et al., 2001). Increases in metabolic rates of mysids have been demonstrated following exposure to pesticides and hydrocarbons (Carr et al., 1985; McKenney, 1985; Mckenney and Matthews, 1990; Reitsema, 1981; Roast et al., 1999b). Since no seasonal changes were observed in our study for the sampled periods, and energy consumption in *N. integer* is apparently relatively unaffected by changes in temperature and salinity, spatial differences might be indicative of toxicant-induced effects.

### *10.4.5. Cellular energy allocation*

*Neomysis integer* from the Scheldt estuary had a significantly higher cellular energy allocation (CEA) in spring than in winter, indicating that they were allocating more energy towards their energy reserves in spring (Fig. 10.6a). This effect must be considered with

reference to breeding, food supply and metabolic rate. Energy consumption was not significantly different between spring and winter, and total available energy content was also unaffected by season, indicating no significant differences in energy acquisition. Since reproduction is stopped during winter months (and starts again around April; Mees et al., 1994), it can be expected that the increase in CEA is a direct result of a lower energy demand for reproduction during winter months. It can thus be expected that *N. integer* are most susceptible to toxic stress during periods of high reproductive activity, which would be during April, July and early winter, when energy demand to fuel these processes is maximal. It was, for instance, remarkable that CEA was significantly reduced in the more upstream sites during the two winter campaigns (November 2001 and December 2002), but that this effect was reversed in the two summer campaigns (June 2001 and June 2002). However, the overall data suggest that CEA decreases in the more upstream locations, especially at the DO and AP sites where pollution is highest and *N. integer* was previously not found due to oxygen depletion (Fig. 10.6b). It is also important to mention that mysid weight was significantly reduced in the AP site, which could indicate that the observed effects on CEA resulted in a reduced growth efficiency (Fig. 10.3b). Since we did not sample the DO and AP locations in the first 5 campaigns, unaware of the fact that mysids had effectively moved upstream of BA in recent years, we remain cautious in drawing conclusions on potential effects on energy allocation at these sites. We will further assess the potential effects of pollutants, more specifically endocrine disruptors, on the CEA in *N. integer* of the Scheldt estuary in the ENDIS-RISKS project. In this project, the 5 sites from the present study will be sampled three times a year from 2002 until 2005. In addition, other biomarker responses will be investigated in *N. integer* of the Scheldt estuary and a large number of endocrine disruptors will be measured in mysid, water, sediment and suspended solids and correlated with population level data.

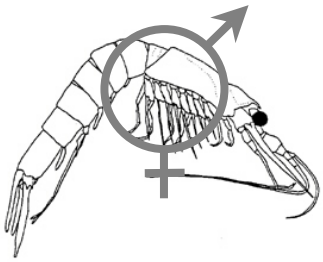
In a previous study, we investigated the variability of the CEA under different combinations of salinity (5 and 25‰), temperature (5 and 20°C) and dissolved oxygen (60 and 110% saturation) in the laboratory, chosen within the range which occurs in the Scheldt estuary (Verslycke and Janssen, 2002). The average available energy of all mysids in this study was 3334 ± 719 mJ/mg ww which is very similar to what we found in the present study for *N. integer* of the Scheldt estuary (3172 ± 888 mJ/mg ww). However average energy consumption was lower in mysids from the Scheldt estuary (31 ± 17 mJ/mg ww/h), than energy consumption in the laboratory experiment (54 ± 16 mJ/mg ww/h), resulting in a higher average CEA in the field (135 ± 79) as compared to the laboratory-exposed mysids (66 ± 20). Although it is obviously difficult to directly correlate biomaker responses from field and laboratory experiments, the similarities in energy content are remarkable. Since average CEA values for mysids from our laboratory culture (132 ± 39) are very similar to the average

CEA value found in the Scheldt estuary, it may be deduced that acute changes in the abiotic environment result in significant effects on the CEA, due to an increase in energy consumption to cope with these changes. However, these changes in abiotic environment occur more gradually in the field, allowing the mysids to acclimate.

### 10.5. Conclusion

The spatial and seasonal patterns in energy allocation were described for *Neomysis integer* of the Scheldt estuary. Seasonal effects were apparent for mysid wet weight and were related to the population biology, whereas spatial effects were probably related to pollution-induced effects on energy allocation in the two most upstream sites (BA and AP). Total available energy was relatively unaffected by season or location, while energy consumption was clearly induced in the BA and AP site, resulting in a significant effect on the cellular energy allocation (CEA) of *N. integer* at these sites. Due to a recent amelioration in the oxygen concentration at these sites, it can be expected that *N. integer* will migrate further upstream, similar to what is observed in other European estuaries. It will be important to assess the physiological and potential population effects of this migration to more polluted sites in the Scheldt estuary.

The CEA assay provides a valuable tool for assessing toxicant and abiotic stress-induced physiological effects in an organism in the laboratory. This study provides evidence that this assay has similar potential under field conditions where it can provide valuable information on the energy fluxes in ecosystems, and the *in situ* effects of chemicals on organisms. However, extended baseline laboratory and long-term field data are the key to the successful use of physiological biomarkers such as the CEA.



# CHAPTER 11

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# Testosterone metabolism in field populations of *Neomysis integer* of the Scheldt estuary

**Redrafted after :**

Verslycke T, Poelmans S, Ghekiere A, De Brabander HF, Janssen CR (submitted) Testosterone metabolism in field populations of the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) of the Scheldt estuary (The Netherlands). *Environmental Pollution*.

Chapter

# 11

## *Testosterone metabolism in field populations of Neomysis integer of the Scheldt estuary*

**Abstract**

Testosterone metabolism in *Neomysis integer* (Leach, 1814) has been used as a biomarker of exposure to endocrine disruptors in the laboratory (Verslycke et al., 2003a, submitted-Chapter 7,8). The present study investigates the applicability of the described techniques under field conditions in the Scheldt estuary. Mysids were sampled in three campaigns during 2001 and 2002, and metabolic assays were performed with testosterone as a substrate. To detect effects on phase I and phase II biotransformations in field-exposed mysids, testosterone elimination as polar hydroxylated, nonpolar oxido-reduced, and glucose- and sulfate-conjugated metabolites was examined. In addition, the vertebrate-type androgens androstenedione (AED), dihydrotestosterone (DHT) and testosterone (TST) were measured in extracts of homogenized mysids. The integrated effect on testosterone metabolism was evaluated using the metabolic androgenization ratio (MAR), which is the ratio of nonpolar to hydroxylated and conjugated derivatives of testosterone. The effects on phase I and II testosterone metabolism were significantly different between sampling campaigns. The spatial effects on hydroxylation were unclear, whereas the production of oxido-reduced testosterone metabolites was lower in more upstream sites during all campaigns, indicating that mysids from these sites have significantly different metabolic capacities. This reduction in reductase activity at upstream sites results in lower endogenous concentrations of AED, DHT and TST, and a lower MAR. Presently, the lack of a sufficiently large dataset on testosterone metabolic assays in mysids hinder a conclusive interpretation of the observed responses. The continued application of these assays in laboratory and field experiments are, therefore, needed to validate their use in detecting *in situ* effects of endocrine disruptors.

### 11.1. Introduction

Mysid crustaceans (Crustacea: Mysidacea) are shrimp-like animals which are predominantly coastal, shallow water organisms, living in close proximity to the sediment surface. Because of their high abundance and widespread distribution and their different feeding methods, mysids form an important link in marine food webs (Winkler and Greve, 2002). Mysids

are also frequently used in toxicity testing and there is growing interest in developing toxicity tests with mysids that are indigenous to local ecosystems (Roast et al., 1998a). In this context, *Neomysis integer* has been evaluated as a potential test organism for determining the effects of chemicals on northern European estuarine biota (Roast et al., 1998a, 1999a,c, 2000a,c, 2001a,b; 2002; Verslycke et al., 2003a,b,c). In addition, mysids may serve as a viable surrogate for many crustaceans and have been put forward as suitable test organisms for the evaluation of endocrine disruption (DeFur et al., 1999; LeBlanc, 1999; Verslycke et al., submitted-Chapter 2).

Baseline studies on the spatial and temporal patterns in the hyperbenthic component of the Scheldt estuary have identified the mysid *N. integer* as a dominant and key species in the brackish part of the Scheldt estuary (Mauchline, 1980; Mees and Hamerlynck 1992; Mees et al., 1993a,b, 1994, 1995b). The Scheldt estuary is heavily polluted with a wide range of man-made chemicals, many of which are known to have endocrine-disruptive properties (Verslycke et al., submitted-Chapter 9). *N. integer*, inhabiting the brackish and most polluted part of this estuary, accumulate potential endocrine disruptors via the water, sediment and suspended material in the water column, which results in a build-up of these compounds in the tissues of exposed mysids (Fockedey and Mees, 1999; Verslycke et al., submitted-Chapter 9). A recent study on the energy metabolism in field populations of *N. integer* of the Scheldt estuary suggested that this exposure leads to an induced physiological challenge, which results in adverse effects on the overall energy allocation, which is significant in the most upstream (i.e. most polluted) sites (Verslycke et al., submitted-Chapter 10). It was, therefore, hypothesized that the biotransformation capacity of field-exposed mysids from the Scheldt estuary may also be affected to cope with this chemical exposure stress.

Alterations in the steroid metabolism is one of the few relatively well-documented biomarkers of endocrine disruption in invertebrates (DeFur et al., 1999). However, this biomarker has been mainly used in laboratory experiments in the context of tributyltin-induced imposex in gastropod mollusks, which is believed to involve changes in the steroid metabolism resulting in a build-up of androgens and leads to masculinization of females (Bettin et al., 1996; Matthiessen and Gibbs, 1998; Spooner et al., 1991; Stroben et al., 1991). Changes in testosterone metabolism of daphnids have proven to be sensitive indicators of the acute and chronic effects of a number of endocrine disruptors in the laboratory (Baldwin et al., 1997; 1998; LeBlanc and McLachlan, 2000; Oberdörster et al., 1998a). Furthermore, it has been demonstrated that environmental chemicals which can interfere with the metabolic clearance of testosterone by daphnids also interfere with embryo development through anti-ecdysteroidal activity of testosterone (Kast-Hutcheson et al., 2001; LeBlanc et al., 2000; Mu and LeBlanc, 2002a). The paucity of data, however, raises the question if observed effects of vertebrate-type steroids, such as testosterone, reflect the disruption of endocrine pathways

normally used by other hormones rather than the stimulation of pathways that are specifically responsive to the administered hormone (LeBlanc et al., 2000). Vertebrate-type sex steroids (i.e. testosterone) have been measured in *N. integer* (Verslycke et al., 2002) and the question remains as to whether these compounds function as true signaling molecules (i.e. hormones), indirect regulators of physiological processes (i.e. modulators of enzyme activity), or inactive products of endogenous steroid hormones in mysids. Clearly, testosterone metabolism offers a valuable research tool to investigate these processes in mysids. It has been demonstrated that *N. integer* is capable of extensively biotransforming testosterone to various hydroxylated, oxido-reduced, and conjugated derivatives (De Wasch et al., 2002, Verslycke et al., 2002) and that these metabolic processes are susceptible to xenobiotic exposure (Verslycke et al., 2003a; Verslycke et al., submitted-Chapter 8). Hydroxylated and oxido-reduced derivatives of testosterone are considered phase I metabolites and can either be directly eliminated from the organism or may undergo additional biotransformation to glucose- or sulfate-conjugated derivatives (phase II metabolism). As mentioned earlier, several other studies have investigated the metabolic elimination of testosterone following xenobiotic exposure in daphnids (Baldwin et al., 1997; 1998; LeBlanc and McLachlan, 2000; LeBlanc et al., 2000; Oberdörster et al., 1998a), but also in other crustaceans (Oberdörster et al., 1998c) and in gastropods (Gooding and LeBlanc, 2001; Oberdörster et al., 1998b; Ronis and Mason, 1996). Still, the limited data on testosterone metabolism remain suggestive and more studies that investigate the effects of a wide range of chemicals in various invertebrates are needed to determine the value of alterations in steroid metabolism as a good biomarker for endocrine disruption in invertebrates. Finally, the strong evidence of alterations in the steroid metabolism which have led to imposex in field-exposed gastropods, should stimulate the use of this biomarker in field studies. However, until now, only one study has assessed the applicability of these techniques in the field with field-exposed mud snails, *Ilyanassa obsoleta* (Oberdörster et al., 1998b). The present study is the first to evaluate alterations in the metabolic elimination of testosterone in a field-exposed crustacean species.

## 11.2. Material and Methods

### *11.2.1. Study area*

The river Scheldt rises at Saint-Quentin in France about 350 km upstream of Vlissingen (The Netherlands) where it discharges into the North Sea (Fig. 11.1). The physical, chemical and biological characteristics of the Scheldt estuary were discussed in Chapter 10 (§ 10.2.1).

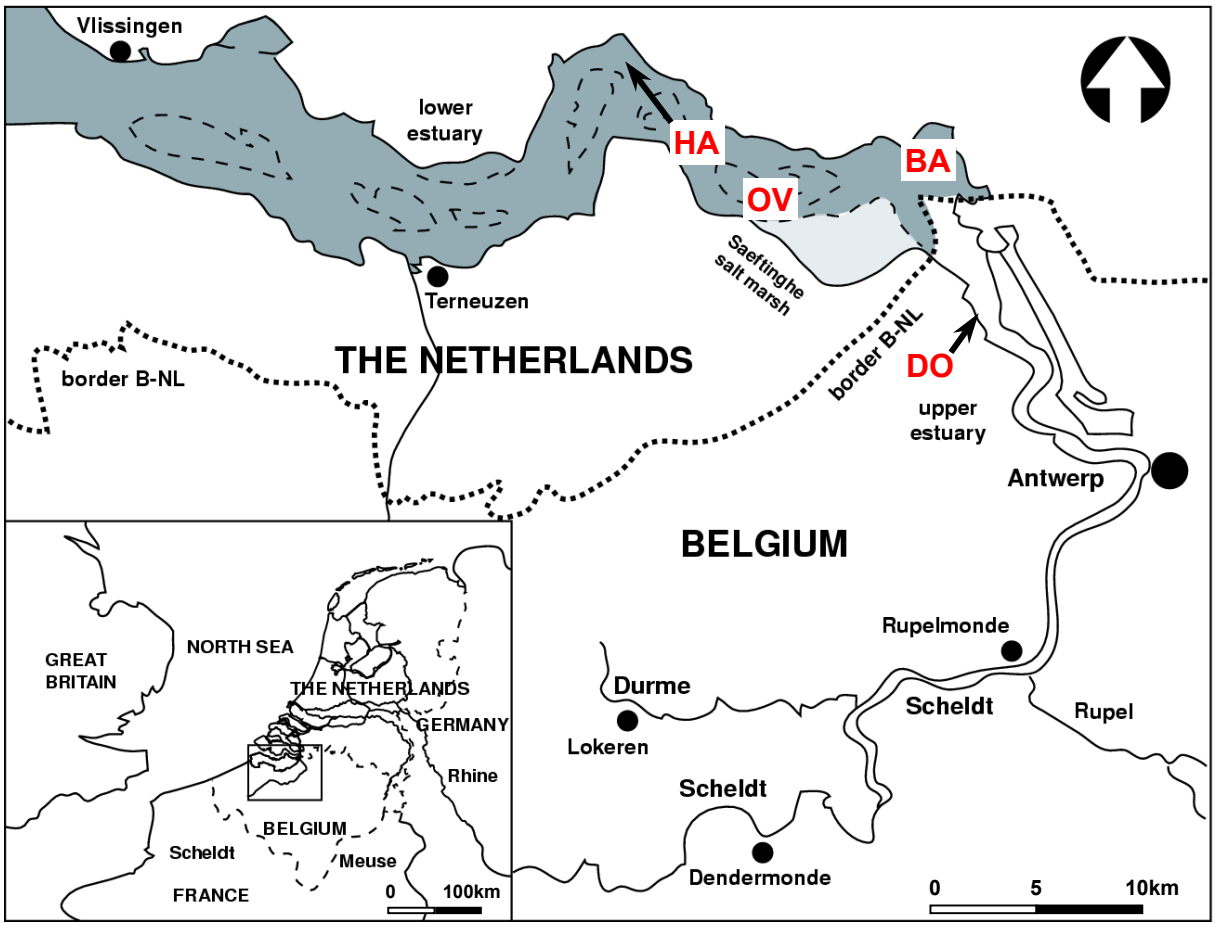



**Figure 11.1.** Map of the Scheldt estuary with location of the different sampling sites, representative of the major distribution area of the estuarine mysid *N. integer* (HA, Hansweert; OV, Overloop van Valkenisse; BA, Bath; DO, Doel).

### *11.2.2. Sampling*

Mysids were sampled during one spring (March 2002) and two winter campaigns (November 2001, December 2002). Sampling was done from the RV 'Zeeleeuw' or the RV 'Belgica'. The environmental variables during the different campaigns are given in Table 10.1 (Chapter 10). Each sampling period, 3 different stations were sampled (HA, OV and BA or DO). Mysid samples were collected with a hyperbenthic sledge as described in Chapter 10 (§ 10.2.2; Fig. 10.2). At least 20 juvenile mysids were directly transferred to plastic 5-l aquaria containing artificial sea water (Instant Ocean®; Aquarium Systems, Sarrebourg, France), diluted with aerated, MilliQ water to a final salinity of 5‰. Mysids were acclimated for a few hours to the test salinity (5‰) and test temperature (ambient temperature). The assays were performed on board of the research vessel.

### *11.2.3. Testosterone metabolism and LC-MS$^n$ analysis*

The testosterone metabolism assay and the liquid chromatography with multiple mass spectrometry (LC-MS$^n$) analyses were performed as described in Chapter 6 and 7. In short, *Neomysis integer* were exposed for 6 h in 5-ml glass tubes containing 2 ml test medium to which 2 µg of testosterone (in 10 µl methanol) was added. During the first campaign (November 2001), only phase I derivatives of testosterone were analyzed in the medium. Mysids from the other campaigns (March 2002, December 2002) were also evaluated for their capacity to eliminate testosterone as sulfate- and glucose conjugates (phase II). In addition, endogenous concentrations of the vertebrate-type androgens androstenedione, testosterone and dihydrotestosterone were quantified in mysids collected during the last two campaigns.

Average wet weight (ww) of all juvenile mysids collected from the Scheldt estuary in the present study was 8.5 ± 4.4 mg ww. The wet weight of mysids (9.7 ± 4.2 mg ww) used for the metabolic assays during the winter campaigns was significantly higher ($p < 0.01$) compared with mysids of the spring campaign (4.6 ± 2.1 mg ww). No significant spatial differences in wet weights were detected within a single campaign, except during the last campaign where mysids from the Doel site had a significantly lower wet weight ($p < 0.05$) than mysids from the Bath and Overloop sites. To eliminate variation caused by differences in weight of the mysids, all metabolite concentrations were normalized for wet weight.

A number of the testosterone derivatives, especially the polar monohydroxy metabolites, were only detected in low concentrations in some samples and were below the LC-MS$^n$'s limit of detection in other samples from the same location (at least 5 replicate assays were analyzed for each location). Metabolites which were below detection limit were set equal to zero to allow calculation of averages and statistically compare averages between locations. Alternatively, the value for an undetected metabolite could be replaced by the lowest detected concentration in the replicate samples for a certain location. Since this value would, therefore, be variable between locations, it was considered appropriate to replace all undetected values by zero. Thus, the calculated averages for the production of testosterone derivatives per location are potentially higher.

### *11.2.4. Statistical analysis*

All analyses were done with the software package Statistica$^{TM}$ (Statsoft, Tulsa, OK, USA). Data were checked for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene's test, respectively, with an $\alpha$ of 0.05. The effects of location on the metabolic elimination of testosterone in mysids were examined statistically using one-way

analysis of variance (ANOVA). Due to the limited number of campaigns, it was not possible to statistically examine seasonal effects on the testosterone metabolism in mysids. Where significant *F*-ratios were calculated by ANOVA, Tukey's Honestly Significant Difference (HSD) test was applied to identify which datasets were different. All box-plots were created with Statistica™ and show the mean (small square), standard error (box), and the standard deviation (whisker).

## 11.3. Results

### *11.3.1. Metabolic elimination of testosterone by field-exposed N. integer*

As previously described, *Neomysis integer* produces a range of testosterone metabolites that can be divided into oxido-reduced/hydroxylated (phase I biotransformation) and conjugated (phase II biotransformation) derivatives. *N. integer* collected from different locations in the Scheldt estuary during three sampling periods were evaluated for their ability to eliminate testosterone (Table 11.1). The major metabolites detected in the medium were the nonpolar testosterone derivatives androstenedione and dihydrotestosterone, although the latter was only detected during the first campaign (November 2001). Minor metabolites in the medium were the polar compounds boldenone, 2α-, 6α-, 7α/15α-, 11α-, 11β-, and 16α-hydroxytestosterone. The medium was also analyzed for phase II conjugates of testosterone and its metabolites. In mysids of the Scheldt estuary, sulfation and conjugation to β-glucose of testosterone and its derivatives were, in general, equally important. It was previoulsy demonstrated that addition of testosterone and its metabolites to α-glucose is negligible in *N. integer* (Verslycke et al., 2003a).

### *11.3.2. Spatial effects on the testosterone metabolism of field-exposed N. integer*

The phase I (Fig 11.2) and phase II (Fig. 11.3) testosterone metabolism of mysids from different sites in the Scheldt estuary were compared to evaluate spatial differences. The most obvious effect was a lower reductase activity in the most upstream sites, which was statistically significant for the last two campaigns. In addition, total conjugation activity was significantly lower in mysids from upstream sites during the last campaign. The spatial differences in oxidative metabolism were extremely variable between the different campaigns.

**Table 11.1.** Metabolic elimination of testosterone by *N. integer* of the Scheldt estuary (refer to Fig. 11.1 for sampling locations)

| Station | Date | Polar metabolites[a] | | | | | | | | Nonpolar metabolites[a] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | boldenone | 2α-OH[b] | 6α-OH | 7α/15α-OH | 11α-OH | 11β-OH | 16α-OH | Total | AED[b] | DHT[b] | TST[b] | Total |
| **Phase I** | | | | | | | | | | | | | |
| HA | 6-7/11/01 | 5.5 ± 5.7 | 0.3 ± 0.6 | 8.6 ± 6.5 | 9.7 ± 7.0 | 4.1 ± 3.0 | 27 ± 10 | x[c] | 48 ± 23 | 2258 ± 719 | 693 ± 585 | | 2951 ± 1180 |
| | 4-5/3/02 | 23 ± 11 | 3.4 ± 3.8 | 41 ± 12 | 19 ± 5.1 | — | 35 ± 21 | x | 124 ± 41 | 11511 ± 3321 | — | | 11511 ± 3321 |
| | 4/12/02 | 22 ± 21 | — | 2.5 ± 1.8 | 0.3 ± 0.5 | x | 9.9 ± 9.5 | — | 35 ± 22 | 368 ± 252 | — | | 368 ± 252 |
| OV | 6-7/11/01 | 7.3 ± 6.8 | 1.4 ± 0.9 | 7.2 ± 5.0 | 23 ± 14 | 20 ± 19 | 68 ± 25 | 0.6 ± 1.0 | 111 ± 44 | 2515 ± 1358 | 663 ± 576 | | 3177 ± 1191 |
| | 4-5/3/02 | 10 ± 3.5 | — | 20 ± 10 | 11 ± 6.2 | — | 14 ± 4.4 | 18.4 ± 15.7 | 71 ± 29 | 924 ± 288 | — | | 924 ± 288 |
| | 4/12/02 | ND | — | 5.3 ± 1.6 | 3.1 ± 1.7 | x | 21 ± 8.3 | — | 29 ± 8.2 | 151 ± 74 | — | | 151 ± 74 |
| BA | 6-7/11/01 | 7.1 ± 8.8 | — | 7.6 ± 5.0 | 55 ± 9.1 | 38 ± 6.7 | 38 ± 24 | — | 99 ± 30 | 2524 ± 1512 | 18 ± 17 | | 2543 ± 1511 |
| | 4-5/3/02 | 22 ± 8.9 | — | 23 ± 7.2 | 14 ± 5.1 | — | 25 ± 7.5 | 19.0 ± 12.1 | 106 ± 21 | 1180 ± 153 | — | | 1180 ± 153 |
| DO | 4/12/02 | x | — | 2.4 ± 2.6 | 0.6 ± 0.9 | — | 0.9 ± 2.2 | — | 3.9 ± 4.5 | 33 ± 28 | — | | 33 ± 28 |
| **Phase II glycosylation** | | | | | | | | | | | | | |
| HA | 4-5/3/02 | — | — | — | x | — | 30 ± 14 | — | 31 ± 16 | — | — | 5232 ± 1714 | 5232 ± 1714 |
| | 4/12/02 | — | — | x | — | 1.6 ± 2.6 | 58 ± 14 | — | 60 ± 14 | 5.1 ± 8.4 | x | 15630 ± 3727 | 15634 ± 3726 |
| OV | 4-5/3/02 | — | — | — | x | 1.2 ± 1.4 | 16 ± 5.8 | — | 17 ± 6.5 | x | — | 4844 ± 1558 | 4854 ± 1561 |
| | 4/12/02 | x | x | x | — | — | 51 ± 16 | — | 45 ± 23 | x | — | 2576 ± 1155 | 2598 ± 1140 |
| BA | 4-5/3/02 | — | — | — | x | x | 27 ± 7.8 | — | 28 ± 7.7 | 40 ± 43 | — | 5560 ± 2348 | 5600 ± 2319 |
| DO | 4/12/02 | — | — | x | — | x | 26 ± 16 | x | 27 ± 15 | 5.4 ± 9.2 | x | 9232 ± 3034 | 9257 ± 3038 |
| **Phase II sulfation** | | | | | | | | | | | | | |
| HA | 4-5/3/02 | — | x | x | 2.8 ± 3.1 | 3.1 ± 5.9 | 39 ± 20 | — | 47 ± 27 | 70 ± 81 | — | 6563 ± 2315 | 6633 ± 2378 |
| | 4/12/02 | x | — | x | x | 3.1 ± 6.4 | 54.3 ± 35.1 | 2.4 ± 3.4 | 47 ± 30 | x | x | 12059 ± 9666 | 12106 ± 9734 |
| OV | 4-5/3/02 | — | — | — | 2.0 ± 1.3 | x | 25 ± 6.7 | — | 27 ± 7 | 90 ± 80 | — | 6137 ± 1506 | 6228 ± 1528 |
| | 4/12/02 | x | x | — | — | x | 44.1 ± 39.0 | — | 37 ± 19 | x | — | 2796 ± 1912 | 2798 ± 1917 |
| BA | 4-5/3/02 | — | x | — | 2.6 ± 0.9 | 0.5 ± 1.0 | 29 ± 8.6 | — | 33 ± 10 | - | — | 5752 ± 1457 | 5752 ± 1457 |
| DO | 4/12/02 | x | — | x | 5.3 ± 0.3 | — | 33.2 ± 13.8 | — | 21 ± 12 | x | x | 7388 ± 1828 | 7423 ± 1783 |

[a] Values obtained from LC-MS$^n$ analysis. Data are presented as mean ± standard deviation in ng/g wet weight.
[b] OH: hydroxytestosterone; DHT: dihydrotestosterone; AED: androstenedione; TST: testosterone.
[c] x: metabolite was only detected in one replicate sample.

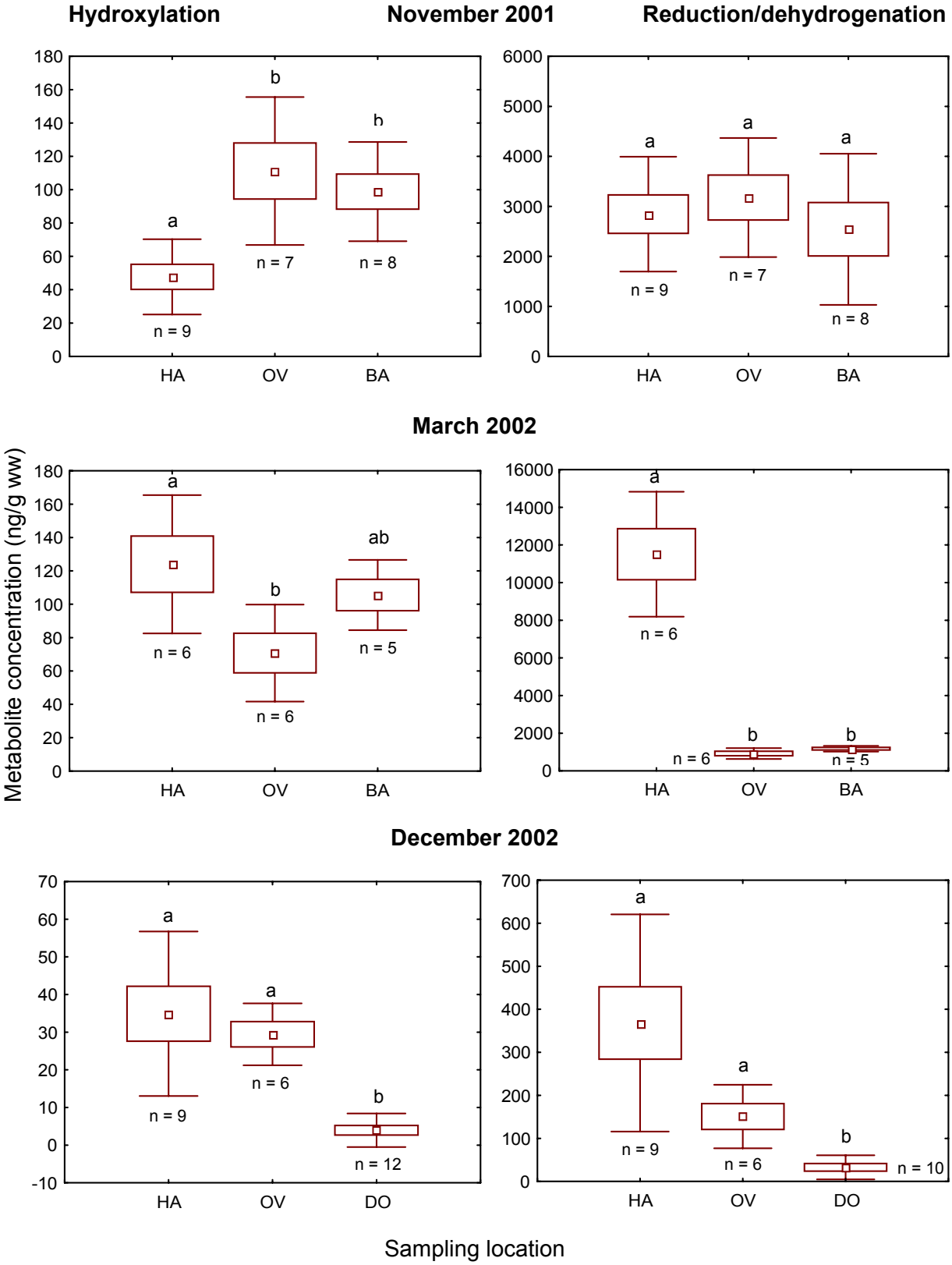



**Figure 11.2.** Metabolic elimination of testosterone in *N. integer* of the Scheldt estuary as polar hydroxylated (left) and nonpolar reduced/dehydrogenated (right) phase I metabolites. Different letters indicate significant differences ($p < 0.05$). Number below the whisker indicates the number of observations. Refer to Fig. 11.1 for sampling locations.

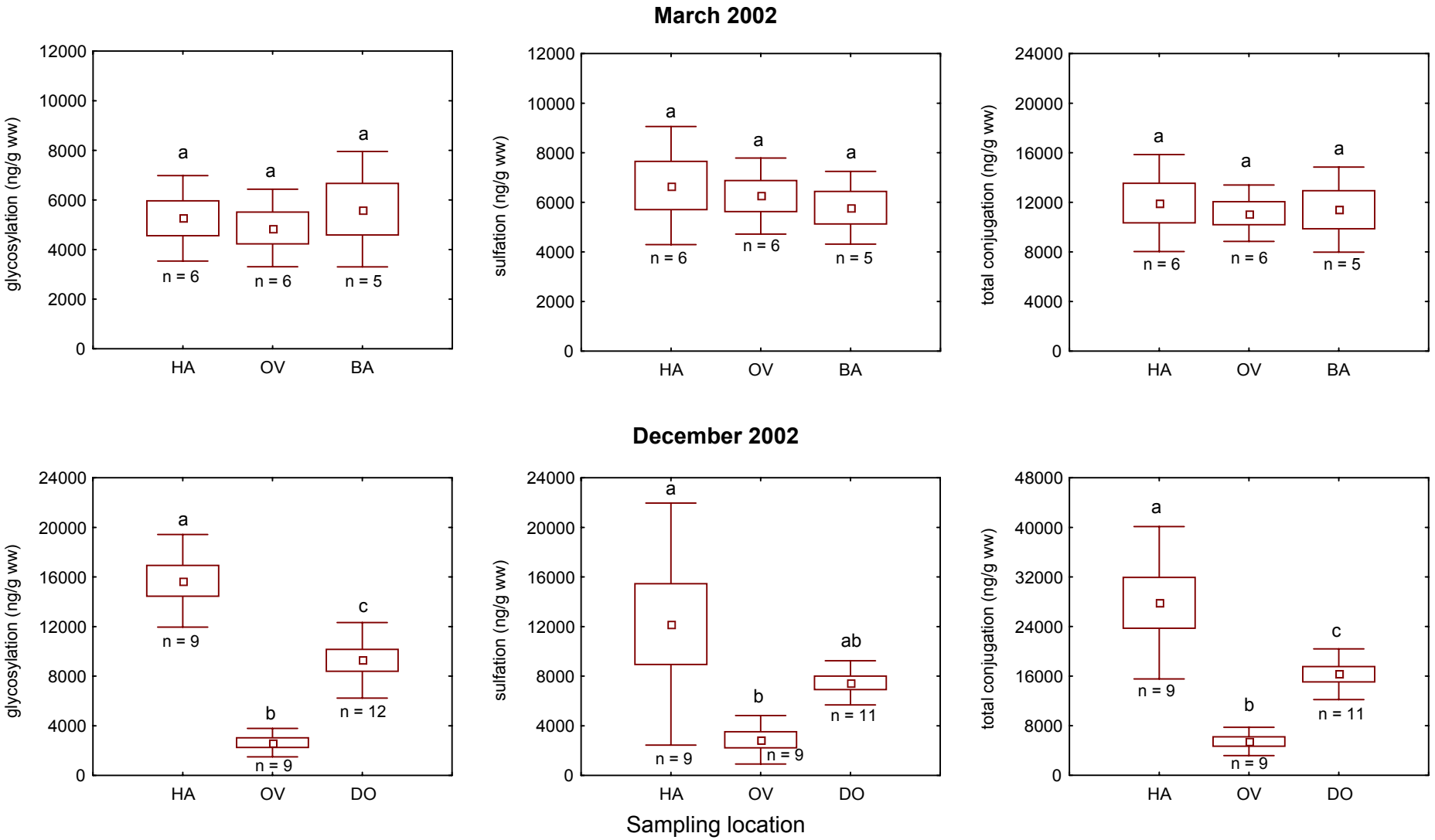



**Figure 11.3.** Metabolic elimination of testosterone in *N. integer* of the Scheldt estuary as phase II metabolites. Different letters indicate significant differences ($p < 0.05$). Number below the whisker indicates the number of observations. Refer to Fig. 11.1 for sampling locations.

*11.3.3. Endogenous concentrations of vertebrate-type androgens in mysid*

Following the metabolic assays, endogenous concentrations of a number of vertebrate-type androgens were measured in homogenized mysids (March, December 2002) (Fig. 11.4). Androstenedione and testosterone were present in mysids collected from all sites. Dihydrotestosterone was only detected during the December campaign. In general, testosterone was quantitatively the most important endogenous vertebrate-type androgen, although this varied strongly between both campaigns. In March 2002, average endogenous testosterone and androstenedione concentrations accounted for 57% and 43% of the total, respectively. Mysids from the December campaign contained 90% testosterone, 6% dihydrotestosterone and 4% androstenedione.

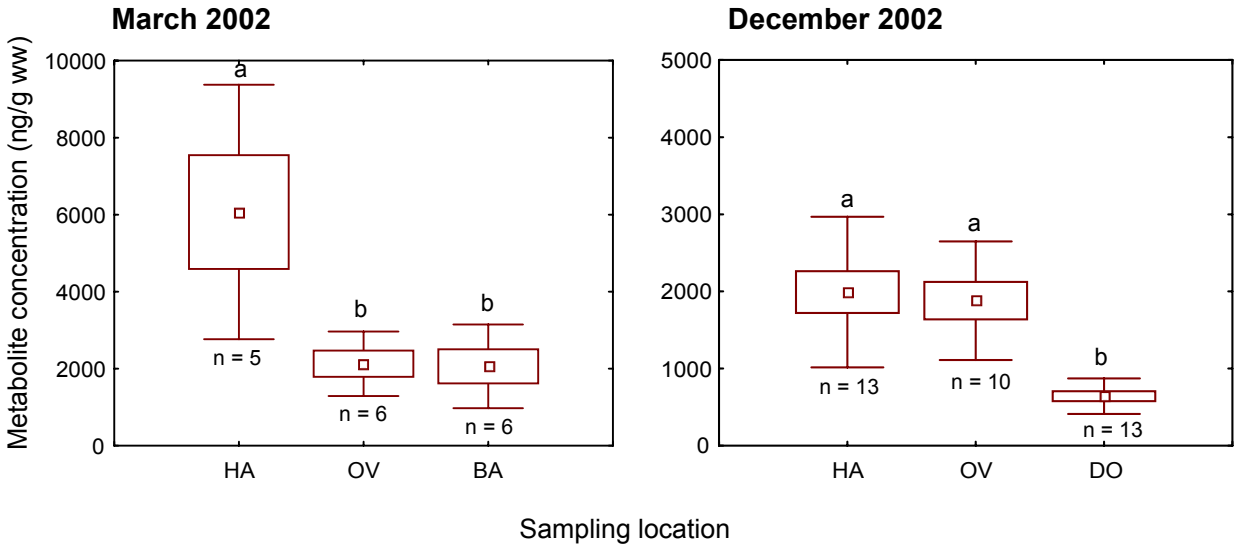



**Figure 11.4.** Retention of vertebrate-type androgens (sum of androstenedione, testosterone and dihydrotestosterone) in *N. integer* of the Scheldt estuary. Different letters indicate significant differences ($p < 0.05$). Number below the whisker indicates the number of observations. Refer to Fig. 11.1 for sampling locations.

*11.3.4. Metabolic androgenization in field-exposed mysids*

Following Baldwin and co-workers (1997), we calculated the metabolic androgenization ratio (MAR) to evaluate the overall metabolic elimination of testosterone in the field-collected mysids (Fig. 11.5). The MAR is the ratio of the eliminated reduced/dehydrogenated products and the polar products (hydroxylated plus conjugated) (Baldwin et al., 1997). The MAR integrates the various metabolic processes contributing in both the production of androgenic derivatives and inactivated products of testosterone (Oberdörster et al., 1998a).

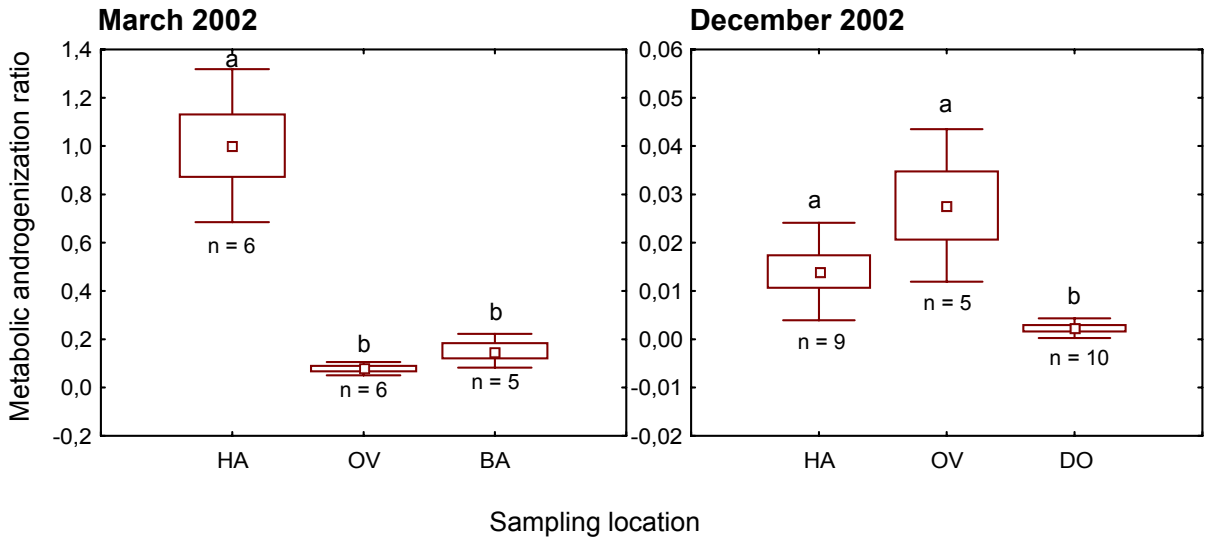



**Figure 11.5.** Metabolic androgenization ratio in *N. integer* of the Scheldt estuary. Different letters indicate significant differences ($p < 0.05$). Number below the whisker indicates the number of observations. Refer to Fig. 11.1 for sampling locations.

## 11.4. Discussion

Metabolic elimination of testosterone as polar phase I derivatives was differentially affected in the three campaigns. However, the total amount of polar testosterone metabolites produced was relatively stable. Steroid hydroxylation reactions are catalyzed by P450 isozymes. Consequently, testosterone metabolism is a tool to study the activity of P450 isozymes. In this perspective, we previously demonstrated that *Neomysis integer* produces monohydroxymetabolites of testosterone which are mainly linked to the CYP2 family (Verslycke et al., 2002, 2003a). Xenobiotics, such as polycyclic aromatic hydrocarbons can induce the expression of P450s (Fent, 1996; Oberdörster et al., 1998c). Induced expression of P450s results in higher monooxygenase activity and the production of more polar derivatives of exogenous compounds, which are more easily excreted from the body. If field-exposed mysids upregulate P450s to cope with chemical exposure, the activity of these enzymes should also increase. However, no clear induction was observed in field-exposed mysids, although significant spatial differences in the amount of monohydroxytestosterone metabolites were apparent. All collected mysids in the Scheldt estuary are exposed to a mixture of chemicals which are capable of up- or downregulating P450 activity. In absence of a control site in this estuary, it is therefore difficult to interpret spatial differences in the oxidative metabolism of mysids. It was observed from previous laboratory experiments with compounds such as tributyltin, methoprene and nonylphenol that hydroxylation of testosterone in *N. integer* is relatively stable, with total monohydroxytestosterone concentrations of around 50-70 ng/g ww (Verslycke et al., submitted-Chapter 8). The

average amount of these polar testosterone derivatives produced in field-exposed mysids was 68 ng/g ww, with high activities during the first two campaigns (83 and 100 ng/g ww, in November 2001 and March 2002, respectively) and low activities during the last campaign (20 ng/g ww). The reason for these seasonal differences remain unclear. Still, from these results, it can be concluded that no clear induction of P450 activity is observed in field-exposed mysids, as compared to control mysids from laboratory experiments. Although there is considerable interest in being able to relate induction of specific cytochrome P450 enzymes in crustaceans to exposure to environmental pollutants, the results from many studies with crustaceans exposed to a wide range of chemicals have been contradictory. In general, the responses of the cytochrome P450 system of crustaceans to chemical exposure suggest that crustaceans appear to be generally unresponsive (for a review refer to James and Boyle, 1998). The present study corroborates these findings, although, most probably other cytochrome P450, i.e. different from the typical P4501A induction in vertebrate species, may be inducible in crustaceans.

In contrast with the relatively stable production of polar metabolites, oxido/reduced nonpolar derivatives of testosterone were lower in more upstream sites during all campaigns. This effect was significant in the last two campaigns, with mysids from the Hansweert location (most downstream site) producing significantly higher amounts of nonpolar metabolites compared with mysids from the more upstream sites (OV, BA and DO). It was also remarkable that very significant differences were observed in the total production of these nonpolar metabolites between the three campaigns. The reason for this effect remains unclear. Only one previous study investigated testosterone metabolism in field-exposed and tributyltin-induced imposex invertebrates, i.e. the mud snail *Ilyanasse obsoleta* (Oberdörster et al., 1998b). Tributyltin-induced imposex, a pseudohermaphroditic condition mainly observed in gastropod mollusks, is believed to involve changes in the steroid metabolism, especially P450 hydroxylation, reduction/dehydrogenation and conjugation by transferases. Oberdörster et al. (1998b) observed a decrease in the levels of reduced/dehydrogenated products in field-collected imposex snails which is similar to our observations in mysids from the most polluted upstream sites of the Scheldt estuary. Contrary to this, most laboratory exposure studies with invertebrates have demonstrated an increase in the phase I production of nonpolar metabolites, such as dihydrotestosterone and androstenedione (LeBlanc and McLachlan, 2000; Oberdörster et al., 1998a; Ronis and Mason, 1996). Previous studies in the laboratory with *N. integer* indicated that the reductive metabolism of testosterone in mysids was induced at low exposure concentrations of tributyltin and nonylphenol, whereas no significant induction was present at higher concentrations (Verslycke et al., 2003a; Verslycke et al., submitted-Chapter 8).

Phase II conjugation in *N. integer* involves glycosylation and sulfation (Verslycke et al.,

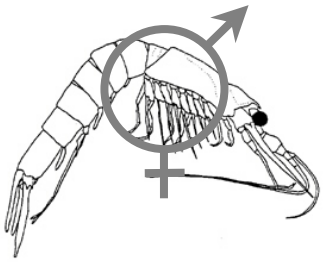
2002, 2003a). In field-exposed mysids elimination of testosterone as glucose- and sulfate conjugates was unaffected in the March 2002 campaign, whereas conjugation was significantly higher in the HA site, compared to the more upstream sites (OV, BA and DO) in the last campaign (December 2002). We previously demonstrated that sulfation and glucosylation of testosterone are decreased in tributyltin- and methoprene-exposed mysids compared to control organisms (Verslycke et al., 2003a, Verslycke et al., submitted-Chapter 8). The effects of chemicals on elimination of testosterone as phase II conjugates has mainly been studied in daphnids. Sulfation of testosterone in daphnids was decreased following exposure to nonylphenol, nonylphenol polyethoxylate and pentachlorophenol, although glucose conjugation appears to be the major elimination process in these animals (Baldwin et al., 1997, 1998; Parks and LeBlanc, 1996). Unfortunately, almost no data are available on the effects of other chemicals or chemical mixtures on testosterone conjugation in daphnids. Furthermore, data is lacking on the effects of xenobiotics on steroid conjugation in other invertebrates, which hinders a conclusive interpretation of the observed effects in our study.

Reduced/dehydrogenated metabolites of testosterone are preferentially retained in the organism and may serve as androgens or androgen precursors. On the other hand, hydroxylation and conjugation of testosterone lead to inactivation and preferential excretion (Baldwin and LeBlanc, 1994b). The overall impact of a chemical on the metabolic elimination of testosterone can be calculated by the metabolic androgenization ratio (MAR), which is the ratio of the rates of production of the eliminated reduced/dehydrogenated products and the polar products (hydroxylated plus conjugated) (Baldwin et al., 1997). The MAR was significantly lower in the upstream sites of OV and BA in March 2002 and DO in December 2002 (Fig. 11.5). Since total conjugation was unaffected and the relative hydroxylation activity was low in the March 2002 campaign, the high production of nonpolar metabolites -which are preferentially retained in the organism- was the main factor explaining the increase in the MAR at the HA site. This resulted in a build-up of androgens in the tissues of mysids from this site (Fig. 11.4). Similarly, endogenous concentrations of testosterone and androgenic derivatives of testosterone in the tissues of field-exposed mysids correlated well with the MAR of mysids from the December 2002 campaign. During this campaign, the overall elimination of testosterone was reduced at the DO site, e.g. hydroxylation, reduction/dehydrogenation and total conjugation were significantly lower compared to the other sites. Although only based on the two campaigns in this study, it can be concluded that mysids which live upstream of the HA site have a decreased reductase pathway which results in lower endogenous concentrations of androgens and a lower MAR. It should be noted that, although reductase activity was lower in December 2002 at the OV site compared to the HA site, this effect was not significant and the MAR was not lower at the OV site. More data are, therefore, needed to confirm this effect.

A decrease in reductase activity and the MAR in mysids found in upstream sites of the Scheldt estuary can be interpreted in the context of a higher pollution level at these sites. It was previously demonstrated that mysids from these upstream sites have higher chemical body burdens which results in an induced energy demand and altered energy allocation (Verslycke et al., submitted-Chapter 9,10). A higher excretion to retention activity can be considered as an adaptive mechanism of mysids to minimize the build-up of chemicals in their tissues. As mentioned earlier, a similar effect was observed in field-exposed snails which had a significantly lower MAR than laboratory-exposed animals (Oberdörster et al., 1998b).

## 11.5. Conclusion

Significant spatial differences were observed in the testosterone elimination capacity of *Neomysis integer* of the Scheldt estuary, indicating that mysids along a pollution gradient in the Scheldt estuary have significantly different metabolic capacities. The most obvious effect was a reduction in reductase activity in the most upstream sites which correlated with a lower metabolic androgenization ratio and lower endogenous concentrations of androgen-active compounds. A reduction in the production of nonpolar metabolites, which are preferentially retained in the body, may be a reflection of an adaptive mechanism in mysids at these polluted sites to minimize the build-up of chemicals. Furthermore, these data corroborate the observed effects on energy allocation of mysids collected from these sites during the same samplings, i.e. spatial differences on energy metabolism were apparent and mysids from upstream sites had a significantly altered energy allocation (Verslycke et al., submitted-Chapter 10). In the absence of more data, the observed seasonal differences in testosterone metabolism are difficult to interpret. The present data are insufficient to make conclusions on the applicability of testosterone metabolism as a valuable biomarker for endocrine disruption in field studies. The continued application of these assays in laboratory and field experiments are, therefore, needed to validate their use in detecting *in situ* effects of endocrine disruptors.



# CHAPTER 12

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# General conclusions and research perspectives

Chapter

# 12

## *General conclusions and research perspectives*

The ultimate goal of ecotoxicity testing is to monitor or predict the effects of toxicants on the long-term health of individual organisms, populations, communities and ecosystems. Chemical toxicity to endocrine processes is recognized as a means by which exposure to low, environmentally-relevant levels of chemicals may result in profound effects at both the organism and population level. Accordingly, ecotoxicologists are faced with the daunting task of developing effective screening tools for the detection of endocrine-related toxicity. Invertebrates utilize processes not found in vertebrates and thus must be included in such screening processes (LeBlanc, 1999).

Addressing the research needs mentioned in the introduction to this doctoral thesis, the overall aim of the present study was to investigate endocrine disruption in an ecologically relevant invertebrate test species, the mysid shrimp *Neomysis integer* (Crustacea: Mysidacea), through laboratory and field research. More specifically the research goals of this doctoral study were:

- A fundamental study of the hormone-regulated processes of energy and testosterone metabolism in *N. integer*;
- Evaluation and validation of the use of these processes as endpoints to evaluate environmental endocrine toxicity in the laboratory;
- Evaluation of the exposure and effects of endocrine disruptors in the Scheldt estuary on the energy and testosterone metabolism in the resident *N. integer* population.

As discussed in the introduction of this doctoral thesis (*chapter 1*), there are inherent dangers associated with the use of surrogate or 'representative' invertebrate species in toxicity testing with the intent of defining safe exposure levels of a chemical for invertebrates as a whole. The protostome mollusks and crustaceans have little more in common than do the deuterostome starfish and humans, and starfish are unlikely to ever be used to estimate chemical toxicity to humans. However, the use of surrogates can be invaluable when screening chemicals for endocrine-disrupting properties. With sufficient knowledge of the susceptibility of different invertebrate groups to specific types of endocrine toxicity, the information derived with these standard surrogate species can then be used to identify compounds with which toxicity to non-traditional invertebrate species should be evaluated

(LeBlanc, 1999).

The literature review in *chapter 2* clearly identified the ecological relevance and the potential use of mysids as a viable surrogate species for many crustaceans for the evaluation of chemically-induced endocrine disruption. Despite the long-standing use of mysids in toxicity testing, little information is available on their endocrinology and few studies have focused on the potential use of these animals for evaluating the effects of hormone-disrupting compounds. However, the highly standardized use of mysids in toxicity testing is an important advantage and research should be directed at evaluating the current standardized endpoints, such as survival, growth, and reproduction preferably through an entire life cycle, with a number of endocrine disruptors. In this context, a number of reference chemicals, chosen for their possible mode of action (i.e. ecdysone agonist, estrogen antagonist, juvenile hormone agonists, etc.) was proposed in DeFur et al. (1999) for evaluating relative endpoint sensitivity to potential endocrine-disrupting compounds. Furthermore, the review in *chapter 2* provides an extensive list of non-standardized endpoints which should be investigated further. Some of these endpoints, such as disruption of the ecdysteroid metabolism and embryonic development, might differentiate for invertebrate-specific effects of chemicals.

As chronic toxicity testing with mysids (i.e. *Americamysis bahia*) and daphnids (i.e. *Daphnia* and *Ceriodaphnia*) has been required for decades, extensive toxicological background data is available for these organisms. Furthermore, a number of endocrine-regulated endpoints in these organisms have been used in the past, although not directly aimed at evaluating endocrine toxicity. In this perspective, energy metabolism as a biomarker of toxicant exposure has been evaluated in both daphnids (e.g. De Coen, 1999) and mysids (e.g. works of McKenney et al. and Roast et al.). Although the link between a physiological response and the exposure may not always be direct, changes in growth, reproduction or development cannot occur without changes in physiology (Munkittrick and Van Der Kraak, 1999). Consequently, it is necessary to establish what significant whole organism changes occur, what the physiological changes mean to the organism, whether the physiological changes are the cause of, or caused by the whole organism changes. Thus, the requirement for a good physiological indicator is actually very simple: ‘the observed change should mean something useful’. Simple indeed, but very complex when put to the test. A good example of a meaningful physiological indicator, is the cellular energy allocation (CEA) assay, recently developed for *Daphnia magna*. This assay offers the following fundamental possibilities: assessing effects on energy budgets and providing insights to the mode of action of chemicals (De Coen, 1999). Furthermore, this methodology is appropriate for predicting long-term effects at the population level, justifying its use as a biomarker to investigate reproductive toxicity.

In this doctoral study the CEA assay was used to investigate the energy metabolism of *N. integer* with the aim of developing an understanding of the interactions between chemical availability and physiological responses. The CEA assay was first adopted for use with the test species in our study, *N. integer* (*chapter 3*). Throughout this doctoral study many different methodological aspects of this assay were optimized, most notably feeding procedures, replication, age of the animals, exposure temperature, exposure equipment, CEA calculation, etc. (not all these data were described in detail in this study). The major limiting factor in all uses of physiological endpoints is the understanding of the background levels of variability, which have three main sources: organismal (e.g. sex, age, season,…), environmental (food, temperature,…), and methodological (tissue used, sampling method,…) (Mayer et al., 1992). Organismal and methodological variability in mysids can been closely monitored and minimized through standardization of testing procedures in the laboratory.

An important aspect was the evaluation of environmental variability related to abiotic stress in the laboratory (*chapter 3*). The CEA proved to be relatively insensitive to changes in the abiotic environment within the physiological limitations of *N. integer*, which are determining factors of its spatial distribution in the field (i.e. Scheldt estuary).

Next, the CEA assay was used to evaluate effects of a number of toxicants (i.e. tributyltinchloride, chlorpyrifos, nonylphenol and methoprene) on the energetic processes of *N. integer* in the laboratory (*chapter 4, 8*). From these studies it was concluded that the CEA is a sensitive sublethal biomarker of exposure to toxicants at environmentally relevant concentrations next to being a very useful physiological research tool to study the energy metabolism of an organism.

Scope for growth (SFG) is probably the most developed and field-validated physiological bioassay (Huggett et al., 1992). In this perspective, we compared the CEA with the SFG assay in an interlaboratory validation effort, with *N. integer* exposed to the pesticide chlorpyrifos (*chapter 5*). In contrast to the SFG assay, the CEA methodology offers the possibility to assess (separately) both energy conversion and energy allocation, and can help to elucidate different modes of action of toxicants. Furthermore, the CEA appeared to be a more sensitive biomarker than SFG [perhaps because it measures effects at a lower level of biological organization (cellular) than SFG (sub-organismal)]. In contrast to SFG, CEA did not show a concentration-dependent response in the sub-lethal to lethal range. This study should, therefore, stimulate the future use of CEA as an alternative or complementary approach to measure physiological aberrations in animals.

Finally, the CEA assay proved to have similar potential under field conditions where it may provide valuable information on the energy fluxes in ecosystems, and the *in situ* effects of chemicals on organisms (*chapter 10*). Extended baseline laboratory and long-term field data will be the key to the successful use of physiological biomarkers such as the CEA.

Future studies with the CEA in *N. integer* should be directed at long-term exposures, linking organismal and population endpoints with the observed cellular effects on energy allocation. A continued monitoring of the CEA in the Scheldt estuary will lead to a sufficiently large dataset to make firm conclusions on the spatial and seasonal effects observed in the present study. These should be linked to *in situ* contamination, or ideally chemical body burdens in mysids from the collected sites. Especially the observation that mysids have migrated further upstream over the last decade to more polluted sites warrants future investigation into the potential effects of this migration on the energetic processes of mysids. Indeed, we demonstrated that *N. integer* in the Scheldt estuary are exposed to very high concentrations of known endocrine disruptors (i.e. organotins, flame retardants, surfactants) (*chapter 9*), and that *N. integer* has a high potential of bioaccumulating these compounds (*chapter 4*), which probably explains their high sensitivity to many of these compounds (*chapter 8*).

A number of environmental contaminants act as cellular signal transduction modifiers (McLachlan, 1993; McLachlan and Arnold, 1996, McLachlan et al., 1992). The majority of the molecular and cellular research on endocrine disruptors has focused on their ability to bind with the vertebrate estrogen receptor (Guillette et al., 1996; Soto et al., 1995). However, endocrine disruptors can also interact with other cellular receptors, such as the androgen receptor (Gray et al., 1996; Kelce et al., 1995), the progesterone receptor (Vonier et al., 1996) or retinoic acid receptor (Harmon et al., 1995). Alterations in plasma steroid hormone concentration have been reported following contaminant exposure in representative species of most vertebrate classes (Crain and Guillette, 1997). Furthermore, disruption of the steroid metabolism is one of the few relatively well-documented biomarkers of endocrine disruption in invertebrates (DeFur et al., 1999). These biomarkers correlate well with effects on steroid hormone-dependent processes such as growth and reproduction (Parks and LeBlanc, 1996).

In this doctoral study, the testosterone metabolism of *N. integer* was assessed to obtain data on its metabolic capacity (*chapter 6*). Identification of *in vivo* produced testosterone metabolites and endogenous vertebrate-type steroids was performed using thin-layer chromatography (TLC) and liquid chromatography with multiple mass spectrometry (LC-$MS^n$). The results from these *in vivo* biotransformation experiments with *N. integer* demonstrated the presence of a complex steroid hydroxylase system consisting of different P450 isozymes. The remarkable diversity of testosterone hydroxylation exhibited should stimulate further studies on the induction, stereo-specificity, and regulation of the enzyme systems of *N. integer*. Furthermore, the developed LC-$MS^n$ method proved to be sensitive and more convenient for use in routine biomonitoring than previously published TLC-based methods (Baldwin and Leblanc, 1994a,b; Baldwin et al., 1997, 1998; Morcillo et al., 1998; Oberdörster et al., 1998a,b,c;

Ronis and Mason, 1996).

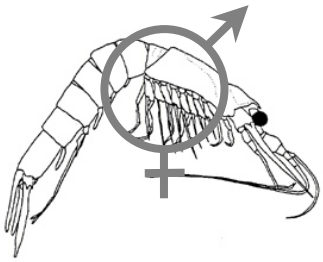
Additional exposure experiments in the laboratory demonstrated that the developed bioassay was sufficiently sensitive to detect alterations in the steroid metabolism at environmentally relevant concentrations of a number of toxicants (*chapter 7, 8*). Apparently, glycosylation may be the most sensitive steroid metabolizing pathway in mysids following toxic exposure. Similar findings are reported for daphnids (Baldwin et al., 1997; LeBlanc and McLachlan, 2000). However, we also found that sulfation was most strongly affected in *N. integer* following tributyltin exposure (*chapter 7*). Future studies will have to determine which of these processes is most important in the detoxification of chemicals in mysids, or if these processes might be differentially affected depending on the chemical. These studies suggest that steroidal androgens have a specific target site of toxicity in mysids. Although a functional role for steroidal androgens has not been firmly established, testosterone has been reported to have androgen-like activity in some crustaceans (LeBlanc, 1999). We reported endogenous concentrations of testosterone and other vertebrate-type androgens (e.g. testosterone, androstenedione) in *N. integer* and have also observed a sex-specific steroid metabolism in these animals (*chapter 6*). Thus, it can be hypothesized that several chemicals are likely to interact with the steroid metabolism in mysids which as a result could lead to functional abnormalities. Future studies should focus on the understanding of a potential physiological role of vertebrate-type steroids in *N. integer* (chapter 4). In addition, the developed assay should be adopted to evaluate endogenous metabolism of physiologically relevant substrates, such as ecdysteroids.

Finally, a preliminary field study (*chapter 11*) of *N. integer* occurring in the Scheldt estuary demonstrated significant spatial differences in the testosterone elimination capacity along a pollution gradient. Since this field study was too restricted to make conclusions on the applicability of testosterone metabolism as a valuable biomarker for endocrine disruption in field studies, the continued application of these assays in laboratory and field experiments is, therefore, needed to validate their use in detecting *in situ* effects of endocrine disruptors.

Testosterone and energy metabolism proved to be interesting and sensitive research tools to investigate endocrine disruption in mysids. However, future studies on endocrine disruption in invertebrates should also focus on the identification of invertebrate-specific endpoints. For example, in some countries the issue of endocrine disruption has been driven largely by the induction of the vitellogenin protein in male fish (Matthiessen, 1998; Sumpter and Jobling, 1995). Some studies have investigated vitellogenin in invertebrates as well, but there have been few studies investigating whether xenobiotics induce vitellogenin concentrations in invertebrates (Oberdörster et al., 2000b; Tuberty et al., 2002). Recently, vitellogenin was purified from *A. bahia* (Tuberty et al., 2002) and *N. integer* (Ghekiere et al., unpublished data).

Since reproduction in crustaceans is closely related with molting and ecdysteroid metabolism, different approaches will be needed to use vitellogenin as a biomarker of exposure in these organisms. Vitellogenin might, for instance, prove to be a sensitive biomarker of exposure in invertebrates, although of compounds with a very different mode of action (i.e. juvenile-hormone analog, ecdysteroid analog,…) than those interfering with vitellogenin production in vertebrates (i.e. estrogen-analogs). The fundamental understanding of ecdysteroid metabolism in relation to vitellogenesis in *N. integer* and the potential disruption of these processes by endocrine disruptors is presently being investigated in our laboratory. These studies will add to the energy and testosterone metabolism endpoints investigated in the present study, and to the holistic understanding of hormone disruption in *N. integer*. Clearly, long-term integrated studies of (ecdy)steroid and energy metabolism in relation to the processes of molting and reproduction in *N. integer* are the next research steps. While the fundamental understanding of these processes in mysids is the most important research need at this time, efforts should also be directed at implementing these assays in long-term field studies. However, it should be borne in mind that scarce research resources may be better invested in understanding the causes or relevance of the responses, than in developing several new indicators for a plethora of species and using them in a variety of receiving environments. Most probably, the lack of a fundamental understanding of the used responses is the main reason why, despite the fact that environmental hormone disruption has been increasingly investigated over the last decades, no clear examples of field-effects on natural populations of invertebrates have been reported (aside of the imposex phenomenon caused by tributyltin exposure).

The field studies in the present doctoral study were a first step in the implementation of laboratory-based biomarkers for endocrine disruption in *N. integer* in the field. In the meantime, these studies have led to a large interdisciplinary research project on endocrine disruption in mysids, the ENDIS-RISKS project (http://www.vliz.be/projects/endis). This four-year project will assess the distribution, exposure and effects of endocrine disruptors on mysid shrimp in the Scheldt estuary. An extended list of endocrine disruptors will be monitored in water, sediment, suspended solids and mysid and will be linked with biomarker responses in these animals. The availability of a comprehensive historical dataset on the population structure and spatial distribution of mysids in this estuary, will hopefully allow us to answer a question which remained unresolved in this doctoral study: is environmental endocrine disruption occurring in field-exposed mysids?



# Summary

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# Samenvatting

## *Summary*

This doctoral research is situated in the field of aquatic toxicology. The ultimate goal of aquatic ecotoxicity testing is to monitor or predict the effects of toxicants on the individual aquatic organism, populations, communities and ecosystems. Chemical toxicity to endocrine processes is recognized as a means by which exposure to low levels of chemicals, so called endocrine disruptors, may result in environmentally-relevant effects at both the organism and population level. The potential for endocrine toxicity of chemicals has been relatively well documented in vertebrates, including mammals, fish, birds, and reptiles. Less well understood is whether invertebrates are similarly susceptible to endocrine-disrupting toxicity.

Accordingly, the aim of present study was to investigate endocrine disruption in an ecologically relevant invertebrate test species, the mysid shrimp *Neomysis integer* (Crustacea: Mysidacea), through laboratory and field research. More specifically, the steroid and energy metabolism of *N. integer* were explored as endpoints to evaluate environmental endocrine disruption.

In **chapter 1**, an introduction to the environmental endocrine hypothesis is given, with particular reference to the situation of invertebrates, and more specifically crustaceans. The potential application of the biomarker approach in the research field of endocrine disruption is discussed. Finally, the current research needs are presented together with the conceptual framework of this study.

In **chapter 2**, a global assessment is given of the state-of-the-art in science on endocrine disruption in mysid shrimp. This review demonstrated clearly the ecological relevance and the potential use of mysids as a test species for the evaluation of environmental endocrine disruption, and as a potential surrogate for many other crustaceans. The highly standardized use of mysids in toxicity testing is an important advantage and research should be directed at evaluating the current standardized endpoints, such as survival, growth, and reproduction preferably through the entire life cycle, with a number of endocrine disruptors. In addition, an extensive list of potential non-standardized endpoints in mysids is presented, as well as criteria for the selection of suitable mysid test species.

In **chapter 3**, a new methodology, the cellular energy allocation (CEA), was adopted to assess the energy budget for *N. integer*. The biochemical composition of *N. integer* was determined. In addition, the effects of natural variability on the energy metabolic processes of *N. integer* were investigated using a factorial test design with different naturally (Scheldt

estuary, The Netherlands) occurring combinations of temperature, salinity and dissolved oxygen. The different abiotic factors had no significant effect on the CEA of *N. integer* within the tested range, although significant effects were observed on the energy reserves and energy expenditure. Temperature and dissolved oxygen, in general, had the strongest effect on the energy allocation in *N. integer*. The present study demonstrated that *N. integer* efficiently regulates its energy metabolism in response to a variable environment to minimize changes in the CEA.

In **chapter 4**, the CEA methodology was evaluated using adult *N. integer* exposed for 96 h to the antifoulant tributyltinchloride (TBTCl). From a range-finding experiment with juvenile *N. integer*, a 96-h median lethal concentration (LC50) of 164 ng TBTCl/l was calculated. The energy metabolism of *N. integer*, as summarized by the CEA, was significantly altered by TBTCl exposure. These changes at the cellular level occurred at environmentally relevant concentrations of TBTCl. The high sensitivity of mysids towards the sublethal and lethal effects of TBT may be a result of the low *in vivo* metabolism of this compound in mysids leading to high TBT body burdens, as was demonstrated by an additional uptake experiment with TBT and *N. integer*.

In **chapter 5**, the responses of *N. integer* following exposure to environmentally realistic concentrations of the organophosphate pesticide chlorpyrifos were compared using the CEA and scope for growth (SFG) assays. Oxygen consumption in the SFG assay was significantly correlated with cellular respiration rate in the CEA assay, and both were significantly increased by chlorpyrifos exposure. In addition, the protein, sugar, lipid and total energy content in the CEA assay and the egestion rate in the SFG assay were significantly different in chlorpyrifos-exposed mysids compared with control mysids. SFG was significantly reduced at near-lethal concentrations (72 and 100 ng chlorpyrifos/l), whereas CEA was reduced in all chlorpyrifos-exposed mysids (38, 56, 72 and 100 ng chlorpyrifos/l). Differences in sensitivity between these assays may be a reflection of the effects of chlorpyrifos at different levels of biological organization (e.g. CEA, cellular and SFG, organismal). This study, however, did not permit a conclusive statement as to whether one assay is better than the other, as both assays have inherent strengths and weaknesses.

In **chapter 6**, testosterone metabolism by *N. integer* was assessed to obtain initial data on its metabolic capacity. Identification of *in vivo* produced testosterone metabolites and endogenous vertebrate-type steroids was performed using thin-layer chromatography (TLC) and liquid chromatography with multiple mass spectrometry (LC-$MS^n$). In addition, endogenous production of testosterone in mysids was detected for the first time, and the

anabolic steroid β-boldenone was identified for the first time in invertebrates. A sex-specific testosterone metabolism was also observed in mysids, although this observation requires further confirmation. The results of this study revealed interesting similarities in enzyme systems in invertebrate and vertebrate species. Furthermore, the developed LC-$MS^n$ method proved to be sensitive and more convenient for use in routine biomonitoring than previously published TLC-based methods by other researchers.

In **chapter 7**, the effects of TBTCl on the phase I and phase II testosterone metabolism of *N. integer* were evaluated. Therefore, testosterone elimination as polar hydroxylated, nonpolar oxido-reduced, and glucose- and sulfate-conjugated metabolites was examined. TBTCl differentially affected testosterone metabolism. The effect of TBTCl on phase I metabolism was unclear and has been shown to vary among species, likely depending on the inducibility or presence of certain P450 isozyme families. Reductase activity and metabolic androgenization were induced in the 10 ng/l treatment, whereas higher concentrations resulted in a reduction of sulfate conjugation. However, the exact mechanisms underlying TBT-induced imposex and alterations in the steroid metabolism need to be further elucidated.

In **chapter 8**, a diverse set of reference compounds suspected of having an endocrine-disrupting mode of action were tested for acute toxicity, i.e. testosterone, flutamide, ethinylestradiol, precocene, nonylphenol, fenoxycarb and methoprene. *N. integer* was very sensitive to all tested compounds, with 96-h LC50s in a narrow range between 0.32 and 1.95 mg/l. In addition, the short-term sublethal effects of methoprene and nonylphenol on the energy and steroid metabolism of *N. integer* were evaluated. Both compounds significantly affected energy and testosterone metabolism of *N. integer* at concentrations below acute toxicity levels. Consequently, this study indicate that energy and testosterone metabolism of mysids, as endpoints, are able to detect endocrine disruptive activity of chemicals following short-term exposure to environmental realistic levels of endocrine disruptors.

In **chapter 9**, sediment and mysids (*N. integer*) from the Scheldt estuary, one of the largest and most polluted estuaries in Western Europe, were analyzed for a number of endocrine disruptors, i.e. organotins, polybrominated diphenyl ethers, hexabromocyclo-dodecane, tetrabromobisphenol A, nonylphenol ethoxylates, and transformation products nonylphenol and nonylphenol ether carboxylates. In addition, *in vitro* estrogenic and androgenic potencies of water and sediment extracts were determined. Significant estrogenic potency, as analyzed using the yeast estrogen assay, was detected in sediment and water samples from the Scheldt estuary, but no androgenic activity was found. This study was the

first to report high levels of endocrine disruptors in estuarine mysids and, therefore, warrants further research into the potential effects of these chemicals on field-exposed mysid populations.

In **chapter 10**, the seasonal and spatial patterns in energy allocation of the *N. integer* were investigated in the Scheldt estuary over a 2-year period using the CEA assay. Total energy reserves were relatively unaffected by sampling season or location, whereas individual energy reserve fractions of *N. integer* were differentially influenced by sampling location and season. Seasonal effects were apparent for mysid weight and were related to the population biology, whereas spatial effects on the weight of *N. integer* may depend on pollution-induced effects on energy allocation in the two most upstream sites (Doel and Antwerp). These upstream sites coincide with the most polluted part of the sampled area and were characterized by a significant increase in energy consumption, resulting in a significantly lower CEA. Due to the recent amelioration in the oxygen concentration at these sites, it can be expected that *N. integer* will migrate further upstream, similar to what is observed in other European estuaries. It will, therefore, be important to assess the physiological consequences and potential population effects on mysids from these polluted areas in the Scheldt estuary. In conclusion, this study provided evidence that the CEA assay has potential as an *in situ* biomarker of pollutant effects.

In **chapter 11**, the applicability of the testosterone metabolism assay in *N. integer* was investigated under field conditions in the Scheldt estuary. Mysids were sampled in three campaigns during 2001 and 2002, and metabolic assays were performed with testosterone as a substrate. The effects on phase I and II testosterone metabolism were significantly different between sampling campaigns. The spatial effects on hydroxylation were unclear, whereas the production of oxido-reduced testosterone metabolites was lower in more upstream sites during all campaigns, indicating that mysids from these sites have significantly different metabolic capacities. It was concluded that the continued application of these assays is needed to validate their use in detecting *in situ* effects of endocrine disruptors.

In **chapter 12**, general conclusions and future perspectives of this doctoral study were formulated. Summarizing, this doctoral study was able to demonstrate the susceptibility of the hormone-regulated processes of testosterone and energy metabolism in *N. integer* towards the effects of endocrine disruptors and the use of these mechanisms as sensitive biomarkers.

## *Samenvatting*

Dit doctoraatsonderzoek situeert zich in het onderzoeksdomein van de aquatische toxicologie. Deze onderzoeksdiscipline houdt zich bezig met de evaluatie of voorspelling van de effecten van toxische stoffen op individuele aquatische organismen, populaties en ecosystemen. Chemische toxiciteit ten opzichte van hormoongestuurde processen wordt aanzien als een mechanisme waarbij blootstelling aan lage concentraties van chemicaliën (hormoonverstoorders) kan leiden tot belangrijke milieurelevante effecten op het niveau van het organisme, en op het niveau van de populatie. De werking van deze hormoon-verstoorders werd reeds vrij uitgebreid onderzocht bij gewervelde soorten, zoals zoogdieren, vissen, vogels en reptielen. De kennis over de mogelijke gevoeligheid van ongewervelde diersoorten voor deze stoffen is echter beperkt.

Het huidig doctoraatsonderzoek kadert binnen deze problematiek en heeft een fundamentele studie van de hormoonverstoring bij een ecologisch belangrijk ongewerveld testorganisme, namelijk de aasgarnaal *Neomysis integer* (Crustacea: Mysidacea), door middel van laboratorium -en veldexperimenten, tot doel. Meer specifiek wordt de bruikbaarheid van het testosteron -en energiemetabolisme van *N. integer* als eindpunten voor de evaluatie van hormoonverstoring door milieurelevante stoffen onderzocht.

In **hoofdstuk 1**, werd een inleiding gegeven tot de milieuproblematiek van de hormoonverstoring door milieuverontreiniging. Hierbij werd de nadruk gelegd op de situatie bij ongewervelde dieren en meer specifiek bij crustaceeën met bespreking van de potentiële bruikbaarheid van biomarkers in het onderzoeksdomein van de hormoonverstoring. Bovendien werden de belangrijkste onderzoeksnoden geschetst evenals het conceptueel kader van dit doctoraatsonderzoek.

In **hoofdstuk 2**, werd een overzicht gegeven van de huidige kennis omtrent hormoonverstoring bij aasgarnalen. Dit literatuuroverzicht toonde duidelijk de ecologische relevantie en bruikbaarheid van deze organismen aan in het kader van de problematiek van de hormoonverstoring. Bovendien werd aangetoond dat de aasgarnaal bij de evaluatie van hormoonverstoring kan beschouwd worden als een representatieve soort voor andere crustaceeën. De hoge graad van standaardisatie van mysidaceeën in toxiciteitstesten is een belangrijk voordeel. Het onderzoek moet worden gericht naar de evaluatie van bestaande gestandaardiseerde eindpunten, zoals overleving, groei en reproductie en dit bij voorkeur gedurende een volledige levenscyclus met verschillende hormoonverstoorders. Bovendien werd in dit hoofdstuk een overzicht gegeven van additionele niet-gestandaardiseerde

eindpunten die potentiële toepassingen kunnen hebben binnen dit onderzoeksdomein. Er werden eveneens aanbevelingen gedaan over de selectiecriteria van aasgarnalen als testorganisme.

In **hoofdstuk 3**, werd de cellulaire energie allocatie (CEA)-bepaling ontwikkeld om het energiemetabolisme van *N. integer* te bestuderen. De biochemische samenstelling van *N. integer* werd bepaald en de effecten van een veranderlijke omgeving op de CEA respons werden bestudeerd. Hiervoor werd een factorieel testopzet gebruikt waarbij de invloed van verschillende milieurelevante (Schelde estuarium, Nederland) combinaties van temperatuur, saliniteit en opgeloste zuurstof op de CEA werden onderzocht. De verschillende abiotische factoren hadden geen significant effect op de CEA van *N. integer*. Nochtans werden significante effecten op de energie opname en het energieverbruik aangetoond. Temperatuur en opgeloste zuurstof hadden de belangrijkste invloed op de energie allocatie bij *N. integer*. Deze studie toonde aan dat *N. integer* in staat is om zijn energiemetabolisme efficiënt te reguleren in een veranderlijke omgeving zodat de uiteindelijke effecten op de CEA minimaal zijn.

In **hoofdstuk 4**, werd de CEA methodologie geëvalueerd bij volwassen *N. integer* die gedurende 96 u werden blootgesteld aan tributyltinchloride (TBTCl), een stof die wordt gebruikt als aangroeiwerend middel op schepen. Uitgaande van een range-finding test met juveniele *N. integer*, werd een mediaan letale concentratie (LC50) van 164 ng TBTCl/l bekomen. Zoals bleek uit de CEA-analyses had TBTCl een significante invloed op het energiemetabolisme van *N. integer*. De effecten op cellulair niveau traden op bij milieurelevante concentraties van TBTCl. De hoge gevoeligheid van mysidaceeën voor de subletale en letale effecten van TBT zijn mogelijks gerelateerd aan het lage *in vivo* metabolisme van deze toxische stof bij aasgarnalen. Hierdoor accumuleren mysidaceeën hoge concentraties van deze organotinverbindingen zoals bleek uit een aanvullend opname experiment met TBT.

In **hoofdstuk 5**, werden de CEA en de ‘scope for growth’ (SFG) methodes vergeleken na blootstelling van *N. integer* aan milieurelevante concentraties van het organofosfaat pesticide chloorpyrifos. Zuurstofverbruik in de SFG test was significant gecorreleerd met de cellulaire respiratie in de CEA bepaling en beide werden significant beïnvloed door blootstelling aan chloorpyrifos. Bovendien waren de suiker-, vet-, en totale energie-inhoud in de CEA bepaling en de egestie-snelheid in de SFG bepaling significant verschillend bij chloorpyrifos-blootgestelde aasgarnalen ten opzichte van controle organismen. SFG was significant lager bij concentraties die dicht aanleunden bij de LC50 (72 en 100 ng

chloorpyrifos/l), in tegenstelling tot de effecten op CEA die significant verschillend waren ten opzichte van de controles in alle blootstellingsconcentratie (38, 56, 72 en 100 ng chloorpyrifos/l). Deze verschillen in gevoeligheid zijn mogelijks een reflectie van de effecten van chloorpyrifos op de verschillende niveaus van biologische organisatie (e.g. CEA, cellulair and SFG, organisme). Uit deze studie bleek dat beide bepalingen inherente voor -en nadelen hebben.

In **hoofdstuk 6**, werd het testosteronmetabolisme van *N. integer* bestudeerd om initiële data te genereren over de metabolisatiecapaciteit van deze soort. Identificatie van *in vivo* geproduceerde testosteronmetabolieten en endogene vertebraat-type steroïden gebeurde via dunnelaagchromatografie (TLC) en vloeistofchromatografie gekoppeld aan multiple massa spectrometrie (LC-$MS^n$). Endogene productie van testosteron werd voor de eerste maal vastgesteld in mysidaceeën. Bovendien werd het anabool steroïd β-boldenone voor de eerste maal gedetecteerd in een ongewervelde soort. Er waren bovendien indicaties van een geslachtsspecifiek testosteronmetabolisme, maar deze vaststelling dient nog verder onderzocht te worden. De resultaten van deze studie toonden opmerkelijke overeenkomsten aan in de enzymsystemen van ongewervelde en gewervelde diersoorten. Bovendien bleek uit deze studie dat de ontwikkelde LC-$MS^n$ methode gevoeliger en eenvoudiger is dan de bestaande TLC-methodes zoals deze door andere onderzoekers werden gepubliceerd.

In **hoofdstuk 7**, werden de effecten van TBTCl op het fase I en fase II testosteron-metabolisme van *N. integer* bestudeerd. Hiervoor werd de eliminatie van testosteron als polaire gehydroxyleerde, apolair oxido-gereduceerde en glucose- en sulfaatgeconjugeerde metabolieten gekwantificeerd. TBTCl had verschillende effecten op het testosteron-metabolisme van *N. integer*. De effecten op het fase I metabolisme waren onduidelijk en lijken te verschillen tussen diersoorten, afhankelijk van de induceerbaarheid of aanwezigheid van bepaalde cytochroom P450 isoenzyme families. Reductase activiteit en metabole androgenisatie werden geïnduceerd in de 10 ng/l behandeling, terwijl hogere TBTCl concentraties resulteerden in een reductie van de sulfaatconjugatie. Er werd besloten dat de exacte mechanismen die aan de basis liggen van het TBT-geïnduceerde fenomeen van imposex en de effecten van TBT op het steroïdmetabolisme verder onderzoek vereisen.

In **hoofdstuk 8**, werd de toxiciteit van een reeks chemicaliën met potentieel hormoonverstorende werking getest bij *N. integer*. De teststoffen waren testosteron, flutamide, ethinylestradiol, precoceen, nonylfenol, fenoxycarb en methopreen. *N. integer* was uiterst gevoelig voor de verschillende teststoffen en de LC50 voor deze stoffen varieerde tussen 0.32 en 1.95 mg/l. Bovendien werden de korte-termijn subletale effecten van

methopreen en nonylfenol bepaald op het testosteron -en energiemetabolisme van *N. integer*. Beide toxicanten hadden significante effecten op het testosteron -en energie-metabolisme van *N. integer* bij concentraties die niet acuut toxisch waren. Hieruit kon besloten worden dat deze eindpunten in staat zijn om hormoonverstoring vast te stellen bij aasgarnalen na een korte blootstelling aan milieurelevante concentraties.

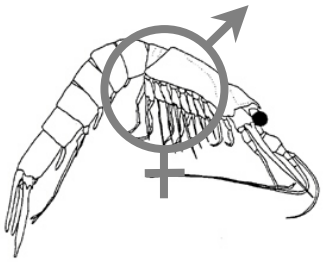
In **hoofdstuk 9**, werden sediment en mysidaceeën (*N. integer*) bemonsterd in het Schelde estuarium, een van de grootste en meest vervuilde estuaria van West-Europa. De concentraties van een reeks gekende hormoonverstoorders werd bepaald in deze stalen, namelijk organotins, polygebromeerde difenylethers, hexabromocyclododecane, tetrabromobisfenol-A, nonylfenolethoxylaten en hun transformatieproducten nonylfenol en nonylfenolethercarboxylaten. Bovendien werd de *in vitro* oestrogene en androgene activiteit bepaald in extracten van water en sediment van het Schelde estuarium. Significante oestrogene potentie, zoals bepaald door de gisttest, werd teruggevonden in sediment -en waterstalen van de verschillende bemonsteringsplaatsen. Er werd echter geen androgene activiteit vastgesteld. Dit was de eerste gerapporteerde veldstudie naar concentraties van hormoonverstoorders in estuariene mysidaceeën. De hoge concentraties die werden teruggevonden zijn een belangrijke indicatie dat verder onderzoek noodzakelijk is om de effecten van deze stoffen te bestuderen in veldpopulaties van aasgarnalen.

In **hoofdstuk 10**, werden de seizoens -en plaatsgebonden patronen in energie allocatie van *N. integer* uit het Schelde estuarium bestudeerd gedurende een periode van twee jaar met behulp van de CEA bepaling. De totale energie-inhoud was relatief constant, terwijl de individuele energiefracties van *N. integer* differentieel beïnvloed werden door staalnameseizoen en staalnameplaats. Seizoenseffecten op het gewicht van *N. integer* werden vastgesteld en konden gerelateerd worden aan de populatiebiologie van deze soort in de Schelde, terwijl plaatsgebonden effecten op het gewicht van deze soort mogelijks het gevolg zijn van site-specifieke vervuiling en de effecten hiervan op de energie allocatie. Deze effecten werden vastgesteld in de twee meest stroomopwaarts gelegen staalnameplaatsen, Doel en Antwerpen. Deze plaatsen worden beschouwd als zijnde het meest vervuild en werden gekarakteriseerd door significante toenames in energieconsumptie, wat aanleiding gaf tot een verlaagde CEA. Door de recente verbetering in het zuurstofregime van de Schelde ter hoogte van de bemonsterde sites in deze studie, kan verwacht worden dat *N. integer* verder stroomopwaarts zal migreren, analoog aan de verspreidingspatronen van deze soort in andere Europese estuaria. Het zal daarom belangrijk zijn om de fysiologische effecten en de potentiële effecten op populatieniveau te bestuderen in deze vervuilde sites. Deze studie toonde aan dat de CEA bepaling interessante perspectieven biedt in het veld als

*in situ* biomarker voor toxicant-geinduceerde effecten.

In **hoofdstuk 11**, werd de bruikbaarheid van de testosteron metabolisatie bepaling geëvalueerd bij *N. integer* in het Schelde estuarium. Mysidaceeën werden bemonsterd gedurende drie campagnes in de periode 2001-2002 en metabolisatie-experimenten werden opgezet met testosteron als substraat. De effecten op het fase I en II metabolisme waren significant verschillend tussen de verschillende campagnes. De plaatsgebonden effecten op de hydroxylatie waren onduidelijk, terwijl de productie van oxido-gereduceerde testosteron-metabolieten lager was in meer stroomopwaarts gelegen locaties tijdens alle campagnes. Deze studie toonde aan dat mysidaceeën van verschillende plaatsen in het Schelde estuarium over een verschillende metabolisatiecapaciteit beschikken. De huidige dataset is echter te beperkt om sluitende conclusies toe te laten. Een verdere doorgedreven toepassing van deze technieken in het laboratorium en in het veld moet echter toelaten om in de toekomst *in situ* effecten van hormoonverstoorders te detecteren.

In **hoofdstuk 12**, werden de algemene conclusies en toekomstperspectieven van dit doctoraatsonderzoek geformuleerd. Samenvattend werd in deze doctoraatsstudie de gevoeligheid van een aantal hormoongestuurde processen, namelijk het testosteron -en energiemetabolisme, voor de effecten van hormoonverstoorders aangetoond bij *N. integer*. Deze eindpunten kunnen bijgevolg gebruikt worden als bioassays voor de evaluatie van de effecten van hormoonverstoorders.



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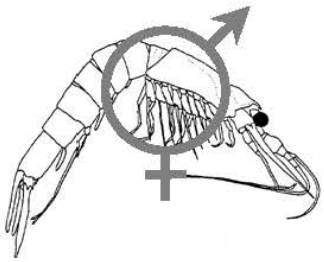

# Curriculum vitae

## *Curriculum vitae*

### Personalia

Name: Tim Verslycke

Date of Birth: October 5th, 1975

Place of Birth: Sint-Amandsberg, Belgium

### Record of education

1987-1993 St-Jan Berchmanscollege, St-Amandsberg, Belgium. Mathematics-Sciences. Honors

1993-1994 Northern Bedford County High School (AFS exchange student), Loysburg, PA, USA. College-Prep. High Honors

1994-1999 Ghent University, Ghent, Belgium. Bio-engineer, option environmental technology. Honors

Thesis: Study of the endocrine metabolism of *Lymnaea stagnalis* exposed to endocrine-disrupting chemicals (in Dutch)
*Promotor*: Prof. Dr. Colin Janssen; *Co-promotor*: Prof. Dr. Wim De Coen

1999-2003 Ghent University, Ghent, Belgium. Doctoral training program in the agricultural and applied biological sciences

Thesis: Endocrine disruption in the estuarine invertebrate *Neomysis integer* (*Crustacea: Mysidacea*)
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### Professional Employment

1999-2003 Ghent University, Ghent, Belgium. PhD student. Laboratory of Environmental Toxicology and Aquatic Ecology

Grant from the Flemish Institute for the Promotion of Innovation by Science and Technology (IWT-V, 1999-2003)

2003-2005 Woods Hole Oceanographic Institute, Woods Hole, MA, USA. Ocean Life Institute

Ocean Life Institute Postdoctoral Fellowship (WHOI, 2003-2005)

## Publications

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Lock K, Verslycke T, Janssen CR (2003) Energy allocation in brachypterous versus macropterous morphs of the pygmy grasshopper *Tetrix subulata*. *Journal of Insect Physiology* (submitted).

Verslycke T, Roast SD, Widdows J, Jones MB, Jansen CR (2003) Cellular energy allocation and scope for growth in the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) following chlorpyrifos exposure: a method comparison. *Journal of Experimental Marine Biology and Ecology* (submitted).

Verslycke T, Vercauteren J, Moens L, Sandra P, Janssen CR (2003) Uptake and effects of tributyltin in the estuarine mysid shrimp *Neomysis integer* (Crustacea: Mysidacea). *Marine Environmental Research* (submitted)

Verslycke T, Poelmans S, De Wasch K, De Brabander HF, Janssen CR. Testosterone metabolism in field populations of the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) of the Scheldt estuary. *Environmental Pollution* (submitted).

Verslycke T, Poelmans S, De Wasch K, De Brabander HF, Janssen CR (2003) Testosterone and energy metabolism in the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) following exposure to endocrine disruptors. *Environmental Toxicology and Chemistry* (submitted).

## Reports

Vandenbergh G, Verslycke T, Janssen C, De Coen W, Comhaire F, Dhooge W and Versonnen B, Arijs K, Vandenbergh G, Du Four V, Verslycke T, Janssen C (2000) Community Programme of Research on Environmental Hormones and Endocrine Disrupters (COMPREHEND): Technical Report on *in situ* exposures in Belgium.

Vandenbergh G, Verslycke T, Janssen C, De Coen W, Comhaire F, Dhooge W, Callebaut K (2001) Evaluation of the impact of endocrine disruptors on the North Sea ecosystem: Final report. Plan for Scientific Support for A Policy of Sustainable Development (DWTC - PODO II). Programme 'Sustainable management of the North Sea'.

## Oral presentations

Verslycke T, De Wasch K, De Brabander HF, Janssen CR (2001) The testosterone metabolism of the estuarine invertebrate *Neomysis integer*: How different are we from shrimp? SETAC Europe 11$^{th}$ Annual Meeting, May 6$^{th}$-10$^{th}$ 2001, Madrid, Spain.

Vandenbergh G, Verslycke T*, Janssen C, De Coen W, Comhaire F, Dhooge W, Callebaut K (* presenter) (2002) Evaluation of the possible impact of endocrine disruptors on the North Sea ecosystem. DWTC. Sustainable management of the North Sea: presentation of the results of the 'Plan for Scientific Support for a Policy of Sustainable Development', January 21$^{st}$-22$^{nd}$ 2002, Brussels, Belgium

Verslycke T, Janssen CR (2002) Can mysid shrimp help us asses possible endocrine disruption in marine environments? VLIZ Young Scientists' Day. March 13$^{th}$ 2002, Sint-Andries (Brugge), Belgium.

Verslycke T, Janssen CR (2002) Can mysid shrimp help us asses possible endocrine disruption in marine environments? SETAC Europe 12$^{th}$ Annual Meeting, May 12$^{th}$-16$^{th}$ 2002, Vienna, Austria.

Verslycke T, Poelmans S, De Wasch K, De Brabander HF, Janssen CR (2002) The steroid metabolism of the European mysid *Neomysis integer* as a potential biomarker for endocrine disruption: lab and field results. Workshop: Recent progress in endocrine disruption and toxicity testing, September 16th-17th 2002, Leuven, Belgium.

Verslycke T, Poelmans S, De Wasch K, De Brabander HF, Janssen CR (2002) The steroid metabolism of the European mysid *Neomysis integer* as a potential biomarker for endocrine disruption. SETAC North America 23rd Annual Meeting, November 16th-20th 2002, Salt Lake City, USA.

Verslycke T, Goethals P, Vandenbergh G, Callebaut K, Janssen CR (2002) Application of rule detection techniques for detecting the possible impact of endocrine disruptors on the North Sea ecosystem. The Colour of Ocean Data: International symposium on oceanographic data and information management with special attention on biological data, November 25th-27th 2002, Brussels, Belgium.

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 CR (2003) The ENDIS-RISKS project: Endocrine disruption in the Scheldt estuary; distribution, exposure and effects. SETAC UK/SETAC Europe Joint meeting on endocrine disruptors in the environment - Linking research and policy, March 31st-April 1st 2003., 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 CR (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.

Poelmans S, De Wasch K, Noppe H, Verslycke T, Ghekiere A, Van Hoof N, De Brabander HF, Janssen CR (2003) Derivatisation in LC-$MS^n$ and GC-$MS^n$: Necessary approaches to meet low detection limits in environmental analysis. SETAC Europe 13th Annual Meeting, April 27th-May 1st 2003, Hamburg, Germany.

Verslycke T, Janssen CR (2003). Endocrine disruption in the estuarine invertebrate *Neomysis integer*: laboratory and field evidence. 2nd Beltox meeting, September 5th 2003, UCL-Woluwe, Belgium.

## Poster presentations

Verslycke T, De Wasch K, De Brabander HF, Janssen CR (2001) The testosterone metabolism of the estuarine invertebrate *Neomysis integer*: How different are we from shrimp? VLIZ Young Scientists' Day, February 23rd 2001, Brugge, Belgium.

Verslycke T, Janssen CR (2001) The energy metabolism of *Neomysis* integer: a new biomarker for endocrine disruption in estuaries? SETAC Europe 11th Annual Meeting, May 6th-10th 2001, Madrid, Spain.

Verslycke T, De Wasch K, De Brabander HF, Janssen CR (2001) The testosterone metabolism of the estuarine invertebrate *Neomysis integer*: How different are we from shrimp? SETAC Europe 11th Annual Meeting, May 6th-10th 2001, Madrid, Spain.

Verslycke T, De Wasch K, De Brabander HF, Janssen CR (2001) The testosterone metabolism of the estuarine invertebrate *Neomysis integer*: How different are we from shrimp? B-IWA Happy Hour, October 22nd 2001, Brussels, Belgium.

Verslycke T, De Wasch K, Poelmans S, De Brabander HF, Janssen CR (2002) The testosterone metabolism of *Neomysis integer*: the quest continues... VLIZ Young Scientists' Day, March 13th 2002, Sint-Andries (Brugge), Belgium

Arijs K, Verslycke T, Versonnen B, Vandenbergh G, Slabbert L, Janssen CR (2002) Estrogenic activity in South African inland waters assessed by a yeast estrogenic screen. SETAC Europe 12th Annual Meeting, May 12th-16th 2002, Vienna, Austria.

Versonnen B, Arijs K, Verslycke T, Janssen CR (2002) Estrogenic effects of ethinylestradiol and dichlorobenzene on zebrafish (*Danio rerio*). SETAC Europe 12th Annual Meeting, May 12th-16th 2002, Vienna, Austria.

Verslycke T, Vandenbergh GF, Versonnen B, Arijs K, Janssen CR (2002) Induction of vitellogenesis in rainbow trout exposed to 17$\alpha$-ethinylestradiol: a method comparison. SETAC Europe 12th Annual Meeting, May 12th-16th 2002, Vienna, Austria.

Verslycke T, De Wasch K, Poelmans S, De Brabander HF, Janssen CR (2002) The testosterone metabolism of *Neomysis integer*: the quest continues... SETAC Europe 12th Annual Meeting, May 12th-16th 2002, Vienna, Austria.

Verslycke T, De Wasch K, Poelmans S, De Brabander HF, Janssen CR (2002) The testosterone metabolism of *Neomysis integer*: the quest continues... Recent progress in endocrine disruption and toxicity testing, September 16th-17th 2002, KUL, Leuven, Belgium.

Arijs K, Verslycke T, Versonnen B, Vandenbergh G, Slabbert L, Janssen CR (2002) Estrogenic activity in South African inland waters assessed by a yeast estrogenic screen. Recent progress in endocrine disruption and toxicity testing, September 16th-17th 2002, KUL, Leuven, Belgium.

Verslycke T, Vandenbergh GF, Versonnen B, Arijs K, Janssen CR (2002) Induction of vitellogenesis in rainbow trout exposed to 17$\alpha$-ethinylestradiol: a method comparison. Recent progress in endocrine disruption and toxicity testing, September 16th-17th 2002, KUL, Leuven, Belgium.

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 CR (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 CR (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.

Versonnen BJ, Goemans G, Verslycke T, Arijs K, Belpaire C, Janssen CR (2003) First monitoring of the occurrence of endocrine disruption in inland populations of eel (*Anguilla anguilla*), roach (*Rutilus rutilus*), rudd (*Scardinius erythrophtalmus*) and tench (*Tinca tinca*) in Flanders (Belgium). SETAC UK/SETAC Europe Joint meeting on endocrine disrupters in the environment - Linking research and policy, March 31st-April 1st 2003, York, U.K.

Ghekiere A, Verslycke T, Janssen CR (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.

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 CR (2003) The ENDIS-RISKS project: Endocrine disruption in the Scheldt estuary; distribution, exposure and effects. SETAC Europe 13th Annual Meeting, April 27th-May 1st 2003, Hamburg, Germany.

Poelmans S, De Wasch K, Schilt R, Van Hoof N, Noppe H, Verslycke T, Janssen C, Courtheyn D, De Brabander HF (2003) The possible transformation of phytosterols to boldenone. EURO FOOD CHEM 12th Annual Meeting, September 24th-27th 2003, Brugge, Belgium.

## Foreign research visits

CSIR, Pretoria, South-Africa: February 14th-March 6th 2000, April 22th-April 29th 2001, November 29th-December 14th 2001.

Plymouth Environmental Research Centre and Plymouth Marine Laboratory, Plymouth, UK: November 19th 2001.

Woods Hole Oceanographic institution, MA, USA: July 11th-July 19th 2003.

## Awards

2001: Best Poster Award: VLIZ Young Scientists' Day, February 23rd 2001, Brugge, Belgium.

2002: SETAC North America Student Travel Award.

## Memberships

Member of the Society for Environmental Toxicology and Chemistry (SETAC)

Member of the Scientific Committee of the Flanders Marine Institute (VLIZ), Belgium

Member of the Scientific Expert Group of the Centre for Environment and Health, Belgium