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

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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 long-term health of individual aquatic 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, so called endocrine disruptors, may result in profound 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 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 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 special reference to the situation for invertebrates, and more specifically crustaceans. The potential application of the biomarker approach in the research field of endocrine disruption is discussed. Finally, the present 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-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 an 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 (cellular energy allocation, CEA) to assess the energy budget was adopted 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 fractional 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 96h 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 their own 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-MSn). 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-MSn 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 indicated 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 cellular 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 cellular 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 under field conditions 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. 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.

In **chapter 12**, general conclusions and future perspectives of this doctoral study were formulated.