

AN GHEKIERE

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

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

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

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CHAPTER 8

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

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ABSTRACT-----

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

8.1. INTRODUCTION

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

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

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

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

8.2. MATERIAL AND METHODS

8.2.1. Study area

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

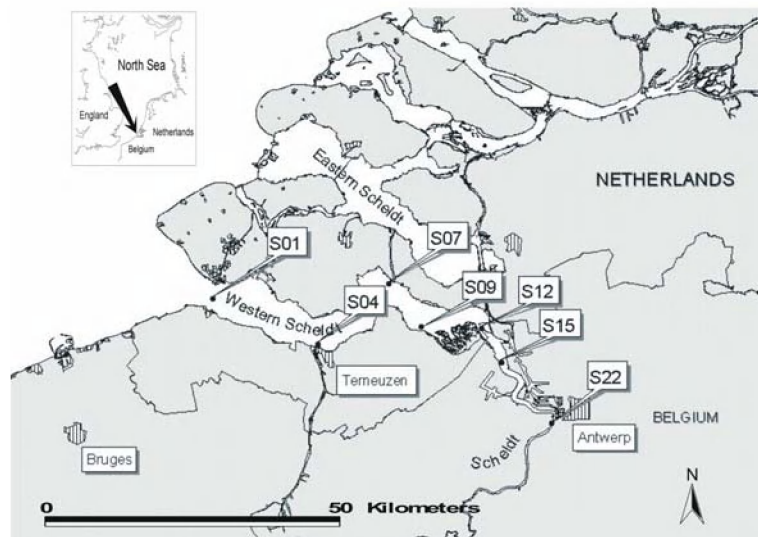


Figure 8.1: Map of the Scheldt estuary with location of the different sampling sites used in the ENDIS-RISKS project (S01, Vlissingen; S04, Terneuzen; S07, Hansweert; S09, Saeftinge; S12, Bath; S15, Doel and S22, Antwerp). Samples from S09, S12, S15, and S22 were analyzed in the present study.

8.2.2. Mysid sampling

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



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

8.2.3. Quantification of vitellin

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

8.2.4. Brood size and standard length determination

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

8.2.5. Statistical analyses

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

8.3. RESULTS

8.3.1. Abiotic measurements

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

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

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

8.3.2. Vitellin levels in mysids collected from different sites

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

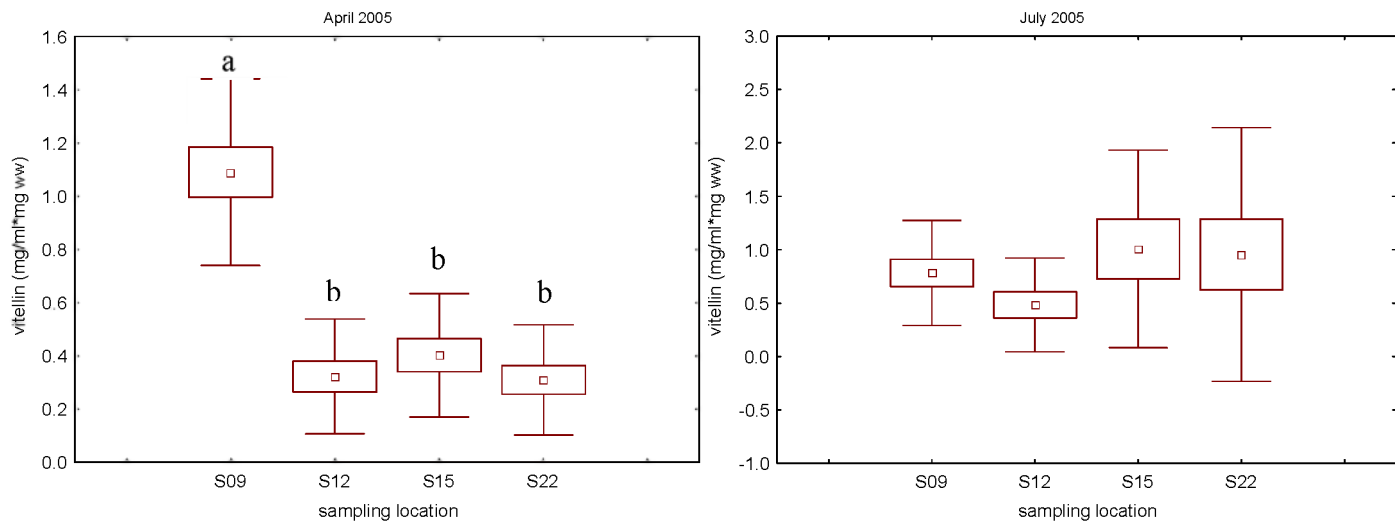


Figure 8.3: Vitellin levels in ovigerous *N. integer* of the Scheldt estuary carrying stage I broods during the sampling campaigns in April (left) and July (right) 2005. Different letters indicate significant differences ($p < 0.05$). Refer to Figure 8.1 for sampling locations.

8.3.2. Brood size and standard length

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

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

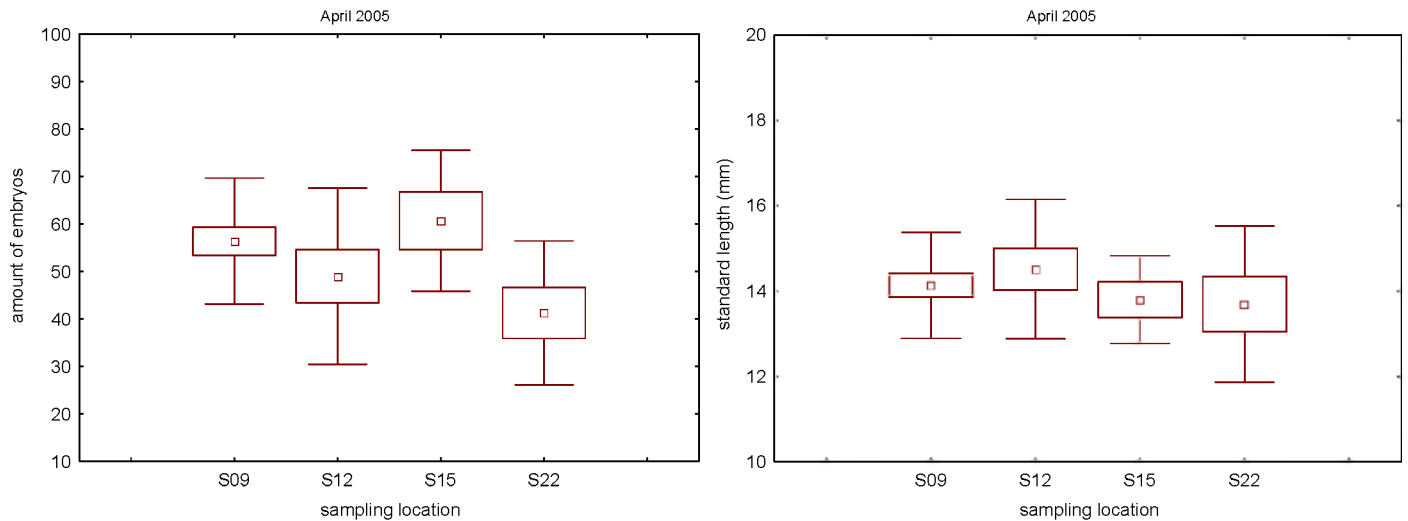


Figure 8.4: Brood size (left) and standard length (right) of female *N. integer* of the Scheldt estuary carrying stage I embryos in April 2005. Refer to Figure 8.1 for sampling locations.

8.3.3. Vitellin levels in ovigerous females with different developmental stages

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

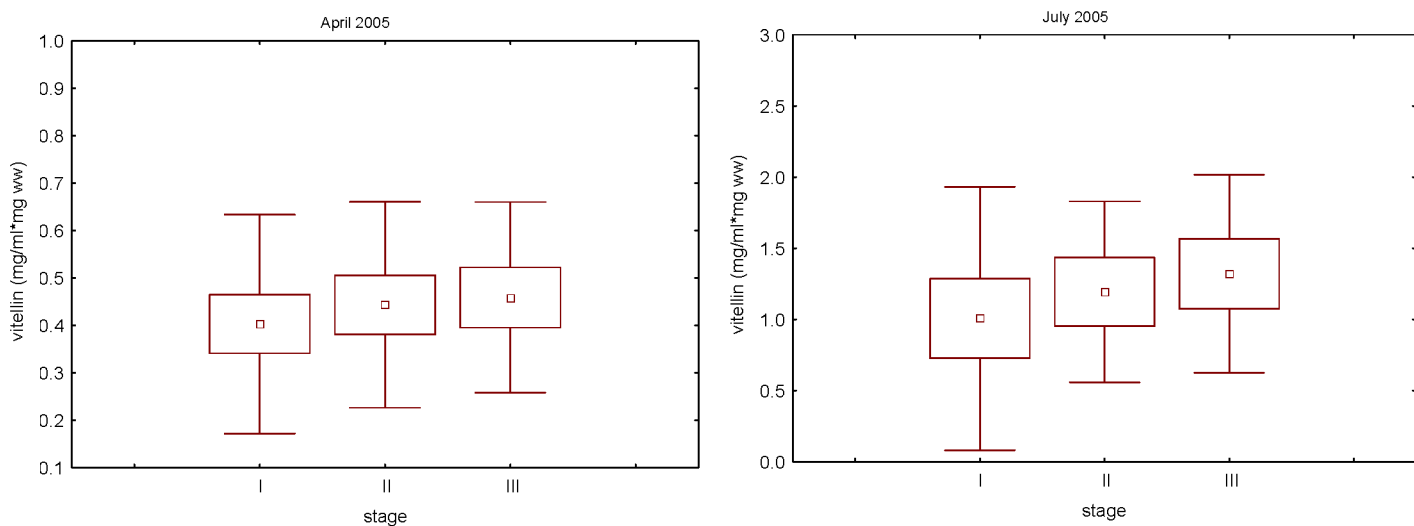


Figure 8.5: Vitellin levels of *N. integer* of the Scheldt estuary carrying embryos in three different developmental stages. Animals were collected at the site S15 (Doel) during the April (above) and July (below) campaigns in 2005. The different developmental stages are described in detail in Chapter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

8.4. DISCUSSION

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

Spatial differences in vitellin levels of ovigorous mysids carrying stage I embryos were evaluated by sampling animals from different sites (S09, S12, S15, S22; see Fig. 8.1) along a salinity gradient in the Scheldt estuary. At these sites, we also collected abiotic (temperature, salinity, and dissolved oxygen) and biotic (brood size and standard length) information. Significant difference in vitellin levels were observed in April 2005, and these could not be correlated with differences in either brood size or standard length. Similarly, no obvious correlations were observed between the abiotic measurements (see Table 8.1) and the *in situ* vitellin levels. The most striking finding was significantly lower levels of vitellin in animals collected from the most upstream sites (S12, S15 and S22) compared to the most downstream site (S09). Verslycke et al. (2004b) previously studied seasonal and spatial patterns in energy allocation of *N. integer* in the Scheldt estuary at the same sites. *N. integer* in this study had less energy at the more upstream sites S15 and S22, where pollution is highest. Lower energy

levels would be expected to impact a high energy-demanding process such as vitellogenesis. This would lead to eggs with lower vitellin levels and of lower quality (Arcos et al., 2003). While these spatial differences were significant in the spring campaign, no such differences were observed during the sampling campaign in July 2005. A more comprehensive dataset needs to be compiled in the future to confirm the spatial trends observed in this preliminary study.

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

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