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Effects of environmental stress on the condition of *Littorina littorea* along the Scheldt estuary (The Netherlands)

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Abstract

The condition of the periwinkle *Littorina littorea*, expressed in terms of its shell morphology, reproductive impairment (i.e. female sterility/intersex, male penis shedding), trematode infestation load, lipid reserves and dry/wet weight ratio, was determined in function of environmental stress along the polluted Western and relatively clean Eastern Scheldt estuary (The Netherlands). The upstream increasing pollution and decreasing salinity levels along the Western Scheldt estuary (Fig. 1) are reflected in the dry/wet weight ratio and lipid content of the periwinkles. Compared to the Eastern Scheldt, female intersex (i.e. indicator of TBT pollution) and sterility occurred more frequently in the Western Scheldt estuary, while male penis shedding was even restricted to the latter estuary. The highest population intersex and sterility incidence was found near the harbour of Vlissingen and reflects potential nautical activities. The number of trematode infested periwinkles did not differ between both estuaries, although local sampling site differences were detected within each estuary, reflecting the complex interactions that exist among parasites, hosts and the local environment. Finally, both estuaries were maximally discriminated from each other based on the shell weight of the periwinkles using a canonical discriminant analysis. Periwinkles with the heaviest shells were found in the Western Scheldt estuary and may reflect growth rate or structural population differences caused by the less favourable living conditions in the Western Scheldt estuary.

Keywords: Condition; Environmental stress; *Littorina littorea*; Morphology; Scheldt estuary

1. Introduction

Natural and anthropogenic pressures often decrease the health and stability of ecosystems, although the precise effects of these stressors and of their interactions on the individual ecosystem components remain largely unknown (O'Connor, 1996; Connell et al., 1999). The complex stress–effect relationships are even more difficult to assess in dynamic environments were natural and/or anthropogenic pressures are known to vary in space and time (Wilson, 2003). Estuarine systems, for example, display an extreme level of spatio-temporal variability in their physicochemical properties. As a result, estuaries are regarded as highly complex and dynamic environments that are governed by a variety of natural and anthropogenic stress related gradients (O'Connor, 1996; Telesh, 2004; Elsdon and Gillanders, 2006). Animals that inhabit such an extreme environment must be capable of maintaining normal metabolic function despite the constant changes in their external

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environment (Verslycke et al., 2004). In general, sublethal stress will induce compensatory changes in the organism's energy metabolism (Widdows and Donkin, 1991; De Coen and Janssen, 2003; Smolders et al., 2004b). Because the majority of the organism's energy budget is used for growth, reproduction and basal metabolism, an increase in energy consumption in the basal metabolism caused by stress will affect the animals condition, reducing its growth, reproduction potential and susceptibility to other stressors (De Coen and Janssen, 2003).

Situated in the south of the Netherlands, the Scheldt estuary forms an ideal setting to assess anthropogenic and/or natural stress related eco(toxico)logical effects. Indeed, the Scheldt estuary consists of two adjacent, but separated arms (i.e. Western and Eastern Scheldt estuary) of which the Eastern Scheldt is relatively clean while the Western Scheldt is highly polluted (e.g. metals, perfluorooctane sulfonic acid, polychlorinated biphenyls, organochlorine pesticides) (De Wolf et al., 2000, 2005; Du Laing et al., 2002; Hoff et al., 2003; Van de Vijver et al., 2003; Danis et al., 2004; Voorspoels et al., 2004; Mubiana et al., 2005). In 1998 the Western Scheldt estuary was ranked among the most polluted estuaries worldwide for metals, especially cadmium, in the dissolved, particulate (Baeyens, 1998) and sediment phase (Zwolsman et al., 1996). As a result, it has been a preferred study site for many heavy metal related ecotoxicological studies (De Wolf et al., 2000, 2005; Du Laing et al., 2002; Danis et al., 2004; Mubiana et al., 2005; Mubiana and Blust, 2006). Environmental heavy metal levels are known to follow a concentration gradient in the Western Scheldt which decreases from the mainland towards the North Sea (i.e. downstream) (Fig. 1) and which is reflected in the tissues of many marine biota (e.g. asteroids, mussels, periwinkles) (De Wolf et al., 2000, 2005; Danis et al., 2004; Mubiana et al., 2005; Mubiana and Blust, 2006). In addition, these heavy metal gradients are opposed by a natural occurring salinity gradient which decreases in an upstream orientated fashion (Fig. 1) and which is known to set the distribution limits of a variety of marine taxa. In contrast to the Western Scheldt, the Eastern Scheldt has a uniform salinity profile and, as pointed out above, is far less polluted (Gerringa et al., 1996).

The periwinkle, *Littorina littorea* occurs in both estuaries and because of its low salinity tolerance limit (i.e. 9.5‰) is able to reach far upstream located sites (Reid, 1996). This periwinkle has been used throughout its North Atlantic distribution area (i.e. White Sea to Southern Portugal and Newfoundland to Virginia) as a heavy metal and TBT sentinel species (e.g. Bryan et al., 1983; Bauer et al., 1997; Minchin et al., 1997; Soto et al., 1997). Indeed, *L. littorea* is able to accumulate a variety of metals in an apparently unregulated fashion while at 15 ng TBT (as Sn l^{-1}) intersex is known to occur (Bauer et al., 1995). The latter masculinisation condition is described as the gradual transformation of the female pallial oviduct into male reproductive characteristics. Four distinct intersex stages have been described and were used as TBT bio-indicator in the Joint Assessment and Monitoring Programme (JAMP) of the OSPAR Commission (ICES, 2004). Recently the shell morphology, intersex and soft tissue heavy metal content were described in *L. littorea* along the Western Scheldt estuary (e.g. De Wolf et al., 2000, 2001a,b, 2004, 2005). Soft tissue heavy metal levels followed the ambient downstream decreasing concentration gradient, the intersex incidence was highest in the close vicinity of the harbour of Vlissingen and Antwerp, while the shell size/weight of *L. littorea* decreased in an upstream orientated fashion (De Wolf et al., 2000, 2001a,b, 2004, 2005). Although the condition of this periwinkle, in terms of its female reproductive impairment (i.e. intersex) and shell size/weight characteristics shows a clear relationship with the ambient stress levels in the Western Scheldt estuary, we want to assess female reproductive impairment and shell morphology in order to compare them with previous studies performed by De Wolf et al. (2001a,b, 2004). Furthermore, we will include trematode infestation incidence, male reproductive impairment (i.e. penis shedding), water content (i.e. dry/wet weight ratio) and energy reserves (i.e. lipid content) as condition parameters. Finally, we will extend the research towards the Eastern Scheldt estuary enabling us to compare both estuaries. Hence, in this study we will assess the condition of *L. littorea* both within and between the Western and Eastern Scheldt estuary, using shell morphological, reproductive impairment (i.e. female intersex and male penis shedding), trematode infestation incidence, dry/wet weight ratio and lipid content data. Given the extreme living conditions in the upstream parts of the Western Scheldt estuary, we hypothesize that periwinkles in that area will have the poorest condition. In addition, we postulate that the condition of *L. littorea* will gradually increase along the downstream parts of the Western Scheldt, ultimately reaching levels that become indistinguishable from the condition status of periwinkles from the Eastern Scheldt estuary.

2. Materials and methods

2.1. Sample collection

In February and March 2004, *L. littorea* was collected at ten sites in the Western (W) and Eastern

(E) Scheldt estuary. In the Western Scheldt these sites included, in order of decreasing pollution and increasing salinity: Hansweert (W1), Hoedekenskerke (W2), Ellewoutsdijk (W3), Borssele (W4), Vlissingen (W5) and Westkapelle (W6). The sampling sites along the Eastern Scheldt were, from east to west: Krabbendijke (E1), Yerseke (E2), Wemeldinge (E3) and Kattendijke (E4) (Fig. 1). At each site 40 animals were collected, except for Hoedekenskerke where only 35 specimens were sampled.

2.2. Morphology

Each of the 395 specimens was morphometrically characterised. Five shell traits were measured to the nearest 0.05 mm using callipers: shell height (HS), shell width (WS), aperture height (HA), aperture width (WA) and shell top height (HT). In addition, total weight (TW) (i.e. shell+soft body parts) and body weight (BW) (soft body parts) were determined to the nearest 0.1 mg and all individuals were sexed based on the presence/absence of the vesicula seminalis after which the sex ratio (male:female) was calculated. Male penis shedding and female intersex were determined. Intersex females were classified to one of the four intersex stages. At stage 1, the female genital opening is enlarged by a proximal slit and the copulatory bursa is split, while at stage 2, the rest of the pallial oviduct is opened ventrally, exposing the internal lobes. At stage 3 the pallial oviduct glands are partially or totally supplanted by a prostate gland, while a penis and a seminal groove can be detected in stage 4 (Bauer et al., 1995; Oehlmann et al., 1998). The intersex index (ISI), measuring the average intersex stage in a particular population (Bauer et al., 1995), and the sterility percentage (i.e. intersex stages 2, 3 and 4) were determined at each site. Finally each animal was inspected for the presence/absence of trematodes.

2.3. Condition-indices

The whole specimen, except the radula and operculum, was homogenised in 4 aliquots of buffer (10 mM TRIS–HCl, 85 mM NaCl, pH 7.4). This homogenate was used to assess the condition of the periwinkles. The dry/wet weight ratio (dw/ww) was calculated after drying a fraction of the homogenate at 60 °C until stable weight to estimate the water content. Total lipids were extracted following the method of Bligh and Dyer (1959). Lipid concentrations were calculated by means of a tripalmitin standard curve.

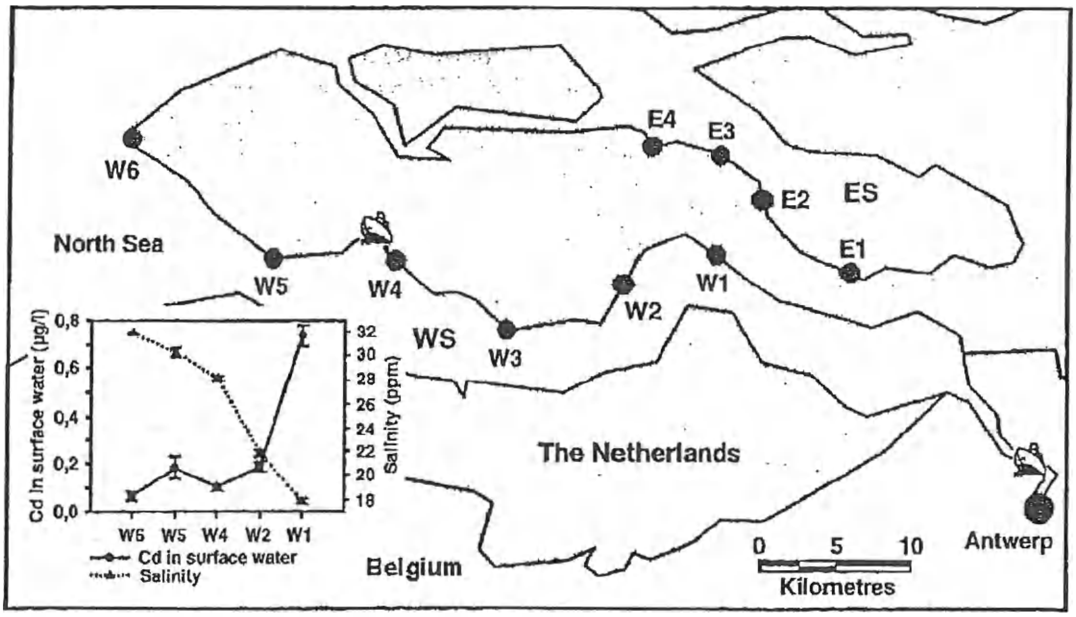

Fig. 1. Study area and sampling sites: Hansweert (W1), Hoedekenskerke (W2), Ellewoutsdijk (W3), Borssele (W4), Vlissingen (W5) and Westkapelle (W6) in the Western Scheldt (WS) and Krabbendijke (E1), Yerseke (E2), Wemeldinge (E3) and Kattendijke (E4) in the Eastern Scheldt (ES) estuary. The full and dashed line plots represent respectively the mean cadmium surface water concentrations, mean salinity values and their standard errors along the Western Scheldt estuary (taken from http://www.waterbase.nl/).

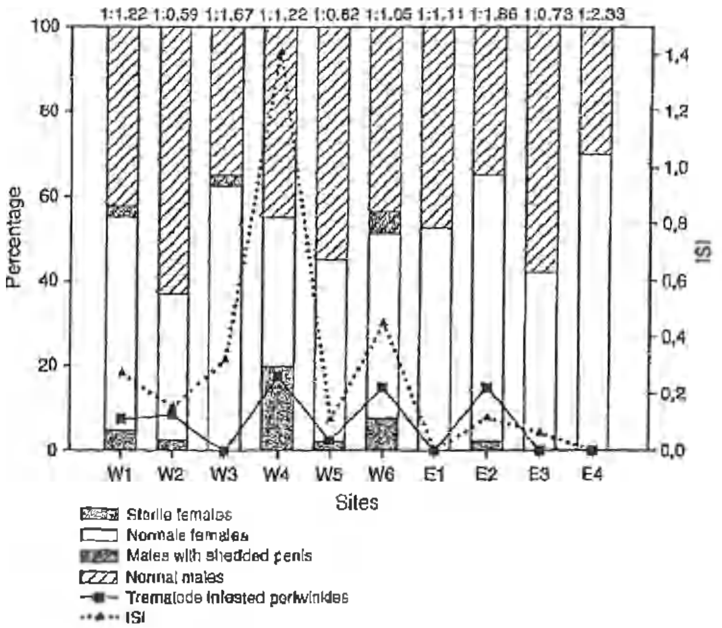

Fig. 2. Distribution of female (normal and sterile) and male (normal and penis shedded) *Littorina littorea* along the Western (W1 Hansweert; W2 Hoedekenskerke; W3 Ellewoutsdijk; W4 Borssele; W5 Vlissingen; W6 Westkapelle) and Eastern (E1 Krabbendijke; E2 Yerseke; E3 Wemeldinge; E4 Kattendijke) Scheldt estuary. Sex ratios (male:female) are given on top of the columns. Percentage of trematode infested individuals and intersex index (ISI) of females is also given.

2.4. Data analysis

To test whether (1) sex ratios, (2) distribution of trematode infested periwinkles, (3) male penis shedding, (4) intersex stage and (5) female sterility differed within and between both estuaries, contingency tables were constructed. The RxC package (Miller, 1997), which employs the Metropolis algorithm to obtain estimates of the exact P value was used to perform the contingency table analysis.

Morphometric patterns were investigated by means of a two-way multivariate analysis of variance (MANOVA) using the factors "sex" and "site" to test differences within and between estuaries. Hereafter, post hoc Tukey tests were performed to detect differences among sites. These analyses were performed using the software package STATISTICA, version 6 for Windows (Statsoft, 2001).

Differences in dw/ww ratio and lipid concentrations were analysed separately, with shell height as a covariate, using the PROC MIXED module of the SAS/STAT® software package, Version 9.1 of the SAS System for Windows (SAS Institute, 2004). Unlike classical analysis of variance (ANOVA) techniques which are based on ordinary least squares computations, PROC MIXED is a likelihood based method. The covariance parameters are estimated via the maximum likelihood method (REML), while the parameter means are estimated via the generalized least squares method (Littell, 2002). The PROC MIXED model included the dependent variables: site, estuary (est), sex, site*sex and est*sex, with site nested within est as a random variable. Post hoc Tukey–Kramer tests were subsequently performed, in order to establish differences among sites within the Western and within the Eastern Scheldt estuary.

Table 1
Results of the contingency tables constructed to test for differences in *sex ratio distribution, trematode infestation, penis shedding, intersex* stage and sterility within and between the estuaries

	Within Western Scheldt estuary	Within Eastern Scheldt estuary	Between estuaries
Sex	0.2986	0.0517	0.2621
Trematode infestation	0.0220*	0.0005*	0.0700
Shedding	0.2311	1.0000	0.3014
Intersex stage (0/1/2/3/4)	<0.0001*	0.5220	0.0006*
Sterility	0.0052*	0.6889	0.0011*

*$P<0.05$.

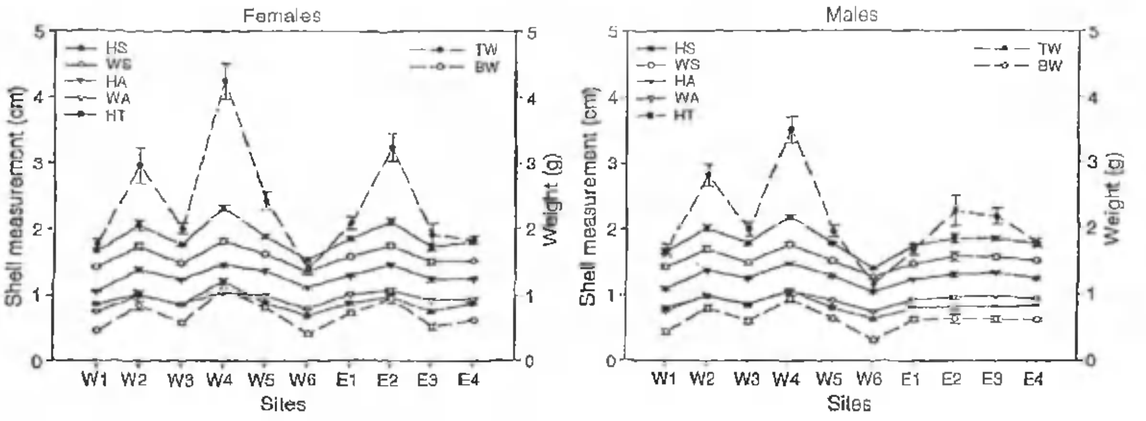

Fig. 3. Mean shell height (HS), shell width (WS), aperture height (HA), aperture width (WA), shell top height (HT), total weight (TW) (i.e. shell+soft body parts), body weight (BW) (soft body parts) and standard errors for females (left) and males (right) of *Littorina littorea* collected along the Western (W1 Hansweert; W2 Hoedekenskerke; W3 Ellewoutsdijk; W4 Borssele; W5 Vlissingen; W6 Westkapelle) and Eastern (E1 Krabbendijke; E2 Yerseke; E3 Wemeldinge; E4 Kattendijke) Scheldt estuary.

Finally, a standard canonical discriminant analysis (CDA) was performed on the complete morphometric and condition index data set. The mean values of the first two canonical variables (CV's) were used to plot the different sites in a biplot superimposing the morphometric and condition-related data as vectors, which were used to perform the CDA analysis. The CDA was performed using the software package STATISTICA version 6 for Windows (Statsoft, 2001).

3. Results

3.1. Sex, sterility, penis shedding, trematode infestation incidence and intersex

An overview of the sex ratios, female sterility, penis shedding, percentage of trematode infested individuals and intersex stage are given in Fig. 2. Within and between the estuaries no significant differences were found for the sex ratio distribution nor for the male penis shedding distribution (Table 1). However, males with a shedded penis were only found in sites along the Western Scheldt estuary. Significant differences in female sterility and intersex stage distribution were found within the Western Scheldt estuary and between both estuaries (Table 1). Along the Western Scheldt estuary W4 had the highest number of sterile females (i.e. 36%), the highest ISI (i.e. 1.41) and was the only site where intersex stage 4 females were observed. In comparison to the Eastern Scheldt estuary, the Western Scheldt estuary revealed (1) higher sterility percentage (i.e. 13% vs. 1%) and (2) more females that displayed the various intersex stages (i.e. 22% vs. 3%). Furthermore, along the Western Scheldt estuary all four intersex stage were found, while in the Eastern Scheldt estuary only intersex stage one and two could be detected. Significant differences in distribution of trematode infested periwinkles were found within the two estuaries (Table 1). Within the Western Scheldt estuary periwinkles from sampling sites W4 and W6 had the highest trematode load, 17.5% and 15% respectively, while in W3 no trematodes could be detected. Furthermore, no trematode infested specimens were found in the Eastern Scheldt estuary except for E2 (i.e. 15%). Finally,

Table 2
Results of the two-way MANOVA for the morphological data (i.e. shell height, shell width, aperture height, aperture width, shell top height, total weight and body weight) within and between the estuaries, contrasting the factors "site/est" and "sex"

Effect	Wilks value	Df1	Df2	*P* value
Within Western Scheldt estuary				
Site	0.0934	35	911	<0.0001*
Sex	0.8815	7	216	0.0003*
Site*Sex	0.7470	35	911	0.0018*
Within Eastern Scheldt estuary				
Site	0.2284	21	414	<0.0001*
Sex	0.9204	7	144	0.0960
Site*Sex	0.7461	21	414	0.0030*
Between estuaries				
Est	0.6982	7	382	<0.0001*
Sex	0.9300	7	382	0.0002*
Est*Sex	0.9867	7	382	0.6442

*$P<0.05$.

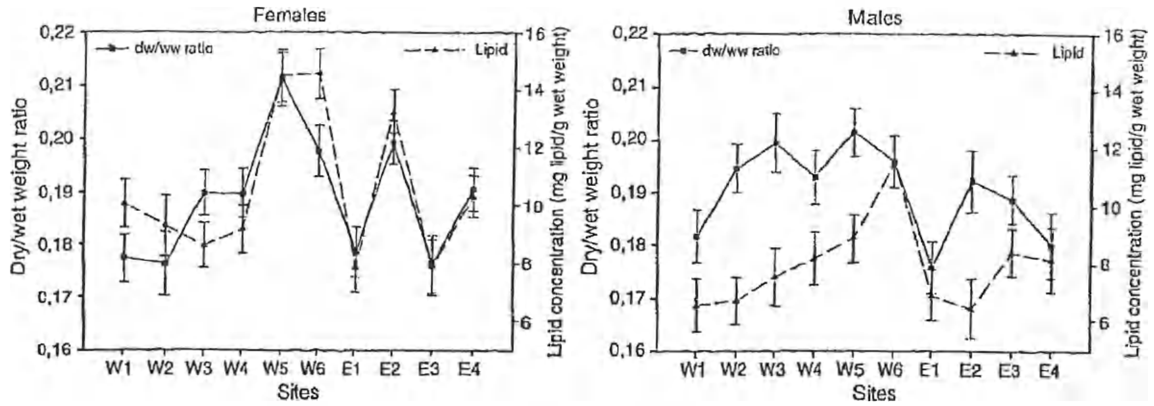

Fig. 4. Mean dry/wet weight ratio (dw/ww), lipid concentration and standard errors for female (left) and male (right) *Littorina littorea* collected along the Western (W1 Hansweert; W2 Hoedekenskerke; W3 Ellewoutsdijk; W4 Borssele; W5 Vlissingen; W6 Westkapelle) and Eastern (E1 Krabbendijke; E2 Yerseke; E3 Wemeldinge; E4 Kattendijke) Scheldt estuary.

female sterility, ISI and periwinkle trematode infestation followed a similar distribution pattern along the entire Scheldt estuary (Fig. 2).

3.2. Morphometric data

Mean values of the morphometric data (i.e. HS, WS, HA, WA, HT, TW and BW) and standard errors for female and male *L. littorea* are presented in Fig. 3. Within the Western Scheldt estuary significant morphometric differences were detected for the factors "site" and "sex" using a two-way MANOVA (Table 2). Post hoc Tukey tests indicated that individuals from W4 were significantly larger and heavier than individuals from the other sites, except for those from W2, while animals from W6 were significantly smaller and lighter compared to animals sampled at other sites, except for those at W1. Furthermore, females were larger and heavier than males. In contrast, within the Eastern Scheldt estuary significant morphometric differences could only be detected among sites, but not between the sexes (Table 2). Post hoc Tukey tests showed that individuals from E2 were significantly larger and heavier than those from other sites. Moreover, within

Table 3
Results of the PROC MIXED model performed by SAS/STAT to test for differences in dry/wet weight ratio (left side) and lipid concentration (right side) within and between the estuaries using sex and site nested in estuary (est) as variables and shell height as covariable

Effect	Dry/wet weight ratio				Lipid concentration			
	Df1	Df2	*F* value	*P* value	Df1	Df2	*F* value	*P* value
Within Western Scheldt estuary								
HS	1	221	4.70	0.0312*	1	221	3.79	0.0528
Sex	1	221	3.54	0.0611	1	221	28.24	<0.0001*
Site	5	221	8.08	<0.0001*	5	221	14.91	<0.0001*
Site*Sex	5	221	1.56	0.1729	5	221	2.34	0.0430*
Within Eastern Scheldt estuary								
HS	1	149	43.96	<0.0001*	1	149	18.78	<0.0001*
Sex	1	149	0.48	0.4897	1	149	16.57	<0.0001*
Site	3	149	1.74	0.1620	3	149	2.25	0.0854
Site*Sex	3	149	1.30	0.2759	3	149	3.56	0.0159*
Between estuaries								
HS	1	9	30.81	0.0004*	1	9	15.92	0.0032*
Sex	1	8	2.36	0.1628	1	8	14.96	0.0048*
Est	1	8	4.03	0.0795	1	8	1.95	0.1999
Est*Sex	1	8	1.33	0.2828	1	8	0.00	0.9771

*$P<0.05$.

Table 4
P values derived from the post hoc Tukey–Kramer tests for the Western Scheldt estuary (W1 Hansweert; W2 Hoedekenskerke; W3 Ellewoutsdijk; W4 Borssele; W5 Vlissingen; W6 Westkapelle)

	W1	W2	W3	W4	W5	W6
W1	X	1.0000	0.0824	0.9998	<0.0001*	0.0010*
W2	0.6490	X	0.1426	0.9990	0.0001*	0.0265*
W3	0.9949	0.8700	X	0.4257	0.2561	0.7457
W4	0.9216	0.9982	0.9881	X	0.0025*	0.1581
W5	0.0022*	<0.0001*	0.0002*	0.0002*	X	0.9937
W6	<0.0001*	<0.0001*	<0.0001*	<0.0001*	0.1550	X

The P values obtained for the dry/wet weight ratio are given above the diagonal and the P values for the lipid concentrations are shown below the diagonal. *$P<0.05$.

the Western and within the Eastern Scheldt estuary the interaction between the factors "site" and "sex" was significant, indicating that morphometric differences between the sexes are site-dependent at both estuaries.

3.3. Condition-indices

Female and male mean dw/ww ratio and lipid concentrations are given together with their standard errors in Fig. 4. Both condition-indices display a similar pattern.

Within the Western Scheldt estuary significant dw/ww ratio and lipid concentration differences could be detected among the sampled sites (Table 3) and reveal a downstream increasing gradient. Indeed, post hoc testing indicated that the two sites closest to the North Sea (i.e. W5 and W6) had a significantly higher dw/ww ratio and lipid content than the two most inland located sites (i.e. W1 and W2). Furthermore, W4 had a significantly lower dw/ww ratio compared to W5 (Table 4). Finally, sex related site dependent lipid concentration differences were detected within the Western Scheldt while dw/ww ratio did not reveal differences between male and female periwinkles (Table 3).

In contrast to the Western Scheldt estuary, no significant differences for dw/ww ratio could be found within the Eastern Scheldt estuary nor between both estuaries. However, within the Eastern Scheldt estuary significant lipid concentration differences were found between the sexes and a significant interaction between "site" and "sex" was detected as well (Table 3). This resulted from higher female lipid levels at all sites except at E3. Lipid concentrations between the estuaries only differed significantly for the factor "sex" (Table 3) which implies that females contain higher lipid levels.

3.4. Combined analysis

A CDA was performed for the two sexes separately. The mean values of the first two CV's were used to plot all sampling sites, while the morphometric and condition-related dependent variables were superimposed as vectors (Fig. 5).

The first CV of the female CDA described 36.9% of the total variation and is mainly an expression of the shell weight, contrasting TW (−3.2940) and BW (1.7390) with negative values indicating specimens

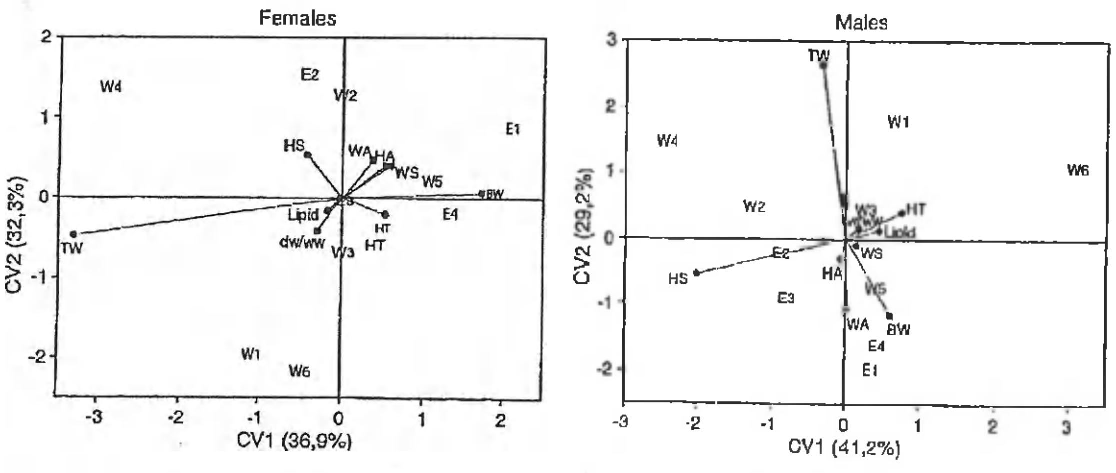

Fig. 5. Graphical representation of the first two canonical variables for female (left) and male (right) *Littorina littorea*, representing all sampling sites collected along the Western (W1 Hansweert; W2 Hoedekenskerke; W3 Ellewoutsdijk; W4 Borssele; W5 Vlissingen; W6 Westkapelle) and Eastern (E1 Krabbendijke; E2 Yerseke; E3 Wemeldinge; E4 Kattendijke) Scheldt estuary.

with heavier shells (i.e. heavy TW with low BW). Specimens from the Western Scheldt estuary have a wider shell weight range compared to specimens from the Eastern Scheldt estuary. The heaviest shells were found in female periwinkles from W4 and at the Western Scheldt in general, while females from the Eastern Scheldt estuary tend to have lighter shells and relatively higher BW. CV2 described an additional 32.3% of the variation and appeared to reflect shell shape, contrasting HS (0.5313) and HT (−0.2102). Positive values along this axes indicated specimens with large shell sizes, relatively small shell top heights and large and broad aperture sizes. Again, specimens from the Western Scheldt estuary have a much wider shell shape range compared to specimens from the Eastern Scheldt estuary. Finally, the lipid, dw/ww ratio and TW are highly correlated.

The first CV of the male CDA explained 41.2% of the total variation and is mainly an expression of shell shape, contrasting HS (−2.0171) and HT (0.7584). Negative CV1 values indicated more elongated periwinkles with large shell and aperture heights but with relatively small shell top heights and narrow shells and aperture widths. Specimens from the Western Scheldt have a wider shell shape range compared to specimens from the Eastern Scheldt estuary. The second CV described an additional 29.2% of the variation and is mainly an expression of shell weight, contrasting TW (2.6265) and BW (−1.1574). The heaviest shells are found in W1 and W4 and along the Western Scheldt in general, while male periwinkles from the Eastern Scheldt estuary have lighter shells and have relatively higher body weights. Yet again, specimens from the Western Scheldt estuary have a wider shell weight range compared to specimens from the Eastern Scheldt estuary. Finally, the vectors lipid, dw/ww ratio and HT are highly correlated.

In summary, the CDA for both females and males show that the main difference between the estuaries can be explained by shell weight, which is relatively higher in the Western Scheldt than in the Eastern Scheldt estuary. Furthermore specimens from the Western Scheldt always have a broader shell weight and shell shape range compared to the specimens from the Eastern Scheldt estuary, while the vectors of both condition indices (i.e. dw/ww ratio and lipid) are highly correlated independent of the sex which is considered.

4. Discussion

Within the Western Scheldt estuary significant differences were detected in the condition of the periwinkles. Indeed, shell morphology, reproductive impairment (i.e. intersex and female sterility distribution), trematode infestation incidence and condition-indices (i.e. dw/ww ratio and lipid content) differed within the Western Scheldt estuary. In contrast, within the Eastern Scheldt estuary only significant differences in shell morphology characteristics and trematode infestation incidence could be detected. Finally, shell morphology and reproductive impairment (i.e. intersex and female sterility distribution) differed significantly among periwinkles from both estuaries.

No sex ratio differences were detected within and between the estuaries, which is in accordance with previous studies performed by De Wolf et al. (2001b, 2004) on *L. littorea* along the Western Scheldt estuary. In addition, penis shedding did not differ within and between both estuaries. Nevertheless, the observation that penis shedding occurs solely in the Western Scheldt estuary may be related to environmental stress levels which are expected to be higher in the Western Scheldt estuary. Indeed, Deutsch and Fioroni (1992) described stress induced penis shedding in *L. littorea*.

Intersex and the resulting sterility, which are known to be induced by TBT as antifouling agent used in boat paints (Bauer et al., 1995, 1997; Matthiessen and Gibbs, 1998; Barroso et al., 2000), reflect potential ship yard activities. Indeed, within the polluted Western Scheldt estuary W4, which is nearest to the harbour of Vlissingen (Fig. 1), has the highest ISI (i.e. 1.41) and sterility percentage (i.e. 36.36%). This ISI can be considered as an average value when compared to other studies (i.e. min–max) (Oehlmann et al., 1998; Barroso et al., 2000; Evans et al., 2001). Previous intersex/sterility studies performed on *L. littorea* along the Western Scheldt estuary during 1998 (De Wolf et al., 2001b), 2000 and 2002 (De Wolf et al., 2004) also indicated Borssele (i.e. W4) as the site with the highest ISI (i.e. 1.26, 1.30 and 0.53) and highest sterility percentage (i.e. 33%, 30.40% and 15.78%). Furthermore De Wolf et al. (2004) reported an ISI and sterility percentage decline along the Western Scheldt, which may be the result of the Dutch ban on the usage of TBT-containing antifouling paints for ships and vessels up to 25 m in length. The only exceptions were Hansweert (i.e. W1) and Vlissingen (i.e. W5) where there was an ISI increase in 2002, after it had dropped in 2001 compared to 2000. Our results indicate that the ISI values in W1 and W5 have decreased even further compared to 2002. However, compared to 2002 ISI values in W2, W3 and W4 have increased and in W2, W4 and W5 the sterility percentages also augmented. Our study reports the highest ISI and sterility percentage ever detected at W4, which contradicts the previous described decreasing ISI value trend in the Western Scheldt estuary (De Wolf et al., 2004). Nevertheless, when interpreting ISI values one has to be cautious because (1) intersex can only be induced in juvenile and sexually immature females and its intensity depends on the ontogenetic stage of development during TBT exposure (Bauer et al., 1997), (2) intersex is an irreversible process (Oehlmann et al., 1998) and (3) ISI values in *L. littorea* exhibit a kind of background noise with values between 0 and 0.4 (Oehlmann et al., 1998). Hence ISI values of animals collected in a particular year, do not necessarily reflect the TBT pollution at that specific point in time. Knowing that *L. littorea* reaches maturity at 2–3 years and its life span is 5–10 years (Jackson, 2005), the ISI values are a result of the pollution that spans a maximum period of 8 years prior to the sampling date. Nonetheless, it is clear that W4 is a hot-spot for TBT pollution. Since 01/01/2003 there is a ban on the use of TBT based antifouling paints throughout the world and by 01/01/2008 all TBT based coatings on ships will have to be removed (Omae, 2003). Hence, we predict that ISI values will decline still further along the Western Scheldt estuary in the future.

Within the Eastern Scheldt estuary neither ISI nor sterility differences were detected among the sites. Despite our assumption that this estuary is relatively clean, we did detect intersex in the sampled females of sites E2 (i.e. 7.69%) and E3 (i.e. 6.25%). Moreover, effects of TBT pollution have already been reported for the Eastern Scheldt estuary in studies by Mensink et al. (1996, 1997). They reported imposex in the common whelk, *Buccinum undatum*, between September 1992 and March 1995 (Mensink et al., 1996) and during several seasons in 1995 (Mensink et al., 1997).

Trematode infestation incidence did not differ between the two estuaries, which can be explained by the fact that pollutants affect the susceptibility to parasitism in multiple ways. Pollutants can increase parasitism by increasing the host susceptibility or by increasing the abundance of intermediate hosts and vectors (Lafferty and Kuris, 1999). They can also decrease parasitism if (1) infected hosts suffer differentially high mortality, (2) parasites are more susceptible to pollution than the hosts or (3) the pollutants negatively affect intermediate hosts or vectors (Lafferty and Kuris, 1999). Besides pollutants, there are other factors that influence parasitism. A study by Krist et al. (2004) stated that the host condition does not affect the susceptibility to infection, but hosts in poor condition have higher parasite-induced mortality than hosts in good condition. Moreover, for intertidal species that live at the lowest limit of their stress tolerance limit, parasite infestation may result in reduced survivorship (Mouritsen and Poulin, 2002). Parasitized *L. littorea* have an overall lower survival rate than uninfected specimens (Mouritsen and Poulin, 2002) and are known to move slower and cover shorter distances, making them more susceptible to predation (Davies and Knowles, 2001). Interactions between parasites (i.e. trematodes), hosts and environment probably caused individuals from each site to react uniquely and as a result no clear pattern can be observed along the pollution gradient of the Western Scheldt estuary nor between the two estuaries. Nonetheless parasitism may have affected the morphological outcome and/or female sterility observations. Indeed, parasitic infestation may effect the growth rate and causes gigantism in gastropods (Mouritsen and Jensen, 1994; Reid, 1996; Krist, 2000; McCarthy et al., 2004). The sites with the largest specimens in the Western Scheldt (W4) and the Eastern Scheldt (E2) estuary were also the most heavily trematode infested, thus supporting this hypothesis. However, at W6 the smallest sized periwinkles were found, despite W4 and W6 shared comparable trematode infestation incidence rates. In addition to the impact on growth rate, parasites will damage their hosts to a certain degree, with effects ranging from minor metabolic changes to severe tissue destruction (Mouritsen and Poulin, 2002), even shrinkage or complete disappearance of the pallial oviduct has been reported (Reid, 1996). This latter might explain the similar patterns that are found among percentage of trematode infested specimens, intersex and female sterility. It is, indeed, possible that tissue damage of the pallial oviduct caused by the parasites is mistakenly interpreted as intersex, since this is also an abnormality of the pallial oviduct (Bauer et al., 1997; Casey et al., 1998). However, the morphological and ISI/sterility patterns remain when the trematode infested periwinkles are removed from the analysis.

The morphological variation that was observed among populations may be attributed to a variety of factors including genetic and/or environmental effects (Kemp and Bertness, 1984). In *L. littorea* however, the genetic differences among populations is reduced by its planktonic development (Reid, 1996). Therefore, differences in shell morphology in this species are expected to be largely determined by non-genetically based phenotypic responses to the environment (i.e. parasitism, pollution, population density, predation, salinity, wave action, water temperature, etc.), which are manifested through growth rate and/or population structure differences. A study performed by De Wolf et al. (2001a) on shell morphology along the Western Scheldt estuary stated that the shell size showed a clear transition between two salinity ranges (i.e. 10–20‰ and 21–30‰)

and animals would attain their largest size within a salinity range of 21–30‰. In our study however, *L. littorea* collected within a salinity range of 21–30‰ (i.e. W2–W6) did not always reach their largest size. Periwinkles that were gathered at W6 (i.e. highest salinity) were smaller than periwinkles that were sampled within the same salinity range, but did not significantly differ from the specimens sampled within a salinity range of 10–20‰ (i.e. W1). Although we expected the largest periwinkles at the least polluted sites such as W5 and W6 because of conditional restraints, the largest animals along the Western Scheldt estuary were found in W4 (intermediate pollution), which was not significantly different from W2 (relatively polluted). The extreme shell size of the specimens at W4 may be explained by the high percentage of trematode infested animals, which can lead to gigantism as discussed above or by the higher water temperature at this site. Indeed, sampling of this site was performed near the outflow of cooling water from a nuclear power plant. At higher water temperatures calcium carbonate is more available and deposition and maintenance of shells is less difficult (Trussell and Etter, 2001) than at low temperatures where calcification is more costly (Reid, 1996). Comparison between the two estuaries showed that periwinkles from the Eastern Scheldt estuary were larger than those from the Western Scheldt estuary which may be caused by the more favourable living conditions in the former estuary since there is no salinity nor pollution gradient present there. It is known that a high growth rate leads to the production of thin-walled shells since the rate at which shell material can be deposited is limited (Kemp and Bertness, 1984; Reid, 1996). This is reflected in the CDA analysis which showed that periwinkles from the Western Scheldt have relatively heavier shells than their conspecifics from the Eastern Scheldt estuary. Sexual dimorphism in morphology was detected within the Western Scheldt estuary and between the estuaries. Although some studies on *L. littorea* detected no sexual dimorphism (references in Reid, 1996; De Wolf et al., 2001a), there are other studies that suggest the opposite (Moore, 1937; references in Reid, 1996). These latter studies ascribed the sexual dimorphism to a higher female growth rate (Moore, 1937), female longevity or because males mate preferentially and for longer with larger, thus more fecund females (Reid, 1996). In our study the detected sexual dimorphism in morphology might be stress-induced if the sexes reacted differently to environmental stress as suggested by the sex related lipid content level differences.

The condition of an organism is expected to decrease when it has to live in a suboptimal environment because responses to environmental stress are costly (Smolders et al., 2002, 2004b). Previous studies on the condition of rock crabs and zebra mussels as expressed in terms of their water content showed an increase with increasing contamination of field sites (Depledge and Lundebye, 1996; Smolders et al., 2002). In our study we detected an increase in water content (i.e. decrease in dw/ww ratio) land inwards along the Western Scheldt estuary, whereby the most polluted and least saline sites (i.e. W1 and W2) had a significantly higher water content than the least polluted and most saline sites (i.e. W5 and W6). Nevertheless, pollution may not be the only factor influencing the water content of *L. littorea*. Taylor and Andrews (1988) ascertained that the body water content of *L. littorea* significantly increased when they were acclimated to 60% sea water. The periwinkles also seemed incapable to regulate their water volume after salinity transfer, because they did not return towards their initial water volume after the swelling phase. Therefore, the osmotic behaviour of *L. littorea* is characterised by passive tolerance to large changes in cell volume rather than intracellular osmoregulation (Taylor and Andrews, 1988). Thus, it is difficult to assign the current water content increase to an increase in pollution or a decrease in salinity. Hence, most likely the combination of these two factors resulted in our observed dw/ww ratios. Condition expressed in terms of lipid levels are known to display different patterns depending on the studied pollutants. Sakellarides et al. (2006) stated that the tissue concentration of liposoluble toxicants (e.g. organochlorines) can cause toxic effects that are inversely related to the amount of lipid in an organism. Thus, for a given wet or dry weight tissue concentration, the higher the lipid content, the higher the resistance to the toxicant because a higher proportion of the hydrophobic compound is associated with the lipid and is not fully available to cause toxicity (Sakellarides et al., 2006). A field study by Pastor et al. (1996) using red mullet (*Mullus barbatus*), sea mullet (*Mugil cephalus*) and sea bass (*Dicentrarchus labrax*) reported that higher levels of the organochlorinated contaminants PCBs and DDTs were generally found in red mullet and could be related to the higher lipid content of this species. Another field study in Puget Sound, Washington, USA showed an increased accumulation of lysosomal and cytoplasmic unsaturated neutral lipids in mussels from urban-associated sites (i.e. areas with elevated sediment concentration of anthropogenic contaminants such as PAHs, PCBs, DDTs and toxic elements) (Krishnakumar et al., 1994). Other field studies report a decrease in lipid content with increasing pollution, which can be explained in terms of the energy budget and energy allocation processes of the exposed organisms. For instance Smolders et al. (2004a, 2004b) revealed a negative effect of an industrial effluent on the lipid budget of zebra mussels. Downstream the source of the industrial effluents the lipid budget of the zebra mussel became negative and subsequently restored as the effluents became increasingly diluted. Our study corresponds with the latter study and revealed a gradient in lipid content which opposed the known stress gradient related to pollution increase and salinity decrease in the Western Scheldt estuary. Furthermore, the CDA showed a good correlation between dw/ww ratio and lipid levels. Such a correlation has previously been established between the energy reserves and dw/ww ratio by Smolders et al. (2004b).

5. Conclusion

It is generally accepted that an organism's energy metabolism will alter through stress. The basal metabolism will increase while growth, energy reserves and reproductive potential are diminished. The hypothesis that periwinkles living in the upstream parts of the Western Scheldt estuary would have the poorest condition and that their condition would gradually increase towards the estuary's mouth, is supported by dw/ww ratio and lipid content. Indeed, the condition-indices (i.e. dw/ww ratio and lipid content) follow a pattern that clearly opposes the known pollution and salinity gradient in the Western Scheldt estuary. Additionally, male reproductive impairment (i.e. penis shedding) is only detected in the Western Scheldt estuary and could be related to the increased stress level in this estuary in comparison to the Eastern Scheldt estuary. Furthermore, female reproductive impairment (i.e. intersex and sterility) was observed in both estuaries and could be linked to potential nautical activities. The detected morphological differences between both estuaries might be related to a decrease in growth rate or population structure differences in *L. littorea* caused by the less favourable living conditions along the Western Scheldt estuary.

Nevertheless, further research is necessary to investigate if the condition of *L. littorea* is affected by the metal pollution, by the salinity effects or by a combination of both. Consequently, heavy metals will be measured in the soft tissue of the studied specimens. In addition metallothionein levels (i.e. detoxification protein) will be assessed to study the response of the organism to metal pollution. Finally, this field data set will be viewed in the context of single stressor/response laboratory experiments, enabling us to mathematically quantify adverse condition related effects/risks to specific environmental stressors in the field.

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