# A Field Survey of Estrogenic Effects in Freshwater and Marine Fish in the Netherlands

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## Abstract

Endocrine-active substances (EASs) have been shown to affect several species of fish in different parts of the world. In particular, feminizing effects have been observed in male fish. The large-scale field study in the Netherlands, on which we report here, focused on the potential effects of estrogenic compounds on wild fish. The freshwater bream (*Abramis brama*) and the estuarine flounder (*Platichthys flesus*) were sampled at a large number of locations in the spring and fall of 1999. Average concentrations of the yolk protein vitellogenin (VTG) in blood plasma of male flounders at most investigated locations were not higher than at reference locations. At 2 locations, however, moderately higher concentrations of VTG were observed in the fall. Both locations were situated in the same industrial harbor that also receives effluent from sewage treatment plants (STPs). At some locations, particularly in the spring, VTG concentrations in male bream were significantly higher than in males from a reference location that received little direct contamination. The highest concentrations were observed in individuals collected from a small stream, close to the discharge of a relatively large municipal STP. This was also the only location where considerable histologically visible hermaphroditism occurred, with ovotestes observed in 38% of male bream collected at the location. Hermaphrodotism was not observed in any of the 400 male flounders examined. Because few flounder exhibited elevated plasma concentrations of VTG and hermaphroditism was not observed in the present survey, there seems to be little reason for concern of severe estrogenic effects in flounder that

pass most of their time at open sea and in Dutch estuaries connected to the open sea. In some larger inland waters, moderate estrogenic effects may occur in both flounder and bream. However, the extreme effects observed in male bream from the small stream indicate that, locally in smaller waters, estrogenicity of the aquatic environment is a potential threat to the presence and functioning of fish populations.

## Introduction

This chapter describes some of the findings of the 1999 field survey of the Dutch National Investigation into the occurrence and effects of estrogenic substances in the aquatic environment (in Dutch: Landelijk Onderzoek oEstrogene Stoffen [LOES]), conducted to assess estrogenic effects in wild fish. The primary aim was to apply estrogen-specific response techniques to identify areas of specific concern. Some preliminary results have been published previously by Vethaak, Lahr et al. (2002).

The freshwater bream (*Abramis brama*; *Cyprinidae*) and the euryhaline flounder (*Platichthys flesus*; *Pleuronectidae*) were chosen as indicator species. Both species have already been used in monitoring programs of Dutch waters, so background information was available. Bream commonly occur in large freshwater systems, including both lentic and lotic systems, and spawn from April to June. Bream live and migrate in schools, and although it tends to be a more resident species of fish than flounder, bream migrate on a seasonal basis (albeit over shorter distances than flounder that spawn off shore). Bream deposit their eggs in dense vegetation near the banks of lakes and small, slow-running streams. During this time, males occupy a territory. After spawning, bream migrate back to more open waters such as larger lakes and rivers where they remain until the next spawning season (e.g., Grift et al. 2001). Bream is a suitable freshwater sentinel species for detecting site-specific estrogenic effects, especially in fall and winter when they stay in a relatively limited area.

Adult flounder leave the Dutch inland waters and estuaries to collect in the coastal zone during November and December, from where they migrate to their offshore spawning areas, some 40 kilometers into the North Sea (Rijnsdorp and Vethaak 1989). After spawning in winter, they migrate back from February to April. Flounder usually return to the same feeding grounds as the previous year and move little during the summer. Juvenile fish move predominantly into inland areas where they may stay for up to 4 years before their first reproductive migration. Adult fish also move to inshore feeding zones, but as they age, they tend to increasingly dwell in more open waters of estuaries. Flounder in the Netherlands and else-

where have been studied extensively in the past as indicators for pollution (Vethaak 1993; Janssen 1996; Besselink 1998; Grinwis et al. 2000; Laroche et al. 2002).

Fish were captured at various freshwater and marine locations during both spring and fall of 1999 (see Chapter 1 for a map of LOES sampling locations). While conducting the survey work, most of the guidelines and recommendations of the SETAC-Europe/OECD/EC expert workshop on the assessment and testing of endocrine modulators in wildlife were followed (Vethaak et al. 1997). The induction of estrogen-mediated vitellogenin synthesis in male specimens and the presence of gonadal abnormalities in both male and female specimens were the principal parameters for estrogenicity investigated in both species.

Vitellogenin is a precursor of yolk proteins that is produced under control of estrogens in the liver of female oviparous animals such as fish. Vitellogenin is normally produced in the liver of females and transported in the blood to the ovaries where it is incorporated in the oocytes. Male fish do not usually produce VTG, but they can be stimulated to do so when exposed to some estrogen agonists. Concentration of VTG in blood plasma of male fish is a sensitive and suitable biomarker for exposure to estrogen agonists or compounds that elevate endogenous concentrations of estrogens (Sumpter and Jobling 1995; Tyler et al. 1996; Hylland and Haux 1997; Jones et al. 2000).

The male gonad consists of seminiferous tubules, separated by varying amounts of connective tissue. The tubules are lined by spermatogonia, large cells with basophilic cytoplasm and large, round nuclei. In between the spermatogonia, Sertoli cells are present, characterized mainly by their basal localization. Depending on the stage of development and reproductive activity, the tubules are filled with a varying amount of small sperm cells. The occurrence of oocytes in the testes of male fish can, dependant on the fish species, reflect a gonadal abnormality that may be due to estrogenic substances, termed an ovotestis or testis-ova. Often, the condition is also referred to as intersex, but according to Van Tienhoven (1983), the term rudimentary hermaphroditism is most appropriate (i.e., the individual functions as a male [the testis produces sperm], but ovarian tissue is present). All male fish from the survey were screened for the presence of ovotestes.

The use of VTG in plasma and occurrence of ovotestes in male fish have been successfully applied to detect exposure of fish to estrogenic compounds. In the UK, it has been shown that male rainbow trout (*Oncorhynchus mykiss*) placed in cages and submerged in effluents from municipal STPs had higher VTG levels than fish in reference areas that did not

receive sewage effluent (Purdom et al. 1994). Similarly, wild fish in rivers receiving sewage effluent exhibited higher concentrations of VTG as well as the presence of ovotestes (Harries et al. 1996, 1997). Roach (*Rutilus rutilus*) and gudgeon (*Gobio gobio*) collected from some rivers in the UK exhibited elevated plasma concentrations of VTG and an incidence of ovotestes or female reproductive ducts in male fish of up to 100% in some places (Jobling et al. 1998; Van Aerle et al. 2001). Flounder collected from estuaries along the coast of Wales and England also exhibited elevated VTG concentrations, and up to 20% of the male flounders in the most polluted estuaries contained oocytes in their testes (Allen, Matthiessen et al. 1999; Allen, Scott et al. 1999). Ovotestes and slightly increased VTG concentrations were also observed by Hashimoto et al. (2000) in the flounder *Pleuronectes yokohamae* from the inner part of Tokyo Bay, which receives a large amount of industrial and domestic sewage effluent. Anomalously great VTG concentrations were also observed in female winter flounder (*Pleuronectes americanus*) from polluted estuarine environments on the East coast of North America (Pereira et al. 1993).

## Methods and Materials

### Fish sampling

Fish were captured in the spring (March 2 to April 22) or fall (September 9 to November 1) of 1999. Depending on the accessibility and other features of the sampling locations, different capture methods were used: seine nets, standing gill, or entangling nets, trawl nets (with or without a beam), or fyke nets. At sea, larger research or commercial fishing vessels were employed. Smaller boats were used inland. On several occasions, fish were kept overnight in the flow-through fish wells of the vessels or in creels at the locations before sacrificing and processing. In spring, fish were further processed on the vessel or jetty. In the fall, live fish were transported to the laboratory in large oxygenated tanks.

Fish were anaesthetized with MS 222 (tricaine methane sulphonate; Sigma-Aldrich, Zwijndrecht, NL) until death. The total length (cm) was measured, and in some cases, incisions were made to determine the gender of the fish. The skin of the fish was examined for the presence of skin lesions. Blood samples ranging from 0.5 to 2.0 milliliters were taken from the caudal vein using 2.5-mL syringes and transferred into heparinized glass tubes. Then, 0.03 mL of a 0.1 mg·mL$^{-1}$ solution of the protease inhibitor aprotinin (Merck, Amsterdam) in 0.9% v/v NaCl solution was added per mL of blood to inhibit breakdown of VTG. The tubes with blood samples were

thoroughly shaken and centrifuged at 3000 rpm, at 4 °C, for 10 min. The supernatant was decanted into 2-mL Eppendorf vials and frozen at –80 °C until analysis. Plasma samples prepared in the field were transported to the laboratory on dry ice. After removing other internal organs, the gutted body weight (g) was recorded. The internal organs were screened for (gross) pathological lesions. Sections of the liver and of both gonads (5×5×5 mm) were excised and fixed in neutral buffered 4% formalin for histological examination.

## General parameters and gross pathology

The condition factor (CF) and hepatosomatic and gonadosomatic indices (HSI and GSI) were calculated (equations 1 through 4).

$$CF_{bream} = bodyweight / (0.053 \times length^{3.1997}) \qquad (1)$$

$$CF_{flounder} = 100 \times bodyweight / length^3 \qquad (2)$$

$$HSI_{bream/flounder} = (liver\ weight / total\ bodyweight) \times 100\% \qquad (3)$$

$$GSI_{bream/flounder} = (gonad\ weight / total\ bodyweight) \times 100\% \qquad (4)$$

The formula to calculate the CF for bream was derived empirically during previous monitoring (unpublished data). A higher CF indicates a better general condition of the fish. Higher HSI values may indicate good nutritional status, but this may also be due to increased liver activity because of exposure to organic pollutants. A higher GSI is an indication of increased reproductive activity.

The fish were also examined for external lesions and abnormalities. Gross pathologies were described, classified and enumerated (Bucke et al. 1996).

Phenotypic gender of the fish determined by visual inspection of the gonads was later verified by histological examination. During the spring sampling, fish that were determined visually to be females were released, and males were collected. Fish sampling in the fall, on the contrary, was conducted in a non-selective manner, to allow assessment of sex ratio (ratio between females and males) in fish populations at the various locations.

## Plasma vitellogenin

The method for VTG measurement applied by AquaSense during the LOES project was a slightly modified version of the method used by Smeets (1999) who, in turn, based his method on a protocol developed and later published by the Michigan State University (see Nichols et al. 2001). VTG in fish plasma was analyzed using a competitive enzyme-linked immuno sorbent assay (ELISA) in 96-well micro titer plates. A different coating, primary anti-VTG antiserum and VTG standard for calibration, was used for each fish species (see Table 7-1). VTG standards

Table 7-1 Details of vitellogenin (VTG) analysis in flounder and bream

| Fish species | Primary antibody (AB 1) | Final dilution of AB 1 in wells | Dilution of E2-induced female plasma used as coating | Standard used for calibration | Detection limit in male plasma (ng·mL⁻¹) | Intra-assay CV (%)[a] | Inter-assay CV (%)[a] |
|---|---|---|---|---|---|---|---|
| Flounder (*Platichthys flesus*) | Polyclonal rabbit anti-flounder VTG antiserum; CEFAS, Lowestoft, UK (see Allen, Matthiessen et al. 1999) | 510,000× | 70,000× | Purified lyophilized flounder VTG; CEFAS, Lowestoft, UK | 313–625 | 3.8–11.7 | 5.5–7.7 |
| Bream (*Abramis brama*) | Polyclonal rabbit anti-goldfish VTG antiserum; Michigan State University, East Lansing, USA (see Nichols et al. 2001) | 120,000× | 70,000× | Plasma of E2-induced female bream[b]; RIKZ, the Netherlands (14.5–16.7 mg VTG·mL⁻¹ plasma) | 14–114 | 2.8–15.8[c] 6.1–10.0[d] | 5.6–14.4[c] 6.9–17.5[d] |

[a] the range represents lowest and highest values on the linear part of the calibration curve

[b] for spring samples of bream, the coating and standard consisted of a pool of plasma samples of several E2-induced female bream; for the fall samples, the plasma of a single E2-induced bream was used

[c] using the plasma pool of several E2-induced females as a coating and standard

[d] using plasma of a single E2-induced female as a coating and standard

were produced for both species studied. Production of VTG was induced by injecting female bream or flounder intraperitoneally with 5 mg·mL⁻¹ 17ß-estradiol (E2; Sigma-Aldrich, Zwijndrecht, NL) dissolved in an inert vegetable oil. The quantities injected were adjusted to obtain a nominal concentration of 1 mg E2·kg⁻¹ ww per individual fish. Maximum amounts of the solution in oil, injected, were 0.2 mL in bream and approximately 0.06 mL in flounder. The fish were injected twice with a 1-wk interval and sacrificed after 2 weeks. Samples of induced plasma were drawn according to the procedure described above. This plasma was used for coating and as a VTG-standard in the analyses (see below).

The ELISA took 3 days. On the first day, high-binding affinity flat-bottom 96-well enzyme-linked immunoassay/radio immunoassay (EIA/RIA) plates (Costar, Badhoevedorp, NL) were coated with 150 µL per well of a E2-induced female plasma solution in a 50 mM sodium bicarbonate buffer (SBB; pH 9.6). At the same time, regular flat-bottom 96-well plates (Greiner, Alphen a/d Rijn, NL) were blocked with 200 µL per well of a 2.5 g·L⁻¹ Bovine Serum Albumin solution (BSA, Sigma-Aldrich) in a wash buffer (TBS-T: 10 mM Tris, 0.15 M NaCl, 0.1 % Tween-20, 5.0 mg·L⁻¹ gentamicin, pH 7.5) to serve as pre-incubation plates. Both types of plates were left overnight at room temperature.

On the second day, dilution series of plasma samples of male fish and VTG calibration solutions in Tris buffered saline Tween bovine serum albumin (TBS-T-BSA) containing 2 µg·mL⁻¹ aprotinin were made. Depending on the plasma volume available, the original plasmas were pre-diluted 2 to 4 times; a factor of 1.5 to 4 between successive dilutions of plasma samples was used. This resulted in a logarithmically increasing series of 12 dilutions. Sixty µL volumes per well of the sample and calibration dilutions were introduced in the pre-incubation plates, and the plates were shaken for at least 1 h with 60 µL per well extra TBS-T-BSA containing aprotinin at a concentration of 18 µg·mL⁻¹. Then, 60 µL per well of the serum solution containing the primary anti-VTG antibody (AB1) in TBS-T-BSA was added, and the pre-incubation plate was shaken for another 30 min. Separately, the coated ELISA (EIA/RIA) plates were emptied, washed 3 times with Tris buffered saline Tween (TBS-T) and any unbound locations were blocked with 200 µL TBS-T-BSA per well for at least 30 min at 37 °C. Following blocking, the ELISA plates were emptied, after which 150 µL of the solutions in the pre-incubation plates was transferred to each well on the ELISA plates. The plates were then incubated overnight at room temperature.

On day 3, the ELISA plates were emptied and washed 3 times with TBS-T. The secondary antibody, monoclonal mouse anti-rabbit IgG conjugated

with alkaline phosphatase (Sigma-Aldrich, Zwijndrecht, NL), was diluted 5000-fold in TBS-T-BSA and 150 µL of this solution was added to each well. The ELISA plates were then incubated for 2 h at 37 °C. After incubation, the plates were emptied and washed twice with TBS-T, once with sodium bicarbonate buffer (SBB) containing 1 mM $MgCl_2$, and immediately emptied. Buffered solutions of the substrate Methyl Umbeferyl Phosphate (MUP, Sigma-Aldrich, Zwijndrecht, NL) were prepared freshly on day 3 (0.2 mM MUP, 1 M di-ethanol amine [DEA], 1 mM $MgCl_2$, pH 9.8). Next, 150 µL MUP solution was added to each ELISA well and fluorescence at 460 nm was immediately measured on a Victor-2 1420 multi-label counter (Wallac, Breda, NL) at room temperature. Measurements were repeated after exactly 15 min. The difference in fluorescence (i.e., a measure for the hydrolysis rate of MUP by the bound enzyme) was used to quantify the plasma vitellogenin (VTG) concentration in the original plasma sample.

On each ELISA plate, 3 duplicate rows with sample dilution series and 1 duplicate row with a dilution series of the VTG standard were run. All (duplicate) measurements within the linear range of the calibration curve were pooled to calculate the overall VTG concentration per plasma sample, expressed as $ng \cdot mL^{-1}$. Sample analyses were repeated when none of the measurements fell within the linear part of the calibration curve. Detection limits ranged from 14 to 114 ng $VTG \cdot mL^{-1}$ plasma for bream and from 313 to 625 $ng \cdot mL^{-1}$ for flounder (Table 7-1). Ranges of intra-assay variation (replicates on the same 96-well plate) and inter-assay variation (replicates measured on different plates) are given (Table 7-1).

## Histological analysis

After routine processing and embedding of the tissue samples in paraffin, 3–5 µm sections were cut and mounted on microscopy slides. Before microscopic examination, slides were routinely stained with haematoxylin and eosin (H&E). The slides were examined by viewing entire sections under Olympus BX40 and BHB light microscopes at magnifications ranging between 40× and 400×. Liver tissue was examined histologically for lesions with an emphasis on possible hepatocyte basophilia potentially caused by VTG synthesis.

A general score for maturation was used, based on the overall appearance of the gonads of both sexes for both species of fish. Male gonads were specifically examined for the presence of oocytes. Classification of oocytes for both males and females was based on the system of Yamamoto (1969; reviewed by Nagahama 1983). Oocytes were classified as follows: Class I corresponded to all perinucleolar stages preceding class II, the yolk vesicle

stage; class III included stages with abundant yolk and fatty globules and mature oocytes. Maturation of the ovary was classified according to the predominant class of oocytes present.

## Statistical analysis

Data were checked for normality and homogeneity of variances with Lilliefors' test and Bartlett's test, respectively. Mean parameter values from each location were compared to the mean values of the reference locations using one-way analysis of variance (ANOVA), followed by Tukey's mean comparison test. The analyses were performed using the statistical software package SYSTAT (1992). The parameters that were statistically analyzed for both species were CF, HSI (males and females together for both parameters), GSI (males and females separately), and plasma VTG levels in males. VTG concentrations that were less than the detection limit were assigned the value of the detection limit. Plasma concentrations of VTG were log-transformed prior to statistical analysis. The reference location for bream was VRO in Lake IJssel (fresh water). The reference for flounder was the relatively clean coastal location of NWK (prior to the study, it was not known if flounder captured at the freshwater reference VRO would not have been affected by pollution during their migration through less clean inland waters).

# Results

A total of 798 bream and 1403 flounder were examined. The target sample size (25 mature males and 25 mature females in the spring, and 20 mature males and 20 mature females in the fall) for each location was not always reached (Table 7-2). Notable exceptions were bream at EIJS, in the River Dommel (DOM) and at LOB (only in the fall), and flounder at AMS (only in the spring).

## General health

The results of the general health survey are published elsewhere (Vethaak, Rijs et al. 2002). The results are briefly discussed here. The mean length of bream sampled at the various locations varied between 39.5 cm (AMS) and 51.6 cm (VRO). The mean length of bream showed no important variations at the different locations between the spring and fall. The mean length of the flounder also varied considerably between sampling locations (23.6 to 32.0 cm), but again there were no major seasonal differences with perhaps the notable exception of a location in the Wadden Sea (OEV), where flounder was considerably larger in spring (32.0 ± 2.9 cm) than in

Table 7-2 Numbers of fish used for analysis and the sex ratios of the whole catch. The sex ratio was only determined in the fall because of selective sampling during spring.

| Location code[a] | Number of fish[b] | | Sex ratio[c] of catch (% females) |
|---|---|---|---|
| | Male | Female | |
| **Bream** | | | |
| Spring | | | |
| AMS | 22 | 26 | — |
| BER | 17 | 25 | — |
| DOM | 14 | 21 | — |
| EYS | 13 | 12 | — |
| HAR | 24 | 25 | — |
| KOU | 25 | 25 | — |
| LOB | 25 | 25 | — |
| VRO | 24 | 26 | — |
| Fall | | | |
| AMS | 19 | 20 | 27 |
| APK | 20 | 20 | 52 |
| BER | 17 | 22 | 75 |
| DOM | 8 | 8 | 50 |
| EYS | 13 | 12 | 77 |
| HAR | 20 | 20 | 42 |
| KOU | 21 | 19 | 66 |
| LOB | 7 | 13 | 65 |
| VRO | 20 | 20 | 49 |
| | | Mean: | 55.9 |
| | | Standard error of mean: | 5.4 |
| **Flounder** | | | |
| Spring | | | |
| AMS | 16 | 12 | — |
| NWK | 25 | 25 | — |
| OEG | 25 | 21 | — |
| OEV | 22 | 14 | — |
| SOD | 22 | 12 | — |
| SPL | 25 | 25 | — |
| VLI | 21 | 19 | — |
| VRO | 20 | 20 | — |
| Fall | | | |
| AMS | 13 | 23 | 64 |
| BVW | 20 | 20 | 34 |
| DAN | 18 | 20 | 59 |
| HAM | 20 | 20 | 54 |
| MAA | 23 | 17 | 43 |
| NWK | 20 | 20 | 58 |
| OEV | 22 | 14 | 39 |
| SOD | 22 | 12 | 35 |
| VLI | 21 | 19 | 48 |
| VRO | 20 | 20 | 50 |
| WST[d] | 18 | 20 | 53 |
| IJM | 18 | 22 | 55 |
| | | Mean: | 49.3 |
| | | Standard error of mean: | 2.8 |

[a]For a list of LOES sampling location codes, see Appendix A

[b]the numbers of males and females mentioned represent only the fish that were used for further processing and analysis

[c]sex ratios are based on all fish captured per location

[d]surface water in the direct vicinity of the Westpoort STP near Amsterdam

fall (23.6 ± 2.1). This size difference may be due to a high number of large post-spawning flounder that attempt to migrate from the Wadden Sea into the freshwater Lake IJssel in spring but are hindered from doing so by sluices.

Average CF values of bream ranged from 0.90 to 1.14 while those of flounder ranged from 0.83 to 1.14. The greatest CF values for bream during both seasons were observed in the River Dommel (close to the STP). The greatest CFs for flounder were observed at VRO in the spring and at MAA (River Rhine) in the fall. CF values varied among seasons and locations, and there was no consistent trend. There was no clear evidence of differences in condition between males and females for either species at most of the sampling locations.

Mean relative liver weights (HSI) in flounder and bream varied among locations. Significantly higher values for bream were found at known polluted inland locations such as the AMS, APK, the River Dommel (DOM) and the River Rhine at LOB. For flounder, the HSI was the highest at the reference location (NWK). HSI values for both fish species were higher in spring than in fall but showed no clear spatial trend. No obvious differences between sexes were observed, with the exception of significantly higher values in female flounder than in males observed at some coastal locations in spring, such as NWK, OEG (where flounders spawn during winter), and OEV. The reason for this difference remains unknown.

## Gross pathology

Macroscopic examination of the external body of the fish indicated that almost all bream were free from gross lesions and anomalies, except for bream from location VRO which showed growths resembling epidermal papilloma (8% of the catch in spring) and skin ulcers (2% in spring and 2.5% in fall). Skin ulcers were prevalent in flounder with the greatest incidence observed during the fall in the North Sea Canal (at IJM and AMS near the WST STP outfall: 16.7% to 18.4%) and in the Wadden Sea (OEV) (16.7%). The occurrence of skin ulcers in flounder at these locations is probably due to discharges of fresh water and associated salinity fluctuations as reported by Vethaak (1992). Visible signs of gonadal disorders were observed occasionally during the present survey, including 1 male bream with a single gonad (2.4% of the catch from location BER in spring) and 1 female bream with gonads containing hard nodules (2.9%; River Dommel in spring). The latter observation was not confirmed through histological analysis.

## Gonadosomatic Index

The average GSI values for male and female bream were considerably higher in the spring than in the fall (Figure 7-1, parts a and b respectively), reflecting the spawning season (April-June) and reproductive status of the species. Exceptions were the locations HAR (males and females), AMS (females), and VRO (females) where seasonal variation was less. These are larger waters that were sampled early in spring. They may therefore still have been too cold to trigger the spring female gonadal development. Average GSI for both males and females differed by no more than a factor of approximately 2 between sampling locations, but the variation among individuals from the same location was also large. At some locations in spring and many locations in fall, the average GSI of female bream was significantly less than at the reference location (VRO). These differences, however, are likely due to a relatively great GSI in female bream collected at VRO (see Figure 7-1 part b). The cause of this increase is unknown. It is probably not an effect caused by local exposure to estrogens because male bream captured at the same location on the same day contained very low plasma concentrations of VTG. Location VRO was therefore still considered a suitable reference location for estrogenic effects in bream.
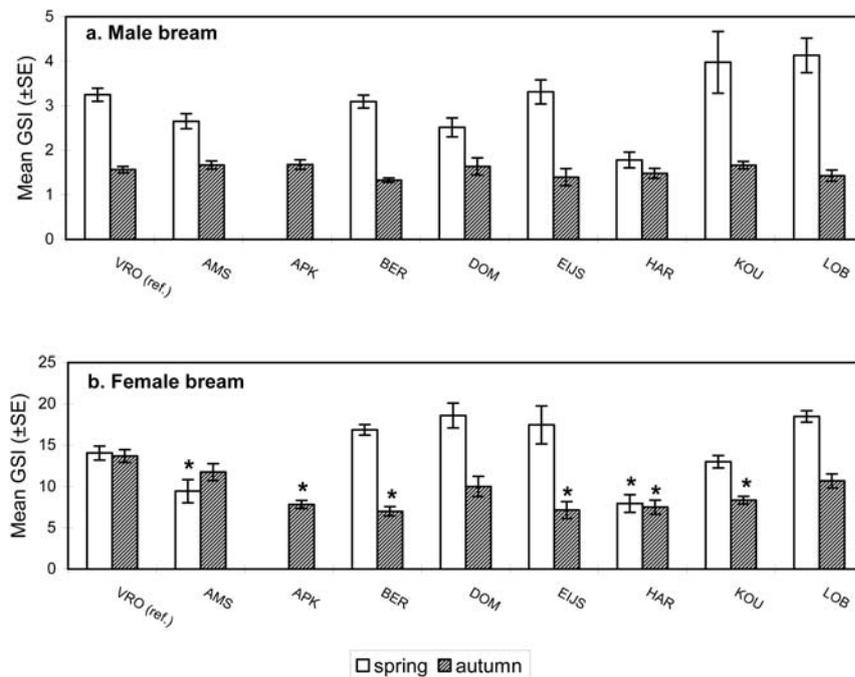


Figure 7-1 Average relative gonadal weight (gonadosomatic index: GSI) of male (a) and female (b) bream (*Abramis brama*) captured at various freshwater locations in the Netherlands during the spring and fall of 1999. Asterisks indicate significant differences from the reference location ($p < 0.05$).

The mean GSI of female flounder in spring was higher at OEG ($p <$ 0.0001) and, to a lesser extent, at AMS (non-significant) compared to the reference location (Figure 7-2 part b). It is very likely that this finding is not due to estrogenic effects but reflects the reproductive status and associated migratory behavior of this species which spawns in the open sea (OEG) during winter. Male GSIs varied considerably between the spring and fall and between locations (Figure 7-2 part a). Significantly higher GSI values ($p < 0.0001$) were observed at locations OEG and AMS in the spring. These results are similar to those for female flounder.

## Vitellogenin

Concentrations of plasma VTG of male bream varied among individuals at each location and average levels were often higher in spring than in fall (Figure 7-3 part a). Significantly higher average plasma concentrations of VTG compared to the reference location (with concentrations in some individuals up to 1 000 000 ng·mL$^{-1}$) were measured where the Rivers Rhine and Meuse enter the Netherlands (LOB and EIJS), in the North Sea Canal
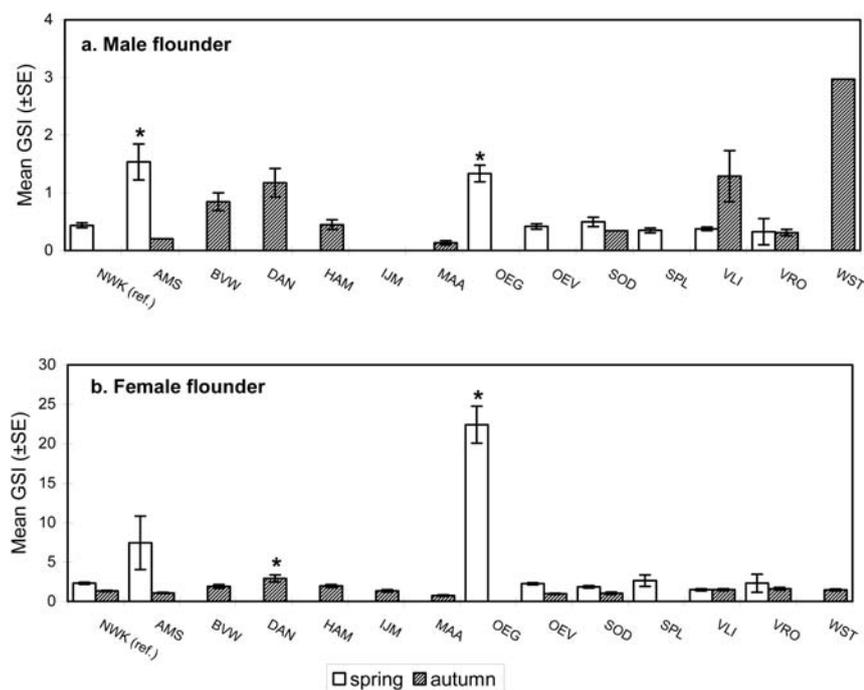


Figure 7-2 Average relative gonadal weight (gonadosomatic index: GSI) of male (a) and female (b) flounder (*Platichthys flesus*) captured at various marine, estuarine, and freshwater locations in the Netherlands during the spring and fall of 1999. WST refers to the surface water in the direct vicinity of the Westpoort STP near Amsterdam. Asterisks indicate significant differences from the reference location ($p < 0.05$).
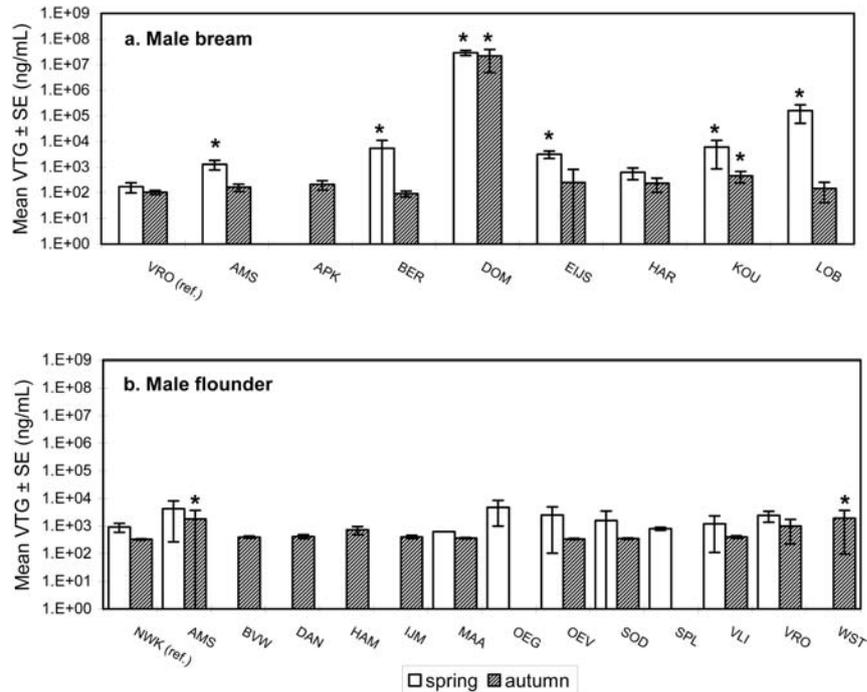
Figure 7-3 Average concentration of the yolk protein vitellogenin (VTG) in blood plasma of male bream (*Abramis brama*) (a) and male flounder (*Platichthys flesus*) (b) captured at various locations in the Netherlands during the spring and fall of 1999. WST refers to the surface water in the direct vicinity of the Westpoort STP near Amsterdam. Asterisks indicate significant differences from the reference location ($p < 0.05$).

(AMS), and in 2 freshwater locations (BER, KOU). These increased concentrations occurred mostly in the spring.

The greatest plasma concentrations of VTG during the LOES survey were observed in male bream from the River Dommel (DOM), which is a small inland stream. In both the spring and autumn, all of the plasma samples contained concentrations of VTG that were equal to or higher than $1\,000\,000$ ng·mL$^{-1}$. More than 80% of the individuals contained concentrations that were higher than $10\,000\,000$ ng VTG·mL$^{-1}$. Average VTG concentrations were significantly higher than that of fish from the reference location in both the spring and fall.

At most locations, plasma concentrations of VTG of individual flounders were less than 1000 ng VTG·mL$^{-1}$ (Figure 7-3 part b). At some locations, 1 or 2 individuals captured contained levels higher than $1\,000\,000$ ng VTG·mL$^{-1}$ plasma (Figure 7-3 part b). In the spring, this phenomenon was observed at locations SOD, OEG, OEV, and NWK, and in the autumn at VRO, VLI, AMS, and WST near the outlet of STP. The deviant specimens in the spring were therefore found only at locations that were

directly influenced by the North Sea, while in the autumn such individuals were encountered only in brackish inland waters and freshwater environments. Histological analysis of the gonads of these specimens confirmed that they were all (phenotypic) males. In the autumn, significantly increased average VTG concentrations in plasma of male flounder were observed at AMS and WST near the outlet of STP.

## Histopathology

The occurrence of parasitic granulomas, melanomacrophages, and general basophilia in livers of the fish were evenly distributed among the locations (data not shown). Increased basophilia of hepatocytes, the result of active synthesis of yolk precursor proteins (Aida et al. 1973), was not associated with males that exhibited higher concentrations of plasma vitellogenin.

The gonads of female and male fish were in different stages of maturation as determined by the amounts of ripe ova and sperm, respectively. No differences in maturation stage among groups that were sampled at varying locations were observed (data not shown). In general, it was observed that both species had more mature gonads in the spring than in fall.

In the spring, the testes of 43% of male bream from the River Dommel contained oocytes inside the seminiferous tubules (Figure 7-4; Table 7-3). In fall, the incidence of ovotestes was 33%. Oocytes in testicular tissue were of Class I type (perinucleolar) and were never surrounded by granulosa cells. Ovotestes were also observed in male bream from a canal (KOU) and the reference location (VRO; see Table 7-3), but at a lesser frequency (4% and 9%, respectively) and only in the spring. In 1 male bream captured in the River Dommel during spring, extensive squamous metaplasia of the seminiferous tubules was observed. These structures were distinct from parasitic granulomas; the epithelium showed signs of keratinization and no inflammatory cells were present. Contrary to what usually occurs when gonads are affected by parasitic infestation, no parasites were found in the liver of this individual.

No ovotestes were observed in any of the male flounders captured at the various offshore, estuarine and inland locations.

## Sex ratio

The mean sex ratios of bream and flounder, expressed as the percentage of female fish, were 56% and 49% for bream and flounder, respectively. Bream from the River Dommel, where the greatest plasma VTG concentrations and incidence of ovotestes in males were observed, had a sex ratio of 50%. The sex ratios varied among locations, but no trend was observed, and there seemed to be no correlation with the type of water or with any
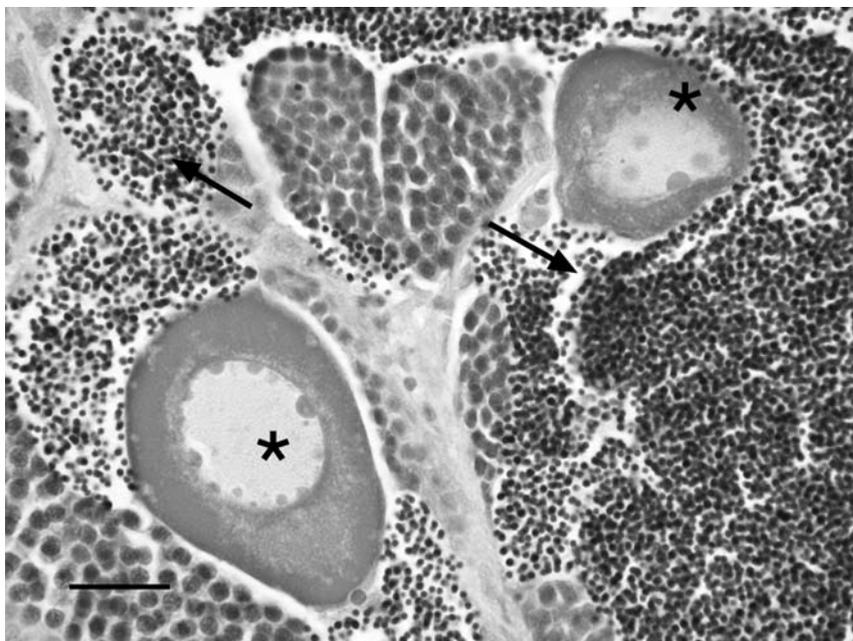
Figure 7-4 Ovotestis in a bream (*Abramis brama*) captured in the Netherlands during 1999. The picture shows testis tissue with oocytes in the testicular tubules (asterisks) and testicular tubules containing (few) spermatocytes (arrows) (bar = 34 μm). The follicular epithelium that differentiates into granulose cells is absent, and no yolk granules are deposited in these oocytes. Tissues were stained with haematoxylin-eosin.

Table 7-3 Incidence of ovotestes in male bream. Ovotestes were only found at the locations shown.

| Location code[a] | Season | *n* males examined | Number of testes with oocytes[b] | | | Ovotestes in males (%) |
|---|---|---|---|---|---|---|
| | | | − | + | ++ | |
| KOU | Spring | 25 | 24 | 1 | 0 | 4 |
| VRO | Spring | 23 | 21 | 2 | 0 | 9 |
| DOM | Spring | 14 | 8 | 4 | 2 | 43 |
| DOM | Fall | 9 | 6 | 3 | 0 | 33 |

[a]For a list of LOES sampling location codes, see Appendix A

[b]− : no oocytes in testicular tissue
 + : sporadic to several oocytes in testicular tissue
 ++ : numerous oocytes in testicular tissue

known general degree of pollution by classic toxic substances (see Chapter 1 and also Lahr et al. 2003).

# Discussion

## Increased plasma VTG and its effects

Several studies on the effects of estrogenic compounds in fish and surveys of fish responses to exposure of estrogenic compounds under field conditions have been conducted with rainbow trout (*Oncorhynchus mykiss*). The effects of STP discharges on caged rainbow trout in small streams in the UK were among the first to be reported and have triggered much of the current concern about estrogenic contamination in the aquatic environment (e.g., Purdom et al. 1994; Sumpter and Jobling 1995; Harries et al. 1996, 1997, 1999; Routledge et al. 1998). Concentrations of plasma VTG in males has been a key biomarker in these studies. The average VTG concentration in plasma of male rainbow trout that could be discriminated from background was approximately 1000 to 10 000 ng·mL$^{-1}$. The same approximate threshold was observed in a field survey of male flounder conducted in estuaries of the UK (Allen, Matthiessen et al. 1999; Allen, Scott et al. 1999) as well as in laboratory studies where carp (*Cyprinus carpio*) were exposed to estrogenic substances (Gimeno 1997), and during field surveys of carp near STPs in the USA and Spain (Folmar et al. 1996; Petrovic et al. 2002).

Natural concentrations of VTG in the plasma of various male cyprinid fish species such as carp are generally less than 20 ng·mL$^{-1}$, whereas the concentrations in plasma of females are always higher than 200 ng·mL$^{-1}$, even in immature specimens (Tyler et al. 1996). According to the same authors, levels in mature females range from 100 000 to 1 000 000 ng VTG·mL$^{-1}$ plasma. VTG concentrations in females, however, change during the annual recrudescence cycle. Concentrations of VTG as great as 100 000 ng VTG·mL$^{-1}$ have been observed in plasma of female rainbow trout in May and 12 900 000 ng·mL$^{-1}$ in November during ovulation and spawning (Van Bohemen et al. 1981). Maximum concentrations of VTG in plasma higher than 50 000 000 ng·mL$^{-1}$ were observed at different times of the year in rainbow trout of fall-spawning or winter-spawning strains (Scott and Sumpter 1983). Besides a seasonal effect, plasma concentrations of VTG also depend on the age of the fish. VTG concentrations of female rainbow trout plasma may increase as much as a million-fold during the 2 or 3 years that are needed to reach sexual maturity, whereas they do not often exceed a concentration of 1000 ng·mL$^{-1}$ plasma in mature males (Copeland et al. 1986; Bon et al. 1997). Similar differences in plasma VTG

concentration have been observed among life stages of female flounder (Korsgaard Emmersen and Petersen 1976). It thus seems reasonable to assume that plasma concentrations of VTG in male fish that are higher than 1000 to 10 000 ng·mL$^{-1}$ would be considered anomalous and indicate exposure to estrogenic compounds. Hence, in the present study, average concentrations of VTG in plasma of males between 1000 and 1 000 000 ng VTG·mL$^{-1}$ of plasma were considered to be moderately increased, and levels higher than 1 000 000 ng VTG·mL$^{-1}$ plasma are regarded as highly increased, relative to what would be expected in unexposed fish.

Unlike females, male fish are unable to transform VTG into the yolk protein that is incorporated in eggs and therefore, VTG can accumulate in the blood plasma. It has been demonstrated that the production of VTG in male fish may cause kidney damage (Wester and Canton 1986). It has also been suggested that VTG production in males may decrease metabolic expenditure for growth and spermatogenesis (Herman and Kincaid 1988; also see Sheahan et al. 1994). In female fish, an unnaturally increased level of VTG has been associated via a feedback mechanism with reduced estradiol production (Reis-Henriques et al. 1997), which in turn may negatively influence egg quality. Induction of the production of VTG in female fathead minnows (*Pimephales promelas*) exposed to E2-17α and E2 has been associated with decreased egg production (Laenge et al. 1997; Kramer et al. 1998).

The cause of the occasionally greater concentrations of VTG in plasma of individual male flounder from sites in the present study where most other specimens did not have elevated concentrations of estrogenic compounds is unknown. The fact that most of these observations were made in plasma samples taken during the spring suggests that the phenomenon may be associated with seasonal and/or migratory factors. Individual fish captured in spring at these locations may have migrated from elsewhere, where they may have been exposed to estrogens and xenoestrogens. These observations may also indicate that a fraction of the male flounder population consists of specimens with a genetically determined high natural VTG concentration or increased sensitivity to estrogens and xenoestrogens. Despite large variations among individual fish, average plasma VTG concentrations at 2 locations in the North Sea Canal of the present study were still found to be significantly higher than at the reference locations.

## Occurrence and significance of hermaphroditism in male fish

Substances that induce VTG production in male fish may also reduce testicular growth (Jobling et al. 1996) or cause morphological abnormali-

ties in testes (Lye et al. 1997). Elevated concentrations of VTG in plasma of male fish sometimes coincide with the observation of ovotestes (roach: Jobling et al. 1998; flounder: Allen, Matthiessen et al. 1999; Allen, Scott et al. 1999). Exposure to high concentrations of E2 may even result in a total sex reversal of genetically male carp (*C. carpio*; Gimeno et al. 1996).

It is known that hermaphroditism in males is a naturally occurring phenomenon in various species of fish. In the present study, where males with ovotestes were observed, such as in the River Dommel, this feature was entirely due to the occurrence of primary (Class I) oocytes containing no yolk granules. In females that also exhibited secondary and tertiary oocytes, a normal granulosa layer was present. The failure of the "male" oocytes to develop beyond the primary stage is probably due to the absence of a surrounding granulosa cell layer. This feature of testicular oocytes without a granulosa layer has also been observed in medaka (*Oryzias latipes*; Oka 1931). A small, probably natural, incidence of Class I oocytes in the testes has been observed in channel catfish *Channa punctata* (Joshi and Sathyanesan 1980).

The consequences of hermaphroditism for the reproductive fitness of males are relatively unknown. Recent work by Jobling et al. (2002) has demonstrated, however, the fact that wild roach (*Rutilus rutilus*) exposed to STP effluents were reproductively compromised and that lesser rates of fertilization were probably due to the inferior sperm quality of males with ovotestes in these populations.

The incidence of ovotestes observed in the present study was greatest in bream from the River Dommel, which was also the location where the greatest concentrations of plasma VTG were observed. Based on the results of earlier studies, a correlation between plasma VTG concentrations and incidence of ovotestes might be expected, but this correlation is not necessarily the case. The presence of ovotestes has been observed in male flounder when plasma VTG concentrations were higher than approximately $100\,000$ ng·mL$^{-1}$ plasma (Allen, Matthiessen et al. 1999; Allen, Scott et al. 1999). However, relatively high concentrations of VTG were also observed at locations where no ovotestes occurred. Thus, they concluded that there was no consistent pattern between induction of VTG and the presence of ovotestes in males. Furthermore, ovotestes were observed in the flounder *Pleuronectes yokohamae* from Japan that contained relatively small concentrations of plasma VTG (25 to 2200 ng·mL$^{-1}$; Hashimoto et al. 2000). It is conceivable that rudimentary hermaphroditism in adult male fish reflects exposure to estrogens and xenoestrogens during younger life stages when sexual differentiation takes place (also see Chapter 11), while elevated levels of VTG in the plasma of adult males is caused by more recent exposure.

Because the presence of hermaphroditism is thought to be induced at a young stage, there may be a difference in the occurrence of ovotestes in flounder populations between those that breed in (more contaminated) estuaries and those that breed in the (cleaner) open sea because of differences in exposure to estrogens and xenoestrogens during these sensitive stages (Allen, Matthiessen et al. 1999). The fact that most Dutch flounder breed at sea, instead of in estuaries, which seems to be the case in the UK, may therefore explain why no hermaphroditism was observed in flounder from the Netherlands (i.e., the youngest stages of these fish remain longer in supposedly cleaner open sea areas).

## Other effects

There were no apparent relationships between values of CF, HIS, or GSI and specific measures of estrogen-specific responses such as plasma concentrations of VTG, presence of ovotestes (this chapter) or concentrations of estrogenic compounds in fish bile measured by the ER-CALUX assay (reported by Legler et al. 2002). These general parameters are useful, however, as measures of condition, reproductive status, and exposure to other pollutants and therefore, may facilitate interpretation of the results for research on estrogenic effects in fish.

Squamous metaplasia of male accessory reproductive organs resulting from estrogenic stimulation is a well-known feature in mammals (Kroes and Teppema 1972). To the best of our knowledge, estrogen-induced metaplasia of the reproductive tract has never been reported in fish. The single animal in which this feature was found was from the River Dommel and indeed showed a relatively high concentration of plasma vitellogenin (approximately $26\,000\,000$ ng·mL$^{-1}$). This finding may therefore indicate that metaplasia related to estrogenic activity similar to that found in mammals may also occur in fish. However, in view of its low frequency (one specimen), this finding may be a coincidence. Further studies will be required to establish the prevalence of this abnormality in estrogen and xenoestrogen-rich environments.

Reliable estimation of the sex ratios of fish requires that a large number of fish are captured and examined. This was not always possible in our study. It has generally been suggested that estrogenic compounds that interfere with sexual differentiation may possibly affect the sex ratio of fish populations, and thus, their reproductive potential. However, data from this study suggest that the sex ratios observed in wild fish were largely determined by small sample sizes and other interfering factors such as season-related and possibly sex-related migratory behavior.

## Extent of estrogenic effects in fish in the Netherlands

The concentrations of plasma VTG of male fish observed in the LOES study showed that several areas in the Netherlands may exist where moderate estrogenic effects in male fish occur. That is where plasma VTG concentrations between 1000 and 1 000 000 ng·mL$^{-1}$ are found in flounder and/or bream. Most of these locations were situated inland and may be influenced by industrial, agricultural, and/or domestic pollution sources: the North Sea Canal near Amsterdam (AMS and WST near the outlet of STP), the lowland rivers Rhine and Meuse, especially where they enter The Netherlands (LOB and EYS), and the two investigated waters in a rural area (KOU and BER). The plasma concentrations of VTG in both fish species were higher in the spring than in fall. But for flounder, the only significant increases in VTG were observed during the fall, in the North Sea Canal (AMS and WST near the outlet of STP). Assuming that sublethal estrogenic effects by contaminants, such as induction of VTG, may take some time to develop, flounder captured in the spring probably represent a population that is less influenced by local pollution conditions than flounder captured during the fall. In the spring, flounder captured may have just arrived back from the spawning grounds, especially at sampling locations in close connection to the sea, such as the North Sea Canal. In fall, flounder captured in most areas have probably been near where they were collected for several months. The moderately elevated concentrations of plasma VTG in male bream from the mentioned waters were somewhat higher than those observed in male bream from the River Elbe in Germany, where median values per location ranged from 10 to 1000 ng VTG·mL$^{-1}$ (Hecker et al. 2002).

Except for occasionally high concentrations of plasma VTG in a few individuals, male flounder from the tidal Wadden Sea, the North Sea, and in the Rhine-Meuse-Scheldt estuaries seem relatively unaffected by estrogens even where anthropogenic influence is considered to be great, such as in the Western Scheldt, the New Waterway and the Ems estuary. In general, the results for flounder from Dutch coastal waters exhibited lower concentrations of plasma VTG than did flounder collected from various estuaries in the UK (Allen, Matthiessen et al. 1999; Allen, Scott et al. 1999; Kirby et al. 2004). In addition, none of the male flounder from the Dutch locations had ovotestes, whereas in the UK this condition was observed in estuaries of the Rivers Mersey, Tyne, and Clyde (Allen, Matthiessen et al. 1999; Allen, Scott et al. 1999; Stentiford et al. 2003; Kirby et al. 2004).

Relatively high concentrations of VTG, of between 7 and 79·10$^6$ ng mL$^{-1}$ plasma, were measured in male bream from the only small stream where fish were captured during the LOES study (i.e., the River Dommel).

These concentrations exceeded the concentrations in plasma of some female bream that were captured at the same location ($2.5 \cdot 10^6$ to $10.5 \cdot 10^6$ ng·mL$^{-1}$). The location was also the only location where a high prevalence of ovotestes was observed. The River Dommel is heavily polluted with metals from a zinc smelter, notably with cadmium and zinc (e.g., Lahr et al. 2003). However, the fish were collected downstream of the large STP of Eindhoven (EHV). The final impact of STP effluent on fish populations may largely depend on the dilution that occurs in the receiving surface water. Fish in smaller effluent-receiving streams seem relatively vulnerable to estrogenic effects (Harries et al. 1996, 1997). The volumetric contribution from the effluent of the Eindhoven STP is equal to that from the flow of the River Dommel, and the effluent will therefore have a considerable impact on the surface water quality in this stream, indicating that the estrogenic effects observed may indeed be related to the discharge of the EHV STP effluent. Experimental exposure of rainbow trout (in situ; Chapter 10) and zebrafish (in the laboratory; Chapters 11 and 12) confirmed a high estrogenicity of the Eindhoven effluent.

As a result of their effects on individuals, estrogens and xenoestrogens potentially affect reproduction and, therefore, the survival of populations (Arukwe and Goksøyr 1998). However, the link between estrogenic effects on individual fish and population-level effects remains elusive because of the complex nature of contamination in the aquatic environment and because of the scarcity of data on the status of fish populations. It has not yet been investigated if the observed physiological and histopathological effects observed in flounder and especially bream result in any decrease in fecundity. Both species of fish studied produce large numbers of eggs, and there is a great deal of population density-dependent competition and mortality. Thus, it is largely unknown if the changes observed would be likely to translate into population-level effects. A previous study of bream conducted in the Netherlands between 1965 and 1999 reported that average gonad weight was decreasing and that there seemed to be earlier maturation and a higher proportion of females in the population (Winter and Sluis 2000). However, the authors concluded that these findings could be explained by a wide range of environmental factors and presented only circumstantial evidence for the effects of any given type of stressor. Long-term (1960–1995) monitoring of populations of North Sea plaice (*Pleuronectes platessa*) and sole (*Solea solea*) indicated a decrease in size and age to maturation but no change in sex ratios (Rijnsdorp and Vethaak 1997). Increased proportions of females in some populations of dab (*Limanda limanda*) collected from the North Sea have been observed (Lang et al. 1995). However, it seems unlikely that these changes in North Sea plaice, sole, and dab are solely related to specific contaminants, but may rather be caused by

changes in population dynamics because of pressing factors such as food availability, changes in habitat, and fishing.

The findings of the present study have demonstrated that exposure to estrogen-active substances and biologically significant effects of such exposure are occurring in Dutch waters. However, based on this first survey, the exposure seems to be confined to smaller waters and possibly to only a limited number of contaminated locations. The situation in the Netherlands seems therefore different from the well-known example of the UK. A factor that may contribute to this difference is the predominant size of the water bodies in both countries. In the Netherlands, many waters consist of large branches of lowland rivers where estrogenic effluents and other emissions of estrogens and xenoestrogens are more rapidly diluted. It is also important to note that the freshwater indicator species in this study (bream) was different from the ones used in the UK approach (roach, gudgeon). The results of further investigations that focused specifically on small Dutch inland waters where anthropogenic stress was thought to be high are reported in Chapter 8. Chapters 9 through 12 address the possible estrogens that caused the effects observed in this field study, from both a statistical and experimental point of view.

## Acknowledgements

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