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Evidence for Endocrine Disruption in Perch (*Perca fluviatilis*) and Roach (*Rutilus rutilus*) in a Remote Swedish Lake in the Vicinity of a Public Refuse Dump

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A two-year study on perch (*Perca fluviatilis*) in Lake Molnbyggen, Sweden, located in a pristine area but with a public refuse dump in the vicinity, has been conducted. The mechanistic approach through a set of biomarkers during the first year included age, condition, somatic growth, liver, gonad, and spleen weights, and a number of other physiological variables, in addition to ethoxyresorufin *O*-deethylase, glutathione-*S*-transferase, glutathione reductase, catalase, and the formation of DNA adducts in the liver. Perch from the uncontaminated Lake Djursjön, located in a neighboring drainage area, were used as reference fish. The most pronounced effect was a 80% reduction in the gonadosomatic index (GSI) for females and a corresponding 36% reduction in males. Fin erosion and shallow open sores were also frequently observed. Biomarkers and later chemical analysis employed indicated that exposure to well-known environmental pollutants was low, suggesting that less well-known antrophogenic substances are responsible for the effects observed in perch from Lake Molnbyggen. During the second year, roach (*Rutilus rutilus*) of both sexes were also included in this study. In addition, aromatase (P450arom) activity in the brain and testosterone and 17β-estradiol levels in blood plasma were analyzed. Only one-fourth of the female perch were found to be sexually mature, which was associated with decreased GSI, lower P450arom activity, and reduced circulating levels of steroids. The reproductive disorders observed indicates disturbed endocrine function(s), arresting the majority of the female perch in a sexually nonreproducible immature stage. This novel study is the first to report evidence for endocrine disruption in wild populations of fish living in a lake exposed to leakage water from a public refuse dump. © 2001 Academic Press

Key Words: **public refuse dump; field study; teleost; reproductive disorder; endocrine disruption; P450arom; testosterone; 17β-estradiol; EROD; DNA adducts.**

The increasing numbers of refuse dumps and volumes of refuse dumped are among the most visible signs that many societies have not yet succeeded in achieving sustainable management of natural resources, including recycling of materials. Many countries today work actively to decrease industrial release of environmental pollutants. However, a neglected area in this connection are refuse dumps, which are also potential point sources for various pollutants. In fact, refuse dumps are not only potential point sources of antrophogenic substances presently in use, but also for substances used previously, including substances whose use is now banned by regulations and laws.

The relatively small country of Sweden was estimated in 1993 to have more than 500 refuse dumps in use and approximately 6000 that are no longer used, many unregistered (Swedish Environmental Protection Agency, 1998). Especially with regard to unregistered dumps, their contents of toxic substances and possible toxicological effects on humans and wildlife are presently unknown. The leakage water from most registered refuse dumps in Sweden is routed to nearby sewage treatment plants (Swedish Environmental Protection Agency, 1998; Rihm, 1998), but in many other cases this leakage water diffuses into the surrounding environment in an uncontrolled manner. It is therefore of urgent necessity to investigate the potential adverse biological effects of leakage water from refuse dumps more extensively, as well as the chemicals responsible for such effects.

Previous studies regarding biological effects of leakage water from refuse dumps or waste landfills have focused primarily on acute toxic effects on nonvertebrate organisms. The possible effects of leach water from landfills on growth and mortality have been examined using a number of different systems, e.g., green algae (Lambolez *et al.*, 1994; Baun *et al.*, 1999), plants and luminescent bacteria (Devare and Bahadir, 1994), and small crustaceans such as *Daphnia magna* (Atwater *et al.*, 1983; Lambolez *et al.*, 1994; Baun *et al.*, 1999). Various species of fish, including rainbow trout (*Salmo gairdneri*), sockeye salmon (*Oncorhynchus nerka*), and tilapia (*Sarotherodon mossambicus*) (Atwater *et al.*, 1983; Wong, 1989), have

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also been used for acute toxicity measurements in this connection. Genotoxicity has been assessed employing the Ames test (Schrab *et al.*, 1993; Lambolez *et al.*, 1994) as well as *Bacillus subtilis* DNA repair and the *Aspergillus nidulans* diploid chromosome damage bioassay (Scrab *et al.*, 1993). Many leachate samples were found to be both highly acutely toxic and genotoxic in the above employed toxicity tests. However, sublethal biological effects in wild populations of organisms inhabiting the waters that are recipients for these same leachates have not been investigated.

Xenobiotics that give rise to negative endocrine effects on humans and in wildlife populations have received increasing attention during recent years (Colborn *et al.*, 1993; Colborn, 1995; Cooper and Kavlock, 1997). Undesirable effects of environmental pollution on growth, behavior, and reproduction, including reduced sperm count and shortened penis length at puberty in humans (Carlsen *et al.*, 1992; Guo *et al.*, 1994); female–female mating in colonial nesting birds (Shugart, 1980; Fox, 1992); growth of oviductal tissue in male birds (Fry and Toone, 1981; Fry *et al.*, 1987); and feminization of alligators (Guillette *et al.*, 1994) have been reported. Reproductive disorders and/or endocrine effects due to antrophogenic substances have also been studied in different species of fish exposed to bleached kraft mill effluents (BKME) from pulp and paper mills (Andersson *et al.*, 1988; Sandström *et al.*, 1988; Munkittrick *et al.*, 1992; Van Der Kraak *et al.*, 1992; Sandström, 1994; McMaster *et al.*, 1995) or to effluents from sewage treatment plants (Purdom *et al.*, 1994; Folmar *et al.*, 1996). Most of the concern in this respect has centered around effects on male reproduction, so-called estrogenic effects. However, there is absolutely no reason to believe that female reproduction should be less affected, e.g., by antiestrogenic xenobiotics.

In 1994–1995, people living around the freshwater Lake Molnbyggen, in the central part of Sweden, started to report that, in particular, perch (*Perca fluviatilis*) and northern pike (*Esox lucius*) in this lake showed signs of illness, demonstrating increased frequencies of various skin lesions. There was great concern that drainage water from a public municipal refuse dump, used from 1980, was contaminating the stream Våtån, which empties into Lake Molnbyggen.

The present study was initiated to determine whether perch, roach (*Rutilus rutilus*), and, to some extent, northern pike and burbot (*Lota lota*) in Lake Molnbyggen are indeed ill and, if so, what the main symptoms are. A set of different biomarkers, known to respond to specific antrophogenic substances and representing different biological functions in the fish, was employed to mechanistically examine the toxicological effects. The study period extended over two consecutive years and the data obtained from the first year served as a basis for sampling and the selection of appropriate biomarkers during the second year.

MATERIALS AND METHODS

Chemicals and materials. Standard DNA (salmon sperm, D-1626), spermidine (S-2626), RNase A (R-9134), micrococcal endonuclease (N-3755), spleen phosphodiesterase (P-6897), resorufin (R-3257), ethoxyresorufin (E-3763), 1-chloro-2,4-dinitrobenzene (C-6396), glutathione (oxidized) (G-4626), glutathione (reduced) (G-4251), NADPH (N-7505), bovine serum albumin (A-7030), androst-4-ene-3,17-dione (A-9630), polyethylenimine (P-3143), and activated charcoal (C-5260) were purchased from Sigma Chemical Company (St. Louis, MO). RNase T_1 (109 193), nuclease P1 (236 225), T4 polynucleotide kinase (3′-phosphatase-free, 838 292), proteinase K (1000 144), and phenol (100 300) were obtained from Boehringer Mannheim, Scandinavia AB, Sweden. The standard adduct 7*R*,8*S*,9*S*-trihydroxy-10*R*-(N^2-deoxyguanosyl-3′-phosphate)-7,8,9,10-tetrahydrobenzo[*a*]pyrene (B*a*PDE-dG-3′p) was procured from Midwest Research Institute (Kansas City, MO). [γ-^{32}P]ATP (specific activity $\geq$ 5000 Ci/mmol, PB 10218) was obtained from Amersham Sweden AB (Solna, Sweden). Cellulose (MN-301) and vinyl strips (PVC foil frosted/frosted, 0.2 mm), used for the preparation of the polyethylenimine (PEI)-cellulose sheets, were purchased from Machery-Nagel (Düren, Germany) and Andren & Söner (Stockholm, Sweden), respectively. Androst-4-ene-3,17-dione, [1β-^{3}H(*N*)]- (NET-926) (23.1 Ci/mmol) was bought from NEN, Life Science Products (Boston, MA). Ultima gold scintilation fluid was obtained from CIAB (Lidingö, Sweden). Reagents, antibodies, and other chemicals for the testosterone (T) and 17β-estradiol (E2) assays were procured from Bayer Health Care, Bayer B.V., Division Diagnostics (Mijdrecht, The Netherlands). Solvents used for preparation of chemical extracts and fractions thereof were of analytical grade and obtained from KEBO (Stockholm, Sweden). All other chemicals were purchased from common commercial sources and were of analytical purity.

Equipment and apparatus. DNA was quantitated on the basis of its absorption at 260 nm using microcuvettes and a GeneQuant spectrophotometer from Pharmacia (Uppsala, Sweden). Autoradiography was performed using Xomat AR-5 films from Kodak AB (Stockholm, Sweden), with intensifying screens from DuPont (Wilmington, DE). Liquid scintillation spectroscopy was performed employing a Packard Tri-Carb 2100TR liquid scintillation counter from Packard Instrument Company. A Jasco ST 777 fluorimeter and a Hitachi U-3200 spectrophotometer were used for enzyme activity assays. Quantitative determination of blood hemoglobin and glucose was performed using specially designed photometers obtained from HemoCue AB, (Ängelholm, Sweden). T and E2 were measured using the Chiron Diagnostics ACS:180 Automated Chemiluminescence Systems (East Walpole, MA). Gas chromatography/mass spectrometry (GC/MS) analysis was performed using a Fison MD 800 mass selective detector connected to a Fison GC 8000 gas chromatograph (Fison, Manchester, UK) with a 30 m $\times$ 0.25 mm $\times$ 0.25 μm film thickness capillary column (PTE-5) from Supelco (Bellefonte, PA). The mass spectrometer was operated in electron impact mode at 70 eV.

Study site. The body of water investigated, Lake Molnbyggen (67°28′N, 14°53′E), is located in the municipality of Leksand in the county of Dalarna, Sweden (Fig. 1). Molnbyggen is an oligotrophic lake with an approximate area of 3 km^2, a maximum depth of 22 m, and an average pH of 7. The main teleost species in the lake are northern pike, perch, roach, burbot, vendace (*Coregonus albula*), bleak (*Alburnus alburnus*), and ruffe (*Gymnocephalus cernuus*).

A 20-year-old municipal dump for household refuse, but with partially unknown content, is located at Lindbodarna on a mountainside approximately 3 km from Lake Molnbyggen. The Våtån stream is located in the dump drainage area and later empties into the lake (Fig. 1). It has been estimated that the leakage water from this refuse dump has an annual volume of 18,000 m^3 and one-third of this water reaches Lake Molnbyggen via the Våtån stream (T. Lundgren, unpublished data).

Lake Djursjön (67°25′N, 14°47′E), located in a neighboring drainage area 7 km southwest of Molnbyggen, was chosen as the reference lake (Fig. 1). Djursjön is an oligitrophic lake with an approximate area of 4 km^2, a maximum depth of 37 m, and an average pH of 7. The distribution of teleost species in Lake Djursjön is similar to that in Molnbyggen.

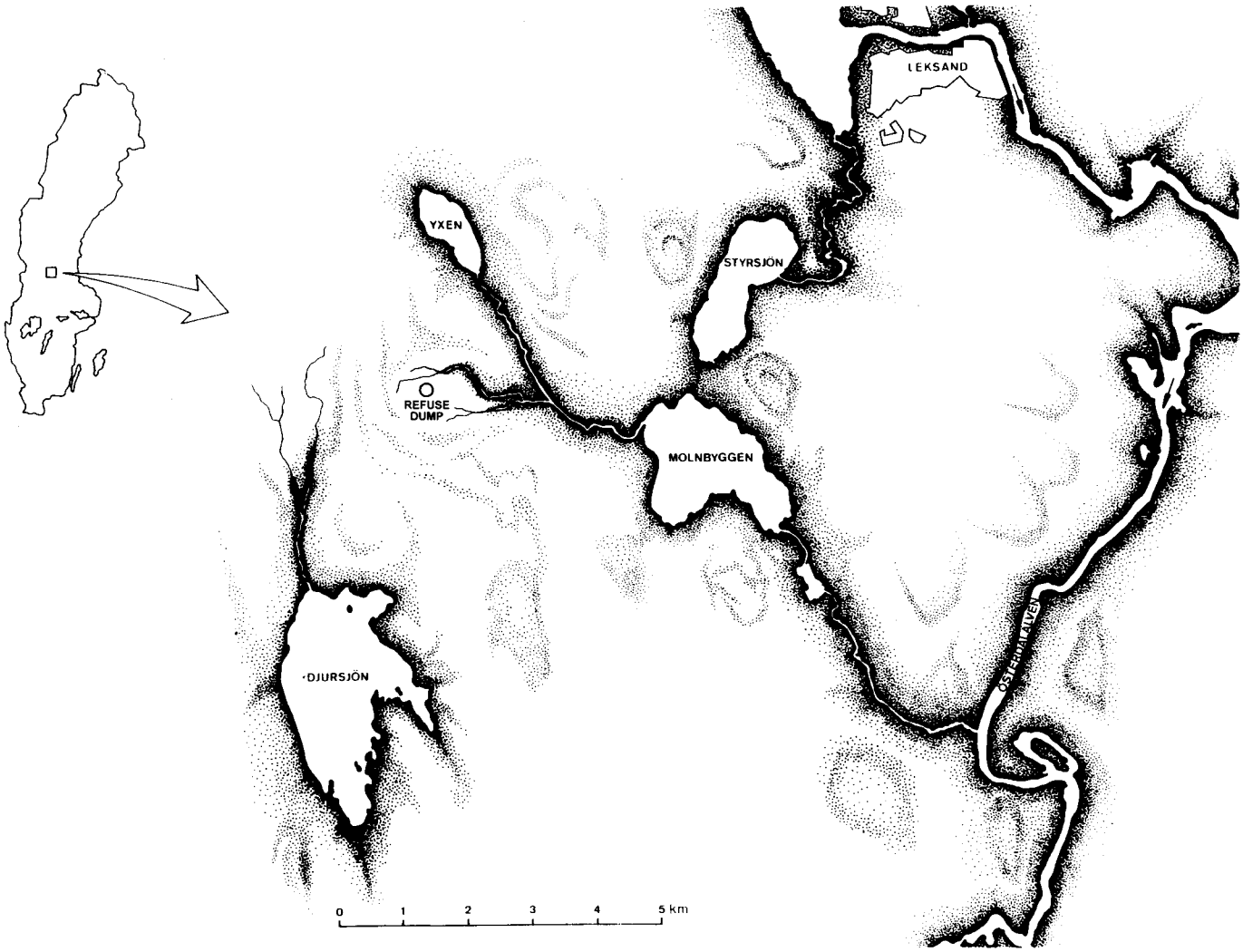

FIG. 1. The study site: the lake investigated, Lake Molnbyggen; the reference lake, Lake Djursjön; and the city of Leksand, Sweden, are shown on the map. The public refuse dump located at Lindbodarna, approximately 3 km away from Lake Molnbyggen, as well as the Våtån stream, located in the dump drainage area and emptying into Lake Molnbyggen, are also shown.

Collection of fish and tissue sampling. The first part of this study involved perch caught in Lakes Molnbyggen and Djursjön in late October 1996 with gill nets (mesh size 30–33 mm), as well as a few specimens of pike captured by hoop net in April 1997 and stored frozen at −20°C. The second part included perch and roach caught at the end of October and beginning of November 1997 with gill nets (mesh size 30–33 mm) and a few specimens of burbot captured during this same period and stored frozen at −20°C. The weather and average temperatures in this area of Sweden were normal during both the summer and fall of 1996 and 1997, when these investigations were performed.

In both periods of study, the fish were placed in a cage in the lake where they were caught. In 1996, the fish were allowed to recover for 1–3 days before euthanasia and tissue sampling at the cage as described earlier (Balk *et al.*, 1993, 1996). However, tissue samples from fish caught in 1997 were taken within 24 h of capture in order to minimize any negative effects on the level of circulating steroids or aromatase activity due to sampling stress. No significant effects on either circulating steroids or production of gonadal sex steroids (McMaster *et al.*, 1994, 1995), as well as on brain aromatase activity (Callard *et al.*, 1981), have been observed within this timeframe of tissue sampling. Fish found dead in the nets were frozen (−20°C) separately with a small amount of water in plastic bags for later analysis of morphological disorders and organ weights.

At the cage, blood was removed directly from the dorsal aorta employing a heparinized syringe. From the perch caught in 1996, aliquots of whole blood were analyzed for hematocrit (Dave *et al.*, 1975), glucose, hemoglobin, and blood cell number. Differential counting of leukocytes (percentage of total blood cells), lymphocytes (percentage of leukocytes), granulocytes (percentage of leukocytes), and thrombocytes (percentage of leukocytes) was performed according to Lehman and Sturenberg (1975) using the Pappenheim stain (Sandoz Ltd, 1973). Glucose was measured using a modified enzymatic procedure involving glucose dehydrogenase (Bergmeyer, 1974; Banauch *et al.*, 1975) and hemoglobin with modified Vanzetti's reagents (Vanzetti, 1966). In the case of perch and roach caught in 1997, the blood was centrifuged for 2 min ($4000 g_{av}$) and the plasma thus obtained was frozen in liquid nitrogen for later steroid analysis. After blood sampling, each fish was carefully examined visually for disorders such as fin erosion, deformations, and skin lesions and the disorders observed were photographed.

In perch, the total length (mm), total body weight (g), and sex, as well as liver, spleen (only for 1996), and gonad weights (g) were recorded for each

fish. For perch caught in 1996, the fish were only recorded as being female or male. However, for 1997, female perch were divided into sexually mature (SM) and sexually immature (SIM) fish based on the occurrence of maturing oocytes in the gonad. This could be determined because of the distinct difference in the morphology of the female ovary (a single structure) and male testis (paired bilaterally) in perch.

The age of the perch was determined from examination of the opercular bone according to LeCren (1947). The somatic weight, somatic growth (average growth per year), somatic condition factor (SCF = somatic body weight (g) expressed as a percentage of cubic length (cm)), gonadosomatic index (GSI = gonad weight expressed as a percentage of somatic body weight), liver–somatic index (LSI = liver weight expressed as a percentage of somatic body weight), and spleen–somatic index (SSI = spleen weight expressed as a percentage of somatic weight) were recorded for each perch. These same variables were also documented for frozen perch and partly for pike and burbot. A control experiment revealed that freezing at −20°C for 6 weeks (the longest storage time) had no effect on these variables. The same variables (with the exception of age and somatic growth) were also determined for roach. Several frozen roach livers could not be adequately dissected (due to the effects of freezing on the structure of the liver) and their weights were therefore not used.

A slice (50–100 mg) from the middle of the liver of perch caught in 1996 was immediately frozen in liquid nitrogen and thereafter stored at −80°C for subsequent analysis of DNA adducts employing the ^{32}P-postlabeling procedure (Randerath *et al.*, 1981; Gupta *et al.*, 1982). The rest of the liver was homogenized in a Potter–Elvehjelm homogenizer in an equal volume of 0.25 M sucrose using four up-and-down strokes at 420 rpm. This liver homogenate was mixed in order to obtain a homogeneous mixture and aliquots were then immediately frozen in liquid nitrogen.

The brains from female perch and roach caught in 1997 were carefully removed and weighed. This tissue was homogenized in nine times its weight of buffer (0.25 M sucrose–0.1 M sodium phosphate, pH 7.4) in a Potter–Elvehjelm homogenizer using four up-and-down strokes at 400 rpm. The resulting homogenate was centrifuged at 2250g_{av} for 10 min. The gonads from SM female perch caught in 1997 were homogenized in an equal volume of buffer (0.25 M sucrose–0.2 M sodium phosphate, pH 7.4) and this homogenate was mixed with an equal volume of another buffer (0.25 M sucrose–0.1 M sodium phosphate, pH 7.4) prior to centrifugation (2250*g* for 10 min). The resulting supernatants obtained were each transferred to a 10-ml glass tube and then mixed with 20% glycerol (final concentration), to minimize the adverse effects of freezing and thawing. Aliquots of these mixtures were placed into cryo vials and immediately frozen in liquid nitrogen.

All the tissue preparation steps described above were performed at 0–4°C in an ice bath. Supernatants employed for various enzyme assays were stored at −120°C until use.

Testosterone and 17β-estradiol measurements. Total testosterone and 17β-estradiol levels in blood plasma were quantified employing the Chiron Diagnostics ACS:180 competitive immunoassays involving chemiluminescence and polyclonal rabbit anti-testosterone and rabbit anti-estradiol antibodies, respectively. The detection limits for these assay procedures are 0.35 nmol T/L and 0.037 nmol E2/L. Samples containing steroid levels below these detection limits were considered as having 50% of the minimal values detectable.

Enzyme assays. Ethoxyresorufin *O*-deethylase (EROD) activity was measured essentially according to the method of Burke and Mayer (1974) and expressed as pmol resorufin formed per min/mg protein. Glutathione reductase (GR) activity was assayed according to Carlberg and Mannervik (1975) and expressed as nmol NADPH oxidized per min/mg protein. Glutathione *S*-transferase (GST) activity was determined using 1-chloro-2,4-dinitrobenzene as the second substrate (Habig *et al.*, 1974) and expressed as μmol conjugate formed per min/mg protein. Catalase was assayed with hydrogen peroxide as substrate according to Bergmeyer (1955) and the activity was expressed as μmol hydrogen peroxide consumed per min/mg protein. The homogenates were rapidly thawed immediately prior to the performance of these enzymatic measurements, in order to avoid adverse thawing effects. Finally, protein was quantified according to Lowry *et al.* (1951) with bovine serum albumin as standard.

Aromatase (P450arom) activity was measured as the specific release of tritiated water accompanying conversion of 1β-H^3-androstenedione to estrone, primarily as described by Jeyasuria *et al.* (1994) with certain modifications. Each 300 μl incubation routinely contained 2 mM NADPH, 300–525 μg protein, and 146 nM (5×10^5 dpm) 1β-H^3-androstenedione (added as a solution in 5 μl ethanol) in 0.1 M sodium phosphate, 0.25 M sucrose, and 20% glycerol buffer, pH 7.4. Progesterone was omitted, since appropriate controls revealed no significant loss of substrate via the 5α-reductase pathway (Berkovitz *et al.*, 1984) under the conditions employed here. These reaction mixtures were generally incubated for 20 min at 30°C in a shaking waterbath. An external standard was used to correct for quenching. Control samples showed no significant decrease in aromatase activity after up to 12 months of storage (results not shown).

All enzyme assays were demonstrated to be linear with time and amount of protein under the conditions employed and appropriate background and control incubations were routinely performed. All enzymatic measurements were carried out at least in duplicate and the replicate values obtained agreed within ±20%.

Genotoxicological methodology. In the livers of perch caught in 1996, large hydrophobic DNA adducts were analyzed according to Gupta *et al.* (1982) and Beach and Gupta (1992), with the modifications described by Ericson *et al.* (1998, 1999). Briefly, this procedure involves homogenization, incubation with α-amylase, sequential extraction with phenol and chloroform: isoamylalcohol, and ethanol precipitation. The redissolved DNA was subsequently incubated with RNase A and RNase T_1 in order to degrade any RNA still present and the DNA was then reprecipitated with cold ethanol. The concentration of DNA was determined spectrophotometrically.

An aliquot (12.5 μg) of the isolated DNA was digested completely to deoxynucleoside-3′-monophosphates (dN3′P(s)) by treatment with micrococcal endonuclease and spleen phosphodiesterase. This digest was then enriched for aromatic/hydrophobic adducts by selective dephosphorylation of normal dN3′Ps with nuclease P1 (Reddy and Randerath, 1986). The remaining nucleotides were labeled with ^{32}P, using γ-[^{32}P]ATP and polynucleotide kinase according to Ericson *et al.* (1998). Apyrase treatment was omitted.

Nucleotide adducts were subsequently separated from the remaining traces of normal nucleotides and from excess ATP by chromatography on PEI cellulose thin-layer chromatography plates prepared in our own laboratory (Randerath and Randerath, 1964), employing resolution in two dimensions as described by Ericson *et al.* (1999). ^{32}P-Postlabeled adducts were localized by screen-enhanced autoradiography and the appropriate areas were excised from the TLC plates and their content of radioactivity was determined by liquid scintillation counting. In order to ensure that all steps in this ^{32}P-postlabeling analysis were performed as expected, standard unlabeled DNA (from salmon sperm) was used as a negative control. In addition, as both a positive control and internal chromatography standard, the adduct B*a*PDE-dG-3′p was labeled and run in parallel with the samples examined.

Chemical analysis of lake sediment. Surface lake sediment was collected at one of the deepest parts in both Lakes Molnbyggen (19 m) and Djursjön (29 m). A Kayak-corer (8.5 cm in diameter) (Kajak *et al.*, 1965) was employed to collect the top layer (1–1.5 cm) of sediment. Five to six sampled sediments from each site were pooled and later extracted by Soxhlet extraction (Soxhlet, 1879). The organic extract obtained was later fractionated on a silica column and the hexane and hexane:dichloromethane fractions, containing polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), respectively, were collected. The 15 dominating nonsubstituted PAH compounds (three to six rings) and the six dominating penta- and hexa-chlorinated PCBs were later analyzed by GC/MS (Zebühr *et al.*, 1993; Bandh *et al.*, 1996).

Statistical analysis. The data are presented as means ± SE of the values obtained with a number (n = 4–129) of fish. The data on perch caught in 1996, as well as on male perch and female and male roach caught in 1997, were analyzed using the Mann–Whitney *U* test. Data regarding SM and SIM female

TABLE 1
Parameters Examined in Female and Male Perch (*Perca fluviatilis*) Caught in Lake Molnbyggen in October 1996 in Comparison to Reference Fish from Lake Djursjön

Parameter	Female		Male	
	Lake Djursjön	Lake Molnbyggen	Lake Djursjön	Lake Molnbyggen
Total weight (g)	86.5 ± 9.18[a] (19)	74.5 ± 12.1 (21)	57.0 ± 7.61 (13)	56.5 ± 6.89 (24)
Somatic weight (g)	81.2 ± 8.60 (19)	73.6 ± 11.9 (21)	53.7 ± 7.17 (13)	54.5 ± 6.64 (24)
Total length (mm)	197 ± 6.37 (19)	183 ± 8.63 (21)	174 ± 8.14 (13)	174 ± 5.97 (24)
Age (year)	5.3 ± 0.41 (14)	3.0 ± 0.28* (20)	5.5 ± 0.64 (12)	4.2 ± 0.27 (20)
Somatic condition factor (%)	0.995 ± 0.0133 (19)	0.981 ± 0.0341 (21)	0.957 ± 0.0179 (13)	0.922 ± 0.0248 (24)
Somatic growth (g/year)	15 ± 1.5 (14)	21 ± 2.4 (20)	11 ± 1.3 (12)	15 ± 1.2* (20)
Fin erosion and sores[b] (%)	0 (19)	4.8 (21)	0 (13)	33.3* (24)
Liver–somatic index (%)	1.30 ± 0.0637 (19)	1.09 ± 0.0719* (21)	0.854 ± 0.0547 (13)	1.03 ± 0.0702 (24)
Gonadosomatic index (%)	6.54 ± 0.224 (19)	1.31 ± 0.411* (21)	6.05 ± 0.219 (13)	3.88 ± 0.243* (24)
Spleen–somatic index (%)	0.05 ± 0.004 (19)	0.05 ± 0.005 (21)	0.06 ± 0.007 (12)	0.07 ± 0.007 (24)
Hematocrit (%)	22.1 ± 1.02 (12)	20.7 ± 2.06 (10)	23.0 ± 1.57 (5)	21.9 ± 1.41 (7)
Leukocytes (% of total blood cells)	6.94 ± 0.554 (12)	5.69 ± 0.330 (9)	4.89 ± 0.556 (5)	5.04 ± 0.377 (7)
Lymphocytes (% of leukocytes)	24.2 ± 3.78 (12)	25.6 ± 4.76 (9)	22.4 ± 7.21 (5)	23.6 ± 3.73 (7)
Granulocytes (% of leukocytes)	22.0 ± 1.88 (12)	22.7 ± 4.60 (9)	13.1 ± 1.68 (5)	17.8 ± 1.51 (7)
Thrombocytes (% of leukocytes)	53.9 ± 3.66 (12)	51.7 ± 6.08 (9)	64.5 ± 8.61 (5)	58.6 ± 3.45 (7)
Blood hemoglobin (mg/ml)	53.5 ± 2.11 (17)	51.2 ± 3.48 (10)	55.7 ± 3.51 (5)	52.4 ± 2.82 (9)
Blood glucose (mg/100 ml)	12.7 ± 2.14 (12)	3.54 ± 0.468* (9)	7.10 ± 1.59 (4)	6.40 ± 0.802 (9)
Ethoxyresorufin *O*-deethylase (pmol/min/mg protein)	83.1 ± 5.20 (11)	114 ± 10.3* (12)	51.0 ± 13.7 (5)	130 ± 20.2* (9)
Glutathione-*S*-transferase (μmol/min/mg protein)	0.612 ± 0.0237 (11)	0.824 ± 0.0697* (12)	0.855 ± 0.0604 (5)	0.765 ± 0.0272 (9)
Glutathione reductase (nmol/min/mg protein)	12.4 ± 0.559 (11)	14.0 ± 0.523 (12)	14.1 ± 1.29 (5)	13.2 ± 0.465 (9)
Catalase (μmol/min/mg protein)	180 ± 7.72 (11)	336 ± 45.9* (12)	315 ± 37.5 (5)	345 ± 38.3 (9)
Hydrophobic DNA adducts (nmol adducts/mol normal nucleotides)	1.0 ± 0.34 (10)	0.63 ± 0.066 (12)	na[c]	0.54 ± 0.072 (9)

[a] Means ± SE of values derived from (*n*) fish. Each sex was subjected separately to statistical analysis.
[b] The χ^2 test was used to test for associations between the lake of origin and the frequency of fin erosion and sores.
[c] na, not analyzed.
* Significantly different from the corresponding value for fish from Lake Djursjön ($p < 0.05$) according to the Mann–Whitney *U* test.

perch, caught in Lake Molnbyggen in 1997, were analyzed by the Kruskal–Wallis test and the major differences thus observed were examined further using the two-tailed nonparametric multiple comparison test of Dunn (1964), with SM female perch from Lake Djursjön as the control. Data obtained for SIM female perch from Lake Djursjön were not included in the Kruskal–Wallis test, since the number of individuals in this group was naturally low. In order to keep the statistics uniform, nonparametric tests were employed due to sometimes unequal variances and variations in sample size between the groups. The chemical analysis of lake sediments was only performed on one pooled single sample from each lake. The level of significance in all statistical tests was set at $p < 0.05$, whereas a tendency was considered to be demonstrated when $0.05 \leq p \leq 0.20$.

RESULTS

Body and organ parameters in 1996. As documented in Table 1, in October 1996, no differences between female perch from Lakes Djursjön and Molnbyggen with respect to total weight, somatic weight, total length, SCF, somatic growth, or SSI were observed. However, female perch from Lake Molnbyggen were found to have significantly lower LSI (16%) and GSI (80% decreased) values. The female perch from Lake Molnbyggen were also younger and showed a tendency ($p = 0.16$) toward increased somatic growth.

Nor were differences between male perch from these two lakes with respect to total weight, somatic weight, total length, age, SCF, LSI, or SSI observed (Table 1). However, male perch in Lake Molnbyggen demonstrated significantly lower GSI values (36% decreased), as well as significantly increased somatic growth. Furthermore, male perch from Lake Molnbyggen revealed a tendency ($p = 0.08$) toward increased LSI.

The combined frequency of fin erosion and shallow open sores in male perch from Lake Molnbyggen was 33%, whereas none of the male perch from Lake Djursjön demonstrated such abnormalities. Of the female perch in Lake Molnbyggen, 4.8% showed either fin erosion and/or shallow open sores, which were not seen in any of the female perch from Lake Djursjön. However, this later difference was not statistically significant. One characteristic example of a shallow open sore on a perch is depicted in Fig. 2A. These sores, 3–12 mm in diameter, were usually located on the tail, between the anal opening and the tail fin.

Blood variables. Of the different blood variables examined in female and male perch from Lakes Molnbyggen and Djursjön, the only significant difference observed was a lower

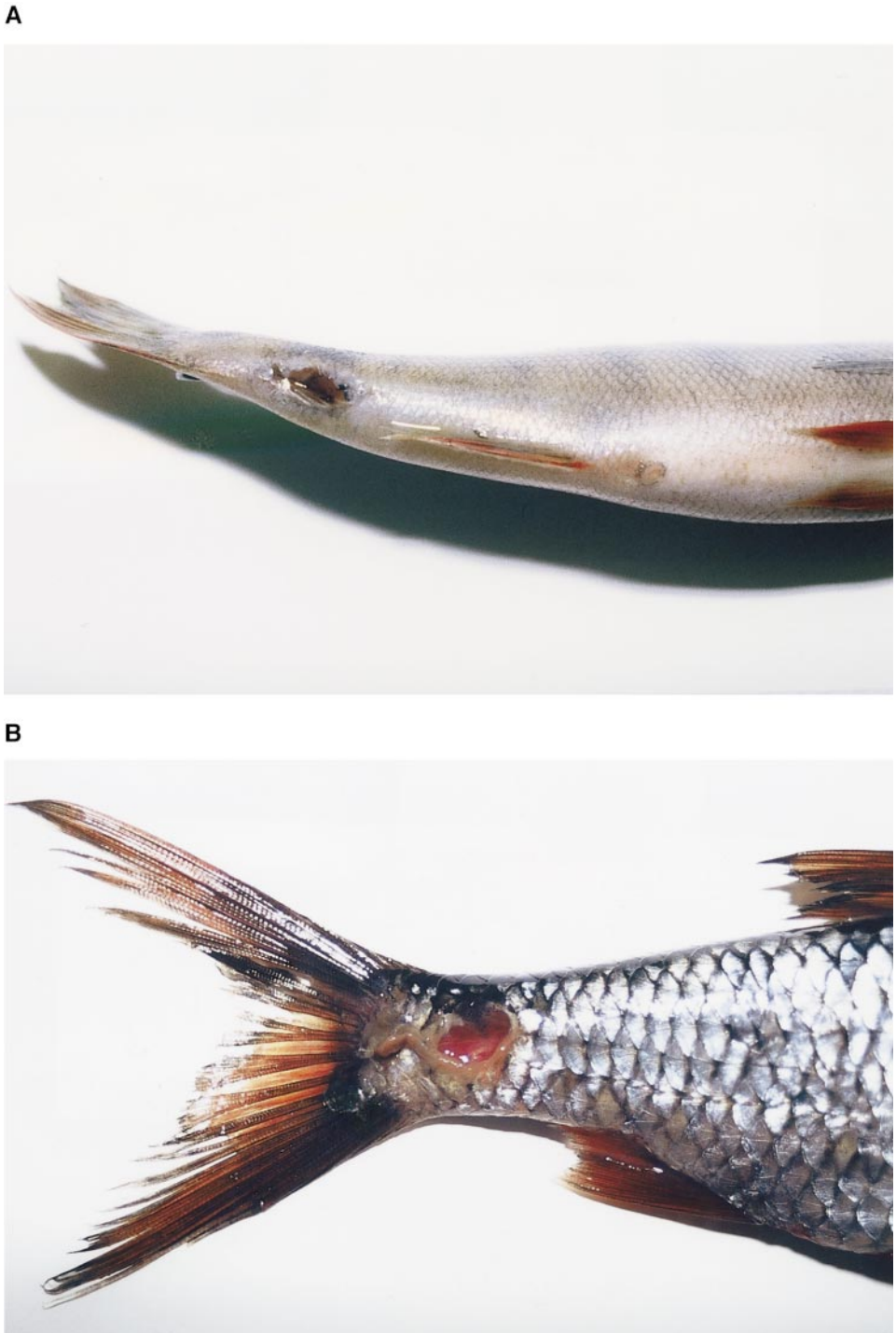

FIG. 2. Characteristic examples of shallow open sores observed on both perch (*Perca fluviatilis*) (A) and roach (*Rutilus rutilus*) (B) from Lake Molnbyggen. These sores, 3–12 mm in diameter, were usually found on the tail between the anal opening and the tail fin.

blood glucose level in the fish from Lake Molnbyggen (Table 1).

Xenobiotic-metabolizing enzyme activities. In female perch from Lake Molnbyggen, the hepatic activities of EROD, GST, and catalase were significantly higher than those in female perch from Lake Djursjön (Table 1). No difference in GR activity was observed. In the case of male perch, a significantly higher hepatic EROD activity was found in the fish from Lake Molnbyggen, but no differences in GST, GR, or catalase activity were seen.

TABLE 2
Parameters Examined in Female Perch (*Perca fluviatilis*) and Roach (*Rutilus rutilus*) Caught in Lake Molnbyggen in October/November 1997 in Comparison to Reference Fish from Lake Djursjön

	Perch[a]				Roach[b]	
	Mature (SM)		Immature (SIM)		All mature	
Parameter	Lake Djursjön	Lake Molnbyggen	Lake Djursjön	Lake Molnbyggen	Lake Djursjön	Lake Molnbyggen
Total weight (g)	138 ± 5.39[c] (93)	183 ± 32.1 (19)	111 ± 18.3 (4)	97.3 ± 7.66* (58)	123 ± 5.63 (65)	109 ± 4.46* (129)
Somatic weight (g)	131 ± 5.12 (93)	175 ± 30.7 (19)	111 ± 18.2 (4)	96.9 ± 7.64* (58)	111 ± 5.05 (65)	101 ± 4.14 (129)
Total length (mm)	220 ± 2.40 (93)	229 ± 11.2 (19)	213 ± 10.3 (4)	197 ± 4.13* (58)	211 ± 3.31 (65)	208 ± 2.70 (129)
Age (year)	7.3 ± 0.20 (93)	7.8 ± 0.89 (19)	8.0 ± 0.50 (4)	6.1 ± 0.27* (58)	na[d]	na
Somatic condition factor (%)	1.18 ± 0.0120 (93)	1.22 ± 0.0369 (19)	1.11 ± 0.0459 (4)	1.15 ± 0.0147 (58)	1.13 ± 0.0116 (65)	1.05 ± 0.00817* (129)
Somatic growth (g/year)	18 ± 0.61 (93)	21 ± 1.6 (19)	14 ± 2.3 (4)	15 ± 0.65* (58)	na	na
Fin erosion and sores[e] (%)	2.2 (93)	21.0* (19)	0 (4)	37.9* (58)	1.5 (65)	7.0 (129)
Liver–somatic index (%)	1.56 ± 0.0301 (93)	1.43 ± 0.0775 (19)	1.10 ± 0.206 (4)	1.32 ± 0.0490* (58)	1.55 ± 0.0536 (65)	1.43 ± 0.0397 (98)
Percent of fish sample[e] (%)	95.9 (93)	24.7* (19)	4.1 (4)	75.3* (58)	100 (65)	100 (129)

[a] The data regarding SM and SIM female perch from Lake Molnbyggen were compared employing the Kruskal–Wallis test and the major differences thus observed examined further using the nonparameteric comparison test of Dunn (1964), with SM female perch from Lake Djursjön as control. SIM perch from Lake Djursjön were not included in this analysis.

[b] Data on female roach were tested with the Mann–Whitney *U* test.

[c] Means ± SE for the values derived from (n) fish.

[d] na, not analyzed.

[e] The χ^2 test was used to test for associations. SIM perch from Lake Djursjön were not included in these tests.

* The level of significance in all of these tests was set at $p < 0.05$.

Levels of hydrophobic DNA adducts. The autoradiographic pattern of hepatic DNA adducts in perch from both lakes induced three to five very faint spots, as well as a general weak shading (not shown). The overall level of hydrophobic DNA adducts was low, i.e., close to the background levels observed in fish living in pristine waters. There were no significant differences in the total hepatic adduct levels in female perch from Lakes Molnbyggen and Djursjön (Table 1).

Body and organ parameters in 1997. As shown in Table 2, for fish caught in 1997, no differences between SM female perch from Lakes Djursjön and Molnbyggen with respect to total weight, somatic weight, total length, age, SCF, somatic growth, or LSI were observed. However, the SM female perch in Lake Molnbyggen demonstrated a significantly lower (17%) GSI value (Fig. 3). SIM female perch in Lake Molnbyggen were significantly smaller (lower total and somatic weights), shorter, and younger and showed decreased somatic growth compared to SM female perch from Lake Djursjön (Table 2). Furthermore, the SIM female perch from Lake Molnbyggen had significantly lower LSI and GSI (93% decrease) values (Table 2 and Fig. 3). The combined average value for GSI in both SM and SIM female perch from Lake Molnbyggen was 73% lower than the corresponding value for Lake Djursjön (compared to 80% lower in 1996).

Both SM and SIM female perch in Lake Molnbyggen demonstrated statistically significantly higher frequencies of fin erosion and shallow open sores (21.0 and 37.9%, respectively) than did female perch from Lake Djursjön (2.2%). The most pronounced effect observed was the extremely low proportion of SM female perch living in Lake Molnbyggen (Table 2). The gonad of only 24.7% of the female perch in Lake Molnbyggen contained maturing oocytes, i.e., 75.3% of these fish were SIM. In the reference Lake Djursjön, 95.9% of the female perch were SM and only 4.1% were SIM ($p < 0.05$).

Since female perch caught in 1996 and 1997 in Lake Molnbyggen tended to be both younger and smaller than those caught in Lake Djursjön, a more detailed analysis of the SIM female perch from both of these lakes was performed. In Table 3, the SM and SIM female perch caught in Lakes Molnbyggen and Djursjön in 1997 have been divided into three different age and weight classes. For these classes the proportion of SIM females in Lake Djursjön ranged between 0.0 and 7.7%, whereas in Lake Molnbyggen this frequency ranged between 44.4 and 93.3% ($p < 0.05$ for all age and weight classes). The lowest proportion (6.7%) of SM female perch in Lake Molnbyggen was observed in the age class of 6.5–8.5 years. It is also seen that the proportion of SIM female perch has a tendency to be slightly lower among older (9.5–14.5 years) and heavier (200–500 g) fish in Lake Molnbyggen. In summary, in all age and weight classes investigated, a significantly larger proportion (by a factor of 10 or more) of SIM female perch were living in Lake Molnbyggen than in Lake Djursjön.

No differences in the somatic weight and total length of female roach in Lakes Djursjön and Molnbyggen were observed. A tendency ($p = 0.12$) toward a lower LSI in female roach from Lake Molnbyggen was found. Furthermore, female roach in Lake Molnbyggen demonstrated a significantly de-

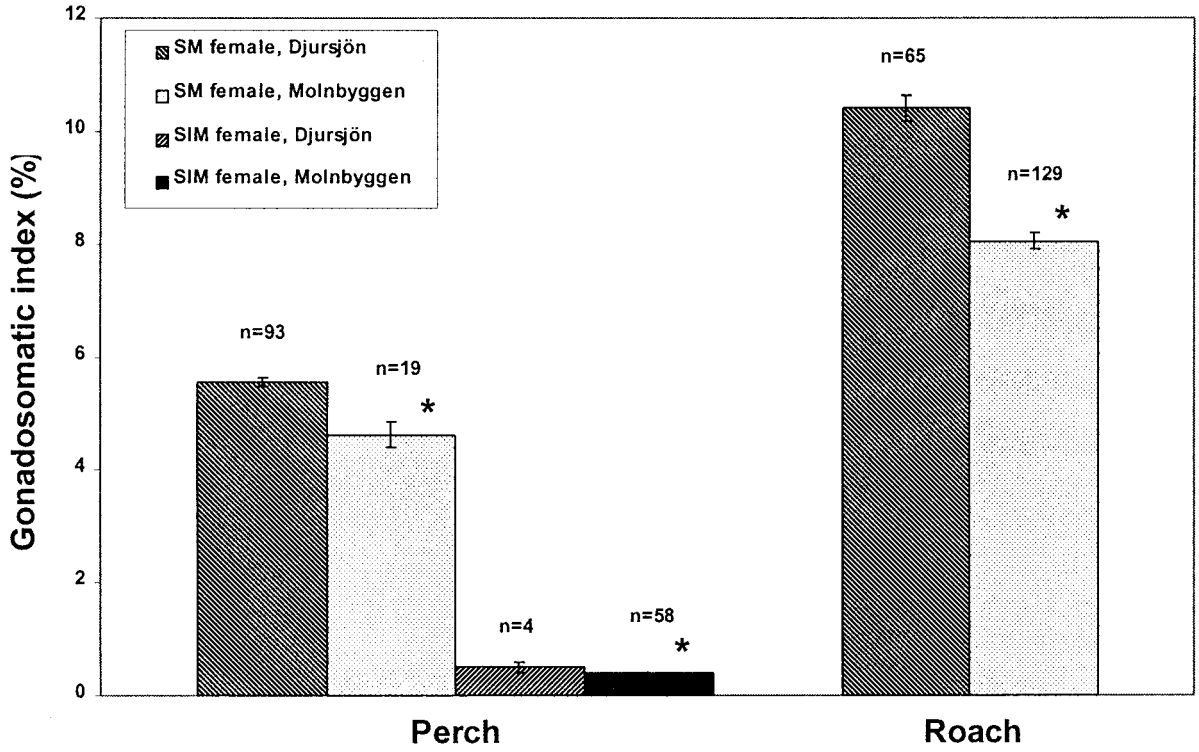

FIG. 3. The gonadosomatic index (GSI) for sexually mature (SM) and sexually immature (SIM) female perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) in Lakes Molnbyggen and Djursjön. All of the female roach caught in both lakes were sexually mature. The data are presented as means ± SE for the values derived from *n* fish. The data on SM and SIM female perch from Lake Molnbyggen were compared employing the Kruskal–Wallis test and the major differences thus obtained were examined further using the nonparametric comparison test of Dunn (1964), with SM female perch from Lake Djursjön as the control. The data obtained with SIM perch from Lake Djursjön were not included in this analysis. Data on female roach were compared using the Mann–Whitney *U* test. *The level of significance in all of these tests was set at $p < 0.05$.

creased total weight and SCF as well as a tendency ($p = 0.20$) toward higher frequencies of fin erosion and shallow open sores (7.0%) compared to female roach in Lake Djursjön (1.5%) (Table 2). With respect to both size and location, these sores were very similar to those seen on perch in Lake Molnbyggen (Figs. 2A and B). The gonads of all female roach in both Lakes Djursjön and Molnbyggen contained sexually maturing oocytes. As was the case for SM female perch in Lake Molnbyggen, female roach in this lake also had a GSI value that was lower (23% decrease) than that for female roach in Lake Djursjön (Fig. 3).

The data on male perch and roach caught in 1997 are

TABLE 3
Proportions of Sexually Mature and Sexually Immature Female Perch (*Perca fluviatilis*) Caught in October/November 1997 in Lake Molnbyggen and in the Reference Lake Djursjön

		Lake Djursjön		Lake Molnbyggen	
Class	Subclass	Mature (SM)	Immature (SIM)	Mature (SM)	Immature (SIM)
Age (years)	3.5–5.5[a]	100 (19)	0.0 (0)	22.9* (11)	77.1* (37)
	6.5–8.5	94.8 (55)	5.2 (3)	6.7* (1)	93.3* (14)
	9.5–14.5	95.0 (19)	5.0 (1)	50.0* (7)	50.0* (7)
Weight (g)	45–99	92.3 (24)	7.7 (2)	15.9* (7)	84.1* (37)
	100–199	96.8 (60)	3.2 (2)	29.2* (7)	70.8* (17)
	200–500	100 (9)	0.0 (0)	55.6* (5)	44.4* (4)

Note. Values are percentages with number of fish in parentheses.
[a] The χ^2 test was used to analyze for associations between the subclass and proportions of SM and SIM female perch in the two lakes.
* The level of significance was set at $p < 0.05$.

TABLE 4
Parameters Examined in Male Perch (*Perca fluviatilis*) and Roach (*Rutilus rutilus*) Caught in Lake Molnbyggen in October/November 1997 in Comparison to Reference Fish from Lake Djursjön

	Perch[a]		Roach	
Parameter	Lake Djursjön	Lake Molnbyggen	Lake Djursjön	Lake Molnbyggen
Total weight (g)	96.0 ± 7.43[b] (27)	180 ± 22.5* (8)	62.0 ± 9.48 (8)	63.6 ± 9.12 (13)
Somatic weight (g)	90.1 ± 6.98 (27)	172 ± 21.3* (8)	59.5 ± 9.07 (8)	61.5 ± 8.77 (13)
Total length (mm)	196 ± 4.54 (27)	238 ± 10.0* (8)	174 ± 7.04 (8)	178 ± 5.21 (13)
Age (year)	7.3 ± 0.35 (27)	9.1 ± 0.60* (8)	na[c]	na
Somatic condition factor (%)	1.13 ± 0.0150 (27)	1.21 ± 0.0395 (8)	1.09 ± 0.0271 (8)	1.04 ± 0.0236 (13)
Somatic growth (g/year)	13 ± 0.88 (27)	18 ± 1.9* (8)	na	na
Fin erosion and sores[d] (%)	0 (27)	25.0* (8)	0 (8)	0 (13)
Liver–somatic index (%)	0.928 ± 0.0709 (27)	1.22 ± 0.118* (8)	1.64 ± 0.163 (8)	1.13 ± 0.152 (6)

[a] The data on male perch and roach were analyzed separately using the Mann–Whitney *U* test.
[b] Means ± SE for the values derived from (n) fish.
[c] na, not analyzed.
[d] The χ^2 test was used to test for an association between the lakes of origin and frequency of fin erosion and sores.
* The level of significance in all of these tests was set at $p < 0.05$.

presented in Table 4. Male perch from Lake Molnbyggen were significantly larger (higher total and somatic weights), longer, and older than the male perch in Lake Djursjön. The somatic growth and LSI were higher in male perch from Lake Molnbyggen, whereas the GSI was significantly lower (24% decreased) than for male perch from Lake Djursjön (Fig. 4). All of these male perch were found to have sexually maturing testes. Male perch in Lake Molnbyggen demonstrated significantly higher frequencies (25%) of fin erosion and/or shallow open sores than did male perch from Lake Djursjön (0.0%).

In the case of male roach, no differences with respect to total and somatic weights, total length, or SCF for fish from Lakes Djursjön and Molnbyggen were seen. However, a significantly lower GSI (19% decrease; see Fig. 4) as well as a tendency ($p = 0.05$) toward a lower LSI was found in male roach from Lake Molnbyggen (Table 4). All of these male roach were found to have sexually maturing testes. None of the male roach in Lakes Molnbyggen and Djursjön exhibited fin erosion or shallow open sores.

Aromatase activity. As depicted in Fig. 5, the brain P450arom activity in SIM female perch from Lake Molnbyggen was significantly lower than the corresponding value for SM female perch in Lake Djursjön. A tendency toward a lower P450arom activity could also be seen in SM female perch from Lake Molnbyggen, although this difference was not statistically significant ($p = 0.10$). Although P450arom activity could be measured in only one SIM female perch from Lake Djursjön, this activity was the same as that seen in SIM female perch from Lake Molnbyggen. A tendency ($p = 0.07$) toward lower P450arom activity in the brain of female roach in Lake Molnbyggen compared to Lake Djursjön was observed (Fig. 4).

When the specific P450arom activity in the gonad of SM female perch from Lakes Djursjön ($n = 15$) and Molnbyggen ($n = 8$) was assayed, no differences were observed (results not shown). The specific P450arom activity in the gonad was 6.4- and 4.1-fold lower than in the brain of SM female perch in Lakes Djursjön and Molnbyggen, respectively.

Testosterone and E2 levels. As shown in Fig. 6, the level of T in the blood plasma of SM perch caught in Lake Molnbyggen in 1997 was significantly lower than in SM perch in Lake Djursjön. Furthermore, SIM perch in Lake Molnbyggen also demonstrated significantly lower levels of T than did SM perch from Lake Djursjön. The only SIM perch from Lake Djursjön whose blood plasma was analyzed for T had a value similar to the mean level in SIM perch from Lake Molnbyggen.

The mean blood level of E2 was lower in SM perch from Lake Molnbyggen than from Lake Djursjön, although this difference was not statistically significant ($p = 0.10$). However, significantly lower levels of E2 were present in SIM perch from Lake Molnbyggen compared to SM perch in Lake Djursjön (Fig. 6). The single SIM perch from Lake Djursjön that was analyzed demonstrated an E2 level similar to the mean value for SIM perch in Lake Molnbyggen.

The level of T in the blood plasma of female roach caught in Lake Molnbyggen in 1997 was significantly lower than the corresponding value for Lake Djursjön (Table 5). No difference in the levels of E2 was seen.

A single male perch from Lake Molnbyggen was found to have 5.9 nmol T/L and 2.1 nmol E2/L in comparison with values of 16.4 ± 1.95 nmol T/L ($n = 7$) and 1.6 ± 0.28 nmol E2/L ($n = 7$) in male perch from Lake Djursjön.

Chemical analysis of lake sediment. The total level of the 15 dominating nonsubstituted three- to six-ringed PAHs analyzed in lake surface sediment from Lake Molnbyggen was 1.9 μg/g dry wt sediment. The corresponding total level of PAHs

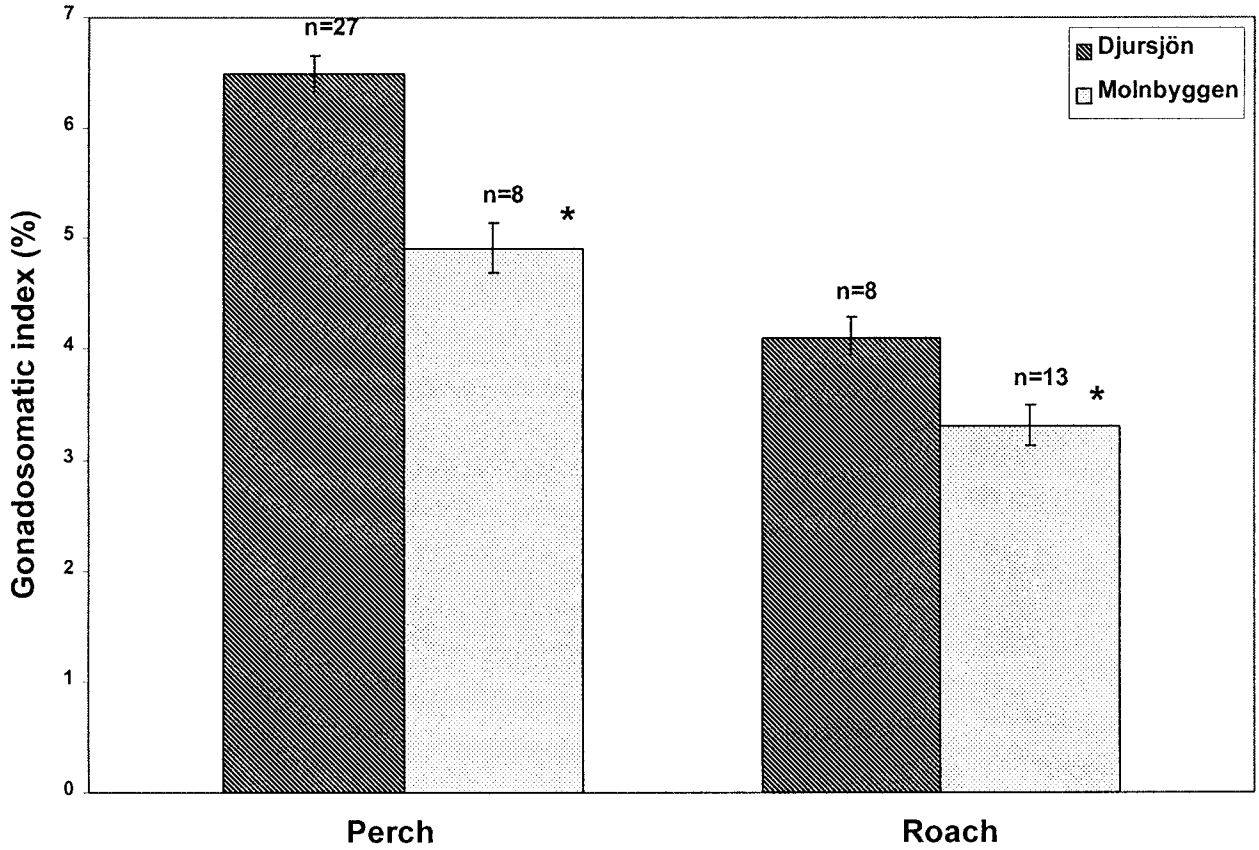

FIG. 4. The gonadosomatic index (GSI) for male perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) from Lakes Molnbyggen and Djursjön. The data are means ± SE of the values derived from *n* fish. Male perch and roach were compared using the Mann–Whitney *U* test. *The level of significance was set at $p < 0.05$.

in Lake Djursjön was 2.1 μg/g dry wt sediment; hence very similar total levels of PAHs were found in Lakes Molnbyggen and Djursjön. The individual levels of the 15 PAHs analyzed were also very similar between the two lakes (results not shown).

A total level of 5.8 ng/g dry wt sediment of the six dominating penta- and hexa- chlorinated PCBs was found in lake sediment from Lake Molnbyggen. The sediment in Lake Djursjön contained a total level of 3.9 ng/g dry wt sediment of the corresponding penta- and hexa-chlorinated PCBs. Very similar levels of the six individual penta- and hexa-chlorinated PCBs analyzed were observed in Lakes Molnbyggen and Djursjön (results not shown).

DISCUSSION

In 1994–1995 local fishermen began to report that several species of fish in Lake Molnbyggen showed signs of illness. A limited number of perch and pike from this lake, caught in late August 1995, demonstrated increased frequencies of fin erosion and shallow open sores (26.3%) as well as small and undeveloped gonads (L. Balk, unpublished data). However, this pilot study involved only a small number of fish stored frozen and sampled at the end of the summer.

There was serious local concern about the possibility that the leakage water from the nearby public refuse dump in Lindbodarna, Leksand, was exerting toxic effects on the fish in Lake Molnbyggen. No industrial activities are or have been located in the pristine area surrounding this lake, which is characterized by small hills covered with large forests of pine and spruce. Lake Djursjön was selected as the reference lake because of its great similarity to Lake Molnbyggen with respect to parameters including habitat type, water temperature, and very little past or present human encroachment. However, Lake Djursjön is located in another, nearby drainage area that does not receive leakage water from the public refuse dump.

In perch caught in Lake Molnbyggen in 1996, very high frequencies of fin erosion and shallow open sores were seen, as well as significantly increased hepatic activities of catalase and GST in the female fish. However, only a weak significant induction of EROD activity and low levels of DNA adducts (not significant) could be observed in these female and male perch. The most pronounced effect was the extreme decrease in the size of the gonads, especially in the female perch (80%),

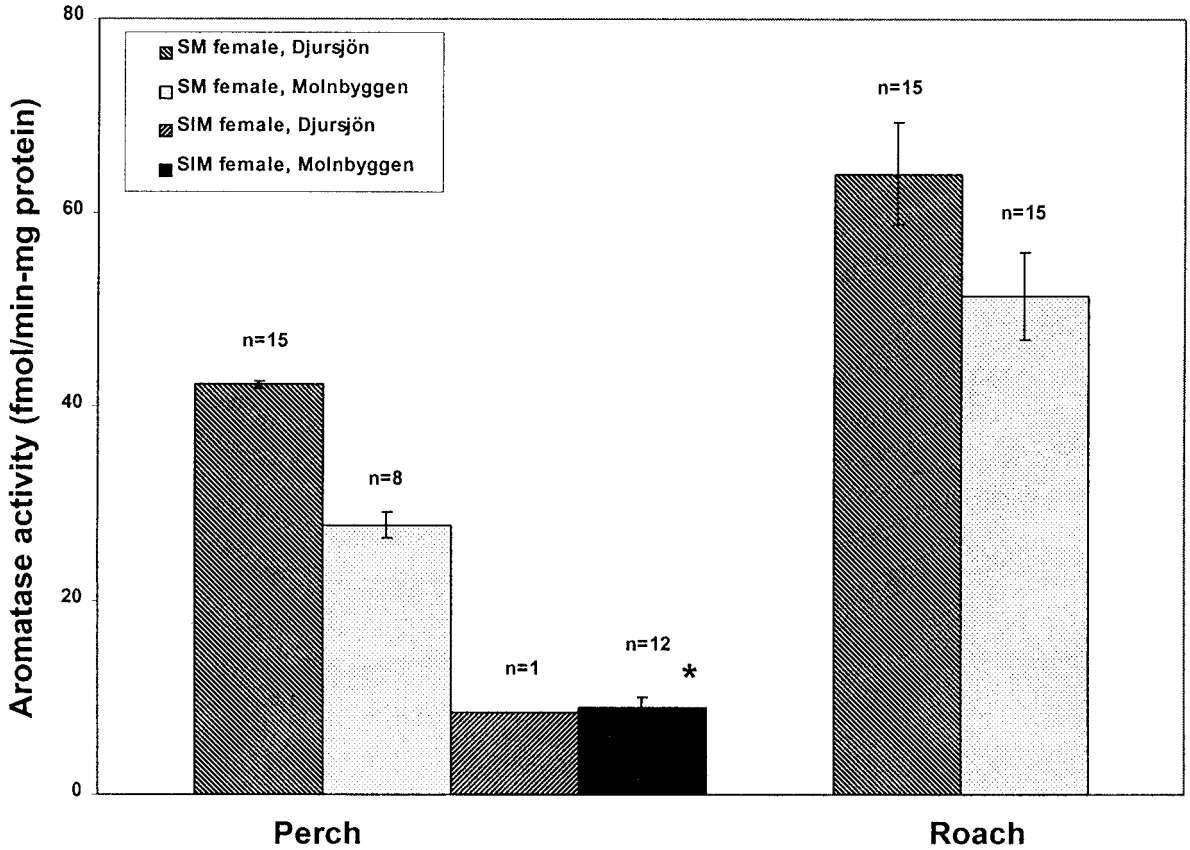

FIG. 5. Brain aromatase (P450arom) activity (fmol/min/mg protein) in sexually mature (SM) and sexually immature (SIM) female perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) from Lakes Molnbyggen and Djursjön. All of the female roach caught in both lakes were sexually mature. The data are presented as means ± SE for the values derived from *n* fish. SM and SIM female perch from Lake Molnbyggen were compared employing the Kruskal–Wallis test and the major differences thus indicated were examined further using the nonparametric comparison test of Dunn (1964), with SM female perch from Lake Djursjön as control. SIM perch from Lake Djursjön were not included in this analysis. Female roach were analyzed using the Mann–Whitney *U* test. *The level of significance in all of these tests was set at $p < 0.05$.

but also in the males (36%). Similarly, female and male perch inhabiting Swedish coastal waters polluted by BKME and caught in 1984–1985 were also found to have a lower GSI and elevated frequencies of eroded and shortened tail fins, perhaps as a direct consequence of an impairment of the immune system (Andersson *et al.*, 1988). However, in this other case, the effects on GSI were generally less and more pronounced in male perch. Furthermore, in an investigation of female and male lake whitefish (*Coregonus clupeaformis*) exposed to BKME at Jackfish Bay, Lake Superior, Munkittrick *et al.* (1992) also reported high frequencies of lateral, elongated, slash-like lesions penetrating the body cavity. Although 20% of the lake whitefish demonstrated this kind of lesion, these sores were very different morphologically from those seen on both perch and roach in the present study. It should be stressed that BKME are not suspected to be present in the leakage water from the refuse dump, since no pulp and paper mills are located in this part of Sweden.

In Lake Molnbyggen, the decrease in GSI was accompanied by a significantly lower LSI and a tendency toward increased somatic growth in female perch, as well as significantly increased somatic growth and a tendency toward lower LSI in male perch caught in 1996. Since the development of sexually mature gonads in both female and male fish is an energy-requiring process, the increase in somatic growth might thus be a result of energy reserves available related to the lower GSI. A lower production of vitellogenin in the liver might explain the lower LSI (fewer hepatocytes) in female perch from Lake Molnbyggen. Vitellogenin is produced by the liver in response to E2, which is in turn synthesized by ovarian follicle cells in response to increased gonadotropin levels in the blood (Ho, 1991; Nagahama, 1994). Oocyte growth in fish involves uptake of circulating vitellogenin, which is then modified and deposited as yolk in the oocyte (Wallace *et al.*, 1985). The decrease in blood levels of glucose could also reflect impaired hormonal control (Andersson *et al.*, 1988).

There were no signs of parasites or of other pathogens in the perch studied here. Furthermore, the values for the different blood parameters analyzed indicated that these perch did not suffer from severe infection. However, the involvement of

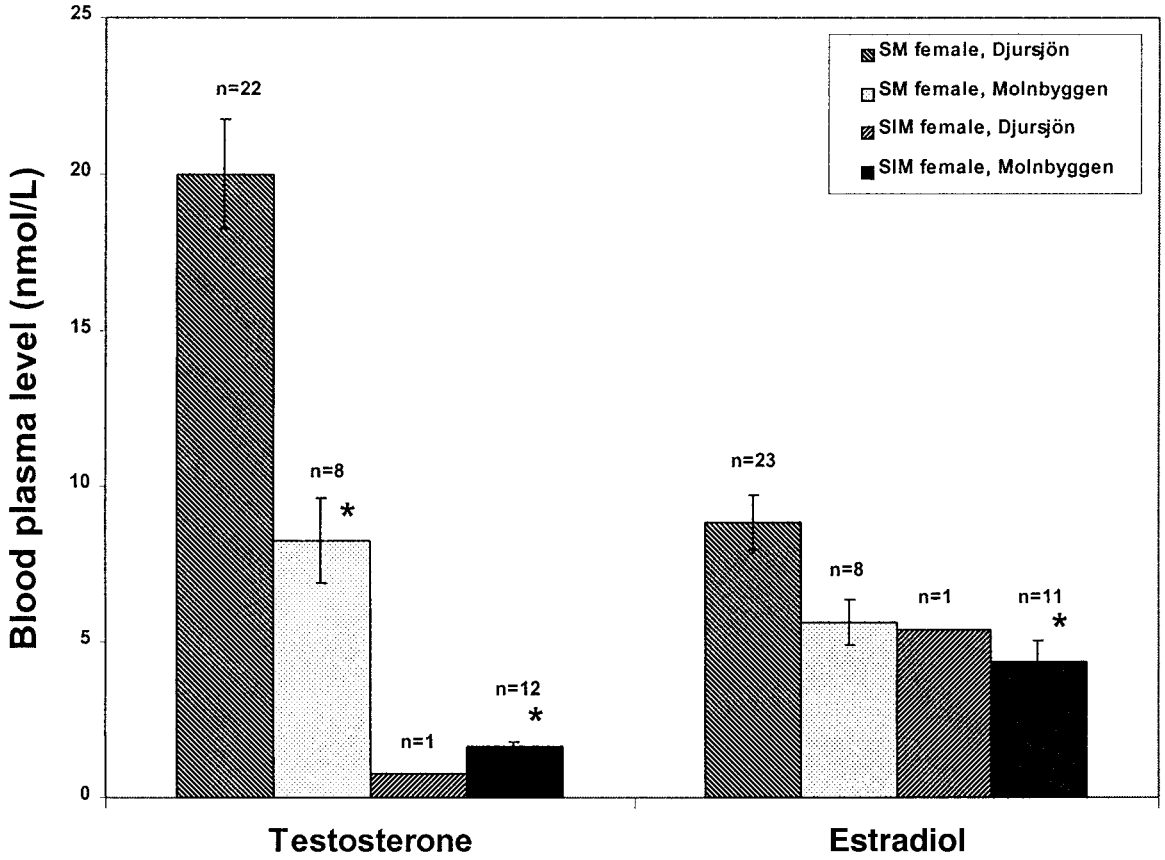

FIG. 6. Levels of testosterone and 17β-estradiol (nmol/L) in the blood plasma of sexually mature (SM) and sexually immature (SIM) female perch (*Perca fluviatilis*) from Lakes Molnbyggen and Djursjön. The data are presented as means ± SE of the values derived from *n* fish. SM and SIM female perch from Lake Molnbyggen were analyzed employing the Kruskal–Wallis test and the major differences thus indicated examined further by the nonparametric comparison test of Dunn (1964), with SM female perch from Lake Djursjön as controls. SIM perch from Lake Djursjön were not included in this analysis. *The level of significance in all of these tests was set at $p < 0.05$.

infection in some of our findings cannot at present be ruled out completely.

All together, the data from 1996 with comparatively little induction of EROD and low levels of DNA adducts in both sexes and only female perch demonstrating changes in GST and catalase activities also indicated that the perch in Lake Molnbyggen were not exposed to significant levels of well-known environmental pollutants, e.g., PAHs or PCBs. If the very high frequencies of fin erosion and sores, as well as the very low GSI observed were due to exposure to PAH and/or PCB, these biomarkers would most likely have responded differently and in a more similar manner in both sexes. For instance, a strong correlation between adduct levels and the level of PAH contamination have been found previously in the liver of perch caught at different distance from an aluminum smelter on the Swedish Baltic coast (Ericson *et al.*, 1998). Such graded responses have also been observed in other fish species living in PAH-contaminated waters (Collier *et al.*, 1993; Van der Oost *et al.*, 1994; Eufemia *et al.*, 1997). The low levels of the 15 dominating nonsubstituted PAHs and the six dominating penta- and hexa-PCBs analyzed in lake sediments from Lakes Molnbyggen and Djursjön also confirmed this hypothesis. The levels of PAHs and PCBs in both lakes were very similar to what recently has been observed in lake sediments from 80 pristine Swedish freshwater lakes (M. Söderström and T. Alsberg, unpublished data).

Today, EROD induction is an established and sensitive biological response to and early warning signal for exposure to

TABLE 5

Levels of Testosterone and 17β-Estradiol in the Blood Plasma of Female Roach (*Rutilus rutilus*) Caught in Lakes Molnbyggen and Djursjön in October/November 1997

Steroid	Lake Djursjön (nmol/L)	Lake Molnbyggen (nmol/L)
Testosterone[a]	1.3 ± 0.11[b] (15)	0.83 ± 0.11* (15)
17β-Estradiol	8.4 ± 0.55 (15)	9.4 ± 0.61 (15)

[a] Female roach were analyzed employing the Mann–Whitney *U* test.
[b] Means ± SE of the values derived from (n) fish.
* The level of significance was set at $p < 0.05$.

planar PCBs and several PAHs. EROD induction in the liver of female and male perch in Lake Molnbyggen was relatively weak, i.e., 1.4- and 2.5-fold, respectively. Female perch from a Swedish lake highly contaminated with PCB demonstrated low EROD activity similar to that seen in female perch from a reference lake (Förlin and Norrgren, 1998). These female perch were subsequently found to be nonresponsive to the CYP1A inducers PCB 77 and β-naphthoflavone (Förlin and Celander, 1995). The weak induction of EROD in the perch in Lake Molnbyggen would seem to indicate that this fish are indeed responsive to EROD-inducing compounds. All of these findings, in combination with the very low GSI observed in both female and male perch from Lake Molnbyggen, indicate that these fish are not generally exposed to well-known environmental pollutants in the leakage water from the refuse dump but to an organic pollutant(s) causing severe reproductive disorder, possibly as a result of endocrine disruption.

One promising biomarker for reproductive disorders is decreased aromatase (P450arom) activity (Monod *et al.*, 1993; Piferrer *et al.*, 1994). Aromatase is the enzyme complex that catalyzes conversion of C19 androgens into C18 estrogens. Brain P450arom activity in teleost fish is exceptionally high compared to the corresponding activities in rat, mouse, rabbit, and hamster (Callard and Pasmanik, 1987). The brain of teleost fish has therefore been suggested to be a suitable tissue for studies on P450arom activity (Callard and Pasmanik, 1987; Callard *et al.*, 1995). Moreover, several imidazole compounds, used as potent antifungal agents and fungicides, are potent inhibitors of P450arom (Monod *et al.*, 1993). In addition, changes in the levels of steroid hormones involved in reproduction, e.g., testosterone and 17β-estradiol, have been suggested to be good predictors of the effects of BKME on maturity, egg production, and secondary sex characteristics in various species of fish (Munkittrick *et al.*, 1998).

In order to mechanistically investigate the reproductive disorders observed here and the associated hypothesis that perch in Lake Molnbyggen are exposed to endocrine-disrupting substances, we analyzed the number of sexually mature perch of different ages and weights, as well as specific endocrine biomarkers, i.e., brain P450arom activity and plasma levels of testosterone and 17β-estradiol. Roach of both sexes and a limited number of pike and burbot were also included in this analysis.

SM and SIM female and male perch caught in Lake Molnbyggen in 1997 again demonstrated very high frequencies of fin erosion and shallow open sores, similar to the findings in 1996. However, the most pronounced difference was the extremely low frequency (25%) of SM female perch in Lake Molnbyggen compared to Lake Djursjön (96%). This reproductive disorder must be considered very severe, since the majority (75%) of the female perch in Lake Molnbyggen do not develop a gonad containing sexually mature oocytes. This high frequency of SIM female perch and significantly lower GSI of both SM female and male perch can, in some respects, be compared to findings on perch in waters outside a pulp and paper mill in Sweden during the 1980s. However, in these earlier studies, the effects on male perch were more pronounced and the frequency of SIM female perch was in general much lower (Sandström *et al.*, 1988; Sandström, 1994). Reduced size of the gonad has also been found on lake whitefish and white sucker (*Catostomus commersoni*) exposed to BKME in Canada (Munkittrick *et al.*, 1992; McMaster *et al.*, 1995).

Less than 7.8% of the female perch of all ages and weights caught in Lake Djursjön during both 1996 (not shown) and 1997 were SIM. A certain percentage of female perch with SIM or resting gonads appears to be a natural phenomena also occurring in waters minimally contaminated by anthrophogenic substances. Two important natural factors affecting the frequency of SIM female perch (Luksiene *et al.*, 2000), including the closely related yellow perch (*Perca flavescens*) (Dabrowski *et al.*, 1996), have been suggested to be photoperiod and temperature. In previous studies, the frequency of SIM female perch in minimally contaminated waters in northern Europe has been reported to be 0–3% (Sandström, 1994; Luksiene *et al.*, 2000), which are in good agreement with our present findings with respect to Lake Djursjön.

It should be pointed out that the normal SCF value and somatic growth in perch, from both Lakes Djursjön and Molnbyggen in 1996 and 1997, indicated that the adverse effects on gonad development in Lake Molnbyggen are not due to a shortage of food, nor could the adverse effects on the gonad development simply reflect biased sampling of juvenile, sexually nonmature perch from Lake Molnbyggen since we observed the effects on female perch of all ages and weights. According to Sandström *et al.* (1988), female and male perch in the southwestern Bothnian Sea exceeding 170 and 150 mm in length, respectively, are normally sexually mature. The perch studied here were larger than this by a good margin.

Furthermore, both female and male roach caught in Lake Molnbyggen were found to have lower GSI values than roach in Lake Djursjön. However, all female roach were found to be SM. Reduced gonad growth in roach exposed to BKME in Sweden was also reported in the earlier study by Sandström *et al.* (1988). However, no mechanistic explanation for the reduced gonad growth was given. A tendency toward higher frequencies of fin erosion and shallow open sores was also seen on female roach. These lesions were of the same kind as those seen on perch from Lake Molnbyggen, suggesting a common cause.

Several female pike and female and male burbot from Lake Molnbyggen were also analyzed and found to have high frequencies of fin erosion and sores, as well as very low GSI values, in comparison to control fish from various reference waters (results not shown). These effects were similar to those observed in perch and roach from Lake Molnbyggen, suggesting that most teleost species in Lake Molnbyggen might be exposed and affected in a similar manner. Pulliainen *et al.* (1992) reported high frequencies of SIM burbot in estuaries on

the northern coast of the Bothnian bay. The cause of this reproductive disorder is unknown, but environmental pollutants were considered to be a possible factor in this regard.

In order to determine whether the extremely high frequency of SIM female perch found in Lake Molnbyggen might be due to a inhibition of P450arom, this enzymatic activity was assayed in the brain of female perch and roach. Interestingly, specific P450arom activity in the brain of SIM female perch from Lake Molnbyggen was lower than in SM female perch in Lake Djursjön. SM female perch in Lake Molnbyggen demonstrated intermediate P450arom activity, while SIM female perch in both lakes showed similar activities. In addition, the P450arom activity in the brain of female roach from Lake Molnbyggen also showed a tendency to be lower than in Lake Djursjön.

These findings indicate that the low frequency of SM female perch in Lake Molnbyggen might reflect reduced production of E2, perhaps resulting in lowered production of vitellogenin and, thus, disrupted development of the oocytes. Indeed, SIM female perch from Lake Molnbyggen had significantly lower plasma levels of T and E2 and SM female perch from this same water showed a significantly lower T level and a tendency toward a lower E2 level in comparison to SM female perch from Lake Djursjön. Reduced circulating levels of T and E2 have also been observed in lake whitefish and white sucker exposed to BKME at Jackfish Bay, Lake Superior, and these changes were associated with impaired gonadal development and delayed maturity (Munkittrick *et al.*, 1992; McMaster *et al.*, 1995).

McMaster *et al.* (1995) suggested that downstream disruption of pregnenolone formation was responsible for the reduced steroid production in the ovarian follicles of fish exposed to BKME. The lower levels of E2 observed here in female perch from Lake Molnbyggen could reflect inhibition of P450arom activity in the brain. However, the very low levels of T suggest that there might be a dysfunction in steroid synthesis somewhere prior to the formation of T and that the lower P450arom activities might be due to down-regulation at the mRNA and/or protein level rather than to inhibition. Indeed, several of the imidazole antifungal agents, which are known inhibitors of P450arom, are also potent inhibitors of several other steroid synthesizing P450s (Kan *et al.*, 1985; Ayub and Levell, 1987). A key enzyme for the formation of androgens is the microsomal cytochrome P450-dependent monooxygenase 17α-hydroxylase/17,20-lyase (P450 17α) enzyme. Rajfer *et al.* (1986) observed that ketoconazole, by inhibition of the P450 17α activities, decreased testicular T production in humans. However, another possible mechanism behind the very low levels of T might be an increased metabolism and excretion of T.

Very similar patterns of steroids were observed in SIM female perch in both lakes, again indicating that the majority of the female perch in Lake Molnbyggen are arrested in a juvenile, sexually nonmature stage. In addition, there were no differences in the morphologies of the gonads of SIM female perch in the two lakes and no differences were observed regarding P450arom activity and the levels of T and E2 between female perch with or without fin erosion and/or sores from Lake Molnbyggen (not shown). The high frequency of fin erosion and skin lesions is at present assumed to be associated with an impairment of the immune system due to endocrine disruption. Alternative possibilities should however not be excluded. The same tendency toward lower P450arom activity and a lower level of T could also be seen in female roach from Lake Molnbyggen. The single male perch caught in Lake Molnbyggen had a much lower plasma level of T than the average value of male perch from Lake Djursjön. Munkittrick *et al.* (1992) also found that male lake whitefish exposed to BKME demonstrated significantly lower levels of T.

Interestingly, no difference in gonadal P450arom activity in SM female perch from Lakes Molnbyggen and Djursjön was observed. Steroid production *in vitro* by ovarian follicles from unexposed fish and fish exposed to BKME in Jackfish Bay, Lake Superior, was found to parallel the observed differences in circulating steroid levels, suggesting that alteration of ovarian steroid production is a major action of BKME (Van Der Kraak *et al.*, 1992; McMaster *et al.*, 1995). In the present study, the low levels of T and E2 in blood plasma paralleled the P450arom activity in brain, suggesting that alteration of steroid production in the brain of female perch might be a major action of pollutants in Lake Molnbyggen. In contrast, gonadal P450arom activity did not reflect circulating levels of T and E2.

The influence of the effects observed here on individual fish with respect to the entire fish population has not yet been studied. However, since we were able to obtain perch of different ages from Lake Molnbyggen, the SM female perch in this lake must still be capable of producing viable offspring. Since our data indicate that piscivores fish, e.g., pike, burbot, and, especially, perch, are most strongly affected (compared to roach), both intra- and interspecies competition would appear to be low. It can therefore be assumed that the offspring produced by SM female perch in Lake Molnbyggen have a greater chance to survive than would be the case in most other waters. Thus, there is no obvious reason to believe that effects at the population level are manifest and/or easily identifiable at present. However, the sublethal effects, especially on the circulating levels of steroids and gonad development, strongly suggest that an irreversible negative impact is being exerted on the future of this ecosystem.

It has been agreed that "*An endocrine disrupter is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function*" (European Commission, 1996). The present study, with a mechanistic approach through a set of various biomarkers, documents strong evidence that the fish living in Lake Molnbyggen are exposed to an unidentified endocrine-disrupting substance(s) (EDS(s)), which affects several species of fish. The fact that female fish and, in particular, female perch from

Lake Molnbyggen are differently and more seriously affected than male fish also provide strong evidence for exposure to EDSs. To our knowledge, this novel and important finding of endocrine disruption in fish exposed to leakage water from a public refuse dump has not been reported previously.

Our future studies will include laboratory experiments and field sampling of fish to elucidate the underlying molecular mechanism in more detail. Such studies will facilitate identification of the EDS(s) present in the leakage water from the public refuse dump at Lindbodarna, Leksand. This will, in turn, allow us to determine how common these kind of endocrine-disrupting effects on fish in other lakes receiving leakage water from Swedish refuse dumps might be.

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