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Female-biased sex ratios and delayed puberty in two fish species with different Ecologies in an Anthropogenically affected urban lake

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ABSTRACT

In aquatic ecosystems, endocrine-disrupting compounds (EDCs) pose a growing concern for their potential adverse effects on fish reproduction and development. In lake Pyhäjärvi, located in the urban boreal region of Tampere, Finland, a significant number of sexually immature pikeperch (*Sander lucioperca*) individuals have been identified in size and age categories that are expected to be sexually mature. To explore if this phenomenon is attributed to estrogenic endocrine disruption, we conducted a comprehensive study comparing fish from lake Pyhäjärvi with those from a nearby reference lake, lake Näsijärvi. Roach (*Rutilus rutilus*), known for its susceptibility to EDCs, was also included for comparison. We examined various parameters in both pikeperch and roach, including size, condition factor, age, reproductive indicators, biometric indices and gonadal histology. We also assessed liver vitellogenin mRNA levels and genetic sex in roach, and measured estrogen levels in lake waters and wastewater treatment plant effluents. Results revealed that approximately one-third of fish in both species exhibited sexual immaturity in lake Pyhäjärvi, with a female-biased sex ratio. Surprisingly, we found no signs of estrogenic endocrine disruption, indicated by the absence of intersex fish in both species. Furthermore, vitellogenin levels in roach closely resembled those in the reference lake. Estrogens were undetectable in the lake waters, suggesting that factors other than estrogenic EDCs, including other potential endocrine disruptors such as PCBs or heavy metals, may be influencing delayed sexual maturity and skewed sex ratios. Further inquiry is needed to pinpoint these underlying causes. Our study provides essential baseline information on fish sexual development in lake Pyhäjärvi, emphasizing the need for ongoing monitoring and research to understand delayed sexual maturity and biased sex ratios. This is vital given the increasing concern about EDC impacts on aquatic ecosystems and the necessity for effective management strategies to protect these ecosystems' health and integrity.

1. Introduction

Chemical pollutants from anthropogenic activities can impose adverse health and reproductive outcomes in non-human animals and humans. Pollutants such as pesticides, pharmaceuticals, personal care items, and human prescription residues can contaminate soil, groundwater, and rivers through run-off, affecting both agricultural and wild populations (La Merrill et al., 2020). Endocrine disrupting chemicals (EDCs) are of particular concern as they have the ability to mimic, block, or interfere with hormones in the endocrine system (Ahn and Jeung, 2023; Kahn et al., 2020). These chemicals are prevalent in the environment and aquatic organisms are continuously exposed to them. EDC exposure can impact multiple hormonal pathways, potentially leading to developmental and reproductive abnormalities in both humans and animals (Marlatt et al., 2022; Meeker, 2012; Roig et al., 2013). Evidence suggests that EDC exposure causes the following effects in fish: 1) the disruption of gonad development and sex differentiation, inducing intersex; 2) abnormal gonad differentiation leading to episodes of sterility due to germinal cell damage, and 3) alterations in the timing of puberty (Delbes et al., 2022).

Wastewater treatment plants (WWTPs) release a broad range of compounds, including both natural and synthetic steroid estrogens (Aerni et al., 2004; Välitalo et al., 2016). EDCs classified as estrogenic chemicals (ECs) have received particular attention due to the significant

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environmental pollution they cause and the wide range of effects they have on aquatic ecosystems. Both the natural estrogen such as estrone (E1) and 17β-estradiol (E2) and the synthetic estrogen 17α-ethynylestradiol (EE2) have been shown to produce feminine responses in male fish, such as the formation of eggs in male testes and the generation of vitellogenin (VTG) mRNA and protein in males (Alves da Silva et al., 2018; Bahamonde et al., 2013; Kidd et al., 2007). Vitellogenin is synthesized and secreted by the liver under estrogen stimulation, transported in the blood to the ovary where they are taken up by oocytes and processed into their derivative yolk proteins (YP). Intersex, the presence of both male and female characteristics in the same fish, has been identified in 37 species in lakes and rivers across North America, Europe, and other regions of the world (Bahamonde et al., 2013). There is a growing concern that intersex fish have lower sperm quality and quantity, which leads to a detrimental influence on fish populations over time. Disruption of endocrine function may adversely impact the sustainability of wild populations in fish populations or communities, as has been evidenced by (Kidd et al., 2007). In addition to these reproductive effects, EE2 has been reported to cause a number of detrimental effects, including liver and kidney lesions, modulation of the inflammatory response and permanent dysfunction of the immune system (Colli;Dula et al., 2014). Altogether, EDCs are known to impact fertility, sexual maturation, somatic growth, stress reactions, and create cellular damage in fish, primarily through changes in hormone and receptor levels. Effects on reproductive behaviour, secondary sex characteristics, hepato-somatic index (HSI), gonadosomatic index (GSI), gonadal histology, and plasma concentrations of vitellogenin and sex hormones (17β-estradiol, testosterone, 11-ketotestosterone) have been proposed as desirable endpoints for EDC screening in fish models (Hutchinson et al., 2006). It is worth noting that the effects of EDCs can be influenced by a variety of factors, including the duration and frequency of exposure, as well as the age and sex of the organism. Therefore, it is important to continue monitoring the levels of EDCs in aquatic environments and assess the potential risks they pose to both human and animal health.

The risks posed by the estrogenic hormones have been acknowledged in the EU environmental administration as well, and the EU Commission has proposed EE2, E2 and E1 (estrone) to be new priority substances for the update of the Water Framework Directive (EC, 2022). The proposed Annual Average Environmental Quality Standards (AA-EQS) for the surface waters are 0,017 ng/l, 0, 18 ng/l and 0,36 ng/l for EE2, E2 and E1, respectively (Scheer, 2022). Moreover, as many other chemicals can act as endocrine disrupting contaminants in wildlife, mixture effects should be evaluated. The EU Commission has suggested obligatory monitoring practices where Effect-Based methods are used for detection of estrogenic risks (EC, 2022).

Pikeperch (*Sander lucioperca*) is a predatory percid fish living in lakes and Baltic Sea coastal areas in Europe. In Finland, pikeperch is one of the most valuable freshwater fish species for both recreational and commercial fisheries (Heikinheimo et al., 2006). Pikeperch prefer deep-water bodies with rocky or sandy bottoms, such as large lakes and rivers. They are a predatory fish and primarily feed on other fish, including roach, perch, smelt and vendace. Pikeperch are also able to tolerate low oxygen levels in the water, and they can forage efficiently even in low visibility conditions thanks to the retina's particular tapetum lucidum layer (Ali et al., 1977; Jokela-;Määttä et al., 2019). Pikeperch are classified as eurytopic since they live in both pelagic and benthic water habitats. After larval stages, they are a freshwater species that lives in turbid, eutrophic, and brackish settings.

Roach, (*Rutilus rutilus*) on the other hand, are more adaptable to different water conditions and are commonly found in both lakes and rivers across Europe. They prefer slow-moving or standing water bodies but can also be found in faster-moving streams and rivers. Roach, a typical generalist, appear to integrate both pelagic and littoral resources during their ontogeny (Hayden et al., 2014; Hayden et al.2014). Roach are omnivorous and will eat a variety of food sources including insects, crustaceans, and plants. The biomass and size distribution of the roach may be regulated by pelagic predatory fish, such as the pikeperch, as the roach as a relatively slender cyprinid is vulnerable to predation and reaches size refuge at relatively old age (Lammens et al., 1992). However, in highly eutrophic lakes, the effective reproduction of the roach may override the consumption by piscivores (Mehner et al., 2005; M Olin et al., 2002).

Both pikeperch and roach begin their lifecycle with undifferentiated gonads. Sex differentiation is influenced by genetic and environmental factors during the early larval stages. As juveniles grow, their gonadal development progresses under the guidance of environmental conditions such as temperature. Histological examinations reveal the formation of spermatogenic cells in males and ovarian follicles in females, which mature into structures capable of gamete production. In the Baltic Sea, pikeperch usually become sexually mature between the ages of 4 and 6 years, reaching lengths of 35–44 cm. Males often attain maturity sooner and at smaller sizes compared to females (Kosior and Wandzel, 2001; Lappalainen et al. 2003). In Finland, particularly in the Tampere region, it is uncommon for 2-year-old pikeperch to be mature. Studies have shown that 2-year-old pikeperch are generally around 20+ cm and do not spawn at this size and age. In a survey of several lakes in the Tampere region, including Lake Pyhäjärvi, numerous specimens (n = 2726) ranging from 30 to 60 cm were observed, but none were 2-year--old. Very few 3-year-old pikeperch were found to be mature, indicating that the typical maturation age for pikeperch in this region is later than previously thought (Kolari and Westermark, 2017). Roach of both sexes generally mature between 2 and 3 years (https://www.fishbase.se/summary/rutilus-rutilus.html). The capability to produce viable gametes marks maturity in these species, with environmental cues such as photoperiod and temperature playing essential roles in triggering reproductive readiness and spawning.

The gonochoristic species such as roach and pikeperch normally display distinct sexes without intersex characteristics. However, studies have shown that exposing freshly hatched fish to natural and synthetic hormones (estrogens or androgens) and/or aromatase inhibitors may induce intersex or sex reversal (Jobling et al., 2002; Nolan et al., 2001; Piferrer and Donaldson, 1992). Exposure to estrogens in gonochoristic fish like roach and pikeperch can disrupt their normal sexual development, leading to issues such as sexual immaturity. In these cases, genetic sex and histological sex can differ due to sex reversion, where an individual's phenotypic (histological) sex does not align with their chromosomal (genetic) sex. This misalignment not only impacts the individual's reproductive capability but also poses broader ecological and evolutionary concerns for populations exposed to environmental contaminants.

The pikeperch population in lake Pyhäjärvi in Pirkanmaa, southwestern Finland, had shown more sexually immature individuals than expected based on their size and age (Kolari and Westermark, 2017), which raised the question whether EDCs are causing problems for fish reproduction. There had also been infrequent findings of elevated concentrations of estrogenic hormones in the lake (VESLA database; https://ckan.ymparisto.fi/en/dataset/pintavesien-tilan-tietojarjestelma-vedenlaatu-vesla), probably originating from two municipal wastewater treatment plants (WWTPs) that release their effluent in the lake. We therefore aimed to study whether fish in the area show signs of estrogenic endocrine disruption. We assessed the sexual maturity, sex ratio, and gonadal structure in pikeperch and roach in lake Pyhäjärvi). We compared our findings with those from lake Näsijärvi, which is known for its cleaner conditions, enabling a comprehensive assessment of EDC effects across species with varying susceptibilities and reproductive strategies. We also studied condition factor (CF), gonado-somatic index (GSI), hepato-somatic index (HSI), and fecundity in both species, as well as vitellogenin mRNA levels in the liver and genetic sex in roach. Estrogens were also analyzed in both lake water and wastewater samples.

2. Materials and methods

2.1. Area

Lake Pyhäjärvi, in Tampere, south-western Finland, is situated in the heart of a heavily populated region. Lake Pyhäjärvi is 121.6 km^2, eutrophic, shallow (mean depth 5.5 m), and has high humic content (water color ca. 60 mg Pt L^{-1}, syke.fi/avoindata) (Fig. 1) (Olin et al., 2022). Heavy nutrient inputs from sources like pulp and paper mills and wastewater treatment plants have historically had an impact on lake Pyhäjärvi (Niemi, J. & Eloranta, J., 1984). Currently, two wastewater treatment plants (sum of population equivalent 380,000) release treated effluent into Lake Pyhäjärvi. Lake Pyhäjärvi has also received wastewaters from various mills over the past two centuries, and the sediments contain historical contamination of PCBs, PCDD/Fs, TBT-TPTs, metals and petroleum hydrocarbons (Supplementary Table 1, Hertta database; HERTTA is an Internet technology-based system, and it is the basic tool for environmental control, monitoring and assessment used by the Environment Authorities at all levels of organization throughout the Finnish Environment Network; https://www.syke.fi/fi-FI/Avoin_tieto/Ymparistotietojarjestelma). Of the various sediment contaminants, PCBs are thought to be of main concern (Hyttinen, 2022). In 2001, sediments in the study area contained up to 160 mg PCBs/kg sediment, which is the highest measured PCBs concentration in Finnish lake sediment (Frisk et al. 2007). It is thought that the main source for PCBs is historical contamination with Aroclor 1242 and Aroclor 1260 by a condenser manufacturer in the area in the 1960s and 1970s (Hyttinen, 2022; Anonymous, 2024). In addition, waste and contaminated land materials have been used when filling the Viinikanlahti bay in the 20th century (Anonymous, 2024). Other contaminants are thought to originate from that era as well. The contaminants are leaking from the sediments e.g. due to changes in water flow in the river Tammerkoski, as also upper layers of sediments may contain rather high concentrations of contaminants, and traces can be found in fish (Frisk et al. 2007; Hyttinen, 2022, Kerty database: https://ckan.ymparisto.fi/dataset/kertymarekisteri-kerty). Chemicals are thus still bioavailable to biota.

The environmental administration data on concentrations of estrogenic hormones in the Lake Pyhäjärvi are scarce and only few, infrequent above the detection limit observations have been reported ranging from 0,06 ng/l (EE2) to 0,6 ng/l (E1) (VESLA online database August 11, 2023). There is no data on estrogenic hormones in the reference lake, lake Näsijärvi.

2.2. Fish sampling

In February 2021, pikeperch were caught using nets, while roach were captured in April 2021 using wire fish-traps. After capture, both species underwent similar procedures for data collection. The fish were euthanized with MS-222 (200 mg/L) and then weighed to the nearest gram and measured for length to the nearest millimeter. The spine was cut at the neck using a knife, and the fish were subsequently opened. Sex determination was performed, and the gonads and livers were weighed to the nearest 0.1 g. The intestines and other organs were removed, and the remaining fish body was weighed. Ovaries were frozen at −20 °C for later fecundity analyses, while the anterior and posterior parts of the testes were fixed in neutral buffered 10% formalin for 24 h and subsequently transferred to 70% ethanol. Scales (20–30) were preserved for future age analyses. Additionally, the livers were placed in microcentrifuge tubes, frozen in liquid nitrogen, and preserved at −80 °C until analysis.

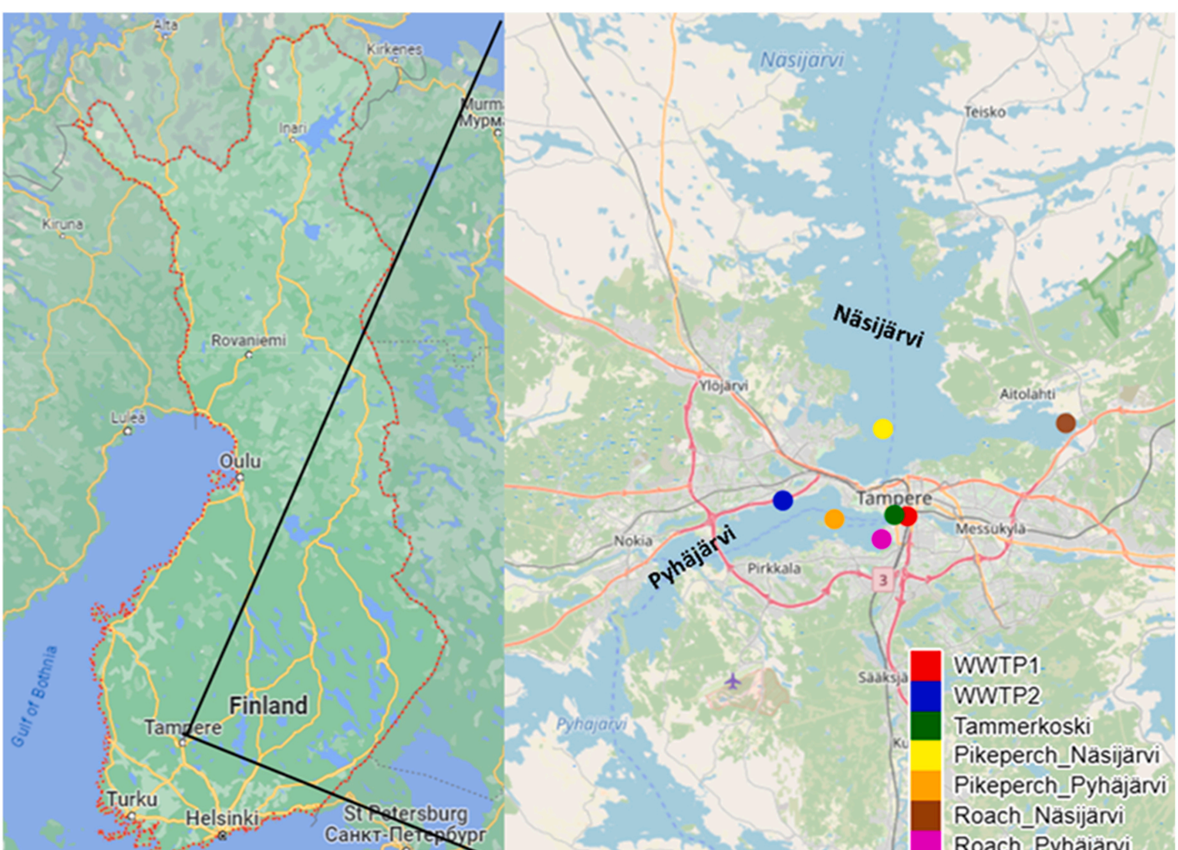

Fig. 1. Map of Finland showcasing Tampere, with emphasis on the study lakes - Näsijärvi, designated as the reference lake, and Pyhäjärvi, identified as the contaminated lake. The map also highlights wastewater treatment facilities in the vicinity of Pyhäjärvi and the various sampling points. Additionally, the location where these two lakes connect, Tammerkoski, is distinctly marked.

2.3. Age determination

Age was determined by counting the annual growth rings microscopically in scales taken from the dorsal trunk region.

2.4. Condition factor, gonado-somatic index, and hepato-somatic index

The condition factor (CF) was calculated as Fulton's CF using this equation CF=W/(l^3)*100 in which W is the weight of fish (g) and l is the length of fish (cm).

The gonado-somatic index (GSI) was calculated using following equation:

GSI=W(gonad)/W(fish) *100

The hepato-somatic index (HSI) was calculated using this equation:

HSI=W(liver)/W(fish) *100

2.5. Fecundity analysis

The ovaries were fixed in neutral buffered 10 % formalin for 24 h, after which the eggs were separated from connective tissue and air-dried on plastic netting. After measuring the total weight of the eggs in an ovary, three subsamples of eggs were weighed and the number of eggs in the subsample counted under microscope. These values were used to calculate the weight of one egg, which was used to calculate the total number of eggs in a female.

2.6. Histology

After fixation the testis samples were dehydrated in graded series of ethanol and embedded in paraffin. Sections of 7 μm were cut with a microtome and stained with Mayer's hematoxylin – eosin stain. At least nine sections of each sample were observed under microscope, making a minimum total of 36 sections per individual (samples from anterior and posterior ends of each testis).

2.7. Chemical analyses of water samples

Water samples were taken from lake Pyhäjärvi and lake Näsijärvi at the time of fish sampling. In addition, samples were taken of the effluent from both wastewater treatment plants releasing their effluents in Lake Pyhäjärvi. They were analyzed for estrone, 17β-estradiol, 17α-estradiol, estriol and 17α-ethinylestradiol at the Finnish Environmental Institute (Välitalo et al., 2016).

2.8. Vitellogenin mRNA measurement from roach liver

Total RNA was extracted from 50 to 70 mg liver tissue sample using 0.8 mL Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. After tissue homogenization, samples were incubated for 5 min at room temperature, and then 160 μL chloroform was added into these tubes. The mixture was shaken vigorously by hand for 15 s and incubated for 10 min at room temperature. Next, the sample was centrifuged at 12,000×*g* for 15 min at 4 °C. The upper aqueous phase was transferred to a new tube and 400 μL isopropanol was added to the supernatant. After mixing and incubating at room temperature for 10 min, the mixture was centrifuged at 12,000×*g* for 8 min at 4 °C. The RNA pellet was washed with 1 mL ethanol (75%) after removal of the supernatant. Then, the tube was centrifuged at 7500×*g* for 5 min at 4 °C, the wash discarded, and the RNA pellet air dried for 5–10 min at room temperature. Finally, the pellet was dissolved in RNase-free water and incubated at 60 °C for 3 min. RNA concentration and purity of all RNA samples were assessed by measuring the absorbance ratio at 260/280 nm and at 230/260 nm in a NanoDrop 2000 spectrophotometer (Thermo Scientific). The integrity of all RNA samples was assessed the quality of the RNA samples were judged by their RNA integrity number (RIN) calculated by the Agilent 2100 Bioanalyzer. In this study only RNA samples with RIN values ≥ 8 was used for subsequent analysis.

In order to attain DNA-free total RNA, one 1 μg RNA (8 μL of diluted sample to 125 ng/μl) was treated with DNase I (Thermo Scientific™) working solution, consisting of 1 μL reaction buffer (10x) and 1 μL DNase I (1 unit/μL). After incubation at 37 °C for 30 min, DNase I was inactivated using 1 μl of 25 mM EDTA (ethylenediaminetetraacetic acid) and heated for 10 min at 65 °C.

Total DNase-treated RNA was used as template for cDNA synthesis by iScript (BioRad) (reverse transcriptase). cDNA synthesis was carried out with 450 ng of total RNA to which was added 4 μl of 5x iScript Reaction Mix, 1 μl of iScript reverse transcriptase and nuclease-free water up to 20 μl. The mixture was incubated as described in the protocol of the manufacturer: 5 min at 25 °C,30 min at 42 °C and 5 min at 85 °C to conclude the synthesis. All cDNA reactions were diluted 10-fold with molecular-grade water and stored at −20 °C.

We amplified Vitellogenin (*vtg*) cDNA in roach using a species-specific primer pair developed by Oveysi et al. (2017). To normalize vitellogenin expression, we searched for previously validated reference genes in roach using RT-qPCR studies. Among the eight candidate reference genes, ribosomal protein L8 and β2-microglobulin, which were found to perform well in the study by (Kroupova et al., 2011), were included in our study (Table 1). In our study, we evaluated the stability of the reference genes using Bio-Rad CFX Maestro™ software. We calculated the average expression stability (M) using the formula [Ln (1/AvgM)], where both genes had values lower than 0.5, indicating their stable expression.

RT-qPCR reactions were performed in triplicate in 96-well reaction plates including negative controls without cDNA. Each sample well contained a final volume of 20 μl, including 10 μl of Biorad iQ™ SYBR® Green supermix (2x), 1 μl of primer mix (6 μM each forward and reverse primers), 4 μl of nuclease-free water and 5 μl of 1:10 diluted cDNA sample. RT-qPCR analyses were conducted under the following conditions: 5 min at 95 °C for initial denaturation followed by 29 cycles of 35 s for denaturation at 95 °C, 1 min for annealing at 55 °C and extension for 2 min at 72 °C. The final extension step was carried out at 72 °C for 2 min (Oveysi et al., 2017). Specificity of amplification reaction was checked by melting curve analysis. The high-resolution melting analysis was performed immediately afterwards by increasing the temperature from 55 °C to 95 °C by steps of 0.5 °C maintained for 10 s each. In the case of roach, existing literature provides extensive validation for these biomarkers, thereby ensuring the reliability and accuracy of our results. However, for pikeperch, standardized methods for measuring these specific biomarkers have not yet been firmly established. This was a key consideration in our study design, as we aimed to utilize the most reliable and scientifically validated techniques available.

2.9. Genetic sex determination of roach

DNA was extracted from liver tissue using the HotSHOT method (Truett et al., 2000). Briefly, a small section of liver tissue was incubated in 75 μl alkaline lysis reagent (25 mM NaOH, 0.2 mM Na_2EDTA) at 95 °C for 45 min. The samples were placed on ice for 5 min before the adding 75 μl neutralising reagent (40 mM Tris-HCl, pH 5.0).

Samples from each fish were analyzed in three separate PCR reactions using previously used three different primer combinations of two sense and three antisense primers (Lange et al., 2020) (Table 2). An individual was assigned as a genetic male if the PCR product could be seen in at least two out of the three different PCR reactions (Lange et al., 2020). PCR reactions were carried out using 1 μl of DreamTaq™ Green Buffer (10X) (Thermo Scientific™), 0.2 mM dNTP mix (Thermo Scientific, UK), 0.2 μM of each forward and reverse primer, 1.25 U of

Table 1
Sequence of primers used for quantitative PCR.

Target Gene	Target Name	NCBI accession number	Primer	5′–3′ Sequence	Amplicon size
rpl8	ribosomal protein L8	FJ769335	Forward	ATCCCGAGACCAAGAAATCCAGAG	94
			Reverse	CCAGCAACAACACCAACAACAG	
β2m	β2-microglobulin	EU930849	Forward	TATGTCTGACGCCCAGCAG	82
			Reverse	GACGCTCTTGGTGAGGTGAAAC	

Table 2
PCR primers used to amplify sex-specific marker in roach (Lange et al., 2020).

Primer	Sequence (5′–3′)	Rr_780797_r1	Rr_780797_r2	Rr_780797_r3
Rr_780797_f1	AGGGGCACCATGTGAAAATCC	247 bp	381 bp	–
Rr_780797_f2	AGAGATGTCTGGAGTTATATAGGGG	–	–	400 bp
Rr_780797_r1	TATGCCTCCTCCCAGCACAA	–	–	–
Rr_780797_r2	ACAGCCTTATAGTTGCTTGCTC	–	–	–
Rr_780797_r3	CAGCCTTATAGTTGCTTGCTCC	–	–	–

DreamTaq DNA Polymerase (5U μL−1 Thermo Fisher Scientific™), 0.1 mg/ml Bovine serum albumin (BSA) and 1 μl DNA in a total volume of 20 μl. An initial denaturing step at 95 °C for 5 min was followed by 30 cycles of denaturation (1 min at 95 °C), annealing (30 s at 60 °C) and extension (45 s at 72 °C), followed by a final extension of 5 min at 72 °C. Amplicons were resolved on 1.5% agarose gels.

2.10. Statistical analyses

For statistical analysis and data representation, R version 4.3.1 was used. The study evaluated differences in body length, weight, condition factor, fecundity, HSI, and GSI among fish sampled from various lakes. In cases where assumptions of normality and equal variance were not met, a non-parametric Kruskal-Wallis test was employed. Subsequently, a Dunn's post hoc test was conducted separately for different sex categories (female, male, immature). To determine the relative (normalised) expression level of the vitellogenin (VG1) gene, we used the ΔΔCt method (Pfaffl, 2001). Statistical significance ($P < 0.05$) of log-transformed data was determined by a one-way analysis of variance (ANOVA) followed by Tukey HSD post hoc test.

3. Results

3.1. Sex, biometric data and age of fish samples

A total of 61 pikeperch and 60 roach were captured during the study. Among the pikeperch, 24 were females and 23 were males, while 24 of the roach were females and 23 were males. we assessed sexual maturity through direct visual inspection of gonads during fish dissection. Mature males displayed enlarged seminiferous tubules filled with mature sperm, and mature females exhibited various stages of oocyte development. Conversely, immature fish had minimally differentiated gonadal strands, lacking the developed structures characteristic of maturity.

Visual inspection of the gonads revealed that 14 of the pikeperch and 13 of the roach were immature (exhibiting reduced gonad size in age categories that typically correspond to sexual maturity). Further histological analysis of the immature fish showed that 10 of the immature pikeperch were female, and in the contaminated lake, the majority of the immature fish were female. Similarly, among the immature roach, the contaminated lake exhibited a striking majority of female individuals, with 11 out of 12 immature females originating from that particular location. Overall, the study found evidence of reproductive abnormalities in both pikeperch and roach, with a higher incidence of immature females in the contaminated lake. Furthermore, the study identified two immature male pikeperch in the reference lake and two in the contaminated lake, as well as one immature male roach found in the contaminated lake.

The mean age for pikeperch was 8.1 years for females, 7.6 years for males, and 6.6 years for immature individuals. For roach, the mean age was 9 years for females, 6.4 years for males, and 8 years for immature individuals. The age and sex distribution for each lake is plotted in Fig. 2. In the reference lake, a total of 8 age classes of pikeperch were observed, with the majority falling into age-classes 6 to 8, covering an age range of 5–12 years. The contaminated lake featured 7 age classes of pikeperch (age range: 5–11), with a prevalence of individuals in age-classes 6 and 8. Regarding roach, the reference lake contained representatives from 7 age classes, with most belonging to age-classes 7 and 8, spanning from 5 to 11 years old. Similarly, the contaminated lake exhibited a predominance of age-class 7 and also had 7 age classes (age range: 5–12).

Additionally, individuals with delayed puberty in older ages were found at the contaminated site for both species (Fig. 2).

The total length of pikeperch ranged from 37.9 to 63.1 cm (mean: 46.4 ± 4.9 S.D.) for all individuals. Males had a length range of 38.3–63.1 cm (mean: 46.6 ± 6.2 S.D.), females ranged from 37.9 to 54.8 cm (mean: 47.1 ± 4.4 S.D.), and immature individuals had a range of 41.2–52.5 cm (mean: 45.2 ± 3 S.D.). For roach, the total length ranged from 13.2 to 22.9 cm (mean: 18.5 ± 2.1 S.D.) for all individuals. Males had a length range of 13.2–20.1 cm (mean: 16.7 ± 1.6 S.D.), females ranged from 16.5 to 21.8 cm (mean: 19.1 ± 1.5 S.D.), and immature individuals had a range of 17.8–22.9 cm (mean: 20.4 ± 1.5 S.D.).

The mean total body mass for pikeperch was 906.2 g (S.D. = 402) for all individuals, with females weighing 942 g (S.D. = 281.4), males weighing 956.8 g (S.D. = 558), and immature individuals weighing 762 g (S.D. = 172). For roach, the mean total body mass was 60.8 g (S.D. = 23) for all individuals, with females weighing 68.5 g (S.D. = 18.7), males weighing 42.1 g (S.D. = 13.1), and immature individuals weighing 79.6 g (S.D. = 21.2).

In our study, we utilized the Kruskal-Wallis test followed by Dunn's test with Bonferroni correction to evaluate differences in body length and weight among pikeperch and roach populations from contaminated and reference lakes. For the pikeperch species, significant variations were discerned through pairwise post-hoc Dunn testing. Notably, when comparing males from the reference lake to males from the contaminated lake, significant differences emerged in both body length and weight. Males from the reference lake were found to be significantly longer and heavier ($p = 0.003$ for both length and weight) than their counterparts in the contaminated lake. Similarly, among females, those from the reference lake exhibited significantly greater body length ($p = 0.03$) and weight ($p = 0.01$) compared to females from the contaminated lake. In contrast, within the roach population, the only significant difference observed was in body weight among females; those from the contaminated lake were significantly heavier than those from the reference lake ($p = 0.01$) (Fig. 3).

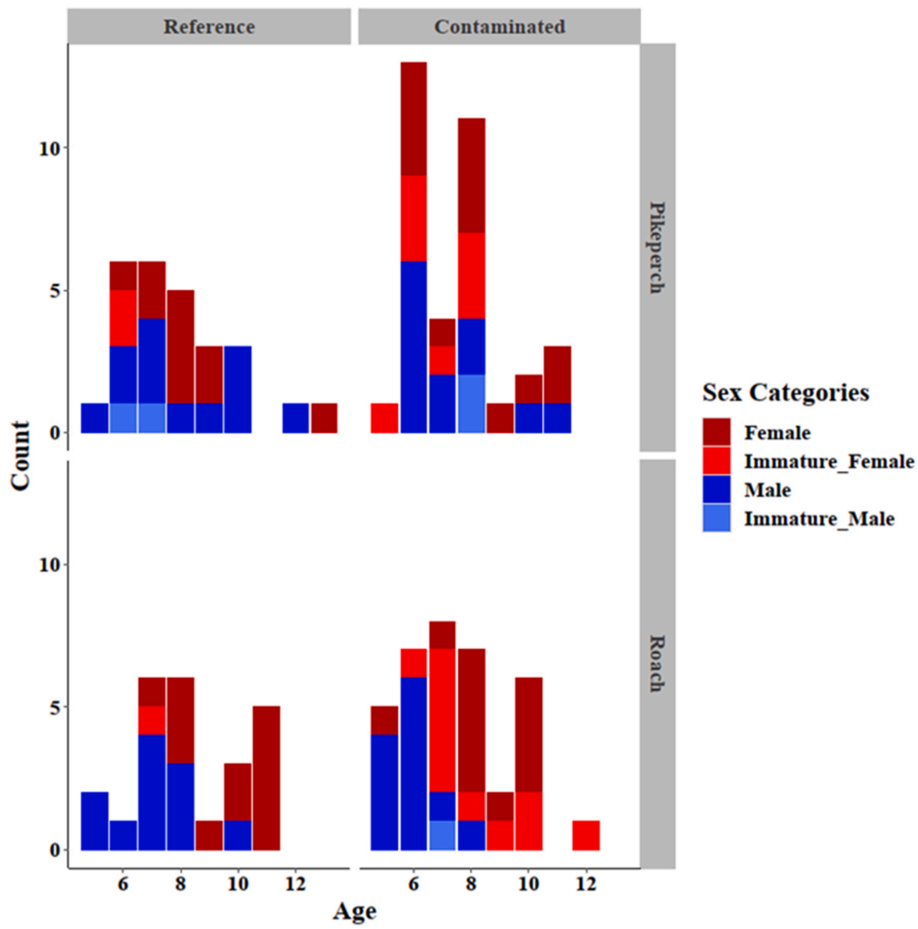

Fig. 2. Age distribution of each sex category in pikeperch and roach populations in the reference and contaminated lakes. The figure shows the proportion of individuals in each age class for females, males, and immature individuals for each species in both lakes.

In addition to the sex-based comparisons, we also investigated differences between immature and mature individuals (combining both females and males) in both species. For pikeperch, the analysis yielded varying results across the two sites. At the contaminated site, no significant differences were observed between immature and mature individuals in terms of age ($p = 0.49$), weight ($p = 0.92$), and length ($p = 0.54$). However, at the reference site, significant differences were found: age showed a significant difference ($p = 0.033$), as did weight ($p = 0.020$) and length ($p = 0.027$), indicating that mature pikeperch at the reference site were significantly older, heavier, and longer than their immature counterparts (Supplementary Fig. 1).

Similar comparison was not possible for roach, as there was only one immature individual at the reference site.

3.2. Condition factor, gonado-somatic index, and hepato-somatic index

The GSI and HSI were used to evaluate the reproductive and health status of pikeperch and roach from contaminated and reference lakes. There were no differences in the mean GSI for males or females between lakes in pikeperch, but roach females from the contaminated site had marginally higher GSI relative to females from the reference site ($p = 0.04$, Kruskal-Wallis followed by Dunn's test). In pikeperch, both females and males from the contaminated lake had a significantly lower mean HSI compared to those from the reference site ($p_{\text{Females}} = 0.02$, $p_{\text{Males}} = 0.001$, Kruskal-Wallis followed by Dunn's test), although HSI was similar between the two sites for all sex categories in roach. Additionally, the condition factor of pikeperch females and males from the contaminated lake showed marginally lower values than those from the reference lake ($p_{\text{Females}} = 0.06$, $p_{\text{Males}} = 0.05$, Kruskal-Wallis followed by Dunn's test). Interestingly, the condition factor of female roach was higher in the contaminated site than in the reference site ($p = 0.04$, Kruskal-Wallis followed by Dunn's test) (Fig. 3).

3.3. Fecundity

In pikeperch, fecundity was not significantly affected in the contaminated lake, although it was still lower than in the reference lake. However, in roach, females from the contaminated site had significantly higher fecundity than those from the reference site (Kruskal-Wallis chi-squared = 6.75, df = 1, $p = 0.009$) (Fig. 4).

3.4. Gonadal histology

The gonads of most males had enlarged seminiferous tubules that were completely filled with mature sperm and had a relatively thin germinal epithelium. Immature females showed different stages of oocytes, and some had atretic follicles whereas the immature females from the reference lake showed only previtellogenic oocytes (Fig. 5).

3.5. Genetic sex identification in roach

The study involved collecting 60 wild roach fish, 23 of which were identified as males and 37 as females based on their histological gonadal phenotype. Subsequently, a sex marker was applied to determine their genetic sex, and the results showed that the genetic sex of 59 of the 60 fish (99%) aligned with their phenotypic sex. It is worth noting that 12 immature fish could not be visually assigned to either male or female initially, but upon further histological and genetic marker studies, 12

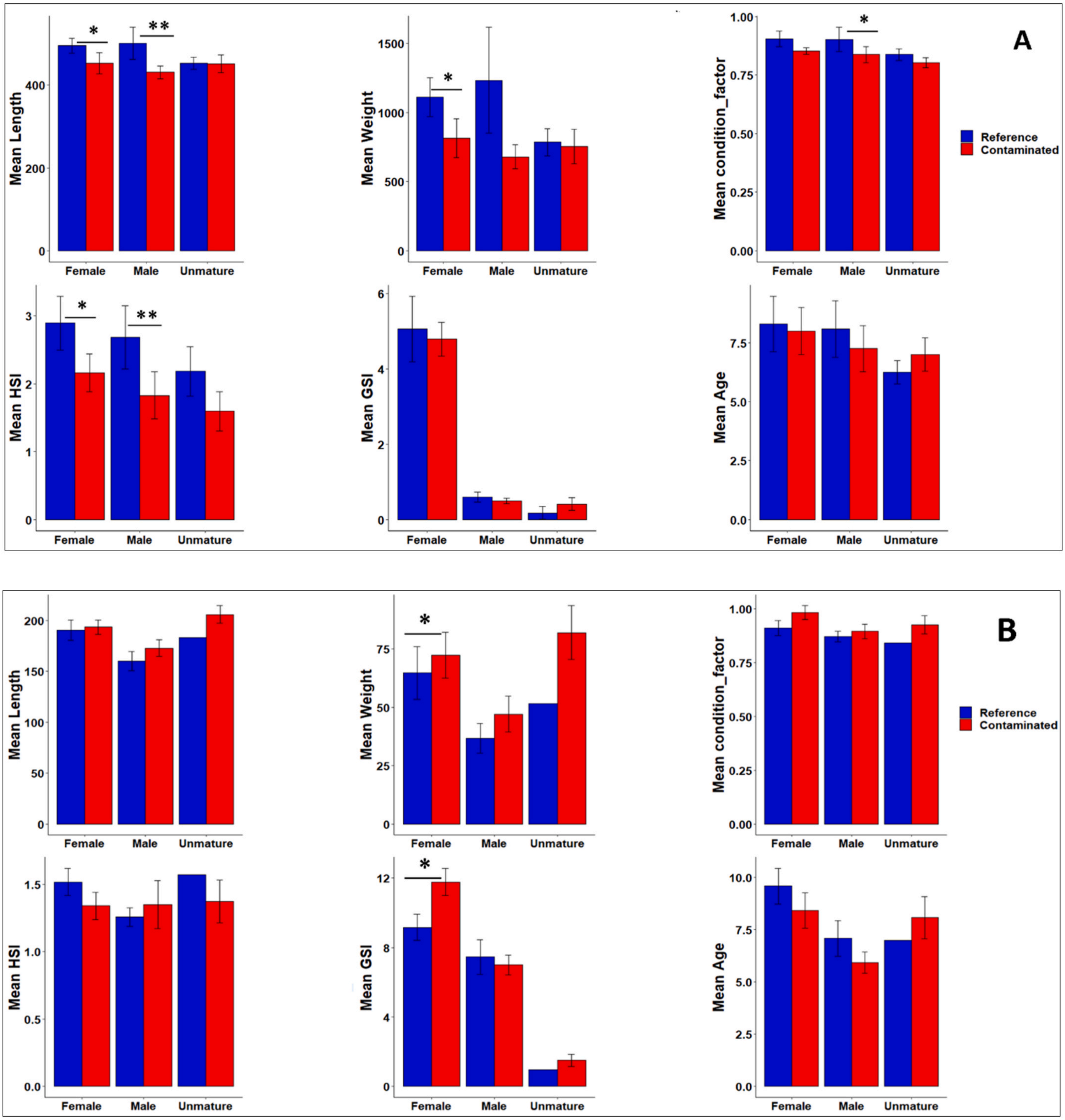

Fig. 3. Bar plots comparing the mean weight, length, condition factor, HSI (hepatosomatic index), GSI (gonadosomatic index), and age of fish populations between the reference and contaminated lakes. Panel **A** presents data for pikeperch, with sample sizes as follows: contaminated lake (Females: 13, Males: 12, Immature: 10) and reference lake (Females: 10, Males: 12, Immature: 4). Panel **B** presents data for roach, with sample sizes as follows: contaminated lake (Females: 12, Males: 12, Immature: 11) and reference lake (Females: 12, Males: 11, Immature: 1). Significance levels are indicated on the plots where applicable.

were assigned to be females. However, one immature fish was identified as male histologically, but female based on genetic markers.

3.6. Liver vitellogenin mRNA in roach

The vitellogenin mRNA levels were highest in females, and much lower in males and immature individuals (Fig. 6). The individual differences were large in all study groups, and there were no statistical differences between sites (Fig. 6).

3.7. Hormone content of water

No hormones (estrone, 17β-estradiol, 17α-estradiol, estriol, 17α-ethinylestradiol) were detected in our lake water samples. The only estrogen present in WWTP effluent was estrone, which was detected at 2.1 ng/L in the effluent of WWTP1. The effluent from WWTP2 did not

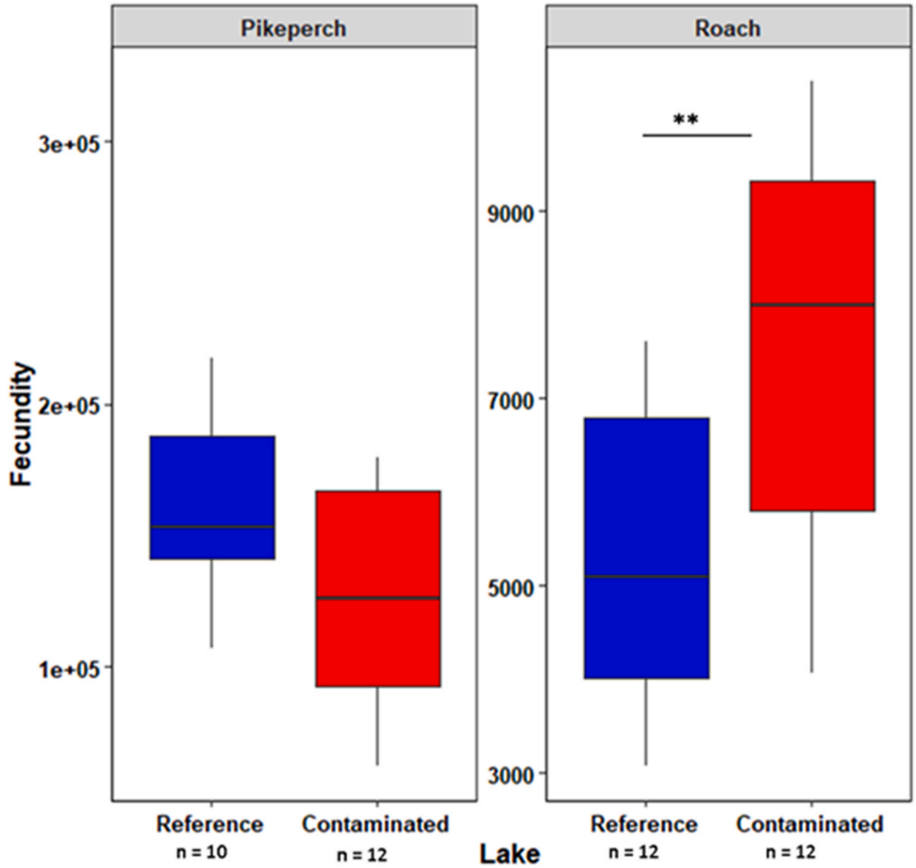

Fig. 4. Fecundity (number of eggs/female) of female pikeperch and roach populations in the reference and contaminated lakes. Significance levels are marked on the plots when applicable.

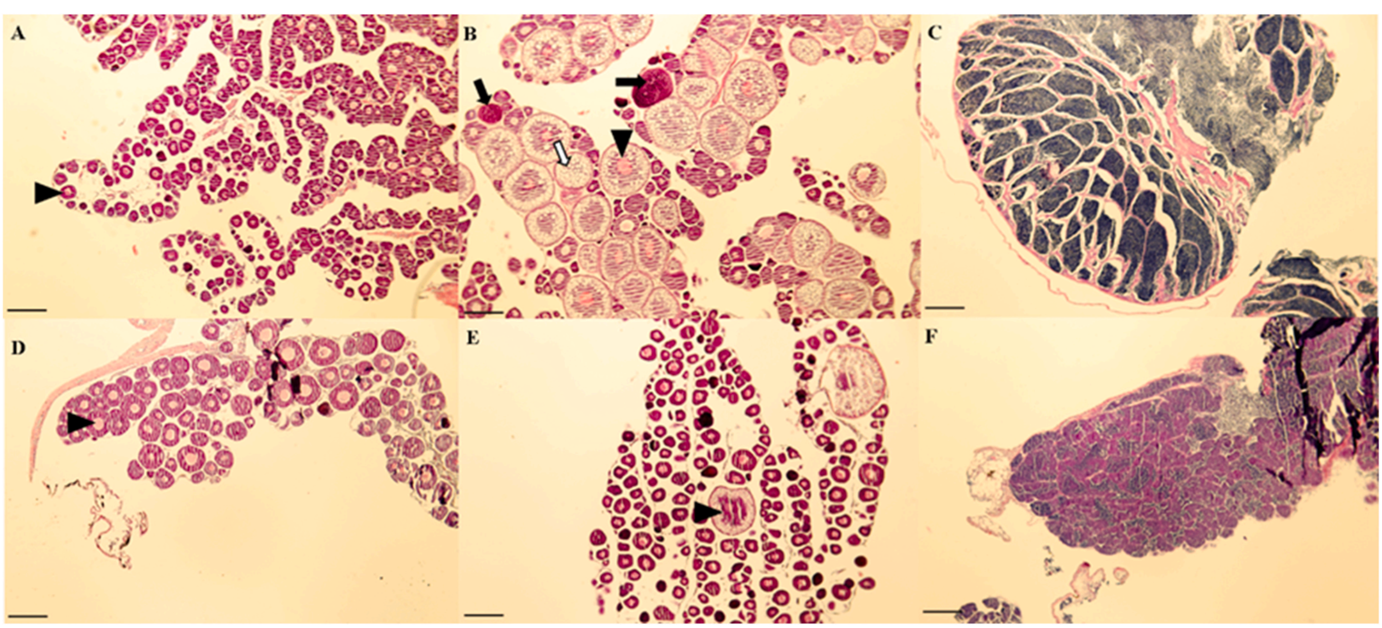

Fig. 5. Photomicrographs of ovaries and testes from roach and pikeperch: A) Immature female pikeperch from the reference site with numerous previtellogenic oocytes (indicated by arrowheads); **B)** Immature female pikeperch from the contaminated site with vitellogenic oocytes (arrowhead), atretic follicles (black arrow), and mature oocytes (white arrow); **C)** Mature male pikeperch with enlarged seminiferous tubules; **D)** Immature female roach from the reference site with numerous previtellogenic oocytes (indicated by arrowheads); **E)** Immature female roach from the contaminated site with vitellogenic oocytes (arrowhead); **F)** Mature male roach. Scale bar = 250 μm.

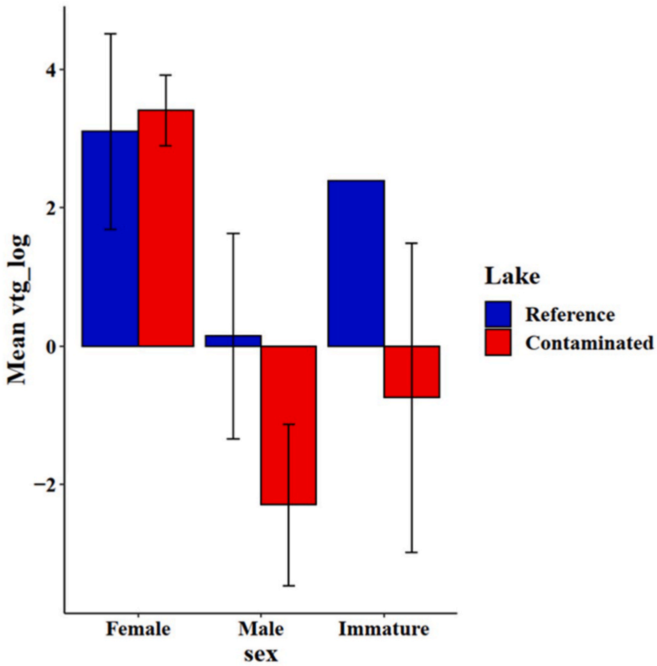

Fig. 6. The log10-transformed liver vitellogenin mRNA expression levels in the liver of male, female, and immature roach individuals from both the reference and contaminated lakes. Sample sizes are as follows: Roach in contaminated lake (Females: 9, Males: 11, Immature: 11), reference lake (Females: 8, Males: 8, Immature: 1).

contain estrogens.

4. Discussion

The primary goal of this study was to establish whether sexual immaturity observed in pikeperch in lake Pyhäjärvi in Finland is a true phenomenon and if it is connected to EDCs exposure. We compared condition factor, liver and gonad somatic indexes and histology of gonads of fish sampled from lakes located in both contaminated and reference conditions.

Our study revealed that both pikeperch and roach in the contaminated site exhibited delayed puberty. Furthermore, we found a higher incidence of females among the immature fish in the contaminated site, making the sex ratio female biased.

Pikeperch caught in lake Pyhäjärvi, which is more contaminated, tended to be smaller than those caught in the cleaner lake Näsijärvi. This size difference could be attributed to the greater availability of food resources in lake Näsijärvi. Pikeperch primarily consume smaller prey like zooplankton before transitioning to a fish-based diet. Eutrophic lakes with cyanobacteria blooms have been shown to decrease copepods and large zooplankton, potentially leading to smaller pikeperch in the contaminated lake due to their nutrient-poor diet during their juvenile stage (Taipale et al., 2022; Ginter et al., 2011; Frankiewicz et al., 1997). Furthermore, vendace, the primary prey of pikeperch, may be less abundant in eutrophic lakes due to higher oxygen levels and relatively lower trophic conditions. However, some other prey species might be more abundant in eutrophic lakes. It is important to note that increased water turbidity resulting from eutrophication can also hinder the fish's ability to capture prey, potentially further contributing to their smaller size. In addition, the absence of significant differences in age, weight, and length between mature and immature pikeperch at the contaminated site is intriguing. Conventionally, we would expect immature fish to be distinctly younger and smaller. Yet, the possibility that immature pikeperch at the contaminated site share the same size and age as mature ones suggests alternatives beyond just size and age impacting their maturation. This could be indicative of the disruptive effects of EDCs on the endocrine system, potentially delaying or altering the natural maturation process despite physical growth.

On the other hand, it is worth considering that Pyhäjärvi might offer more favorable conditions for efficient fishing, both in terms of net fishing and rod fishing, compared to the vast and open waters of Näsijärvi. In the context of the observed size differences between pikeperch populations in lake Pyhäjärvi and lake Näsijärvi, fishing pressure may indeed be a significant contributing factor. It is conceivable that over time, larger and older pikeperch individuals have been selectively harvested from lake Pyhäjärvi, leading to a population dominated by smaller and younger individuals. This targeted removal of larger individuals can have profound implications for the overall size distribution of the population and provides a plausible explanation for the observed size disparities. Both pikeperch and roach become sexually mature as they reach a certain age and size. In the reference lake, the immature pikeperch females were smaller in weight and younger than mature females, suggesting that they may not yet have reached maturity because of smaller size and age. In the study lake, lake Pyhäjärvi, however, the immature pikeperch had a similar size and age to the mature females. If food is scarce, female fish may skip a year of spawning, as egg production consumes energy reserves. As the pikeperch in lake Pyhäjärvi were smaller in size, they might not have had enough energy to reproduce. It is important to note, however, that whereas poorer diet might be the cause for sexual immaturity in pikeperch in lake Pyhäjärvi, this would not be the cause for sexual immaturity observed in roach, since the female roach from the contaminated site had a larger weight and GSI, than those from the reference site. The poorer energy reserves also cannot explain the female-biased sex ratios observed in both species. Nevertheless, if the size difference were solely due to fishing pressure, we might expect to see a skewed sex ratio with more males in the lake Pyhäjärvi population, as larger females are often the primary target for harvesting.

While the significantly larger size and weight of pikeperch from the reference lake suggest that these fish are healthier and better able to allocate resources towards growth and reproduction, the opposite was true for roach. The female roach from the contaminated site had a larger weight and GSI, than those from the reference site. We believe that the abundance of food in the contaminated site may have contributed to the better weight and GSI of the roach population. This could have allowed the roach to allocate more resources towards growth and reproduction. It is crucial to highlight that although the roach population in lake Pyhäjärvi seems to be flourishing, this prosperity does not automatically imply a robust and healthy ecosystem. There is a looming concern that contamination may still exert adverse effects on this species. For instance, an analysis of the age distribution of the fish populations in the reference and contaminated lakes revealed noteworthy disparities. In the contaminated lake, a higher proportion of younger individuals was observed, although this difference did not reach statistical significance. This intriguing pattern was consistently observed in both female and male populations, suggesting that contamination may indeed be exerting an influence on the survival, growth, or recruitment of all individuals within the population.

The liver is an important organ for detoxifying pollutants, and the hepatosomatic index (HSI) is commonly used to assess liver health. HSI values can increase due to vitellogenesis, and pathological changes caused by pollutants. However, the liver weight can also decrease due to starvation, exposure to sublethal levels of crude oil, or aquatic environments with low pH (Goede and Barton, 1990; Chambers and Yarbrough, 1979; Jacobsen, 1977; Lee et al., 1983). In most studies, high HSI values have been associated with disturbed zones, indicating that the liver is actively detoxifying pollutants. Conversely, in some cases, low HSI values have been observed in zones adjacent to sewage treatment plant effluent discharges, suggesting that the effluent may be causing sub-lethal toxicity to the liver (Ma et al., 2005; Sadekarpawar and Parikh, 2013). Our study is consistent with these findings, as we observed significant declines in mean HSI values in contaminated sites for both male and female individuals, as well as immature ones. HSI can also indicate fat accumulation and energy storage in fish, so a decrease in HSI, along with other biometric data such as height, weight, and condition factor, may suggest a depletion of energy reserves due to a poor diet in a polluted area rather than direct evidence of the ecological impact of pollution. It is known that parasitic infestation in fish can cause significant damage to fish liver size and function. Therefore, the presence of parasites that we observed during sampling in both sites may contribute to the decreased mean HSI values seen in the contaminated lake in our study.

Exposure to estrogens can interfere with reproductive development in fish (Jobling et al., 2002). In laboratory experiments, estrogen-type EDC exposure causes sex reversal followed by a skewed sex ratio toward females, as complete sex reversal has been observed in medaka (Scholz and Gutzeit, 2000), fathead minnow (Länge et al., 2001), and sheepshead minnow (Zillioux et al., 2001). Histological analysis revealed that the majority of the individuals of both fish species in the contaminated area were female, indicating a notable skew in the sex ratio favoring females. However, despite this skewed sex ratio, we found no evidence of intersex fish in the contaminated lake, and the genetic and histological sex generally matched in roach. These findings suggest that the skewed sex ratio and prolonged puberty are not caused by chemicals that act on female or male sex hormonal system. It is important to note that our analysis did not detect estrogens in the lake water. Nevertheless, sporadic exposure to estrogens has been reported previously (VESLA database), implying that contamination events may occur intermittently. Therefore, the potential effects of such sporadic exposure should not be dismissed, as they could accumulate over time and possibly lead to intersex conditions in the future.

Vitellogenin is an egg protein produced in the liver of fish, regulated by estrogen. Its induction in males or juvenile individuals serves as a biomarker for estrogenic endocrine disruption in fish. Although generally considered highly specific, some reports indicate that vitellogenin may also be induced by non-chemical stressors in certain fish species (Brown et al. 2023; Burden et al. 2023). Vitellogenin protein levels can be measured from plasma or mucus using immunological methods (e.g., ELISA), or its mRNA levels from the liver (Burden et al. 2023). These measurements have been shown to correlate well, though the response persists longer at the protein level (See et al. 2022). Due to significant individual variation, even under laboratory conditions, the response must be substantial to stand out from natural variation—after estrogen exposure, induction can indeed be several orders of magnitude (Brown et al. 2023; Burden et al. 2023). In this study, liver vitellogenin mRNA levels in roach were consistent across different areas, reinforcing the conclusion that the reproductive effects were not caused by estrogenic compounds. However, the large inter-individual variation and relatively small sample size may have obscured any subtle induction.

Our results suggest that factors other than estrogenic EDCs might be influencing delayed sexual maturity and the skewed sex ratios observed in the fish populations of the contaminated area. Contaminants such as polychlorinated biphenyls (PCBs) and heavy metals have been identified in the sediment of lake Pyhäjärvi (Hertta database) and are known to act as endocrine disruptors. For instance, heavy metals like mercury have been shown to cause significant reproductive toxicity. Dey and Bhattacharya (1989) reported ovarian damage in Channa punctatus exposed to low concentrations of mercury. Similarly, the EPA (2003) has discussed how PCBs disrupt fish reproduction and development, emphasizing the importance of understanding both the concentration ranges and the mechanisms through which these disruptions occur. Further supporting this, research in the Hudson River Basin by Baldigo et al. (2006) found that PCBs and mercury were significantly correlated with endocrine biomarkers, indicating disrupted hormone levels and reproductive issues in various fish species. Compounds that affect the thyroid system also play a crucial role. For example, studies by Dang et al. (2021), Wang et al. (2011), and Ismail et al. (2017) indicate that thyroid-disrupting chemicals, such as certain industrial intermediates, PAHs, and PCBs, can significantly impact fish development and reproduction by altering hormone regulation and metabolism (see also Iwamatsu, 2000). Moreover, fish living near sewage treatment plants, pulp and paper mills, and areas with high industrial activity often exhibit endocrine disruption, including altered hormone levels and reproductive abnormalities (Pait and Nelson, 2002).

While dose addition is a practical approach to assessing mixture effects, it should be noted that EDC mixtures may cause non-additive responses (Hamid et al., 2021; Martin et al., 2021; Kortenkamp et al., 2022). Given the area's history of inorganic and organic contamination from various sources, complex mixture effects may be occurring. Contaminants like PCBs, heavy metals, and estrogens can interact synergistically, leading to more severe endocrine disruption than when these chemicals are present individually. Therefore, it is essential to consider the potential interactions between different types of contaminants and their cumulative effects on fish health. Recent research by Pierron et al. (2021, 2022) has highlighted the potential epigenetic effects of metals on aquatic organisms, which could contribute to biased sex ratios. The sensitivity of early-life stages to stressors, such as pulp mill effluent (Orrego et al., 2021), may also play a significant role in shaping the observed sex distribution. Furthermore, hormonally active chemicals in mixtures have been observed to interact and affect fish egg production (Thrupp et al., 2018). Faheem and Bhandari (2021) reviewed the detrimental effects of bisphenol compounds on fish, noting that these contaminants can interact synergistically to exacerbate endocrine disruption. These findings underscore the need for comprehensive monitoring and assessment of multiple contaminants to fully understand their combined effects on aquatic ecosystems. These complex interactions highlight the need for a comprehensive investigation into the causes of the skewed sex ratio and delayed puberty in this fish population.

Our findings suggest that while sexually mature individuals in lake Pyhäjärvi currently appear to have normal gonads and are likely capable of reproducing, there are still observed issues such as skewed sex ratios and delayed puberty. This indicates that, despite outward signs of normal reproductive capacity, underlying disruptions may be present. Therefore, it is crucial to remain vigilant and continue monitoring the reproduction of fish in the area. The potential long-term consequences of sporadic contamination exposure and the interplay of various endocrine-disrupting factors should be investigated in more detail to ensure the health and stability of these fish populations in the face of environmental challenges. To protect these fish populations, we recommend several key strategies. The causes underlying the delayed puberty and skewed sex ratio should be solved, and thereafter possible mitigation strategies should be considered. Regular water and sediment quality assessments are currently tracking EDC levels, especially PCBs and heavy metals, and monitoring of polyfluorinated organic compound levels has been started as well (Perälä, 2021). Since steroidal hormones and hormonally active chemicals induce effects at very low concentrations (ng/l levels), the sensitivity of water samples should be increased by obtaining larger sampling volumes and concentrating samples using SPE methods. Additionally, passive samplers could be applied in routine monitoring to enhance analysis sensitivity. The extracts should be analyzed with quantitative in vitro endocrine disruption assays to relate concentrations to suggested Environmental Quality Standards for hormonal effects. Lake restoration projects focusing on sediment removal or capping can further reduce the bioavailability of PCBs and heavy metals, thereby lowering the overall contaminant burden on fish populations (EPA, 2003). This activity is already underway in the lake Pyhäjärvi watershed (Anonymous, 2024). Raising awareness and involving local communities in conservation efforts can improve the effectiveness of management strategies. Engaging stakeholders in pollution reduction initiatives is vital (Faheem and Bhandari, 2021; Prno et al., 2021). Further research on EDCs' long-term effects and stricter environmental regulations based on scientific findings are necessary to protect aquatic ecosystems (Dang et al., 2021; EPA, 2003). By implementing these strategies, we can mitigate the adverse effects of EDCs and heavy metals, ensuring the long-term health of lake Pyhäjärvi's fish populations and aquatic environment.

5. Conclusion

Our investigation into the health and reproductive status of pikeperch and roach populations in lake Pyhäjärvi, Finland, underscores the multifaceted impacts of environmental contamination on aquatic life. Through comparative analyses with specimens from the less contaminated lake Näsijärvi, we have illuminated the nuanced interplay between habitat quality, endocrine-disrupting chemicals (EDCs), and the biological responses of these fish species. The delayed puberty observed in both species within the more contaminated environment, alongside a female-biased sex ratio and no significant size or age difference between mature and immature pikeperch, suggests a profound disruption of natural maturation and reproductive processes, potentially attributable to EDC exposure. The absence of intersex fish, despite a skewed sex ratio towards females, prompts a critical evaluation of the subtleties of EDC exposure and other pollutants like PCBs and heavy metals that disrupt hormone systems. Studies have shown that PCBs and heavy metals can significantly affect endocrine function, leading to developmental and reproductive abnormalities. These complex interactions emphasize the need for continued monitoring and a comprehensive investigation into the causes of these observed effects to ensure the long-term well-being of this fish population in the face of environmental challenges. Furthermore, considering the potential interactions between different types of contaminants, a holistic approach to environmental monitoring is essential. Future studies should aim to disentangle the effects of multiple contaminants and their cumulative impacts on fish health. This approach will help identify the most critical factors contributing to endocrine disruption and guide targeted mitigation strategies. By broadening the scope of our investigation to include a variety of potential endocrine disruptors and their cumulative effects, we can develop a more holistic understanding of the challenges facing fish populations in contaminated environments.

CRediT authorship contribution statement

Roghaieh Ashrafi: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ari Westermark:** Writing – review & editing, Resources, Methodology, Data curation, Conceptualization. **Matti T. Leppänen:** Writing – review & editing, Resources, Methodology, Data curation, Conceptualization. **Eeva-Riikka Vehniäinen:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Eeva-Riikka Vehniainen reports financial support was provided by Kone Foundation (Koneen Säätiö). Eeva-Riikka Vehniainen reports financial support was provided by Pirkkalan kalatalousalue (The Pirkkala fisheries region). Eeva-Riikka Vehniainen reports financial support was provided by City of Tampere and Tampereen Vesi (Tampere water). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2024.119844.

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