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Gonadal Malformations in Whitefish from Lake Thun: Defining the Case and Evaluating the Role of EDCs

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Abstract: The objectives of this project were to evaluate i) whether the gonad alterations of whitefish (*Coregonus lavaretus* spp.) in Lake Thun represent abnormal morphological variations specific to this lake, and, if so, ii) whether the malformations are related to chemical exposure, in particular to exposure to endocrine-disrupting compounds (EDCs). Large-scale monitoring data revealed that, although whitefish in other lakes display some background variation of gonad morphology, the situation in Lake Thun, is unique because of the significantly higher prevalence of gonad malformations. The abnormal variations of whitefish gonad morphology include aplasias, compartmentations, fusions, and intersex. In the search for the factor(s) causing the gonad malformations, coregonids were exposed from fertilization up to maturity to Lake Thun water and plankton or to contaminants possibly being present in the lake, including trinitrotoluenes, and naphtalene sulfonates. Since these experiments are still ongoing, a conclusive answer cannot be given yet, but initial observations point to a role of the lake plankton. The possible presence of EDCs in Lake Thun was assessed using bioanalytics and biomarkers. The bioanalytical studies found estrogenic activities in concentrated plankton extracts of Lake Thun, however, estrogenic activities occurred also in plankton extracts of reference lakes. Bioassay-directed fractionation of the plankton samples points to degradation products of natural substances as a cause of the estrogenic activity. Examination of Lake Thun whitefish for EDC biomarkers such as vitellogenin, sex steroid levels or intersex frequency yielded no indications of exposure to EDCs, neither in fish with normal nor in fish with abnormal gonad morphology. Long-term laboratory exposure of developing coregonids to the prototype estrogenic compound, 17β-estradiol, resulted in an increased frequency of intersex gonads, but did not induce the other gonad malformations typical for Lake Thun coregonids. In summing up, the currently available evidence does not support an EDC or chemical etiology of the gonad malformations, however, this preliminary conclusion needs to be substantiated in the ongoing investigations. The project also highlights the need for more detailed knowledge of natural variation in wildlife populations to be able to recognize anthropogenically caused variation.

Keywords: *Coregonus* · Endocrine disruption · Gonad malformation · Intersex

Introduction

Lake Thun is a prealpine, oligotrophic lake of 47 km^2 with a maximal depth of 217 m. Whitefish (*Coregonus lavaretus* spp.) represent the main fish species caught by commercial fishermen. Several morphologically and genetically distinct ecotypes of whitefish occupying different ecological niches occur in the lake.[1]

In 2000, commercial fishermen observed a high number of whitefish with morphologically altered gonads in their catches from Lake Thun. According to the fishermen, corresponding alterations of whitefish gonads were not observed in previous years. A subsequent detailed investigation[2] classified the gonadal morphological variations into distinguishable categories including adhesions/fusions to the peritoneal wall and the lateral trunk musculature, compartmentations, asymmetry of the left or right gonad strand, atrophy/aplasia, constrictions, and hermaphroditism (Fig. 1). Since wild fish serve as indicator for the quality of the aquatic environment and since Lake Thun serves as drinking water reservoir for nearly half a million people, it is of great public interest to evaluate to what degree the suspected gonad malformations in whitefish from Lake Thun indicate abnormal variation in gonad morphology. Moreover, if the alterations are abnormal, it will also be important to identify the underlying causes.

The high prevalence of gonadal morphological variations in Lake Thun coregonids raises the following questions:

i) To what extent do the gonad alterations of Lake Thun coregonids represent normal morphological variations in

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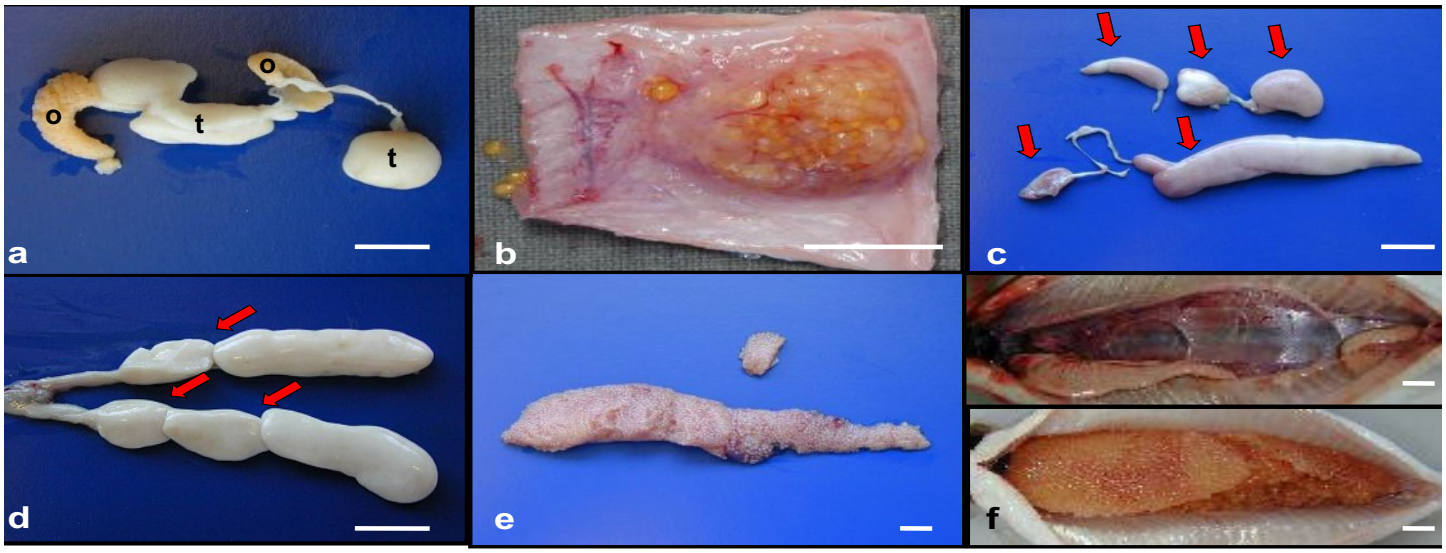

Fig. 1. Gross morphology of gonad alterations in whitefish from Lake Thun. a) Hermaphrodite gonad: Ovarian (o) and testicular lobes (t) on the same gonadal strand. b) Fusions of the gonads to the peritoneal wall and the muscles. c) Compartmented testes: The testicular strand is separated into several lobes (red arrows). d) Constrictions: Development of several lobes that are not separated from each other. e) Asymmetrical ovaries. f) Aplasia of one gonad strand. Above: The left testicular strand is missing. Below: Only the left ovarian strand is present. Bars = 1 cm.

wild whitefish populations, and is the situation in Lake Thun different to other lakes?

ii) If the alterations are abnormal, what is the factor(s) causing the alterations?

These questions were addressed through a series of experiments starting in 2004 and partly still ongoing today. In the following, a short overview of the currently available results will be given.

Are the Gonad Alterations of Lake Thun Coregonids 'Abnormal' and is the Situation in Lake Thun Unique?

Most morphological traits show some degree of quantitative and/or qualitative variation and, as stated by Sumpter and Johnson,[3] "it is axiomatic that it is not possible to conclude something is abnormal unless one knows what is normal". Thus it is crucial to clarify whether the extent of variation in gonad morphology observed in whitefish from Lake Thun goes beyond a 'natural' background level.

When initiating our studies on Lake Thun whitefish, the available information from either the literature,[4–7] or from responses to questionnaires that we had sent out to researchers all over the world, did not provide any clue with regard to 'normal' levels of variation in coregonid gonad morphology. To overcome this situation we

i) performed a regular monthly monitoring of the whitefish catch of commercial fishermen in Lake Thun,

ii) extended the monitoring to two neighboring lakes, Lake Brienz and Lake Biel, in which morphologically altered gonads of whitefish had not been reported so far, and

iii) included a second monitoring strategy by sampling mature fish on the spawning sites in all three lakes.

We analyzed the variation in gonad morphology at three hierarchical levels

i) among the lakes,

ii) among whitefish ecoforms within lakes, and

iii) among spawning sites within the coregonid ecoforms.

With both monitoring strategies it was found that gonad morphological variations were not restricted to coregonids of Lake Thun but were also present in whitefish populations of the two neighboring lakes. Asymmetries, which were most prevalent in females, and constrictions, which were most prevalent in males, occurred at comparable frequencies in the whitefish populations of all three lakes.[8] The situation in Lake Thun, however, was different because of significantly higher prevalence of aplasia, compartmentations, fusions, and intersex. Among the four ecoforms of coregonids being present in Lake Thun, the so-called 'Brienzlig' showed the highest frequency of gonad alterations,[2,8] and males were generally more affected than females (Fig. 2). These findings enabled discrimination between normal and abnormal variation in gonad morphology: Aplasia, compartmentations, fusions, and intersex are apparently 'abnormal' variations or malformations, whereas asymmetries and constrictions appear to represent natural variations of coregonid gonad morphology.[8]

What is the Factor Causing the Alterations?

Gross alterations of gonad morphology have been reported for a variety of fish species from a number of field studies.[9–13] In many of the published cases, the etiology of the gonadal alteration is not known. As a matter of fact, gonad morphology of fish is susceptible to many environmental factors. For instance, infestation by the parasite *Pleistophora mirandellae* can lead to gonad histological alterations.[14] In Lake Thun coregonids, gonad parasites were never observed among the approximately 400 individuals examined histologically. Interestingly, however, *Pleistophora mirandellae*-induced gonad alterations were observed in brown trout (*Salmo trutta fario*) populations from tributaries to Lake Thun (Bernet, unpublished).

Another environmental factor that frequently has been associated with morphological alterations of fish gonads are contaminants[*e.g.*11,15,16]. Particularly the so-called endocrine-disrupting compounds (EDCs), *i.e.* substances that mimic endogenous hormones or modulate their metabolism, are able to modify the morphology of developing gonads or of already differentiated gonads. This has been shown in numerous laboratory experiments[*e.g.*17,18] as well as in field studies.[*e.g.*19,20] Probably the best documented example of an association between EDC exposure and gonad morphological alterations are the findings on roach, *Rutilus rutilus*, showing high frequencies of intersexuality (*i.e.* the presence of both male and female gonadal characteristics within the same gonad) in many populations of UK rivers.[21] The strong correlation of intersex frequency with the proximity of the roach populations to sewage treatment plants (STPs) point to a causal relationship between estrogenic chemicals in the effluents and the presence of the intersex gonads.[22] Also

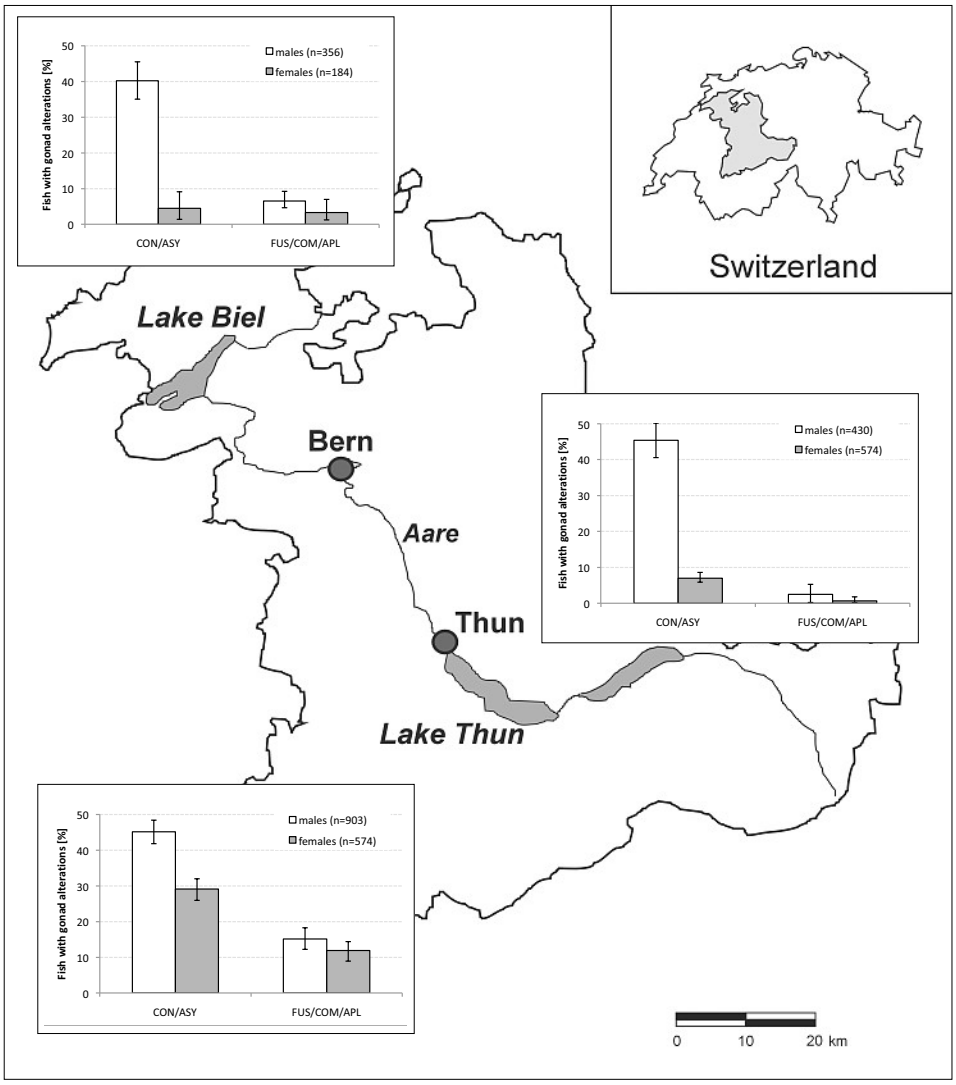

Fig. 2: Frequency of whitefish with gonad malformations in Lake Thun, Lake Brienz and Lake Biel. Data originate from a monitoring campaign in 2004 where all known spawning sites in the lakes were sampled.[8] Abbreviations: ASY = Asymmetries, CON = constrictions, FUS = Fusions (FUS), COM = Compartmentations, APL = Aplasia or atrophy.

for coregonids, environmental EDCs have been suspected as cause of gonad anomalies.[7]

The findings from field and laboratory studies on the induction of gonad morphological alterations by exposure of fish to EDCs, as well as the fact that morphological alterations of Lake Thun whitefish were restricted to the reproductive organs put the hypothesis forward that EDCs may be a causative factor. This leads to the question on potential sources of EDC contamination in the lake. An impact of effluents from STPs is unlikely, as there is only one substantial STP (population equivalent: 68'000) in the immediate vicinity of Lake Thun that releases its effluents directly into the lake. Atmospheric deposition is a major input pathway for polybrominated diphenyl ethers and related compounds which are suspected as endocrine disruptors, but their levels in Lake Thun are lower than in other lakes in which coregonid populations show no gonad alterations.[23]

One important group of contaminants could be explosives from ammunition deposits. From the 1920s until the 1960s, the Swiss Army dumped approximately 4'600 tons of ammunition into Lake Thun. Certain explosive constituents including trinitrotoluenes were reported to have weak endocrine activities.[24] A further source of chemical contamination is the wastewater from the Lötschberg tunnel works which reaches Lake Thun *via* the River Kander, a main tributary of the lake. Chemicals used for the tunnel works include a wide range of substances, with various explosives and naphthalene sulfonates being major components (Cantonal Office for Water and Soil Protection, unpublished). Although the wastewater is pre-treated before release into the Kander, and diluted during the 25 km passage until the lake, it still can be a source of low-level contamination, as indicated from model calculations.

Experimental Approaches to Test the EDC Hypothesis for Lake Thun Whitefish

Based on the aforementioned considerations, the hypothesis was tested that the gonad malformations of Lake Thun whitefish are related to exposure to EDCs in the lake environment, be it in the water, in the food (lake plankton; coregonids rely on gill raker filter feeding) or in the sediment. Since to date the vast majority of known EDCs are those that interfere with the parts of the endocrine system associated with the sex steroid system, we focused on compounds acting as agonists or antagonists of the estrogen and androgen receptors. Additionally, we investigated the effects of key toxicants from potential contamination sources, *i.e.* ammunition dumps and Lötschberg tunnel wastewater.

Experimentally, we used the following approaches to test the EDC hypothesis:

Bio-analytical Studies on the Presence of Endocrine Activities in Water, Sediment, Plankton, and Whitefish of Lake Thun

Due to the fact that the identity of the compounds causing the malformations is unknown, the combination of bioanalytical screening together with effect-oriented analysis appears to be the most promising approach to search for the presence of EDCs in Lake Thun. Using this strategy, we performed a comprehensive monitoring of the habitat (Lake Thun water and sediment), the food chain (plankton) and fish (muscle and bile of whitefish with normal or malformed gonads). Reference samples were taken from Lake Brienz which hosts a coregonid population showing significantly fewer abnormal gonad morphological variations than Lake Thun.[8] For monitoring of the water compartment, passive samplers of the POCIS type were used.[25] Sediment samples were collected from the surface layers of coregonid spawning sites in Lake Thun and Lake Brienz.

After extraction of the samples, they were tested in the recombinant yeast estrogen/androgen screen (YES/YAS[26,27]). Samples with endocrine activity were then chemically analyzed for the target steroids estrone, 17β-estradiol and 17α-ethinylestradiol and further fractionated according to physicochemical properties in order to reduce the complexity of the sample. The obtained fractions were tested again in the YES/YAS system and positive fractions were analyzed by means of mass spectrometry. In order to be able to detect biological activities beyond the YES/YAS system, we additionally implemented the E-Screen, based on estrogen-sensitive breast cancer cells,[28] the Mol-

Table 1. Hepatic levels of vitellogenin (VTG) mRNA levels (quantified by means of real time RT-PCR) and circulating levels of androgens (quantified by means of ELISA) in male whitefish from four different sites in Lake Thun. Males are categorized into males with normal or with abnormal gonad morphology. Gonads were classified as abnormal if one or more of the following deviation types were present: aplasia, compartmentation, fusion and intersexuality. The values are medians ± SD, number of analysed fish, minimum and maximum values [N; min-max].

		VTG [Copies Vtg-mRNA/1000 copies 18s-RNA]		Testosteron [ng/ml plasma]		11-Keto-Testosteron [ng/ml plasma]	
Morph	Site	Normal males	Abnormal males	Normal males	Abnormal males	Normal males	Abnormal males
Brienzlig	Merligen	1.31 ± 21.45	0.87 ± 28.45		30.81		36.37
		[24; 0.01 - 106.36]	[15; 0.04 - 99.21]		[1; 30.81 - 30.81]		[1; 36.37 - 36.37]
	Faulensee	2.89 ± 20.45	0.85 ± 9.24	26.54 ± 49.40	20.67 ± 12.46	20.37 ± 6.51	15.53 ± 10.68
		[27; 0.02 - 70.19]	[17; 0.01 - 27.95]	[6; 19.28 - 30.65]	[13; 0.11 - 48.28]	[6; 8.9 - 27.92]	[13; 0.49 - 33.44]
Albock	Schifflätti	0.33 ± 3.92	3.89 ± 4.28	43.36 ± 19.65		62.81 ± 26.70	
		[11; 0.05 - 10.07]	[7; 0.004 - 12.10]	[8; 16.42 - 69.31]		[8; 19.44 - 96.83]	
	Gwatt	3.99 ± 4.61	9.86 ± 20.65	29.86 ± 22.5	29.89 ± 16.42	33.00 ± 19.59	30.53 ± 29.01
		[6; 0.31 - 12.26]	[3; 7.01 - 44.14]	[15; 8.24 - 77.61]	[5; 11.79 - 50.97]	[15; 0.29 - 74.43]	[5; 0.68 - 75.65]

DarT assay based on molecular marker responses in zebrafish embryos,[29] and the sediment contact assay with zebrafish early life stages.[30]

Extracts of passive samplers from Lake Thun, Lake Brienz and River Kander showed neither estrogenic nor androgenic potencies. Extracts of the sediment samples taken in 2005 showed no estrogenic or androgenic activity. In 2006, again no androgenic activity was found but two out of five samples from the Beatenbucht, a former ammunition dump site, showed estrogenic activity (Liedtke and Suter, unpublished). According to chemical analyses, the two samples contained neither estrone, 17β-estradiol nor 17α-ethinylestradiol; thus, the nature of the receptor-activating substances remains unknown. The sediments were also screened for heavy metals because metals could interfere with the endocrine system, but, metal concentrations were generally low and site differences were not found. Concentrated extracts of plankton (mixed zoo- and phytoplankton) showed no androgenic potencies, but estrogenic activities were detected in three bioassays (YES, E-screen, MolDarT assay). However, estrogenic activities were not only present in plankton extracts of Lake Thun but also in plankton extracts of reference lakes with coregonid populations showing normal gonad morphology. The obviously more widespread presence of estrogenic activities in plankton extracts argues against a causative relationship between plankton estrogenicity and gonad malformations in Lake Thun. Preliminary results from bioassay-directed fractionation of the plankton extracts point to degradation products of natural substances to be responsible for the activity observed in the YES assay (Liedtke and Suter, unpublished).

Biomarker Studies to Indicate Exposure of Whitefish in Lake Thun to EDCs

Several biomarkers that are indicative of exposure of fish to EDCs were analyzed in coregonids of Lake Thun:

- *Vitellogenin:* The induction of vitellogenin (VTG) is a well-established biomarker of exposure of male fish to estrogen receptor ligands.[31]
- *Sex steroids (*testosterone and 11-keto testosterone): Disturbances in sex steroid metabolism and levels of circulating sex steroids have been repeatedly observed in fish exposed to EDCs.[32]
- *Prevalence of intersex fish*: A histological feature frequently observed in fish exposed to estrogenic compounds is the presence of intersex gonads, or more specifically, ovotestis, *i.e.* gonads with predominantly testicular morphology but containing also ovarian morphology.[21,22]

For biomarker measurements, a total of 878 whitefish were collected in September and December 2005 and 2006 on four spawning sites, two of them ('Merligen' which is in close proximity to an ammunition dump site, and 'Faulensee') used by the summer spawning ecotypes 'Brienzlig' (the form with the highest prevalence of malformations, see above), and two other sites ('Schifflätti', 'Gwatt') used by the winter-spawning ecotypes 'Albock'. Despite distinct differences in the frequency of gonad alterations between Brienzlig and Albock, the two ecotypes showed no significant differences in hepatic VTG mRNA levels (Table 1). Also plasma levels of testosterone and 11-keto-testosterone did not differ significantly between male Brienzlig

Table 2. Frequencies of macroscopically abnormal gonads and intersex in male (M) and female (F) whitefish from four different sites in Lake Thun. Data indicate number of analyzed fish (N), frequency (f) of gonad malformations and its 95% confidence intervals (CI_{95}). Gonads were classified as abnormal if one or more of the following deviation types were present: aplasia, compartmentation, fusion and intersexuality.

			Macroscopy			Histology		
			Abnormal fish			Mosaic Intersex		
Morph	Site	Sex	N	*f*	CI_{95}	N	*f*	CI_{95}
Brienzlig	Merligen	M	100	0.37	0.28-0.47	39	0.15	0.06-0.31
		F	163	0.30	0.23-0.38	12	0	0.00-0.25
	Faulensee	M	249	0.37	0.31-0.43	44	0	0.00-0.08
		F	206	0.12	0.07-0.17	13	0	0.00-0.25
Albock	Schifflätti	M	63	0.19	0.10-0.31	18	0.02	0.00-0.27
		F	22	0.05	0.00-0.23	5	0	0.00-0.52
	Gwatt	M	40	0.15	0.06-0.30	9	0	0.00-0.34
		F	35	0.03	0.00-0.15	4	0	0.00-0.60

and male Albock (Table 1). Moreover, there were no significant differences in VTG mRNA or sex steroid levels between fish with malformed testes and fish with normal testes.

Intersex gonads were found at all sites with very low frequency in fish except at Merligen, where 15% of male whitefish displayed ovotestis (Table 2). The intersex gonads were of a mild form, with one or few oocytes scattered in normal testicular tissue, and they were neither correlated with elevated vitellogenin expression nor with altered plasma androgen concentrations.

Both the results of the bioanalytical and the biomarker studies do not point to relevant chemical or EDC exposure of fishes in the Lake Thun environment. This finding agrees well with the results of chemical-analytical studies showing that levels of chemical contamination in Lake Thun tends to be lower than in other lakes.[23,33]

Long-term Rearing of Coregonids under Exposure to Either Lake Thun Water or Plankton

The aim of the rearing experiments was to evaluate whether exposure of developing whitefish to Lake Thun water or plankton results in induction of gonad abnormalities, thereby indicating the presence of a causative factor in the Lake Thun ecosystem. The coregonids were reared from fertilization until early maturity at 2–3 years of age under exposure to either water or plankton from Lake Thun. For the experiments, both offspring from Lake Thun and Lake Biel were used in order to differentiate environmental and genetic factors. As negative controls, coregonids were reared in either spring water or in water of Lake Lucerne, and they were fed with dry feeds instead of lake plankton. A controlled breeding scheme was used with a view to obtain a multitude of known parental combinations. This allowed us to generate offspring from pairs of parent fish with normal or abnormal gonad morphology, and from mixed pairs.

Two sets of experiments were initiated, one in 2004 and, after part of the 2004 experiments had been lost due to a flooding event, a second set was started in 2005. While the latter experiments are still ongoing, the remaining groups of the first set were sampled in October 2007. A striking observation from this experiment is that the feed significantly influenced the development of gonad alterations: Among the males fed with Lake Thun plankton, 28% showed malformed testes, while among the females, 12% had malformed ovaries. In contrast, whitefish reared on dry feed displayed significantly lower percentages of malformations (males: 2–8%; females: 1–2%), independent of fish origin and water quality (spring water, Lake Lucerne water). It needs now to be clarified whether the results of the second rearing experiment (started in 2005) will confirm these findings.

Long-term Rearing of Coregonids under Exposure to Prototypic EDCs and Lake Thun Contaminants

Two types of contaminants were tested in these experiments. First, we investigated whether chemicals bound to the sediments may have organizational effects on gonad development of whitefish. The fertilized eggs of whitefish develop on the sediments until they reach the swim-up stage. During this period of life, the fish therefore could be exposed to bioavailable sediment-borne toxicants. Candidate contaminants in sediments of Lake Thun are chemicals originating from the dumped ammunition, for instance, trinitrotoluenes, as well as lipophilic chemicals originating from Loetschberg tunnel wastewaters that are transported *via* River Kander to Lake Thun and may bind to the sediments. In this context, naphthalene sulfonates (NSF) are of concern, as these compounds have been used in large volumes as additives to concrete constructions in the tunnel. Environmentally persistent NSF monomers leach out during concrete hardening[34] and, according to environmental fate modelling, could reach Lake Thun in the low microgram/l range (Cantonal Office for Water and Soil Protection, unpublished).

A novel incubation system had to be developed to be able to expose whitefish eggs on sediments. This system was used to incubate whitefish eggs from fertilization until hatching on artificial control sediments, on natural Lake Thun sediments, or on Lake Thun sediments spiked with either 2,4,6,-trinitrotoluene (TNT), a major ammunition constituent, or a mixture of four environmentally persistent congeners of NSF. After hatching, the fish were transferred into non-contaminated tap water and they are now reared with dry feeds. The fishes will reach the sampling age (2.5 years) in October 2008.

In a second long-term rearing experiment, we investigated the impact of the natural estrogen, 17β-estradiol (E2) – as prototype estrogen receptor ligand – on gonad development of whitefish. The questions to be answered in this experiment were

i) whether long-term estrogen exposure is able to induce gonadal morphological alterations in whitefish, and
ii) whether these alterations are identical or similar to those found in whitefish from Lake Thun.

Whitefish were reared from start-feeding up to an age of 2.5 years with either an estrogen-free control diet or with estrogen-spiked diets. Measurement of hepatic vitellogenin (VTG) mRNA was used to test the efficiency of the dietary E2 treatment and the results showed a dose-dependent induction. The estrogen treatment also led to a dose-dependent increase in the prevalence of intersex gonads (Kipfer *et al.*, in prep.). Importantly, however, while intersex is the gonad malformation showing the lowest frequency in whitefish from Lake Thun,[8] those malformations that are more frequent in wild fishes, *e.g.* fusions or aplasia, were not induced by the estrogen treatment. This finding argues against a causative role of estrogen receptor ligands in the induction of the gonad alterations of Lake Thun coregonids.

Conclusion

The objectives of this project were to evaluate i) whether the gonad alterations of whitefish in Lake Thun represent abnormal morphological variations specific to this lake, and, if so, ii) whether the malformations are related to chemical exposure, in particular to exposure to EDCs. Although the investigations are not fully completed yet, the results obtained so far provide strong evidence that certain alterations of the gonad morphology are indeed abnormal variations, and that the high frequency of malformations is a feature unique to coregonids of Lake Thun. The comparison of gonad morphologies of whitefish from the Lakes Thun, Brienz and Biel revealed that coregonids in all three lakes express some level of gonad morphological variation, but whitefish from Lake Thun, in particular the ecotype 'Brienzlig', show significantly higher prevalence of aplasias, compartmentations, fusions, and intersex gonads. A causative role of estrogen- or androgen-active EDCs in the development of the gonad malformations is not supported by the currently available data. The bioanalytical investigations found estrogenic activities in concentrated plankton extracts, but this was not a specific finding for Lake Thun and was observed in plankton extracts of other lakes as well. Biomarkers such as vitellogenin or gonad histopathology neither indicated estrogenic exposure of Lake Thun coregonids nor did they show differences between fish with normal and with malformed gonads. Exposure experiments with the positive control substance, 17β-estradiol, failed to induce the Lake Thun-typical gonad malformations. Initial results from feeding experiments with plankton of Lake Thun point to a role of the plankton in the formation of gonad malformations, however, this finding on the one hand needs to be confirmed in the ongoing experiments, and on the other hand does not necessarily implicate a role of EDCs. Overall, the current state of knowledge does not support an EDC or chemical etiology of the gonad malformations, but this preliminary conclusion needs to be further substantiated.

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