Field and laboratory approaches to assess "estrogen disruption" in the brown trout *Salmo trutta*.

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Prof. Dr. Hans-Peter Hauri (Dekan)

"If you want to build a ship, don't drum up people together to collect wood and don't assign them tasks and work, but rather teach them to long for the endless immensity of the sea".

(*Antoine de Saint-Exupery*, 1900 – 1944)

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Summary

In recent years, the annual catches of brown trout and other native fish species have been declining in Switzerland about 60%. One hypothesis was that the reduced catch is linked to estrogen-active chemicals entering the aquatic environment via waste water effluents. These so–called environmental estrogens have the potential to mimic the actions of endogenous hormones and impair the reproductive fitness of fish. The present thesis aimed to assess the reproductive health of brown trout in Swiss rivers and to link putative reproductive disturbances with the exposure to waterborne estrogens. In this context, we tracked field as well as laboratory based approaches.

In order to assess whether the reproductive health of feral brown trout is disturbed, we applied two different sampling strategies - namely passive and active monitoring approaches. In the first approach, we sampled feral fish at three sites along four rivers with a well documented catch decline. These rivers are affected by inputs of wastewater effluents. The sampling was conducted during two years; we measured plasma vitellogenin (Vtg) concentrations and surveyed gonadal histology. In general, our data indicate that effects of environmental estrogens in Swiss rivers are low. In only 5% of the analyzed males, we found plasma Vtg concentrations higher than 1 µg/mL. Also the incidence of ovarian atresia was low and we found no male intersex fish. In contrast to males, females caught along two rivers had spermatogenic activity in ovarian tissue. However, this intersex condition does not appear to be linked to environmental estrogens. In our second field trial, we developed a mini–caging method to suit the hydrological conditions in small rivers and to improve upon the often poor survival of salmonids in caging trials. After three weeks of exposure, we measured plasma yolk protein and linked the Vtg concentrations with the bioaccumulation of estrogens in bile of caged fish. Because of the estrogenicity of river water is highly variable and it is difficult to obtain an average measure of the estrogenicity we additionally tested the use of passive sampling by means of polar organic chemical integrative samplers (POCIS). The POCISs were positioned upstream and downstream of wastewater treatment works. Concurrently, water grab samples were taken at each site. Concentrations of estrogens were determined using a yeast-based reporter gene assay and chemical analysis. Results from grab sampling, passive sampling, and

bioaccumulation were correlated; however, plasma vitellogenin concentrations were elevated at only 1 of 5 sites. The POCISs provided an integrated and biologically meaningful measure of estrogenicity in that they accumulated estrogens in a pattern similar to that of brown trout. The mini caging appears a significant methodological advance; no fish were lost, moreover, all fish survived in excellent health. On the basis of our field data, we conclude that impaired reproductive health does not appear to be a major factor contributing to the marked decline of brown trout catches in the four investigated rivers. In addition to the potential risk of environmental estrogens, increasing water temperatures as a result of global warming has become a serious problem in many Swiss rivers and streams. In particular low mountain range rivers frequently reach temperatures that are suboptimal for many salmonid species. In our field surveys, we used the analysis of Vtg as an indicator of estrogenic exposure. Little, however, is known regarding the potential interaction between ambient water temperature and the Vtg production induced by waterborne environmental estrogens. In order to test the influence of temperature on Vtg synthesis, we exposed juvenile brown trout to ethinylestradiol (EE_2) and hold them either at low or high temperatures (12°C and 19°C, respectively), but also at temperature cycles of 12°-19°C to simulate the field situation. The EE_2 exposure caused a 7 to 74-fold increase of hepatic Vtg mRNA and the synthesis Vtg mRNA was clearly stimulated in fish hold at higher water temperatures. On the protein level, Vtg showed a similar pattern; the higher the temperature, the higher the concentration of Vtg in the plasma. The experiment further revealed a temperature dependent increasing amount of hepatic estrogen receptor alpha mRNA after exposure to waterborne EE_2. The gene expression of estrogen receptor beta-1 and the glucocorticoid receptor in the liver of EE_2 exposed fish, however, showed no treatment related alterations. In line with observed constant bile cortisol concentrations, our data do not indicate any stress related effects on hepatic Vtg production. The present experiment, however, clearly demonstrated that ambient temperature significantly change the estrogen-induced expression of Vtg and therefore may alter the interpretation of environmental monitoring studies under field conditions.

Changing water temperature alters the permeability of the gills and result in a disturbed mineral balance in fish. The branchial sodium pump (Na^+/K^+–ATPase)

enables teleosts to cope with such varying environmental conditions and compensates for the temperature–related loss of ions by active ion uptake from the ambient water. Estrogens have the potential to interfere with the endocrine regulation of Na^+/K^+–ATPase and may affect the molecular expression of sodium pump mRNA and related branchial steroid receptors (mineralocorticoid and glucocorticoid receptor). In the light of a recently observed warming of Swiss rivers as well as the occurrence of estrogen-active chemicals in river water, such interactions may have detrimental effects on the general health of brown trout in Switzerland. To test the influence of temperature on the regulation of Na^+/K^+–ATPase we used the same juvenile brown trout as described above in the Vtg study. Data obtained from quantitative PCR evidenced a significant down regulation of Na^+/K^+-ATPase gene expression in gills from estrogen–treated brown trout held at low and fluctuating temperatures. However, the expression of Na^+/K^+–ATPase in estrogen-treated fish from the EE_2–high temperature group were not significant lower than the control groups – indicating a response to the elevated water temperatures. No significant effects on the number of immunoreactive chloride cells were found; though, estrogen treatment tend to reduce the protein abundance of Na^+/K^+–ATPase in the gills. The synthesis of mineralocorticoid receptor mRNA correlated significantly with the expression of Na^+/K^+–ATPase. In contrast, bile cortisol levels and the glucocorticoid receptor gene expression were not affected by estrogen treatment alone or in combination with elevated temperatures. This suggests that the expression of Na^+/K^+–ATPase is probably regulated via the mineralocorticoid receptor. In addition, the lack of cortisol response as well as the absence of effects on higher levels of biological organization (e.g. histology or condition factor) suggests that the temperature regimes used in the present study were insufficient to cause stressful conditions in brown trout.

Introduction (Chapter 1)

Fish catch decline in Switzerland

Since the early eighties of the last century, fishermen as well as corresponding cantonal authorities observed an alarming catch decline of freshwater fish in numerous rivers and streams all across Switzerland (Burkhardt-Holm et al., 2005). In particular, the annual catch of brown trout (*Salmo trutta* fario), a native salmonid species in European low mountain range rivers, has declined about 50% over a twenty years period (Fig. 1)

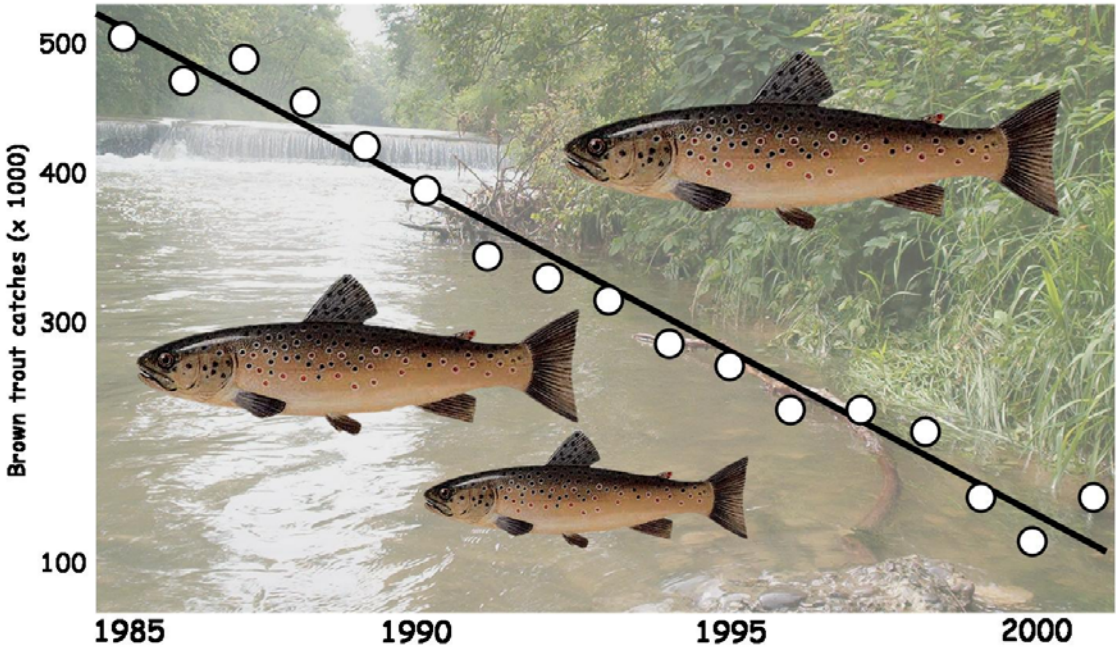


Fig.1 Catches of brown trout in Swiss river systems documenting a continuous reduction over a fifteen year period. Data are obtained from Burkhardt-Holm et al. (2002).

In addition, catches of grayling (*Thymallus thymallus*), nase (*Chondrostoma nasus*) and other species were also considerably reduced over two decade period (Burkhardt-Holm et al., 2002). A follow up study revealed a catch decline in 20 out of 26 surveyed cantons (Burkhardt-Holm et al., 2005). Some of these areas are characterized by a high human population density (380 inhabitants km–2), extensive agriculture activities or input of industrial and municipal wastewater effluents. In addition, the majority of Swiss streams and rivers and their tributaries are canalized and affected by human flow regime alterations (Peter et al., 2005).

Along with water pollution and river morphology, informations on impaired fish health (e.g. Bernet et al., 2001; Schmidt-Posthaus et al., 2001) in rivers and streams across the country caused public concern about potential risk on endemic fish species.

To identify the causes of the catch decline, an interdisciplinary project FISCHNETZ (engl. FISHNET – Project on declining fish catch in Switzerland) was initiated in 1998 and started one year later (Burkhardt-Holm et al., 2002). By evaluating the catch, fish abundance as well as diverse biotic and abiotic parameters, FISCHNETZ aimed to reveal the causes of the catch decline and to propose measures to ensure the future viability of this ecological and economical important resource. In order to structure the search for the causes, FISCHNETZ provided twelve hypotheses developed specific research questions and compared them to the existing knowledge (Burkhardt-Holm, 2002; Burkhardt-Holm, 2007). The working hypotheses were:

1. The decline in fish is due to more than one of the factors that follow, with each factor having a different significance depending on the geographical region involved.
2. The fish population is suffering from reproductive failure of adult fishes.
3. The fish population is suffering from reduced recruitment of young stages.
4. The health of fish and their fitness is impaired.
5. Chemical pollution (both nutrients and synthetic compounds) is responsible for the fish decline and impairment of health.
6. Poor morphological quality of the streams and a lack of longitudinal connectivity (restricted upstream migration of fish) are responsible for the decline in fish.
7. An increased amount of fine sediments in streams is responsible for the decline in fish.
8. Reduced amount of food leads to the decline in fish.
9. The decline in fish is caused by inadequate management of fisheries.
10. The decline in fish is the result of an increased removal of fish, or reflects altered behavior of anglers.
11. Changes in the water temperature have led to a decline in fish population, abundance and fish catch.
12. An altered hydrological regime and modified sediment transport are responsible for the decline in fish.

In this context, the present thesis addresses the hypothesis that observed catch decline is linked to impaired reproductive fitness of brown trout especially in regard to the exposure to hormonal active substances, which is generally called "endocrine disruption".

One central study of FISHNETZ was a comprehensive field monitoring survey (called TESTGEBIETE; engl. test areas) at four selected rivers in Switzerland and aimed to assess the significance of different stressors on brown trout populations (Fischnetz, 2004). These areas (namely Venoge, Emme, Necker and Liechtensteiner Binnenkanal) are known for a 25 to 90% decline in brown trout catches during a 20 years period and have been therefore considered as representative rivers in Switzerland (Fig. 2).

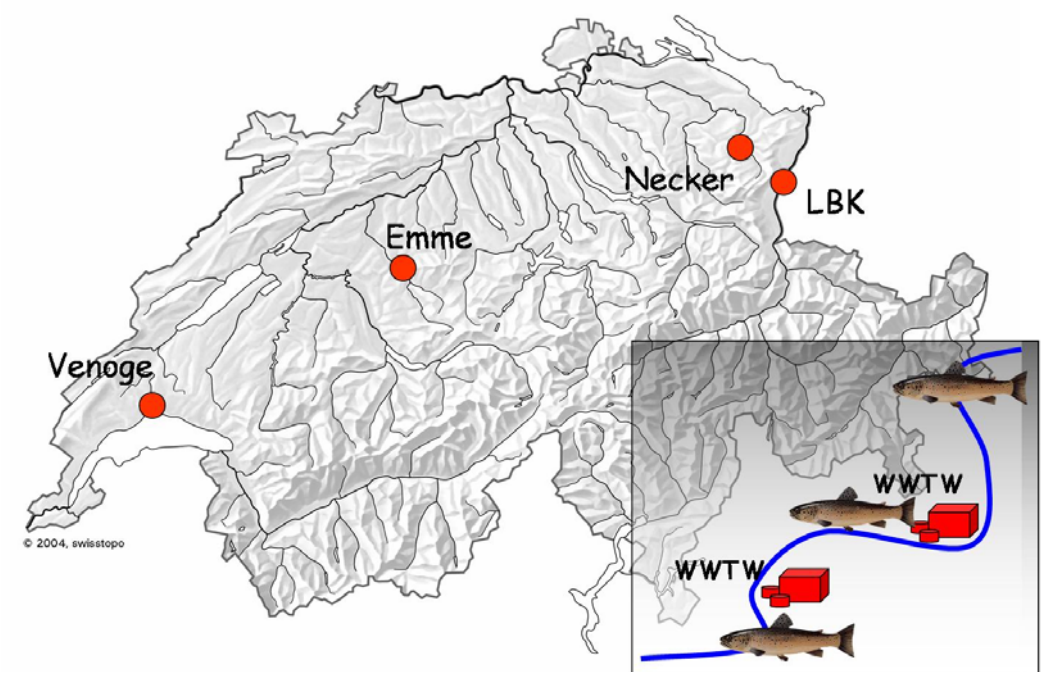


Fig. 2 Map of Switzerland showing the four TESTGEBIETE Venoge, Emme, Necker and LBK (signified by red dot). The rivers were selected on the basis of a previous observed catch decline. Each river was sampled at one upstream site (control site) and two sampling areas downstream from wastewater treatment works (WWTW). The map was generated using the software swisstopo 2004.

Within TESTGEBIETE, different hypotheses suggested to be responsible for the observed catch decline were tested including general fish health (Zimmerli et al., 2007), fish recruitment (Schager et al., 2007); fish disease (Wahli et al., 2007; Zimmerli et al., 2007), pollution (Götz et al., unpublished data; Suter et al., unpublished data) as well as fish reproduction (the present thesis).

Fish reproduction in teleost fish – a short summary

Fish have always posed a special challenge for the study of reproduction because there are about 25.000 species and they have diverse strategies to produce offspring. In general, the reproduction of teleosts is regulated via the hypothalamus-pituitary-gonad axis and comprised a complex interaction of hormones, receptors and proteins (see Fig. 3).

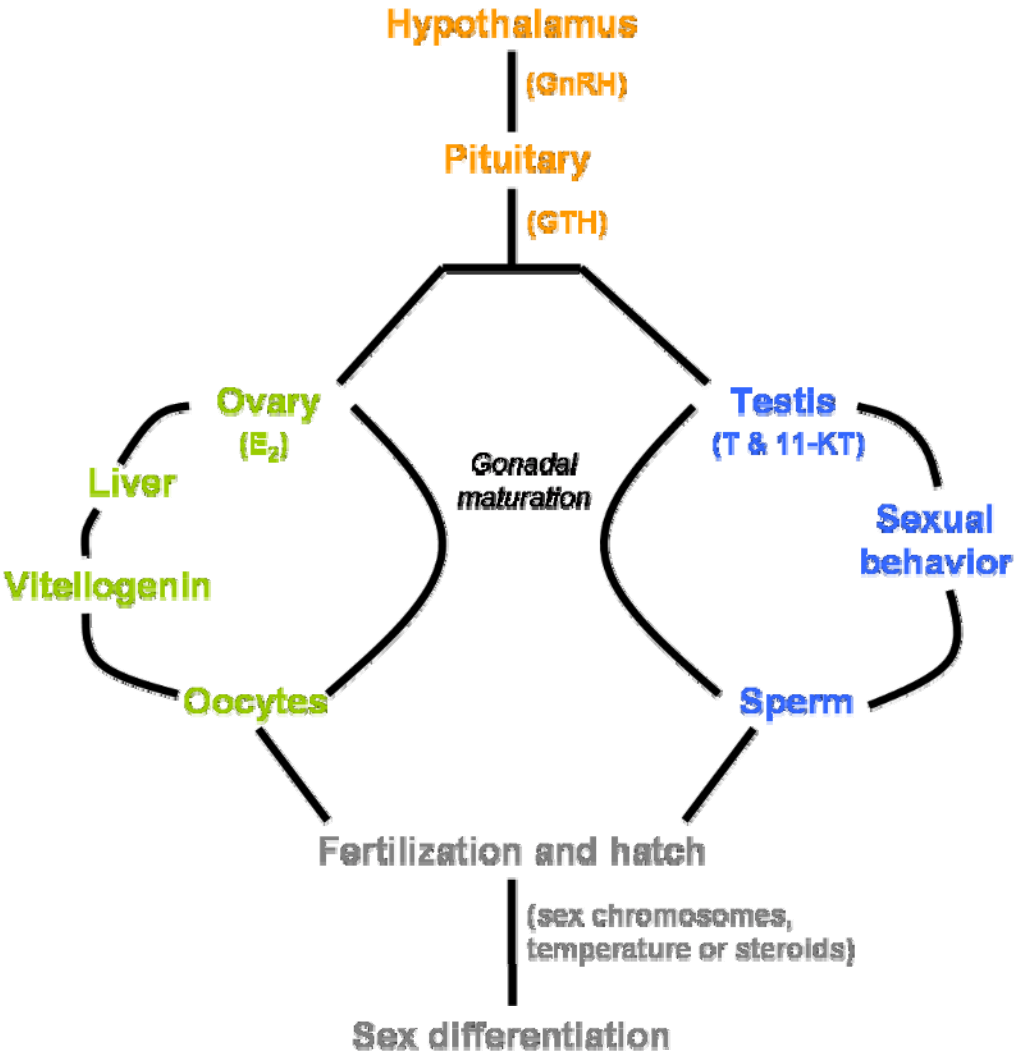


Fig. 3: Schematic illustration of reproductive system of fish; modified after Kime (1998). GnRH = gonadotropin releasing hormone; GTH = gonadotropin; E2 = 17β–estradiol; T = testosterone; 11–KT = 11–ketotestosterone.

In response to an external stimuli (e.g. temperature, photoperiod), the hypothalamus initiates the secretion of GnRH (gonadotropin releasing hormone)

which leads to a release of two different kinds of gonadotropic hormones - namely GTH–I and GTH–II. The GTH-I is homolog to mammalian follicle stimulating hormone (FSH) and stimulates the sexual development and gonadogenesis of both, female ovaries and male testis. In contrast, the GTH–II (homolog to the mammalian luteinizing hormone, LH) is responsible for the final gonad maturation including ovulation and sperm release (Kime, 1998).

One additional crucial function of gonadotropins is the stimulation of steroidogenesis of 17β-estradiol, 11-ketotestosterone and testosterone in ovarian and testicular tissue. The three steroid hormones are present in both sexes - though, the plasma concentrations of these hormones vary considerable between males and females. In males, 11-ketotestosterone and testosterone are involved in sexual maturation, development of secondary sex characteristics and reproductive behavior (Kime, 1998). The female sex steroid 17β-estradiol promotes particularly ovarian development and maturation as well as the hepatic vitellogenin (Vtg) production (Kime, 1998; Tyler and Sumpter, 1998). In particular ovarian development is strongly linked to the synthesis of Vtg and vitellogenesis contributes to the strong increase of ovarian weight during ovarian recrudescence (Tyler and Sumpter, 1996). Vitellogenin is normally synthesized in the female liver, excreted to the blood stream and transported to its target organ – the ovaries. After receptor-mediated endocytosis, Vtg is incorporated into the developing oocytes, converted into yolk and serves as nutrition source for the embryo (Tyler and Sumpter, 1996). In male fish, Vtg concentrations are usually in the lower ng/mL range (ca. 10 – 100 ng/mL) and therefore below the levels reported for females (1 – 100000 µg/mL; Wheeler et al., 2005).

In mammals, sex is genetically determined by XX and XY chromosomes. In fish, sex determination is less clear. Teleosts are evolutionarily highly labile in respect to sex determination and sex can be determined by sex chromosomes, temperature, social interactions or exogenous steroid hormones (Strüssmann and Nakamura, 2002; Godwin et al., 2003; Munday et al., 2006). Today, several gene families – known to be involved in sex determination of higher vertebrates – are shown to be functional in fish as well, which suggests conservative pathways (Devlin and Nagahama, 2002), Though, so far in only 10% of the examined fish species sex chromosomes were found and the sex determining factors are often located on

autosomes (Devlin and Nagahama, 2002). Once the particular developmental profile in gonochoristic species is determined, the sex is stable throughout the subsequent sexual development (Strüssmann and Nakamura, 2002; Devlin and Nagahama, 2002). Gonochoristic fish species (e.g. brown trout) are characterized by morphological differences between sexes and developed distinct testicular or ovarian germ cells. In contrast, hermaphroditic fish species (e.g. sea bream) are able to alter the way of sexual differentiation in order to maximize the reproductive fitness. Two different types of hermaphroditisms in fish are described, sequential (i.e. sex is changed after a certain time) and synchronous hermaphroditisms (testicular and ovarian germ cells in one gonad at the same time). The latter condition is usually called intersex and has been also documented in numerous gonochoristic species - although a reproductive function is still unknown (Mousa and Mousa, 1999; Gercken and Sordyl, 2002; Bernet et al., 2004). Furthermore, the degree of such intersex as well as the number of intersexed fish in a given gonochoristic fish population, however, is normally very low.

Endocrine disruption

Over recent decades, increasing evidence exist that certain compounds entering the environment interfere with the endocrine system of wildlife animals and consequently impair important reproductive and developmental processes (Oetken et al., 2004; Edwards et al., 2006; Jenssen, 2006). A wide range of marine mollusc species (e.g. dog whelk *Nucella lapillus*) inhabiting harbors and areas with extensive shipping traffic, are suffering from imposex. Imposex is a condition in which male sex organs in females are superimposed after exposure to tributyltin, the major component of antifouling products (Gibbs and Bryan, 1986). Alligators living in areas contaminated with pesticides have high incidences of altered sexual differentiations of male reproductive tract and showed feminized steroid concentration profiles (Guillette et al., 1995). Colonial fish-eating birds nesting in the Great Lakes basin exhibited severe reproductive impairments, which were characterized by high embryonic and chick mortality, oedema, growth retardation as well as morphological malformations (Gilbertson et al., 1991). Male fish inhabiting areas downstream from waste water treatment work (WWTW) effluents showed elevated plasma concentrations of vitellogenin (Vtg) comparable to levels found in females (Purdom et al., 1994; Jobling et al., 1995). In

addition, numerous reproductive disorders in humans linked to endocrine disruptors have been reported - including reduced semen quality, cryptorchidism, testicular cancer and others (reviewed in Edwards et al., 2006). Therefore, well-founded apprehensions are present that endocrine disruptors seriously affect reproductive health and fitness of humans as well.

Since Purdom and colleagues (1994) established a correlation between elevated plasma Vtg concentrations in male fish and hormonal active substances in wastewater effluents, the scientific attention has focused on estrogenic compounds on fish. Subsequent studies, particularly performed in North America and Europe, revealed additional reproductive disruptions in feral fish populations. Alongside with induction of Vtg in males, altered steroid concentrations, high incidences of oocyte atresia, retardation of gonadal development, decreasing fertility and frequent intersex were reported (Jobling and Tyler, 2003). Intersex is characterized by simultaneous occurrence of male and female germ cells in one gonad. As mentioned before, some species exhibit intersex naturally to a certain, although variable degree (Sumpter and Johnson, 2005). In the United Kingdom, for instance, intersex exist in up to 4% of roach (*Rutilus rutilus*) caught at uncontaminated control sites, whereas 100% of male roach caught downstream of WWTWs showed intersex conditions (Jobling et al., 1998). Similar observations were in addition made in flounder (Lye et al., 1997), gudgeon (van Aerle et al., 2001) or common carp (Sole et al., 2003b). High incidences of intersex conditions were mostly found in geographical vicinity to WWTW effluents (Jobling et al., 1998; van Aerle et al., 2001). It is therefore most likely that intersex is caused by hormonal active chemicals discharged via WWTW effluents in the environment (Jobling and Tyler, 2003; Sumpter, 2005). Indeed, numerous laboratory studies confirmed the close relation between intersex and estrogenic compounds (Gimeno et al., 1996; Gray and Metcalfe, 1997). Nevertheless, there are no field surveys in which a specific intersex condition in wild fish has been causally linked with exposure to a specific compound (Jobling and Tyler, 2003). Alongside with Vtg induction and intersex in male fish from areas near discharging WWTWs, female fish showed an increase number of atretic oocytes (Jobling et al., 2002). Atresia is a degenerative process, characterized by vitelline envelope breakdown, nucleolus disintegration and increased follicular cells (Blazer, 2002). Although it is a common physiological

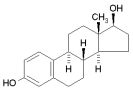
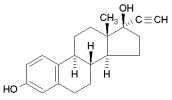
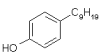
event in ovarian development, increased incidence of atresia has been associated to water pollution and other biotic stressors (Janz et al., 1997; van den Belt, 2002; Blazer, 2002).

The ecological implications of intersex on population level still remain unclear (Arcand-Hoy and Benson, 1998). Jobling et al., (2002) reported impaired sperm motility in intersexed roach compared with normal male fish that resulted in lower number of fertilized eggs and offspring. In severely feminized fish such reduction was as much as 50%, and sperm quality (in terms of fertilization success) was reduced by 75%. The results suggest that endocrine-disrupting chemicals discharged into the aquatic environment may have an effect on the long term stability of fish populations (Sumpter, 2005).

Vitellogenin as biomarker of estrogenic exposure

In males, the gene expression of Vtg is on the basis of low endogenous estrogen concentrations inactivated and therefore baseline plasma concentrations are in the lower ng/mL range (Wheeler et al., 2005). Because male fish contain the genetic disposition to synthesize Vtg, exposure to environmental estrogens strongly increases hepatic Vtg production. Therefore, Vtg has been repeatedly used as an indicator of estrogens exposure in field and laboratory studies and is accepted as a reliable and sensitive biomarker (Sumpter and Jobling, 1995; Denslow et al., 1999). To date, a great number of chemicals are identified to induce Vtg production in fish (see Table 2) and enter the aquatic system via WWTW effluents.

Table 2: Collection of estrogenic chemicals with the potential to cause hepatic vitellogenin production in male fish. Each compound has been previously detected in Swiss surface waters (e.g. Aerni et al.. 2004).

Chemical compound	Chemical structure	Fish species	LOEC [ng/L]	Reference
17β-estradiol		trout	20	van den Belt et al. (2003)
		medaka	36	Imai et al. (2005)
		zebra fish	21.4	Rose et al. (2002)
17α-ethinylestradiol		minnow	1	Pawlowski et al. (2004)
		minnow	0.8	Liao et al. (2006)
		zebra fish	1.67	Fenske et al. (2001)
4-Nonylphenol		trout	20.3×10^3	Jobling et al. (1996)
		minnow	10×10^3	Zha et al. (2007)

LOEC = lowest observed effect concentration

With respect to the situation in the environment, natural and synthetic estrogens (estradiol, estrone and ethinylestradiol) are the major agents of Vtg induction in feral fish (Desbrow et al., 1998; Routledge et al., 1998). Though, it is important to note that wastewater effluents do not contain single estrogenic substances. Rather, WWTWs discharge a complex mixture of estrogens in the environment – resulting in additive, antagonistic or synergistic effects on Vtg synthesis (e.g. Thorpe et al., 2001; Thorpe et al., 2003). Despite the clear association between environmental estrogens and vitellogenic response in male fish, it is still difficult to link Vtg induction with effects on higher level of biological organization (e.g. population). Jobling et al. (2002) found a positive correlation between the proportion of ovarian tissue in the gonads of male intersex fish and their plasma vitellogenin concentration, whereas other studies failed to detect such a relationship, albeit some found considerable Vtg induction (Faller et al., 2002; Kleinkauf et al., 2004; Bjerregaard et al., 2006). By virtue of these inconsistent results, the measurement of Vtg, but also other, such as intersex conditions, should be considered as an indicator of estrogenic exposure in aquatic systems, but not as an indicator of population related effects in fish.

Climate change and global warming

In recent decades, it became more and more evident that man-made climate change is influencing air temperature, rainfall and frequency of storms (IPCC, 1995). Although we are at an early stage in the projected trends of global warming, ecological responses to recent climate change are already visible (Walther et al., 2002). For instance, phenological activities in spring started earlier since the 1960s, including earlier breeding of birds, earlier arrival of migrant birds as well as earlier spawning of amphibians (Menzel et al., 2006). Additionally, global warming has produced shifts in distributions and abundance of species and has implicated in species extinction (Thomas et al., 2004). Based on computer simulations, Europe will experience an increase of mean annual air temperatures of approximately 2 to 4°C due to continuous inputs of greenhouse gases like methane or carbon dioxide (IPCC, 1995). Such increases in air temperature result in elevated surface and groundwater temperatures and thus have profound effects on aquatic ecosystems (Rahel et al., 1996). In Switzerland as well, scientists already reported rising water temperature in numerous rivers and streams (Hari et

al., 2006). That is, the mean annual temperature of the rivers increased about 1.5°C during the last 20 years (Hari et al., 2006). Because upstream migration of fish is most often hampered by artificial barriers (e.g. dams), the reported river warming implies a considerable upstream shift of river zonations to higher altitudes and hence reduces the habitat that is suitable for brown trout in Switzerland (Hari et al., 2006). Additionally, water temperature shows a distinct diurnal cycle particularly in lower river stretches, with differences between day and night of up to 8°C (Körner et al., 2007; see chapter 3 for details). Suchlike changes in diurnal temperature regimes, however, are not linked to global warming, rather is the result from other anthropogenic activities like water diversion, river regulations or the removal of riparian buffer zones (Meier et al., 2003). Particularly with regard to the anthropogenic removal of ecological important buffer zones, the unscreened solar radiation heats up the river water and cause high temperature peaks during summer day. Additionally, elevated temperatures promote susceptibility of fish to various parasites. A recent Swiss field survey documented that in 56% of investigated river sampling sites salmonids are suffering from PKD (proliferative kidney disease). The disease is caused by the myxozoa *Tetracapsuloides bryosalmonae* and clinical disease of PKD is found during summer, when water temperature is higher than 15°C for more than 15°C (Wahli et al., 2007). Based on the conclusions made by Hari et al. (2006) and Wahli et al. (2007), river warming has been considered as very likely single parameter for the decline of brown trout abundance in some areas of Switzerland (Burkhardt-Holm and Scheurer, 2007),

Thesis objectives and key questions

The major objective of the present thesis was to evaluate selected parameters of endocrine status in field studies and laboratory experiments in brown trout under the influence of stressor combinations. In particular, we focused on interactions between estrogenic substances and different temperatures on selected endocrine parameters of reproductive, stress and mineral physiology.

Field Approach (Chapters 2 – 4)

Key question: Do brown trout in Switzerland show indications of estrogenic disruption in areas influenced by wastewater effluents?

In a first step, the study aimed to characterize the current situation in Swiss midland rivers that received input from WWTW. On the basis of previous surveys in Switzerland, the chemical burden of Swiss rivers and streams with environmental estrogens is well documented (Aerni et al., 2004; Rutishauser et al., 2004, Vermeirssen et al., 2006). In addition, some studies reported elevated plasma Vtg concentrations in fish caught downstream from WWTWs as well as sporadic appearance of intersex in certain fish species (Wahli et al., 1998; Faller et al., 2003; Bernet et al., 2004). Hence, potential effects on reproductive health of brown trout after exposure to estrogens may contribute to the declining fish catches in Switzerland. To link declined brown trout catches to impaired reproduction we investigated Vtg and gonadal histology in feral trout from four Swiss rivers with a well documented catch decline (chapter 2 and 3). By means of passive monitoring, each river was sampled for feral brown trout at three representative sites - one head water site (control) and two sampling sites downstream of WWTWs (FISCHNETZ - TESTGEBIETE; Fig. 2). In a second step, the study tested the practicability of mini caging devices as a promising alternative to passive monitoring in environmental risk assessment (Chapter 4). Both, passive and active monitoring approaches have several drawbacks and advantages (Burki et al., 2006). The most important benefit of active monitoring consists in the fact that the life history of fish is known and exposure is restricted to a specific area. In contrast, feral fish may have experienced migration and the previous exposure scenario is dissimilar from the sampling site. The new mini cages suit the variable hydrological conditions in low mountain range rivers in Switzerland. In combination with active

grab samples and passive sampling using polar organic chemical integrative samplers (POCIS), the field survey presented in chapter 4 tied the estrogenic burden of river water with internal estrogen exposure and Vtg concentrations in caged fish.

Laboratory approach (Chapters 5 – 6)

Within the laboratory approach, we addressed two different question (*Key question I* and *II*). In both studies, we focused not only on constant temperature conditions. Rather, we additionally applied daily temperature cycles that were recently measured during our field survey in the frame of the FISCHNETZ (TESTGEBIETE, see chapter 3). The experimental set up for these chapters are illustrated in Fig. 4.

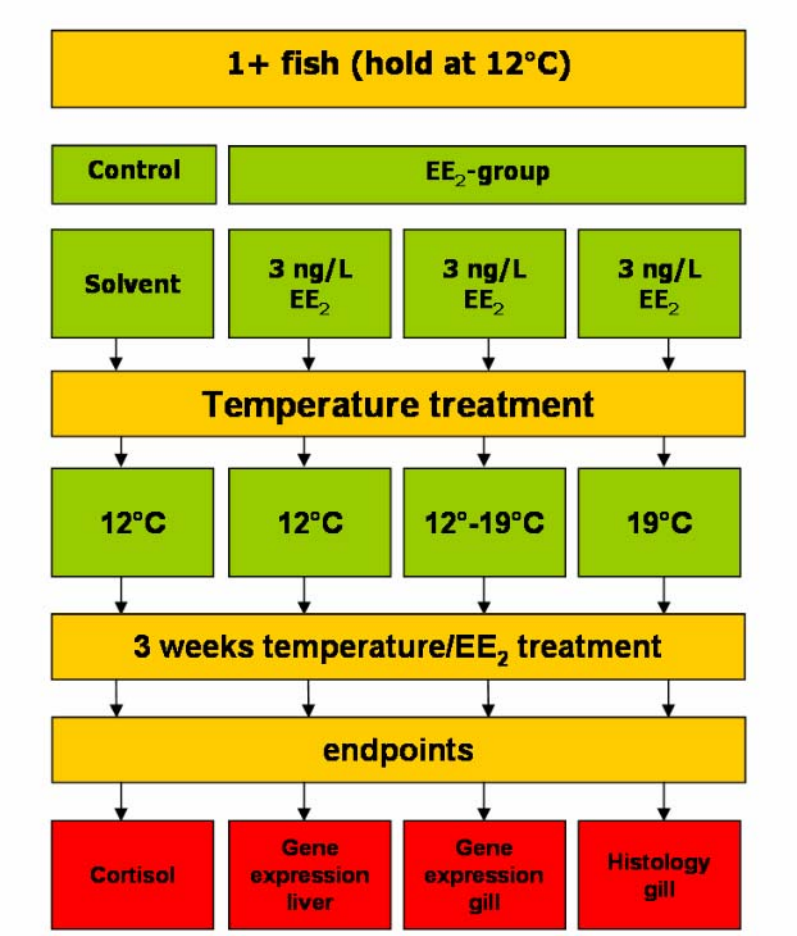


Fig. 4: Schematic illustration of the experimental design of the experiments described in chapter 5 and 6. (EE_2 = 17α-ethinylestradiol)

Key question I: Does temperature alterations influence environmental risk assessment using Vtg as biomarker of exposure? (Chapter 5)

On the basis of data obtained from field work (see chapter 3), the question came up, whether increasing river temperature affect the expression of Vtg - the major biomarker of estrogen exposure in numerous monitoring studies. Long-term exposures of fish to various stressors (e.g. increased temperatures) result in a steady suppression of the reproductive system, including the reduction of Vtg concentrations (Wendelaar Bonga, 1997; Mommsen et al., 1999). On the other hand, *in vitro* studies evidenced stimulating effects of temperature on hepatic Vtg production (Pawlowski et al., 2000; Kim and Takemura, 2003).

Irrespective of the mode of effects, such putative temperature effects could significantly influence environmental risk assessment studies – an issue already discussed in some surveys (Sole et al., 2003a; Snyder et al., 2004). For example, the lack and/or reduced degree of Vtg induction in exposed males could be wrongly interpreted as "not exposed" and therefore would lead to an underestimation of the actual exposure (for details refer Fig. 5). Potential alterations of Vtg expression were discussed in relation to variations in bile cortisol concentrations and hepatic steroid receptors expression (estrogen receptor alpha, estrogen receptor beta-1 and glucocorticoid receptor). Great effort was made to precisely simulate the temperature conditions in the field – including daily temperature cycles.

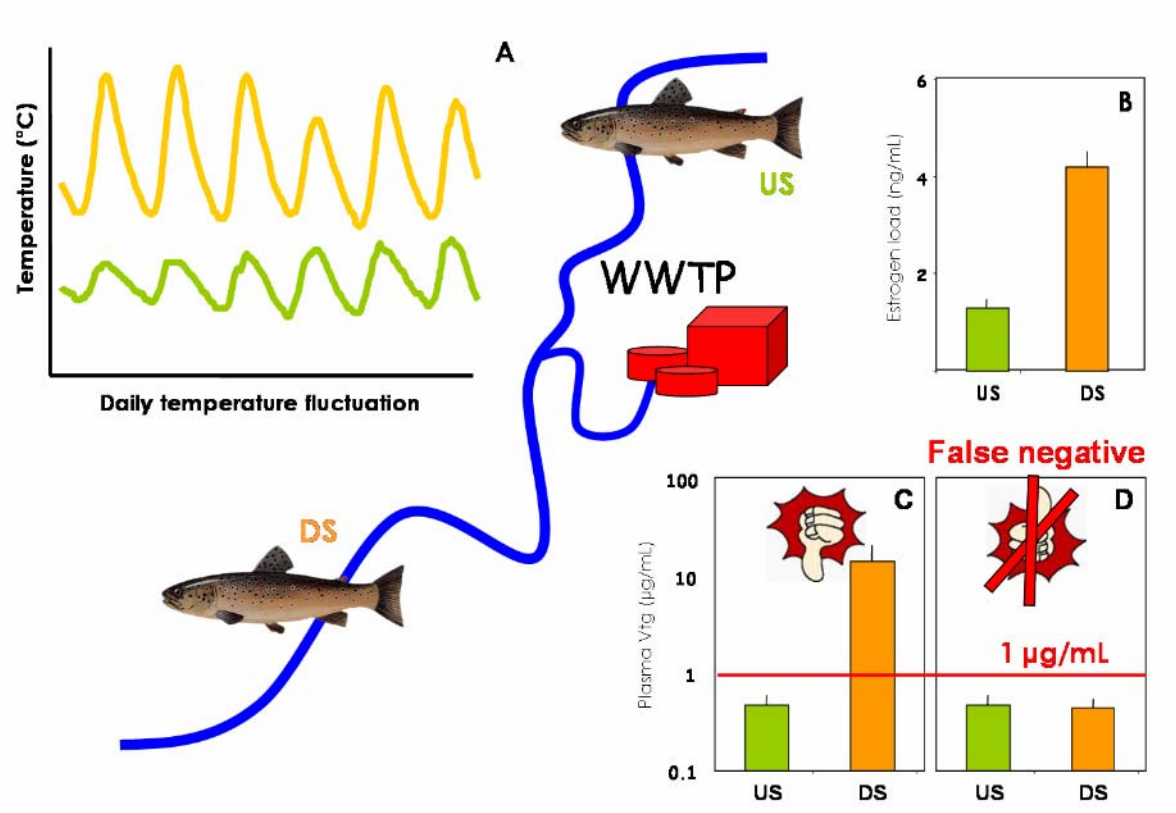


Fig. 5: One hypothetical consequence of temperature effects on the expression of male vitellogenin (Vtg) concentrations after exposure to estrogenic compounds. Male fish living upstream (US) from wastewater treatment works (WWTW) are not exposed to estrogenic compounds (Fig. 3A and Fig. 3B). Consequently the plasma Vtg concentrations are below the threshold concentrations of 1µg/mL. In contrast, fish inhabiting the downstream (DS) area experience a considerable estrogenic exposure and one may suggest that male fish will show a vitellogenic response (i.e. Vtg concentrations ≥ 1µg/mL; Fig. 3C). In the case that increased temperatures at the downstream area (Fig. 3A) negatively affects the expression of Vtg in estrogen exposed fish; such clear vitellogenic response will not appear (Fig. 3D). The conclusion made from this observation would be: fish living in the DS area are not exposed to estrogenic compounds – which is a false negative conclusion.

Key question II: Do environmental estrogens impair the endocrine regulation of mineral balance? (Chapter 6)

Environmental estrogens are known to interfere with various aspects of fish reproduction and may result in lower reproductive fitness of feral fish populations. Along with well described effects on reproduction, less information is available regarding potential interactions between environmental pollutants and additional endocrine processes (Sumpter, 2005). Water temperature alterations influence the mineral balance of fish by changing the influx of water and elevated efflux of important ions (e.g. Na^+; Cl^- or Ca^{2+}) across the body surface. In response to temperature induced hydromineral disturbances, freshwater fish activates specific enzyme-driven ion transport mechanism in the gills to regain the physiological homeostasis. The best characterized enzyme is the sodium/potassium-activated adenosine triphosphatase (Na^+/K^+–ATPase), which is located in chloride cells of gills. It facilitates the active transport of Na^+ and Cl^- via the gill epithelium and matches the passive loss of ions. There is accumulating evidence that regulation of Na^+/K^+–ATPase is a complex interaction between various steroids, receptors and transcription factors including estrogens, cortisol, estrogen receptors and corticoid receptors (Lou et al., 2005; Singer et al., 2007; Kiilerich et al., 2007). In the light of the recently documented river warming in Switzerland, estrogen related disturbances of osmoregulation via Na^+/K^+–ATPase pathways may have detrimental effects on mineral balance and consequently influence the general fitness of trout. Therefore, chapter 6 aimed to explore the interactions between waterborne estrogens and the endocrine regulation of branchial Na^+/K^+–ATPase in trout hold at temperature conditions that are common in Swiss midland rivers. The study did not only measure gene expression of Na^+/K^+–ATPase, but also determined the temperature-dependent transcription of gill mineralocorticoid receptor and glucocorticoid receptor in the gills. These steroid receptors have been recently suggested to regulate both, the expression and activity of branchial Na^+/K^+–ATPase (Kiilerich et al., 2007).

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Intersex in feral brown trout from Swiss midland rivers (Chapter 2)

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I don't mind that you think slowly, but I do mind that you are publishing faster than you think.
(*Wolfgang Pauli, 1900 – 1958*)

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Intersex in feral brown trout from Swiss midland rivers

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The incidence of intersex in feral populations of brown trout *Salmo trutta* in Switzerland is reported. The affected brown trout showed spermatogenic activity in ovarian tissue.

Key words: brown trout; endocrine disruption; gonad; intersex; ovotestis; Salmonidae.

Several studies over the last 20 years conducted particularly in North America and Europe have documented a widespread reproductive impairment of feral fish populations. Commonly, decreased steroid levels, increased oocyte atresia, induction of vitellogenin (VTG) in males and juveniles, retardation of gonad development as well as reduced egg fertility were reported (Jobling & Tyler, 2003). Additionally, incidences of intersex have been reported, mainly in cyprinids (Jobling *et al.*, 1998; van Aerle *et al.*, 2001; Faller *et al.*, 2003). It appears that some species exhibit intersex naturally to a certain, although variable degree (Jobling *et al.*, 1998). Most of the fish species investigated so far, however, are naturally gonochoristic, *i.e.* existing as either male or female. Therefore, intersex in male gonochoristic fishes is ascribed to oestrogenic compounds (*e.g.* 17β-oestradiol or 17α-ethinyloestradiol), which enter the aquatic ecosystem *via* sewage treatment works (STW) effluents (Jobling & Tyler, 2003). Indeed, several studies revealed a close connection between exposure to oestrogenic compounds and intersex under laboratory conditions (Gimeno *et al.*, 1996; Gray & Metcalfe, 1997). In addition, Jobling *et al.* (1998) showed that a strong correlation exists between the proportion of intersex in roach *Rutilus rutilus* (L.) and the concentration of STW effluent in river water.

The resident brown trout *Salmo trutta fario* L. is the dominant freshwater fish in European low mountain range rivers and has a high commercial value. Over

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the last 20 years, however, its catch has declined in Switzerland (Burkhardt-Holm *et al*., 2002). As part of an interdisciplinary project, called FISHNET, an extensive field monitoring at four rivers [Emme (47°04′ N; 7°38′ E), Liechtensteiner Binnenkanal (47°10′ N; 9°30′ E), Necker (47°22′ N; 9°08′ E) and Venoge (46°34′ N; 6°32′ E)] was carried out to assess the significance of different stressors (*e.g*. environmental contamination) on brown trout (Fischnetz, 2004). Each river was sampled at three sites, one near the source of the river (head water) and two further downstream; each site showing differential exposure to the STW effluent (Table I). A total of 425 brown trout were caught in summer (2002 and 2003) by means of electrofishing. After anaesthesia with MS-222 the fish were sacrificed; the gonads were removed and then preserved in 10% neutral buffered formaldehyde. The gonads were embedded in paraffin wax and longitudinally sectioned (3–5 μm) for histological analysis. Two longitudinal sections of each gonad were stained with haematoxylin and eosin (H&E) as well as Putt's carbol fuchsin according to Berg (1953) (a selective marker for spermatozoa) and examined by light microscope.

Histological examination of the gonads revealed spermatogenic activity in ovarian tissue of 13 out of 64 and 14 out of 57 macroscopically classified females caught at Liechtensteiner Binnenkanal and Venoge, respectively. Multiple spermatogenic nests (occupying <1% of gonadal surface area) were incorporated between the oocytes and located nearby the ovarian lamellae. The nests were dominantly filled with spermatocytes and spermatids, but also contained some spermatozoa [Fig. 1(a)], as confirmed by the selective staining (Putt's carbol fuchsin). In control male testis [Fig. 1(b)] and in the affected ovaries [Fig. 1(c)] spermatozoa were conspicuously stained red, in contrast to the remaining blue, counterstained tissue. The proportion of intersex fish at Liechtensteiner Binnenkanal and at the Venoge ranged from 4·5 to 26·7% (Fig. 2), whereas no intersex was observed at Emme and Necker. Furthermore, occurrence of intersex was not restricted to the downstream sites, but also appeared at one site with no effluent load.

Salmonids, like the brown trout are, in contrast to some cyprinids, believed to be strictly gonochoristic (Woram *et al*., 2003) and therefore intersex is an exceptional occurrence in this species. Only two records of intersex in wild brown trout were found. O'Ferrall & Peirce (1989) observed one gonad (macroscopically), which was part male and part female. L.B. Christiansen & T. Plesner (unpubl. data) describe the presence of spermatogenic cell nests in 'ovaries' (similar to the present observations) but they did not use a specific staining for spermatozoa. In a recent study L.B. Christiansen & C. Lindholst (unpubl. data) report the occurrence of spermatogenic cell nests in 'ovaries' of hatchery reared brown trout, similar to observations made by Ashby (1965). Further histological field studies reporting intersex in salmonids are rare and restricted to the genus Coregonus (Mikaelian *et al*., 2002; Bernet *et al*., 2004). In both studies, some fishes contained either randomly scattered oocytes throughout the testis, or intermingled ovarian and testicular tissue in the same fish, similar to the intersex described for other fish species (Jobling *et al*., 1998; van Aerle *et al*., 2001). The study from Mikaelian *et al*. (2002), however, revealed additional nests filled with spermatogonia and spermatids in 11·7% of the ovaries of lake whitefish *Coregonus clupeaformis* (Mitchell). This finding is

TABLE I. Characterization of the headwater (HW) and downstream sites (D1 and D2) of the four investigated rivers (Waadt, Bern, St Gallen cantons and the principality of Liechtenstein, unpubl. data)

	Venoge			Emme			Necker			LBK		
Sampling site	HW	D1	D2	HW	D1	D2	HW	D1	D2	HW	D1	D2
STW	Ballens	Penthaz	Bussigny	No STW	Ruegsau	Aeflingen	No STW	Mogelsberg	Necker	No STW	Balzers	No STW
Population equivalents[a]	1750	10 200	21 250	–	26 000	40 000	–	1500	4500	–	4500	–
Distance from STW (km)	6	1	2	–	4	3	–	1	3	–	1	9
Q_{347} ($m^3 s^{-1}$)[b]	0	0·5	0·6	0·2	4·1	5·3	0·1	0·5	0·6	0·2	0·4	1·0
Q_{median} ($m^3 s^{-1}$)[b]	0·2	2·3	2·5	1·1	11·9	14·1	0·3	1·9	2·7	0·2	0·8	1·7
Q_T ($L s^{-1}$)[c]	4	25	34	–	104	176	–	5	10	–	37	–
Per cent effluent at sampling site[d]	2[e]	5	6	–	2	3	–	1	2	–	10	4

STW, sewage treatment works.

[a]Population equivalents (PE) were calculated on basis of biochemical oxygen demand after 5 days (1 PE = 60 g O_2 day^{-1}).

[b]Q_{347} signifies the flow of the river at times of drought; Q_{median} is the median flow rate of the river.

[c]Q_T signifies the average dry weather effluent discharge from the STW.

[d]Per cent effluent = $Q_T (Q_T + Q_{347})^{-1}$.

[e]As a result of karst formation in the upper region of the River Venoge, the Q_{347} there is 0 $m^3 s^{-1}$. Therefore, the per cent effluent was calculated using Q_{median}: per cent effluent = $Q_T (Q_T + Q_{median})^{-1}$.

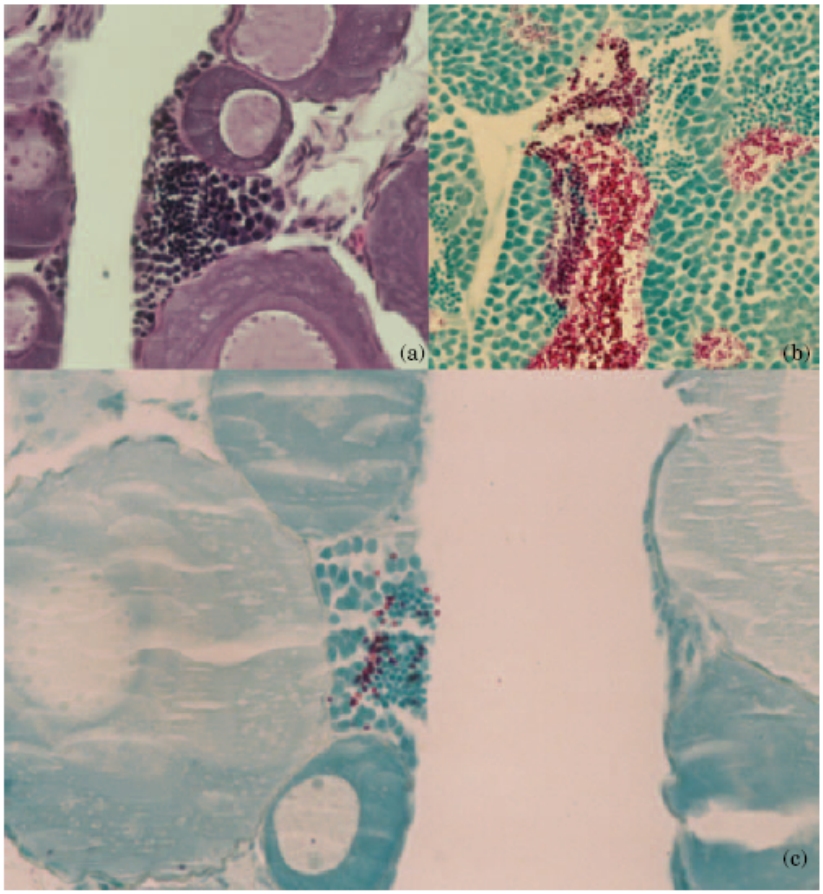

FIG. 1. Gonadal histology of spermatogenic activity in feral brown trout. (a) Nests filled with male germ cells in a gonad of an intersex fish. H&E stain (×200). (b) Mid-spermatogenic testis (control) comprising spermatocytes, spermatids and red stained spermatozoa. Putt's carbol fuchsin stain (×200). (c) Intersex gonad containing nests filled with spermatocytes, spermatids and red stained spermatozoa. Putt's carbol fuchsin stain (×400).

similar to the present observations, leading to an assumption that the described structure is a characteristic form of intersex in salmonids. That hypothesis is supported by a transgenerational study in rainbow trout *Oncorhynchus mykiss* (Walbaum) conducted by Schwaiger *et al.* (2002).

In most fish species sex cannot be easily determined due to the lack of sex chromosomes. As a genetic sex marker for brown trout is not yet available it could not be determined whether the affected fish in the present study were genotypic male or female. Furthermore, the reason for the observed intersex remains to be clarified. Some authors detected spermatogenic nests in control fish, indicating a natural phenomenon (Ashby, 1965; Schwaiger *et al.*, 2002; L.B. Christiansen & C. Lindholst, unpubl. data). Intersex, however, was clearly restricted to the Venoge and Liechtensteiner Rivers and therefore river specific reasons are conceivable. It is well known that exposure of salmonids to exogenous androgens and oestrogens during sensitive developmental periods (in

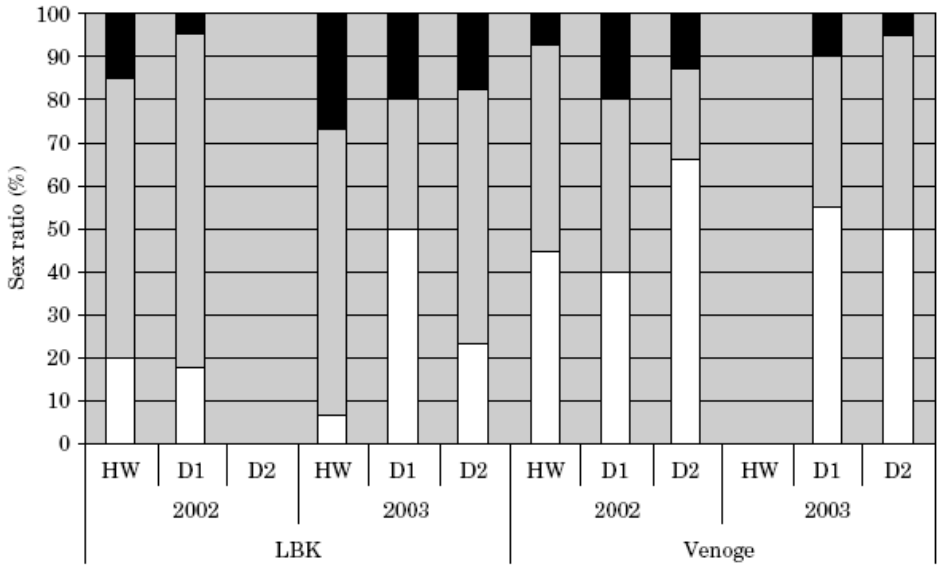

FIG. 2. Proportions of males (□), females (▒) and intersex (■) feral brown trout at three sites [headwater (HW) and two downstream sites (DI and D2)] along the Liechtensteiner Binnenkanal (LBK) and Venoge in the years 2002 and 2003 ($n = 10$–27). Gonads were classified histologically as male, female or intersex (phenotypically female with sperm nests).

particular between hatching and first feeding) results in varying degrees of sex reversal or intersex (Piferrer, 2001; Devlin & Nagahama, 2002). Therefore, exposure of brown trout alevins to endocrine disruptors during sensitive periods could be responsible for the observed spermatogenic activity. Previous studies provide evidence of environmental oestrogens in Swiss rivers and STW effluents (Ahel *et al.*, 2000; Aerni *et al.*, 2004; Fischnetz, 2004), though no data are available with respect to emissions of androgenic or anti-oestrogenic substances. The involvement of STW effluents, however, is less likely because of three specific reasons. First, concentrations of environmental oestrogens in Swiss rivers are lower compared to other countries (Ternes *et al.*, 1999). Second, the analysis of plasma from male brown trout caught at the four rivers revealed no significant VTG induction (O. Körner, unpubl. data). Third, although intersex was found at five sites influenced by relatively high effluent load, intersex also appeared at one site without any effluent input.

Besides a potential involvement of natural and synthetic steroids, another explanation could be polybrominated diphenyl ethers (PBDE). These flame retardants appeared to be elevated in liver and bile of brown trout caught at the Liechtensteiner Binnenkanal and Venoge Rivers (Fischnetz, 2004). The effect of PBDE on sex differentiation and development in brown trout, however, has not been studied in detail. Therefore further studies are needed to clarify whether the intersex observed is a natural phenomenon as shown by Jobling *et al.* (1998) or that environmental factors (*e.g.* chemicals) are involved.

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Reproductive health of brown trout inhabiting Swiss rivers with declining fish catch (Chapter 3)

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"Like all Holmes' reasoning," Dr. Watson says, "the thing seemed simplicity itself when it was once explained."
(*Sir Arthur Conan Doyle, 1859 – 1930*)

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Aquatic Sciences

Research Article

Reproductive health of brown trout inhabiting Swiss rivers with declining fish catch

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Abstract. In recent years, brown trout catches have been declining in many Swiss rivers. One hypothesis is that this declining catch is linked to environmental estrogens, known to have the potential to adversely affect fish reproduction. In order to assess if the reproductive health of brown trout is impaired, we sampled fish at three sites along four rivers with a well documented catch decline. These rivers are affected by inputs of treated sewage effluent. The sampling was conducted during two years; we measured plasma vitellogenin (Vtg) concentrations and surveyed gonadal histology. Analysis of plasma Vtg revealed elevated concentrations (up to 8 µg/mL) in only 10 out of 197 males. Furthermore, there were no site specific patterns to this induction. These results indicate that the exposure to estrogenic compounds is low. Also the incidence of ovarian atresia was low and we found no male intersex fish. In contrast to males, females caught along two rivers had spermatogenic activity in ovarian tissue. However, this intersex condition does not appear to be connected to exposure to environmental estrogens. At one of 12 sites there was a high incidence of gonadal parasites in ovarian tissue, which may affect reproductive output but was not a general problem across sites. In conclusion, the exposure to estrogenic compounds does not appear to significantly affect the reproductive parameters we investigated in Swiss brown trout.

Key words. Trout; endocrine disruptors; temperature stress; vitellogenin; intersex; atresia.

Introduction

Since the 1980ies of the past century several indications caused concern about declining fish populations in Switzerland. Yearly records of anglers indicated an up to 50 % reduced fish catch of primarily brown trout (*Salmo trutta fario*), but also grayling (*Thymallus thymallus*) and the nase (*Chondrostoma nasus*), between 1980 and 1997 (Friedl, 1999). These observations prompted a more detailed study, which revealed a reduced catch in 20 of 26 cantons (Frick et al., 2002). Some of the affected rivers and streams were located in the midlands and the northern part of the country, where several anthropogenic impacts (e.g. degradation of habitat structure or impaired water quality) affect the fish habitat. In order to establish the causes of the catch decline, an interdisciplinary project FISCHNETZ (engl. FISHNET – Project on declining fish catch in Switzerland) was initiated in 1998 (Burkhardt-Holm et al., 2002). By evaluating the catch, fish abundance as well as diverse biotic and abiotic parameters, FISCHNETZ aimed to reveal the causes of the catch decline and to propose measures to ensure the future viability of this ecological and economical important resource.

Within FISCHNETZ various hypotheses were put forward to explain the reduced catch (Burkhardt-Holm et al.,

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2002). Among others, a disturbed reproductive health as a consequence of endocrine disruption has been considered. This hypothesis mostly relies on several reports, particularly from Europe as well as North America, indicating widespread reproductive impairments in feral fish. Effects on reproduction range from an induction of vitellogenin (Vtg, a precursor of yolk protein) in male fish, intersex in gonochoristic fish species, retardation of gonad development or high incidences of oocyte atresia (Jobling and Tyler, 2003a). Although the ecological implications of these effects on fish populations still remain unclear (Arcand-Hoy and Benson, 1998), exposure to estrogenic compounds may affect the long term stability of fish populations (Rolland, 2000). The catch decline observed in Switzerland could be a manifestation of such effects.

Vitellogenin is the major precursor of yolk protein in oviparous vertebrates and has been repeatedly used as an indicator of exposure of fish to estrogens in field and lab studies (Sumpter and Jobling, 1995; Denslow et al., 1999). Vitellogenin is normally expressed in the female liver under estrogenic control, excreted to the blood stream and transported to its target organ – the ovaries. In male fish, base levels of Vtg are usually in the lower ng/mL range (ca. 10–100 ng/mL) and therefore well below background levels (10–1,000 µg/mL) reported for females (Wheeler et al., 2005). However, evidence of elevated Vtg values in feral male fish downstream from sewage treatment works (STW) has been reported for various fish species (Folmar et al., 1996; Jobling et al., 1998). Subsequent surveys provided evidence that certain natural and synthetic compounds, entering the aquatic ecosystem via STW effluents, provoke a vitellogenic response in males (Routledge et al., 1998).

Besides the induction of Vtg, manifold observations of intersex (testicular and ovarian germ cells in one gonad) in geographical vicinity to STWs have been reported. In the United Kingdom, a study by Jobling et al. (1998) revealed intersex in up to 100 % of male roach (*Rutilus rutilus*) living downstream of STWs, compared to 0–4 % in fish of control sites. Intersex has also been reported in flounder (Lye et al., 1997), tilapia (Mousa and Mousa, 1999), barbel (Vigano et al., 2001) gudgeon (van Aerle et al., 2001; Faller et al., 2003), whitefish (Mikaelian et al., 2002), stickleback and European perch (Gercken and Sordyl, 2002), carp (Solé et al., 2003b); white perch (Kavanagh et al., 2004) and catfish (Barnhoorn, et al. 2004) – indicating a widespread issue in feral fish populations worldwide. Although STW effluents carry a large variety of micropollutants besides environmental estrogens (e.g. androgens and anti-androgens), certain effects such as Vtg induction and intersex can be mimicked by estrogens in lab studies (e.g. Gimeno et al., 1996; Gray et al., 1999).

Alongside with the occurrence of Vtg induction and intersex in male roach captured in the proximity of STW effluent discharges, female fish showed an increase of ovarian atresia (Jobling et al., 2002). Atresia is a degenerative process, characterized by nucleolus disintegration, vitelline envelope breakdown and increased follicular cells (Blazer, 2002). Although it is a common physiological event in ovarian development, increased incidence of atresia, particularly in previtellogenic oocytes, has been mainly associated with environmental endocrine disruptors (Janz et al., 1997; Van den Belt et al., 2002), but also environmental stressors like temperature or nutritional deficits (Blazer, 2002).

The study presented here aimed to clarify, whether feral brown trout are suffering from a disturbed reproductive health as a result of exposure to estrogenic compounds. We discuss effects on reproductive health in relation to the declining fish catches in Switzerland. In order to make this link we investigated vitellogenin and gonadal histology in fish from four Swiss rivers that receive treated sewage effluent and have a well documented catch decline.

Materials and methods

Test areas

Passive biomonitoring on brown trout was carried out in four rivers (Venoge, Emme, Necker and Liechtensteiner Binnenkanal (LBK) – for detailed information please refer to Table 1 as well as Burkhardt-Holm and Scheurer, 2007). Each river was sampled at three representative sites. Two sampling sites downstream of STWs (D1 and D2) as well as one head water site (HW). The HW site on the Venoge is affected by a small STW further upstream (Table 1). Most sites are separated by at least one physical barrier that prevents upstream migration but still allows downstream movements of fish. Where sampling sites are not separated by a migration barrier, we selected sites that are reasonably far apart from each other, making upstream migration of brown trout between these sampling sites unlikely (Borsuk et al., 2006).

Venoge. The Venoge represents a mid-sized river located in the western part of the Swiss midland arising from two springs. Until the river finally enters Lake Geneva, the Venoge is draining an overall area of 231 km^2 on a total length of 70 km. While the downstream sites were located on the Venoge, the head water site was situated on a tributary stream (La Veyron). This upper area is geologically characterized by a distinct karst formation. Due to that, in summer La Veyron is occasionally desiccated (Table 1). According to Borsuk et al. (2006), the downstream area of the Venoge is mainly dominated by farmland (58 %), while the La Veyron represents a less agrarian area (40 %) dominated mostly by forests (56 %). A total of seven STWs with a population equivalent (PE) of 1,750 to 50,000 are discharging into the Venoge.

Table 1. Characterisation of the headwater (HW) and downstream sites (D1 and D2) of Venoge, Emme, Necker and LBK (Lichtensteiner Binnenkanal).

	Venoge			Emme			Necker			LBK		
Sampling site	**HW**	**D1**	**D2**	**HW**	**D1**	**D2**	**HW**	**D1**	**D2**	**HW**	**D1**	**D2**
STW	Ballens	Penthaz	Bussigny	–	Ruegsau	Aeffligen	–	Mogelsberg	Necker	–	Balzers	–
Population equivalents [1]	1'750	10'200	21'250	–	26'000	40'000	–	1'500	4'500	–	4'500	–
Distance from STW (km)	6	1	2	–	4	3	–	1	3	–	1	9
EEQ (ng/L) [2]	n.d.	0.2	0.3	0	0.1	0.2	0	0.1	0.1	n.d.	n.d.	n.d.
Q_{347} (m^3/s) [3]	0	0.5	0.6	0.2	4.1	5.3	0.1	0.5	0.6	0.2	0.4	1.0
Q_{median} (m^3/s) [3]	0.2	2.3	2.5	1.1	11.9	14.1	0.3	1.9	2.7	0.2	0.8	1.7
Q_T (L/s) [3]	4	25	34	–	104	176	–	5	10	–	37	–
STW effluent (%) [4,5]	2[5]	5	6	–	2	3	–	1	2	–	10	4
P_{tot} (mg/L) [6]	0.04	0.08	0.09	0.02	0.05	0.11	0.02	0.06	0.03	0.02	0.03	0.01
NO_3-N (mg/L) [6]	2.39	3.71	3.95	0.40	2.51	3.76	0.52	1.24	1.27	1.00	1.50	0.89
NH_4-N (mg/L) [6]	0.02	0.09	0.16	0.01	0.09	0.31	< 0.01	0.03	0.03	0.08	0.45	0.02
Atrazine (ng/L) [7]	n.d.	n.d.	23–764	n.d.	n.d.	7–728	n.d.	n.d.	BDL-23	n.d.	n.d.	BDL-11
Diazinon (ng/L) [7]	n.d.	n.d.	1–16	n.d.	n.d.	BDL-26	n.d.	n.d.	BDL-5	n.d.	n.d.	BDL-27
T (°C) summer 2002 [8]	10–14	12–19	12–20	11–23	11–18	9–20	9–16	n.d.	n.d.	10–13	10–15	9–15
T (°C) summer 2003 [8]	12–17	16–24	17–24	11–23	13–22	13–25	9–17	10–24	11–26	11–15	11–18	10–16

STW = sewage treatment works; n.d. = not determined; HW = head water; D1 = downstream 1; D2 = downstream 2; BDL = below detection limit

[1] Population equivalents (PE) were calculated on the basis of biochemical oxygen demand after 5 days (1 PE = 60 g oxygen per 5 days).

[2] 17 beta-estradiol equivalent (EEQ) values were calculated on the basis of Q_{median}. Data kindly provided by Suter et al. (Eawag Dübendorf, unpublished data).

[3] Q_{347} signifies the flow of the river at times of drought; Q_{median} is the median flow rate of the river; Q_T signifies the average dry weather effluent discharge from the STW.

[4] Percent effluent = $Q_T/(Q_T+Q_{347})$.

[5] As a result of karst formation in the upper region of the river Venoge the Q_{347} there is 0 m^3/s. Therefore, we calculated the percent effluent using Q_{median}: percent effluent = $Q_T/(Q_T+Q_{median})$.

[6] Phosphorus and nitrogen values are presented as 80th percentile of monthly measured samples in 2002. Data kindly provided by the cantonal authorities of Waadt, Berne, St.Gallen and the Principality of Liechtenstein.

[7] Pesticides are presented as minimum-maximum values (data from Götz et al., 2003).

[8] Temperature variations during the sampling period (July to August). Temperature data kindly provided by Eva Schager (Eawag Kastanienbaum).

Emme. With a total catchment area of 963 km^2, the Emme represents the largest river of the present survey. Over 80 km long, the Emme is first flowing through a sub alpine region and subsequently through the Swiss midland where it joins the Aare River. The Emme River is considerably influenced by seasonal flow fluctuations which led to intense river management activities in the past centuries (Borsuk et al., 2006). The catchment area is mainly characterized by farmland (35 %) as well as forests (40 %), but also has a residential area (6 %) of partly high density. In total, three large STWs (PE ≤ 40,000) as well as some smaller STWs (PE ≥ 30) are discharging into the river.

Necker. The smallest river of the present study, the Necker, originates from a spring in the prealps and flows into the Thur River nearby Lütisburg. The river is 31 km long and its catchment area corresponds to 123 km^2 (Borsuk et al., 2006). About 38 % of the catchment area is covered by forest, whereas 35 % is used as farmland. Residential areas and public infrastructure are covering approximately 4 % of the examined region. The Necker is influenced by effluents from three STWs with a PE of 1,500 to 4,500.

Liechtensteiner Binnenkanal. The Liechtensteiner Binnenkanal (LBK) is a mid-sized canal with a total length of 29 km. It was engineered in the 1930s for flood protection, but also to drain the Rhine valley of Liechtenstein. Today, the LBK drains roughly 138 km^2 (Borsuk et al., 2006). Near 50 % of the area is covered with forest and only 18 % occupies intensive farmland. At least 10 % is covered by buildings, streets and railroads. Furthermore, almost 10 % of the catchment area is fallow land and therefore not in use. One STW (PE = 4,500) discharges into the LBK.

Fish sampling and processing

A total number of 424 brown trout were collected by means of electro fishing in summer 2002 and 2003 (Table 2). Upon collection, the fish were anesthetized with MS222 (Sigma Aldrich, Germany) and blood was sampled via the caudal vein by heparinized syringes (Monovette®, Sarstedt, Nürnbrecht, Germany). The plasma samples were centrifuged at 4 °C (Eppendorff, Centrifuge 5415R) at 10,000 g for 5 min, transferred to cryogenic tubes, frozen in liquid nitrogen and stored at –80 °C. After blood sampling the fish was killed by a blow to the head followed by the determination of weight and length. The gonads were removed, their weights recorded and preserved in 10 % buffered formalin. The gonadosomatic index (GSI) and condition factor (CF) was calculated as follows:

$$CF = (\text{weight} \times 100)/\text{length}^3$$

$$GSI = 100 \times \text{gonad weight}/(\text{body weight} - \text{gonad weight})$$

Table 2. The dates when field sampling took place in four rivers in 2002 and 2003.

Test area	Site	2002	2003
Venoge	**HW**	16.08.2002	B
	D1	21.08.2002	05.08.2003
	D2	21.08.2002	05.08.2003
Emme	**HW**	26.08.2002	23.07.2003
	D1	09.08.2002	24.07.2003
	D2	09.08.2002	A
Necker	**HW**	27.08.2002	26.08.2003
	D1	28.08.2002	27.08.2003
	D2	28.08.2002	27.08.2003
LBK	**HW**	02.08.2002	07.08.2003
	D1	19.08.2002	07.08.2003
	D2	A	05.08.2003

A = no fish was caught at sites LBK D2 in 2002 and Emme D2 in 2003.
B = due to drought no sampling took place at Venoge HW (2003).
HW = head water; D1 = downstream 1; D2 = downstream 2.

Some scales of each fish were removed and age was determined by cantonal fishery authorities.

Histological analysis

The gonads were processed further for histological analysis (longitudinally sectioned at 3–5 µm; stained with haematoxylin and eosin) as described by Körner et al. (2005). Both, male and female gonads were microscopically examined with a Nikon ECLIPSE E400 (magnifications ranged from 20x to 400x) for histopathological alterations (e.g. intersex, parasites, etc). The percent of atresia was quantified according to Blazer (2002) by counting the number of atretic follicles out of 150 oocytes. To evaluate the stage of male spermatogenesis (Fig. 1a and Fig. 1b), the testes were scored semi-quantitatively according to the criteria described in Blazer (2002). Female gonads were classified according to the most advanced stage of oocytes present (Fig. 1c and Fig. 1d) as described by Blazer (2002).

Vitellogenin analysis

Plasma Vtg was analyzed by means of a competitive brown trout Vtg enzyme linked immunosorbent assay (ELISA). Purified Vtg for coating and standard was isolated as described by Burki et al. (2006). Primary Vtg antibody (Vtg AB) against *Salmo salar* Vtg was raised in rabbit and was kindly provided by Birgitta Norberg (IMR, Austevoll Aquaculture Research Station, Storebø, Norway). Secondary AB (goat-anti-rabbit IgG, horse-radish peroxidase conjugated) was supplied by BIO-RAD, Germany.

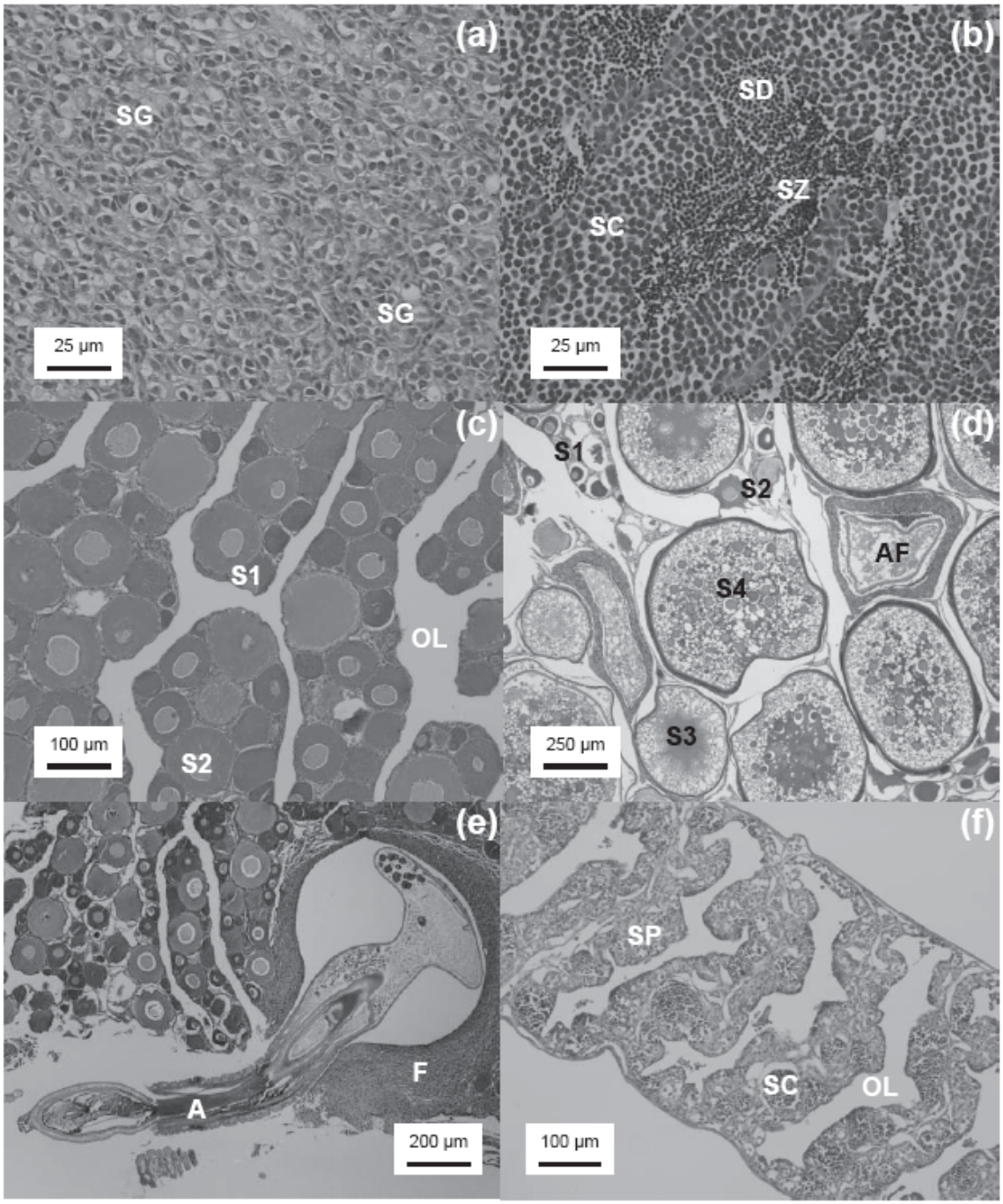

Figure 1. Histological sections of brown trout gonads from Swiss rivers. (a) Immature testis filled with spermatogonia (SG). (b) Mid spermatogenic testis comprising spermatocytes (SC), spermatids (SD) and spermatozoa (SZ). (c) Previtellogenic ovary containing early (S1) and late (S2) perinuclear oocytes as well as the characteristic ovarian lamellae structure (OL). (d) Vitellogenic ovary consisting of early and late perinuclear oocytes (S1 and S2), cortical alveolar stages (S3) as well as vitellogenic oocytes (S4). An atretic follicles is labelled as AF. (e) Acanthocephalan parasite in female ovary (A) as well as pronounced fibrosis (F) in surrounding tissue. (f) Male gonad – filled with spermatogonia (SG) and spermatocytes (SC) showing ovarian lamellae structure (OL). Sections were stained with haematoxylin and eosin.

Ninety-six well plates (Nunc™ F96 Maxisorp Immuno Plate, Nunc, Wiesbaden, Germany) were coated with 15 ng/well Vtg in carbonate buffer (0.05 M sodium carbonate, pH 9.6), sealed and incubated overnight at 4 °C in a humid plastic container. For the standard, bt-Vtg was thawed and diluted in 0.1 M PBS-T (8.1 mM sodium hydrogen phosphate, 2.7 mM potassium dihydrogen phosphate, 1.5 mM potassium chloride, 137 mM sodium chloride, 0.005 % Tween20) to concentrations of 1 µg/mL Vtg to 0.004 µg/mL Vtg. Plasma samples were also diluted in 0.1 M PBS-T. One hundred µL of both, standards and samples, were incubated overnight at 4 °C in sealed non-coated 96-well plates with Vtg-AB (100 µL/well, 1:10,000 in 0.1 M PBS-T). The coated plates were washed three times with 0.05 M PBS-T washing buffer and blocked 1 h at room temperature (RT) with blocking solution (1 % non fat dry milk in PBS-T, w/v). Subsequently the plates were rinsed three times with washing buffer. One hundred µL of standard/AB and sample/AB were transferred to the wells and incubated for 2 h at RT. The plates were rinsed three times with washing buffer, then the secondary AB was added (100 µL/well, 1:1,500 in PBS-T). After incubation for 2 h at RT, plates were rinsed five times with washing buffer and 100 µL substrate solution was added to each well (0.5 mg/mL ortho-phenylene diamine and 0.5 µL /mL 30 % hydrogen peroxide in 0.05 M dibasic sodium phosphate and 0.024 M citrate acid, pH 5.0). The plates were incubated in the dark at RT for at least 30 min. The reaction was stopped by adding 100 µL 0.5 M sulfuric acid to each well. Absorbance was read at 490 nm with a micro plate reader (Spectra Rainbow, Tekka).

The inter assay CV (coefficient of variation) was 9.5 % and the intra assay CV was 11.7 %. The detection limit was 7 ng/mL Vtg. However, due to matrix effects plasma samples had to be diluted at least 1:50. This resulted in a detection limit of 0.35 µg/mL Vtg. For statistical analysis, values below the detection limit were set at 0.35 µg/mL Vtg. According to Vethaak et al. (2002), concentrations of 1 µg/mL Vtg were defined as threshold of Vtg induction in male trout and therefore considered as induced male fish.

Data analysis

Most of the data did not meet statistical requirements for normality and were analyzed by nonparametric techniques. To compare the values between the different sites, the Kruskal-Wallis test was applied. Subsequent multiple comparisons were carried out using the Mann-Whitney-U test (Bonferroni corrected). Significance levels were $p \leq 0.05$. Statistical analysis was carried out using SPSS for Windows (Version 11.0.1).

Results

Despite our efforts to obtain fish of a similar age class (preferred immature fish), this was not always achieved. The age of brown trout sampled in the four test areas ranged from 0+ to 3+ (Table 3). Though, most of the fish were young of the year (0+) or one year (1+) old. The mean CFs in males ranged from 0.91 to 1.22 and in females from 0.90 to 1.04. For almost all areas, the mean CFs were lower at the head water sites compared to the downstream sites (Table 3), though significant differences between sites were restricted to the year 2002 (Table 3). The comparison of male and female CFs revealed higher values in males compared to females at almost all sites (Wilcoxon signed ranks test, $n = 21$; $Z = -3.228$; $p = 0.001$). However, when comparing male and female fish from each site, this trend was only significant at Necker D1 ($p = 0.024$) and LBK D1 ($p = 0.016$) in the year 2003.

Plasma vitellogenin concentrations in male brown trout did not differ between the sampling sites (Table 4). The Vtg concentrations were almost all near the detection limit (DL = 0.35 µg/mL) or lower. In only 10 out of 197 males (~5 %) Vtg values exceeded 1.0 µg/mL; the highest Vtg value (8.3 µg/mL) was found at Necker D1.

In females, the plasma Vtg values ranged from 0.35 to 70 µg/mL. Plasma Vtg concentrations of female fish caught in 2002 at Venoge D1 and D2 were significantly lower compared to Venoge HW (Table 4). In females captured in 2003 at Necker D2 (but not 2002), concentrations of Vtg were significantly lower than in females from the HW site (Table 3). Female Vtg was associated with the stage of ovarian development (Fig. 2a), but not with the age of fish (Fig. 2b).

Large variations in GSI values were noted predominantly in male fish (mean male GSI = 0.05–1.43), while variability in females was relatively low (mean female GSI = 0.16–0.78). In five out of seven samplings (males) as well as in five out of seven cases (females) the mean GSI were highest in the HW region. These differences were significantly different for certain sampling sites (Table 3). However, most of the observed differences were associated with the age of the examined specimen (Table 2 and Table 3; e.g. 1+ fish with a GSI of 0.3 were caught at Necker HW whereas 0+ fish with a GSI < 0.2 were caught at Necker D1 and D2).

The overall sex ratio was arround 1:1, although in five out of 21 sampling events more females were caught – in particular at LBK, but also at one site in the Necker River (Table 4). None of the males showed indications of intersex or other histological alterations in their testes, except for a one year old male fish from Venoge D1. The testes of this fish had a lamellar structure, which is a common histological feature of female gonads (Fig. 1f). The affected gonad did not contain oocytes, however, and was mostly filled with spermatocytes and spermatids. Therefore, it was regarded as a male fish. Most of the males remained in the pre-spermatogenic stage (66 %), whereas 26 % (early-spermatogenic stage) and 8 % (mid-spermatogenic) comprised advanced stages of spermatogenic

Table 3. Age, length, weight and condition factor (CF) of male and female brown trout sampled at Venoge, Emme, Necker and LBK (Liechtensteiner Binnenkanal) in 2002 and 2003. The data are presented as mean ± standard deviation.

Test area	Site	N		Age (year)		Length (cm)		Weight (g)		CF	
		male	female	male	female	male	female	male	female	male	female
2002											
Venoge	**HW**	12	15	1 ± 0	1 ± 0	15.0 ± 1.1	14.5 ± 1.3	33 ± 6	28 ± 7	0.96 ± 0.1	0.94 ± 0.1
	D1	10	15	1 ± 0	1 ± 0	16.2 ± 0.7	15.5 ± 1.4	43 ± 9	38 ± 10	1.00 ± 0.1	1.00 ± 0.1
	D2	16	8	1 ± 0	1 ± 0	15.7 ± 1.4	15.1 ± 1.9	36 ± 8	34 ± 13	0.92 ± 0.1	0.91 ± 0.1
Emme	**HW**	10	13	1 ± 0	1 ± 0	13.6 ± 0.7	13.2 ± 1.0	25 ± 4	22 ± 4	0.96 ± 0.1[1]	0.95 ± 0.1
	D1	12	7	1.4 ± 0.7	1.7 ± 0.5	19.8 ± 1.4	20.2 ± 2.1	81 ± 15	85 ± 27	1.04 ± 0.1[1]	1.00 ± 0.1
	D2	4	2	1.5 ± 0.6	1.5 ± 0.7	18.5 ± 1.7	20.1 ± 2.3	66 ± 20	86 ± 27	1.03 ± 0.1	1.04 ± 0.1
Necker	**HW**	10	10	1 ± 0	1 ± 0	11.0 ± 0.7	10.5 ± 1.1	12 ± 2	11 ± 3	0.91 ± 0.1[2]	0.90 ± 0.1[3]
	D1	12	10	0 ± 0	0 ± 0	10.1 ± 0.5	10.2 ± 0.6	10 ± 2	11 ± 2	0.99 ± 0.1[2]	0.98 ± 0.1[3,4]
	D2	11	19	0 ± 0	0 ± 0	9.7 ± 0.8	10.1 ± 0.6	9 ± 2	10 ± 2	0.94 ± 0.1	0.93 ± 0.1[4]
LBK	**HW**	9	16	1.8 ± 0.7	1.5 ± 0.6	12.9 ± 1.9	12.3 ± 1.7	22 ± 9	20 ± 9	0.99 ± 0.1[5]	1.00 ± 0.1[6]
	D1	9	18	1.9 ± 0.3	2.1 ± 0.6	14.8 ± 1.7	15.2 ± 1.9	36 ± 13	37 ± 12	1.07 ± 0.1[5]	1.03 ± 0.1[6]
	D2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2003											
Venoge	**HW**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	D1	11	9	1 ± 0	1 ± 0	14.8 ± 1.7	15.4 ± 1.6	32 ± 10	36 ± 10	0.95 ± 0.1	0.97 ± 0.1
	D2	10	10	1 ± 0	1 ± 0	15.0 ± 2.3	14.2 ± 1.4	32 ± 12	27 ± 9	0.92 ± 0.1	0.91 ± 0.1
Emme	**HW**	8	6	1 ± 0	1 ± 0	13.5 ± 0.8	13.7 ± 0.8	25 ± 4	25 ± 4	0.99 ± 0.1	0.96 ± 0.1
	D1	9	11	1 ± 0	1 ± 0	18.3 ± 1.2	18.7 ± 1.7	64 ± 13	67 ± 17	1.03 ± 0.1	1.01 ± 0.1
	D2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Necker	**HW**	9	10	1.4 ± 0.5	1.3 ± 0.5	14.0 ± 1.9	14.4 ± 1.3	27 ± 9	28 ± 7	0.96 ± 0.1	0.93 ± 0.1
	D1	9	11	0.4 ± 0.5	0.3 ± 0.5	12.6 ± 0.5	12.7 ± 1.5	20 ± 2	21 ± 9	1.01 ± 0.1[7]	0.96 ± 0.1[7]
	D2	7	9	0.7 ± 0.5	0.6 ± 0.5	14.2 ± 2.2	15.6 ± 3.3	28 ± 12	39 ± 21	0.94 ± 0.1	0.91 ± 0.1
LBK	**HW**	5	14	1.4 ± 0.6	1.4 ± 0.7	13.3 ± 1.3	12.6 ± 1.3	22 ± 6	19 ± 6	0.95 ± 0.1	0.92 ± 0.1
	D1	8	5	1.9 ± 0.4	1.6 ± 0.6	14.8 ± 1.3	15.3 ± 1.0	41 ± 18	35 ± 7	1.22 ± 0.4[8*]	0.96 ± 0.1[8]
	D2	4	11	1.3 ± 0.5	1.3 ± 0.5	12.5 ± 2.3	14.2 ± 1.2	22 ± 11	28 ± 7	1.03 ± 0.1	0.98 ± 0.1

HW = head water; D1 = downstream 1; D2 = downstream 2; n.d. = not determined.
CF (condition factor) = (weight × 100)/length^3.
Values with same super fix are significantly different from each other ($p \leq 0.05$).
* We excluded two points, because of some uncertainty (CF = 1.03 ± 0.1), but the difference between males and females remained significant ($p \leq 0.05$).

Table 4. Gonadosomatic index (GSI), gonadal development and vitellogenin (Vtg) in male and female brown trout sampled at Venoge, Emme, Necker and LBK (Liechtensteiner Binnenkanal) in 2002 and 2003. The data are presented as mean ± standard deviation, except for Vtg. The Vtg values were presented as median and (minimum-maximum).

Test area	Site	N		GSI	testis stage	GSI	oocyte stage	Vtg (µg/mL)		Number of fish below LOD (%)	
		male	female	male		female		male	female	male	female
2002											
Venoge	**HW**	12	15	$1.29 \pm 1.2^{1,2}$	1–3	0.30 ± 0.1	2–4	0.35 (0.35–2.52)	1.21 (0.35–62.8)[3,4]	83	13
	D1	10	15	0.09 ± 0.1^{1}	1–2	0.23 ± 0.7	1–2	0.35 (0.35–0.53)	0.53 (0.35–2.49)[3]	90	26
	D2	16	8	0.06 ± 0.1^{2}	1–2	0.24 ± 0.5	2	0.35 (0.35–1.35)	0.35 (0.35–0.45)[4]	81	63
Emme	**HW**	10	13	1.06 ± 1.9	1–3	0.17 ± 0.1^{5}	2	0.35 (0.35–0.52)	0.96 (0.35–0.52)	90	7
	D1	12	7	0.38 ± 0.5	1–3	0.78 ± 0.8^{5}	3–4	0.35 (0.35–0.46)	3.27 (0.35–58.41)	67	14
	D2	4	2	0.21 ± 0.2	1–2	0.21 ± 0.1	3	0.35 (0.35–0.35)	0.35 (0.35–0.35)	100	100
Necker	**HW**	10	10	$0.26 \pm 0.3^{6,7}$	1–2	$0.30 \pm 0.1^{8,9}$	2–3	0.35 (0.35–1.41)	1.38 (0.49–6.33)	70	0
	D1	12	10	0.05 ± 0.1^{6}	1	0.14 ± 0.1^{8}	2	0.35 (0.35–4.37)	2.13 (0.80–14.82)	58	0
	D2	11	19	0.06 ± 0.0^{7}	1	0.13 ± 0.1^{9}	2–3	0.35 (0.35–0.48)	1.98 (0.53–5.12)	90	0
LBK	**HW**	9	16	0.29 ± 0.5	1–2	0.39 ± 0.3	2–4	0.35 (0.35–0.98)	1.38 (0.35–70.96)	33	6
	D1	9	18	1.43 ± 1.71	1–3	0.27 ± 0.1	2–3	0.38 (0.35–0.81)	2.13 (0.40–12.27)	33	0
	D2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
2003											
Venoge	**HW**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
	D1	11	9	0.08 ± 0.1	1	0.22 ± 0.1^{10}	2	0.37 (0.35–1.01)	0.69 (0.41–2.06)	46	0
	D2	10	10	0.05 ± 0.1	1	0.30 ± 0.1^{10}	2–3	0.50 (0.35–5.00)	0.54 (0.35–1.02)	40	40
Emme	**HW**	8	6	0.33 ± 0.8	1–2	0.16 ± 0.1	2	0.35 (0.35–0.42)	1.65 (0.89–4.47)	50	0
	D1	9	11	0.10 ± 0.1	1–2	0.17 ± 0.1	2–4	0.36 (0.35–1.42)	0.83 (0.38–14.62)	89	0
	D2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
Necker	**HW**	9	10	$2.07 \pm 2.4^{11,12}$	1–2	$0.38 \pm 0.1^{13,14}$	2–4	0.35 (0.35–0.39)	5.53 (0.64–57.29)[15]	78	0
	D1	9	11	0.03 ± 0.1^{11}	1	0.22 ± 0.3^{13}	2	0.35 (0.35–8.34)	3.46 (2.14–24.34)	88	0
	D2	7	9	0.03 ± 0.1^{12}	1	0.16 ± 0.1^{14}	2–3	0.35 (0.35–0.61)	1.11 (0.35–4.29)[15]	71	11
LBK	**HW**	5	14	0.50 ± 0.6	1–2	0.32 ± 0.2	2–4	0.35 (0.35–1.93)	1.41 (0.35–4.63)	80	7
	D1	8	5	1.82 ± 1.5	1–2	0.25 ± 0.1	2	0.35 (0.35–1.61)	1.82 (0.35–3.12)	75	20
	D2	4	11	1.84 ± 3.5	1–2	0.26 ± 0.1	2–4	0.35 (0.35–0.35)	1.65 (0.46–5.97)	100	0

HW = head water; D1 = downstream 1; D2 = downstream 2 n.d. = not determined.
GSI (gonadosomatic index) = 100 × gonad weight/(body weight – gonad weight).
LOD = Limit of detection (Vtg = 0.35 µg/mL).
[1–17] Values with same super fix are significantly different ($p \leq 0.05$).

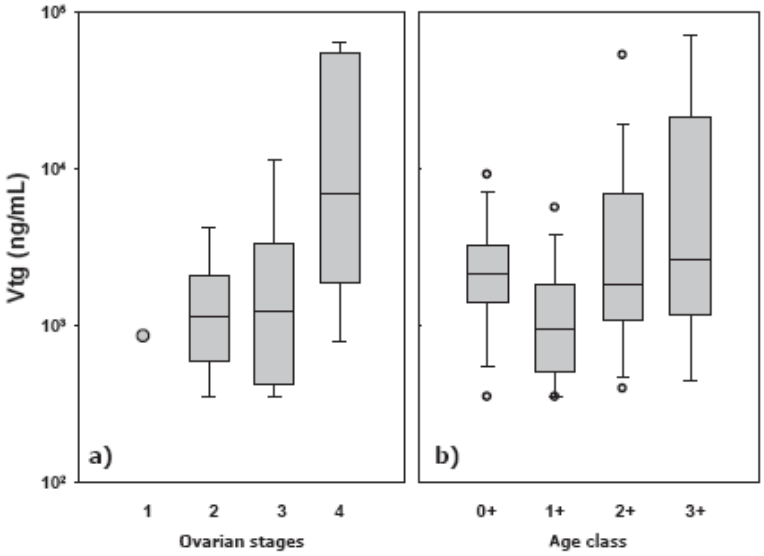

Figure 2. Plasma vitellogenin (Vtg) in dependence on stage of (a) ovarian development (ovarian stage) and (b) age of female brown trout sampled at Venoge, Emme, Necker and LBK (Liechtensteiner Binnenkanal) in 2002 and 2003 (n = 1-182). The data are presented as box plots indicating the median, 75th and 95th percentiles. Gray dot represents one single data point (n = 1) and white circles signify outliers. (1 = early perinuclear stage; 2 = late perinuclear stage; 3 = cortical alveolar stages; 4 = vitellogenic stage).

development. None of the males had testes in late spermatogenic or matured stages.

In contrast to males, we found intersex in females caught at LBK and Venoge in both years. Some ovaries contained several spermatogenic nests, mostly located near the ovarian lamellae. The nests were filled predominately with spermatocytes and spermatids, but also contained some spermatozoa as evidenced by selective staining. The occurrence of intersex (prevalence between 5 and 27 %) was not solely restricted to the downstream sites, but also appeared at one site with no STW effluent input (for details, please see figures and tables in Körner et al., 2005).

Most of the female fish remained at the late perinucleolar stage (80 %), whereas only 12 % (cortical alveolar stage) and 8 % (vitellogenic stage) of the females showed a higher stage of ovarian development. One female at Venoge D1 showed no indications of ovarian development. All her oocytes were at the early perinucleolar stage.

The occurrence of ovarian atresia was relatively low at almost all examined sites and ranged from 0 to 28 %. The highest values of atresia were found in fish from Emme D1 in 2002. Atresia at Emme D1 was significantly elevated compared to Emme HW in 2002 ($p < 0.001$) and 2003 ($p = 0.044$; Fig. 3). This downstream increase of atretic follicles also appeared at the Necker in 2003. Atresia at Necker D1 and D2 was significantly higher than at the corresponding HW site (Fig. 3). The severity of atresia in females caught in 2002 was significantly higher ($p < 0.001$) than in 2003 (Fig. 4), but a clear connection between atresia and stage of ovarian development was absent (Fig. 5).

In ovaries of female brown trout at Venoge D1 we observed high incidences of parasites with an occurrence of 47 % in 2002 and 44 % in 2003. Linked to the occurrence of the parasites, the surrounding tissue showed severe fibrosis (Fig.1e). Females from all other sampling sites as well as all male fish showed no indications of gonadal parasitism. On the basis of several characteristics (e.g. everted proboscis with non-spined collar, spines on the cuticle at the posterior end as well as thick hypodermal layer of felted and cross fibers), the parasites were assigned to the phylum acanthocephalan (Dr. Sarah Poynton, IGB Berlin, Germany, personal communication). A more detailed determination (e.g. genus) would have required the preparation of complete parasites out of ovarian tissue.

Discussion

The present study aimed to assess the hypothesis that certain reproductive parameters in brown trout are impaired and that reduced reproductive health could contribute to the declining fish catch in Swiss rivers. In general, our results do not indicate any significant perturbations of brown trout reproductive health.

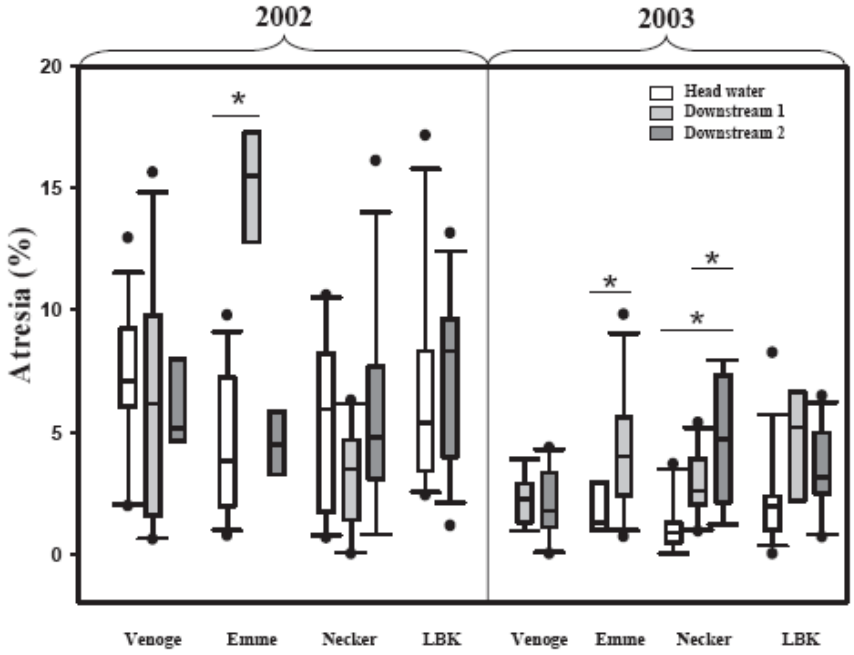

Figure 3. Ovarian atresia in female brown trout sampled at Venoge, Emme, Necker and LBK (Liechtensteiner Binnenkanal) in 2002 and 2003 (n = 2–18). The data are presented as box plots indicating the median, 75th and 95th percentiles. The black dots signify outliers. Asterisks denote significant differences between sampling sites ($p \leq 0.05$).

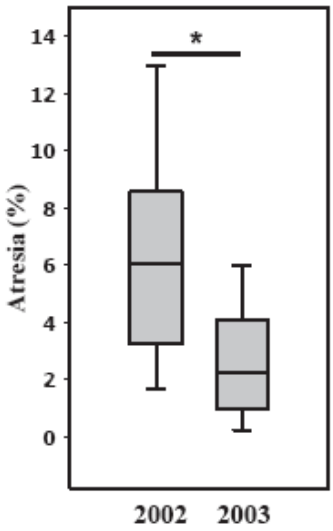

Figure 4. Ovarian atresia in female brown trout sampled in 2002 (n = 126) and 2003 (n = 93). The data are presented as box plots indicating the median, 75th and 95th percentiles. Asterisk denotes a significant difference between groups ($p \leq 0.001$).

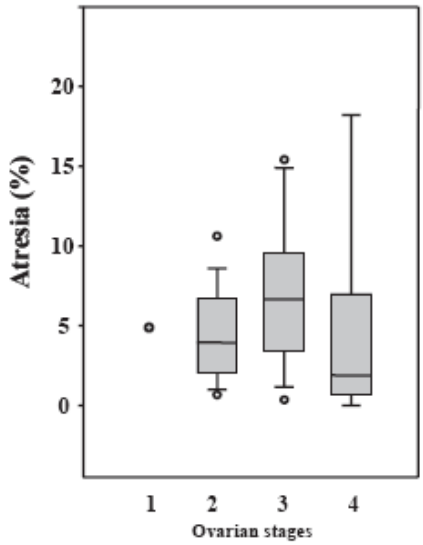

Figure 5. Female atresia in relation to the stage of ovarian development in brown trout sampled at Venoge, Emme, Necker and LBK (Liechtensteiner Binnenkanal) (1 = early perinuclear stage (n = 1); 2 = late perinuclear stage (n = 179); 3 = cortical alveolar stages (n = 25); 4 = vitellogenic stage (n = 17)). The data are presented as box plots indicating the median, 75th and 95th percentiles. Gray dot represents one single data point (n = 1) and white circles signify outliers.

In order to assess a potential exposure of brown trout to estrogenic compounds in STW effluents we measured the estrogen dependent yolk precursor Vtg in plasma of feral brown trout. We only found a few induced males (~5 %) and there was no site specific pattern to this induction. This indicates that the exposure of brown trout to environmental estrogens is low - at least in the four rivers we investigated. It may be that brown trout are not sensitive enough to be an adequate sentinel for environmental estrogens. The sensitivity of fish to certain xenoestrogens varies between different species (Van den Belt et al., 2003; Tyler et al., 2005). However, previous studies on brown trout in Switzerland provided evidence of Vtg induction in males exposed to STW effluents at various hot spots (Wahli et al., 1998; Vermeirssen et al., 2005a, Vermeirssen et al., 2005b). The lack of Vtg in our survey is probably connected with the relatively low effluent load at the investigated downstream sites. In a study by Harries et al. (1999) a significant increase of Vtg in rainbow trout was observed after exposure to at least 25 % effluent. Even under dry weather conditions the effluent percentage at our downstream sampling sites only reaches up to 10 % (LBK), which is quite low. Consequently, the concentrations of steroidal estrogens – thought to be the main contributors to estrogenicity of domestic effluent (Desbrow et al., 1998; Routledge et al., 1998) – in river water are low. This assumption is supported by calculations made by Suter and colleagues (unpublished data, see also Table 1). They modeled the estrogenicity (calEEQs, calculated estradiol equivalents) in three test areas (Venoge, Emme and Necker). The calEEQs only reached values up to 0.3 ng/L at Q_{median} (Table 1) and were in the low range compared to EEQ values measured in Swiss rivers using a recombinant yeast assay (Vermeirssen et al., 2005a; Vermeirssen et al., 2005b). These surveys observed induced Vtg in feral male brown trout at EEQs exceeding ca. 0.8 ng/L.

The female Vtg values observed in the present study appeared to be within the range previously reported for brown trout (Crim and Idler, 1978; Norberg et al., 1989; Burki et al., 2006) and showed a strong association with the stage of ovarian development. However, females from the downstream Venoge (in 2002) and Necker (in 2003) sites revealed significantly lower Vtg concentrations compared to females caught at the HW sites. Reduced female plasma Vtg has recently been used as an indicator of endocrine disruption (Solé et al., 2003a). In that study, female carp collected in the vicinity of STW showed lower Vtg concentrations than fish from upstream and downstream sites as a result of disturbed estrogen homeostasis. Prior to our sampling periods, the temperature in the lower parts of the rivers were considerably higher than in the colder headwaters. Therefore, reduced Vtg at downstream sites in the present study may be linked to this elevated water temperature, which is known to alter the physiology of fish considerably (Wendelaar Bonga, 1997). Previous studies showed that a high summer temperature during natural vitellogenesis of female Atlantic salmon (*Salmo salar*) is associated with a reduction of plasma Vtg (King et al., 2003) - an effect primarily linked to increased plasma cortisol levels (Mommsen et al., 1999; Berg et al., 2004). Ojanguren et al. (2001) reported an upper temperature optimum of brown trout around 20 °C. The river water temperature in the test areas often exceeded 20 °C and in 2003 it reached values up to 25 °C (Hari et al., 2005) – a temperature range known to stimulate a cortisol response in salmonids (Strange et al., 1977). Therefore, the elevated water temperatures during summer probably stressed the fish and may have provoked an inhibition of Vtg synthesis at downstream sites similar to the effects observed by King et al. (2003).

An inhibition of Vtg synthesis may not only influence female fish, but would also affect the assessment of exposure to environmental estrogens using male Vtg as biomarker. A lack of Vtg induction in exposed males could be wrongly interpreted as "not exposed" and therefore lead to an underestimation of the actual exposure. Therefore, subsequent studies are needed to test the influence of temperature stress on Vtg expression in males after exposure to environmental estrogens and finally the applicability of brown trout monitoring studies during the summer months.

The abundant appearance of intersex among gonochoristic fish populations is increasingly ascribed to environmental estrogens entering the aquatic system via STW effluents (Jobling et al., 1998). Although we observed intersex in female brown trout in two of the four rivers, this intersex does not appear to be linked with estrogenic exposure (Körner et al., 2005). This is supported by the absence of Vtg induction in males as well as the low values of atretic follicles in female trout. Additionally, the intersex described in Körner et al. (2005) is completely different from the type commonly observed in estrogen exposed fish. Usually, single or clusters of follicular oocytes randomly distributed within testicular tissue (ovotestis), but also clear zoned ovarian and testicular tissues within one gonad, are reported (Jobling et al., 1998; Nolan et al., 2001; Blazer, 2002). In contrast, Körner et al. (2005) observed small nests filled with all stages of spermatogenesis occupying less than 1 % of the gonads. This finding, with a prevalence up to 27 %, is consistent with field observations in Danish brown trout (Christiansen and Plesner, 2001), but also whitefish (Mikaelian et al., 2002) and pike (Vine et al., 2005), reporting a prevalence between 12 % and 26 %.

The assessment of potential implications of intersex on feral brown trout population remains difficult for several reasons. Although brown trout at LBK showed a female biased population, this situation was not present at the River Venoge. Beyond this, the number of fish (n =

6–30) per sampling date is too low to make reliable statements regarding altered sex ratios. As sex markers of brown trout are still missing, the genetic sex of the affected fish could not be determined. While many laboratory studies established a strong association between certain environmental estrogens and ovotestis (i.e. feminization), relatively little is known about effects of environmental androgenic and anti-estrogenic substances on histological masculinisation of fish. In particularly, with our measurement of Vtg we focused on a possible role of environmental estrogens. However, the fish at most of our sampling sites were obviously exposed to a variety of micropollutants that are carried to the aquatic ecosystem by STW effluents and diffuse sources.

Finally, it is not clear, whether the intersex has a chemical etiology or is a natural phenomenon. The latter does seem to be more likely, because some types of intersex often seen in male fish (i.e. feminization, ovotestis) were not solely restricted to areas in the vicinity of STWs, but also at sites with low or no effluent load (e.g. van Aerle et al., 2001; Faller et al., 2003; Bernet et al., 2004). Additional research will be necessary to provide more details about the natural occurrence of intersex in salmonids (e.g. Kinnison et al., 2000), which are commonly considered as strictly gonochoristic fish species (Woram et al., 2003).

The incidence of ovarian atresia in brown trout was generally low and can be considered as being natural background levels for most of the sites (Billard, 1987; Leino et al., 2005). Highest values were found in females caught at site Emme D1 in 2002 (mean = 15 %). Wood and Van Der Kraak (2001) discussed atresia (and apoptosis respectively) in the context of natural ovarian growth and post ovulatory regression. In our study we did not observe a correlation between ovarian stage and atresia. This may mean that atresia is indicative of stressful conditions for fish inhabiting Emme D1 in 2002. However, the impact of increased atresia on population relevant parameters (e.g. fecundity or egg size) remains vague. For instance, the study by Janz et al. (1997) observed elevated atresia after exposure to bleached kraft mill effluent, but observed no significant reduction in egg fecundity. Ovarian atresia has been associated with different stress factors including starvation, water pollution or unfavorable temperature regimes (Blazer, 2002). Condition factors were all above 0.8, thus little evidence of nutrition deficits was seen. High frequencies of follicular atresia in females inhabiting polluted river water have been reported (Janz et al., 1997; Adams et al., 1999; Jobling et al., 2002). In these studies, atresia was mainly related to certain chemicals like environmental estrogens or pesticides. Indeed, certain environmental estrogens (e.g. nonylphenol or quercetin) as well as pesticides (e.g. atrazine) are known to enhance atresia in fish (Miles-Richardson et al., 1999; Spano et al., 2004; Weber et al., 2002). We did not measure concentrations of estrogens; however, on the basis of low plasma Vtg levels in males, the concentrations of estrogenic contaminants appear to be low. This is the same with respect to the measured pesticides. Although chemical analysis provided evidence of various pesticides (Table 1) in the Emme, the highest concentrations of e.g. atrazine are approximately 100 fold lower than the concentrations used by Spano et al. (2004).

Given the argument that elevated temperatures tend to increase ovarian atresia (Wallace and Selman, 1981; Blazer, 2002), one would expect, that atresia in 2003 would be higher than in 2002 due to the high water temperature. For unknown reasons, atretic oocytes were less abundant in summer 2003. Therefore, higher temperatures in 2003 had apparently no adverse effects on female gonads. This is in contrast to the finding in other fish species like sturgeon or medaka. White sturgeons (*Acipenser transmontanus*) exposed to an increased water temperature showed an increase of follicular atresia (Linares-Casenave et al., 2002). Furthermore, Koger et al. (1999) observed severe incidences of atresia in the medaka (*Oryzias latipes*) after increasing water temperatures.

Gonadal alterations caused by parasites or diseases may provoke significant adverse effects on the reproductive fitness of fish (Barber et al., 2000; Hecker and Karbe, 2005). Previous studies reported a great variety of metazoan parasites, which were found in gonadal tissue of feral fish. The major groups identified in these investigations were trematodes, cestodes as well as nematodes (Blazer, 2002). In contrast, species belonging to the phylum acanthocephala are predominantly parasites inhabiting the gastrointestinal tract of fish (Schäperclaus, 1990; Bakke and Harris, 1998). Therefore, the observation made in the present study describing acanthocephalan parasites in the ovary of feral salmonids is at least something uncommon if not unique. Interestingly, the parasites were only found at one site (Venoge D1). In addition, their prevalence was obviously restricted to females. The reasons for the apparent selection of female trout as well as potential consequences on the female reproductive fitness are not yet assessable. In female Bucchich's goby (*Gobius bucchichi*), however, the abundance of acanthocephalan parasites was negatively correlated with the GSI and parasitism reduces the number of eggs of the host (Sasal et al., 2001). The well studied cestode *Ligula intestinalis* interferes with normal gonadal hormone production of cyprinids resulting in a suppressed development of the gonads (Jobling and Tyler, 2003b). Furthermore, sticklebacks infected with the cestode *Schistocephalus sp.* had smaller gonads and were less reproductively active than uninfected fish (Barber et al., 2000).

The histological evaluation of gonadal development as well as the results of GSI in males and females revealed similar data as previously reported for brown trout (Billard, 1987). While females start their first reproductive cycle as 2+ or 3+ fish, males usually start their first

gametogenesis as 1+ fish (Billard, 1987). This is consistent with the data of the current survey; apart from one 1+ female caught at Venoge HW which showed vitellogenic oocytes and elevated plasma Vtg. As the subjects of our study were predominantly juvenile brown trout, most of the surveyed brown trout were reproductively inactive or just at the beginning of gonadal recrudescence. In addition, particularly in 2002 significant differences of GSI originated mainly from the separated day of sampling.

Condition factors of male and female brown trout in the four test areas were within the range previously reported for feral brown trout in Switzerland (Kobler, 2004; Burki et al., 2006). Interestingly, almost all HW sites showed lower mean CFs than sites downstream of STWs. Weatherly and Gill (1987) proposed that low CFs may be indicators of a low feeding status by reason of food limitation. Indeed, Kobler (2005) also observed higher CF values in fish caught downstream of STWs compared to fish living in the upper river reaches. Data in Table 1 show that the availability of nutrients (nitrogen and phosphor) was generally (and noticeably) higher at the downstream sites. Hence the higher CFs at the downstream sites might be related to higher concentrations of nutrients and possibly an increased food supply. Nevertheless, the mean CF observed in the present survey was well above 0.8 at all sites, indicating no severe lack of food, even at the HW sites. The higher mean CF in males compared to females is probably due to sex dimorphisms (in respect to morphometric parameters) often seen in salmonid species (Reyes-Gavilan et al., 1997; Casselman and Schulte-Hostedde, 2004).

In conclusion, male Vtg and female ovarian atresia data indicate that the effects of estrogenic compounds on brown trout in the examined rivers are relatively minor. Consequently, impaired reproductive health, as a result of exposure to environmental estrogens, does not appear to be a major factor contributing to the marked decline of brown trout catch in the four rivers in our study, though other micropollutants may play a role. In this light, it must also be noted that we did not assess population relevant parameters such as gamete quality or recruitment. In addition, although previous studies documented the occurrence of estrogenic compounds in several Swiss STW effluents (Aerni et al., 2004; Rutishauser et al., 2004), the effects are more likely restricted to a few hot spots (see also Vermeirssen et al., 2005a) which were not investigated here.

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Characterization of environmental estrogens in river water using a three pronged approach: Active and passive water sampling and the analysis of accumulated estrogens in the bile of caged fish (Chapter 4)

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Every technology really needs to be shipped with a special manual, not how to use it, but why, when and for what.
(*Alan Kay*, 1940)

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Characterization of Environmental Estrogens in River Water Using a Three Pronged Approach: Active and Passive Water Sampling and the Analysis of Accumulated Estrogens in the Bile of Caged Fish

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Estrogenicity of river water is highly variable and it is difficult to obtain an average measure of the estrogenicity. Consequently it is difficult to tie the estrogenic effects observed in fish to their level of exposure to estrogens. To get a better handle on average estrogenic exposure we tested a recently developed passive sampling system (polar organic chemical integrative sampler, POCIS). In addition, we investigated the bioaccumulation of estrogens in caged brown trout and measured plasma vitellogenin in males as a bioindicator of estrogenic effects. We developed a mini-caging method to suit the hydrological conditions in small rivers and to improve upon the often poor survival of salmonids in caging trials. POCISs were positioned upstream and downstream of 5 sewage treatment works' discharges and left on site for 3 weeks (as were the caged fish), during which period 3 water grab samples were taken at each site. Concentrations of estrogens were determined using a yeast-based reporter gene assay and chemical analysis. Results from grab sampling, passive sampling, and bioaccumulation were correlated; however, plasma vitellogenin concentrations were elevated at only 1 of 5 sites. POCISs provide an integrated and biologically meaningful measure of estrogenicity in that they accumulate estrogens in a pattern similar to that of brown trout. Mini-caging appears a significant methodological advance; no fish were lost, moreover, all fish survived in excellent health.

Introduction

Sampling the average chemical load in the aquatic environment, particularly in rivers, is difficult because riverine ecosystems are highly variable. River flow rates are strongly affected by rainfall, and the breakdown of chemicals is often dependent on weather conditions (e.g., UV radiation and temperature; *1*, *2*) and microbial activity (*3*, *4*). Consequently, it is difficult to gauge the chemical burden that aquatic organisms face in their environment.

A common way to obtain an estimate of the average chemical load is the use of autosamplers to collect and pool multiple small samples taken over a long time window (e.g., days or weeks). However, there are numerous logistical issues that complicate the use of autosamplers to collect water from rivers. One example is freezing in the tubing that feeds the autosampler; this is a concern for a large number of days in our study areas. Another method to obtain a more integrated measure of concentrations of chemicals is the use of passive sampling (*5*). Particularly, semipermeable membrane devices (SPMDs) have been shown to be useful in assessing the chemical load in rivers and lakes (*6*). An alternative approach is the analysis of accumulated chemicals in organisms, for example in liver, bile, or fat (*7*). However, not all chemicals accumulate equally well in fish and the internal concentrations do not always reflect the factual chemical exposure of organisms (e.g., *8*, *9*). Finally, models can be used to estimate the input and subsequent chemical load in water (*10*). However, to calibrate models and to take into account, for example, the sorption of chemicals to soil or biota as well as the breakdown of chemicals, measurements of chemicals in the environment remain essential.

Our main focus is on environmental estrogens in river water; these compounds mainly enter rivers via effluent from sewage treatment works (STW). When we performed a broad survey on the estrogenicity of Swiss midland rivers (by means of grab sampling; $n = 20$ STWs), we observed large variations in estrogenicity at a given site; variability was particularly high in the smaller rivers (*11*). To get around this variation—and obtain a more integrated measure of the estrogenicity—we employed both passive sampling and bioaccumulation in our present study. The primary objective of our study was to find an appropriate and biologically meaningful way to determine the degree of exposure to estrogens of our species of interest, the brown trout, in our study area, small Swiss midland rivers.

As a passive sampling device we used the polar organic chemical integrative sampler (POCIS), a promising novel passive sampler (*12*). One particular positive aspect of POCISs is that sample preparation is much easier compared to SPMDs. Previously, POCIS samples had tested positive for estrogens in an in vitro yeast reporter gene assay (yeast estrogen screen, YES; *13*) and chemical analysis (*12*; our unpublished data). One aim of our study was to see if the average estrogenicity of a site—as obtained through repeated grab sampling—correlated with levels of estrogens trapped by the POCISs. To quantify environmental estrogens we used the YES and chemical analysis. The chemical analysis specifically focused on steroidal estrogens, as these have been shown to be the main contributors to the estrogenicity of domestic effluent (*14*).

Alongside the passive samplers we used caged fish to investigate the bioaccumulation of estrogenic substances in the bile (*15*, *16*). We used a salmonid, the brown trout, as it is a dominant species in Swiss midland rivers. Furthermore, salmonids are a popular species for exposure experiments (e.g., *17*). However, mortality is a frequent problem in cages that are stocked with salmonids and the health status of surviving fish is rarely reported (*16*, *18*, *19*). We hypothesized that aggression may be a significant cause of this mortality and hence we devised and tested a method in which we kept individual fish in small cages (*20*). Another reason to adopt the use of small cages is that many of the rivers that we

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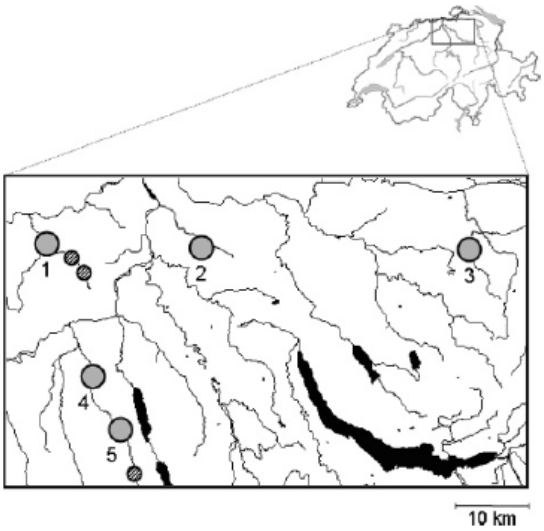

FIGURE 1. Location of the 5 sampling sites (large circles) on a map of Swiss rivers and lakes (reprinted with permission from swisstopo): (1) sewage treatment works (STW) Fricktal on the river Sissle; (2) STW Oberes Surbtal on the river Surb; (3) STW Lützelmurgtal on the river Lützelmurg; (4) STW Mittleres Wynental; and (5) STW Oberwynental on the river Wyna. Small hatched circles indicate additional STWs that discharge into the rivers Sissle and Wyna.

sample are shallow and have low flow rates (<500 L/s) with frequent extreme peak flows (10−20 times base flow rates); therefore, it is often not possible to find good and secure sites to fix larger cages. Besides the analysis of estrogens in the bile we also used plasma samples from the caged fish to examine whether the classic biomarker for exposure of male fish to estrogens—vitellogenin (*21*)—was induced (*16*).

Materials and Methods

Site Selection. In an earlier study we assessed the estrogenicity of river water downstream from where an STW discharges effluent (*11*), this study involved 20 sampling sites. From these sites we selected 1 as low (Oberes Surbtal) and 4 as moderate to highly estrogenic (Lützelmurgtal, Oberwynental, Mittleres Wynental, and Fricktal; *11*). Two sites (Oberwynental and Mittleres Wynental) are on 1 river, the Wyna; this river has a further STW upstream. Two additional STWs discharge into the Sissle upstream from the sampling site at Fricktal. For a situation map see Figure 1; for general parameters of the rivers and STW see ref *11*.

Polar Organic Chemical Integrative Sampler: POCIS. POCISs (EST, St. Joseph, MO) were taken to the field in their original containers. Containers were opened upon arrival at the field site and the POCISs were given a protective cover of stainless steel plates with 5-mm holes. Each POCIS was attached to a 0.5-m stainless steel pin and the pin was partially fixed into the gravel so that the POCIS remained free of the river bed. Blank POCISs were wrapped in aluminum foil and kept in the original transport container in the lab until the end of the trial. Two POCIS varieties were tested: "pharmaceuticals" (containing Oasis HLB as a solid phase, 100 mg/POCIS) and "pesticides" (containing a solid phase mixture of Isolute ENV+ and Ambersorb 1500 dispersed on S-X3 Bio Beads, 100 mg/POCIS; see *12* for a detailed description of solid-phase materials). For each POCIS configuration we placed individual samplers upstream (100−300 m) and duplicate samplers downstream (350−800 m) of the STW discharges.

Mini Cages for Brown Trout. Mini cages were made by cutting stainless steel tubes (100 mm diameter, 2.5 mm wall) to 0.5 m lengths. Ends were sanded to remove sharp edges and capped with detachable stainless steel wire mesh (1 mm wire, 5 mm squares). Stainless steel rings were welded onto the sides of the tubes; these rings were used to anchor the tubes on the river bed via a chain and 2 concrete weights (ca. 20 kg each). Six mini cages were placed at each downstream site and stocked with 1 brown trout each (Fischzucht Nadler, Rohr, Switzerland). The fish were ca. 13 months old with an average weight (± standard error of the mean) of 28 ± 2.0 g and an average length (±SEM) of 140 ± 3.5 mm.

At the end of the trial each cage was opened and each fish was anesthetized in MS222. Blood was sampled and centrifuged, and the plasma was snap-frozen in liquid nitrogen. Fish were killed by a blow on the head and the body cavity was opened to determine the gender. The gallbladder was removed and frozen in liquid nitrogen.

Water Sampling, Solid-Phase Extraction, and Processing of POCISs. In parallel to the passive sampling we collected 3 grab samples at the 10 sites where the POCISs were positioned. A grab sample consisted of ca. 10 scoops of 0.5 L of water taken across the river bed. Water samples were collected on days 3, 10, and 17 of the 22-day POCIS and fish exposure period. Grab samples at the 10 sites were sampled within a period of 4 h in order to reduce possible variability due to STW discharge cycles (*22*). A 1-L portion of each water sample was processed to enrich environmental estrogens by means of solid-phase extraction (*11*). Briefly, water was filtered, acidified to a pH of 6.5, and passed over a 0.5-g bed of carbopack (Carbopack X 120/400 mesh, Supelco, Bellefonte, PA). The solid phase was washed with distilled water (pH 6.5), acidified methanol, and 10% dichloromethane in methanol. Estrogens were eluted with 80% dichloromethane in methanol, the solvent was evaporated under nitrogen, and the sample was taken up in 500 μL of ethanol. Part of the sample was used in the YES analysis, and 340 μL of sample were completely dried down and taken up in 100 μL of a mixture of water/methanol/acetone (4:5:1, v:v:v) prior to LC/MS/MS analysis.

At the end of the trial the POCISs were removed, rinsed in river water, wrapped in aluminum foil, and stored in their original containers. When the POCISs were returned to the lab they were stored frozen at −20 °C. Prior to processing, the POCISs were allowed to reach room temperature and remaining debris was rinsed away with distilled water. Each POCIS was opened and methanol was used to wash the solid phase into a glass syringe cartridge fitted with a Teflon frit and some glass wool. The solid phase was eluted with either 40 mL of methanol (pharmaceuticals) or 50 mL of dichloromethane/methanol/toluene (8:1:1, v:v:v; pesticides) according to the manufacturer's instructions. The eluate was collected in a flask and reduced in a rotary evaporator to about 1 mL. The eluate was transferred to a glass vial and the flask was washed with 1 mL of methanol. Subsequently, the combined eluate and the methanol wash was completely dried down under a stream of nitrogen and the sample was taken up in either 1.5 mL of methanol (pharmaceuticals) or 1.5 mL of dichloromethane/methanol/toluene (8:1:1, v:v:v; pesticides). A 500-μL aliquot of the POCIS extract was completely dried down and taken up in 170 μL of ethanol prior to YES analysis. A second aliquot of 500 μL of POCIS extract was completely dried down and taken up in 100 μL of a mixture of water/methanol/acetone (4:5:1, v:v:v) prior to LC/MS/MS analysis.

Yeast Estrogen Screen. The yeast strain for the in vitro assay was kindly provided by J. Sumpter (Brunel University, Uxbridge, U.K.; *23*). This assay uses 17β-estradiol as a standard and the estrogenicity of a sample is expressed as 17β-estradiol equivalents (EEQ, ng/L). Grab samples were analyzed at 4

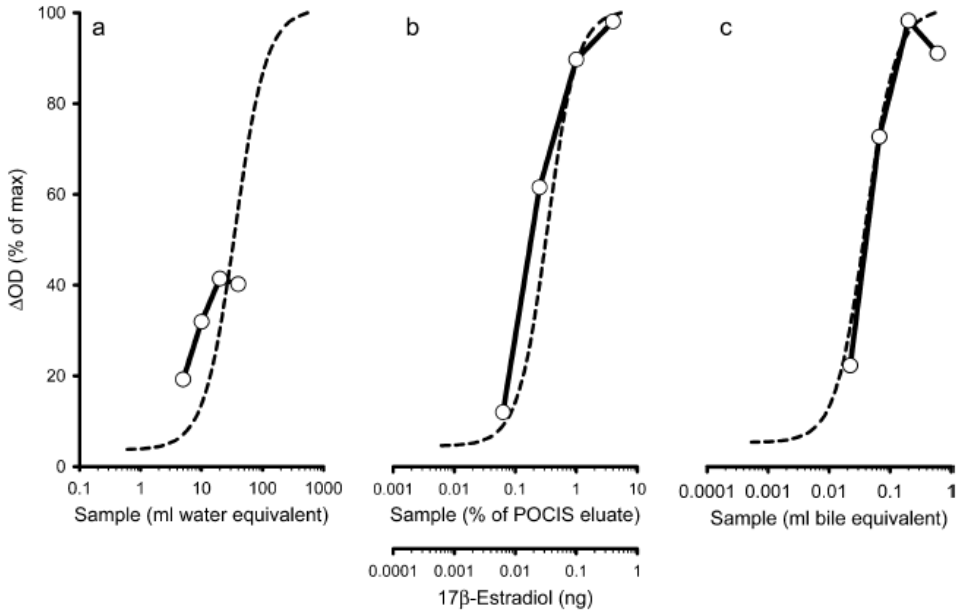

FIGURE 2. Dilution curves of: river water (a) 2-fold dilution steps; POCIS pesticides (b) 4-fold dilution steps; and bile (c) 3-fold dilution steps) in the yeast estrogen screen (YES). Samples are shown by open circles connected with solid lines; the 17β-estradiol standard dilution curves (2-fold dilution steps) of the assays are shown as dashed lines. The response of the YES to estrogens is expressed as ΔOD = optical density at 620 nm − optical density at 540 nm.

dilutions and tested in triplicate (the amount of sample extract used was equivalent to 40, 20, 10, and 5 mL of river water). EEQ values are dependent on the dilution used in the assay (i.e., the dilution curve of a sample is not parallel to that of the standard, Figure 2a) and all samples were evaluated at the lowest sample dose (see also ref *11*). POCIS samples were tested at 4 dilutions equivalent to 4, 1, 0.25, and 0.06% of the total sample. POCIS samples did not usually dilute parallel to the standard at the 2 highest doses, but sample dilution curves and the standard curve showed good parallelism at the 2 lowest sample doses (Figure 2b).

Chemical Analysis. Steroidal estrogens were quantified in both POCIS and grab sample extracts using a HP Series 1100 high-performance liquid chromatograph (Hewlett-Packard, Waldbronn, Germany), equipped with an online vacuum degasser (DG4, Henggeler Analytic Instruments, Riehen, Switzerland), a binary high-pressure gradient pump, an autosampler, and a heated column compartment (column temperature was kept at 23 °C). Separation was achieved with a 100 × 2.1 mm Waters XTerra, MS C18, 3.5-μm column (Waters, Rupperswil, Switzerland). A 10-μL aliquot of sample extract was injected and eluted off the column with 0.25 mL/min of 10% acetonitrile in Nanopure water for 2 min, followed by a linear increase to 90% acetonitrile at 19 min, and a 3 min hold at 90% acetonitrile.

A triple quadrupole mass spectrometer (API4000, Applied Biosystems, Rotkreuz, Switzerland) was operated in negative ion mode electrospray. Ionization efficiency was increased by postcolumn addition of a 2.5% ammonia solution at 10 μL/min using a micro-HPLC pump (Phönix 20 CU, Carlo Erba Instruments). Nitrogen was used as drying and nebulizer gas.

We monitored the MRM ions 269 to 145 for estrone, 271 to 145 for 17β-estradiol, and 295 to 145 for 17α-ethynylestradiol. Quantitation limits were determined at a signal-to-noise ratio of 10:1 and were typically 0.2 ng/L for estrone, 0.9 ng/L for 17β-estradiol, and 0.9 ng/L for 17α-ethynylestradiol. Recovery of the SPE procedure was assessed by adding deuterated estrogens as internal standards to river water samples (90% for estrone, 92% for 17β-estradiol, and 79% for 17α-ethynylestradiol).

Estradiol equivalents were calculated (calEEQ) by multiplying the concentration of each estrogen with its relative potency in the YES (estrone = 0.4 and 17α-ethynylestradiol = 1.2, both relative to 17β-estradiol; *24*) and adding up the values for the 3 steroids.

Estrogens in Bile. A 15-μL aliquot of bile was pipetted into a glass tube and 10 μL of snail juice (an enzyme preparation that contains glucuronidase and sulfatase; G7017, Sigma-Aldrich, Buchs, Switzerland) and 500 μL of a 50 mM sodium acetate buffer (pH 5.0) were added, and the tubes were incubated at 37 °C (*25*). After 18 h the samples were passed through C18 Sep-Pak cartridges (Waters, Rupperswil, Switzerland) which were previously primed with 5 mL of methanol and 5 mL of distilled water. Samples were eluted with 5 mL of methanol and the methanol was evaporated under a flow of nitrogen at 30 °C. Finally the samples were taken up in 500 μL of ethanol. The efficiency of the deconjugation enzymes was verified with 17β-estradiol 17-glucuronide in sodium acetate buffer, and more than 80% of conjugated 17β-estradiol was recovered as free 17β-estradiol.

Samples were tested at 4 dilutions in the YES, equivalent to 0.6, 0.2, 0.07, and 0.02 μL of bile. Most samples showed good parallelism with the standard curve (Figure 2c); a few samples with small amounts of estrogens were less parallel. All data presented here were derived from a sample dose equivalent to 0.07 μL of bile.

Vitellogenin in Blood Plasma. Plasma vitellogenin was analyzed by means of a competitive enzyme linked immunosorbent assay (ELISA). Brown trout vitellogenin for coating ELISA plates and for use as a standard was isolated as described elsewhere (*11*). The antibody was raised in rabbit against salmon vitellogenin and was a gift of B. Norberg (IMR, Austevoll Aquaculture Research Station, Storebø, Norway). The secondary antibody (goat-anti-rabbit IgG, HRP-conju-

TABLE 1. Location of the Sampling Sites and Amount of Estrogens in Grab Samples (Average of 3 Grab Samples ± SEM Collected over 22 d) and Passive Samples (Collected with Polar Organic Chemical Integrative Samplers POCISs over 22 d)[a]

		YES (EEQ ng/L; ng/POCIS)			LC/MS/MS (calEEQ ng/L; ng/POCIS)		
river	STW (town)	grab samples ± SEM	POCIS pesticides	POCIS pharmaceuticals	grab samples ± SEM	POCIS pesticides	POCIS pharmaceuticals
Sissle US	Fricktal	4.9 ± 3.11	22.0	22.8	3.7 ± 2.39	22.2	23.0
Sissle DS	(Frick)	12.6 ± 5.24	55.9−76.7	16.8−24.7	9.7 ± 4.85	41.2−43.8	15.8−19.6
Surb US	Oberes Surbtal	0.5 ± 0.03	0.4	0.4	0.1 ± 0.01	0.6	1.0
Surb DS	(Unterehrendingen)	0.6 ± 0.12	1.9−2.0	1.7−1.9	0.2 ± 0.05	2.1−2.2	1.8−2.1
Lützelmurg US	Lützelmurgtal	0.4 ± 0.12	0.8	0.9	0.2 ± 0.07	0.7	0.8
Lützelmurg DS	(Aadorf)	1.5 ± 0.52	3.0−6.5	2.5−3.9	0.5 ± 0.11	2.7−4.0	1.8−2.9
Wyna US	Mittleres Wynental	1.6 ± 0.23	8.6	9.2	0.7 ± 0.30	9.4	8.0
Wyna DS	(Teufenthal)	2.5 ± 0.15	12.9−18.2	12.9−14.8	1.4 ± 0.47	6.6−9.6	14.3−15.5
Wyna US	Oberwynental	0.9 ± 0.29	1.7	2.0	0.3 ± 0.04	6.0	5.9
Wyna DS	(Reinach)	1.3 ± 0.23	5.8−7.0	5.4−7.5	0.4 ± 0.02	5.2−6.4	5.6−6.2

[a] Samples were collected upstream (US) and downstream (DS) from sewage treatment works (STWs). Samples were analyzed with a bioassay (yeast estrogen screen, YES) and estrogenicity was expressed as 17β-estradiol equivalents (EEQ). Estrogenicity of the samples in the YES was calculated (calEEQ), based on chemical analysis of steroidal estrogens (LC/MS/MS).

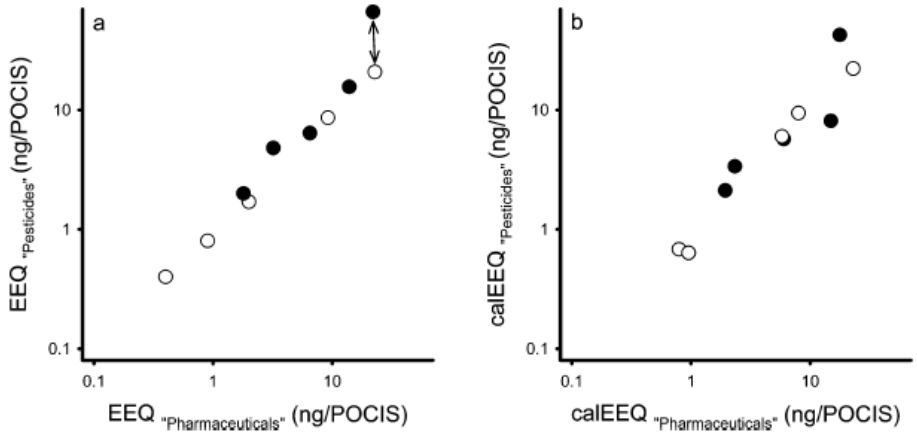

FIGURE 3. Amount of estrogens accumulated on POCIS pharmaceuticals and pesticides that had been exposed immediately upstream (open circles) and downstream (filled circles) of 5 sewage treatment works for a period of 22 days. Estrogen data extracted from the in vitro assay (17β-estradiol equivalents, EEQ) are shown on the left, and calculated estrogenicity (calEEQ), based on chemical analysis of steroidal estrogens, is shown on the right. The arrow links the data pair where the downstream EEQ value for POCIS pharmaceuticals (filled circle) is attenuated as compared to the upstream value (open circle) and compared to EEQ values for POCIS pesticides.

gated) was supplied by BIO-RAD (München, Germany). Male plasma samples were tested at a dilution of 1:50 or higher (samples were not parallel with the vitellogenin standard at dilutions lower than 1:50); the detection limit was 0.35 μg/mL. Inter- and intra-assay coefficients of variation were 9.5% and 11.7%, respectively (further assay details will be published elsewhere).

Results

Comparison of 2 POCIS Varieties. EEQ values of the POCIS samples ranged between 0.4 and 77 ng/sampler (pesticides) and 0.4 and 25 ng/sampler (pharmaceuticals; Table 1). For POCIS pesticides, EEQ and calEEQ values were always higher downstream of the STWs. This was not the case for POCIS pharmaceuticals samples from 1 STW (STW Fricktal on the river Sissle). This site showed the highest estrogenic activity (POCIS pesticides: upstream, 21 ng; downstream, 56 and 77 ng), however, at this site the downstream POCIS pharmaceuticals EEQ values appeared to be attenuated compared to results from POCIS pesticides (Figure 3) and grab water samples (see below; Figure 4).

Both EEQ and calEEQ data from POCIS pharmaceuticals and pesticides samples were highly correlated (EEQ, Figure 3a, upstream r_P [Pearson correlation coefficient on log transformed data] = 1.00, $P < 0.001$, $n = 5$; downstream $r_P = 0.96$, $P = 0.010$, $n = 5$; calEEQ, Figure 3b, upstream $r_P = 1.00$, $P < 0.001$, $n = 5$; downstream $r_P = 0.88$, $P = 0.049$, $n = 5$). Furthermore, EEQ and calEEQ data from the duplicate POCIS at the downstream sites were highly correlated (EEQ, pesticides, $r_P = 0.95$, $P = 0.014$, $n = 5$; pharmaceuticals, $r_P = 0.95$, $P = 0.012$, $n = 5$; calEEQ, pesticides, $r_P = 0.97$, $P = 0.006$, $n = 5$; pharmaceuticals, $r_P = 0.98$, $P = 0.004$, $n = 5$).

POCISs Provide an Integrated Measure of Environmental Estrogens. EEQ values in the 30 grab samples ranged between 0.2 and 18.3 ng/L. Average EEQ values of the repeated grab samples correlated well with the EEQ values from the POCIS samples (pharmaceuticals, upstream $r_P = 0.89$, $P = 0.041$; downstream $r_P = 0.95$, $P = 0.014$; pesticides, upstream $r_P = 0.98$, $P = 0.002$; downstream $r_P = 0.95$, $P = 0.013$; Figure 4a). This was also the case for the steroidal estrogens (calEEQ, pharmaceuticals, upstream $r_P = 0.94$, $P = 0.019$; downstream $r_P = 0.86$, $P = 0.063$; pesticides, upstream $r_P = 0.93$, $P = 0.023$; downstream $r_P = 0.98$, $P = 0.004$; Figure 4b). Moreover, results from the YES (EEQ) were correlated with those of the chemical analysis (calEEQ). This was the case both for the grab samples as well as for the passive samples (Figure 5; r_P values > 0.92).

The main steroidal estrogen in both active and passive samples was estrone. In fact, in most samples only estrone was found at levels exceeding the detection limit. In the 4

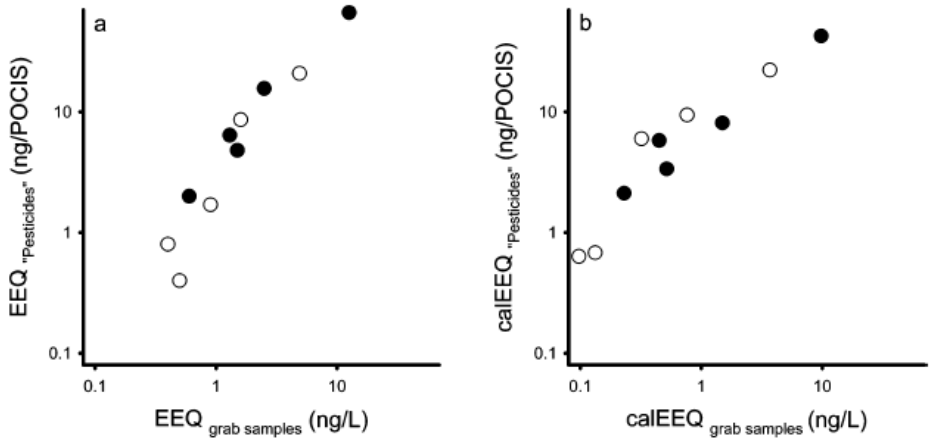

FIGURE 4. Correlation between estrogenicity of active samples (average of 3 grab samples) and passive samples (POCIS pesticides). Sampling sites immediately upstream from a sewage treatment works are shown as open circles, and downstream sites are shown as filled circles. Estrogen data extracted from the in vitro assay (17β-estradiol equivalents, EEQ) are shown on the left, and calculated estrogenicity (calEEQ), based on chemical analysis of steroidal estrogens, is shown on the right.

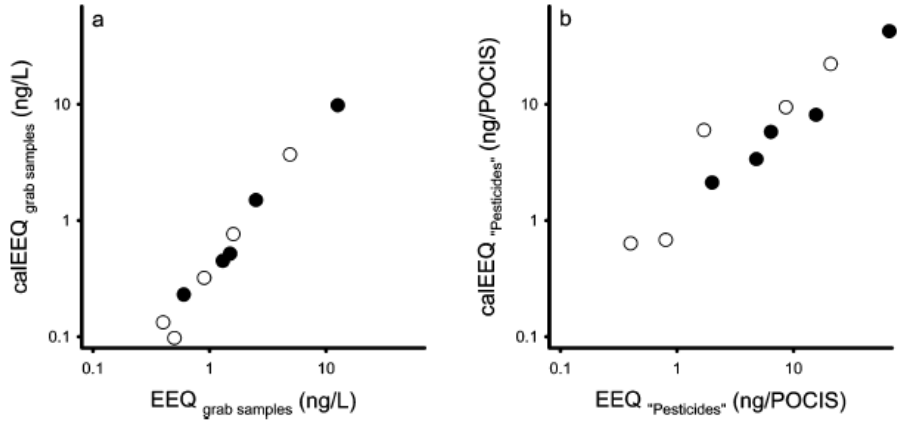

FIGURE 5. Correlation between estrogenicity data obtained with the in vitro assay (17β-estradiol equivalents, EEQ) and calculated estrogenicity (calEEQ), based on chemical analysis of steroidal estrogens, for both active samples (average of 3 grab samples; left graph) and passive samples (POCIS pesticides; right graph). Sampling sites immediately upstream from a sewage treatment works are shown as open circles, and downstream sites are shown as filled circles.

grab samples where 17β-estradiol was found (i.e., samples from the river Sissle; >0.7 ng/L) the ratio of estrone and 17β-estradiol was identical in active and passive samples (estrone/17β-estradiol: upstream, 4:1; downstream, 12:1).

At the sites Fricktal and Oberwynental upstream estrogenicity was clearly elevated compared to the near background levels observed at Lützelmurgtal and Oberes Surbtal (Table 1). This is likely the result of discharges by the additional STWs that are present upstream from Fricktal and Oberwynental (see Figure 1).

Mini Caging: Bioaccumulation of Estrogens and Vitellogenin Induction in Male Fish. Survival of the fish was 100%; moreover, the fish were in excellent condition at the end of the trial. Their condition factor was reduced compared to the initial control (from 0.99 ± 0.01, n = 10, to 0.90 ± 0.01, n = 30) but similar to that of wild fish (0.92 ± 0.02, n = 23) caught in these rivers during an earlier study (*11*). Most fish had been feeding (visible stomach contents) and none showed any sign of damage or disease.

EEQ values in the bile reached 500 ng/mL in male fish and 646 ng/mL in females. Average male and female bile EEQ values were highly correlated (r_P = 0.99, P = 0.001, n = 5) and associated with EEQ values from the POCIS pesticides (r_P = 0.88, P = 0.048, n = 5; Figure 6) and grab samples (r_P = 0.93, P = 0.024, n = 5).

Average plasma vitellogenin concentrations was highest at the STW where EEQ was highest (STW Fricktal, males, 8.3 ± 2.9 μg/mL; females, 39.3 ± 15.4 μg/mL). At the other 4 STWs, male plasma vitellogenin concentrations were below 1 μg/mL (females: <4 μg/mL). Male plasma vitellogenin concentrations and EEQ values of POCIS pesticides samples were not significantly correlated (Figure 7; r_P = 0.73, P = 0.16, n = 5); male plasma vitellogenin concentrations and EEQ values of bile (internal exposure) were also not significantly correlated (r_P = 0.85, P = 0.07, n = 5).

Discussion

Our experiments show that POCISs are integratively sampling environmental estrogens and provide data similar to those obtained with repeated grab samples. This makes POCISs particularly useful in ecosystems that experience very dynamic hydrological conditions and where it is otherwise difficult to assess the estrogenic exposure of wildlife. Moreover, POCISs appear to accumulate estrogens in a way very similar to the brown trout. Taken together this makes

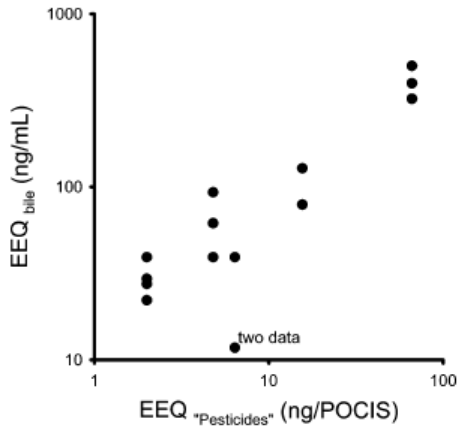

FIGURE 6. Correlation between estrogenicity of river water (POCIS pesticides) and bile from male fish caged at 5 sites downstream from sewage treatment works' discharges. Each point signifies 1 male; estrogenicity data were obtained with an in vitro assay (17β-estradiol equivalents, EEQ).

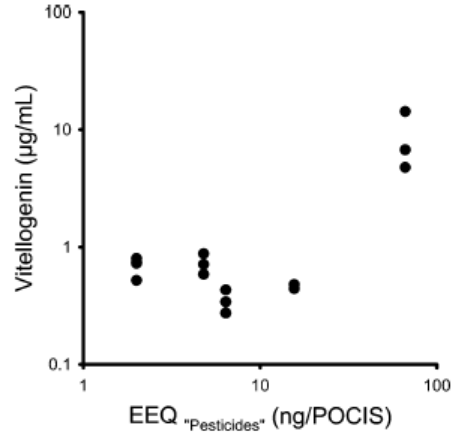

FIGURE 7. Correlation between estrogenicity of river water (POCIS pesticides) and plasma vitellogenin concentrations in male fish caged at 5 sites downstream from sewage treatment works' discharges.

the POCIS a biologically meaningful sampling tool with respect to environmental estrogens that originate from STW with mainly domestic inputs (i.e.. the main estrogenic input being steroidal estrogens). Furthermore, the brown trout mini-caging method provided excellent results in that we observed no mortality and all fish emerged in healthy condition after a 3-week exposure period.

Preference for POCIS Pesticides for Future Studies. The 2 types of POCIS provided very similar results, except for 1 sampling site (i.e., STW Fricktal) where the POCIS pharmaceuticals result appears attenuated. When the 2 data sets are regressed without this 1 point the relationship between POCIS pesticides and POCIS pharmaceuticals is almost 1:1 (actual slope is 0.97 with the intercept forced through 0). We do not know why the POCIS pharmaceuticals EEQ result appears to be attenuated at this particular downstream sampling site. The 2 duplicates gave almost identical results in the YES (EEQ, 17 and 25 ng/POCIS pharmaceuticals; Table 1) as well as with chemical analysis (calEEQ, 16 and 20 ng/POCIS pharmaceuticals); so a sampling or sample processing error seems unlikely. Interestingly, in a preliminary test of the 2 POCIS phases we also observed this attenuation for the pharmaceuticals POCIS type (unpublished data). However, for that test—single POCIS at 2 upstream and 2 downstream sites—we had neither grab sample nor fish data to compare with the passive sampler data.

Data from grab sampling as well as the bioaccumulation data support the POCIS pesticides data more than those of the POCIS pharmaceuticals. On the basis of these observations (and the preliminary trial) we selected the POCIS pesticides for further studies; in the remaining discussion we will mainly focus on data obtained with this POCIS type.

Integration of Environmental Estrogens on POCISs. EEQ amounts eluted from the POCISs showed a very good association with average EEQ concentrations in the grab samples, though EEQ concentrations from the grab samples were roughly a third of the amount of EEQs eluted from POCIS. Comparable relationships were obtained for calEEQ. This accumulation of chemicals from the water into the POCISs is obviously a desired aspect of a passive sampler. This feature is particularly well explored for SPMDs, where the accumulation rate is dependent on factors such as the log K_{OW} and the size and the polarity of the chemical (*26*). To our knowledge, there are no data yet on the sampling efficiency and sampling rates of estrone and 17β-estradiol by POCISs (the steroidal estrogens that we could measure in our samples)—although some data are available for herbicides (*12*; and see *27* for data on 17α-ethynylestradiol—a compound we did not find in our samples). Average EEQ levels in the passive samplers were approximately 3-fold higher than average EEQ ng/L values in the active samples. This would indicate that the POCIS had sequestered estrogens from about 3 L of water over the 22 day sampling period (0.14 L/d). However, we cannot judge from our data set if the uptake rate is continuous or changes over time and if the uptake of estrogens is in any way dependent on temperature (as with SPMDs, *28*) or other factors such as pH or organic matter content of the water. To be able to extrapolate ng/POCIS values to whole water concentrations (ng/L), these aspects would need to be established under field and experimental conditions (e.g., stirring/flow rate, *12*) but are beyond the scope of the work presented here.

In our previous study (*11*) we observed that EEQ values are highly variable at a given site. Therefore we were somewhat surprised to find such a good relationship between the active and passive samples—we expected a much higher variability for the active sampling data. One reason the results were better than expected may be that the average coefficient of variation (CV) of the EEQ of active samples (41%) was much lower compared to our earlier study. In our previous data set the average CV for these 5 rivers was 58% (winter 2003; $n = 5$ rivers sampled 3 times) and 85% (summer 2003; $n = 4$ rivers sampled 3 times, Oberwynental was not sampled in summer; *11*). Therefore, given that in the present study the variability was 30−50% lower, the average of 3 grab samples may be a good reflection of actual average EEQ levels and thus correlate well with EEQ values from passive samples.

Chemical Analysis Confirms the Presence of Steroidal Estrogens and In Vitro Assay Data. Previous work in our laboratory showed a good correlation between YES data and chemical analysis of steroidal estrogens (*29*; $r^2 = 0.60$, n = 70). Our current data set is smaller but shows a much better association between EEQ and calEEQ ($r^2 > 0.85$), both for the water grab samples and the POCIS samples. We think that our improved results may originate in a more uniform analysis of the samples in the YES (all samples were analyzed in 1 assay and they were compared at the same sample dilution). Furthermore, we measured both EEQ and calEEQ in the same sample and not in samples from 2 independent enrichments. These aspects probably reduced method-derived variability in our data sets.

We mainly found estrone in both types of POCIS and in the water grab samples; amounts of 17β-estradiol were often

low or undetectable (17α-ethynylestradiol was not found). Where we detected both steroids, both water sampling methods gave similar steroidal estrogen signatures (predominantly estrone). It is interesting to note that the only other study on steroidal estrogens in POCIS river water samples reports 17β-estradiol as the major estrogen (*12*). This contrasts clearly with our data. Our observations are substantiated by the fact that it is widely reported that estrone is the main estrogen in STW effluent (*14*, *30*) and river water (*31*), and that our earlier studies always showed higher concentrations for estrone compared to 17β-estradiol.

In our previous study on estrogens in Swiss rivers we observed that EEQ values increase with sample dilution and decrease with incubation time of the assay (*11*). We again saw the dilution effect in the grab water samples collected in this trial but the LC/MS/MS data show that the results obtained with the YES are very reliable, at least in relative terms (Figure 5). The dilution effect observed with grab samples was much smaller or absent with either POCIS or bile samples. We sometimes observed suppression of the YES signal at high doses of POCIS sample due to toxicity (i.e., cell death; see also *13*), however, at higher dilution all samples tested so far were parallel or very close to parallel with the 17β-estradiol standard in the YES. This facilitates the interpretation of YES results considerably. Also, the fact that there are more estrogens present in a POCIS sample than in a 1-L water sample facilitates their measurement in the YES (i.e., one can dilute POCIS samples further without running into problems with the detection limit in the YES) and chemical analysis.

For both the grab water samples and the passive samples the steroidal estrogens (calEEQ) did not quite reach the EEQ values obtained with the YES. It remains to be seen if this difference was merely caused by the use of 2 different methods to analyze the same sample; as discussed earlier, it is difficult to obtain absolute EEQ values, particularly for grab water samples (Figure 2; *11*). However, it could be that our samples contained compounds that were active in the YES but that were not analyzed in the LC/MS/MS (*23*).

Advantages of Mini Cages for Field Trials using Salmonids. The caging technique provided exceptional results—no fish were lost and the health status of the fish was excellent. Although we cannot assess the deleterious affect of confinement in terms of stress (*32*) or reproductive physiology (i.e., confinement can cause a decrease in plasma vitellogenin concentrations; *33*), in terms of survival and physical appearance mini caging appears to be a significant improvement over established caging methods where mortality is a common problem (*16*, *18*, *19*). This success may lead to a reduction in required animals, as one does not need to cover for the loss of testing animals. Given the good health status of the fish the data may also be more biologically meaningful (e.g., variability in our bile EEQ and plasma vitellogenin data was fairly low). Also, sampling from mini-caging is very quick and only the fish in the sampled cage is disturbed—these aspects further enhance data from mini-caging, particularly with respect to fast responding biomarkers (e.g., mRNA, hormones). One further positive aspect of mini cages is that the resulting data are somewhat more independent than when a group of fish at a site reside in a single large cage. In statistical terms, data from fish in a single cage can only be taken as a single average data point, regardless of the number of animals that make up the average. With respect to the statistical analysis for our study we also treated the data from the mini cages from 1 site as an average (single) data point because the cages were positioned at the same site (*34*). Nonetheless, fish in mini cages will obviously not experience typical cage effects such as disease transmitted by contact or stress and injuries through the presence of particularly aggressive animals or the competition for food. Furthermore, the mini cages can be scattered in an area to further enhance the aspect of "independence", and thus a better indication may be obtained for the average exposure within a certain area rather than at a restricted point site.

Given our positive experiences with the mini caging method we were able to obtain permission for 2 follow-up studies with a 60- and a 30-day exposure regime. These studies were conducted in the summer and we observed that after these trials the health status of the fish was not as good as in the study presented here. It appears that the longer exposure, possibly combined with the higher water temperatures in summer, led to skin lesions on the belly of the fish and damage to the tip of the jaw, particularly in the 60-day trial. Still, mortality in the 60-day trial was also very low (<5%, excluding a site where an algae mass drifted on and covered a set of cages). Despite the health concerns with respect to longer caging periods at higher temperatures, mini caging still appears to be a significant improvement over more traditional caging methods.

Bioaccumulation of Estrogens and Vitellogenin Induction in Male Fish. The EEQ values of bile samples correlated well with the levels of estrogens in the water (Figure 6). Obviously the n of 5 sites in our study is low and the P-value was only just below 0.05 (the limitation to 5 sites—30 fish—was a restriction imposed within the animal license for our trial). The relationship between internal exposure (EEQ of bile) and estrogen-induced effect (vitellogenin) was not significant ($P = 0.07$). Again, also for this analysis we used an n of 5. However, if we follow the same approach as Legler and co-workers (*15*) (i.e., when we use each fish as an individual data point) we observe the same significance for the association between internal exposure and effect (5 locations, $n = 2-4$ fish per location: $r_P = 0.82$, $P = 0.0002$; compare reference *15*, 8 locations, $n = 1-4$ fish per location: $r_P = 0.81$, $P = 0.0002$).

Plasma vitellogenin concentrations in males were generally low and relatively similar within each of the sites. Vethaak and co-workers (*35*) interpreted plasma vitellogenin levels in males exceeding 1 μg/mL as a sign of estrogen-induced effect; only at 1 site did vitellogenin levels in males exceed that level (Figure 7). Approximately 6 months earlier (August 2003) we assessed the estrogenicity of river water (3 samples in 4 weeks) and measured plasma vitellogenin in the plasma of male fish (September 2003; *11*). Then, EEQ values were 40% lower, at the same time feral males had 8-fold higher vitellogenin levels than the caged males in the current study. The results obtained with mini caging (bile EEQ values and male plasma vitellogenin concentrations) confirm this site as an estrogenic hot spot for brown trout. The fact that vitellogenin in caged fish did not reach (or exceed) VTG levels of feral fish—despite apparent higher levels of EEQ—may be a consequence of stress (*33*) or the fact that the exposure period was relatively short (*36*).

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Water temperature affects the vitellogenin expression in juvenile brown trout (Salmo trutta) after waterborne ethinylestradiol exposure? (Chapter 5)

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Abstract

Environmental estrogens have the potential to considerably affect the reproduction and development of aquatic vertebrates by interfering with the endocrine system. In addition to the potential risk of environmental estrogens, increasing water temperatures as a result of global warming has become a serious problem in many rivers and streams. Some rivers frequently reach temperatures that are suboptimal for many salmonid species like the brown trout. Additionally, during the summer, some low mountain range rivers show temperature differences of up to 8°C between day and night. To assess the degree of estrogenic exposure, the analysis of the estrogen-dependent protein vitellogenin (Vtg) is a frequently used biomarker in field studies. Little, however, is known regarding the potential interaction between ambient water temperature and the Vtg production induced by waterborne environmental estrogens. In order to test the influence of temperature on Vtg synthesis, we exposed juvenile brown trout to environmental relevant concentrations of ethinylestradiol (EE_2) and hold them either at low or high temperatures (12°C and 19°C, respectively), but also at temperature cycles of 12°-19°C in order to simulate the field situation. The EE_2 exposure caused a 7 to 74-fold increase of hepatic Vtg mRNA. The synthesis Vtg mRNA was clearly stimulated in fish hold at higher water temperatures (12°-19°C and 19°C, respectively). On the protein level, Vtg showed a similar pattern; the higher the temperature, the higher the concentration of Vtg in the plasma. The experiment further revealed a temperature dependent increasing amount of hepatic estrogen receptor alpha mRNA after exposure to waterborne EE_2. The gene expression of estrogen receptor beta-1 and the glucocorticoid receptor in the liver of EE_2 exposed fish, however, showed no treatment related alterations. In line with observed constant bile cortisol concentrations, our data do not indicate corresponding stress related effects on hepatic Vtg production. The present survey, however, clearly demonstrate that ambient temperature significantly change the estrogen-induced expression of Vtg and therefore have to be considered when interpreting environmental monitoring studies.

Introduction

The increasing inputs of pesticides, pharmaceuticals and industrial chemicals in aquatic ecosystems have generated a global concern regarding potential, sustainable threats to wildlife. These apprehensions have been enforced by a growing literature reporting impaired reproductive and developmental mechanisms in wild populations of molluscs, teleosts, amphibians, reptiles, birds and mammals (Oetken et al., 2004; Edwards et al., 2006; Jenssen, 2006).

During the last decade, growing scientific attention has been focused on the effects of environmental estrogenic compounds on the reproduction of marine and freshwater fish species. A number of these 'environmental estrogens' are known to enter the aquatic ecosystem via sewage treatment works, but also via diffuse inputs such as agricultural run off events from areas treated with pesticides or farmyard manures (Routledge et al., 1996; Okoumassoun et al., 2002; Orlando et al., 2004). Exposure of fish to these chemicals are linked to various abnormalities, such as intersex in gonochoristic species and retardation of gonadal development in combination with reduced fertility, that may affect the integrity of exposed fish populations (Jobling and Tyler, 2003). Given that recent studies in Switzerland, Germany and France provided evidence of environmental estrogens in rivers and streams, detrimental effects on endemic fish species are likely (Aerni et al., 2004; Rutishauser et al., 2004; Vermeirssen et al., 2006). Indeed, observations of intersex in fish species like gudgeon (Faller et al., 2003), whitefish (Bernet et al., 2004) and brown trout (Körner et al., 2005) as well as induction of vitellogenin (Vtg) in male brown trout downstream from STWs (Vermeirssen et al., 2005; Burki et al., 2006; Körner et al., 2007) have been suggested to corroborate this supposition.

Vitellogenins are major precursors of yolk protein in oviparous vertebrates and synthesized in the liver after stimulation of the estrogen receptor by endogenous 17β-estradiol. In juveniles and males, plasma Vtg concentrations are at baseline protein levels or undetectable - though exposure to exogenous estrogens strongly increases hepatic Vtg production. Due to the specific association between estrogens and the induction of Vtg, it has been frequently used as an indicator of estrogen exposure in field and lab studies (Sumpter and Jobling, 1995; Denslow et al., 1999). To date, three different Vtg gene clusters are well described (Vtg-A, Vtg-B and a phosvitin-less Vtg-C) and recent work suggests that additional Vtg isoforms

are likely (Hiramatsu et al., 2006). These genes differed in most instances by less than 3% at the nucleotide level and suggesting they produce indistinguishable protein products (Hiramatsu et al., 2006). Whereas minor differences in the sensitivity of induction between the various Vtg isoforms have been observed, the magnitude of Vtg synthesis between the isoforms may vary considerably (Knoebl et al., 2004; Wang et al., 2005). In minnows, for instance, the isoform Vtg-A showed a 10-fold higher expression levels compared to isoform Vtg-B (Knoebl et al., 2004). In brown trout it is documented that Vtg-A is responsive to estrogens and therefore an appropriate isoform to assess estrogenic exposure (Burki et al., 2006).

In addition to a potential risk of environmental estrogens, global warming has become a severe issue for fish populations worldwide (Caissie, 2006). Recently, scientists in Switzerland reported rising water temperatures in numerous Swiss rivers (Hari et al., 2006). The mean annual temperature of the studied rivers increased approximately ~1.5°C during the last 20 years. During the summer, some Swiss rivers frequently reach the upper temperature optima (around 19°C) for brown trout (Körner et al., 2007). Additionally, water temperature shows a distinct diurnal cycle particularly in lower river stretches, with differences between day and night of up to 8°C (Körner et al, 2007; Fig. 1-A). The implications of rising river temperatures, especially in regard to the sustainability of existing populations, are well documented. In 1996, Rahel and co-workers postulated a noticeable decline of thermally suitable habitats for cold water fish even with a modest increase in temperature. Indeed, increased water temperature resulted in a significant reduction of brown trout habitats in Switzerland (Burkhardt-Holm and Scheurer, 2007). Within their optimal temperature range, the survival and growth of salmonids, however, are often more tolerant to high temperatures than more sensitive endpoints, such as reproduction and development (Jobling, 1997). In female rainbow trout (*Oncorhynchus mykiss*), exposure to high temperatures leads to lower steroid levels, reduced egg production as well as lower survival of developing embryos (Pankhurst et al., 1996). Additionally, elevated temperatures promote susceptibility of fish to various parasites. Salmonids in more than 50% of Swiss rivers are suffering from PKD (proliferative kidney disease; caused by *Tetracapsuloides bryosalmonae*) - a disease whose clinical outbreak is linked with water temperatures above 15°C for more than 2 weeks (Wahli et al., 2007).

In a recent field study, Körner et al. (2007) observed significantly higher Vtg protein concentrations in female brown trout caught in cooler headwaters compared to females from warmer downstream stretches. Elevated temperatures were hypothesized to be the causal agent for the reduced Vtg levels. During natural vitellogenesis, female salmon (*Salmo salar*) respond to high temperatures with a reduction in plasma Vtg compared to fish held at lower temperatures (King et al., 2003). In general, fish respond to a variety of stressors by increasing the release of cortisol from the adrenal gland (Mommsen et al., 1999). Hence, we further hypothesized that the observed reduction in vitellogenin was linked to elevated plasma cortisol. It is known that chronic exposure to stressors – associated with elevated plasma cortisol concentrations - results in a steady suppression of the reproductive system (Wendelaar Bonga, 1997; Mommsen et al., 1999). For instance, cortisol implants that mimic chronic stress caused lower plasma sex steroid concentrations, smaller gonads and reduced plasma Vtg concentrations (Campbell et al., 1994; Berg et al., 2004). Subsequent experiments demonstrated that cortisol related effects on Vtg synthesis were mediated via glucocorticoid receptor pathways (Leitmonier et al., 2000).

Although it is likely that environmental estrogens and temperature individually influence the development and functioning of the reproductive system of fish, the interaction of such biotic and abiotic factors has rarely been studied. Specifically, little is known concerning the interaction between ambient temperature and its effect on Vtg production induced by waterborne environmental estrogens in vivo in any fish species. Such putative temperature related effects could significantly influence environmental risk assessment studies – an issue already discussed in diverse surveys (Sole et al., 2003a; Snyder et al., 2004; Körner et al., 2007).

Therefore, the present study tested the hypothesis that temperature affects the expression of Vtg in brown trout exposed to ethinylestradiol (EE_2). We tested this by exposing brown trout to EE_2 that were held at low (12°), high temperatures (19°C) or a diurnal temperature cycle (12 - 19°C) that simulated the situation in some European rivers. The study not only examined effects on Vtg expression, but also determined temperature-dependent variations in bile cortisol levels and expression of mRNA quantitatively for the steroid receptors linked to reproduction

and stress - namely estrogen receptor alpha (ERα) and beta-1 (ERβ-1) and the glucocorticoid receptor (GR).

Material and Methods

Animal Care

The experiment was conducted in accordance with the Swiss guidelines for experiments on animal use. One year old (1+) immature brown trout (*Salmo trutta fario*, mixed sex) with a mean body weight of 21.82 ± 0.11 g (± standard error of the mean) and average body length of 13.26 ± 0.02 cm were obtained from a local fish farm (Nadler, Rohr, Switzerland) and brought to the MGU fish facility at the University of Basel, Switzerland. After arrival, fish were randomly distributed to flow through tanks (volume = 65 liter; flow rate = 4 liter per hour) with a density of 5 fish/tank and allowed to acclimate at 12°C for 8 days. The photoperiod was maintained in a 12 h light / 12 h dark regime and fish were fed daily with frozen chironomids (~1% of body weight). Each treatment was replicated (2 test tank/treatment; n = 5 per tank).

Treatment

Temperature treatments were generated by housing tanks in 12°C climate chambers and heating tanks to appropriate temperature regime by submersible aquaria heaters. The estrogen 17α-ethinylestradiol (SIGMA-Aldrich, Switzerland) was dissolved in 99.9% ethanol (SIGMA-Aldrich, Buchs, Switzerland); the stock solution was stored in a refrigerator at 4°C and used throughout the study. Dilution of the EE_2 treatment was carried out via computer driven Hamilton diluters (Hamilton, Bonaduz, Switzerland) using activated-charcoal filtered tap water. The three exposure groups were treated with nominal concentrations of 3 ng EE_2/L and assigned to low (12°C, T_{low}-EE_2), diurnal fluctuating (12°-19°C, $T_{fluctuate}$-EE_2) or high (19°C, T_{high}-EE_2) temperature regimes. In addition, one group with the solvent ethanol served as control (12°C, $C_{solvent}$); see also Fig. 1-B). Each treatment had an ethanol concentration of 0.003% v/v. Treatment started on day 1 with exposure of fish to EE_2. Simultaneously with switching on the light (6 a.m.), water in the $T_{fluctuate}$-EE_2 and T_{high}-EE_2 groups were heated with a heating rate of approximately 1°C/h. In the $T_{fluctuate}$-EE_2 group, heater was switched off at 2 p.m. allowed the water to cool down to 12°C (at approximately 6 a.m.), while the T_{high}-EE_2 group remained constant at ~19°C (see Fig. 1-B). The oxygen levels (mg/L), conductivity (µS/cm^2) and pH were measured every three to four days throughout the study. The actual

EE_2-concentration in each tank (n = 4) was measured randomly at four days by means of solid phase extraction (SPE) and chromatography-tandem mass spectrometry (LC/MS/MS) as described by Vermeirssen et al. (2005).

Fish sampling

Fish were anesthetized by tricaine methanesulfonate and blood was sampled via the caudal vein by heparinized syringes (Monovette, Sarstedt, Nürnbrecht, Germany). The plasma samples were centrifuged at 4°C at 10000g for 5 min, transferred to cryogenic tubes, frozen in liquid nitrogen and stored at -80°C. After blood sampling, the fish were killed by a spinal cut and the weight and length determined. Liver and bile were removed; their weights recorded and preserved in RNA*later* at -20°C or -80°C, respectively. The condition factor (CF), gonadosomatic index (GSI) and bile somatic index (BSI) was calculated as follows:

$$CF = (\text{weight} \times 100) / \text{length}^3$$

$$GSI = 100 \times \text{gonad weight} / (\text{body weight} - \text{gonad weight})$$

$$BSI = 100 \times \text{bile weight} / (\text{body weight} - \text{bile weight}).$$

Enzyme linked immunosorbent assay (ELISA)

Plasma Vtg was analyzed by means of a competitive ELISA described in Körner et al (2007). A similar protocol with minor modifications was used to measure cortisol concentration in the bile of fish. Primary antibody (monoclonal mouse-anti-cortisol) and secondary antibody (polyclonal rabbit-anti-mouse IgG, horse-radish peroxidase conjugated) were purchased from Abcam Inc., Cambridge (USA). Ninety-six well plates (Nunc F96 Maxisorp Immuno Plate, Nunc, Wiesbaden, Germany) were coated with 1 ng/well cortisol-3-BSA (cortisol conjugated bovine serum albumin; VWR International, Dietikon, Switzerland) in carbonate buffer overnight at 4°C. Cortisol (SIGMA-Aldrich, Buchs, Switzerland) was dissolved in methanol and diluted in PBS (phosphate buffered saline) to concentrations of 100 ng/ mL to 0,195 ng/ mL cortisol. Bile (20 µL) was treated with snail juice (an enzyme preparation that contains glucuronidase and sulfatase; SIGMA-Aldrich, Buchs, Switzerland) and extracted via C18 Sep-Pak cartridges (Waters, Rupperswil) according to Vermeirssen et al. (2005). The extraction efficiency ranged from 106% to 116%. Samples were tested in duplicate at 3 dilutions, equivalent to 5 µL, 0.5 µL

and 0.05 µL of bile and incubated overnight at 4°C with cortisol antibody (final dilution 1:100'000). On the next day, coated plates were blocked (1% non fat dry milk in PBS), incubated two hours with standard/antibody and sample/ antibody solution and finally incubated with secondary antibody (dilution 1:50'000) for 2 h. The color was developed by adding enzyme substrate OPD (ortho-phenylene diamine) according to a commercial protocol (SIGMA ALDRICH Buchs, Switzerland). The enzyme reaction was stopped by adding H_2SO_4 and measured at 485 nm with an ELISA plate reader (Chameleon, Hidex, Turku, Finland). Samples showed good parallelism with the standard. The linear range of the standard curve was between 0.2 and 5 ng/ml and both inter- and intra-assay CV (coefficient of variation) were 10%.

RNA-Isolation, cDNA synthesis and Q-PCR

Total liver RNA was isolated with a total RNA isolation system (Promega, Madison, WI, USA). Concentrations of extracted RNA were measured by spectrophotometry at 260 nm and quality was checked by gel electrophoresis. Extracted RNA was stored at -80°C until analyzed. Five µg of extracted RNA was used for cDNA synthesis in 20 µl of reaction by means of Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with random 6-mer primer for cloning or one µg of total RNA was used in 20 µl of reaction by iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) for Q-PCR. Complementary DNA (cDNA) for Q-PCR was diluted 5-fold with RT buffer (50 mM Tris-HCl (pH 8.3) 75 mM KCl, 3 mM $MgCl_2$) prior to DNA amplification. In order to design gene-specific Q-PCR primers, sequence information were either taken from gene bank databases (Vtg A and GR) or obtained by means of standard PCR techniques using degenerated primers on a basis of known teleost sequences (ERα, ERβ-1 and MR). All primer information and accession numbers for gene sequences are provided in Table 3. Degenerate primers were used for PCR for cDNA cloning. Two µl of cDNA were amplified by PCR (50 µl) with degenerate primers and Ampli Taq Gold Kits (Applied Biosystems, Foster City, CA, USA). The thermo cycle program included an initial denaturation step of 94 °C (5 min), 35 cycles of 94 °C (1 min), 53 °C (1 min) and 72 °C (1 min) and a final elongation step at 72 °C (10 min). Amplified cDNA were run on agarose gel, and the band, which was expected size, was purified by the Wizard® SV Gel

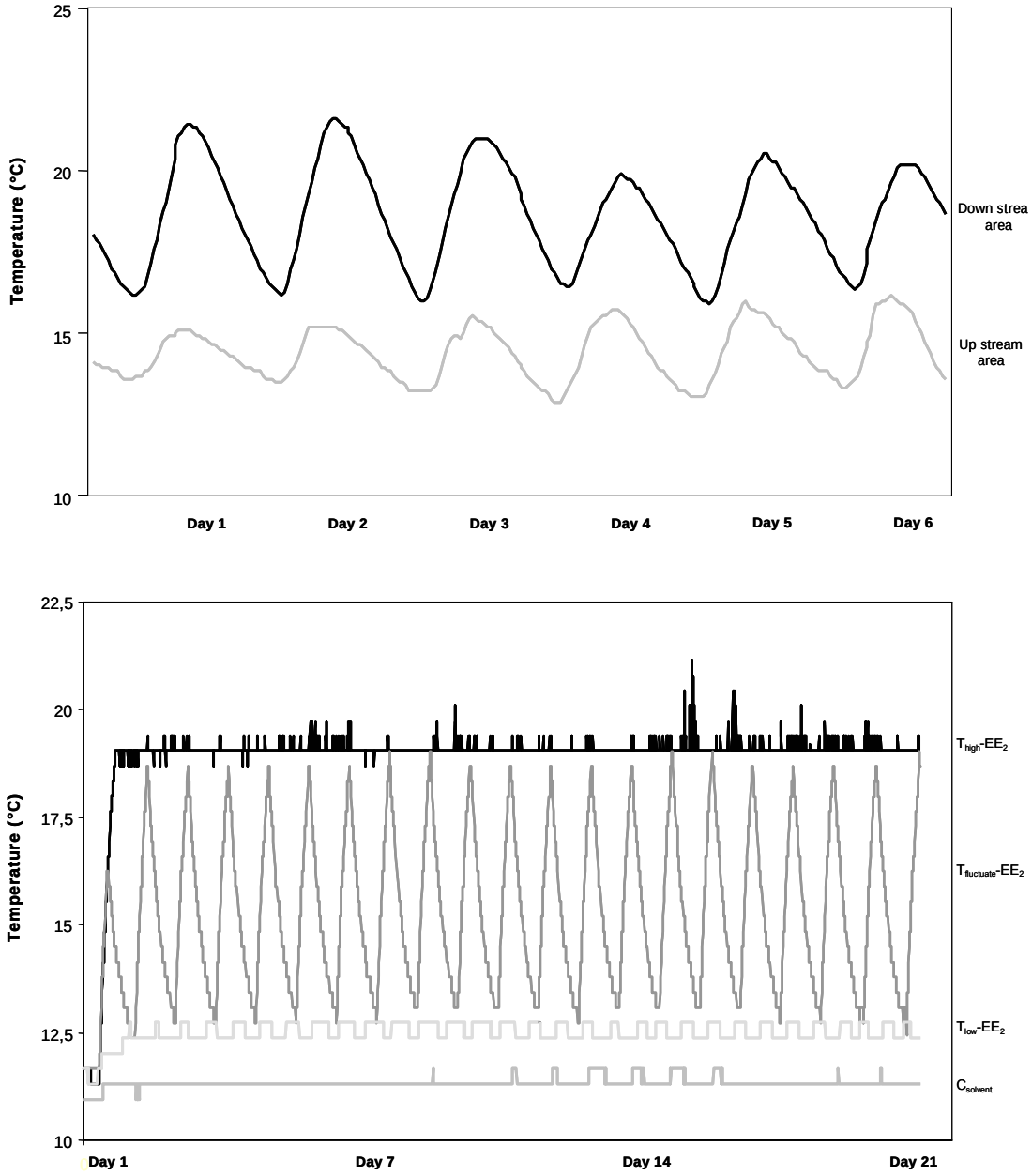
and PCR Clean-Up System (Promega, Madison, WI, USA). Purified cDNA was ligated into vector and transfected into *E. coli* by a pGEM®-T Easy Vector Systems (Promega, Madison, WI, USA). The Plasmid, which contains cloned cDNA fragment, was isolated from the transformed *E. coli* by the Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA). The nucleotide sequences of plasmids were analyzed using the BigDye® Terminator Cycle Sequencing Kits and the ABI PRISM® 3100 Genetic Analyzer provided by Applied Biosystems (Foster City, CA, USA). The homologous sequences for our cloned cDNA fragments were searched by the blast search on NCBI web site (http://www.ncbi.nlm.nih.gov/BLAST). The homologous sequences to teleost ERα, ERβ-1 and ribosomal protein L8 were obtained, and the PCR primers for quantitative real-time PCR were designed by using Primer Express (Applied Biosystems, Foster City, CA, USA).

Quantitative real-time PCR (Q-PCR) was used to determine gene expression of liver Vtg, ERα, ERβ-1 and GR and performed on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Each reaction contained 25 µl homemade SYBR green mix (1x GeneAmp® PCR buffer (Applied Biosystems, Foster City, CA, USA), 3 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4% glycerol, 0.04% Tween-20®, 1% DMSO, 0.2x SYBR Green II (Invitrogen, Carlsbad, CA, USA), 0.01 µM fluorescein, 0.01 U/µl AmpliTaq Gold®), 0.2 µM of each primer and 2 µl of diluted cDNA template. The thermo cycle program for gene targets included an initial enzyme activation step at 95 °C (10 min) followed by 40 cycles of 60 seconds at 95°C (denaturation) and 60 seconds at 61.6° - 66.5°C (annealing, see Table 3). Samples lacking cDNA template were used as negative control. Cycle threshold (C_T) values obtained were converted into copy number using standard plots of C_T versus log copy number. The standard plots were generated for each gene target sequence using known amounts of plasmid containing a template of interest. For each target gene, sample were analyzed in triplicate, averaged and normalized to the ribosomal protein L8 (no significant differences between treatment groups have been observed).

Statistical analysis

Data were tested for normal distribution and homogeneity of variances using Kolmogorov-Smirnov test and Levene test, respectively. Normally distributed data sets were analyzed by one-way ANOVA followed by Bonferroni test for post hoc comparisons. As some data sets could not be transformed to meet the assumption of normality, they were analyzed by non-parametric Kruskal-Wallis rank test or Mann-Whitney U for multiple comparisons. For the statistical comparison of treatments, the solvent control was used as the primary control group and sexes were combined in order to increase the number of samples, if no difference were noted. Significance was set $p \leq 0.05$. Correlations between different parameters were analyzed using Pearson rank correlation coefficient on log transformed data. All statistics were carried out by SPSS for windows (version 13.0.1).

Fig. 1. Daily temperature profiles in (A) upstream and downstream sites of the river Necker during the summer period (August 2003) and (B) in the fish aquaria on the course of the experiments. Temperatures were measured by means of temperature loggers (field data are kindly provided by Eva Schager, Eawag Kastanienbaum)



Results

Chemical and physico-chemical data

Ethinylestradiol concentrations and physico-chemical parameters were, with the exception of oxygen, comparable in each treatment and showed no significant differences (Table 1). Oxygen levels showed significant temperature dependent variations ($p \leq 0.001$); however oxygen saturation was above 90% in all treatments.

Table 1: Measured concentrations of ethinylestradiol (EE_2), oxygen conductivity and pH in the different treatment groups. The sample size of each parameter is n = 8. Data of replicates are pooled and presented as mean ± S.E.M.

	Control	**EE_2 3 ng/L**		
	$C_{solvent}$	T_{low}–EE_2	$T_{fluctuate}$–EE_2	T_{high}–EE_2
EE_2 (ng/mL)	BDL	2.40 ± 0.15	2.12 ± 0.28	2.08 ± 0.04
Oxygen (mg/L)[A]	9.93 ± 0.02	9.09 ± 0.05	8.60 ± 0.02	8.24 ± 0.01
Conductivity (µS/cm²)	358 ± 0.73	354 ± 0.84	358 ± 0.63	354 ± 1.06
pH	7.21 ± 0.02	7.30 ± 0.02	7.23 ± 0.02	7.22 ± 0.02

[A] Nonparametric Kruskal-Wallis test revealed significantly different oxygen levels between treatments ($p \leq 0.001$). These effects, however, are temperature dependent.
BDL = below detection limit of 1 ng/L

Table 2: The morphometric data of brown trout (*Salmo trutta*) from main experiment (n = 10 fish). Data are presented as mean ± S.E.M. Significant differences ($p \leq 0.05$) between treatments are denoted by different uppercase letters.

	Control	**EE_2 3 ng/L**		
	$C_{solvent}$	T_{low}-EE_2	$T_{fluctuate}$-EE_2	T_{high}-EE_2
Weight (g)	21.02 ± 0.68	21.48 ± 0.76	25.04 ± 0.87	22.07 ± 0. 56
Length (cm)	13.27 ± 0.12	13.26 ± 0.14	13.83 ± 0.14	13.20 ± 0.08
CF	0.87 ± 0.01	0.89 ± 0.01	0.91 ± 0.01	0.91 ± 0.02
HSI	1.65 ± 0.02[A]	1.20 ± 0.02[A,B]	1.68 ± 0.04[B]	1.47 ± 0.03
BSI	0.11 ± 0.01	0.17 ± 0.01	0.16 ± 0.01	0.13 ± 0.01

CF = condition factor
HSI = hepato somatic index
BSI = bile somatic index
[A] $p = 0.021$; [B] $p = 0.036$

Effects of treatment on morphometric parameters and indices

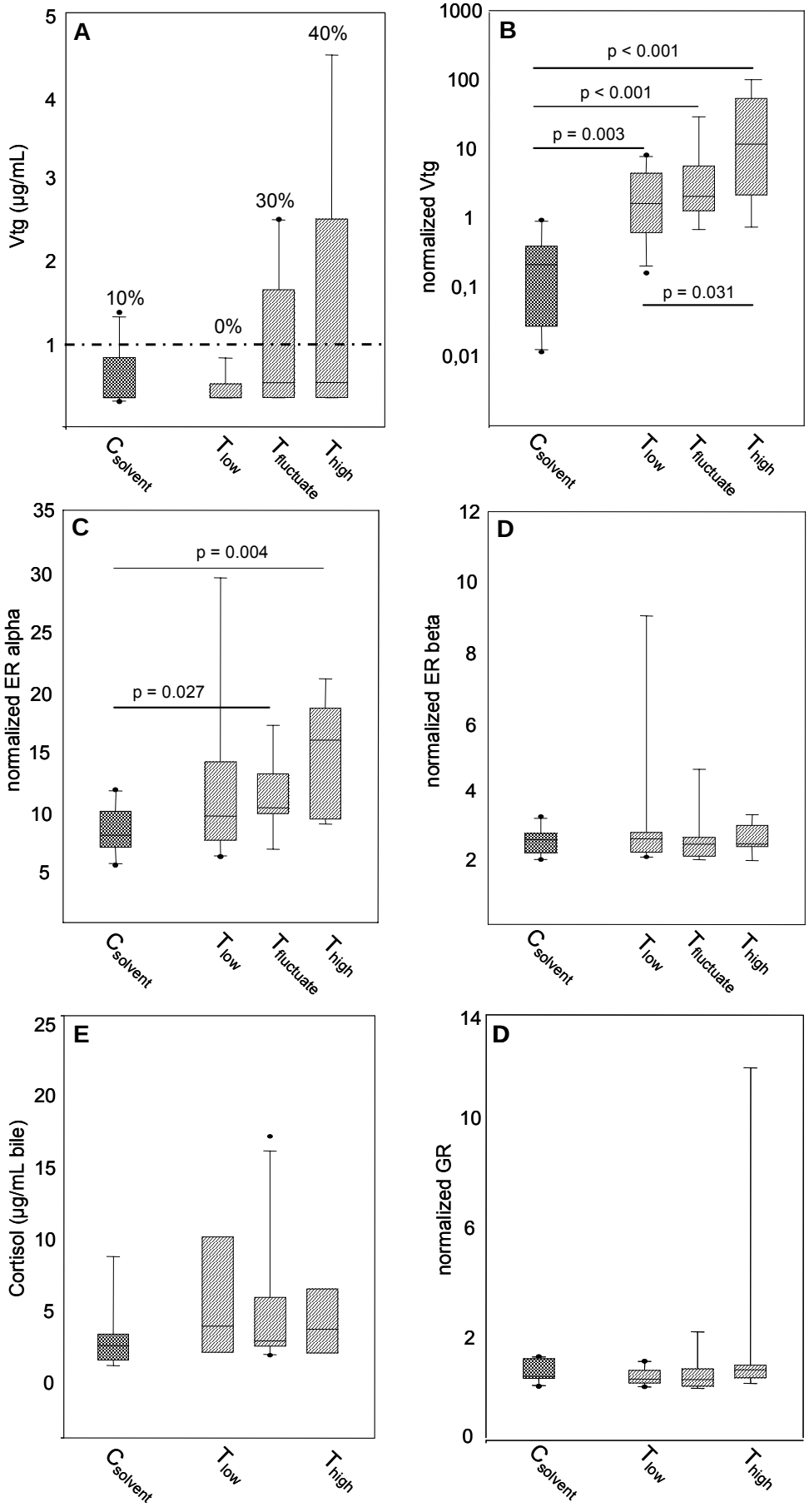
The fish hepatosomatic index (HSI) in the T_{low}-EE_2 group was significantly lower than that observed in the $C_{solvent}$ group ($p = 0.021$) or the $T_{fluctuate}$-EE_2 group ($p = 0.036$; Table 2). No significant effects of treatment on weight, length, condition factor or BSI were observed in the present study (Table 2).

Analysis of Vtg, ERα, ERβ-1, GR and cortisol

Quantitative Real Time-PCR and ELISA techniques were used to evaluate the effects of temperature and EE_2 treatment on plasma Vtg, bile cortisol concentrations and the expression of several target genes in juvenile brown trout. We found no significant differences between immature male and female brown trout in any of the investigated parameters; thus, the data of both sexes were pooled for statistical analysis.

Treatment with EE_2 caused a significant, 7 to 74-fold increase in hepatic Vtg mRNA levels in all EE_2 groups compared to the $C_{solvent}$ group ($p \leq 0.003$ or higher; Fig. 2A). Additionally, water temperature clearly affected the abundance of Vtg mRNA in the liver of EE_2 treated brown trout. This resulted in a significantly higher Vtg mRNA level in the T_{high}-EE_2 treatment compared to the T_{low}-EE_2 group ($p = 0.03$; Fig. 2A). A similar pattern appeared in regard to measured Vtg concentration in the plasma (Fig. 2B). Fish treated with EE_2 and 19°C (T_{high}-EE_2) showed the highest Vtg amount (concentrations ranged from 0.35 - 4.7 µg/mL), but also the $T_{fluctuate}$-EE_2 group reached values up to 2.5 µg/mL. Differences in Vtg protein levels, however, were not statistically different (Fig. 2B). In 21% of all fish, we found plasma Vtg concentrations higher than 1 µg/L, which is generally considered as induced fish. The number of induced fish in the $C_{solvent}$ and T_{low}-EE_2 were comparatively low (10%

Fig. 2; next page. Effects of 17α-ethinylestradiol in combination with different temperature regimes on (A) plasma vitellogenin (Vtg), (B) liver Vtg mRNA, (C) liver estrogen receptor alpha (ERα) and (D) estrogen receptor beta-1 (ERβ-1) mRNA abundance, (E) cortisol titers in the bile as well as (F) hepatic glucocorticoid receptor (GR) gene expression in the brown trout (*Salmo trutta*). The numbers of induced fish (Vtg concentration ≥ 1 µg/mL) are expressed in per cent (A). Gene expression levels (B, C, D and F) were normalized to the ribosomal protein L8. All data are presented as box plots indicating the median, 75th and 95th percentiles. Number of analyzed fish were n = 9 – 10 per treatment; expect for the cortisol data (n = 6 – 10).


A
Vtg (µg/mL)
10%
0%
30%
40%
B
normalized Vtg
p < 0.001
p < 0.001
p = 0.003
p = 0.031
C
normalized ER alpha
p = 0.004
p = 0.027
D
normalized ER beta
E
Cortisol (µg/mL bile)
D
normalized GR
C solvent
T low
T fluctuate
T high

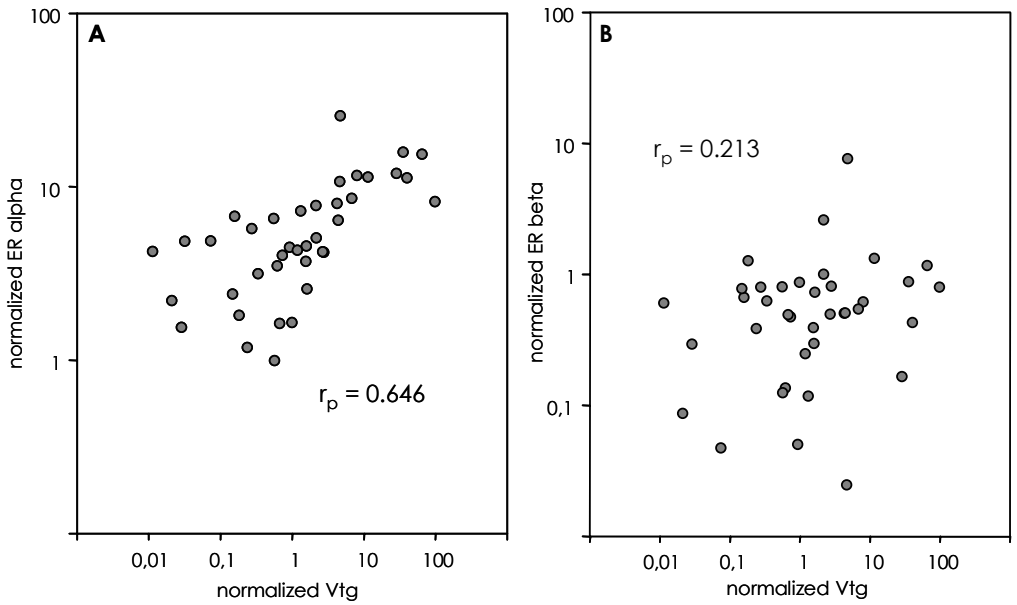


Fig. 3. Pearson correlation coefficient analysis on a logarithmic scatter plots of liver vitellogenin (Vtg) vs. liver estrogen receptor alpha (ERα; r_p = 0.646, p ≤ 0.001, n = 39) and vs. estrogen receptor beta-1 (ERβ-1; r_p = 0.213, p = 0.205, n = 39).

and 0%, respectively), whereas the $T_{fluctuate}$-EE_2 and T_{high}-EE_2 showed an increasing number of induced fish (Fig. 2A). Compared to the $C_{solvent}$ group, hepatic ERα expression was significantly up regulated in the T_{high}-EE_2 and $T_{fluctuate}$-EE_2 groups, but not after EE_2 treatment at low temperature (T_{low}-EE_2; Fig. 2-C). Estrogen receptor beta (ERβ-1) transcription was not affected by any of the treatments and expression were similar among groups (Fig. 2D).

In order to evaluate the association between the expression of the two ER isoforms and the estrogen dependent protein Vtg, a correlation analysis between these parameters were performed using the nonparametric Pearson correlation coefficient method. The scatter plots in Fig. 3A and 3B show log-transformed liver ERα and ERβ-1 gene expression levels versus hepatic Vtg mRNA concentrations. The Vtg and ERα mRNA levels are correlated (r_p [Pearson correlation coefficient] = 0.646, p ≤ 0.001, n = 39), but analysis failed to detect a significant association between Vtg vs. ERβ-1 expression levels (r_p = 0.213, p = 0.205, n = 39).

In some cases, the gallbladder was destroyed during dissection or the content of bile fluid was too low to extract cortisol in an adequate manner. Therefore, numbers of analyzed bile samples were smaller than the fish per treatment. No

significant alterations in bile cortisol concentration were detected among treatments. Median bile cortisol concentrations were around 2.5 µg/mL; some outliners reached concentrations up to 20 µg /mL (Fig. 2E). No variations in liver GR mRNA after EE_2 and temperature treatment were measured (Fig. 2F).

Table 3: Oligonucleotide primers used for cDNA cloning and quantitative Q-PCR.

Name	Direction	degenerate RT-PCR primer (5' to 3')	Q-PCR Primer (5' to 3')	Gene bank accession	Annealing (Q-PCR) T (°C)
Vtg A	Forward Reverse	n.a.	AACGGTGCTGAATGTCCATAG ATTGAGATCCTTGCTCTTGGTC	AY863149	62,9°C
ERα	Forward Reverse	GGCCGACAAGGAGCTGGTNCAYATGAT CGTCCAGCATCTCCAGCARNARRTCRTA	GACATGCTCCTGGCCACTGT TGGCTTTGAGGCACACAAAC	EF589774	61,6°C
ERβ-1	Forward Reverse	CGAGGGCTGCAAGGCNTTYTTYAA CAGGCCCATCATCAGCACYTCNARCCA	TGTGGACCTGTGCCTGTTC ACATGAGCCCTAGCATCAGC	EF589775	66,5°C
GR	Forward Reverse	n.a.	GCTCCTGGCTGTTCCTCATGTCATTCG CATCTGCTCACACTGGTCGGTCATATAGG	AF454750	61,5°C
Ribosomal protein L8	Forward Reverse	GGCATCGTGAAGGACATCATYCAYGAYCC CGAAGGGATGCTCAACAGGRTTCATRGC	TCAGCTGAGCTTTCTTGCCAC AGGACTGAGCTGTTCATTGCG	EF589773	62,9°C

n.a. = not applicable

Discussion

Temperature is one of the most ubiquitous environmental factors that influence aquatic wildlife. Thus, changing temperature profiles within rivers can influence the ecology and physiology of several organisms including invertebrates, small mammals and particularly fish species. In the present study, we examined whether temperature stress - measured as a cortisol response - affects Vtg expression in salmonids after waterborne exposure to the environmental estrogen ethinylestradiol. Our experimental design was based on river temperatures commonly found in Swiss midland rivers during the summer, which are usually between 12 - 21°C (Körner et al., 2007). In general, our data do not indicate significant stress (in terms of cortisol) related effects of these temperature regimes on the expression of Vtg. However, our results clearly indicate that temperature affects estrogen-induced Vtg expression. This is consistent with other studies, both *in vitro* (Pawlowski et al., 2000; Kim and Takemura, 2003) and *in vivo* (Korsgaard et al., 1986; Olin and von der Decken, 1989; MacKay and Lazier, 1993) that also demonstrate a positive temperature relationship with Vtg synthesis. The treatment of primary hepatocytes cultures with estradiol (E_2), at different temperatures, resulted in higher Vtg production with increasing temperatures (Pawlowski et al., 2000; Kim and Takemura, 2003). It is known that environmental temperature determines the rate of metabolic and enzymatic reactions. According to the Van't Hoff rule, every 10°C increase in water temperature provokes a 2-3 fold increase in biochemical or enzymatic activity (Reid et al., 1997, Caissie, 2006). Hence, it can be suggested that elevated Vtg levels at elevated temperatures are the result of a higher synthesis rate. Indeed, similar conclusions were made by Korsgaard et al. (1986), Olin and von der Decken (1989) and MacKay and Lazier (1993) in two juvenile salmonid species, salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Injection of E_2 and subsequent maintenance of the fish at elevated temperatures was associated with a significant increase in Vtg mRNA and protein levels compared to E_2 injected fish held at lower temperatures. This finding strongly supports the assumption that higher Vtg expression in the liver is most likely the result of enhanced synthesis at higher temperatures, presumably due to elevated

enzyme activities. The temperature in the present experiment, but also in Korsgaard et al. (1986), Olin and von der Decken (1989) and MacKay and Lazier (1993) were close to or below the upper optimum temperature of the respective salmonid species. This may signify that increasing temperature stimulates Vtg production up to a certain point and that stress related inhibition of Vtg synthesis is restricted to temperatures exceeding a certain threshold. In the Atlantic salmon (*Salmo salar*) for example, King et al. (2003) determined that threshold to be approximately 18°C.

Although the parameters that we measured showed no stress related effects on Vtg expression in estrogen treated fish, temperature can affect the interpretation of field studies (e.g. Burki et al, 2006; Körner et al. 2007). In a recent field survey, Burki et al. (2006) reported significantly elevated Vtg mRNA levels in brown trout caged downstream from sewage treatment work effluents, but failed to observe a similar pattern in respect to Vtg proteins. On the basis of our data, the results obtained in that field study could be linked to low temperature as that study was conducted between September and October, when water temperatures were between 8 and 12°C (Sara Schubert, Eawag Kastanienbaum, personal communication). This is comparable to the lower temperatures (both, solvent and EE_2 groups) used in our study, which were around 12°C. Like the field study, EE_2 treatment at 12°C provoked significantly higher Vtg gene expression compared to the control group, whereas no differences appeared in regard to Vtg protein concentrations. Hence, one can hypothesize that the heterogeneous response of mRNA and protein observed in the field survey is basically the result of low autumnally temperatures.

Hepatic nuclear estrogen receptors are a major target of endogenous estrogens and are activated upon binding of estrogens to regulate the expression of specific genes including Vtg. In teleosts at least three different receptors (ERα, ERβ-1 and ERβ-2) are known, but only two receptors (ERα and ERβ-1) have been characterized in salmonids to date (Hawkins et al., 2000; Pakdel et al., 2000). Although most natural estrogens (e.g. E_2) and various environmental estrogens (e.g. nonylphenol or EE_2) are potential agonists and/or antagonists of both isoforms, ERα and ERβ-1 do not equally contribute to the gene transcription of

Vtg. Our data demonstrated a significant relationship between ERα and Vtg expression in the liver, whereas no relationship between ERβ-1 and Vtg was found. This is in line with findings of Meucci and Arukwe (2006), who reported a linear relationship between Vtg and ERα, but not between expression of Vtg and ERβ-1. These studies suggest that ERα likely plays a major role in the regulation of Vtg gene expression after exposure to estrogenic substances.

The expression pattern of liver ERα and ERβ-1 exhibited high variation after exposure to EE_2 and its co-treatment with different temperature regimes. For instance, liver ERα mRNA levels in both high temperature EE_2 groups (T_{high}-EE_2 and $T_{fluctuate}$-EE_2) were significant higher than in the control group. However, EE_2 neither alone nor in combination with different temperatures, significantly changed the expression level of ERβ-1 in the liver of brown trout. A similar variation in the expression of ERs after treatment with estrogens has been recently described in several fish species (Sabo-Attwood et al., 2004; Filby et al., 2006; Meucci and Arukwe, 2006). In largemouth bass (*Micropterus salmoides*) as well as in the fathead minnow (*Pimephales promelas*), E_2 caused ERα up-regulation, while ERβ-1 expression remained unchanged (Sabo-Attwood et al., 2004; Filby et al., 2006). As a consequence of nonylphenol exposure, Atlantic salmon respond with a dose dependent increase in ERα mRNA in contrast to down regulation of ERβ-1 expression (Meucci and Arukwe, 2006). Differences in the expression pattern could be linked to distinct differences in the molecular structure of the receptor's promoter or in recruiting cofactors. It is generally assumed that the promoter sequence of ERα in fish contains a functional ERE (estrogen response element) that enables auto-regulation of the receptor (Petit et al., 1999). Molecular characterization of zebra fish ERβ-1 observed that its promoter lacked an ERE and therefore disabling an estrogen dependent auto regulation (Lassiter et al., 2002). It is unknown whether a similar disparity in the promoters of salmonid ERα and ERβ-1 exist, but it could provide an explanation for our observations. In contrast to the well described differences in the expression of ER isoforms following treatment with estrogenic substances, little information is available regarding effects linked to varying temperatures and estrogen treatment. MacKay and Lazier (1993) described a positive correlation

between nuclear ER concentrations in teleost liver extracts and the ambient temperature (4, 9 and 15°C) after E_2-injection. Subsequent experiments using the same liver samples, however, revealed a comparable, significant increase in hepatic ER mRNA in all temperature groups (compared to control group) - indicating no clear relationship between ER mRNA and temperature (MacKay et al., 1997). Additionally, MacKay et al. (1997) were not able to discriminate between the various ER isoforms, in which specific variations of the ER isoforms could be partly masked. Future scientific effort should focus not only on temperature dependent expression of ERα and ERβ-1, but also to discover potential post translational modifications on ERs up to functional proteins.

In general, fish respond to stressful conditions (e.g. dramatic temperature changes) with a rapid increase of plasma cortisol concentration in order to readjust physiological homeostasis (Wendelaar Bonga, 1997; Mommsen et al., 1999). Thus, quantification of plasma cortisol is a common method to evaluate acute stress in teleosts. Plasma cortisol concentrations are, however, extremely prone to sampling stress and thereby could cover treatment related effects (Laidley and Leatherland, 1988; Biron and Benfey, 1994). Previous studies have demonstrated that corticosteroid levels in the bile represent a practical indicator of physiological impairments and have, therefore, been recommended for the detection of chronic stress in teleosts (Truscott, 1979; Pottinger et al., 1992). In the present study, neither trout from the high temperature group (T_{high}-EE_2), nor from the fluctuating temperature ($T_{fluctuate}$-EE_2) group had significant elevated cortisol concentrations in the bile. Rather, measured concentrations of deconjugated cortisol were in the same range as those measured in unstressed, control rainbow trout (Pottinger et al., 1992). This finding suggests that the temperatures and EE_2 concentrations applied in our experiment were insufficient to elicit an apparent cortisol response. Indeed, coho salmon *Oncorhynchus kisutch*, that inhabit temperate rivers and streams, showed a distinct cortisol response with diel temperature cycles of 6.5 - 20°C (Thomas et al., 1986). Fish exposed to diel temperature cycles with a lower amplitude (e.g. 9 - 15°C or 8 - 17°C) failed to show such a response. The major genomic pathway for cortisol signaling is mediated by perinuclear GRs and includes various processes such as modulation

of gluconeogenesis, regulation of phosphoenolpyruvate carboxykinase activity or the transcriptional inhibition of fish vitellogenesis (Lethimonier et al., 2000; Boone and Vijayan, 2002). Despite the essential role of GRs in the regeneration of cellular homeostasis after stressful situations, temperature dependent variations of hepatic GR expression have not been studied in detail to date. Cortisol is known to provoke a noticeable increase in GR mRNA in hepatic cell lines after treatment with physiologically relevant cortisol concentrations (Sathiyaa and Vijayan, 2003). In the present study, however, no significant alterations in GR mRNA expression in the liver of brown trout were observed. The unchanged GR mRNA expression in liver in concert with constant cortisol concentrations in the bile could support the hypothesis that the experimental regimes used in this study were insufficient to cause a stress response. Similar to the combinative treatment of trout with EE_2 and temperature, the exposure to EE_2 alone does not alter the production of GR mRNA. This is in line with data recently published by Filby et al. (2006). Waterborne exposure of female fathead minnow to E_2 resulted in significantly altered GR expression levels in the ovary and the pituitary, but not in liver, intestine and gill. Interestingly, immunohistochemical studies in the rainbow trout revealed a consistent appearance of GR in certain neuroendocrine regions of the diencephalon and pituitary (Teitsma et al., 1998; Teitsma et al., 1999) - areas that are already known for their important role in fish reproduction (e.g. regulation of gonadotropins and gonadotropin releasing hormones). This may suggest that some impairments of the hypothalamus-pituitary-gonadal axis are also mediated by brain GR - a hypothesis established for other vertebrates (Gore et al., 2006). The fact, that the GnRH (gonadotropin releasing hormone) promoter region of salmon contains a putative glucocorticoid responsive element could support this theory (Klungland et al., 1992; Higa et al., 1997). Such interactions, however, must not be limited to environmental estrogens, but expanded to include other pollutants found in the environment (Heberer, 2002; Baldigo et al., 2006). The pharmaceutical salicylate, for instance, leads to a significant down regulation of brain GR expression in the rainbow trout (Gravel and Vijayan, 2006). Furthermore, Aluru et al. (2004) reported a significant down regulation of brain GR mRNA after treatment to Aroclor 1254 - which is a highly

toxic PCB (polychlorinated biphenyl). Nevertheless, to tie specific interactions between environmental pollutants on GR expression in the brain and reproductive impairment on higher biological levels, further research is needed.

The present study aimed to assess the effects of static or fluctuating temperature variations on estrogen-induced expression of Vtg, ERα, ERβ-1, GR and steroidal stress hormone cortisol. We observed a clear interaction between temperature, the environmental xenoestrogen EE_2 and the expression of various genes, which are involved in some aspects of brown trout reproduction. As a consequence, temperature (among other environmental factors) must be taken into consideration during future environmental monitoring studies in order to avoid possible misinterpretations of the exposure situation.

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Combined effects of different temperature regimes and environmental estrogens on the gills of freshwater brown trout Salmo trutta (Chapter 6)

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"It isn't pollution that's harming the environment. It's the impurities in our air and water that are doing it."
(*George W. Bush Jr, 1946*)

Abstract

Changing water temperatures alters the permeability of the gills and result in a disturbed mineral balance in fish. The branchial sodium pump (Na^+/K^+–ATPase) enables teleosts to cope with such varying environmental conditions and compensates for the temperature–related loss of ions by active ion uptake from the ambient water. Estrogens have the potential to interfere with the endocrine regulation of Na^+/K^+–ATPase and may affect the molecular expression of sodium pump mRNA and related steroid receptors (mineralocorticoid and glucocorticoid receptor). In the light of a recently observed warming of Swiss rivers as well as the occurrence of estrogen-active chemicals in river water, such interactions may have detrimental effects on the general health of brown trout in Switzerland. To test the influence of temperature on the regulation of Na^+/K^+–ATPase we exposed juvenile brown trout to ethinylestradiol (EE_2) and hold them either at low or high temperatures (12°C and 19°C, respectively). We also maintained them at temperature cycles of 12°-19°C to simulate the differences between day and night. Data obtained from quantitative PCR evidenced a significant down regulation of Na^+/K^+-ATPase gene expression in gills from estrogen–treated brown trout held at low and fluctuating temperature. However, the expression of Na^+/K^+–ATPase in estrogen-treated fish held at high temperatures were not significant lower than the control groups – indicating a response to the elevated water temperatures. No alterations of the number of immunoreactive chloride were found. Though, estrogen treatment tends to reduce the Na^+/K^+–ATPase protein abundance in the gills. The synthesis of mineralocorticoid receptor mRNA correlated significantly with the expression of Na^+/K^+–ATPase. In contrast, bile cortisol levels and the glucocorticoid receptor gene expression were not affected by estrogen treatment alone or in combination with different temperatures. This suggests that the expression of Na^+/K^+–ATPase is probably regulated via the mineralocorticoid receptor. In addition, the lack of cortisol response as well as the absence of effects on higher levels of biological organization (e.g. histology or condition factor) suggests that the experimental regimes used in the present study were insufficient to cause stressful conditions in brown trout.

Introduction

Since the end of the last century, European fishermen and fishery authorities have noticed an alarming catch decline of freshwater fish in numerous rivers and streams all across Europe (Burkhardt-Holm et al., 2005; Keiter et al., 2006). In Switzerland, catches of brown trout (*Salmo trutta*), a native salmonid species in European low mountain range rivers, have declined about 60% over a twenty year period (Burkhardt-Holm et al., 2005). Several hypotheses were put forward to explain the observed catch decline, including temperature changes, chemical pollution or impaired reproduction. To investigate the weight of evidence of these hypotheses, a nationwide project FISCHNETZ was conducted (Burkhardt-Holm et al., 2005). At the end of the five-year project, the study highlighted that not a single factor was responsible for the reduced catch, but rather a combination of various interrelated factors (Burkhardt-Holm and Scheurer, 2007).

In recent years, it has become increasingly evident that man-made activities influencing climate and correspondingly temperature, rainfall and frequency of storms. In particular water temperature affects important processes such as development, growth or reproduction and is a crucial factor in aquatic systems. Brown trout and other salmonid species tend to prefer summer temperatures around 15°C (Crawshaw and O'Connor, 1996). But in Switzerland, yearly mean temperatures in rivers and streams have increased continuously by 1.5°C over the last 25 years (Hari et al., 2006). In some Swiss midland rivers the water temperatures during summer are between 12° and 21°C - but on occasion peaks of 26°C have been reached (Körner et al., 2007). Because upstream migration of fish is most often hampered by artificial barriers (e.g. dams), river warming implies a considerable upstream shift of river zonations to higher altitudes and hence reduces the habitat that is suitable for brown trout (Hari et al., 2006). Additionally, water temperature shows a distinct diurnal cycle particularly in lower river stretches, with differences between day and night of up to 8°C as a result of man-made activities (Körner et al., 2007).

Along with the observed changes in river temperature regimes, water pollution is an important factor that increases stress on aquatic wildlife. Recently a lot of

scientific attention is focused on micro pollutants that interfere with the reproductive system. The contraceptive agent ethinylestradiol (EE_2) is a prominent environmental estrogen that enters the aquatic environment via waste water treatment works (Sumpter and Johnson, 2005). Field surveys provided evidence that rivers may carry concentrations of 3 ng/L EE_2 in some European countries (Williams et al., 2003; Aerni et al., 2004). Such environmental relevant concentrations have been extensively used in the laboratory studies and are known to be sufficient to impair the reproductive fitness of fish (Pawlowski et al., 2004; Parrott and Blunt, 2005). Less attention, however, has been paid to discover interactions between estrogen-active pollutants and additional estrogen-dependent processes like osmoregulation or immunology (Iguchi et al., 2001; Sumpter, 2005; Guillette, 2006).

Teleosts are ectothermic animals and are highly sensitive to thermal changes because various physiological and metabolic processes in fish are linked to environmental temperature. For example, a change in water temperature causes a considerable loss of ions as a result of enhanced passive membranes permeability as well as increased water influx - a condition that consequently leads to impaired ionic balance (Schmidt et al., 1998; Metz et al., 2003). In response to the temperature induced hydromineral disturbances, freshwater fish activates specific enzyme-driven ion transport mechanism in the gills to regain the physiological homeostasis. Several indications suggest that these actions are predominantly mediated by cortisol – the major corticoid stress hormone in lower vertebrates. Firstly, fish respond to dramatic temperature changes with a rapid increase of plasma cortisol concentrations (Mommsen et al., 1999). Secondly, cortisol is known to stimulate proliferation and hyperplasia of branchial chloride cells (McCorrmick, 1990). These cell types are embedded in epithelial layers of the gills and basically responsible for the active uptake of ions (e.g. Na^+, Cl^-or Ca^{2+}) from the ambient water (Sakamoto and McCorrmick, 2006). The responsible enzyme, the branchial Na^+/K^+–ATPase (sodium/potassium-activated adenosine triphosphatase) facilitates the energy dependent uptake of Na^+ ions through membranes. In the past, cortisol has been supposed to act in fish exclusively via glucocorticoid receptor (GR) pathways. The recent detection of

mineralocorticoid receptors (MR) in certain fish species, however, suggests that some corticoid dependent actions (e.g. chloride cell proliferation or gene expression) are potentially mediated via alternative endocrine routes (Colombe et al., 2000; Greenwood et al., 2003). Some studies indeed evidenced that the MR in fish is functional (e.g. Sloman et al., 2001; chloride cell proliferation). Because aldosterone, the general ligand for MR in higher vertebrates, is absent in teleosts, it was considered that cortisol signals both, MR and GR-depending processes (Sloman et al., 2001; Sturm et al., 2005; Prunet et al., 2006). Nothing, however, is known regarding potential interactions of environmental estrogens on the regulation of branchial Na^+/K^+-ATPase expression in the gills of fish which experience different temperature conditions.

The present study explores the interactions between waterborne estrogens and the endocrine regulation of branchial Na^+/K^+–ATPase in brown trout held at temperatures found in Swiss midland rivers during summer. The study not only measured Na^+/K^+–ATPase gene expression, but also determined the temperature–dependent amount of gill mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) mRNA. The concentration of cortisol in the bile was used as integrated measure of stress in fish, but also explores the significance of cortisol in corticoid receptor signaling. Because gene expression only reflects the immediate response of gill tissue to altered environmental conditions, we linked data obtained by quantitative PCR (Q-PCR) with a histological analysis of gill tissue. The histological analysis was performed according to a protocol that was frequently used in Swiss monitoring programs (Bernet et al., 1999; Bernet et al. 2001; Zimmerli et al., 2007).

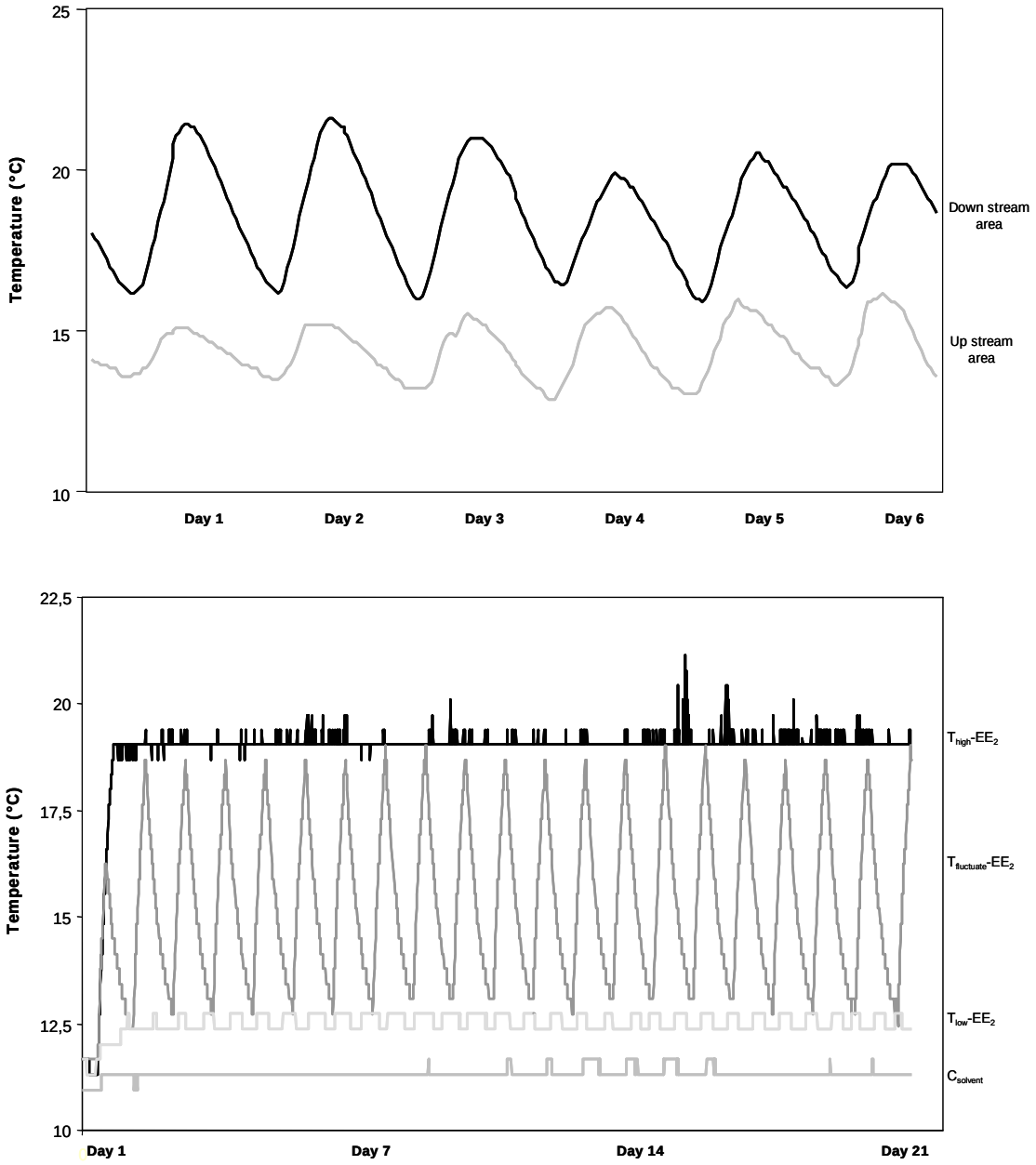
Material and Methods

Animal Care

The experiment was conducted in accordance with the Swiss guidelines for experiments on animals. A mixed sex population of immature brown trout (non-migratory *Salmo trutta*; one year old) with a mean weight of 21.8 ± 5.5 g (± standard deviation) and average length of 13.26 ± 1.1 cm were obtained from a local fish farm (Fischzucht Nadler, Rohr, Switzerland) and brought to the institute's

fish facility at the University of Basel, Switzerland. On arrival, the fish were randomly distributed to flow through tanks (volume = 65 L; flow rate = 4 L per hour; 5 fish/tank) allowed to acclimate at 12°C for 8 days. The photoperiod was a 12 h light / 12 h. Fish were fed daily with frozen chironomids (~1% of body weight). Each treatment was replicated (two test tank/treatment; n = 5 per tank).

Fig. 1. Daily temperature profiles in (A) upstream and downstream sites of the river Necker during the summer period (August 2003) and (B) in the fish aquaria on the course of the experiments. Temperatures were measured by means of temperature loggers (field data are kindly provided by Eva Schager, Eawag Kastanienbaum)



Treatment

Temperature treatments were generated by housing tanks in a 12°C climate chamber and heating of the tanks to the desired temperature regime by submersible aquaria heaters. The estrogen 17α-ethinylestradiol (EE_2, SIGMA-Aldrich, Switzerland) was dissolved in 99.9% ethanol (SIGMA-Aldrich, Buchs, Switzerland); the stock solution was stored in a refrigerator at 4°C and used throughout the study. The dilution of the EE_2 treatment was carried out via computer driven Hamilton diluters (Hamilton, Bonaduz, Switzerland) using activated-charcoal filtered tap water. The three exposure groups were treated with nominal concentrations of 3 ng EE_2/L and assigned to low (12°C, T_{low}-EE_2), diurnal fluctuating (12°-19°C, $T_{fluctuate}$-EE_2) or high (19°C, T_{high}-EE_2) temperature regimes. The fish exposed to ethanol alone (12°C, C_{water}) served as control group. Each treatment had an ethanol concentration of 0.003% v/v. The treatment started on day 1 with exposure of fish to EE_2 and the duration of the experiment was 21 days. Simultaneously with switching on the light (6 a.m.), water in the $T_{fluctuate}$-EE_2 and T_{high}-EE_2 groups was heated with a heating rate of approximately 1°C/h. In the $T_{fluctuate}$-EE_2 group, heating was switched off at 2 p.m. to allow the water to cool down to 12°C (reached at approximately 6 a.m.). Meanwhile, the T_{high}-EE_2 group remained constant at ~19°C. The oxygen levels (mg/L), conductivity (µS/cm^2) and pH were measured every third day throughout the study in each tank. The actual EE_2-concentration in each tank (n = 4) was measured randomly at four days by means of solid phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC/MS/MS) as described by Vermeirssen et al. (2005).

Fish sampling

After 21 days of exposure, the brown trout were anesthetized by tricaine methanesulfonate (SIGMA-Aldrich, Buchs, Switzerland) and killed by a spinal cut. The weight and length of the fish were recorded and the condition factor (CF) calculated using the following formulae:

$$CF = (\text{weight} \times 100) / \text{length}^3$$

The gill arches were removed, preserved in either 4% neutral buffered formaldehyde (Carl Roth, Karlsruhe, Germany) or RNA*later* (Ambion Inc., Austin, USA) and stored at 4°C and -20°C, respectively. The gallbladder was removed *in toto*; the weight of bile was measured, frozen in liquid nitrogen and stored at -80°C until further analysis.

Bile cortisol analysis

Bile cortisol was analyzed using a competitive cortisol ELISA (Körner et al. 2007). The linear range of the standard curve was between 0.2 and 5 ng/ml and the inter- and intra-assay coefficients of variation were 10%. Primary antibody (monoclonal mouse-anti-cortisol) and secondary antibody (polyclonal rabbit-anti-mouse IgG, horse-radish peroxidase conjugated) were purchased from Abcam Inc., Cambridge (USA). To deglucuronidate cortisol metabolites in the bile, samples were treated with snail juice (an enzyme preparation that contains glucuronidase and sulfatase; SIGMA-Aldrich, Buchs, Switzerland) and extracted via C18 Sep-Pak cartridges (Waters, Rupperswil, Switzerland).

RNA-Isolation, cDNA synthesis and Q-PCR

Gill RNA was isolated using a total RNA isolation system (Promega, Madison, WI, USA). Concentrations of extracted RNA were measured by spectrophotometry at 260 nm and quality was checked by gel electrophoresis. Extracted RNA was stored at -80°C until analysis. Five µg of extracted RNA was used for cDNA synthesis in 20 µl of reaction by means of Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with random 6-mer primer for cloning. For Q-PCR, 1 µg of total RNA was used in 20 µl of reaction using the iScript cDNA synthesis kit from Bio-Rad (Hercules, CA, USA).

Complementary DNA for Q-PCR was diluted five-fold with RT buffer (50 mM Tris-HCl (pH 8.3) 75 mM KCl, 3 mM $MgCl_2$) prior to DNA amplification. Gene-specific Q-PCR primers were designed using sequence information taken from gene bank databases (GR) or obtained by means of standard PCR techniques using degenerated primers based on known teleost sequences (Na^+/K^+-ATPase, MR and ribosomal protein L8). All primer information and accession numbers for gene sequences are shown in Table 3. Degenerate primers were used for PCR for cDNA cloning. Two µl of cDNA were amplified by PCR (50 µl) with degenerate primers and Ampli Taq Gold Kits (Applied Biosystems, Foster City, CA, USA). The thermo cycle program included an initial denaturation step of 94 °C (5 min), 35 cycles of 94 °C (1 min), 53 °C (1 min) and 72 °C (1 min) and a final elongation step at 72 °C (10 min). Amplified cDNA were run on agarose gel, and the band with expected size was purified by the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Purified cDNA was ligated into a vector and transfected into *E. coli* by a pGEM®-T Easy Vector Systems (Promega, Madison, WI, USA). The plasmid, which contains cloned cDNA fragment, was isolated from the transformed *E. coli* by the Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA). The nucleotide sequences of plasmids were analyzed using the BigDye® Terminator Cycle Sequencing Kits and the ABI PRISM® 3100 Genetic Analyzer from Applied Biosystems (Foster City, CA, USA). The homologous sequences for cloned cDNA fragments were searched by the blast analysis on NCBI web site (http://www.ncbi.nlm.nih.gov/BLAST) and results are shown in Table 4. Homologous sequences for Na^+/K^+ATPase, MR and ribosomal

protein L8 were used to designed Q-PCR primers using Primer Express (Applied Biosystems, Foster City, CA, USA).

Quantitative real-time PCR (Q-PCR) was used to determine gene expression of gill Na^+/K^+ATPase, MR as well as GR and performed on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Each reaction contained 25 µl homemade SYBR green mix (1x GeneAmp® PCR buffer (Applied Biosystems, Foster City, CA, USA), 3 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4% glycerol, 0.04% Tween-20®, 1% DMSO, 0.2x SYBR Green II (Invitrogen, Carlsbad, CA, USA), 0.01 µM fluorescein, 0.01 U/µl AmpliTaq Gold®), 0.2 µM of each primer and 2 µl of diluted cDNA template. The thermo cycle program for gene targets included an initial enzyme activation step at 95 °C (10 min) followed by 40 cycles of 60 seconds at 95°C (denaturation) and 1 min at 61.6° - 66.5°C (annealing, see Table 3). Samples lacking cDNA template were used as negative control. Cycle threshold (C_T) values obtained were converted into copy number using standard plots of C_T versus log copy number. The standard plots were generated for each gene target sequence using known amounts of plasmid containing a template of interest. Samples were analyzed in triplicate for each target gene, averaged and normalized to the ribosomal protein L8 that has been recently used as house keeping gene (e.g. Kristensen et al., 2007; Körner et al., 2007).

Histology and Immunohistochemistry

The gills of five fish from each treatment were processed according standard protocol embedded in paraffin wax and sections were made at a thickness of 5 µm (Romeis, 1986). The gills were orientated in a standardized way to obtain cross-sections with a most extensive surface area. The sections were deparaffinized and stained with haematoxylin and eosin (Romeis, 1986). The histopathological alterations of gill tissue were microscopically examined with a Nikon ECLIPSE E400 (magnifications ranged from 20x to 400x) and evaluated by a standardized protocol according to Bernet et al. (2001). Mucous cells were stained by means of alcian blue/PAS (pH 2.5) described in Romeis (1986). Five primary lamellae of each fish gill were randomly selected and photographed with a two– hundredfold magnification. The photographs were used to count the

number of stained mucous cells in an area between 15 secondary lamellae. Immunohistological detection of Na+/K+-ATPase was performed according to Schmidt- et al. (1998). A monoclonal IgG antibody directed against the alpha subunit of Na^+/K+-ATPase were purchased from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, USA) and visualization was achieved by a commercial kit (Histostain®-Plus, Zymed Laboratories, San Francisco, CA, USA). Additionally, the intensity of the immune-response was scored semi-quantitatively (1 = low, Fig. 5-A; 2 = medium, Fig. 5-B; 3: high, Fig. 5-C).

Statistical analysis

The data sets were analyzed by non-parametric Kruskal-Wallis rank test or Mann-Whitney U for multiple comparisons. For the statistical comparison of treatments, sexes were combined if no difference were noted. Significance was set $p \leq 0.05$. Correlations between different parameters were analyzed using Pearson rank correlation coefficient on log transformed data sets. All statistics were carried out using SPSS for windows (version 13.0.1).

Results

Chemical and physicochemical parameters

Ethinylestradiol concentrations and physicochemical parameters were, with the exception of oxygen, comparable between treatments (Table 1). Oxygen levels showed significant temperature dependent variations ($p \leq 0.001$); however oxygen saturation was above 90% in all treatments. Temperature regimes of the different experimental groups are presented in Fig. 1.

Table 1: The measured values (mean ± S.D.; n = 8) of 17α–ethinylestradiol (EE_2), oxygen, conductivity and pH in the eight treatment tanks (n = 4 per tank).

	Control	**EE_2 3 ng/L**		
	$C_{solvent}$	T_{low}-EE_2	$T_{fluctuate}$-EE_2	T_{high}-EE_2
EE_2 (ng/mL)	BDL	2.4 ± 1.2	2.1 ± 2.2	2.1 ± 0.3
Oxygen (mg/L)[A]	9.9 ± 0.1	9.1 ± 0.2	8.6 ± 0.2	8.2 ± 0.1
Conductivity (µS/cm²)	358 ± 6	354 ± 7	358 ± 5	354 ± 8
pH	7.2 ± 0.2	7.3 ± 0.2	7.2 ± 0.2	7.2 ± 0.2

17α-ethinylestradiol concentrations were analyzed according Vermeirssen et al. (2005); oxygen levels, conductivity and pH were measured using commercial measurement devices from WTW, Weilheim, Germany.

[A] Nonparametric Kruskal-Wallis test revealed significantly different oxygen levels between treatments ($p \leq 0.001$). These effects, however, were temperature dependent.

Effects of treatment on morphometric parameters and condition factor

Treatments did not affect the body weight and length and condition factor (Table 2).

Table 2: The morphometric data of brown trout (*Salmo trutta*) treated with ethinylestradiol (EE_2) at various temperature conditions. Data are presented as mean ± S.D. (n = 10). There were no differences ($p \leq 0.05$) between treatments.

	Control	**EE_2 3 ng/L**		
	$C_{solvent}$	T_{low}-EE_2	$T_{fluctuate}$-EE_2	T_{high}-EE_2
Body weight (g)	21 ± 7	21 ± 8	25 ± 9	22 ± 6
Body length (cm)	13 ± 1	13 ± 1	14 ± 1	13 ± 1
Condition factor	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.2

Determination of branchial Na^+/K^+–ATPase, glucocorticoid and mineralocorticoid receptor gene expression and bile cortisol content

We used quantitative RT-PCR (Q-PCR) techniques in order to detect a potential impact of EE_2 exposure on branchial Na^+/K^+–ATPase, GR and MR gene expression at various temperature conditions. No significant differences between immature male and female brown trout were found. Thus, the data of males and females were pooled for statistical analysis.

The treatment of brown trout with the synthetic estrogen EE_2 caused a significant down regulation of Na^+/K^+–ATPase gene expression in both, T_{low}-EE_2 and $T_{fluctuate}$-EE_2 groups compared to the $C_{solvent}$ fish group ($p = 0.007$ and $p = 0.009$, respectively; Fig. 2-A). In contrast, the quantity of gill Na^+/K^+–ATPase mRNA in fish from the $C_{solvent}$ group did not differ from that in fish from the T_{high}-EE_2 group. The expression of branchial Na^+/K^+-ATPase, however, was statistical significantly lower in fish from the T_{low}-EE_2 group compared to fish from group T_{high}-EE_2 ($p = 0.045$, Fig. 2-A). We need to note, that PCR primers used in the present study, were designed to recognize highly conservative regions of the sodium pump (Table 3). Therefore, our Na^+/K^+-ATPase alpha subunit primers did not distinguished between the various isoforms.

Table 3: List of oligonucleotide primers used for cDNA cloning and quantitative Q-PCR.

Name	Direction	degenerate RT-PCR primer (5' to 3')	qPCR Primer (5' to 3')	Gene bank accession	Annealing (Q-PCR) T (°C)
Na^+/K^+ ATPase (alpha subunit) [A]	Forward Reverse	AGAATGACCGTGGCTCACATG GCATTCGGCAAACTGGTCA	GGCTGACACCACAGAGAACCA GCACAGGCCAGCGACTCTAG	EF589776	67.5°C
Mineralocorticoid receptor[B]	Forward Reverse	GCGGAATTATGGTCCGAATG CCATCAGGACATCACTGTTGCT	GGACGAAGAGACCAGCAGGAT GGTCCAGGCCTACGTTGAAC	EF589777	66.5°C
Glucocorticoid receptor	Forward Reverse	n.a.	GCTCCTGGCTGTTCCTCATGTCATTCG CATCTGCTCACACTGGTCGGTCATATAGG	AF454750	61.5°C
Ribosomal protein L8[C]	Forward Reverse	GGCATCGTGAAGGACATCATYCAYGAYCC CGAAGGGATGCTCAACAGGRTTCATRGC	TCAGCTGAGCTTTCTTGCCAC AGGACTGAGCTGTTCATTGCG	EF589773	62.9°C

Blast search on http://www.ncbi.nlm.nih.gov/BLAST revealed following sequence identity:
[A] *Salmon salar*: Na^+/K^+ ATPase alpha subunit isoform 1b (99% identity, AY692143); Na^+/K^+ ATPase alpha subunit isoform 1c (95% identity, AY692146); alpha subunit isoform 1a (94% identity, AY692142)
[B] *Oncorhynchus mykiss*: mineralocorticoid receptor form B (98% identity, AY495585); mineralocorticoid receptor form A (96% identity, AY495584)
[C] *Oncorhynchus mykiss*: ribosomal protein L8 (93% identity, AY957563)
n.a. = not applicable

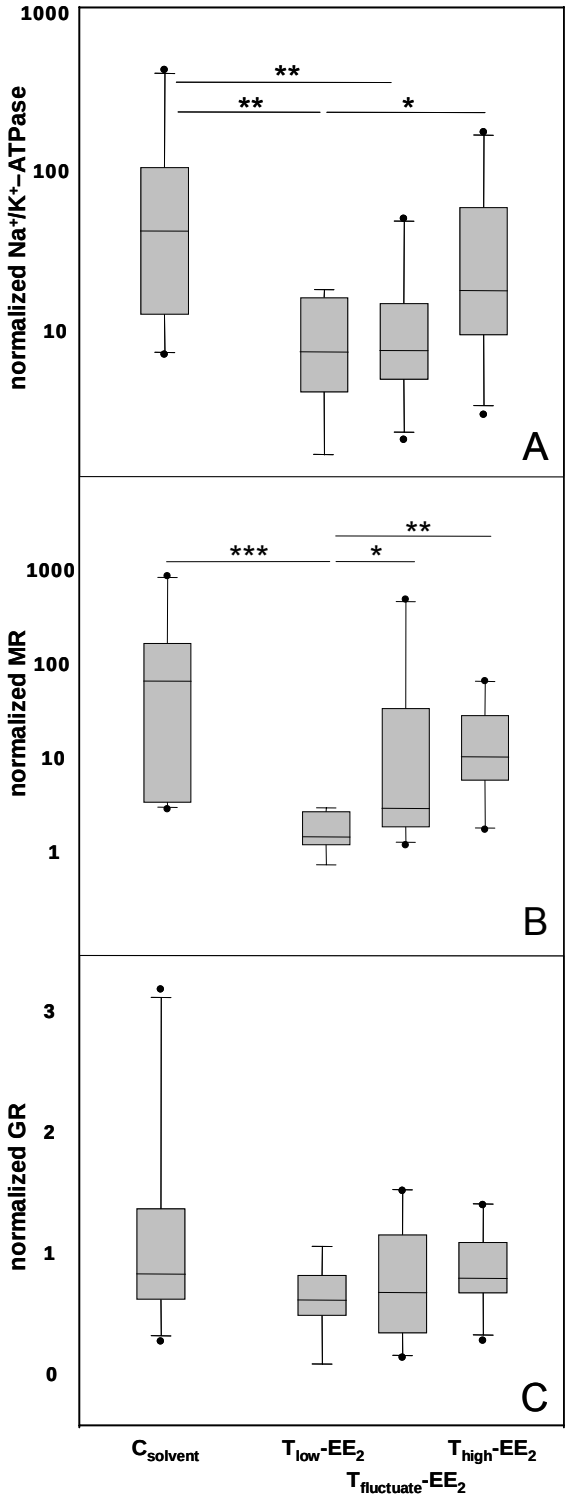


Fig.2 Relative mRNA quantity of sodium–potassium ATPase (Na^+/K^+–ATPase), glucocorticoid (GR) receptor and mineralocorticoid receptor (MR) in gills of 17α–ethinylestradiol treated brown trout held at different temperature conditions. Values were normalized to ribosomal protein L8. All data are presented as box plots indicating the median, 75th and 95th percentiles (n = 6-10). * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ (nonparametric Mann-Whitney U test).

Table 4: Relationship between branchial glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and sodium/potassium-activated adenosine triphosphatase (Na^+/K^+ ATPase) gene expression and cortisol concentration in the bile of brown trout treated with 17α–ethinylestradiol at various temperatures. Relationships were calculated using a bivariate, nonparametric Pearson correlation analysis. The results are presented as Pearson correlation coefficient rp, p value and the number of analyzed samples. Bold marked data sets signify a statistical significant correlation between analyzed parameters.

Parameters	Pearson correlation Coefficient (r_p)	p value	Number of samples
GR vs. MR	0.299	0.065	n = 39
GR vs. Na^+/K^+ ATPase	0.225	0.169	n = 39
MR vs. Na^+/K^+ ATPase	**-0.782**	**≤ 0.001**	**n = 39**
Cortisol vs. GR	**-0.502**	**0.003**	**n = 32**
Cortisol vs. MR	-0.276	0.126	n = 32
Cortisol vs. Na^+/K^+ ATPase	-0.279	0.122	n = 32

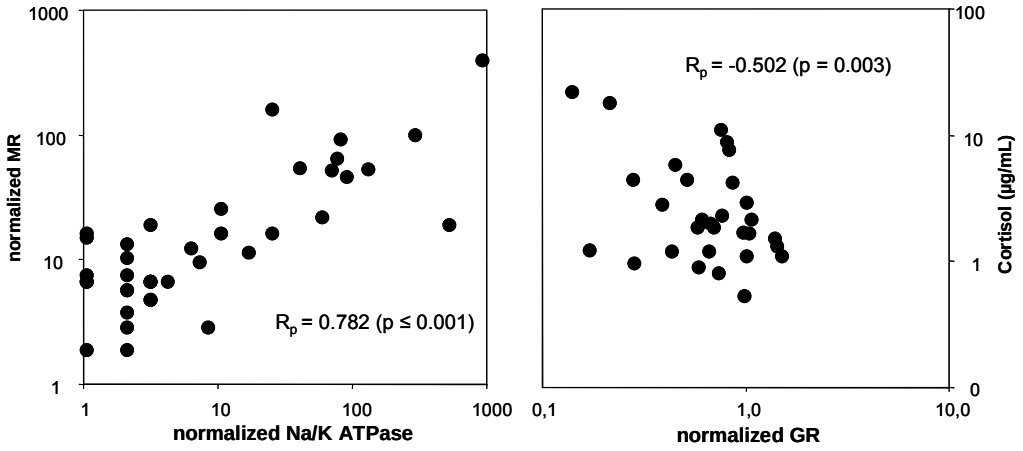


Fig.3. Pearson correlation coefficient analysis on a logarithmic scatter plots of gill mineralocorticoid mRNA (MR) vs. gill sodium-potassium ATPase mRNA (r_p = 0.782, p ≤ 0.001, n = 39) and bile cortisol concentrations vs. GR gene expression (r_p = -0.502, p = 0.003, n = 32). Gene transcription was normalized to ribosomal protein L8.

The treatment of trout with EE_2 at low temperatures resulted in a significant down regulation of MR gene expression in fish from the T_{low}-EE_2 compared to $C_{solvent}$ ($p < 0.001$), but also in comparison to fluctuating and high temperature conditions (T_{low}-EE_2 vs. $T_{fluctuate}$-EE_2, $p = 0.031$; T_{low}-EE_2 vs. T_{high}-EE_2, $p = 0.001$; Fig. 2-B) In contrast, the combination of EE_2 exposure with fluctuating and high temperature treatment did not result in a significant changed MR gene expression levels compared to the $C_{solvent}$ (Fig. 2-B). Branchial glucocorticoid receptor (GR) transcription was not affected by any of the treatments and was similar among groups (Fig. 2-C).

In some cases, the gallbladder was damaged during dissection or the content of bile fluid was too low to extract the cortisol. Therefore, numbers of analyzed bile samples were smaller than the number of analyzed gills. Mean bile cortisol concentrations in estrogen-treated groups (T_{low}-EE_2, $T_{fluctuate}$-EE_2 and T_{high}-EE_2) were slightly, but not significantly higher compared to control group. No significant changes of bile cortisol content in relation to the different temperature regimes were noticed. The bile cortisol levels for most of the analyzed samples ranged between 1 and 10 µg/mL; only three out of 33 samples showed concentrations higher than 10 µg/mL (Table 5).

To evaluate the association between bile cortisol concentrations, the expression of Na^+/K^+-ATPase and both corticoid receptors (GR and MR), we used a Pearson correlation coefficient analysis. The scatter plot (Fig. 3) log-transformed gill MR gene expression levels versus branchial Na^+/K^+-ATPase mRNA concentrations indicates a significant, positive correlation ($r_p = 0.782$; $p \leq 0.001$; $n = 39$) between these two parameters. Furthermore, we found a significant, negative correlation ($r_p = -0.502$; $p = 0.003$; $n = 32$) between the concentration of cortisol in the bile and the GR expression in the gill of brown trout (Fig. 3). This correlation, however, does appear to be mainly related to two data points that comprised cortisol levels of 19 and 23 µg/mL. By excluding these two data points from the analysis, the significance of the relationship disappears ($r_p = -0.337$; $p = 0.065$; $n = 30$). Therefore, the result of this analysis needs to be handled with caution.

Histological analysis

Neither after estrogen treatment alone, nor in combination with fluctuating and elevated temperature condition, a change in the general structure of gill filaments and lamellae was observed. The degree of histological changes was low and pathological alterations were limited to epithelial lifting, hyperplasia as well as epithelial rupture to a lesser extent. The indices of the groups were not statistically different from each other and mean values of $index_{gill}$ ranged from 7.6 ± 2.5 in the T_{high}-EE_2 group to 9.9 ± 1.5 in the $C_{solvent}$ groups (Table 5). An increase in mucous cell number was noted in estrogen treated fish held at fluctuating and constantly elevated temperature ($T_{fluctuate}$-EE_2 and T_{high}-EE_2, respectively), but this increase was not statistically significant (Table 4).

Table 5: Values of histopathological gill index ($Index_{gill}$), the number of mucous cells and the concentration of cortisol in the bile of brown trout treated with 17α–ethinylestradiol (EE_2) at three different temperature conditions. The data are presented as means ± S.D (number of samples). The statistical analysis (nonparametric Kruskal-Wallis test) revealed no significant differences between treatments.

	Control		**EE_2 3 ng/L**	
	$C_{solvent}$	T_{low}-EE_2	$T_{fluctuate}$-EE_2	T_{high}-EE_2
$Index_{gill}$[A]	9.9 ± 1.5 (5)	8.6 ± 1.1 (5)	8.3 ± 1.8 (5)	7.6 ± 2.5 (5)
Mucous cells[B]	9.1 ± 6.7 (5)	8.1 ± 3.1 (5)	13.7 ± 8.3 (5)	11.8 ± 7.1 (5)
Bile cortisol (ng/mL)	2.4 ± 2.4 (8)	6.6 ± 8.1 (9)	4.4 ± 3.0 (6)	3.8 ± 5.0 (10)

[A] Histological gill index was calculated according to a protocol from Bernet et al. (1999).
[B] The number of alcianblue/PAS (pH 2.5) stained mucous cells in an area between 15 primary gill lamellae.

The number of immunopositive chloride cells (labeled with antibody against Na^+/K^+–ATPase alpha subunit) was higher in fish from the $C_{solvent}$ group in comparison to fish treated with estrogens and they contained more immunoreactive Na^+/K^+–ATPase (Fig. 4; Fig. 5 A–C). No differences in degree of immunostaining and the number of immunopositive chloride cells were observed between fish from the T_{low}-EE_2, $T_{fluctuate}$-EE_2 and T_{high}-EE_2 groups.

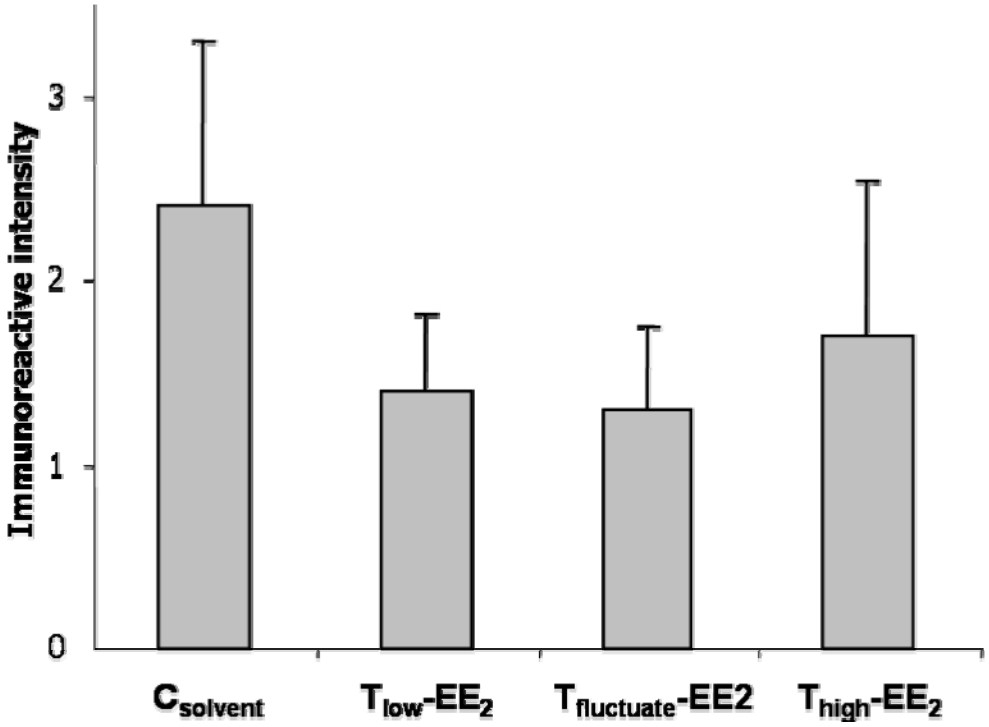


Fig.4. Semi–quantitative analysis of the intensity of Na^+/K^+-ATPase immunostaining in the gills of 17α–ethinylestradiol treated brown trout held at different temperature conditions (mean ± S.D.; n = 5). The intensity was scored according the criteria: 1 = low; 2 = medium; 3 = high. Labelling was done using monoclonal IgG mouse antibody, raised against avian Na^+/K^+-ATPase alpha subunit. There were no differences ($p \leq 0.05$) between treatments.

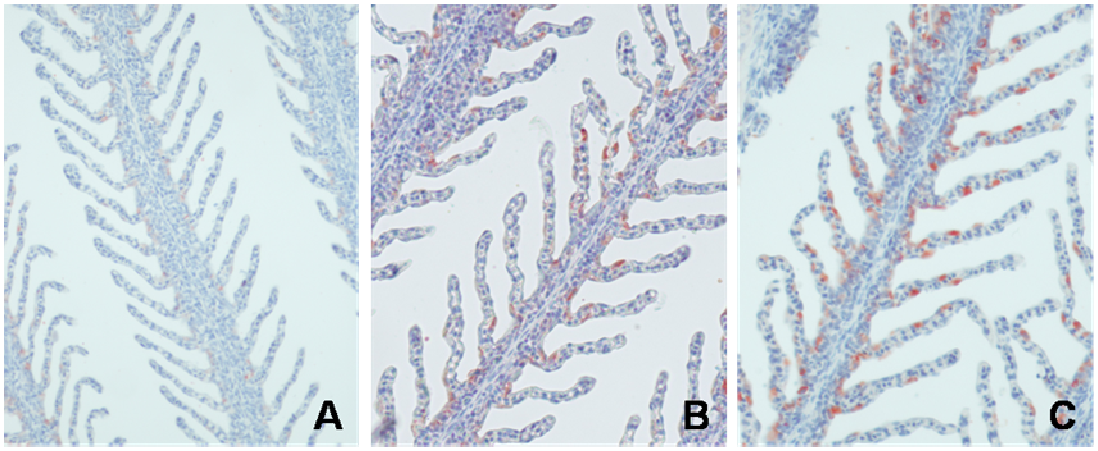


Fig.5. Monographs showing Na^+/K^+-ATPase immunostaining of gills from brown trout. The pictures signify the following scores: (A) low (value = 1) (B) medium (value = 2) and (C) high (value = 3). Labeling was done using monoclonal IgG mouse antibody, raised against avian Na^+/K^+-ATPase alpha subunit; 200x magnification.

Discussion

The ability to cope with varying environmental conditions (e.g. temperature changes) is an important pre-condition of organisms to survive in a dynamic environment. In order to restore the internal homeostasis, fish evolved specific (enzymatic) actions to counteract the disturbed mineral balance after challenging stress events. The present study surveyed the question, whether waterborne exposure to the environmental estrogen EE_2 affects the regulation of branchial Na^+/K^+–ATPase, the major gill enzyme to maintain mineral balance in brown trout.

In general, our data demonstrate a significant down regulation of Na^+/K^+–ATPase gene expression after waterborne treatment of brown trout with estrogens at low but also at fluctuating temperature conditions. This is in line with earlier findings in Atlantic salmon (*Salmo salar*) and sockeye salmon (*Oncorhynchus nerka*) after repeated injections of estradiol (E_2) and 4-nonylphenol (Madsen et al., 2004; Luo et al., 2005). In both studies, the transcription of Na^+/K^+–ATPase alpha subunit mRNA decreased after estrogen treatment, indicating an involvement of estrogens in the regulation of osmoregulative gill function. Indeed, recent work done by Luo et al. (2005) and unpublished work by Madsen et al. (referred in Bangsgaard et al., 2006) provides evidence of ERα and ERβ gene expression in salmon gill tissue - though the expression level was lower than in the liver. The authors therefore concluded that estrogenic compounds could influence, at least in part, the osmoregulatory function in the gills via ER mediated pathways - a hypothesis that needs further verification. The histological evaluation of gill provided no evidence of serious tissue damage in any of our temperature treatments. Indices ($Index_{gill}$) were within the range of values previously reported in control groups (mean $Index_{gill}$ = 6.6 - 9.2) of a field monitoring survey (Bernet et al., 2004). In comparison to control fish, trout exposed to water that received effluents from a WWTWs showed a mean $Index_{gill}$ = 14.2 (Bernet et al., 2004). Wastewater, however, do not contain only estrogenic chemicals. Rather sewage effluent is complex mixture of diverse chemicals (e.g. pharmaceuticals, trace metals or macropollutants) and some of them have the potential to affect gill histology (Mallatt, 1985). Histopathological alterations in the present experiment

were restricted to epithelial cell lifting, rupture of gill epithelium and hyperplasia, but we found no differences in frequency and severity of symptoms between treatments. Bernet et al. (2004) additionally observed epithelial hypertrophy and lamellae fusion in fish from an area that is influenced by WWTW effluents. Given that we did not observe these alterations in the present study, one can suggest that the histopathological alterations observed by Bernet et al. 2004) are less likely linked to environmental estrogens in the water, but rather is the result of the complex mixture condition found downstream from WWTWs.

We observe an increased number of alcianblue/PAS positive mucous cells in fish from fluctuating and high temperature ($T_{fluctuate}$-EE_2 and T_{high}-EE_2) – but not in fish exposed to EE_2 at low temperature which points to a predominant influence of temperature (although a combined effect still cannot be excluded). Similar mucous cell proliferation in fish gills after temperature elevation has been also reported in other fish species. The yellow eel (*Anguilla anguilla*), held at 19°C for instance, showed significantly more mucous cell than fish held at 9°C (Dunelerb et al., 1996). Such mucous cell proliferation may result in advanced mucous production and/or secretion in order to reduce the ion permeability in the gills (Shepard, 1994). Though, extensive accumulation of mucous covering the surface of lamellae was not observed (which can be an artifact during histological process). Along with the missing changes in condition factor, which is a measure of general fitness in fish, it does appear that despite estrogen treatment, fish are still able to respond to temperature changes in an appropriate manner.

The immunohistochemical labelling with the monoclonal antibody against the Na^+/K^+–ATPase alpha subunit is a specific method to detect chloride cells in the gills of teleosts (Schmidt et al., 1998; Metz et al., 2003). The immunohistochemical labelling of chloride cells showed a lower protein abundance in estrogen treated brown trout compared to the $C_{solvent}$ group. No differences, however, were seen between the three temperature treatments - a result that did not match exactly the data of Na^+/K^+–ATPase mRNA obtained by Q-PCR, where the amount of sodium pump transcripts in the T_{high}-EE_2 group was significantly higher than in the T_{low}-EE_2 group. It is important to note, that a change in gene

expression reflects the short-term response of fish to the given stimuli (e.g. stressor). Data obtained from Q-PCR neither allows estimating the amount of the protein, nor gives any information on the enzyme activity in the investigated tissue. Previous experiments with brown trout documented a statistical significant increase in the average number of immunopositive chloride cells after a sudden temperature elevation of 11°C, which is 4°C more than in our study (Schmidt et al., 1998). In addition, they reported that chloride cells from the high temperature group contained more Na^+/K^+–ATPase than fish from the control group. One can hypothesize that the magnitude of temperature elevation may determine the degree of Na^+/K^+–ATPase synthesis. One alternative explanation could be that waterborne ethinylestradiol changes or inhibits the translation of Na^+/K^+–ATPase mRNA to a functional protein. On the basis of the data presented here, however, it is not possible to prove this assumption and needs further investigation.

Fish respond to rapid temperature changes with distinct peaks in plasma cortisol concentration (Wagner et al., 1997) - or at least show enhanced basal plasma cortisol levels after elevated, chronic temperatures exposures (Arends et al., 1998; Lyytikäinen et al., 2002). Due to the fact that sampling stress considerably affects plasma cortisol concentrations, we measured bile cortisol content - an alternative method that has been previously recommended for the detection of chronic stress in teleosts (Truscott, 1979; Pottinger et al., 1992). In the present study, neither trout from the high temperature group, nor from the fluctuating temperature group had significant elevated cortisol concentrations in the bile. Rather, measured (deconjugated) cortisol content in the bile was similar to that measured in unstressed, control rainbow trout (Pottinger et al., 1992). Therefore, it seems that the experimental regime applied in our study was insufficient to elicit a cortisol response in brown trout. This consideration is supported by experiments from Thomas et al. (1986) in the coho salmon (*Oncorhynchus kisutch*) that showed a clear cortisol response after daily temperature cycles of 6.5 - 20°C, but failed to show such a response in temperature cycles with lower amplitude (e.g. 9 - 15°C or 8 - 17°C).

There is a lot of evidence that suggests that GR regulates Na^+/K^+–ATPase actions in the gills of migratory salmonid species during smoltification and seawater

acclimatization (Kiilerich et al., 2007; Singer et al., 2007). For instance, the treatment of rainbow trout with gradual increasing salinity concentrations resulted in a significant stimulation of GR mRNA expression in the gills (Singer et al., 2007). In Atlantic salmon (*Salmo salar*), gill GR gene expression surged with the beginning of smoltification and the associated development of seawater tolerance (Kiilerich et al., 2007). Based on the known relationship between GR and Na^+/K^+–ATPase transcription, we expected alterations of GR gene expression similar to that observed in respect to Na^+/K^+–ATPase gene. The role of branchial GR in response to thermal challenges, however, does appear to be of little importance. Neither estrogen exposure as a single "stressor", nor in combination with varying temperatures produced noticeable GR gene expression changes in the gill of brown trout. We have recently observed a similar GR transcription response in liver of brown trout with no changes after EE_2 exposure at various temperatures (Körner et al., 2007). Similar data were recently published by Filby et al. (2006). Their study revealed no significant alterations of liver and gill GR gene transcription after exposure of fathead minnow to waterborne E_2. The lack of branchial and hepatic GR responses in our study may be related to missing changes in cortisol content after experimental treatment. Indeed, Mazurais et al. (1998) reported a reduction of GR mRNA levels in the gill of Atlantic salmon after short-term treatment with cortisol, whereas Sathiyaa and Vijayan (2003) reported a clear up regulation of GR gene expression in hepatic fish cell lines after application of cortisol. In summary, it would appear that environmental estrogens neither alone nor in combination with temperature elevation do interfere with the expression of GR gene in the gill. Therefore, the observed alteration in sodium pump transcriptions is unlikely to be linked to GR mediated actions. Additionally, the observed correlation between the amount of gill GR mRNA and bile cortisol concentrations is rather a product of two distinct outliers than a validated relationship.

The collective role of cortisol and GR in respect to Na^+/K^+–ATPase regulation during seawater acclimatization in salmonids is well established (Kiilerich et al., 2007; Singer et al., 2007). However, the exact signalling pathways for Na^+/K^+–ATPase gene expression in freshwater salmonids after thermal challenges are still

unidentified. Until the discovery of MR in teleosts by (Colombe et al., 2000), all corticoid effects were considered to be under the control of GR (Shrimpton and McCormick, 1999). We found a significant correlation between branchial Na^+/K^+–ATPase gene expression and the amount of MR mRNA in the gills. Therefore our current data support previous theories by Sloman et al. (2001) and Scott et al. (2005) who hypothesized that some aspects of freshwater osmoregulation are indeed linked to the MR. In the human Na^+/K^+–ATPase alpha gene promoter region a distinct MR/GR response element was previously characterized (Kolla et al., 1999). Although this is not evidenced in lower vertebrates to date, it could provide an explanation for the observed down regulation of Na^+/K^+–ATPase transcription after estrogen exposure and gives a first indication that Na^+/K^+–ATPase gene expression is regulated – depending on the given situation (temperature stress or smoltification) – via either MR or GR. On the contrary; Killerich et al. (2007) suggest a pathway that is different from our conclusions. They measured in the Atlantic salmon a concurrent increase of branchial GR mRNA transcription and Na^+/K^+–ATPase activity, but unchanged MR levels during smolting process. In addition, they observed also a constant increase of 11β–HSD2 (11–β–hydroxysteroid dehydrogenase) gene expression parallel to GR transcription. Transcripts of this enzyme have been previously found in gills of rainbow trout (Kusakabe et al., 2003) and it is known from mammalian studies, that co-localization of 11β–HSD2 with the MR facilitates specific aldosterone signalling by enzymatic inactivation of cortisol (Funder et al., 1988; Sturm et al., 2005). Unfortunately, we have not measured 11β–HSD2 expression in the present study and therefore the role of 11β–HSD2 in the regulation of Na^+/K^+–ATPase during temperature acclimatization remains vague.

The lack of cortisol response and the fact that aldosterone is not present in teleosts indicate that perhaps an alternative ligand is responsible for the putative MR-triggered down regulation of Na^+/K^+–ATPase seen here. Indeed, a previous study suggests that 11-deoxycorticosterone (DOC), a steroidal precursor of cortisol during corticosteroid biosynthesis, has the potential to bind to trout MRs and enhances gene transcription at much lower concentrations than cortisol (Sturm et al., 2005). To date, however, there is no scientific evidence that fish act

in response to temperature alterations with an elevation of DOC synthesis and/or secretion – an important question that needs further scientific verification.

The present study demonstrates that environmental estrogens interfere with Na^+/K^+–ATPase gene expression and could therefore influence the ability of fish to cope with varying environmental conditions (e.g. changing water temperatures). The presented data suggest that Na^+/K^+–ATPase gene expression involved in temperature acclimatization is, at least in part linked to the MR. What is perhaps less clear, given the failure to detect a positive correlation between decreasing Na^+/K^+–ATPase gene transcription and bile cortisol concentration, is the agent which stimulates these actions. The lack of effects on higher level of biological organization (e.g. histology or condition factor) indicates that the used temperature regimes in combination with environmental estrogens did not detrimentally affect the general fitness of brown trout – especially in respect to processes related to branchial Na^+/K^+–ATPase.

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Final discussion and outlook (Chapter 7)

Science never solves a problem without creating ten more.

(*George Bernard Shaw 1856 – 1950*)

The major objective of the present thesis was to evaluate selected parameters of endocrine status in brown trout after treatment with various stressors. In particular, we focused on the interactions between estrogenic substances and different temperatures on selected endocrine parameters of reproductive, stress and mineral physiology. The results were discussed in relation to a recently observed reduction of brown trout catches in Switzerland.

Field Approach (Chapters 2 – 4)

Key question: Do brown trout in Switzerland show indications of estrogenic disruption in areas influenced by wastewater effluents?

The first part of the thesis dealt with the measurement of vitellogenin (Vtg) concentrations and gonadal histology in order to characterize the reproductive health of feral brown trout from rivers which receive WWTW effluents. On the basis of data derived from the present thesis, but also according to conclusions made by Burki (2006), it appears that the effects of environmental estrogens on feral brown trout in the examined rivers are relatively minor. In both field studies, the number of induced fish were low and most fish showed plasma Vtg concentrations below 1 µg/mL, which is commonly used as a threshold concentration for induced fish (Vethaak et al., 2002). Several explanations are to hand, to explain the lack of Vtg induction in brown trout. The most likely one is probably due to low concentrations of estrogen-active pollutants in river water. The chemical burden of Swiss rivers and streams were recently well characterized and appear to be lower compared to other countries (Ahel et al., 2002; Aerni et al., 2004). For instance, in some rivers across the European Union estrogen concentrations up to 9 ng/L 17β–estradiol, 5 ng/L 17α–ethinylestradiol and 180 µg/L nonylphenol were measured (Blackburn and Waldock, 1995; Belfroid et al., 1999; Xiao et al., 2001). Some of these concentrations have the potential to stimulate the hepatic Vtg synthesis in male fish (see Table 2 in chapter 1). In Switzerland, the reported concentrations in most rivers were below the detection limit (~100 pg/L; Aerni et al., 2004). In addition, the estrogenicity in low mountain range rivers is highly variable. Particularly the emission of estrogens via WWTW effluent is not continuous and is affected by various parameters including

hydrology, season or hydraulic retention time in the wastewater treatment works (WWTW; Vermeirssen et al., 2006). In order to overcome the problem of fluctuating estrogen concentrations, we used a passive sampler (POCIS - organic chemical integrative samplers) in combination with a yeast estrogen screen (YES) as a promising alternative to classical analytical sampling approaches (Vermeirssen et al., 2005; chapter 3). Passive sampling not only allows integrating the estrogenic pollution over a certain time period, but also offers the detection of estrogens below the detection limit (BDL), which is ~100 pg/L. For instance, classical sampling strategies (e.g. grab samples) ignore such BDL-concentrations and therefore these substances are not to hand to assess the estrogenicity of the river water. However, these substances may be important in the light of the complex mixture of estrogens in wastewater effluents. Thorpe et al. (2001) observed hepatic Vtg production in rainbow trout after combined 17β–estradiol and nonylphenol exposure even when the concentrations were lower than their individual LOEC (lowest observed effect concentration). The use of the YES accounts for that and integrates potential additive, antagonistic or synergistic effects of structurally different estrogenic pollutants present in river water (Routledge et al., 1996). Indeed, although the individual concentrations of estrogenic active chemicals were in the lower ng/L range (i.e. lower than the Vtg LOECs reported in Table 1, chapter 1), we found at one site an elevated estrogenicity (estradiol equivalents of EEQ ~ 10 ng/L) which was sufficient to induce hepatic Vtg production. In addition, the new developed caging device turned out to be a practical method to explore the effects on fish and to link them with analytical data. The absence of mortality, excellent health status of fish and the low number of animals needed for the survey leads to an improvement of monitoring studies in respect to the 3R strategy (refine, reduce, replace) propagandized by the Swiss Federal Veterinary Office (see www.bvet.admin.ch for details). The external chemical burden (measured as POCIS estrogenicity), correlated very well with the internal exposure in fish (measured as estrogenicity in the bile) and therefore emphasize the suitability of passive sampling. The field study, however, also highlighted that although estrogenic chemicals were present at each sampling site, the general estrogenic

load and estrogenicity in Swiss river water are too low to cause significant Vtg induction. This supports the assumption that estrogen related effects on Swiss brown trout are comparatively small.

Alongside with the low number of Vtg induced male fish, we found no indications of intersex in male fish. The frequent occurrence of intersex in fish in the proximity of WWTWs has been commonly used as an indicator of estrogenic exposure of feral cyprinid fish (Jobling et al., 1998; van Aerle et al., 2001). Under laboratory conditions, putative male brown trout embryos developed gonadal intersex conditions (intersex in terms of oocytes scatter throughout testicular tissue) after continuous exposure to high dosages of estrogens (Burki, 2006). In the light of low estrogen levels in Swiss rivers, Burki (2006) concluded that it is less likely to detect any disruptions of gonadal development in feral brown trout populations – a suggestion that is supported by the result of the present thesis. In contrast to absence of intersex in males, some females caught along two rivers showed spermatogenic activity in ovarian tissue. However, we failed to detect a correlation between intersex and the effluent load in these rivers. This is in line with observations made by Bjerregaard et al. (2006) in brown trout from Danish inland rivers, but also in whitefish from North America (Mikaelian et al., 2002) and pike caught in English rivers (Vine et al., 2005). Each study failed to link the sperm nests in ovarian tissue with the occurrence of WWTP effluent or specific chemicals. Hence, there is evidence to suggest that spermatogenic activity is rather a natural phenomenon than the result of chemical exposure or related to not yet investigated factors.

In conclusion, based on the low number of fish with Vtg induction and the absence of intersex in male trout indicate that the emissions of estrogen-active pollutants via sewage effluents as well as their concentrations are too low to significantly influence fish reproduction. The repeatedly high incidence of gonadal parasites in ovarian tissue at one site as well sporadic occurrence of atresia in some females may affect reproductive output. Though, these observations were not general across sites and therefore contribute little to the observed, nationwide catch decline in Switzerland. Consequently, impaired

reproductive health does not appear to be a major factor contributing to the marked decline of brown trout catches in the four investigated rivers.

Laboratory approach (Chapters 5 – 6)

Key question I: Does temperature alterations influence environmental risk assessment using Vtg as biomarker of exposure? (Chapter 5)

The measurement of Vtg is currently the most common biomarker of exposure to estrogenic pollutants in field monitoring studies. Under field conditions, fish are, however, not exposed to one single stressor only. Rather, fish experience several stressors at the same time and these stressors potentially interact together in a synergistic, antagonistic or additive way. In the view of the recently observed increase of water temperature (as a result of manmade global warming), we tested the influence of different water temperatures on the hepatic expression of Vtg in brown trout concurrently exposure with 17α–ethinylestradiol. The applied temperatures matched those temperature regimes, which were measured during our field campaign in four Swiss rivers (Körner et al., 2007; chapter 3). The exposure of fish to a nominal concentration of 3 ng/L 17α–ethinylestradiol caused a 7 to 74-fold increase of hepatic Vtg mRNA (low and high temperature group, respectively) and was therefore clearly stimulated in fish hold at higher water temperatures. On the protein level, Vtg showed a similar pattern. The higher the temperature, the higher the number of fish with plasma Vtg levels exceeding the threshold concentrations of ≤ 1 µg/mL. Therefore, the present experiment clearly demonstrated that ambient temperature significantly changes the estrogen–induced expression of Vtg. Consequently, water temperature has a considerably influence on the interpretative outcome of field monitoring studies. For example, field work conducted during the warmer spring and summer months will more likely detect an induction of Vtg in male fish than surveys conducted in autumn and winter. For instance, in our field survey during FISCHNETZ, we observed a sporadic induction of Vtg in some few males and this induction is probably potentiated by the summer temperatures during our sampling period. This is, however, contrary to hypothesis evolved in our field study (chapter 3). Based on information from the literature, we hypothesized that Vtg

expression is negatively affected via cortisol mediated processes (Campbell et al., 1994; King et al., 2003; Berg et al., 2004). However, we found no indications of stress response (in terms of cortisol secretion) in fish that experienced different temperature regimes; neither bile cortisol levels nor glucocorticoid receptor gene expression showed any temperature related effects. Hence, the assumed temperature-related covering of estrogenic effects (illustrated in Fig. 5, chapter 1) did not take place during our FISCHNETZ field campaign.

In conclusion, the comparison of plasma Vtg concentrations in fish caught at sampling sites that clearly differ in water temperature, will be great challenge in future field surveys. Along with temperature related effects on Vtg expression, additional "confounding factors" needs to be considered when Vtg is used as biomarker of exposure. Just recently, Burki (2006) evidenced a significant reduction of Vtg expression in trout suffering from proliferative kidney disease (PKD). Additionally, the level of eutrophication has a negative effect on the production of Vtg in fish treated with estrogenic compounds (Gordon et al., 2006).

Key question II: Do environmental estrogens impair the endocrine regulation of mineral balance? (Chapter 6)

Several aspects of fish physiology are under the endocrine control of 17β–estradiol – the major sex steroid in females. Previous work demonstrated a clear association between environmental estrogens and reproductive disorders in teleost fish. Less information, however, is available concerning potential interactions between environmental estrogens and additional endocrine processes. For instance, the branchial sodium pump Na^+/K^+-ATPase facilitate the active transport of Na^+ and Cl^- through epithelial membranes (Perry, 1997). By this, the fish compensates for the temperature–related loss of ions and result in the readjustment the physiological homeostasis (Schmidt et al., 1998) According to Madsen et al. (2004), smolting salmonids respond to estrogen treatment with a reduction in Na^+/K^+-ATPase activity and gene expression. Because increasing river water temperatures are an emerging issue in Switzerland (Hari et al., 2006),

we tested the influence of waterborne estrogens on the endocrine regulation of Na^+/K^+-ATPase at different temperature conditions.

The present data clearly demonstrate an influence of waterborne environmental estrogens on Na^+/K^+-ATPase transcription in gills from brown trout. According to earlier studies, it was thought that Na^+/K^+-ATPase actions are regulated by the cortisol and the glucocorticoid receptor (GR). Based on our results, we suggest that this reduction is regulated via not yet elucidated mineralocorticoid receptor (MR) pathways. This conclusion based on a positive correlation between Na^+/K^+-ATPase transcription and MR gene expression, whereas no relationship occurred between Na^+/K^+-ATPase and GR. There is, however, still need to discover the stimulating steroidal agent of this action. We failed to detect a correlation between Na^+/K^+-ATPase gene transcription and the concentration of cortisol in the bile. One potential candidate is the steroidal precursor of cortisol: the 11-deoxycorticosterone (Sturm et al., 2005). However, it is not known whether fish act in response to temperature variations with increasing 11-deoxycorticosterone (DOC) secretion. The missing cortisol response in fish, however, indicates that the temperature regimes used in our study were insufficient to cause a significant stress (cortisol) response in fish.

In conclusion, although fish were exposed to estrogens, the increase of Na^+/K^+-ATPase gene expression in fish hold at elevated temperatures imply that fish are still able to respond in an appropriate manner. The lack of effects on higher level of biological organization (e.g. histology or condition factor) points to the argumentation that estrogen exposure applied in our experiment does not affect the general fitness of brown trout in respect to gill physiology.

Some remarks on branchial Na^+/K^+-ATPase

According to previous experiment with brown trout, the average numbers of immunopositive chloride cells were significantly higher after a sudden temperature elevation of 11°C, which is 4°C higher than in our study (Schmidt et al., 1998). One can hypothesize that the magnitude of temperature elevation may determine the degree of chloride cell proliferation. On the other hand, it is also conceivable that experimental procedure induced the chloride cell

proliferation (Schmidt et al., 1998). In that experiment, the brown trout were transferred for two hours into tanks that received 19°C tap water and were returned after 2 hours to their former tank which had a water temperature of 8°C. This means that fish experienced two different stressors (temperature and handling stress), whereas the control fish remained in their tank without any disturbances. According to a study by Lyytikäinen et al. (2002), the Arctic charr (*Salvelinus alpinus*) held at various temperatures showed significantly higher plasma cortisol concentrations after handling stress compared to unstressed fish. Given the known stimulation of chloride cell proliferation and Na^+/K^+-ATPase activity by cortisol (Wilson and Perry, 2002), the effect reported in Schmidt et al. (1998) is perhaps linked with handling stress instead of temperature stress. Because handling stress alone does not induce chloride cell proliferation in rainbow trout *per se*, Sloman et al. (2001) made the assumption that cortisol requires an osmotic challenge to considerably alter the number of chloride cells in the gills. In the experiment presented by Schmidt et al. (1998), the temperature stress might be such an osmotic challenge – a question that need further research effort

It is important to note that in experiments with the common carp (*Cyprinus carpio*), both, the total number as well as the intensity of Na^+/K^+-ATPase immunopositive chloride were higher in fish held at lower temperature (15°C) compared to fish maintained at 22° and 29°C. The reported differences in reaction patterns between carp and salmon are perhaps caused by different temperature preferences. Salmonids are stenothermic (coldwater) fish species and prefer summer temperatures around 15°C, whereas cyprinids have a preference to warmer temperatures and show optimal growth conditions around 25°C (Crawshaw and O'Connor, 1996; Metz et al., 2003). This makes it difficult to compare results between different species. Consequently, the effects of environmental estrogens on endocrine ion regulation should be evaluated in each fish group separately.

Future directions

Based on the data and results of the present doctoral thesis, future research efforts should in particular address the following aspects.

- ⇒ Effects of fluctuating exposure scenarios on fish physiology or biomarkers (e.g. receptors, steroids, Vtg or Na^+/K^+–ATPase) in order to simulate real world situations. Such studies may include hormonal-active substances, but also pesticides or trace metals and in combination.
- ⇒ A detailed evaluation of combinatory effects of temperature and hormonal-active chemicals on population relevant parameters (e.g. number of eggs, fecundity, sperm quality or hatching success) in mature brown trout. Such data may provide an important basis for e.g. computer–based simulations (computer models) in order to assess the consequences of global warming on brown trout populations in Swiss rivers.
- ⇒ Continuative studies on the receptor–mediated regulation of branchial Na^+/K^+–ATPase using specific receptor inhibitors (e.g. RU–486, inhibitor of GR; spironolactone, inhibitor of MR) in order to elucidate the detailed pathways. Such studies may also include beside *in vivo* experiments *in vitro* studies (e.g. fish gill cell lines).
- ⇒ Are fish showing an increase in interrenal DOC production after acute or chronic water temperature alterations as well as are such variations in plasma DOC concentrations linked to MR-dependent pathways (e.g. Na^+/K^+-ATPase)?

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