The juvenile three-spined stickleback
– model organism for the study of estrogentic and androgenic endocrine disruption in laboratory and field

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Abstract

Industrial and domestic sewage effluents have been found to cause reproductive disorders in wild fish, often as a result of the interference of compounds in the effluents with the endocrine system. This thesis describes laboratory-based exposure experiments and a field survey that were conducted with juveniles of the three-spined stickleback, *Gasterosteus aculeatus*. This small teleost is a common fish in Swedish coastal waters and was chosen as an alternative to non-native test species commonly used in endocrine disruption studies, which allows the comparison of field data with results from laboratory experiments.

The aim of this thesis was to elucidate 1) if genetic sex determination and differentiation can be disturbed by natural and synthetic steroid hormones and 2) whether this provides an endpoint for the detection of endocrine disruption, 3) to evaluate the applicability of specific estrogen- and androgen-inducible marker proteins in juvenile three-spined sticklebacks, 4) to investigate whether estrogenic and/or androgenic endocrine disrupting activity can be detected in effluents from Swedish pulp mills and domestic sewage treatment plants and 5) whether such activity can be detected in coastal waters receiving these effluents.

Laboratory exposure experiments found juvenile three-spined sticklebacks to be sensitive to water-borne estrogenic and androgenic steroid substances. Intersex – the co-occurrence of ovarian and testicular tissue in gonads – was induced by 17β-estradiol (E2), 17α-ethinylestradiol (EE2), 17α-methyltestosterone (MT) and 5α-dihydrotestosterone (DHT). The first two weeks after hatching was the phase of highest sensitivity. MT was ambivalent by simultaneously eliciting masculinizing and feminizing effects. When applying a DNA-based method for genetic sex identification, it was found that application of MT only during the first two weeks after hatching caused total and apparently irreversible development of testis in genetic females. E2 caused gonad type reversal from male to female. E2 and EE2 induced vitellogenin – the estrogen-responsive yolk precursor protein, while DHT and MT induced spiggin – the androgen-responsive glue protein of the stickleback.

None of the effluents from two pulp mills and two domestic sewage treatment plants had any estrogenic or androgenic activity. Juvenile three-spined sticklebacks were collected during four subsequent summers at the Swedish Baltic Sea coast in recipients of effluents from pulp mills and a domestic sewage treatment plant as well as remote reference sites. No signs of endocrine disruption were observed at any site, when studying gonad development or marker proteins, except for a deviation of sex ratios at a reference site.

The three-spined stickleback – with focus on the juvenile stage – was found to be a sensitive species suitable for the study of estrogenic and androgenic endocrine disruption.
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Abbreviations

DDT  dichlorodiphenyltrichloroethane (insecticide)
DEHP  di(2-ethylhexyl) phthalate
DHT  5α-dihydrotestosterone (non-aromatizable androgen)
dph  days post hatch (days after hatching)
E2  17β-estradiol (estrogen)
EE2  17α-ethinylestradiol (estrogen)
MDHT  17α-methyltrihydrotestosterone (non-aromatizable androgen)
ME2  17α-methylestradiol (estrogen)
MT  17α-methyltestosterone (aromatizable androgen)
PME  pulp mill effluent
STP  sewage treatment plant
TPP  4-tert-pentylphenol
I  The juvenile three-spined stickleback (*Gasterosteus aculeatus* L.) as a model organism for endocrine disruption I - Sexual differentiation  
Edda Hahlbeck, Richard Griffiths & Bengt-Erik Bengtsson  
*Aquatic Toxicology*, in press

II  The juvenile three-spined stickleback (*Gasterosteus aculeatus* L.) as a model organism for endocrine disruption II - kidney hypertrophy, vitellogenin and spiggin induction.  
Edda Hahlbeck, Ioanna Katsiadaki, Ian Mayer, Margareta Adolfsson-Erici, Jonathan James & Bengt-Erik Bengtsson  
*Aquatic Toxicology*, in press

III  The application of DNA sex identification to evaluate intersexuality and to verify gonad type reversal in juvenile three-spined stickleback (*Gasterosteus aculeatus*)  
Edda Hahlbeck, Emma Ehn & Mats Grahn  
submitted

IV  Monitoring androgenic and estrogenic endocrine disruption in sewage and pulp effluents and in receiving coastal waters in Sweden with the juvenile three-spined stickleback (*Gasterosteus aculeatus*)  
Edda Hahlbeck, Ioanna Katsiadaki, Emma Ehn, Eva Ulfsdotter-Turesson, Cecilia Matz, Georgina Rimmer, Alexander Sjögren, Mats Grahn & Bengt-Erik Bengtsson

Statement: I, Edda Hahlbeck, was involved in the following parts of the presented papers: I planned and carried out the exposure experiments for papers I–III. I supervised the experimental work in paper IV, which was carried out by Eva Ulfsdotter-Turesson and Cecilia Matz. Bengt-Erik Bengtsson and I shared the responsibility for the field sampling and selection of field sampling sites. The sampling was carried out with assistance of many persons. I did almost all the histological work and evaluation, but had assistance for parts of paper IV. All statistical analyses and a substantial part of result summaries were carried out by me. I had the main part in writing, discussing and corresponding the papers, of course with valuable input from the co-authors. I was to a lesser degree involved in the RIA and ELISA analyses, which were mainly carried out by Ian Mayer (RIA) and Ioanna Katsiadaki, Jonathan James and Georgina Rimmer (ELISA). I was not involved in genetic sex identification work (Richard Griffiths, Emma Ehn and Mats Grahn) and not in chemical water analyses (Margaretha Adolfsson-Erici) other than in initiating the work and in summarizing and discussing the results.

**Other papers by the author – not included in this thesis**


Reproductive disorders in a variety of fish species have been reported from different regions in Europe (Vos et al. 2000) and other parts of the world. In Canada, insecticides with nonylphenol as a solvent were sprayed onto forest areas, and the proportion of tributaries sprayed showed negative correlation with the return of mature salmon to their native rivers (Fairchild et al. 1999). Also in northwestern USA, salmon species have been declining and have become extinct in some areas (Nagler et al. 2001). In fish in Sweden, reproductive disturbances have been and are still reported along the Baltic Sea coast and in open water (Sandström & Neuman 2003, Bengtsson et al. 1999, Norrgren & Amcoff 1998). In a remote lake and a stream that are contaminated by leachate from a public refuse dump, several fish species showed arrested sexual maturation of females and a general disruption of steroid metabolism (Noaksson et al. 2001, 2003a,b). Many of these reported reproductive disorders have been attributed to environmental contaminants that interfere with the endocrine system – called endocrine disruptors.

In the 1970ies and -80ies anglers in the UK reported “hermaphrodite” roach (Rutilus rutilus) in sewage effluent lagoons, and similarly abnormal fish were later reported from receiving rivers (Matthiessen & Sumpter 1998). Elevated vitellogenin (the yolk precursor protein) levels were induced in rainbow trout (Oncorhynchus mykiss) that were held in cages in sewage effluent streams, indicating that domestic and industrial sewage contains substances that can exert estrogenic effects in fish (Purdom et al. 1994). Similarly, carp (Cyprinus carpio) and walleye (Stizostedion vitreum) collected in the outflow channel of a sewage treatment plant (STP) displayed elevated vitellogenin (Folmar et al. 1996, 2001). Elevated incidence of intersex (co-occurrence of ovarian and testicular tissue in gonads) in roach – probably feminized males – downstream STPs has been shown to be widespread in UK rivers (Jobling et al. 1998). The contraceptive pill ingredient ethinylestradiol (EE2) and degradation products of alkylphenol-polyethoxylate detergents, mainly nonylphenol and octylphenol, were suspected to be the responsible chemicals (Jobling & Sumpter 1993, Purdom et al. 1994, White et al. 1994). Today, estrogens excreted from women, mainly the synthetic estrogen EE2, but also natural estradiol and estrone are considered to be the main responsible compounds (Desbrow et al. 1998, Routledge et al. 1998, Aerni et al. 2004), which does not exclude the possible impact from alkylphenols at least in some STPs (Sheahan et al. 2002). Estrogenic potential of complex effluents on wild fish has been reported from other densely populated industrialized regions in the world. Intersex and elevated vitellogenin levels were found in marbled flounder (Pleuronectes yokohamae) in Tokyo Bay (Hashimoto et al. 2000) and increased incidence of intersex and vitellogenin was reported in bream (Abramis brama) in Elbe river in Germany (Hecker et al. 2002) and in a STP recipient in the Netherlands (Verhaak et al. 2002). Vitellogenin was also induced in carp downstream a large city in Ebro river in Spain (Lavado et al. 2004). However, far from every domestic sewage effluent is estrogenic (Harries et al. 1997, Giesy et al. 2003, Bringolf & Summerfelt 2003). Human population density, dilution factors in receiving waters, season and sewage treatment processes are all factors that play an important role in whether fish will be affected by endocrine active compounds in the receiving waters (Kirk et al. 2002, Bringolf & Summerfelt 2003, Aerni et al. 2004, Hemming et al. 2004, Johnson et al. 2004).

Although it has been known for more than 20 years that effluents from pulp mills can cause masculinization in wild fish by inducing a male anal fin structure (gonopodium) and male sexual behavior in female mosquitofish (Gambusia, Howell et al. 1980, Bortone et al. 1989), this has received surprisingly little attention until the ‘discovery’ of estrogenic endocrine disruption
in UK fish. Still, the sources of androgenic endocrine disruption in pulp mill effluents (PME) have not been satisfactorily identified (Ankley 2004). It could recently be confirmed that the masculinization of the anal fin was correlated with androgen-agonist activity in a river receiving pulp mill effluent (Parks et al. 2001). The androgen androstenedione has been detected in river water that masculinized female mosquitofish and was suggested to be a metabolite of wood extracts (Jenkins et al. 2003), but its responsibility for the androgenic activity of this river has been doubted (Durham et al. 2002).

Disruption of the endocrine system was initially thought to be associated with chlorinated organic compounds formed from elemental chlorine bleaching in pulp mills. However, reproductive effects in wild and laboratory fish populations continued to be reported after industries had introduced chlorine-free bleaching processes and installed secondary treatment of the effluents. Commonly studied effects include liver and gonad size as well as levels and production of steroid hormones (Munkittrick et al. 1994, 2000, Dubé & MacLatchy 2000). Pulp mill effluents are very complex, containing extractives from the raw materials as well as a variety of chemicals from the processing technologies. Plant sterols extracted from wood were already suspected in the early studies, and experiments with soybean extract containing high amounts of plant sterols showed a masculinizing effect on mosquitofish after microbiological degradation (Denton et al. 1985). One of the main plant sterols extracted from wood and detected in pulp mill effluents, β-sitosterol, has been shown to have weak estrogen-agonist properties (Tremblay & van der Kraak 1998, 1999, Mellanen 1996). Pulp and paper mill effluents have in some studies been shown to have androgenic and in other studies estrogenic activity. The latter was indicated by the induction of vitellogenin in rainbow trout (Tremblay & van der Kraak 1999), expression of vitellogenin mRNA in whitefish (Coregonus lavaretus, Mellanen et al. 1999) and development of ovaries in genetic male chinook salmon (Oncorhynchus tshawytscha, Afonso 2002). Moreover, masculinizing and feminizing potential of the same effluent has been reported. The simultaneous induction of male secondary sex characteristics in females and female secondary characteristics in males has been reported in fathead minnows (Pimephales promelas) exposed to effluent from a pulp mill in Canada (Parrott et al. 2003). Similarly, Örn et al. (2001) reported both vitellogenin induction in juvenile zebrafish (Danio rerio) in 50% diluted pulp mill effluent and an increasing proportion of males in a concentration-related manner in 0.7 to 50% effluent. A general inhibition of reproductive function in fish exposed to PMEs has been reported, as for example in largemouth bass (Micropterus salmoides), where gonads were smaller in males and females, vitellogenin production was inhibited in females and sex steroid levels were lower in both males and females (Sepúlveda et al. 2001). Again other studies failed to find significant reproductive impacts (Munkittrick et al. 1994, van den Heuvel et al. 2002). No effect on gonad size but increase in growth and condition of slimy sculpin (Cottus cognatus) downstream a pulp mill indicated a nutrient enrichment or temperature-related rather than toxicological effect (Galloway et al. 2003).
Sex determination in fishes – environmental influences and implications for the study of endocrine disruption

Several papers of this thesis (I, III & IV) deal with the disruption of sex determination and sexual development as an indicator for endocrine disruption. An understanding of the action of chemicals on fish reproduction requires basic knowledge of the reproductive system. Before interpreting data on disruption of sexual differentiation one should be aware of the variety of modes for sex determination displayed in fish, since they can be very different from what is known from mammals.

Mammals are exceptional among vertebrate groups in showing strict genomic determination of sex with male heterogamety. The male and the female mammal chromosomes are microscopically discernible as either X or Y-shaped (XY-pair = male, XX-pair = female, Scherer 1999). Besides mammals, only birds have a strictly genetic sex-determining mechanism, but here it is the female that is the heterogametic sex with clearly distinguishable sex chromosomes (Clinton & Haines 1999). In all other vertebrate groups sex is not always strictly genetically determined, and if, either the female or the male can be the heterogametic sex. Most fish species do not possess morphologically differentiated sex chromosomes (Devlin & Nagahama 2002). Moreover, since DNA-based sex markers are lacking for the majority of fish species, it is often difficult to identify which pattern (male or female heterogamety) is present in a certain species. To make the picture even more complex – the pattern may differ between species even within the same genus, as for example in the guppy and related species: The guppy (Poecilia reticulata) has male heterogamety (Kavumpurath & Pandian 1993), whereas the molly (Poecilia sphenops) has female heterogamety (George & Pandian 1995). Irrespective of male or female heterogamety, strict genetic sex determination with one pair of sex chromosomes should always result in a 1:1 female:male sex ratio. However, there are cases for which sex ratios do not fit perfectly with the expectations of heterogametic systems, suggesting the influence of either minor sex determining genes or environmental factors on the process of sex determination.

Sex ratios different from 1:1 are often found in fish populations both in the wild or held in captivity. Fish populations can suffer from selective mortality with one of the sexes being more susceptible to the effect of a stressor. When wild fish are caught, the fishing method may select predominantly one of the sexes due to sex-specific differences in size, behavior or survival. The sexes may also differ in their habitat preferences or migratory routes. However, even when such selecting factors can be ruled out, as in laboratory-based studies, a number of fish species display sex ratios different from equity. In the sea bass (Dicentrarchus labrax) for example sex ratios of stocks cultured in captivity are consistently skewed in favor of males (about 80% and in some instances over 90%, Blázquez et al. 1999), whereas in natural populations females predominate (Blázquez et al. 1995, 1998b). Undesired dominance of one sex is found in European eel (Anguilla anguilla) where under crowded conditions more males develop (Degani & Kushnirnov 1992). Even though sex determination seems to have a genetic base in most fish species, the genetic mechanisms for sex determination in fishes are primitive and labile. Accordingly, sex in fish is controlled not only by genetic factors, but environmental factors often play an important role (Chan & Yeung 1983). Temperature, pH and social factors such as crowding or relative body size may influence the outcome of sexual differentiation. The whole range from species with almost entirely environmentally determined sex to
species with only marginal influence of environmental factors on sex determination can be found. The variability in patterns is high even among closely related species.

All fish species mentioned here are gonochorists, which means that gonadal sex (either ovary or testis) is determined during a sensitive phase during early sexual development and does not naturally change later in life. The majority of fish species are considered to be gonochorists under natural conditions, although sex determination may be labile and subject to influences by external factors (Chan & Yeung 1983, Patiño 1997). This does not include hermaphroditic species. A species is defined as a hermaphrodite if individuals have the natural potential to function as both sexes, either simultaneously or sequentially, at some time during their life (Sadovy & Shapiro 1987). Many hermaphrodite fish species change sex as a natural part of their life cycle. This sex change is under hormonal control and can be triggered by environmental or social factors and (Yamamoto 1969).

‘Sex’ and ‘sex reversal’

Regardless of genetic sex, the balance between androgens and estrogens may determine which gonadal sex develops. Total functional sex reversal can be induced in a number of gonochorists, if environmental or chemical factors are applied before the phenotypic sex has been defined. The possibility of artificially reversing sex in fish has widely been applied in aquaculture, where often only one of the sexes is desired. Depending on the species, females or males grow faster, and faster growth gives higher output. Females may be preferred if the roe is commercially important. Males may be desired in ornamental fish for their coloration. In other cases, monosex cultures of either males or females prevent fish from reproducing which otherwise e.g. would lead to diminished growth due to overcrowding or undesired inter-breeding (Hunter & Donaldson 1983, Pongthana et al. 1999).

Since in gonochorist species the genetic sex can be overridden under certain conditions and the fish develop the characteristics of the opposite sex, it is difficult to define the term ‘sex’. Should the genetic or the phenotypic (morphological or physiological) sex be the base for the definition? A genetic male can be a phenotypic female and vice versa if the individual is functioning as the opposite sex and is able to spawn and give rise to viable offspring. In the majority of species the actual genetic sex of individuals cannot be identified due to the lack of genetic markers or discernible chromosomes, and sex has therefore been identified with help of gonad appearance or external sexual characteristics. In paper III, a different definition was applied: The term ‘sex’ was reserved for the genetic sex as identified with genetic markers. The phenotypic sex was referred to as the gonad type, i.e. ovary or testis. The term gonad type reversal instead of sex reversal was used for the development of the opposite gonad type in an individual with a given genetic sex – especially since functional sex reversal was not studied. This term also allowed the introduction of other gonad types than ovary and testis, as for example intersex, without implying a certain genetic sex. With new molecular techniques now being available and affordable, DNA-based sex markers will probably be developed for many commercially important fish species, which will make the definition of ‘sex’ more clear in a near future.

Due to the lack of genetic sex markers or discernible sex chromosomes, sex reversal in fish has often been proven indirectly with breeding experiments. Sex reversal is induced by external application or injection of hormones to homogametic parents (depending on species either males or females) that are reared to maturity. The gametes from these sex-inversed homogametic individuals are then joined with the gametes of untreated homogametic fish. If the process of sex determination in the species is strictly sex-chromosome-based and not
polyfactorial (see below), the progeny should all be homogametic and, therefore, of the same genetic sex (Devlin & Nagahama 2002).

If sex ratios are deviating after treatment with exogenous hormones or endocrine disrupters in laboratory studies, this is often seen as proof of sex reversal. However, a deviation in sex ratio might indicate different things: 1) Functional sex reversal: The individuals behave and reproduce as the opposite sex and produce viable offspring when mating with a non-reversed individual of the same genetic sex. This has been applied in aquaculture in order to control sex and reproduction. 2) Gonadal sex reversal without functional sex reversal: As an indicator for endocrine disruption, the confirmation of functional sex reversal is not necessary as it can be assumed that the maldevelopment of gonads is similarly or even more adverse for the reproductive success of an affected population. 3) Delayed or inhibited transition in fish species that are juvenile hermaphrodites – which strictly seen cannot be considered to be sex reversal.

If the visual appearance of the testes occurs much later than that of the ovaries, the appearance of juvenile gonads may not accurately reflect a sex-reversing effect of a treatment (Davis et al. 2000). Generally among fish species, two patterns of gonad development can be distinguished. In so-called ‘differentiated’ species the gonad develops directly into either testis or ovary. In ‘undifferentiated’ species the gonad develops first into an ovary-like gonad, then half (= males) of the individuals develop testes. Those individuals destined to become males apparently pass through a non-functional female phase and then an intersex phase during their juvenile period (Yamamoto 1969, Chan & Yeung 1983). The zebrafish – at least certain strains – displays intersexuality (“transitory hermaphroditism”) during the juvenile period. Gonad differentiation starts with structurally well-defined ovaries, but in only about half of the fish ovaries continue to grow to maturity, while in the other half they begin to transform into testes (Takahashi 1977, Maack & Segner 2003). This transition can be inhibited or delayed by the action of exogenous hormones (Hill et al. 2003, van den Belt et al. 2003).

Intersex

Intersexuality (or intersex) is a condition that sometimes occurs in a number of gonochorist species and that is characterized by the presence of both ovarian and testicular tissue within a gonad. It can occur spontaneously or can be induced under certain environmental conditions, mainly temperature, but it is not considered to be a part of the natural life cycle. Also steroid hormones and endocrine disrupting chemicals can induce the development of intersex. In many fish species populations contain individuals whose gonads contain varying quantities of cells that are generally typical for the opposite sex (Sadovy & Shapiro 1987). This phenomenon of sporadic intersex might be considered natural, although rare. Individuals that display intersex are sometimes also called ‘hermaphrodites’, which they are not according to the definition above. This was for example reported in one specimen of the whitefish from Loch Lomond out of 7500 examined (Brown & Scott 1988). In the pejerrey (Odontesthes bonariensis), three individuals with intersex were found among 3000 examined (Strüssmann et al. 1996b). Especially in cyprinids, a low level of intersexuality seems to be normal (e.g. carp: (Komen et al. 1989), roach: (Jobling et al. 1998), bream (Hecker et al. 2002), and also in sea bass 2–4% intersex was observed in controls (Blázquez et al. 2001). In carp treated with estradiol, ovarian and testicular tissue often appear mixed in the gonads, whereas when intersex occurs in untreated individuals, it is displayed as distinctive areas of ovarian and testicular tissue separated by connective tissue (Komen et al. 1989). Intersex may also be defined in wider terms than solely the simultaneous occurrence of oocytes and spermatocytes (i.e. eggs and sperm). For example, exposure of juvenile roach to treated sewage effluent induces dose-dependent and irreversible feminization of gonadal duct without the development of oocytes in testes (Rodgers-Gray et al. 2001).
Sex chromosomes and polyfactorial sex determination

The evolution of genetic sex determination requires the creation of genes that override environmental cues and makes development of sexual functions less sensitive to disturbances. Close linkage between male and female determining loci is favored by selection, and represents the first step towards the evolution of highly differentiated sex chromosomes (Charlesworth 2002). Initially, sex determination will depend on the combined actions of many different genes. Gradually this system will be replaced by a single locus, a master sex-determining gene, and true sex chromosomes will appear (Devlin & Nagahama 2002, Charlesworth 2002, Peichel et al. 2004).

The susceptibility to external factors might still be under the influence of genes other than major sex genes. In a species with male heterogamety the use of sperm from sex-reversed females to fertilize the ova from untreated females should result in the production of 100% female progeny. However, in chinook salmon, some families produced only 92–99% females (Hunter et al. 1983). Similarly, in Nile tilapia (Oreochromis niloticus) only 94% of homogametic couples produced phenotypic females. This cannot be explained by a simple monogenic sex determination (Calhoun & Shelton 1983). If sex ratios deviate from those expected, polyfactorial (also multigenic or polygenic) sex determination is suggested. The sea bass, appears to have a weak or non-existing genotypic sex determination (Strüssmann et al. 1996b) (Blázquez et al. 1999). In this species – a gonochorist but phylogenetically very close to a family of hermaphrodites (Serranidae) – neither heterogametic male or heterogametic female sex determination could be confirmed despite efforts with progeny testing of sex reversed individuals (Blázquez et al. 1999). Indications of polyfactorial sex determination have been reported also in a number of other species (Devlin & Nagahama 2002).

Environmental sex determination

A pattern of sex determination has been found in a variety of gonochorist fish, in which environmental factors during early development irreversibly determine the phenotypic sex.

Temperature

Temperature is the determination factor in the vast majority of reported cases of environmental sex determination. In the Atlantic silverside (Menidia menidia), a species that shows a high degree of variation in the sex ratios of progeny from different females (Conover & Kynard 1981) the influence of temperature on sex determination might be mediated via polyfactorial sex determination. In this species, temperature during development directly affects progeny sex ratio in an inverse relationship: at lower temperature more females develop. Sex ratios of 0–100% females have been observed, with significant variations between family sex ratios among mothers mated to the same male (Conover & Kynard 1981). Sex determination in the Atlantic silverside is intermediate between complete genetic and strict temperature-dependent (Conover & Heins 1987). Sometimes, sex change appears to be possible only in one direction. In the olive flounder (Paralichthys olivaceus), spontaneous sex reversal to physiological males could be induced by high water temperature, but genetic males never were reversed to females at either high or low water temperature. However, when steroids are given with the food, sex reversal in both directions is possible (Yamamoto 1999).
Other factors

Other physical and social factors affecting sex determination in gonochorist fish seem to be less important or have been much less studied. In the European eel, high population densities result in more males (77%) compared to isolated conditions (20%, Degani & Kushnirov 1992, Colombo & Grandi 1996). Salinity seems not to influence sex determination, at least not in the two species that were studied in this respect: the Atlantic silverside (Conover & Heins 1987) and Nile tilapia (Abucay et al. 1999). In the silverside also photoperiod has no influence (Conover & Heins 1987). The pH, on the other hand had an effect in five cichlid species, where low pH (5-6) skewed sex ratios towards males, whereas sex ratios were skewed towards females at pH 7 (Rubin 1985).

Treatment with steroid hormones

Androgens and estrogens are considered to be the natural sex-inducers (Devlin & Nagahama 2002), and generally, the sex determining mechanism in the embryo can be turned in the opposite direction by the use of appropriate androgens or estrogens. It has been known since the 1930ies that treatment with steroid hormones can cause a change in the gonadal sex in fish. Intersex (ovo-testis or testis-ova) has been described since the 1940ies and 1950ies (Yamamoto 1969).

The ability to control sex in fish is of high importance in aquaculture. This issue has been reviewed several times (Hunter & Donaldson 1983, Pandian & Sheela 1995, Piferrer 2001). In order to induce sex change, Yamamoto (1969) suggested that steroid hormones should be administered consistently during the whole process of sex determination. However, short-term immersions of sometimes only two hours applied during the most sensitive time period are sufficient to induce sex change at least in salmonids (Piferrer & Donaldson 1989, 1992, Piferrer et al. 1993). An inverse relationship may exist between dosage and duration of an effective treatment (Hunter & Donaldson 1983). Steroid hormones are applied via immersion or treated feed. In aquaculture, immersion is mainly used to treat eggs or larvae before the onset of feeding (Pandian & Sheela 1995). Ethinylestradiol (EE2) is often found to be more potent than estradiol (E2) (Yamamoto 1969), but this ratio may change with exposure duration, probably due to differences in uptake and different metabolism rate, and E2 becomes more potent than EE2 as treatment duration increases (Piferrer & Donaldson 1992). Although in most teleosts the gonadal sex can be easily manipulated by the appropriate treatment, sex control by steroid treatment is not fully effective in some species. As an example, in brook trout (Salvelinus fontinalis) androgen treatment did not result in an increase of the proportion of males, nor did it induce intersex (Galbreath & Stocks 1999), although feminization could be achieved by estrogen treatment (Donaldson & Hunter 1982).

Paradoxical feminization

When treating fish with testosterone (T) or MT either at high doses or over extended periods, often a paradoxical feminizing effect of these androgens can be observed. It is now widely accepted that the aromatization of androgens to estrogens, which is the natural pathway for estrogen synthesis, is responsible for this apparent feminizing potential of MT. Vitellogenin (the estrogen-induced yolk precursor) production and vitellogenin mRNA expression were increased by water-borne MT administration in fathead minnow, but not when the aromatase inhibitor fadrozole was administered simultaneously. Instead, premature masculinization was induced as juveniles developed characteristics typical for mature males (Zerulla et al. 2002). Corroborating this, 17α-methylestradiol (ME2) was detected in adult fathead minnow after
treatment with MT, and ME2 displayed a high binding affinity to the estrogen receptor (70% of E2, Hornung et al. 2004). Paradoxical feminization has been observed in a variety of fish species. In the coho salmon (Oncorhynchus kisutch) a single 2h immersion in MT or methylhydrotestosterone (MDHT) had a masculinizing effect, but at high doses of MT the proportion of females increased, which did not occur with the non-aromatizable MDHT (Piferrer & Donaldson 1991). Also in the chinook salmon, MDHT is capable of sustained masculinization even when applied at high doses, whereas MT is not (Piferrer et al. 1993). Intersex in previously masculinized females of sea bass was induced with MT but not MDHT (Blázquez et al. 2001). In Nile tilapia, treatment with aromatase inhibitor during the sensitive phase masculinized genetic females (Kwon et al. 2000). Similarly, application of the aromatase inhibitor fadrozole produced 100% male zebrafish of 40 individuals (Fenske & Segner 2004). Already Hori et al. (1979) described the induction of vitellogenin by high doses of MT in the goldfish (Carassius auratus).

The channel catfish (Ictalurus punctatus) seems to be unusual in its response since masculinization with androgens can apparently not be achieved. Instead, feminization is not only achieved with a variety of estrogens, but also with androgens. However, paradoxical feminization cannot be the cause, since non-aromatizable androgens and aromatase inhibitors also have feminizing effects on channel catfish, albeit less effective than estrogens (Davis et al. 1990, 1992, 2000). Interestingly, in this species, masculinization could also not be achieved with low temperature treatment, despite the induction of feminization at high temperatures (Patiño et al. 1996).

**Sensitive period**

Many gonochorist fish species are particularly susceptible to the action of exogenous steroids or environmental factors that influence the direction of sexual differentiation during a certain sensitive (labile, critical) period during their early life history. The exact timing and duration of this sensitive period differs from species to species, depending on the species-specific time of sex determination, which can lay before or after hatching, before or after onset of feeding (Yamamoto 1969). According to Pandian & Sheela (1995), fish species can in this respect be divided into two groups. In one group differentiation occurs for a short period following hatching, which makes exact timing of any hormonal treatment very important. In the other group, differentiation occurs during the late juvenile stage and is not always restricted to a single physiological stage, extending even to adult stages. These species maintain their sexual bipotentiality for a considerate period and may be influenced by hormonal treatment over several intervals. In a number of species it is known that females differentiate earlier than males, as for example in sea bass (Blázquez et al. 1998a), barfin flounder (Verasper moseri, Goto et al. 1999), and pejerrey (Strüssmann et al. 1996a).

The sensitive period may be quite different even among closely related species, as for example in the viviparous poeciliids (guppies and mollyes). In the molly, this period is restricted to 30 days after hatching, whereas the guppy is sensitive both during embryonic stages and after hatching (George & Pandian 1995). The period for the responsiveness to the action of sex steroids may but does not necessarily need to coincide with the thermo-sensitive period. In Nile tilapia, low temperature (20°C) caused higher female ratio only when applied very early in development at 0-5 days post hatch (dph), earlier than high temperatures caused masculinization (10 dph, Wang & Tsai 2000). The most sensitive time point for chinook salmon was three days after 50% hatching (Piferrer et al. 1993). In coho salmon the labile period lasted three weeks around hatching. The maximum response for estradiol occurred one day before 50% hatching, and that for MT one week later (Piferrer & Donaldson 1989). Estradiol administered to Japanese medaka (Oryzias latipes) eggs 4-10 days before hatching reversed males to functional females (Iwamatsu 1999). Testosterone induced intersex gonads only at one week after
hatching, while estradiol exposure induced intersex both at 7 and 21 day exposure. This suggests that males feminized by estradiol still had bi-potent germ cells present in the testis up to 21 days after hatching, while female fish masculinized by testosterone had sufficient differentiation of germ cells by day 21 to abate the effects of androgen exposure (Koger et al. 2000).
Endocrine disruption

Endocrine disruption has not been predicted from toxicity tests but discovered by accident, as for example by Soto et al. (1991), who found that chemicals were released from laboratory plastics that interfered with the estrogen receptor of cell cultures. Many of the substances that have been discovered to elicit endocrine disrupting potential fit neither into the categories of mutagens nor acute toxicants, so their threat to the health of wildlife had been underestimated until recently (Colborn et al. 1993, Crews et al. 2000). The large number of studies on the estrogenic potential of sewage treatment plant effluents was initiated as a consequence of the accidental observation of feminized fishes in the UK (Matthiessen & Sumpter 1998). Effects of chemicals and effluents on fish in their natural habitat include disturbed gonadal maturation, impaired gonad development (intersex and abnormal development of gonad duct), vitellogenin induction, disturbed steroid metabolism and abnormal development of secondary sex characteristics. Particular phases of the reproductive cycle, such as development of the gonads and sex differentiation, are especially sensitive to xeno-estrogens and -androgens, as these processes are regulated by steroid hormones. An endocrine disrupter is defined as an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism or its progeny (Vos et al. 2000). It exerts its effects through interference with the synthesis, storage/release, transport, metabolism, binding, action or elimination of natural hormones that are responsible for the regulation of homeostasis and the regulation of developmental processes (Cooper & Kavlock 1997). Toxicity tests assessing the hormone-like activity of chemicals released into the environment are just beginning to be developed. Exposure to endocrine disrupting substances in nature is mostly chronic and the effects of exposure may be latent and sometimes not observable for years after exposure. Among substances found to have such effects are the classical chlorinated organic pesticides like DDT and lindane. Alkylphenols (pentylphenol, octylphenol, nonylphenol) as degradation products of detergents and emulsifiers came into focus when their weakly estrogenic properties were discovered (Jobling & Sumpter 1993).

Disruption of sex determination and sexual development in laboratory studies

When males of the Japanese medaka, a species in which spontaneous intersexuality probably does not occur, were exposed to \( \beta \)-HCH (hexachlorocyclohexane, an isomer of the insecticide lindane: \( \gamma \)-HCH) they developed intersex. The effect was recorded although exposures began several weeks after gonadal differentiation, which indicates that bipotentiality of germ cells had not totally disappeared (Wester & Canton 1986). The Japanese medaka has been widely used in a variety of studies on fish biology including toxicity testing. The first week after hatching is the optimal time for induction of intersex, but it can also be induced in adults (Gray et al. 1999). Injection of EE2 into embryos, simulating maternal transfer, induced functional sex reversal as early as seven days after hatching, but no gonadal intersex. Injection of methylestosterone induced sex reversal from females to males (Papoulias et al. 1999, 2000). Scholz et al. 2000 induced sex reversal with EE2 at 0.1 \( \mu \)g/L, but no intersex at lower concentrations. The estrogens estradiol, estrone, EE2 and estriol induced intersex at environmentally relevant concentrations (Metcalfe et al. 2001). In the same species, injection of DDT into embryonic yolk induced functional male-to-female sex reversal, i.e. genetic males developed
ovaries and gave rise to viable larvae (Edmunds et al. 2000). Immersion of medaka fry in water with DDT caused intersex and sex reversal at concentrations below 10 µg/L (Metcalfe et al. 2000, Cheek et al. 2001). Intersex seemed to be an intermediate condition since the proportion of ovaries increased with exposure (Cheek et al. 2001). Nonylphenol caused intersex and sex reversal to females at concentrations only slightly above concentrations reported from effluents (<100 µg/L, Gray & Metcalfe 1997). At environmentally relevant concentrations (<8 µg/L), no intersex was observable (Nimrod & Benson 1998). Bisphenol A at concentrations <2000 µg/L, which are much higher than those detected in the environment, induced intersex and probably sex reversal from male to female (Yokota et al. 2000). Degradation products of nonylphenol ethoxylates caused low incidence of intersex at relatively high concentrations, and di(2-ethylhexyl) phthalate (DEHP) showed no effect (Metcalfe et al. 2001).

Also the fathead minnow has been used for toxicity testing for a long time. Also in this species EE2 treatment induced sex reversal and intersex in a concentration dependent manner. At the highest concentration (64 ng/L) no intersex was recorded, but the population was strongly sex reversed (94% females, Länge et al. 2001). Disruption of gonad duct development (ovarian-like cavity in males), but no intersex was observed in fathead minnow when larvae were exposed to 0.01 µg/L EE2 (Van Aerle et al. 2002).

In the zebrafish, significant changes in sex ratios in female direction were detected at 0.001 to 0.025 µg/L EE2, whereas no intersex fish were found (Örn et al. 2003). Male carp exposed to 4-tert-pentylphenol (TPP) developed oviducts, and the number of primordial germ cells decreased. Some males developed intersex (Gimeno et al. 1996). In Atlantic salmon (Salmo salar) fed DEHP, females were significantly over-represented (64%), whereas with nonylphenol no difference in sex ratio was recorded (Norrgren et al. 1999). So far, intersex in wild fish has almost exclusively been reported in terms of feminization due to xenoestrogens in the environment, with the intersex individuals being assumed to be feminized males (Van Aerle et al. 2001). This assumption has so far not been proven by genetic sex identification of the intersexual individuals.

**Field observations**

In field studies it is difficult to establish the occurrence of skewed sex ratios as a result of the effect of endocrine disrupting substances, because sampling biases such as behavioral differences between the sexes, differences in habitat preference or selective mortalities can seldom be excluded.

Elevated incidences of intersex compared to reference sites have been used as indicator for disruption of sexual differentiation in fish exposed to different kinds of effluents from human activities. The roach and the gudgeon (Gobio gobio) displayed higher incidences of intersex (oviduct in presumed males and ovo-testis) in fish several kilometers downstream from the point of discharge of sewage treatment plants in UK (Jobling et al. 1998, Van Aerle et al. 2001). In the European flounder (Platichthys flesus) in two of the most polluted estuaries in the UK receiving municipal and industrial effluents a low percentage of intersex in male flounder was recorded (Allen et al. 1999). Many studies have been published during recent years that report increased incidence of intersex in wild fish in polluted waters: in marbled flounder in Tokyo Bay (Hashimoto et al. 2000), in bream in Elbe river in Germany (Hecker et al. 2002) and in an STP recipient in the Netherlands (Vethaak et al. 2002). Masculinization has been reported from water close to a pulp mill effluent in Sweden, where a lowered percentage of female embryos (about 42-45%, compared to 50% at larger distance from the point source) was found in the eelpout (Zoarces viviparus, Larsson et al. 2000). Although not evidence of sex reversal, at least sampling biases and differences in behavior or habitat preference could be
excluded as reasons for the deviation in sex ratio since embryos were taken from gravid females of this viviparous fish species.

Genetic markers for sex chromosomes can be invaluably useful for proving the occurrence of sex reversal in field populations. Up to now, such markers have been developed only for very few fish species. In Columbia river and its tributaries, USA, 84% of the females of the chinook salmon that returned to spawn actually contained a genetic marker for the Y chromosome, a feature that was not observed in females raised in hatcheries (Nagler et al. 2001). This could have been the first proof of sex reversal in a wild population, however, subsequent sampling showed that the phenomenon occurred elsewhere in the river basin – also in fish from hatcheries – without an apparent distribution pattern, suggesting that the marker is consequently male-specific only in certain populations of chinook salmon (Chowen & Nagler 2004).

Vitellogenin – the biomarker for feminization in fish

Vitellogenin is the precursor protein for the production of yolk proteins. It is produced by the liver under the stimulation from estrogens and released into the blood stream from where it is incorporated into the oocytes. Both male and female fish, as well as immature juveniles have hepatic estrogen receptors, but only the liver of female fish will normally be exposed to estrogens. Estrogenic xenobiotics can also act on the hepatic receptors to induce the synthesis of vitellogenin. In the UK, rainbow trout exposed to sewage effluent were found with elevated vitellogenin levels (Purdom et al. 1994), and the same effect was reported in a river polluted by textile mill effluents (Harries et al. 1997). A high vitellogenin level in juveniles or males is a good indicator of estrogenic activity, since it does not occur naturally (Denslow et al. 1999). Although the occurrence of elevated vitellogenin itself does not necessarily have relevance to the fertility or health of the fish, it can be indicative of a disruption in sexual differentiation. Vitellogenin induction is in many cases found in combination with the observation of intersex, which does not necessarily mean they are mediated via the same mechanism. For example male roach with intersex (oviduct and ovo-testis) in the UK had also increased vitellogenin levels (Gimeno et al. 1998, Jobling et al. 1998). Vitellogenin induction in the fathead minnow was correlated with developmental and reproductive impairment and kidney failure (Länge et al. 2001). Vitellogenin synthesis induced by octylphenol and reproductive impairment such as inhibited spermatogenesis and the observation of some fish with intersex appear to be closely linked phenomena also in Japanese medaka (Grønen et al. 1999). Sometimes other effects intersex are detectable earlier than vitellogenin increase, which was the least sensitive response of a number of reproduction endpoints (Cheek et al. 2001). On the other hand, in European flounder in UK estuaries the incidence of elevated vitellogenin was much higher than that of intersex, with the latter being found only in the most polluted estuaries (Allen et al. 1999). Concentration dependent induction of effects was found in male carp exposed to TPP, where 36 µg/L of the chemical caused development of oviduct, 90 and 256 µg/L the development of intersex, whereas plasma vitellogenin level were increased at 356 µg/L (Gimeno et al. 1996).

The induction of zona radiata proteins (eggshell proteins, like vitellogenin synthesized in the liver) might be a more sensitive biomarker for environmental estrogens, as has been suggested by Arukwe et al. (2000) and others, but vitellogenin is the by far most widely used biochemical marker for feminization in fish.
Spiggin – an androgen-responsive marker protein

A biochemical marker for androgenic effects has been lacking until recently, when an assay with the protein spiggin was developed in the three-spined stickleback (Katsiadaki et al. 2002). Spiggin is an androgen-induced glue protein produced by the kidneys of male sticklebacks during the breeding season (Jakobsson et al. 1999). In order to cope with the spiggin production, the epithelial cells of kidney tubuli become highly hypertrophied, which results in an enlargement of the whole kidney. While female sticklebacks do not produce spiggin under normal conditions it has recently been demonstrated that the kidneys of female sticklebacks will produce spiggin when exposed to androgens in the water (Katsiadaki et al. 2002). With the development of a sensitive assay for spiggin, the stickleback is currently the first fish species in which biochemical marker for both environmental (anti-) androgens and (anti-) estrogens can be employed on an individual basis (Katsiadaki et al. 2002), since an assay has been developed to measure vitellogenin and spiggin in single individuals (Hahlbeck et al. (paper II of this thesis), Katsiadaki et al. 2002, 2004).
Besides spiggin as the androgen-responsive biomarker, the three-spined stickleback has several advantages that make it suitable for ecotoxicological studies. As an alternative to the use of tropical fish species, the three-spined stickleback is a representative of waters in temperate and boreal climate zones. It is distributed over the northern hemisphere and found in most – if not all – European countries. It can be applied for studies in limnic as well as brackish and marine environments. Some populations are anadromous, others are resident in freshwater throughout the year (Wootton 1984), again other populations can breed in salt water (Bell 1979). Since it spawns easily in the laboratory, this species can be studied at each stage of its life history and serve for studies comparing field and laboratory results. It has hatchability and survival rates close to 100% (papers I, II and IV in this thesis). Compared to many other species in temperate regions it has a relatively short breeding cycle. In nature – due to seasonality – the stickleback has the potential to complete one life cycle per year (Wootton 1984). With adequate care and physical conditions, *G. aculeatus* can reach sexual maturity within 7–8 months in the laboratory (my own observation). In particular, juvenile sticklebacks are suitable for studying endocrine disruption, since sexual differentiation is sensitive to the impact of external factors such as environmental hormones. Furthermore, they can be sampled in large numbers for biomonitoring purposes and can be caught close to the place where they have been exposed during their most sensitive life-stages.

The three-spined stickleback has previously been used for the evaluation of anthropogenic compounds, for example pulp mill effluents, organic tin compounds and halogenated organic compounds, where impacts on reproductive parameters could be observed (Holm et al. 1991, Holm et al. 1993, Holm et al. 1994). For the three-spined stickleback male sex-linked markers have been developed and it has been demonstrated that stickleback has sex chromosomes – although not morphologically distinguishable – and the male is the heterogametic sex (Griffiths et al. 2000, Peichel et al. 2004). The behavior of the stickleback is very well studied and behavioral aspects have been applied in ecotoxicological studies. Males showed decreased aggressive response after exposure to an environmentally relevant concentration of EE2 (Bell 2001). Anti-predator, shoaling and feeding behavior was modulated by exposure to different environmental contaminants (Espmark Wibe 2001, 2002, 2004).
Summary of papers

This thesis deals with the three-spined stickleback as model and indicator organism with relevance for Swedish waters with particular emphasis on juveniles. It comprises five laboratory experiments that were conducted subsequently during autumn of the years 1999 to 2003 and a field survey at the Swedish Baltic Sea coast during summer of the years 2000 to 2003.

Objectives

In laboratory studies, juvenile sticklebacks were exposed to the estrogens $17\beta$-estradiol (E2) and $17\alpha$-ethinylestradiol (EE2) and the androgen $17\alpha$-methyltestosterone (MT) in a semi-static renewal system. The aim of the experiments was to elucidate

if the genetic sex can be overridden in the three-spined stickleback after treatment with natural and synthetic steroid hormones,

and to investigate

whether intersex could provide an endpoint for the detection of endocrine disruption.

E2 and MT were applied during different time windows in order to identify the phase of highest sensitivity to the action of external hormones. Furthermore, the endpoints kidney hypertrophy, spiggin and vitellogenin induction were elucidated for their suitability for the study of both androgen and estrogen-induced endocrine disruption in the juvenile three-spined stickleback. Together, the studies intended to

evaluate the usefulness of juvenile three-spined stickleback as model organism for androgenic and estrogenic modes of endocrine action.

One advantage of the three-spined stickleback is the possibility to identify the genetic sex of individuals. An improved and reliable method for genetic sex identification has been developed as a part of this thesis work, and applied on juvenile sticklebacks from the laboratory studies.

Laboratory experiments were designed to

investigate whether estrogenic and/or androgenic endocrine activity can be detected in effluents from Swedish pulp mills and domestic sewage treatment plants.

For this purpose, juveniles were exposed to dilution series of three pulp mill and two treated domestic sewage effluents. Conditions and studied endpoints were identical to those in experiments 1 and 2 with the exception that diluted effluents and model steroid substances were applied via a flow-through system.

Finally, juvenile three-spined sticklebacks were sampled during four subsequent summers in order to survey

whether estrogenic and/or androgenic endocrine activity can be detected in Swedish coastal waters receiving domestic sewage and pulp mill effluents.
Material and methods

Fish maintenance and exposure regimes
Adult sticklebacks were collected from a wild population in southern Sweden. They were held in the facilities at the Department of Zoology at Stockholm University. The exposure trials were carried out at the Institute of Applied Environmental Research (ITM). Sexual maturity and mating behavior was induced in the laboratory by application of appropriate temperature and photoperiod. Fertilized egg clutches were collected from nests within one day after mating. The fry hatched after six to seven days. On the day of hatching they were divided into a number of different exposure aquaria. Larvae started feeding at day two after hatching. The

Figure 1: A generalized scheme of a typical experimental design. An exposure experiment was conducted with offspring from several mating couples. Each couple mated once. Broods were treated as separate replicates. The same control treatment (water or solvent) was allocated to each brood. Treatments with steroid substances or dilutions of effluents were allocated to the broods. Each brood did not normally receive every treatment, since the number of individuals from each couple was not sufficient for this. Each treatment or control within a brood was carried out in one aquarium containing up to 24 individuals. Deviations from this scheme occurred in the experiments due to practical considerations.

fry and juveniles were fed with freshly hatched brine shrimp (Artemia sp.) nauplii.

Generally, exposures started at the day of hatching. In experiments 1 and 2, some groups were exposed to E2 during the embryo (i.e. egg) stage. Model steroid substances were applied in a semi-static renewal system, in which exposure water was replaced every second day (papers I, II & III). In experiments 3–5 (paper IV, studies 1-3), water with steroids or diluted effluents was delivered to the aquaria in a flow-through system. In order to allow comparison with the semi-static exposures, the flow-through speed was initially set to correspond to a two-days 80–90% water renewal according to the equation \( C = e^{(\alpha/v)*100} \), where \( C \) is the remaining share of spent water in an aquarium (%), \( \alpha \) the flow speed (volume/time), \( v \) the volume of the aquarium and \( t \) the time. Flow speed was doubled in experiment 5 (details on experiments 3–5, studies 1–3 in paper IV). The applied model steroid substances and concentrations are given in table 1. The experiments were terminated when the fish had reached a mean length of approximately 20 mm as judged by eye. The fish had reached this size after 39 to 58 days after hatching. Due to practical limitations, the experiment with domestic sewage effluent (paper IV, study 2) had to be terminated although the fish had only reached an average length...
of 15 mm. Fish were anaesthetized by cooling them in treatment water on ice. Their length was measured, thereafter they were killed by decapitation and their weight (including head) was determined. The condition index was calculated \((weight \times length^3 \times 1000)\).

**Histological assessment of gonads**

According to Swarup (1958), at a size of \(\geq 20\) mm the gonads were expected to be sufficiently differentiated in order to distinguish the gonad types, which also was the case. In the fish that only reached a length of in average 15 mm (experiment 4), gonads were already differentiated enough to discern ovaries and testes. The distinction between intersexual gonads and ovaries, however, was possible with certainty only in combination with genetic sex identification.

In preparation for histological examination, whole bodies were fixed in Bouin-Hollandé. Only in the first experiment, 10% buffered formalin was used as fixative, but the quality of the tissue preservation was poor. In particular, details of spermatogonia were not observable.

![Figure 2: juvenile ovary](image)

After fixation, the body samples were dehydrated stepwise in ethanol, cleared in xylene and embedded in paraffin wax. Longitudinal horizontal sections (\(\geq 4\) \(\mu\)m) were cut from whole fish with 40 \(\mu\)m distance (32 \(\mu\)m in experiment 4) between sections. Control fish were used for the definition of the normal status of ovaries and testes. Gonad types were defined as follows: Gonads were defined as juvenile ovaries if they almost entirely contained oocytes in the perinucleolar stage and if an ovarian cavity wall lined the entire gonad (figure 2). Juvenile testes were defined by the absence of any distinguishable stages of oocytes, and the assembly of gonocytes in a lobulated structure and absence of any ovarian cavity lining (figures 3 and 6). The sectioning scheme of 40 (32) \(\mu\)m intervals did not allow the definition of testes by the presence of a sperm duct, as it was not consistently observable, especially not the attachment point(s) to the mesentery. When observable, the gonad duct appearance was considered in the evaluation of the gonad type. Gonads were defined as intersex when they contained one or more perinucleolar oocytes, but their overall appearance was not ovary-like, i.e. they displayed...
a lobulated structure and no consistent cavity lining (figures 4 and 5). Also another gonad type, named ‘testis with cavities’ or ‘partially sterile’, was observed (figure 7, paper I & III).

Analysis of vitellogenin and spiggin
The ELISA assays have been developed by Ioanna Katsiadaki and co-workers at CEFAS in Weymouth, UK, where the assays also were carried out. Both vitellogenin and spiggin were analyzed in each sampled specimen. Whole bodies were homogenized in liquid nitrogen. The water-soluble fraction of proteins (including vitellogenin) was extracted in phosphate buffer, after which it was centrifuged. The precipitate was digested in a strong urea buffer, heated at 70°C for 60 min and centrifuged. The supernatant was collected. Generally, procedures were as described in Katsiadaki et al. (2002, 2004).

Genetic sex identification
Total genomic DNA was isolated from caudal fin samples that were taken from individuals before processing for histological preparation. The tissue samples were stored in 99.5% ethanol. Initially, the method developed by Griffiths et al. (2000) was applied (paper I). With help of AFLP (Amplified Fragment Length Polymorphism) and PCR (Polymerase Chain Reaction), he established sex-linked markers of probably non-functional DNA. The PCR reactions were carried out with two primers (Ga1F 5’-CTTCTTTCTCTCACCATACTCA-3’ and Ga1R 5’-AGATGACGGGTTGATAAACAG-3’, Ga2F 5’-CACATTATTACAACATACGCA-3’ and Ga2R 5’-ACAGACGCTGAATGACGAAG-3’). However, an additional band suggested as positive control, which should develop in all samples, turned out not to be reliable enough, so for a number of individuals – presumably females – the sex could not be identified. A substantial improvement of the method was made by Emma Ehn and Prof. Mats Grahn at Södertörn University College, Sweden, who added a pair of microsatellite primers, locus Gac 7148 PBBE described by Heckel et al. (2002), as an additional positive control. The improved method was applied in a re-assessment of intersexuality and gonad type reversal (paper III) and in subsequent studies (paper IV).
Field sampling

Juvenile three-spined sticklebacks were collected during July and August. Depending on the topography of the localities, this was done in 0.6 to 1.2 m deep water with a beach seine or a hand net. 12 to 60 juvenile individuals were collected on each site. For comparability with laboratory studies, individuals in the size range 15 to 25 mm were preferred, if available. Ten to 38 individuals were frozen for spiggin and vitellogenin analysis, and the remaining fish were fixed in Bouin-Hollande (10% buffered formalin the first year, see above) for histological analysis.
Seven Swedish pulp mills representing different processes and/or raw material and/or waste treatment were selected. The industries were visited for sampling juvenile sticklebacks in their recipients. At some sampling sites, sticklebacks were not found every year. The recipient of one of the domestic sewage treatment plants was also visited, but sticklebacks could only be caught in low numbers, if at all (year 2000: ten individuals, year 2003: three individuals). The recipient of the other sewage treatment plant was not visited. Localities remote from anthropogenic effluents were chosen as suggested reference sites.

![Testis of genetic female after methyltestosterone treatment during 0-13 days after hatching](image)

**Figure 6**: testis of genetic female after methyltestosterone treatment during 0-13 days after hatching

### Results and discussion

#### Laboratory exposures to steroids (papers I–IV)

Juvenile three-spined sticklebacks are sensitive to both estrogens and androgens. Exposure to estrogens caused the development of intersex and in a few individuals even apparent total gonad type reversal from male to female. The latter was confirmed by the application of DNA sex identification. Estrogens also induced the production of vitellogenin in the juveniles. This yolk precursor protein is normally produced only by sexually mature females. Estradiol at high concentration (10 µg/L) was found to elicit a weak androgenic effect as spiggin was slightly elevated in juveniles and kidney epithelial cells were slightly hypertrophied. Spiggin is normally produced in the kidney of sexually mature males, which is accompanied by the hypertrophy. Both MT and DHT induced kidney hypertrophy and high levels of spiggin in juvenile sticklebacks when applied at effective concentrations, which vindicates their classification as androgens.

MT however, had both androgenic and estrogenic potential when gonadal development was studied. When applied only during the first two weeks after hatching, MT caused total reversal of gonad type from female to male. When applied continuously, MT induced intersex in genetic females, possibly as a result of re-feminization of masculinized individuals. This ‘paradoxical’ feminization is believed to be a result of aromatization of the androgen to the estrogen methylestradiol (see above). In genetic males, MT caused testis abnormalities that were described as cavities or partially sterile testes, when applied after the first two weeks. DHT
induced intersex in genetic females, which was characterized by a masculinization of gonad duct, and the same testis abnormalities as MT in genetic males.

Table 1 summarizes the results and shows threshold concentrations for all tested estrogens and androgens. Generally, only one or very few different concentrations of the steroid substances were applied in each experiment. The sensitivity threshold of juvenile sticklebacks should be better quantified before comparisons with other species can be made with satisfaction. This means that concentration series should be tested of appropriate model substances in flow-through systems with appropriate speed. Substances should either be used that are relatively persistent, or substance concentrations should be monitored throughout the experiment. Establishing dose-response relationships was not the main objective of experiments 4–5. Still, several concentrations were applied since little was known about the potential of the model substances, in particular due to the methodological switches from semi-static to flow-through system and from the aromatizable androgen MT to the non-aromatizable DHT. Indeed, the sensitivity of juvenile sticklebacks to DHT was obviously overestimated, alternatively the degradation rate of this compound in the exposure water was underestimated. Although a trend towards elevated spiggin was observed in DHT (5 µg/L, exp. 4), the only significant effects were detected with DHT 15 µg/L (exp. 5). I also found differences between studies in sensitivity towards estrogens, which are most likely explained by differences in flow through speed. In experiment 4, no effect was seen of EE2 at 0.01 µg/L, whereas in study 3 with doubled flow-through speed elevated vitellogenin was detected already at 0.004 µg/L. The latter result puts the sensitivity of the juvenile stickleback in the same range as in other laboratory sentinel species, as for example the fathead minnow (Länge et al. 2001). The stickleback seems similarly sensitive in disruption of gonad development as in the induction of spiggin and vitellogenin, since threshold levels do not differ between these endpoints within studies.
Sensitive period

Certain phases of gonadal development are particularly sensitive to be disrupted by the action of external steroid hormones. In the three-spined stickleback, this time window of highest – albeit not exclusive – sensitivity is located within the first two weeks after hatching. With estradiol, exposure duration of only two weeks was sufficient to induce intersexuality (developing oocytes and partially developed ovarian cavity) in genetic males. When exposure started later, intersex was still induced to a lower degree (papers I & III). The simultaneous feminizing and masculinizing potential of MT showed differences in the respective sensitive phases. When applied continuously, MT induced intersex in genetic females (testis-like lobulated assembly and absence of ovarian cavity). No intersex was induced when MT treatment started after 14 days after hatching. Exposure to MT only during the first two weeks caused genetic females to develop juvenile testes, which seemed to be irreversible. This indicates that the male gonad might be irreversibly defined earlier than the female gonad. As reported in the medaka (Koger et al. 2000) and the coho salmon (Piferrer & Donaldson 1989), the sensitive time windows for estrogenic and androgenic effects are not necessarily identical.

Table 1: effect threshold concentrations for all studied effects and tested estrogens and androgens in laboratory experiments

<table>
<thead>
<tr>
<th>Estrogens</th>
<th>Applied nominal concentrations (µg/L)</th>
<th>Gonad differentiation and development</th>
<th>Spiggin</th>
<th>Vitellogenin</th>
<th>Experiment</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2</td>
<td>0.01, 1, 10</td>
<td>0.01 1</td>
<td>–</td>
<td>–</td>
<td>1 &amp; 2</td>
<td>I &amp; III</td>
</tr>
<tr>
<td></td>
<td>0.01, 1, 10</td>
<td>– 10</td>
<td>0.01 1</td>
<td></td>
<td>1 &amp; 2</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>&lt; 1</td>
<td>–</td>
<td></td>
<td>3</td>
<td>IV: study 1</td>
</tr>
<tr>
<td>EE2</td>
<td>0.05</td>
<td>&lt; 0.05</td>
<td>–</td>
<td>–</td>
<td>1 &amp; 2</td>
<td>I, III</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>–</td>
<td>0.05 &gt;</td>
<td>&lt; 0.05</td>
<td>1 &amp; 2</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>0.002, 0.01, 0.05</td>
<td>0.01 0.05</td>
<td>0.05 &gt;</td>
<td>0.01 0.05</td>
<td>4</td>
<td>IV: study 2</td>
</tr>
<tr>
<td></td>
<td>0.004, 0.02, 0.1</td>
<td>&lt; 0.1*</td>
<td>0.1 &gt;</td>
<td>&lt; 0.004</td>
<td>5</td>
<td>IV: study 3</td>
</tr>
<tr>
<td>Androgens</td>
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<tr>
<td>MT</td>
<td>1</td>
<td>&lt; 1</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>I &amp; III</td>
</tr>
<tr>
<td></td>
<td>1</td>
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<td>&lt; 1 1</td>
<td></td>
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<td>II</td>
</tr>
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<td></td>
<td>0.1</td>
<td>&lt; 0.1</td>
<td>–</td>
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<td>3</td>
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</tr>
<tr>
<td>DHT</td>
<td>0.2, 1, 5</td>
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<td>5 &gt;</td>
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</tr>
<tr>
<td></td>
<td>0.6, 3, 15</td>
<td>&lt; 15*</td>
<td>3 15</td>
<td>15 &gt;</td>
<td>5</td>
<td>IV: study 3</td>
</tr>
</tbody>
</table>

NOEC (No-observed-effect-concentration) = the highest applied concentration that did not induce the effect
LOEC (Lowest-observed-effect-concentration) = the lowest applied concentration that induced the effect
– = The effect was not studied
* = Gonad differentiation only studied at highest concentration
< = No appropriate concentration, lower than LOEC was applied
> = No appropriate concentration, higher than NOEC, was applied
Laboratory exposures to pulp mill effluents and sewage effluents (paper IV)

Experiment 3 indicated possible disruption of sexual development (intersex) from a pulp mill effluent. However, due to the pilot character of the study, sample were small. Moreover, only one of two tested broods showed this effect (1, 10, and 50% effluent), and this brood also contained a number of sterile individuals even in low-dose treatments. The incidence of sterility was not obviously correlated to concentration, indicating that this brood might have been impacted by another stressor, thus being extra sensitive, and might not be representative for juvenile sticklebacks in general. It cannot be concluded whether the possible effect would be estrogenic or androgenic due to low individual numbers and lack of genetic sex identification. Vitellogenin and spiggin were not analyzed.

Effluents from two other pulp mills (1, 3.7, 13.5 and 50% effluent) did not result in any sign of estrogenic or androgenic endocrine disruption, neither in induction of vitellogenin or spiggin, deviations in sex ratios nor aberrations in gonad development. The only detected effect was reduced somatic growth in 50% effluent from one of the mills.

Two domestic sewage effluents did not result in any sign of endocrine disrupting activity. In this experiment, however, even the positive control substances and elicited none (DHT) or weak (EE2) effect. One explanation might be rapid degradation of the substances in combination with inappropriate flow-through speed of the water. This might have been equally the case for potentially endocrine disrupting substances in the effluents, especially since EE2 is the most potential estrogen in domestic sewage effluents. Nevertheless, concerning relevance for the fish in the receiving water, the effluents cannot be considered as endocrine disrupting because no activity was detected even in 50% effluent.

Field survey (paper IV)

Corroborating the finding from the laboratory exposure studies, estrogenic or androgenic endocrine disruption was not found when studying juvenile three-spined sticklebacks that had been collected in the recipients of pulp mills and a sewage treatment plant along the Swedish Baltic Sea coast. Among 782 analyzed individuals, only one with intersex was detected, which showed very low severity of the intersex condition (i.e. only one single oocyte was detected in the whole testis). Moreover, this individual was collected at a reference site (Askö marine laboratory). Another reference site was the only one showing consistent – albeit small – deviations in sex ratios towards males, accompanied by higher condition indices. Although remote from industrial and domestic effluents, this site might have been impacted by nutrient enrichment from low-intensive cattle farming or degrading red algae (among other species: Polysiphonia spp. and Ceramium spp.), which are known to produce a number of brominated phenolic substances (Pedersen et al. 1974) similar to the chlorinated phenolic compounds found in chlorine bleached pulp mill effluents (Fisher et al. 1996). If and in what way this may have affected the stickleback population is not known. No significantly elevated levels of spiggin and vitellogenin were detected in juvenile sticklebacks from any of the sampled localities, whether they were recipients of effluents or not.

Summary and conclusions

The juvenile three-spined stickleback was found to be sensitive to water-borne natural and synthetic steroids. In a semi-static exposure system, 17β-estradiol (E2: nominal concentration 1 and 10 µg/L) and 17α-ethinylestradiol (EE2: nominal conc. 0.05 µg/L) had estrogenic effect on juvenile G. aculeatus, which resulted in intersex and gonadal sex reversal and the induction of the estrogen-responsive protein vitellogenin. 17α-methyltestosterone (MT: nominal conc.
1 µg/L) induced the stickleback-specific androgen-responsive protein spiggin, but resulted in a more complex picture concerning sex differentiation and sexual development. MT induced intersex in both genetic males and females and testicular abnormality in genetic males. The ambivalent effect of MT can be attributed to aromatization of androgens to estrogens, which is the natural pathway of androgen synthesis. The first two weeks after hatching is the most sensitive time period to the action of exogenous hormones on gonadal differentiation. An advantage of the three-spined stickleback is the possibility to reliably identify the genetic sex with help of male-specific DNA markers. Apparently irreversible gonad type reversal from female to male was confirmed by applying this marker. Intersexuality is potentially suitable as endpoint for the study of endocrine disruption in juvenile sticklebacks, but genetic sex-determination is inevitable for correct distinction between normal ovaries and feminized testes in juveniles and to reveal the direction of the disruption (i.e. masculinization or feminization).

The tested endpoints vitellogenin and spiggin are suitable for the study of estrogenic and androgenic endocrine disruption in juvenile sticklebacks. In flow-through exposure systems, vitellogenin was induced by EE2 as low as 0.004 µg/L (nominal conc.). 5α-dihydrotestosterone (DHT) displayed androgenic effect at >3 to 15 µg/L (nominal conc.), although sensitivity depended on flow speed. Although this indicates that the juvenile three-spined stickleback might not be particularly sensitive to androgenic compounds, the species has the advantage that it is common, abundant and native in Sweden and thus can be collected in large numbers in many recipients of anthropogenic effluents. A field survey with juvenile three-spined sticklebacks revealed no widespread estrogenic or androgenic endocrine disruption in Swedish Baltic Sea coastal waters receiving pulp mill or domestic sewage effluents. This assumption is corroborated by the fact that no such effects were found in relevant dilutions (50% and below) of two domestic sewage and two pulp mill effluents.

### Outlook

Further improvements of the methods described in this thesis could and should be made. If juvenile three-spined sticklebacks are to be used as sentinels in screening and monitoring studies in future their sensitivity needs to be quantified with appropriate model substances in flow-through systems in combination with analysis of concentrations of the active compounds in the water. A slight prolongation of the exposure period might permit the distinction between the juvenile gonad types ovary and intersex even without application of genetic sex identification, as it was found that later stages of spermatogenesis were observable after a few weeks longer exposure than 42 days (not quantified, not published).

The combination of genetic sex identification with gonad histology at the individual level will in future provide a powerful tool for detecting endocrine disrupting activity with the three-spined stickleback in laboratory studies and in particular in the field, as it can be established at the individual level whether deviations in sex ratios are a result of sex reversal. Unless a genetic sex marker is available, large numbers of fish would be required in order to establish statistical significance of slightly skewed sex ratios as a result of endocrine disruption in fish. Furthermore, genetic sex identification can eliminate other bias factors from sampling methodology or the biology of the fish.

Among the small-sized laboratory fish species so far used in endocrine disruption studies the three-spined stickleback is the only European representative, which allows comparison between laboratory and field studies with the same species. Juveniles need even less spatial and material resources than adults, which will be an advantage in large-scale testing of chemicals and effluents, where practical and financial aspects need to be considered. Furthermore, juveniles have the advantage that they can be caught in high numbers close to the place where they
have been exposed during the most sensitive life-stage, since they have probably not yet started to migrate. However, the migratory behavior of juveniles, in particular possible migration distances, needs to be investigated. When juvenile sticklebacks are caught in the field in the search for effluents with endocrine disrupting activity, the actual time point of exposure might be unknown. A disruption of gonad development will indicate an exposure very early in life, possibly only during a very short time during the most sensitive phase, while elevated levels of the protein markers spiggin or vitellogenin will indicate a more recent or still ongoing exposure situation.

Although I did not find severe or widespread effects in Baltic Sea coastal waters, endocrine disruption might still be of local concern in recipients with low dilution rates or in the vicinity of point sources that have not yet been tested. A countrywide survey comprising a large number of representative effluents and recipients will be needed to elucidate this further. Additionally, the influence of natural environmental factors should be studied in more detail.

Very little effort has so far been made to answer the question whether early life-stage exposure to endocrine-disrupting compounds may elicit effects on the reproductive success of individuals or populations. Few studies have addressed whether intersex or other gonadal impairment affect reproductive success, as for example Jobling et al. (2002), who demonstrated that wild roach living in effluent-impacted rivers had a lower proportion of sperm-releasing fish, and in those intersex fish that were releasing sperm, a reduced milt volume and a reduced sperm density were found. Spermatogenesis was delayed in a large proportion of all intersex and exposed male roach. In the sea bass, exposure to E2 after the sensitive period showed no effect on sex ratio, gonadal size or testis function. However, consequences were manifested in dose-dependent reduction in number of mature males during breeding season two years later (Blázquez et al. 1998b). Long-term effects of androgenic endocrine disruption have been studied even less. Little is known whether intersex or gonadal sex reversal as a result of estrogenic exposure during the critical time window will be irreversible over a longer time scale or whether intersexual fish will have a lower reproductive success than normal fish in situ.
References


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