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Behavioural effects of environmental pollution in threespine stickleback *Gasterosteus aculeatus* L.

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Contents

Preface and Acknowledgement

This thesis that is submitted to the Norwegian University of Science and Technology (NTNU) for the degree of Doctor scientiarum is based on four separate studies.

The works have been conducted at Department of Ecotoxicology, Allforsk; Brattøra Research Centre, NTNU; Department of Freshwater Ecology and Water Management, Norwegian Institute for Water Research (NIVA) and Department of Zoology, NTNU.

During the study I have received assistance and knowledge from many important people. First of all I am very thankful to my supervisors, Professor Gunilla Rosenqvist and Professor Bjørn Munro Jenssen. The combination of behavioural ecology and ecotoxicology is an extremely interesting approach, and Nilla and Bjørn have encouraged to many fruitful discussions. I also want to thank my supervisors for MSc, Professor Eivin Røskaft and Dr. Trond Nordtug who were the first to take the challenge of supporting a work at the border of their own fields of research.

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List of Papers

This thesis is based on the following papers:

I. Wibe, Å.E., Nordtug, T. and Jenssen, B.M. (2001). Effects of bis(tributyltin)oxide on antipredator behavior in threespine stickleback *Gasterosteus aculeatus*. *Chemosphere* **44 (3)**, 475-481.

II. Wibe, Å.E., Fjeld, E., Rosenqvist, G. and Jenssen, B.M. (In Press). Post-exposure effects of DDE and butyl benzyl phthalate on feeding behavior in threespine stickleback. *Ecotoxicology and Environmental Safety*.

III. Wibe, Å.E., Billing, A., Rosenqvist, G. and Jenssen, B.M. (2002). Butyl benzyl phthalate affects shoaling behavior and bottom-dwelling behavior in threespine stickleback. *Environmental Research Section A* **89**, 180-187.

IV. Wibe, Å.E., Rosenqvist, G. and Jenssen, B.M. (2002). Disruption of male reproductive behavior in threespine stickleback *Gasterosteus aculeatus* exposed to 17β-oestradiol. *Environmental Research Section A* **90**, 136-141.

Abstract

The aims of this study were to investigate the effects of known environmental contaminants on defined behavioural variables in fish, and to discuss properties of these behavioural traits that make them useful as potential indicators of pollution.

In studying the effects of pollution, the resulting biochemical and physiological alterations are more commonly measured. However, effects of pollution can manifest itself at all levels of biological organisation, including behaviour. In this respect, behaviour can be considered a valid biomarker of pollution in that it is expected to be both susceptible to pollution and of high ecological significance, as it influences the fitness of the affected individuals.

This thesis is based on four individual studies, in which the threespine stickleback *Gasterosteus aculeatus* was used as a model species. Results from these studies show that antipredator behaviour, feeding behaviour, shoaling behaviour, bottom-dwelling behaviour and reproductive behaviour are all sensitive to exposure to sublethal concentrations of defined environmentally relevant chemicals.

The results showed that antipredator behaviour and fright response in threespine stickleback were impaired following exposure to sublethal concentrations of bis(tributyltin)oxide (TBTO). However, for some of the tested antipredator variables the effects were reversed after the ending of exposure. Further, it was shown that feeding motivation in fish exposed to butyl benzyl phthalate (BBP) and/or 2,2-bis(p-chlorophenyl)-1,1-dichloroethylene (DDE) was increased in that exposed fish initiated feeding more often than the controls. Exposure to BBP also caused sticklebacks to aggregate into tight shoals and to spend more time at the bottom of the aquarium compared to the control fish.

The reported significant differences between the controls and BBP-exposed fish with respect to feeding and shoaling behaviour were shown even though the levels of BBP were below the analytical detection limit. Different suggested explanations, for example, too high detection limit, or degradation to its BBP metabolites are given to this result.

17β-Oestradiol (E_2) exposed male sticklebacks started nest building later than non-exposed males, but there were no differences between exposed and control males with respect to the number of males that built nests. Further, the exposed males spent less time displaying paternal care compared to the control males, although there were no differences between the two groups in the number of performed courtship displays. Because of the significant effect upon some but not all reproductive behavioural traits, it was suggested that the different variables might vary in sensitivity, implying that a variety of variables should be studied in order to obtain a more reliable evaluation of the effects of pollution.

Chemicals can cause deleterious effects at one or more levels of biological organisation, from biochemical, physiological, individual, population and through to the ecosystem levels. In contrast to the established hypothesis that a pollutant affects the different biological levels in an escalating time-dependent pattern, starting at the biochemical level, it is here suggested that biomarkers at the biochemical, physiological and behavioural levels often will respond early and simultaneously in the same individual.

Whereas some biochemical responses are specifically related to one class of exposure agents and thus may act as specific indicators of pollution, most behavioural traits may be altered in response to a variety of chemicals. One exception may be alterations in reproductive behaviour caused by endocrine disrupting chemicals, due to effects of the chemicals on hormones that result in immediate reproductive behavioural effects. In spite of the specific action of some biochemical biomarkers, they are often considered to be of little ecological relevance since many of them are not related to individual fitness.

In this thesis, it is argued that behavioural variables can be employed as useful and reliable biomarkers of environmental contamination. It is also important to focus on behaviour to map and quantify the resposes. However, to reliably evaluate the effects of pollution, behavioural variables should be used in association with biochemical and physiological traits. Moreover, optimal combination of results from laboratory and field experiments would enhance the ecological relevance of the study.

1. General Introduction

Nature has been exploited for thousands of years. Natural resources have been utilised, often to an extent that have caused irreversible damage to the environment. It is also a growing public concern over the adverse effects of environmental contaminants on wildlife populations. One of the early scientists that was concerned about the harmful effects of chemicals was M.J.B. Orfilia (1787-1853), who investigated the relationships between the presence of chemicals in organisms and the observed toxic effects. He also made toxicological conclusions (defined as determination of toxic thresholds, e.g. lethality tests) that are valid even today (Manahan, 1989). However, it was not until the 1920`s, when scientists became aware of the noxious effects of food additives, drugs and pesticides, that systematic laboratory studies on effects of pollutants were intensified (Rodricks, 1992).

The use of animal behaviour in applied science is a quite modern approach that requires an extensive knowledge of animal behaviour theory. Charles Darwin expressed some of his hypotheses concerning behaviour and evolution in "On the origin of species" (1859), but it was not until the middle of the 1930s that the discipline of ethology was developed, when Niko Tinbergen and Konrad Lorenz started their investigations on proximate and ultimate questions of behaviour.

The science of animal behaviour has been used in many different disciplines of applied research. Conservation biology is an example (e.g textbook by Clemmons and Buchholz, 1997), but unfortunately behaviour is still considered a neglected topic in conservation (Shumway, 1999). By using behaviour in conservation biology an increased understanding of for example, how to manage wild species, how to manage human – animal conflicts and how to avoid extinctions of endangered species, may be obtained (Shumway, 1999). Applied behaviour has been extensively used in studies on farm animals and animal welfare. The recognition that welfare in animals is associated with the quality of farmed products has led to increased understanding of animal behaviour among people working with domestic animals. Examples where

behaviour has been used to increase welfare in domestic animals include pig farming (e.g. Haskell *et al*., 1996; Vestergaard, 1996), caging of hens (reviews by Appleby and Hughes, 1991; Lewis and Morris, 1998) and foxes (review by Braastad, 1998), and aquaculture (e.g. review by Ruzzante, 1994).

Furthermore, behaviour has also beed used in ecotoxicological investigations (defined as questions concerning the fate and effects of chemicals in ecosystems). Warner and co-workers (1966) were among the first to use behaviour in the investigation of effects of pollution when they studied movement and avoidance behaviour in fish exposed to toxaphene. The main aim of the work by Warner *et al.* (1966) was to identify behavioural variables that could be used to detect effects of sublethal concentrations of pollution. Their hypothesis was that behavioural variables will give an early warning to pollution and that behaviour is a comprehensive variable in the detection of effects of pollution since alterations in behaviour is the consequence of several biochemical and physiological alterations. Studies concerning behavioural effects of pollution have since then often been aimed at identifying stereotyped behaviours that easily and in a standardised manner can be used for detecting effects of pollution.

In ecotoxicological research the aquatic environment is highly relevant since most pollutants, either directly or indirectly, end up into water systems. Since fish, as well as other aquatic organisms, are constantly exposed to pollutants, either directly or via the food chain, they are ideal sentinel species, and have been used as model species in ecotoxicological investigations. To date, in studies on the impact of pollutants in fish, the resulting biochemical, physiological and histological alterations have been of most interest. Examples of reported effects include altered ion balance over the gills (Na^+,K^+-ATPase activity) in juvenile carp *Cyprinus carpio* exposed to cadmium (dellaTorre *et al.*, 1999), and changes in gill structure in mormyrid fish *Gnathonemus perersii* after exposure to heavy metals (Alazemi *et al.*, 1996). In the present thesis, behaviour will be used to detect effects of pollution, and it will be argued that changes in behavioural variables allows for a comprehensive and ecologically relevant evaluation of the effects of pollution on individuals. A short presentation

of some of the behavioural variables that have been used in ecotoxicological studies is given below. The referred case studies are given as relevant examples of the respective behavioural traits, thus no attempts to find original or exceptional case studies have been made.

Avoidance reaction is an example of a stereotyped behaviour (i.e a trait that may be quantified objectively and that shows little variation between individuals) that has been shown to be sensitive to pollution. In a field experiment Saunders and Sprague (1967) showed that Atlantic salmon *Salmo salar* avoided localities that were contaminated with copper and zinc. Also, in laboratory experiments avoidance of pesticide by carp (Ishida and Kobayashi, 1995) and avoidance of acidified water in blacknose dace *Rhinichtys atratulus* and brook char *Salvelinus fontinalis* (Newman and Dulloff, 1995) were documented.

Swimming behaviour is one of the most frequently used behavioural traits in ecotoxicological research on fish, as impaired swimming ability may have severe consequences for the performance of other activities such as feeding, predator avoidance and reproduction. Distinction between swimming capacity and swimming activity is common. Swimming capacity refers to the fish orientation in relation to the water flow, including their capacity for positive rheotaxis (orientation towards water flow), while swimming activity refers to factors such as swimming speed, posture (e.g. head-up swimming), duration of movement, frequency and angle of turns, and position in the water column (Little and Finger, 1990). Experimental studies on several fish species have shown that exposure to commonly used chemicals (i.e. herbicides, cadmium, methylmercury, DDT, TBTO etc.) may severely impair both swimming capacity (e.g. Besch *et al.*, 1977) and activity (e.g. Niki and Farrell, 1993; Triebskorn *et al.*, 1994; Steinberg *et al.*, 1995; Zhou and Weis, 1998; Grillitsch *et al.*, 1999).

Feeding behaviour has also been used to detect effects of pollution in fish. Feeding is a collective term comprising many elements such as detection of prey, identification, prey capture, handling of prey, and consumption (e.g. Endler, 1991). Many of these behavioural elements have been used in studies of pollution effects. For example, in an experiment by Lemly and Smith (1987) it

was found that fathead minnows *Pimephales promelas* exposed to acidified water failed to detect prey due to impaired chemoreception. Furthermore, prey attack has been shown to be impaired in largemouth bass *Micropterus salmoides* after exposure to the biocide pentachlorophenol (Mathers *et al.*, 1985), and in juvenile bluegill *Lepomis macrochirus* after exposure to cadmium (Bryan *et al.*, 1995). In the study by Mathers *et al.*, 1985, the largemouth bass became less efficient at feeding (capture-to-strike ratio), had decreased food conversion rate (weight-gain to food-consumption ratio), and the fish consumed less food.

Antipredator behaviour in fish is a further example of a behavioural trait that has been used to detect effects of chemical pollution. For example, Smith and Weis (1997) and Zhou and Weis (1998,1999) both documented impaired antipredator behaviour in mummichogs *Fundulus heteroclitus* living in polluted habitats. Similarly, increased prey vulnerability following exposure to pollutants have been reported in fathead minnows (Sullivan *et al.*, 1978), juvenile guppies *Poecilia reticulata* (Brown *et al.*, 1985) and juvenile chinook salmon *Onchorhynchus tshawytscha* (Kruzynski and Birtwell, 1994).

An increasing number of studies on the effects of pollution on fish reproductive behaviour have concerned the so-called endocrine-disrupting chemicals (Palanza and Saal, 2002) (defined as exogenous substances that cause adverse effects in an intact organism, or its progeny, consequent to changes in endocrine functions). Endocrine-disrupting chemicals (EDCs) interfere with the endocrine system in both aquatic and terrestrial organisms, by e.g. blocking the receptors, mimicking the natural steroids or interfering with steroid metabolism. Exposure to EDCs is suspected to be the underlying cause of the observed decline in diverse wildlife populations as well as the increased occurrence of reproductive and developmental disturbances in wildlife. Examples of effects include the observed deterioration of courtship behaviour in guppy males exposed to phenol (Schröder and Peters, 1988) and 17β-oestradiol (E_2) (Bayley *et al.*, 1999). E_2 also caused reduced courtship activity in male goldfish *Carassius auratus* (Bjerselius *et al.*, 2001). Other reproductive variables used in pollution studies on fish include fecundity and hatching

success. For example, Shioda and Wakabayashi (2000a, b) found that both egg production and hatching success was impaired in medakas *Oryzias latipes* following exposure to E_2 and other chemicals with oestrogenic properties.

Finally, schooling and aggregation in fish, are traits that have been shown to be susceptible to exposure to some chemicals. For example, DDT-exposed goldfish were less likely to school than unexposed fish (Weis and Weis, 1974), whereas guppies exposed to E_2 aggregated more frequently than control fish (Bayley *et al*., 1999).

1.1 *Biomarkers*

In this thesis the term "indicator of pollution" is used to describe a biological variable that may indicate exposure to or effects of pollution. The term is often used synonymous with "ecotoxicological biomarker", which is defined as:

> *"A biochemical, cellular, physiological or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms (either individuals or populations) that provides evidence of exposure to and/or effects of one or more chemical pollutants (and/or radiations)"*
>
> Depledge (1994)

When discussing biomarkers, it is common to distinguish between four classes: 1) "Exposure biomarkers" indicate that an individual, a population or a community has been exposed to one or more chemicals. 2) "Effect biomarkers" indicate that an individual, a population or a community suffers from effects caused by one or more chemicals. 3) The "exposure/effect biomarkers" link one effect to a specific exposure. 4) "Latent effect biomarkers" refer to changes in the capacity of an individual to adapt to future environmental fluctuations (Depledge, 1994).

Throughout this thesis the definitions of the biomarker concept given above will be used. However, there are alternative definitions in the literature (e.g. Walker, 1998; Adams, 2001), for instance those distinguishing between a biomarker of exposure and a bioindicator of effect (Adams, 2001). In order to develop biomarkers for monitoring programs, evaluation of the biomarkers in controlled and standardised laboratory experiments are normally required. Thus, in addition to being used in natural systems, biomarkers can also be applied in laboratory ecotoxicological tests where the purpose is to test the toxicity of chemicals (e.g. ASTM, 1995).

A further distinction is often made between "special" and "general" biomarkers (Depledge, 1994). Special biomarkers respond to exposure to one chemical or one class of chemicals (e.g. the metal binding proteins, metallothioneines, in response to exposure to some heavy metals, or the response to exposure to lead on aminolevulonic acid dehydratase, ALAD), while general biomarkers will give the same response following exposure to different classes or types of chemicals (e.g. induction of detoxifying enzymes) (Depledge, 1994).

Ecotoxicological biomarkers are found at every level of biological organisation (Fig. 1). The lowest levels of biological organisation where biomarkers can be applied are at the the biochemical and cellular levels. Examples of biochemical biomarkers include the induction of detoxifying enzymes and the formation of DNA adducts (covalent binding of a chemical to a DNA molecule) (Peakall, 1994), whereas cellular biomarkers may be alterations in endoplasmic reticulum and histopathological changes in e.g. liver cells (Moore *et al.*, 1994).

Even though biomarkers at the biochemical and cellular levels are sensitive to pollution, they often do not refelect the ecological significance of exposure at the higher levels of biological organisation, such as reproduction and survival of the individuals (Fossi et al., 1994; Peakall, 1994). It is suggested, as indicated in Fig. 1, that the ecological significance of the biomarker increases as the pollutant affects higher levels of the biological organisation, such as behavioural effects on individual and effects on

population/community (Peakall, 1994). Effects of pollution at the population/community levels are often included in monitoring programs, where effects of chemical pollution on several factors of the ecosystem, such as soil, water, plants and animals are investigated.
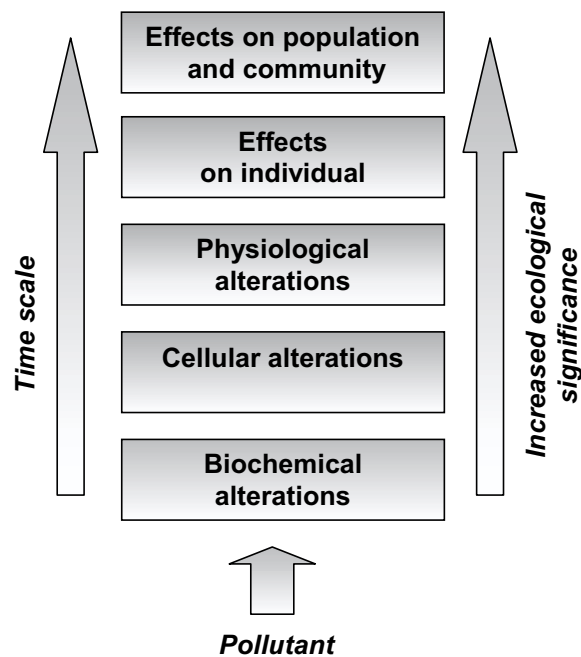
Figure 1. The influence of a pollutant on different levels of biological organisation from the cell to the ecosystem. The pollutant causes effects on higher levels of biological organisation as time passes after the chemical is introduced, and the ecological significance of the effects also increases as the pollutant reaches higher levels of biological organisation (Modified from Peakall, 1994).

1.2 *Aims*

The aims of this study were to investigate the effects of environmentally significant aquatic contaminants, on defined ecologically relevant behavioural variables in fish, and to discuss properties of these behavioural traits that make them useful as indicators of pollution.

To approach these aims, four separate studies were conducted in which the threespine stickleback *Gasterosteus aculeatur* was used as a model species. In the choice of behavioural variables, antipredator behaviour, feeding behaviour, shoaling behaviour, bottom-dwelling behaviour, and reproductive behaviour were selected because of their ecological relevance and suggested sensitivity to exposure to pollution (e.g. Little *et al.*, 1993; Jones and Reynolds, 1997). The test chemicals, bis(tributyltin)oxide (TBTO), 2,2-bis(p-chlorophenyl)-1,1-dichloroethylene (DDE), butyl benzyl phthalate (BBP) and 17β-oestradiol (E_2) were selected because of their significance as aquatic pollutants, and because of their relevance to the respective behavioural variables. The fish were exposed to concentrations of the respective chemicals that were expected to produce behavioural effects, but that simultaneously were expected to be sublethal and to resemble as much as possible the concentrations found in many human influenced waters.

1.3 *Threespine stickleback (Gasterosteus aculeatus* L.*)*

The threespine stickleback (Fig. 2) is a small teleost fish, normally measuring 40 to 60 mm in length. Its name originates from the three spines attached to its dorsal side. It also has two spines on each side near the anal fin. In addition to lateral bony plates, these spines constitute the fish's "body armour" that protects it from predators (Bell and Foster, 1994*b*). The degree of plate covering (low, partial and complete) varies between populations (Bell and Foster, 1994*b*). The geographical distribution of the threespine stickleback is restricted to the northern hemisphere where it is found in marine, brackish or fresh water; some populations are even anadromous (Bell and Foster, 1994*b*).

Several factors make the stickleback suitable for experimental studies. The fish are easily captured in the wild, and adapt readily to laboratory conditions. They also easily adapt to commercial dried food, live *Artemia* or commercially frozen mosquito larvae. Thus, the fish has been used as a model species in several types of experiments, including behavioural and ecotoxicological studies (e.g. Giles and Huntingford, 1984; Milinski and Bakker, 1990; Holm *et al.*, 1991; Sturm *et al.*, 2000), and decades of studies on stickle -

Figure 2. Threespine stickleback *Gasterosteus aculeatus* L. Photo courtesy of Ronny Höglund

back have resulted in a comprehensive knowledge of this species (e.g. Wootton, 1984, Bell and Foster, 1994*a*). Because sticklebacks often inhabit waters in the vicinity of harbours and industries, it is an ideal sentinal species for studying the impact of man-made compounds on aquatic wildlife.

The threespine stickleback is a recognised OECD test species (OECD Guidelines 210).

1.4 *Behavioural variables studied in the project*

Most of the behavioural activities that threespine sticklebacks perform can be classified as antipredator behaviour, feeding behaviour, shoaling behaviour or reproductive behaviour. Other traits are often related to the performance of one or more of these variables. For instance, aggressive behaviour may be triggered by competition for food or mates, and swimming behaviour may be closely related to the fish's ability to avoid predators and catch prey.

Three of the investigated behavioural variables (feeding behaviour, shoaling behaviour and bottom-dwelling behaviour) are described in the "Standard guide for measurement of behaviour during fish toxicity tests" (ASTM, 1995).

Antipredator behaviour

The major threat to young threespine sticklebacks is predation by adult sticklebacks (cannibalism) (Reimchen, 1994). Both young and adult sticklebacks are also vulnerable to predation by mammals, birds, reptiles, fish and macroinvertebrates (Reimchen, 1994).

In addition to their body armour, the stickleback exhibits a variety of antipredator mechanisms, such as shoaling (e.g. Pitcher and Parrish, 1993) and avoidance of locations where predators occur (e.g. Huntingford *et al.*, 1994). The explicit antipredator behaviour chosen depends on a range of factors such as density of predators, water transparency, disease, hunger and former experience (Milinski, 1993; Huntingford *et al.*, 1994). When encountering a predator, sticklebacks perform various escape manoeuvres depending on, for example, the type of predator, the distance to the predator and the individual experiences (Giles and Huntingford, 1984; Huntingford *et al.*, 1994). It has also been suggested that large-sized sticklebacks may avoid predation more successfully compared to small individuals due to predator preference for small fish (e.g. Moodie, 1972).

Predator-prey interactions are of high relevance for survival, and have to some extent been used in the studies of effects of pollution in different species, such as guppies (Brown *et al.,* 1985), mummichogs (Smith and Weis, 1997; Zhou and Weis, 1998; 1999) and fathead minnows (Sullivan *et al.*, 1978).

Feeding behaviour

The majority of prey items consumed by threespine stickleback are zooplankton, larvae and pupae of chironomids (Wootton, 1994). The consumption rate varies between seasons. The feeding rate is highest from May to August/September with a peak in June, and is extremely low from November to March (Allen and Wootton, 1983), when metabolism is low due to low water temperatures (Wootton, 1994).

Feeding motivation is influenced by several factors, such as hunger, presence of predators and presence of suitable prey (Hart, 1993). Undisturbed sticklebacks may forage without breaks until satiated (Tugendhat, 1960). In the

wild, however, foraging fish may be frequently interrupted by predators, or by other activities such as competition or reproductive behaviour (Hart and Gill, 1994; Milinski, 1993). It has also been suggested that fish foraging in groups may increase the possibility of finding food. However, for a low ranked individual the reduced competition ability often makes group-living worse than living alone. Thus, feeding shoals often consist of phenotypically equal individuals (Ranta *et al.*, 1993).

Feeding behaviour is of high ecological significance because of its vital importance to growth, reproduction and survival, and has to some extent been used in ecotoxicological studies in different species. Reported effects of exposure to chemicals on feeding behaviour in fish include both reduced (Mathers *et al.*, 1985), and increased food consumption (MacRury and Johnson, 1999), and reduced prey attack (Bryan *et al.*, 1995). Differences in exposure concentrations and chemicals may explain some of the contradictory results.

Shoaling behaviour

Shoaling in fish is defined as grouping for social reasons, where the structure of the group is less important than in schooling, which refers to a group where the fish swim in a polarised manner (Pitcher and Parrish, 1993). Shoal formation is dependent upon several factors, such as predation pressure (Huntingford *et al*, 1994), hunger and season (Pitcher and Parrish, 1993). Satiated fish tend to prefer larger shoals that are efficient as protection against predators, while hungry fish prefer smaller shoals to minimise food competition (Pitcher and Parrish, 1993). Shoaling in sticklebacks is also season-dependent, with shoals consisting mostly of females and non-reproductive males during spring and summer, and sex-mixed shoals during winter (Whoriskey and FitzGerald, 1994).

Predators attacking a shoal of sticklebacks will suffer from the "confusion effect", since they will find it difficult to select one particular prey out of many equal-sized individuals (Ranta and Lindström, 1990). By grouping, sticklebacks also increase their vigilance as many more eyes may detect a predator better than if each individual remained isolated. As a result the individual fish can

spend more time on other activities. Increased vigilance may also benefit foraging behaviour since more eyes are searching (Ranta and Kaitala, 1991; Pitcher and Parrish, 1993). The equal-sized individuals in a shoal have additionally been explained by the fact that fish of different sizes occupy different ecological nishes (Keenleyside, 1955).

Shoaling behaviour is an example of a variable of high ecological relevance since it may influence predation risk and thus survival (Pitcher and Parrish, 1993). It is a behavioural trait of high complexity that differs considerably between seasons and populations (Pitcher and Parrish, 1993; Krause, 1994). Reported effects of pollution on grouping behaviour include both reduced shoaling behaviour (Weis and Weis, 1974; Besch et al., 1977), and aggregation (Bayley et al., 1999). These contradictory findings are largely explained by differences in exposure chemicals and species.

Reproductive behaviour

The timing of reproduction in sticklebacks varies between geographical localities, but generally breeding starts later at northern latitudes compared to further south. The onset and end of the reproductive period are determined by temperature, food and photoperiod (Whoriskey and FitzGerald, 1994). Sticklebacks have a relatively short life cycle, and the fecundity is low (< 200 eggs). This make quantification of the reproductive end-points possible.

The endogeneous factors controlling the reproductive behaviour in the stickleback are the hormones constituting the hypothalamic-pituitary-gonadal axis (Arcand-Hoy and Benson, 1998). In the stickleback, as in other teleosts, 11-ketotestosterone is physiologically the most important androgen in controlling male reproductive traits. This hormone is of major importance in controlling the devlopment of male secondary sexual characters, including kidney hypertrophy in which the kidney transforms into a glue-secreting organ, and nuptial colouration. The hormone is also important in regulation of male reproductive behaviour (Borg, 1994; Guderly, 1994).

Threespine stickleback males build nests at the bottom using a variety of plant materials. Males attract females by performing a zig-zag dance, and an

interested female responds by performing a head-up posture where she displays her swollen abdomen. The nest consists of a tunnel through which the female swims when spawning. The male follows and fertilises the eggs immediately after spawning. After laying the eggs, the female leaves and the male performs all the parental care. This consists of fanning oxygenated water over the eggs, and protecting them from predators (Foster, 1994). The male further protects the fry for up to two weeks after hatching (Whoriskey and FitzGerald, 1994).

Experiments have shown that female sticklebacks use male courtship and colouration (McLennan and McPhail, 1989; Milinski and Bakker, 1990) and nest quality as cues when assessing male quality (Sargent and Gebler, 1980; Whoriskey and FitzGerald, 1994; Barber *et al.*, 2001). It has also been demonstrated that females prefer males that show an intermediate level of aggressiveness (Ward and FitzGerald, 1987).

Effects of aquatic contaminants on reproductive behaviour have been studied to some extent in different species (reviewed by Jones and Reynolds, 1997). However, there is a growing interest in reproductive effects caused by exposure to E_2 and other EDCs (e.g. Tyler *et al.*, 1998). After the discovery of eggshell thinning in birds exposed to DDT (Ratcliffe, 1967), many cases of reproductive disorders resulting from EDCs have been reported. Recent reports include increased production of the yolk protein vitellogenin in male fathead minnows after exposure to E_2 and oestrone (Panter *et al.*, 1998), and in male platyfish *Xiphophorus maculatus* exposed to nonylphenol and E_2 (Kinnberg *et al.*, 2000). Furthermore, impaired courtship behaviour (Bayley *et al.*, 1999; Bell, 2001), and reduced colour intensity and testis growth (Toft and Baatrup, 2001) have been documented in male guppies exposed to octylphenol and E_2.

In addition to a large number of case studies, several reviews have been published on reproductive effects of EDCs (e.g. Colborn *et al.*, 1993; Jones and Reynolds, 1997; Arcand-Hoy and Benson, 1998; Tyler *et al.*, 1998; Gillesby and Zacharewski, 1998; Jones *et al.*, 2000).

Bottom-dwelling behaviour

In many fish species bottom-dwelling behaviour is a common effect of exposure to pollution. The behaviour may be an effect of impaired swimming activity resulting in the fish spending most of its time at the bottom (Little and Finger, 1990). Fatigue and motionless resting may be one cause of bottom-dwelling, but in some cases the effect is a behavioural stress response. Stress among animals is often observed as a consequence of physiological compensatory mechanisms as a protection against harmfull effects of exposure to sublethal concentrations of chemicals (Depledge, 1994). In the threespine stickleback stress and fright may be expressed by the aggregation into tight groups, and/or bottom-dwelling as the fish want to hide (Wootton, 1984).

Bottom-dwelling behaviour is an easily standardised variable and is considered to be sensitive to pollution (Little and Finger, 1990). Examples where bottom-dwelling is explained by motionless resting include the African fresh water fish *Labeo rohita* after exposure to water extract of the bark of *Buchanania lanzan* L. (Chaudhary *et al.*, 2001). Also, rainbow trout *Oncorhynchus mykiss* sank motionless to the bottom following exposure to carbon dioxide in effluent from an oxygen-activated sludge treatment plant (OConnor *et al*., 2000). Alternatively, bottom-dwelling was explained as a stress response by Israeli-Weinstein and Kimmel (1998) who observed that carp exposed to aluminium dived directly to the bottom of the aquarium and stayed there for a period of time that corresponded positively with the Al-concentration. Bottom-dwelling has also been used as a test variable in the study of effects of linear alkylbenzebne and cadmium in zebra fish *Brachydanio rerio* (Grillitsch *et al*., 1999).

1.5 *Exposure chemicals*

When selecting the chemicals for this study, their environmental significance was considered important. While the use of bis(tributyltin)oxide (TBTO) and 2,2-bis(p-chlorophenyl)-1,1-dichloroethylene (DDE) is restricted today, they (or their metabolites) are still present in the aquatic environment due to their persistance against biological degradation. Butyl benzyl phthalate (BBP)

is produced in huge amounts and is found in numerous products that are in everyday use. Finally, E_2 is normally not thought of as a pollutant, but due to large spills from agriculture, household and municipal wastewater this natural estrogen is considered a significant pollutant. All the chemicals selected for use in this study have previously been implicated as having endocrine-disrupting properties (Tyler *et al.*, 1998).

Bis(tributyltin)oxide (TBTO)

Bis(tributyltin)oxide (Fig. 3) has been widely used as a biocide in antifouling paints for ships and other aquatic equipments. The use of the chemical has been restricted in all European countries since 1990. However, since TBTO is still in use, and is persistent and bioaccumulative (accumulation of a chemical from one trophic level to another), it still represents a major problem (e.g. Triebskorn *et al.*, 1994; Coloso and Borlongan, 1999; Grinwis *et al.*, 2000).

$$C_4H_9-Sn-O-Sn-C_4H_9$$
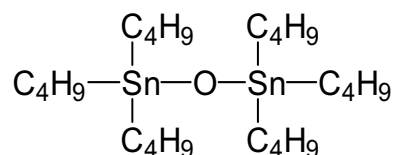
Figure 3. Bis(tributyltin)oxide (TBTO).

The lethal toxicity of TBTO for fish varies considerably (0.96 – 200 ppb) depending on species and age of target individual (Triebskorn *et al.*, 1994). The route of uptake for dissolved TBTO is mainly over the gills, but intake via food may also be of significance (Pärt, 1989; Holm *et al.*, 1991).

TBTO is classified as a pollutant with androgenic effects, as it may cause masculinisation (imposex) of female gastropods (Bryan *et al.*, 1986; Ellis and Pattisina, 1990; SFT, 1993). In fish, exposure to TBTO is shown to have caused histopathological alterations in gill structures, such as fusion of secondary

lamellae and vacuolisation (Holm *et al.*, 1991; Schwaiger *et al.*, 1992). These alterations may disrupt the diffusion distance between blood and water, leading to decreased gas exchange (Holm, 1994). It has also been shown that TBTO inhibits mitochondrial oxidative phosphorylation, resulting in reduced ATP production (Aldridge, 1976). Other effects of TBTO include histopatological abnormalities in the liver, kidney, eye, oral cavity and swim bladder (Wester and Canton, 1987). TBTO has also been reported to be neurotoxic to fish and may thus alter behaviour through neural effects (Holm *et al.*, 1991; Fent and Meier, 1992; Triebskorn *et al.*, 1994).

Even though the bioconcentration factor (BCF = concentration in tissue /concentration in water) of TBTO in fish is in the range of 3200 – 11000 (Yamada and Takayanagi, 1992), the compound may still be metabolised and excreted to some extent. TBTO is metabolised in the liver to dibutyltin (DBT), monobutyltin (MBT) and inorganic tin. Organic tin-compounds are generally more toxic than inorganic tin (Martin *et al.*, 1989). The metabolites are mainly stored in the liver, kidney and gonads, and some of them are further excreted via the bile (Martin *et al.*, 1989).

Reported behavioural effects of TBTO in fish include increased and chaotic swimming activity in rainbow trout (Triebskorn *et al.*, 1994), and reduced appetite in threespine sticklebacks (Holm *et al.*, 1991). Since exposure to TBTO has been reported to alter gonadosomatic index (GSI) in sticklebacks (Holm *et al.*, 1991) and reduced sperm production in guppies (Haubruge *et al.*, 2000), it is also likely that the chemical may affect reproductive behaviour. The fact that tributyltin (TBT) inhibits the conversion of androgens to oestrogens (Tyler *et al.*, 1998) further supports this hypothesis. Indeed, reduced parental care has been observed in mice after exposure to TBTO (Baroncelli *et al.*, 1995).

It was of interest to study the effects of TBTO since behavioural effects of TBTO-exposure are poorly documented. The TBTO concentrations and the exposure time used in the experiment were decided on the basis of earlier studies having comparable aims (e.g. Holm *et al.*, 1991; Schwaiger *et al.*, 1992). Recent field measurements in Norway have revealed high concentrations of TBT in water (up to 12.5 ng/L (Følsvik *et al.*, 2002)), and in

aquatic biota (ranging from 2.4 µg/kg to 9.53 mg/kg (Akvaplan-NIVA, 2000; Elgethun *et al.*, 2000)), indicating that TBT still represents an environmental problem. The Norwegian Pollution Control Authority has recently classified TBTO as a chemical that constitutes a significant environmental problem (SFT, 2001).

2,2-bis(p-chlorophenyl)-1,1-dichloroethylene (DDE)

2,2-bis(p-chlorophenyl)-1,1-dichloroethylene (DDE) (Fig. 4) is one of the metabolites of the insecticide 1,1,1-trichloro-2,2-bis(chlorophenyl)ethane (DDT). The DDT group, often termed total DDTs, constitutes DDT, DDE, DDD (dichlorodiphenyldichloroethane) and DDA (2,2-bis(chlorophenyl)acetic acid). The last three are formed by a series of reductive dechlorination and oxidative reactions (Ecobichon, 1995). DDT is relatively easily metabolised to *p,p´*-DDE (para, para – DDE) which is often the most abundant DDT metabolite in animal tissues (Kozie and Anderson, 1991). DDT and some of its metabolites are extremely persistent, and prone to both bioaccumulation and biomagnification (i.e. accumulation of a chemical throughout the foodweb), the half-life (i.e. time before 50% of the chemical is eliminated) may be 50 years (Tyler *et al.*, 1998). The bioconcentration factor (BCF) of DDE varies between 4770 and 20000 (Nawaz and Kirk, 1995).

The use of DDT has been restricted in the Western world since 1972. However, the pesticide is still in use as an insecticide in some developing countries due to its efficiency and low-cost production, and will thus continue to represent an environmental problem for many years. In most parts of the Western world, the existing water concentrations of DDE are at or under the no-observed-effect-levels, but in developing countries water concentrations of 1 – 10 ppb have been measured (Tyler *et al.*, 1998). In one fjord in nothern part of Norway total DDTs concentrations of 209 ng/g was measured in cod *Gadus morhua*, while the same study reported total DDT concentrations of 2065 ng/g in harbour seal *Phoca vitulina* (Ruus *et al*., 1999).
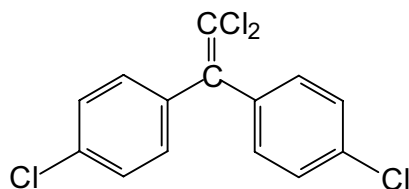
Figure 4. 2,2-bis(p-chlorophenyl)-1,1-dichloroethylene (DDE)

In fish the route of uptake for most dissolved xenobiotics is over the gills (Pärt, 1989). But since DDE is highly lipophilic the food will also constitute a major exposure source. Target organs for DDE accumulation are those with high lipid content such as the nervous system, the reproductive organs, the liver and the kidneys (Kendall *et al.*, 1995). In fish the toxicant will also enter the blood as the fat stores undergo regular turnover (Babin and Vernier, 1989).

The effects caused by DDT and its metabolites are well documented. These organochlorines are examples of chemicals that will cause induction of the hepatic microsomal enzymes (Kendall *et al*., 1995). In the brain the levels of the neurotransmitters serotonin and norepinephrine decrease as a result of exposure to DDTs (Kendall *et al*., 1995). Further, DDE binds to steroid receptors, and in the case of *p,p´*-DDE the affinity is greater for androgen than for oestrogen receptors (Tyler *et al*., 1998). *p,p´*-DDE is also an androgen antagonist in that the binding of *p,p´*-DDE to the androgen receptor inhibits the action of androgen (Kelce *et al*., 1995). As a result of this receptor binding, physiological and behavioural alterations may occur.

The most well known effects of DDTs are probably those reported on reproductive disorders (e.g. Fry and Toone, 1981; Donohoe and Curtis, 1996). One of the best known example includes eggshell thinning in the peregrine falcon *Falco peregrinus* exposed to DDE (Ratcliffe, 1967). In rodents *p,p´*-DDE have caused masculinization effects in females, resulting in abnormalities in vaginal and mammary glands, and enlarged phallus (Gray, 1998). Furthermore, abnormal gonad development and elevated sex hormone concentrations were

observed in alligators *Alligator mississippiensis* in Lake Apopka, Florida, where the animals were exposed to DDT and its metabolites during the 1980`s (Guillette *et al.*, 1994).

Other observed behavioural alterations after exposure to organochlorines include increased feeding rate in largemouth bass (MacRury and Johnson, 1999) and decreased schooling behaviour in goldfish exposed to DDT (Weis and Weis, 1974). Ringed turtle doves *Streptopelia risoria* exposed to DDE showed impaired courtship behaviour, egg laying and hatching (Keith and Mitchell, 1993).

The DDE concentrations used in Paper II (5.0 and 50.0 μg/L) are based on the reported LC_{50} (i.e. lethal concentration for 50 percent of the population) concentrations in e.g. goldfish (30 – 100 ppb) (Odum and Sumerford, 1946), and measured concentrations in salmonids from the wild (5 – 85 ppb) (Datta *et al.*, 1999). The Norwegian Pollution Control Authority (SFT) has classified DDT and its metabolites as an environmental problem (SFT, 1993).

Butyl benzyl phthalate (BBP)

The phthalate ester, butyl benzyl phthalate (BBP) (Fig. 5) is mainly used in the production of plastics to increase the flexibility and workability of the material. Traces of phthalates have been found in food packing materials, and also in food that has been wrapped in plastic (Page and Lacriox, 1995). Because phthalates are not chemically bound to the polymer, they may migrate from the plastic to the environment (Staples *et al.*, 1997). The main release of BBP to surface water is from manufacturing operations (Carr *et al.*, 1997), and the main uptake routes in aquatic animals occur over the gills or through consumed food (Pärt, 1989; Staples *et al.*, 1997).

BBP has shown moderate potential to bioaccumulate in organisms (Jobling *et al.*, 1995; Carr *et al.*, 1997). Reported bioconcentration factors in different fish species varies from 255 – 3171 (Carr *et al.*, 1997). BBP readily forms metabolites such as monobutyl phthalate (MBuP), monobenzyl phthalate

(MBeP), hippuric acid, phthalic acid, benzoic acid and an ω-oxidised metabolite (Nativelle *et al.*, 1999), and some of these are also believed to be toxic (Ema *et*
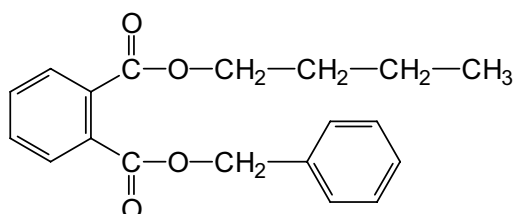
Figure 5. Butyl benzyl phthalate (BBP)

al., 1995; Parkerton and Konkel, 2000). Because of its moderate bioaccumulation ability and its propensity to metabolise (Staples *et al.*, 1997), phthalate esters have been assumed to be of moderate toxicity (e.g. Gledhill *et al.*, 1980). On the other hand, since BBP is the most produced man-made chemical, and has some properties that will be referred to subsequently, there is an increased general concern about the toxicity of phthalates (Mayer *et al.*, 1972; Jobling *et al.*, 1995; Tyler *et al.*, 1998).

The acute toxicity (defined as concentration that cause sudden mortality) of BBP in fish occurs at concentrations between 731 and 6470 ppb (Mayer *et al.*, 1972; Adams *et al.*, 1995). Most Western world waters contain BBP concentrations of 0.3 – 30 ppb (e.g. Sheldon and Hites, 1979; Fatoki and Vernon, 1990; Fromme *et al.,* 2002), but for some waters in developing countries concentrations of 10 – 1500 ppm have been reported (Fatoki and Ogunfowokan, 1993). In Norway, the PVC factory Dynoplast experienced an accidental spill in 1997. Sixteen months after the spill BBP concentrations of 35-320 mg/kg and 210-5600 mg/kg, respectively, were measured in sediments of two nearby lakes (NGI, 1997).

BBP is probably best known for its suggested weak oestrogenic properties (Tyler *et al.*, 1998), and it has been shown that BBP may reduce the binding of E_2 to the steroid receptor (Jobling *et al.*, 1995). Other effects include

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induction of mitosis and also stimulation of transcription activity of E_2 receptors (Jobling *et al.*, 1995). The most likely deposit organs for BBP are the liver and the kidneys that have been shown to increase in weight in rats after exposure to BBP (Piersma *et al.*, 2000). Rats exposed to the BBP metabolite monobutyl phthalate (MBuP) showed reduced food consumption and maternal body weight. The litter size decreased while offspring deformities increased (Ema *et al.*, 1995). Exposed male rats showed reduced testicular size and sperm production (Sharpe *et al.*, 1995).

In the present thesis BBP was used as an exposure chemical in order to document behavioural effects that otherwise have been poorly investigated. The BBP exposure concentrations (0.01 and 0.1 mg/L) were decided on the basis of reported toxicity levels in fish (0.7 – 6.5 ppm) (Mayer *et al.*, 1972; Adams *et al.*, 1995). The Norwegian Pollution Control Authority (SFT) classifies BBP as a contaminant with a possible oestrogenic effect (SFT, 1996).

17β-Oestradiol

The natural oestrogens of vertebrates are E_2 (Fig. 6), oestrone and oestriol. When oestrogens, that are conjugated, enter the water, different bacteria deconjugate the respective oestrogens (Tyler *et al.*, 1998). Since significant levels (2.7 – 48 ng/L) have been found in municipal sewage water and close to sewage treatment plants, connected to agriculture activities (Shore *et al.*, 1993; Brighty, 1996), it has been suggested that E_2 can be classified an environmental pollutant. The bioconcentration factor of E_2 has been calculated to be 174 (Kramer *et al.*, 1998). However, since little is known on the means of uptake of E_2 by aquatic organisms after the hormone has entered the water, the significance of E_2 as an environmental pollutant is not confirmed (Tyler *et al.*, 1998). However, aquatic organisms that live in waters contaminated with E_2 have been shown to suffer from reproductive disorders (Shore *et al.*, 1993; Kramer *et al.*, 1998). It has been shown that E_2 is extremely potent, and biological effects have been recorded after exposure to water concentrations as low as 2-3 ng/L (Tyler *et al.*, 1998).
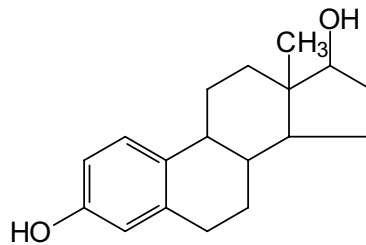
Figure 6. 17β-oestradiol

Inhibited smoltification as a concequence of reduced gill Na^+,K^+-ATPase activity was observed in Atlantic salmon after E_2 exposure (Madsen *et al.*, 1997). Also, Kramer *et al.* (1998) found that decreased hematocrit caused by E_2 may be used as an indirect measure of health. Exposure to E_2 has been shown to reduce liver glycogen content, and to increase the production of liver RNA, liver lipids and protein synthesis in fish (Haux and Norberg, 1985; Ghosh *et al.*, 1989; Madsen and Korsgaard, 1989; Madsen *et al.*, 1997). These liver processes may be linked to the synthesis of the female-specific lipoprotein vitellogenin (e.g. Haux and Norberg, 1985; Washburn *et al.*, 1993; Madsen *et al.*, 1997; Panter *et al.*, 1998). Both the synthesis and release, and subsequent uptake of vitellogenin by developing oocytes is dependent upon E_2 (Wallace, 1978). Male fish normally lack vitellogenin, even though they have the physiological ability to synthesise the protein when exposed to E_2 (Panter *et al.*, 1998). As a consequence of histological alterations in the liver caused by E_2 the size of the liver also increases (Haux and Norberg, 1985; Madsen and Korsgaard, 1989).

Other reproductive effects caused by E_2 include reduced spermatogenesis and regression of the testis (Billard *et al.*, 1981; Miles-Richardson *et al.*, 1999; Kinnberg *et al.*, 2000; Toft and Baatrup, 2001), reduced egg production (Kramer *et al.*, 1998) and poorly developed male secondary sexual characteristics (Miles-Richardson *et al.*, 1999; Toft and Baatrup, 2001). Reported behavioural effects of E_2 include reduced male courtship behaviour

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(Bayley *et al.*, 1999; Bjerselius *et al.*, 2001) and generally reduced male sexual activity (Bjerselius *et al.*, 2001).

In this thesis, E_2 was chosen as an exposure chemical because of two reasons. Firstly, the hormone in itself can be considered to be a major aquatic pollutant, and secondly, the hormone is a suitable model chemical for studies on endocrine disruptive contaminants with oestrogenic properties. If a xenobiotic with suggested endocrine disrupting properties were used in the study (e.g. PCB or phenol) it would have been difficult to exclude the possibility that the observed effects were results of non-endocrine effects of that particular chemical (e.g. morphological alterations of the gonads) since histological or biochemical analysis were not conducted.

The exposure concentration and method of exposure used were decided on the basis of comparable studies (e.g. Haux and Norberg, 1985; Cyr and Eales, 1989; Madsen and Korsgaard, 1989; Washburn *et al.*, 1993; Madsen *et al.*, 1997).

2. General Methods

2.1 *Fish maintenance*

Threespine sticklebacks were caught using plexiglas fry traps (Dolmen, 1982) in freshwater populations in Kindsethtjønna, a small lake in the county of Sør-Trøndelag, central Norway (63.5^{o}25`N, 10$^o$45`E) (Papers I, III, IV), and in Myrdalsvatnet, a lake in the county of Hordaland in south-west Norway (60°18`N, 5$^o$23`E) (Paper II). Before being transported to the laboratory, the fish were disinfected with NaCl (15 minutes) or formalin (10 minutes) to minimise infections with ectoparasites and fungus.

In the laboratory each fish was transferred to separate aquaria (Papers I and IV) or several fish were kept together (Papers II, III) in aquaria containing gravel and material (plants, stones etc) to minimise stress. The fish were kept in the laboratory for 2 months (Papers III, IV), 6 months (Paper I) or more than 12 months (Paper II), depending on the experimental design. All fish were fed daily on commercial dried food, live *Artemia* or *chironomidae* larvae. Laboratory temperature and photoperiod were adjusted to the natural pattern for the time of year and latitude.

2.2 *Exposure*

The sticklebacks were exposed to the contaminants via the water (Papers I, II, III), or by injection (Paper IV). These methods were similar to those used in previous studies on fish (e.g. Madsen and Korsgaard, 1989; Holm *et al.*, 1991; Bayley *et al.*, 1999; Toft and Baatrup, 2001). TBTO, BBP and DDE were dissolved in ethanol or acetone to increase the solubility and hence the likelihood of uptake. The amounts of ethanol and acetone were too low to give any effects on the individuals (Martin *et al.*, 1989). E$_2$ was dispersed in peanut oil before injection, in order to prolong the uptake time (Pankhurst *et al.*, 1986).

TBTO (Paper I) was administered via the water by a multichannel peristralic pump that supplied each aquarium containing one fish with a specific concentration of the chemical. The aquaria were supplied with continuous water flow. The fish were exposed for four consecutive days to 0, 3, 9 or 27 ppb TBTO

from a stock solution prepared with ethanol as a solvent. The respective concentrations were obtained by different flow rates. The control group received the same treatment as the exposed groups, except being exposed to TBTO. The behavioural experiment started immediately after termination of exposure. Since the aim was to study antipredator behaviour on individual fish, and since reliable results required that the fish were not exposed to external noise, the exposure to TBTO and the behavioural observations were conducted in the same aquarium containing one fish.

Butyl benzyl phthalate (BBP) (Papers II, III) and 2,2-bis(p-chlorophenyl)-1,1-dichloroethylene (DDE) (Paper II) were dissolved in acetone and administered manually to the water. As a defined volume of the water in the exposure aquaria was exchanged daily, the chemicals were administered to the aquaria via the daily added water. All aquaria were provided with a stationary water system. The fish used in Paper II were exposed to BBP and/or DDE for 31 consecutive days, and the feeding behavioural experiment started five weeks after terminating the exposure. The fish were divided into seven exposure groups, six of which were exposed to DDE (5.0 or 50.0 μg/l) and/or BBP (0.01 or 0.1 mg/l) in different combinations, and a seventh control group that received acetone only.

The fish used in Paper III were exposed to BBP (0.1 mg/l) for 26 days, while the controls were exposed to acetone only. The experimental fish were divided into four groups; exposed large and exposed small fish, and control large and control small fish. The behavioural experiment started immediately after the exposure was terminated. The exposure concentration and duration was determined on the basis of experiences obtained in Paper II.

During the exposure period the fish in Papers II and III were maintained in large aquaria, and two aquaria per treatment were used. The chemical exposures and the behavioural studies were conducted in separate aquaria, and groups of a pre-determined number of fish were taken out from the exposure aquaria and transferred to aquaria where the behavioural experiments were conducted. Large exposure aquaria enabled a standardised treatment of the test individuals during exposure, to equalise environmental conditions

before the behavioural studies. In order to prevent behavioural differences resulting from non-experimental external conditions, the exposure aquaria were treated identically in respect to factors such as light conditions, external disturbances, food and number of fish per aquaria. The experimental design of using large treatment aquaria has previously been used by amongst others Ranta and Lindström (1990), Ranta *et al.* (1992), Krause (1994), Bayley *et al.* (1999) and Bjerselius *et al.* (2001).

E_2 (Paper IV) was injected intraperitoneally in the fish using a 23-gauge needle. The steroid was first dissolved in peanut oil (Pankhurst *et al.*, 1986), and the stock solution was prepared using an ultrasonic bath (3 x 10 minutes). The fish were injected once a week for four weeks at a concentration of 2.0 μg/g body mass. The control group received only peanut oil but were otherwise treated in the same way as the exposed fish. Each aquarium containing one male were supplied with gravel, plant material and a stationary water system. During injection, each male was taken out from the aquarium, exposed, and immediately transferred to the aquarium again. The behavioural observations were conducted daily during the same period as the exposure (except the days of exposure) and continued for three days after the fourth and last injection. In Paper IV individual exposure was preferred as the behavioural experiment and exposure was conducted in the same aquarium. This method was considered as important since it was believed that in order to successfully reproduce the males require undisturbed conditions that encourage nest building and reproduction. Territorial males kept together in larger aquaria may cause aggressive behaviour and suppression of normal reproductive behaviour (pers. observation).

2.3 *Behavioural experiments*

The experimental studies in this thesis consist of standardised behavioural studies conducted in the laboratory. In Papers I, II and III, video recordings were used to record the behaviour. This method enables studying more variables in the same experiment since each sequence can be replayed.

In Paper I, a dummy heron bill was used to provoke the stickleback's response towards the predator. Behavioural variables, such as the fish's vertical location in the aquarium, the ranking of the response to the heron bill, latency time between start of dummy heronbill stimulation to a response was achieved, and recovery time from start of bill stimulation until the fish resumed its original behaviour, were quantified from the video recordings. Antipredator behaviour may vary between individuals and populations due to factors such as predation risk (Giles and Huntingford, 1984; Wright and Huntingford, 1993), parasite infections (Milinski, 1990; Barber and Huntingford, 1996), sex differences (Giles and Huntingford, 1984) and experience (Giles, 1984). To standardise these factors, only unparasitised males of approximately the same size were used. During introduction of the heronbill all possible efforts were made to standardise the distance from the heronbill to the fish. Each fish was tested once every day for five consecutive days in order to study the temporal changes in behaviour. The possibility for habituation was considered as negligible since the time between the stimulations was approximately 24 hours (Magnhagen and Vestergaard, 1991).

In Paper II, the differences between exposed and control fish in their ability to capture a piece of food (defined amount of commercially frozen mosquito larvae) was investigated. In each trial, one exposed and one control individual were allowed to compete for a food item. The experimental aquarium was divided into three compartments, separated with two removable plexiglas walls. The competing fish were placed in each of the two outer compartments, while the food item was placed in the central compartment, half way between the competing fish. Since satiated fish are shown to be less motivated for feeding than hungry fish (Salvanes and Hart, 1998), feeding motivation was standardised by starving all test fish for 12 hours prior to the start of the behavioural experiment. The objective was to test one individual only once, but some control fish had to be tested more than once since there were fewer controls than exposed fish. A video camera was placed in front of the test aquarium. From the recordings, variables such as identification of the fish initiating feeding, and latency time to feeding, were quantified. The fish used in

each trial were of approximately the same size, thereby minimising individual differences in competitive ability and dominance (Larson, 1976; Rowland, 1989; Olivera and Almada, 1996). Dominance in fish has also been shown to be related to sex (Olivera and Almada, 1996). In the study underlying Paper II the sex of the sticklebacks was unknown since they were captured in late autumn when differences between males and females are inconspicuous. However, it is reason to assume an unbiased sex distribution since sex determination of fish used in the comparable Paper III showed an approximately equal sex distribution. The fish used in Papers II and III were captured at the same time of the year and in comparable habitats.

Video recordings were also used to acquire data for Paper III. Two behavioural tests were conducted and these started immediately after termination of exposure. In the "shoal choice test", one large focal fish was allowed to choose between a shoal of small fish, a shoal of large fish, or to stay in a neutral zone in the central area of the test aquarium. Since one objective of the "shoal choice test" was to investigate differences in preference between exposed and control fish, large focal fish were used since it has been shown that large fish accomplish a choice more readily than small fish (Ranta and Lindström, 1990).

In the "size-assortative shoaling experiment", the structure of a group of mixed-sized fish was quantified once every minute for 10 minutes to investigate the fish's ability to form smaller shoals. In a study by Barber *et al.* (1995), it was shown that satiated sticklebacks formed larger shoals than starved fish, on the basis of the assumption that satiated fish formed shoals to prevent predation, while starved fish are more motivated for feeding and are therefore more ready to form smaller shoals. To prevent starvation in the fish used in Paper III, and thus achieve standardised conditions, the fish were given some food on the morning when the behavioural experiment started. Furthermore, shoaling was expected since the fish were collected in late autumn when they usually form shoals in the wild (Wootton, 1984).

The last study (Paper IV) concerned reproductive behaviour, and was conducted during two consecutive spring seasons. Behavioural traits, such as

nest building, courtship and paternal care were recorded by manually observing each fish for 5 minutes, three times every day. In order to increase the possibility for nestbuilding and courtship each male was provided with suitable nestbuilding material and a receptive female that was ready to spawn. The female was removed after assumed spawning since aggressive behaviour towards her by the male would have interfered with the paternal care. In addition, as the female would be constantly exposed to the male's agressive behaviour after spawning, her life could be threatened. Variation in reproductive behaviour may be due to genetic factors (e.g. Snyder and Dingle, 1989) and experience (Rowland, 1994). Therefore, by using males from the same population, the genetic differences between test individuals may be reduced. Furthermore, by assuming that age correlates with size (e.g. Allen and Wootton, 1982) possible differences in experience were reduced by using equally sized individuals individuals ($t = 1.12$, df = 58, $p = 0.9$). To avoid biased recordings of exposed and control individuals, the aquaria were labelled with blind numbers in all trials. Also, three different observers alternated in conducting the recordings.

After the experiments the sex of the individuals was determined (Paper III) and the fish were stored (- 20 ^{o}C) for later analyses (Paper II and Paper III).

2.4 *Chemical analyses*

The tissue concentrations of BBP (Paper II and Paper III) and DDE (Paper II) were analysed at the Norwegian Institute for Water Research (NIVA) in Oslo, Norway, whereas the residue concentrations of TBTO (Paper I) and E_2 (Paper IV) in the fish were not analysed. To obtain sufficient material for the DDE and BBP analyses, each sample comprised material from a number of fish that was pooled and homogenised prior to analyses.

For analyses of DDE, lipids in the pooled samples were extracted twice using cyclohexane/acetone and an ultrasonification probe. The cyclohexane extract was isolated by adding NaCl solution. The organic extract was evaporated to dryness and the fat content was determined gravimetrically. Approximately 100 mg of the resulting lipid sample was dissolved in dichloromethane, internal standards (PCB-53 and PCB-204) added and cleaned

using size-exclusion chromatography (HPLC/GCP) and concentrated sulphuric acid. DDE was then analysed using a gas chromatograph (Hewlet Packard 5890 Series II) equipped with a splitless autoinjector, a DB-5 capillary column (60 m, i.d. 0,25 mm, film thickness 0,25 μm) and an Electron Capture detector.

Before BBP analyses the lipid extract was cleaned on an ALOX-column according to the EPA-method 606 (EPA, 1984). Di-allyl phthalate was added to the extraction as a recovery standard, while phenantrene was added as an internal standard. The extraction was analysed using gas chromatography (GC), Hewlett Packard (HP) model 5890 Series II, connected to a HP 5970 MSD instrument. The GC was equipped with an on-column injector and a capillary column type DB-5 (length 60 m, i.d. 0.25 mm, film thickness 0.25 μm). BBP and DDE was identified and quantified according to retention time and mass peak signals.

2.5 *Statistics*

The statistical analyses were conducted by using non-parametric tests since the data were not normally distributed. The medians are given with interquatile ranges. More detailed statistical descriptions are given in the individual papers.

2.6 *Ethics*

The National Animal Research Authority approved the experimental works in Papers I - IV. All possible efforts were made in order to avoid unwanted external disturbances. The aquaria were shielded with dark plastic (Paper I), and activities near the aquaria were minimised (Papers I - IV). With approval from the National Animal Research Authority the injection of E_2 (Paper IV) was conducted without anaesthesia, since this would have inflicted considerable stress to the fish.

3. *Summary of the Individual Studies*

Effects of bis(tributyltin)oxide on antipredator behaviour in threespine stickleback *Gasterosteus aculeatus* L. (Paper I)

The aims of Paper I were to investigate the effects of short time exposure to sublethal concentrations of TBTO on fright response and antipredator behaviour in threespine stickleback, and to determine if the effects were reversible after termination of exposure. Effects of TBTO on antipredator behaviour was of interest since TBTO is a significant aquatic pollutant, and the behaviour is well documented (e.g. Wootton, 1984; Huntingford *et al*., 1994; Reimchen, 1994) and is readily induced in the laboratory.

Significant behavioural responses occurred mainly among fish exposed to 9.0 ppb TBTO. TBTO-exposure caused the stickleback to spend significantly more of the observed time at the bottom of the aquaria than the control fish. Furthermore, the exposed fish showed an overall weaker response towards the heronbill compared to the control fish. The time from the start of heronbill stimulation to initial response (latency time) was longer for the exposed than for the control fish. Also, the time from the initial behavioural response to the heronbill stimulus until the fish resumed its normal and original behaviour (recovery time) was shorter for exposed fish than for the controls. After termination of the TBTO-exposure, the location of the fish in the water column (vertical location), the fish response to predator attack, and latency time approached that of the control individuals, indicating that the effects of the exposure were reversible. The results are explained by the biochemical alterations caused by TBTO, and the observed behavioural effects are expected to reduce the fish`s ability to escape a predator.

Post-exposure effects of DDE and butyl benzyl phthalate on feeding behaviour in threespine stickleback (Paper II)

In Paper II the post-exposure effects of *p,p´*-DDE and butyl benzyl phthalate (BBP) on feeding behaviour in sticklebacks were investigated. Post-

exposure effects were studied to evaluate long-term and possible persistent effects of the chemicals after elimination of the exposure source.

Since DDE and BBP are suggested to act mainly as EDCs (Tyler *et al.*, 1998) most studies concerning these chemicals report effects on reproduction. However, because of their central roles as environmental pollutants it is essential to investigate their possible effects on other ecologically significant behavioural variables, such as feeding. Since it was of interest to study possible effects of DDE and BBP separately and in conjunction, the two chemicals were administered either alone or in combination. Fish in natural environments are often exposed to mixtures of chemicals (Tyler *et al.*, 1998). It has been documented that municipal wastewater may contain a mixture of DDT/DDE and phthalate esters (Mayer *et al.*, 1972; Soto *et al.*, 1997; Jobling *et al.*, 1998). However, further investigations of mixture-effects are strongly needed as very little information on the area exist.

The results reported on in Paper II generally showed that the exposed fish initiated feeding more often than did the control fish. This result was significant when fish were exposed to high concentrations of DDE or BBP, and for fish exposed to a mixture of high concentrations of DDE and BBP. In addition, fish exposed to a mixture of high concentrations of DDE and BBP showed a shorter latency time to feeding compared to the controls. In fish exposed to a mixture of low concentrations of DDE and high concentrations of BBP the results were the opposite, i.e the control fish initiated feeding significantly earlier than the exposed fish.

The increased feeding motivation may indicate that exposed fish were hungrier than the controls, due to energy demanding compensatory mechanisms that defend against harmful physiological effects caused by the exposure (Selye, 1956, Beyers *et al.*, 1999). Chemical analyses revealed bioaccumulation of DDT, while traces of BBP was not found in the fish tissue. Thus, the observed behavioural alterations caused by BBP may be due to toxic effects of BBP metabolites. Another possibility may be prolonged or permanent physiological and biochemical alterations in the fish, even after elimination of

BBP, resulting in behavioural changes. Alternatively, the analytical detection limit might have been set too high.

Butyl benzyl phthalate affects shoaling behaviour and bottom-dwelling behaviour in threespine stickleback (Paper III)

The aim of Paper III was to investigate possible changes in shoaling behaviour and bottom-dwelling behaviour in threespine sticklebacks as a result of exposure to BBP. It was of interest to study behavioural effects after termination of exposure in order to investigate possible late effects of BBP.

The purpose of investigating the effects of BBP on shoaling behaviour and bottom-dwelling is analogous to the previous study (Paper II), i.e. to investigate whether BBP may cause other behavioural effects in addition to those reported on reproduction (e.g Jobling *et al.*, 1995; Sharpe *et al.*, 1995; Ema *et al.*, 2000).

In the "shoal choice test", no differences were found between the controls and the BBP exposed fish with respect to their preference for shoals of small and large fish. However, the exposed fish spent significantly less of the observed time in the neutral zone, and more time at the bottom of the aquarium compared to control fish.

In the "size-assortative shoaling experiment", the individuals in the size-mixed shoal did not form smaller size-assortative shoals during the observation period, as found by Ranta *et al*. (1992). However, fish exposed to BBP aggregated more into one shoal compared to the control fish. The exposed fish showed behavioural stress responses, which may occur as a result of sublethal exposure to contaminants (Depledge, 1994). Analyses of fish after the experiment revealed that they contained no detectable BBP, suggesting that the same explanations as given in Paper II may account for the present behavioural effects.

Disruption of male reproductive behaviour in threespine stickleback *Gasterosteus aculeatus* exposed to 17β-oestradiol (Paper IV)

It has been suggested that E_2 may cause reproductive disorders in fish (e.g. Panter *et al.*, 1998; Bayley *et al.*, 1999). Thus, the aims of Paper IV were to investigate the effects of E_2 on different reproductive behaviours in male threespine stickleback, such as nest building, courtship and paternal care, and to study possible variations in these variables with respect to their sensitivity to exposure to E_2.

Threespine stickleback males exposed to E_2 (2.0 µg/g body weight) built nests later than unexposed fish. However, there were no differences between the groups with respect to the number of males that built nest. Furthermore, exposed males spent less of the observed time on paternal care (fanning, guarding of fry, nest-nibbleing) compared to control fish. There were no differences between the two groups with respect to number of courtship displays performed by each male. The recorded behavioural effects showed that fish exposed to E_2 were less able to perform optimal reproductive behaviour with respect to some of the tested variables, while other reproductive behaviours were not altered as a result of E_2 exposure. This finding may indicate that reproductive behaviours vary in sensitivity towards exposure to E_2. Some of the fish, especially those exposed to E_2, also suffered from fungus infections, which may be explained in terms of weaker immune response in exposed compared to unexposed fish.

4. General Discussion

4.1 *Discussion of results in the individual papers*

All four studies underlying this thesis describe changes in the behaviour of threespine sticklebacks as a result of sublethal exposure to chemicals known to be environmental contaminants. Antipredator behaviour was altered after exposure to TBTO (Paper I), feeding behaviour was affected by BBP and DDE (Paper II), and BBP additionally influenced shoaling (Paper III). E_2 caused impairment in reproductive behaviour (Paper IV). Fish that were exposed to TBTO (Paper I) and BBP (Paper III) showed a more pronounced bottom-dwelling behaviour than the control fish. Fish exposed to BBP (Paper III) also formed shoals more readily than did unexposed fish. Fish exposed to sublethal concentrations of contaminants may compensate for toxic effects through physiological and biochemical mechanisms (e.g. Walker, 1998). One consequence of these compensatory mechanisms may be behavioural and physiological stress responses (Carballo *et al.*, 1995; Beyers *et al.*, 1999) that, in sticklebacks, often result in aggregation and/or bottom-dwelling behaviour (Wootton, 1984). Bottom-dwelling, which is an often observed response to chemical exposure (e.g. Grillitsch *et al.*, 1999; OConnor *et al.*, 2000; Chaudhary *et al.*, 2001), may also be a result of the fish's need to rest since the toxic effects caused by the contaminants may involve fatigue (Little and Finger, 1990). The observations of the fish's behaviour in Paper I suggests that the bottom-dwelling behaviour could be explained by resting since the fish sank motionless to the bottom, whereas the bottom-dwelling reported on in Paper III supports the stress explanation since many of the fish rushed to the bottom where they remained motionless for several minutes. The different causes of bottom-dwelling behaviour described in Papers I and III may be that TBTO and BBP have different "modes of action", that is, the two chemicals may affect the same behavioural trait by different mechanisms. Since TBTO is more toxic than BBP (e.g. Mayer *et al.*, 1972; Triebskorn *et al.*, 1994; Adams *et al.*, 1995), and since health status decreases with increasing toxicity (Depledge, 1994), it is also possible that the fatigue response observed in Paper I indicate a worse

health than the stress response observed in Paper III, even though both TBTO and BBP were administred in sublethal concentrations.

Fish exposed to TBTO showed weaker responses to a simulated predator attack than control individuals (Paper I). Furthermore, both the latency and recovery times following the attack were affected by TBTO exposure. The neurotoxic effects of TBTO (Krigman and Silverman, 1984; Schweinfurth, 1985), in addition to its inhibition of ATP production (Aldridge, 1976), may explain some of these effects. A consequence of altered antipredator behaviour in exposed wild-living fish may be increased probability of being caught by predators.

Immediately after termination of the exposure to TBTO, the behaviour of the exposed individuals differed significantly from the controls with respect to some of the tested variables. However, for variables including vertical location, subjective ranking of the response towards the heronbill, and latency time, the differences disappeared within five days after exposure termination. While it is known that TBTO may bioconcentrate in the organism (Yamanda and Takayanagi, 1992), it has also been shown that some species are able to metabolise and excrete the chemical from the tissue after cessation of exposure (Martin *et al.*, 1989; Yamanda and Takayanagi, 1992). The reversibility of the behavioural effects may indicate that changes in behaviour due to short-time exposure to TBTO may not be stable. In the wild, however, sticklebacks often live in harbours where exposure to TBTO may be chronic due to its release from sediments or novel discharge. Even though concentrations at these sites may vary significantly due to variations in release rate, the fish will not have the possibility to recover from the effects provided they do not actively avoid those localities. Thus, the reversible effects reported in Paper I may not apply in the wild since fish living in polluted habitats may not be able to escape.

In the study of shoaling behaviour (Paper III), neither control nor BBP exposed fish showed any preference for either large- or small-fish shoals or the neutral zone between the shoals. The absence of size-assortative shoaling behaviour was unexpected, since earlier studies on sticklebacks have demonstrated preference for different size classes (Ranta *et al.*, 1992; Peuhkuri

et al., 1997). However, in the present study the BBP exposed fish spent more time in front of either shoal than in the neutral zone, and also less time in the neutral zone compared to control fish. This may indicate that BBP exposed fish prefer to stay in aggregations rather than staying alone. BBP exposed fish were observed to rush between each side of the compartments, stopping only momentarely in front of the shoals before rushing was resumed. This observation supports the explanation that the behaviour is a stress related response caused by the chemical exposure.

Feeding behaviour changed as a result of exposure to sublethal concentrations of DDE and BBP (Paper II). The reported increase in feeding motivation may indicate that exposed individuals were more hungry than control fish. This may be a result of a higher metabolic rate due to energy demanding biochemical and physiological compensatory mechanisms against the toxic effects of the pollutants (Selye, 1956; Walker, 1998; Beyers *et al.*, 1999). Previous studies have reported both increased (Piersma *et al.*, 1995; MacRury and Johnson, 1999) and decreased (Bryan *et al.*, 1995; Ema *et al.*, 1995) feeding motivation in chemical-exposed fish. In these examples various chemicals were used. It is likely that different chemicals have different "modes of action", thus resulting in opposing or differing effects. In addition, factors such as exposure concentration, and duration of exposure may be of significance to the effects. It has been shown that hungry sticklebacks are willing to take larger risks to obtain food than satiated fish by feeding closer to a predator (Fraser and Huntingford, 1986; Godin and Crossman, 1994). For the individual, increased time devoted to feeding results in less time being given to other activities, such as predator defence and reproduction, which again may reduce the possibility for survival and reproductive success.

Surprisingly, the response pattern described in Paper II was reversed in sticklebacks exposed to a mixture of low DDE-concentration and high BBP-concentration. Control fish initiated feeding significantly more often than the exposed fish, and the controls also started to feed significantly sooner after being offered food than the exposed individuals. This may be because the reduced condition in the exposed fish made them less motivated for feeding. It

is also possible that exposure to a mixture of low concentration of DDE and high concentration of BBP result in additive or synergistic effecs of the chemicals.

It has been suggested that the variation in behaviour responses between individuals may act as an indicator of pollution (Shulman and Pomory, 2000). Individual variation may be a suitable indicator of pollution if the variation within one group differs from the other. But a large intra-group variation may fail to detect statistical differences between the groups. Although the variation within both control and exposed fish in Paper II was high with respect to latency time to feeding start (Paper II; Tab. 2), no differences were recorded in interquartile ranges between control and exposed fish (t = 0.97, N = 137, p = 0.35). In Paper II, the variation between individuals did not affect the fact that there were significant behavioural differences between control and exposed fish. Thus, in this example, individual variation is not a suitable biomarker of effects of exposure to pollutants.

Although significant changes in behaviour were observed in fish exposed to BBP, the analyses of the chemical (Papers II and III) showed that the tissue concentrations were below detection limit (100 ng/g), One possible explanation is that BBP was metabolised to MBuP, MBeP, hippuric acid, phthalic acid, benzoic acid and/or an ω-oxidised metabolite (Nativelle *et al.*, 1999). Previous studies have suggested that MBuP and MBeP may be toxic (Ema *et al.*, 1995; Nativelle *et al.*, 1999; Parkerton and Konkel, 2000). However, neither of these compounds were analysed. It is also possible that BBP caused physiological/biochemical changes in the animal that persisted even after phthalate had been metabolised and excreted, resulting in the observed effects. A further explanation could be that the detection limit for BBP was set too high, with non-detectable concenrations in fact being anything between 0 – 100 ng/g. However, to my knowledge biological effects of tissue concentrations below 100 ng/g have not been reported in previous studies (e.g. Staples *et al.*, 1997; Harries *et al.*, 2000), and the results reported in Papers II and III might thus be the first indication of biological effects of BBP concentrations below 100 ng/g. This should be verified in repeated experiments employing analytical methods with a lower detection values. While the sticklebacks in Paper II accumulated

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DDE, the concentrations were apparently not sufficiently high to cause lethal or severe health effects.

Reproductive behaviour in male sticklebacks was impaired after exposure to E_2 (Paper IV). The consequence of impaired paternal care in male sticklebacks may be reduced reproductive success. Whoriskey and FitzGerald (1985) showed the significance of paternal care by removing males from their respective nests, resulting in only 19% egg survival. Delayed nest building by exposed males may result in fewer receptive females visiting the nest. As a consequence, late nest builders are less able to successfully compete with males that build their nests earlier (Mori, 1993). Recently, it has also been suggested that the nests might serve as male ornaments, and that stickleback males who built nests early built neater and more compact nests compared to later nest-building males (Barber *et al.,* 2001). Early nest builders may thus have better nest-building capacity than late nest builders. In the study descibed in Paper IV it is suggested that exposure to E_2 may disrupt the development of androgen-dependent male secondary sexual characters such as kidney hypertrophy, and reproductive behaviour (Borg, 1994; Guderly, 1994; Borg and Mayer, 1995; Jakobsson *et al.*, 1996). Males exposed to E_2 were further found to suffer from fungus infection more often than the unexposed males (Paper IV), suggesting that the E_2 exposed males suffered from weaker immune response than the control males (Àlvarez *et al.*, 1995; Carballo *et al.*, 1995).

In conclusion, the results obtained in Papers I-IV show that some behavioural variables are sensitive towards exposure to the respective chemicals, while other variables do not seem to be affected at all. In a forthcoming paragraph it will be suggested that this may be because behavioural traits may differ in sensitivity, rather than because the fish are exposed to different concentrations or exposure durations.

4.2 *The behavioural traits as biomarkers*

Antipredator behaviour, bottom-dwelling, feeding, aggregation, reproductive behaviour and shoal choice are all variables that successfully may be used to evaluate effects of pollution. Some of the variables may be difficult to

standardise due to significant individual differences (e.g. reproductive behaviour), while others are well defined variables that are easily standardised (e.g. bottom-dwelling).

When evaluating the suitability of behavioural variables as biomarkers, it should be taken into account that some behaviours often vary due to environmental factors. Factors such as seasonal influences, sexual and population differences have to a large extent been accounted for in Papers I – IV, as the individuals used in the respective experiments were from the same population, and hunger, sex and size were standardised as much as possible. Reproduction, feeding and shoaling behaviour are all influenced by season. Studies of feeding behaviour at different times of the year are thus likely to yield different results. In laboratory experiments where factors such as light and temperature can be controlled, seasonal influences may be largely eliminated, and by manipulating for example, light and temperature, reproductive behavioural experiments can be conducted even outside the "natural" reproductive period (Bertil Borg, personal communication).

The behavioural biomarkers that are dealt with in this study are mainly general biomarkers, i.e. the same behavioural trait can be affected by different chemical agents. Few, if any, behavioural traits are specific biomarkers that respond to one particular chemical or a class of chemicals, since behaviour is the complex result of several biochemical variables that respond differently to chemicals.

Bis(tributyltin)oxide may cause biochemical and physiological alterations that might explain the behavioural effects reported in Paper I. Some of these alterations, such as altered ATP-ase, gill structure and osmoregulation have also been reported after exposure to several other substances such as arsenic (Hwang and Tsai, 1993), heavy metals (Sola *et al.*, 1994; Muhvich *et al.*, 1995), acid water (Staurnes *et al.*, 1996), and the wood preservative agent 2-(thiocyanomethylthio)benzothiazole (Niki and Farrell, 1993). The antipredator behaviour described in Paper I may thus be considered as a general biomarker that is triggered by several chemical agents acting upon different biochemical variables. Also, altered gill ATP-ase and gill structure may affect other

behavioural responses such as migration behaviour (Pirhonen and Forsman, 1998) and swimming performances (McGeer *et al.*, 2000). The relationships between chemical agents, biochemical/physiological effects, and generally and specifically responding behavioural variables are shown in Figure 7 A and B respectively.
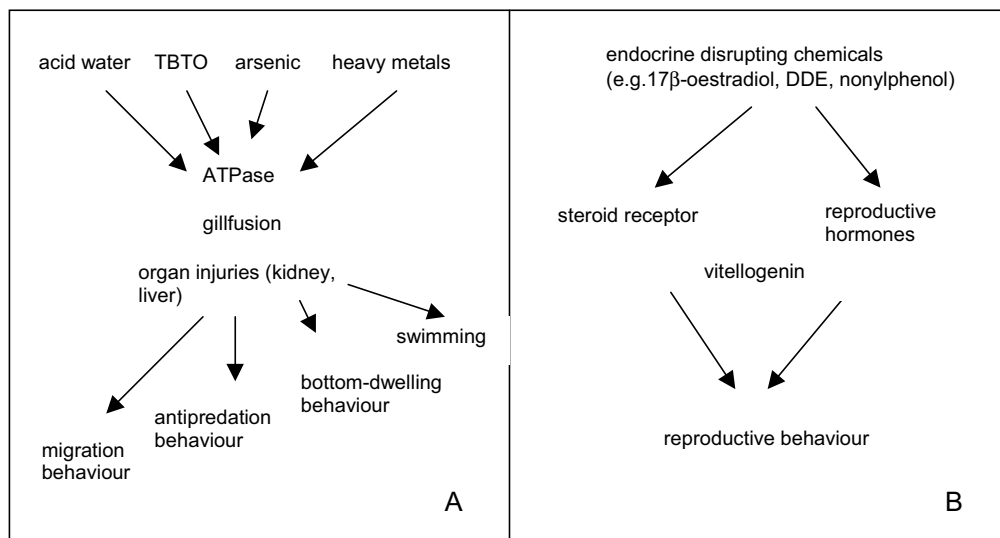

Figure 7. Behavioural variables as biomarkers. Some biomarkers are general in that they respond to several different chemicals (A), while others respond more specificially to one class of chemicals, though without being specific biomarkers by definition (B). In this example, endocrine disruptive chemicals imply chemicals affecting the reproductive system.

Increased feeding in response to DDE- and BBP-exposure (Paper II) is believed to be a general result of elevated feeding motivation. As suggested earlier this is probably due to physiological compensatory mechanisms that protects the individual from the harmful effects of exposure to sublethal concentrations of chemical agents (Selye, 1959; Depledge, 1994; Beyers *et al.*, 1999). The compensatory mechanisms are expected to be energetically costly (Walker, 1998), implying that the individual will be in need of food. The compensation is a general mechanism in fish exposed to sublethal concentrations of most chemicals (Depledge, 1994). DDE and BBP additionally

cause other biochemical alterations that may explain the results in Paper II. An example is the induction of mitosis and transcription activity caused by BBP (Jobling *et al.*, 1995) that may require energy.

Bottom-dwelling and aggregation behaviour reported in Paper III were suggested to result from stress, mainly because of the observations of the fish behaviour. Stress is often the behavioural and physiological consequence of the compensation mechanism described above. Exposure to a variety of chemicals results in a compensatory stress response (Depledge, 1994), and bottom-dwelling and aggregation behaviour can thus be considered as general biomarkers. Also, bottom-dwelling caused by lethargic resting (Paper I) can be considered a general biomarker, since the behaviour may be an effect of very potent chemicals or by chemicals given in high concentrations.

Shoaling behaviour (Paper III) is a complex behaviour that depends on many different factors such as hunger and experience of predators (Pitcher and Parrish, 1993). Since some of these factors, in addition to different biochemical and physiological variables that influence the behavioural trait, are affected by several chemicals, shoaling can also be considered a general biomarker.

One class of chemicals acting as endocrine disrupting chemicals (EDCs) may cause alterations in reproductive behaviour (Paper IV). The action of these chemicals on reproductive behaviour differs from the action of the other referred chemicals (Papers I, II and III) by being more specific, though they are not specific biomarkers by definition (Depledge, 1994). These EDCs may influence steroid receptors or reproductive hormones, and consequently alter reproductive behaviour (Arcand-Hoy and Benson, 1998). They may also induce the synthesis of vitellogenin, both in males and in females outside the reproductive season, which has serious consequences on reproduction (e.g. Haux and Norberg, 1985; Washburn *et al.*, 1993; Madsen *et al.*, 1997; Panter *et al*., 1998) (Fig. 7B). However, reproductive behaviour may also be altered by morphological alterations in the reproductive organs caused by non-endocrine disrupting chemicals. Also, even though several different chemicals can be classified as EDCs, some may in addition cause other biochemical alterations that will result in alternative behavioural disruptions. The specific effect

discussed in this section thus applies only when EDCs affect endocrine variables that alter reproductive behaviour.

In conclusion, since behavioural traits are complex variables they are general biomarkers by definition. However, considering the continuum from specific to general biomarkers, some behavioural traits may respond more specifically following exposure to certain classes of chemicals than other traits that respond generally to many different chemicals.

Sensitivity

It has been claimed that changes in certain behavioural variables, such as avoidance behaviour and antipredator behaviour, occur after exposure to low concentrations of chemicals, and thus may serve as early indicators of some contaminants (Little *et al.*, 1993; Peakall, 1996; Smith and Logan, 1997). Since behavioural traits represent the consequence of a diversity of biochemical and physiological alterations, behaviour is a comprehensive biomarker compared to biochemical or physiological traits alone (Warner *et al.*, 1966; Peakall, 1996).

The continuity from the biochemical level to the population and community levels has been described as a time-dependent process (Peakall, 1994) (Fig. 1), where alterations at the biochemical level are thought to be more sensitive towards pollution than physiological, behavioural and community levels, respectively. However, caution should be made when considering the time-dependent factor, since biochemical, cellular, physiological and behavioural alterations may occur almost simultaneously in a chemically exposed individual. This may be the case for EDCs, as these may affect hormone systems resulting in an immediate behavioural response (Archand-Hoy and Benson, 1998). An example is the influence of a chemical agent on one or more of the hormones in the hypotalamus-pituitary-gonadal axis resulting in an immediate behavioural change as the sex steroids directly influence reproductive behaviour (Archand-Hoy and Benson, 1998).

When a pollutant affects most individuals within a population, changes in the ecosystem will take place (Fig. 1). The propagation of effects from individual through population to ecosystem levels will thus be a time-dependent process.

There are situations where the patterns described in Figure 1 are precise, and where there is a time-dependency in the pathway from induction of pollution through biochemical, physiological to behavioural markers. An example is the effect of TBTO and Zn on Na^+,K^+-ATPase that may cause gill fusion that again may cause impaired antipredator behaviour and swimming performance (McGeer *et al.*, 2000).

All the behavioural traits studied in this thesis are considered to be sensitive to pollution (Little *et al.*, 1993; Arcand-Hoy and Benson, 1998). However, the results reported in Papers I – IV also show that some of these behavioural traits may vary in sensitivity (Little *et al.*, 1993; Smith and Logan, 1997). Examples of variables that remained unaffected after chemical exposure were recovery time (Paper I), size assortative shoaling (Paper III) and courtship behaviour (Paper IV). Since the reproductive variables "paternal care" and "time of nest building" (Paper IV) were affected by exposure to E_2, a measurement of courtship behaviour alone would have caused exclusion of very important information about the suggested reproductive effects of this estrogen. Moreover, by studying the effects of TBTO on antipredator behaviour (Paper I) and BBP on shoaling behaviour (Paper III), it is shown that the investigation of a variety of variables is needed to document effects of exposure to the respective pollutants, effects that would have been missed if only the non-sensitive traits had been tested. The difference in sensitivity may be a result of biochemical and/or physiological variables that regulate behavioural traits differently. Thus, there may be different biochemical and/or physiological processes regulating for example, paternal care and courtship behaviour in threespine stickleback (Paper IV), alternatively, the same biochemical and/or physiological variable may regulate behavioural traits by different mechanisms.

Other studies reporting both the presence and absence of significant behavioural effects of pollution, include significant effects of pentachlorophenol on antipredator behaviour in guppies while at the same time no effects on habitat use or general behaviour were detected (Brown *et al.*, 1985). Zhou and Weis (1998) observed significant effects of methylmercury on swimming behaviour and predator avoidance in larval mummichogs, while other

behavioural traits in the same individuals were not affected. These results may be explained in terms of differences in sensitivity by the different behavioural traits. In conclusion, the results in Papers I-IV demonstrate the importance of investigating several behavioural traits when evaluating the effects of pollution.

Ecological relevance

The behavioural traits studied in Papers I - IV have consequences for the individual fitness, and are thus considered to be of high ecological relevance (Little *et al.*, 1993). The significance of a biomarker is expected to increase with higher biological organisation, with the ecosystem at the highest level (Fig. 1) (Peakall, 1994). Biochemical biomarkers are widely used to indicate exposure to pollutants, but despite their significance in ecotoxicological research, biochemical biomarkers are poor predictors of individual fitness, and they are often considered to be of restricted ecological significance. By including effects on behavioural biomarkers, the ecological significance for individual fitness would be increased, exemplified by the study of disrupted gill structure and reduced swimming performance in salmonids exposed to 2-(thiocyanomethylthio)benzoathiazole (Niki and Farrell, 1993). Similarly, heavy metals were found to disturb gill Na^+,K^+-ATPase, osmoregulation, swimming performance and feeding in rainbow trout (McGeer *et al.*, 2000). Thus, by including biomarkers which enable assessment of individual fitness the ecological significance of a study will be enhanced (review by Peakall *et al.*, 2002).

In the discussion of suitable behavioural traits in ecotoxicological studies, it has been suggested that easily quantifiable variables often have little influence on survival and/or reproductive success and that they are therefore less ecologically significant than complex and less readily measurable variables (Peakall, 1994). Examples of easily quantifiable traits of suggested low ecological importance are operant conditioning (learning by association), and avoidance of chemicals, since there is no documented relationship between these variables and fitness (Peakall, 1994). It has also been argued that the lack of documented relationship between impaired feeding and fitness,

invalidates the use of feeding behaviour as a biomarker (Peakall, 1994). However, reduced feeding may cause reduced survival. Thus hungry sticklebacks are willing to take higher risks and feed closer to predators than satiated fish (Fraser and Huntingford, 1986; Godin and Crossman, 1994). Further, increased time spent on feeding will result in less time devoted to other essential activities, such as reproductive behaviour (Mori, 1993; Barber *et al.*, 2001), antipredator behaviour and shoaling behaviour (Sullivan *et al.*, 1978; Kruzynski and Birtwell, 1994). Impairment of these essential activities entail reduced individual success. Thus, because both avoidance and feeding behaviour will influence survival they are of high ecological relevance and are therefore important behavioural variables to study (Little *et al.*, 1993; Smith and Logan, 1997).

In the studies reported on in Papers I - IV sublethal concentrations of test chemicals were used. The behavioural alterations observed may be unusual in most aquatic habitats since the concentrations of TBTO, DDE, BBP and E_2 will be even lower than the ones used in these studies. However, in man-disturbed habitats, such as harbours and other urban waters, where threespine sticklebacks often live, the concentrations may reach the levels used in this study, or be even higher due to for example, local spills (NGI, 1997; Tyler *et al.*, 1998; NIVA, 2000).

In complex behavioural traits of high ecological relevance, the variations between individuals are usually larger than in more simple and stereotyped traits. In complex traits accurate measurements are difficult to obtain, and individual differences with respect to for example, motivation or experience are more difficult to standardise than for less complex variables. The variation among individuals may impair the possibility of obtaining significant differences between exposure groups, (section 4.1.). However, because of the ecological significance of complex behavioural traits, their susceptibility to environmental pollutants is important to document. By defining variables of complex behaviours that are objective and easily quantifiable, reliable results with respect to effects of chemicals may be obtained, for example, the number of

courtship displays per male (Paper IV) and the number of individuals initiating feeding (Paper II).

Although sensitive to pollution and ecologically significant, behavioural variables are rarely included in ecotoxicological investigations. Whereas biochemical and/or physiological variables are frequently used as biomarkers, few studies give exclusive priority to behavioural variables. The use of behaviour as a biomarker is however important in order to obtain a complete understanding of the effects of pollution. Interfering biotic variables (e.g. individual differences) may be one reason for the scarcity of studies dealing with behavioural changes caused by pollution (Little, 1990).

Examples of the few studies where behaviour has been given priority, include the effects of E_2 and octylphenol on the reproductive behaviour in male guppies (Bayley *et al.*, 1999), the effects of cadmium on parental care in female willow ptarmigan *Lagopus l. lagopus* (Pedersen and Sæther, 1999), the developmental effects of lead on gull chicks *Larus argentatus* (Burger and Gochfeld, 1995), and the effects on antipredator behaviour in guppies exposed to pentachlorophenol (Brown, *et al.*, 1985).

4.3 *Laboratory and field experiments. Reflections of present and future research methods*

If behavioural biomarkers are to be used to objectively evaluate the effects of chemical agents, ecologically relevant and stereotyped traits are required. In the laboratory, it is possible to conduct experiments under standardised conditions where abiotic and some biotic factors can be controlled. Given these conditions, it is possible to compare groups with respect to the effects on defined behavioural traits, when the groups are otherwise identically treated. Furthermore, in the laboratory, individuals can be exposed to one or a few chemicals during a time-limited experiment. On the other hand, laboratory experiments will always be artificial, which should be considered when interpreting the results.

In contrast to laboratory experiments, field investigations provide information about the individuals in their natural habitat. However, free-ranging individuals are likely to be exposed to a mixture of different chemical agents throughout most of their lifetime, and since biotic and abiotic conditions may vary between individuals, standardisation of experiments in the field is difficult or even impossible.

Since biotic and abiotic conditions in the laboratory are not comparable to conditions in nature, extrapolating results from the laboratory to the wild, and vice versa should be made with caution. This may apply not only to behavioural biomarkers but also to physiological and biochemical biomarkers. Also, individuals used in laboratory and field experiments often originate from different populations with different adaptations, gentic pool etc. The dissimilarities between fish from different populations could cause the individuals to behave differently even before the effects of contamination are shown.

In the study of effects of pollutants, behavioural experimentation in the field is important, and there are some reports based on comparative behavioural studies of polluted and unpolluted wild populations (e.g. Smith and Weis, 1997; Zhou and Weis, 1999). Although it can be argued that biotic and abiotic differences between the populations may make this experimental approach suboptimal, it has been an often used method when investigating behavioural effects of pollution in the field.

An optimal, though time consuming approach to the study of the behavioural effects of contamination in the wild is to study a population that is expected to become exposed to pollutants, and perform repeated recordings of the behavioural traits on a long-term basis, before and after contamination. This will minimise the effect of occasional short-term biotic and abiotic variations. These long-term studies should be combined, but not directly compared, with laboratory behavioural studies.

Information about alterations caused by pollution at both the population and ecosystem level is important since biomarkers at these levels represent very high ecological significance (Fig. 1) (Peakall, 1994). Such data are most

reliably obtained in the wild since information of interest includes interactions between the individuals and their environment, between individuals of the same population and between individuals of different species or populations.

In this study behavioural biomarkers have been evaluated, and the properties of the defined traits as indicators of pollution have been discussed. Several advantages but also limitations of using behavioural biomarkers have been discussed, such as high ecological relevance and sensitivity, and the difficulties with individual variation and objective evaluation. However, the importance of using behaviour in studying of effects of pollution implies further development of methods that optimise the use of behavioural biomarkers.

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Doctoral theses in Zoology

from the University of Trondheim / Norwegian University of Science and Technology

	Year	Name	Degree	Title
1	1978	Tore Slagsvold	Dr. philos.	Breeding events of birds in relation to spring temperature and environmental phenology.
2	1980	Arnfinn Langeland	Dr. philos.	Interaction between fish and zooplankton populations and their effects on the material utilization in a freshwater lake.
3	1982	Dag Dolmen	Dr. philos.	Life aspects of two sympartic species of newts (*Triturus, Amphibia*) in Norway, with special emphasis on their ecological niche segregation.
4	1984	Eivin Røskaft	Dr. philos.	Sociobiological studies of the rook *Corvus frugilegus.*
5	1985	Randi E. Reinertsen	Dr. philos.	Energy strategies in the cold: Metabolic and thermoregulatory adaptations in small northern birds.
6	1985	Jarle Mork	Dr. philos.	Biochemical genetic studies in fish.
7	1986	Torleif Holthe	Dr. philos.	Evolution, systematics, nomenclature, and zoogeography in the polychaete orders *Oweniimorpha* and *Terebellomorpha*, with special reference to the Arctic and Scandinavian fauna.
8	1986	John Solem	Dr. philos.	Taxonomy, distribution and ecology of caddisflies (*Trichoptera*) in the Dovrefjell mountains.
9	1986	Bernt Erik Sæther	Dr. philos.	Ecological and evolutionary basis for variation in reproductive traits of some vertebrates: A comparative approach.
10	1987	Olav Hogstad	Dr. philos.	Winter survival strategies of the Willow tit *Parus montanus.*
11	1987	Helene Lampe	Dr. scient.	The function of bird song in mate attraction and territorial defence, and the importance of song repertoires.
12	1987	Bjørn Åge Tømmerås	Dr. scient.	Olfaction in bark beetle communities: Interspecific interactions in regulation of colonization density, predator - prey relationship and host attraction.
13	1988	Tor G. Heggberget	Dr. philos.	Reproduction in Atlantic Salmon (*Salmo salar*): Aspects of spawning, incubation, early life history and population structure.
14	1988	Hans Christian Pedersen	Dr. philos.	Reproductive behaviour in willow ptarmigan with special emphasis on territoriality and parental care.
15	1988	Marianne V. Nielsen	Dr. scient.	The effects of selected environmental factors on carbon allocation/growth of larval and juvenile mussels (*Mytilus edulis*).
16	1988	Ole Kristian Berg	Dr. scient.	The formation of landlocked Atlantic salmon (*Salmo salar* L.).
17	1989	John W. Jensen	Dr. philos.	Crustacean plankton and fish during the first decade of the manmade Nesjø reservoir, with special emphasis on the effects of gill nets and salmonid growth.

18	1989 Reidar Andersen	Dr. scient.	Interactions betweem a generalist herbivore, the moose *Alces alces*, and its winter food resources: a study of behavioural variation.
19	1989 Helga J. Vivås	Dr. scient.	Theoretical models of activity pattern and optimal foraging: Predictions for the Moose *Alces alces*.
20	1990 Arne Johan Jensen	Dr. philos.	Effects of water temperature on early life history, juvenile growth and prespawning migrations of Atlantic salmion (*Salmo salar*) and brown trout (*Salmo trutta*): A summary of studies in Norwegian streams.
21	1990 Tor Jørgen Almaas	Dr. scient.	Pheromone reception in moths: Response characteristics of olfactory receptor neurons to intra- and interspecific chemical cues.
22	1990 Bengt Finstad	Dr. scient.	Osmotic and ionic regulation in Atlantic salmon, rainbow trout and Arctic charr: Effect of temperature, salinity and season.
23	1990 Magne Husby	Dr. scient.	Breeding strategies in birds: Experiments with the Magpie *Pica pica*.
24	1990 Hege Johannesen	Dr. scient.	Respiration and temperature regulation in birds with special emphasis on the oxygen extraction by the lung.
25	1991 Nina Jonsson	Dr. philos.	Aspects of migration and spawning in salmonids.
26	1991 Jan Henning L'Abêe Lund	Dr. philos.	Reproductive biology in freshwater fish, brown trout *Salmo trutta* and roach *Rutilus rutilus* in particular.
27	1991 Odd Terje Sandlund	Dr. philos.	The dynamics of habitat use in the salmonid genera *Coregonus* and *Salvelinus*: Ontogenic niche shifts and polymorphism.
28	1991 Trond Nordtug	Dr. scient.	Reflctometric studies of photomechanical adaptation in superposition eyes of arthropods.
29	1991 Tor Kvam	Dr. scient.	Population biology of the European lynx (*Lynx lynx*) in Norway.
30	1992 Bjørn Munro Jenssen	Dr. philos.	Thermoregulation in aquatic birds in air and water: With special emphasis on the effects of crude oil, chemically treated oil and cleaning on the thermal balance of ducks.
31	1992 Arne Vollan Aarset	Dr. philos.	The ecophysiology of under-ice fauna: Osmotic regulation, low temperature tolerance and metabolism in polar crustaceans.
32	1992 Tycho Anker-Nilssen	Dr. scient.	Food supply as a determinant of reproduction and population development in Norwegian Puffins *Fratercula arctica*
33	1992 Torgrim Breiehagen	Dr. scient.	Mating behaviour and evolutionary aspects of the breeding system of two bird species: the Temminck's stint and the Pied flycatcher.
34	1993 Kjetil Bevanger	Dr. scient.	Avian interactions with utility structures, a biological approach.
35	1993 Thrine L. M. Heggberget	Dr. scient.	Reproductive strategy and feeding ecology of the Eurasian otter *Lutra lutra*.

36	1993 Yngvar Asbjørn Olsen	Dr. scient.	Cortisol dynamics in Atlantic salmon, Salmo salar L.: Basal and stressor-induced variations in plasma levels ans some secondary effects.
37	1993 Tor Fredrik Næsje	Dr. scient.	Habitat shifts in coregonids.
38	1994 Arne Moksnes	Dr. philos.	Host adaptations towards brood parasitism by the Cockoo.
39	1994 Morten Bakken	Dr. scient.	Infanticidal behaviour and reproductive performance in relation to competition capacity among farmed silver fox vixens, *Vulpes vulpes*.
40	1994 Peder Fiske	Dr. scient.	Sexual selection in the lekking great snipe (*Gallinago media*): Male mating success and female behaviour at the lek.
41	1994 Torbjørn Forseth	Dr. scient.	Bioenergetics in ecological and life history studies of fishes.
42	1994 Nils Røv	Dr. scient.	Breeding distribution, population status and regulation of breeding numbers in the northeast-Atlantic Great Cormorant *Phalacrocorax carbo carbo*.
43	1995 Martha Kold Bakkevig	Dr. scient.	The impact of clothing textiles and construction in a clothing system on thermoregulatory responses, sweat accumulation and heat transport.
44	1995 Hanne Christensen	Dr. scient.	Determinants of Otter Lutra lutra distribution in Norway: Effects of harvest, polychlorinated biphenyls (PCBs), human population density and competition with mink *Mustela vision*.
45	1995 Chris Jørgen Jensen	Dr. scient.	The surface electromyographic (EMG) amplitude as an estimate of upper trapezius muscle activity
46	1995 Svein Håkon Lorentsen	Dr. scient.	Reproductive effort in the Antarctic Petrel *Thalassoica antarctica*; the effect of parental body size and condition.
47	1995 Vidar Moen	Dr. scient.	Distribution patterns and adaptations to light in newly introduced populations of *Mysis relicta* and constraints on Cladoceran and Char populations.
48	1996 Ola Ugedal	Dr. scient.	Radiocesium turnover in freshwater fishes
49	1996 Ingibjørg Einarsdottir	Dr. scient.	Production of Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*): A study of some physiological and immunological responses to rearing routines.
50	1996 Christina M. S. Pereira	Dr. scient.	Glucose metabolism in salmonids: Dietary effects and hormonal regulation.
51	1996 Jan Fredrik Børseth	Dr. scient.	The sodium energy gradients in muscle cells of *Mytilus edulis* and the effects of organic xenobiotics.
52	1996 Gunnar Henriksen	Dr. scient.	Status of Grey seal *Halichoerus grypus* and Harbour seal *Phoca vitulina* in the Barents sea region.
53	1997 Ole Reitan	Dr. scient.	Responses of birds to habitat disturbance due to damming.
54	1997 Jon Arne Grøttum	Dr. scient.	Physiological effects of reduced water quality on fish in aquaculture.

55	1997 Per Gustav Thingstad	Dr. scient.	Birds as indicators for studying natural and human-induced variations in the environment, with special emphasis on the suitability of the Pied Flycatcher.
56	1997 Torgeir Nygård	Dr. scient.	Temporal and spatial trends of pollutants in birds in Norway: Birds of prey and Willow Grouse used as Biomonitors.
57	1997 Signe Nybø	Dr. scient.	Impacts of long-range transported air pollution on birds with particular reference to the dipper *Cinclus cinclus* in southern Norway.
58	1997 Atle Wibe	Dr. scient.	Identification of conifer volatiles detected by receptor neurons in the pine weevil (*Hylobius abietis*), analysed by gas chromatography linked to electrophysiology and to mass spectrometry.
59	1997 Rolv Lundheim	Dr. scient.	Adaptive and Incidental Biological Ice Nucleators.
60	1997 Arild Magne Landa	Dr. scient.	Wolverines in Scandinavia: ecology, sheep depredation and conservation.
61	1997 Jarle Tufto	Dr. scient.	Gene flow and Genetic Drift in Geographically Structured Populations: Ecological, Population Genetic, and Statistical Models
62	1997 Trygve Hesthagen	Dr. philos.	Population responces of Arctic charr (*Salvelinus alpinus* (L.)) and brown trout (*Salmo trutta* L.) to acidification in Norwegian inland waters
63	1997 Trygve Sigholt	Dr. philos.	Control of Parr-Smolt Transformation and Seawater Tolerance in Farmed Atlantic Salmon (*Salmo salar*) Effects of photoperiod, temperature, gradual seawater acclimation, NaCl and betaine in the diet
64	1997 Jan Østnes	Dr. scient.	Cold sensation in adult and neonate birds
65	1998 Thor Harald Ringsby	Dr. scient.	Variation in Space and Time: The Biology of a House Sparrow Metapopulation
66	1998 Erling Johan Solberg	Dr. scient.	Variation in population dynamics and life history in a Norwegian moose (Alces alces) population: consequences of harvesting in a variable environment
67	1998 Bente Gunnveig Berg	Dr. scient.	Encoding of pheromone information in two related moth species
68	1999 Kristian Overskaug	Dr. scient.	Behavioural and Morphological Characteristics in Northern Tawny Owls *Strix aluco*: An Intra- and Interspecific Comparative Approach
69	1999 Ingvar Stenberg	Dr. scient.	Habitat selection, reproduction and survival in the White-backed Woodpecker <u>Dendrocopos leucotos</u>
70	1999 Trina Falck Galloway	Dr. scient.	Muscle development and growth in early life stages of the Atlantic cod (*Gadus morhua* L.) and halibut (*Hippoglossus hippoglossus* L.)
71	1999 Marianne Giæver	Dr. scient.	Population genetic studies in three gadoid species: blue whiting (*Micromisistius poutassou*), haddock (*Melanogrammus aeglefinus*) and cod (*Gradus morhua*) in the North-East Atlantic

72	1999 Ingrid Bysveen Mjølnerød	Dr. scient.	Aspects of population genetics, behaviour and performance of wild and farmed Atlantic salmon (*Salmo salar*) revealed by molecular genetic techniques
73	1999 Stein-Are Sæther	Dr. philos.	Mate Choice, Competition for Mates, and Conflicts of Interest in the Lekking Great Snipe
74	1999 Katrine Wangen Rustad	Dr. scient.	Modulation of Glutamatergic Neurotransmission Related to Cognitive Dysfunctions and Alzheimer's Disease
75	1999 Per Terje Smiseth	Dr. scient.	Social evolution in monogamous families: mate choice and conflicts over parental care in the Bluethroat (*Luscinia s. svecica*)
76	1999 Gunnbjørn Bremset	Dr. scient.	Young Atlantic salmon (*Salmo salar* L.) and brown trout (*Salmo trutta* L.) inhabiting the deep pool habitat, with special reference to their habitat use, habitat preferences and competitive interactions
77	1999 Frode Ødegaard	Dr. scient.	Host spesificity as parameter in estimates of arhrophod species richness
78	2000 Ingar Jostein Øien	Dr. scient.	The Cuckoo (*Cuculus canorus*) and its host: adaptions and counteradaptions in a coevolutionary arms race
79	2000 Sigbjørn Stokke	Dr. scient.	Sexual segregation in the African elephant (*Loxodonta africana*)
80	2000 Odd A. Gulseth	Dr. philos.	Seawater Tolerance, Migratory Behaviour and Growth of Charr, (*Salvelinus alpinus*), with Emphasis on the High Arctic Dieset Charr on Spitsbergen, Svalbard
81	2000 Pål A. Olsvik	Dr. scient.	Biochemical impacts of Cd, Cu and Zn on brown trout (*Salmo trutta*) in two mining-contaminated rivers in Central Norway
82	2000 Sigurd Einum	Dr. scient.	Maternal effects in fish: Implications for the evolution of breeding time and egg size
83	2001 Jan Ove Evjemo	Dr. scient.	Production and nutritional adaptation of the brine shrimp *Artemia* sp. as live food organism for larvae of marine cold water fish species
84	2001 Ingebrigt Uglem	Dr. scient.	Male dimorphism and reproductive biology in corkwing wrasse (*Symphodus melops* L.)
85	2001 Bård Gunnar Stokke	Dr. scient.	Coevolutionary adaptations in avian brood parasites and their hosts
86	2002 Ronny Aanes	Dr. scient.	Spatio-temporal dynamics in Svalbard reindeer (*Rangifer tarandus platyrhynchus*)
87	2002 Mariann Sandsund	Dr. scient.	Exercise- and cold-induced asthma. Respiratory and thermoregulatory responses
88	2002 Frank Rosell	Dr. scient	The function of scent marking in beaver (*Castor fiber*)

Paper I

Effects of bis(tributyltin)oxide on antipredator behavior in threespine
stickleback *Gasterosteus aculeatus*

By

Wibe, Å.E., Nordtug, T. and Jenssen, B.M.

Effects of bis(tributyltin)oxide on antipredator behavior in threespine stickleback *Gasterosteus aculeatus* L.

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Abstract

Antipredator behavior was used as a parameter to detect effects caused by exposure to the organotin compound bis(tributyltin)oxide (TBTO). Three groups of threespine sticklebacks (*Gasterosteus aculeatus* L.) were exposed to 3, 9 and 27 ppb TBTO, respectively. A fourth control group was given the same treatment as the other three groups, but no TBTO. Antipredator behavior of the fish was evoked using a dummy heron (*Ardea cinerea*) bill. TBTO exposure caused significant changes in the spatial position of the fish in the aquarium (P_{Loc}), their response to predator attack (P_{Res}), recovery time (P_{Rec}) and latency time (P_{Lat}). Some of the effects were, however, reversible after the exposure was terminated. We suggest that behavior as an indicator of pollution may be used as an ecologically relevant integrative biomarker. © 2001 Elsevier Science Ltd. All rights reserved.

1. Introduction

Most studies concerning biological effects of pollution focus on molecular, biochemical and histological parameters (Wester and Canton, 1987; Schwaiger et al., 1992; Zucker et al., 1992; McCarthy, 1994). Behavioral changes, on the other hand, may represent the consequence of physiological and biochemical alterations (Peakall, 1996), and are of high ecological importance. Since behavior of animals may be affected by very low concentrations of chemicals (Ramade, 1987; Smith and Logan, 1997), it can be used as an early warning to detect pollution (Smith and Logan, 1997).

Bis(tributyltin)oxide (TBTO) is a xenobiotic organotin compound that may affect the nervous system and alter behavior (e.g. Schweinfurth, 1985; Holm et al., 1991; Fent and Meier, 1992; Triebskorn et al., 1994). However, due to metabolism, effects following sublethal TBTO exposure may be reversed when contamination ceases (Martin et al., 1989; Zucker et al., 1992). The organotin is used in antifouling paints, and it is persistent and bioaccumulative (e.g. WHO, 1980; Fent and Hunn, 1995). The biological effects of TBTO will be restricted mainly to harbors where paint leakage from boats and other antifouling treated material may occur (Fent and Hunn, 1995). TBTO is still in use in many countries and continues to present a problem both in marine (e.g. Bryan and Gibbs, 1991; Holm et al., 1991; Danish environmental protection agency, 1995) and freshwater (e.g. Wester and Canton, 1987; Maguire, 1987; Schwaiger et al., 1992; Fent and Hunn, 1995) environments. In natural waters TBTO concentrations usually stay below ppb levels, but in harbors levels of 0.1–1 ppb are regularly found (Holm et al., 1991). Even concentrations up to 11.7 ppb have been observed (Wester and Canton, 1987).

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The species used in this experiment, threespine stickleback (*Gasterosteus aculeatus* L.), often live in freshwater and/or marine harbors and may be vulnerable to TBTO exposure. Intraspecific predation (cannibalism) and interspecific predation are the main causes of mortality in threespine sticklebacks (Reimchen, 1994; Whoriskey and FitzGerald, 1994). Their small body size and relatively slow swimming speed make sticklebacks vulnerable to predation from fish, birds and macroinvertebrates (Reimchen, 1994). In an encounter with a predator, the sticklebacks often freeze to inspect the predator and evaluate the risk. If the predator presents a risk, the sticklebacks will escape to cover (Huntingford et al., 1994). Exposure to chemicals which affect behavior will, in addition to other stressors, make them even more vulnerable to predator attack (Brown et al., 1985; Little et al., 1993).

The aim of this study is to investigate changes in antipredator behavior caused by short term exposure to TBTO and to study the reversibility of these effects. It is also an intention to evaluate the possibility of using antipredator behavior as an indicator parameter for sublethal effects of environmental pollution.

2. Methods

2.1. Fish maintenance

Adult male threespine sticklebacks of uniform size (4.64 ± 0.8 cm) were captured with traps in Kinnsettjønna (fresh water), Sør-Trøndelag county, central Norway (63.5°25′ N, 10°45′ E), in May and June. The experiment was conducted the following autumn. Prior to the experiment the fish were reared in plastic containers (60 l) with continuous fresh water flow. A light regime corresponding to the natural pattern for the latitude and time of the year was maintained. Water temperatures was measured daily and averaged 7.75°C (±0.38)°C, while pH was 7.18 (±0.27). Fish were daily fed commercial dryfood (Tetramin) and Tubifex.

Prior to the start of the experiment, fish were placed individually in glass aquaria ($15 \times 15 \times 10$ cm^3) with continuous fresh water flow (10 ml/min), and were acclimated for one week. To minimize stress during the experiment sheets of colored PVC were placed between the aquaria to avoid visual contact among fish. As hiding places, shelters of dark colored glass were placed at the bottom of each aquarium (Wagner, 1987).

2.2. TBTO exposure

The stock solution containing TBTO was prepared in two steps. Initially TBTO was diluted in ethanol (Zucker et al., 1992) to a concentration of 3.0 g/l (25.5 μl TBTO in 10 ml ethanol). Ethanol, in this concentration, is shown to have no deleterious effects on the organisms (Zucker et al., 1992). A secondary water based stock solution was then prepared by adding 0.23 ml of the primary stock solution to 1 l of water (TBTO concentration = 0.69 mg/l). The secondary stock solution was added into the inlet water (10 ml/min) in each chamber at rates of 0.4 ml/min and 0.133 ml/min, by a multi-channel peristralic pump. The resulting nominal concentrations in the inlet water of the aquaria were then 27 and 9 ppb, respectively. A further tenfold dilution of the secondary stock solution and a pumping rate of 0.4 ml/min achieved a third concentration of 2.7 ppb. Because no analysis of the water content of TBTO was performed, the nominal concentrations referred to denote the maximum possible concentrations. The actual concentrations are somewhat lower because TBTO will adsorb to particles in the water and the walls of the aquarium, however a continuous flow system will reduce the TBTO binding compared to static systems (WHO, 1980; Maguire, 1987). The continuous flow system was also a precaution to maintain stable TBTO concentrations (Peterle, 1991). Because TBTO is degraded by UV-light (WHO, 1980), the stock solution tanks were shielded from light by dark plastic.

A total of 96 sticklebacks were used in the experiment. Fish were randomly divided into four groups; TBTO-27 contained 16 individuals exposed to 27.0 ppb TBTO, TBTO-9 contained 32 individuals exposed to 9.0 ppb TBTO, and TBTO-3 contained 16 individuals exposed to 2.7 ppb TBTO. The TBTO-0 group constituted of 32 individuals. There were unequal numbers of fish in the groups because a concentration of 27 ppb turned out to be lethal to most individuals. It was not possible, therefore, to perform any behavioral observations with this group. Thus, as the experiment went on, the highest concentration was exchanged with the low concentration of 2.7 ppb.

The control fish received the same treatment as the exposed fish, except that the TBTO/ethanol solution was exchanged with ethanol only. The TBTO exposure was terminated after four days of treatment.

2.3. Antipredator behavior

Antipredator behavior of each fish was video recorded once a day. The first recording was done immediately after termination of the exposure (day 1) and continued for the following five days.

Exposing each individual to a simulated predator attack using a dummy heron (*Ardea cinerea*) bill evoked the antipredator response. The dummy was lowered over the aquarium, allowing the tip of the bill to splash the water surface before it was raised back again in its original position (Giles, 1984). This stimulus was designed to simulate a sudden overhead attack from a heron searching for prey (Giles, 1984). To avoid possible

habituation and pseudoreplication, each fish was attacked only once a day for five days. The daily video recordings lasted for 3 min for each aquarium. Each video sequence covered the attack and the following behavioral response of the stickleback. After termination of the entire experiment, the lengths of the fish were measured after they had been anaesthetized with 2-phenoxyethanol. All fish were then decapitated. The animals were further autopsied for endo- and exo-parasites since parasites as well as any disease may cause physiological disruptions in the test animals. This may reduce the possibility that the observed effects are a result of pollution (Hinton et al., 1992).

2.4. Behavioral parameters

When analyzing the video recordings, the observer did not know whether the observed fish were exposed or unexposed, since each aquarium was marked with a number. From the video recordings, four behavior parameters were analysed:

1. The location of the fish in the aquarium prior to the predator attack (P_{Loc}) measured whether the fish stayed in open water (Giles and Huntingford, 1984). P_{Loc} was quantified by dividing the aquarium into three imaginable, vertical levels which were 1 = on the bottom, 2 = open water and 3 = in the surface (Brown et al., 1985). P_{Loc} was not an antipredator response, but illustrated the stickleback condition prior to the bill dummy attack.
2. The fish response to the attack (P_{Res}) was subjectively divided into 4 categories according to the intensity of the response, 1 = no response to the attack, 2 = a small movement following the attack, 3 = the fish jumped, but did not move from the site. 4 = an obvious escape response away from the site.
3. The recovery time (P_{Rec}) was defined as the elapsed time (s) from the attack to the resuming of the fish original swimming behavior (Giles and Huntingford, 1984).
4. The latency time (P_{Lat}) was defined as the elapsed time (s) from the attack until the fish response was achieved (Slater, 1985). The latency time was divided into five categories, where 1 =< 0.5 s, 2 = 0.5–2.0 s, 3 = 2.0–10.0 s, 4 => 10.0 s and 5 = no response.

2.5. Statistical analysis

Differences in frequency among the groups were tested with a χ-square test. Further, Kruskal–Wallis test of variance examined whether the exposure groups differed in their behavior between day 1 and day 5. A Scheffe post hoc test was used to locate the different group. Levels of significance were defined as probability < 0.05 (Zar, 1984).

3. Results

Autopsy of the animals revealed that no animals contained detectable ecto- or endo-parasites. During the entire experimental period 87.5% ($N = 16$) of the TBTO-27 individuals, 37.5% ($N = 32$) of the TBTO-9 individuals, 6.25% ($N = 16$) of the TBTO-3 individuals and 18.75% ($N = 32$) of the control fish died. Due to the high mortality in the TBTO-27 group, it was not possible to perform any behavioral studies with these fish. The results from this group therefore had to be omitted from the rest of the analysis.

3.1. Response frequency

Prior to the simulated heron attack, there were more TBTO-9 than TBTO-3 and TBTO-0 individuals on the bottom of the aquaria ($P_{Loc} = 1$; $\chi^2 = 20.42$, d.f. = 2, $P < 0.001$). Furthermore, there were fewer TBTO-9 individuals swimming in open water ($P_{Loc} = 2$; $\chi^2 = 22.5$, d.f. = 2, $P < 0.001$) (Fig. 1(a)).

With respect to the classification of the fish response (P_{Res}), the frequency of individuals responding to the attack with a small movement differed significantly between the three exposure groups ($P_{Res} = 2$; $\chi^2 = 7.0$, d.f. = 2, $P < 0.05$). It was also documented that more control individuals showed an obvious escape response ($P_{Res} = 4$; $\chi^2 = 11.3$, d.f. = 2, $P = 0.004$). There were no significant differences between exposed and unexposed individuals with respect to $P_{Res} = 1$ or 3 (Fig. 1(b)).

Considering recovery time (P_{Rec}), the majority of the individuals resumed their normal activities after a minimum of 10 s, however more control individuals than exposed individuals showed this pattern ($\chi^2 = 15.73$, d.f. = 2, $P < 0.001$) (Fig. 1(c)).

TBTO-0, TBTO-3 and TBTO-9 individuals differed significantly with respect to the ability to show an immediate response to the bill attack ($P_{Lat} = 1$; $\chi^2 = 18.33$, d.f. = 2, $P < 0.001$). Furthermore, there were more TBTO-9 fish responding after 0.5–2 s ($P_{Lat} = 2$; $\chi^2 = 12.29$, d.f. = 2, $P < 0.01$). There were no differences among the treatments regarding $P_{Lat} = 3$, 4 or 5 (Fig. 1(d)).

3.2. Temporal changes in response

To see whether the effects of TBTO changed from day 1 to day 5, the differences in variance between the groups were measured on the respective days.

On day 1 of the experiment (i.e. the day when the TBTO exposure was terminated) the control individuals were located significantly higher in the aquarium than the exposed individuals (P_{Loc}; $\chi^2 = 8.871$, d.f. = 2, $P = 0.012$) (Scheffe: $P < 0.05$). The TBTO-3 and TBTO-9 fish did not differ (Scheffe: $P > 0.5$). In the last day no
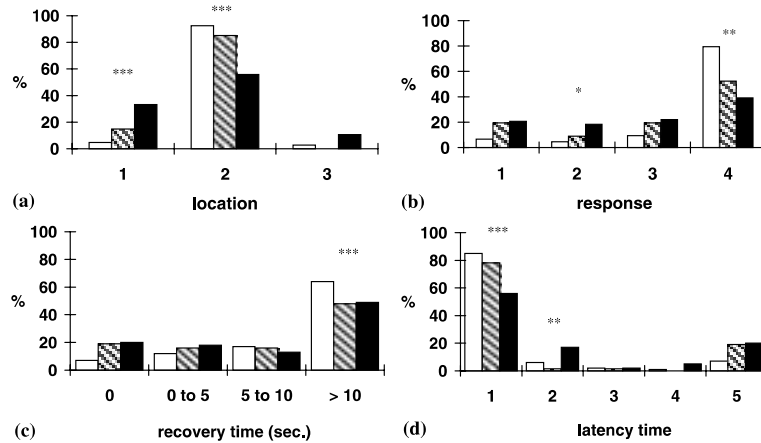
Fig. 1. The frequency (%) of 0.0 ppb (□), 3.0 ppb (▨) and 9.0 ppb (■) TBTO exposed sticklebacks performing respective behavioural patterns. (a) P_{Loc} = the fish vertical position in the aquarium prior to the attack; (b) P_{Res} = a subjective classification of the fish response to the dummy attack, ranging from 1 = no escape response to 4 = very obvious escape response; (c) P_{Rec} = recovery time (s), from the start of the dummy attack to when the fish resumed its original normal behaviour; (d) P_{Lat} = latency time (s), ranging from 1 =< 0.5 s to 5 = no response.

differences occurred between the groups ($\chi^2 = 2.571$, d.f. = 2, $P = 0.276$) (Fig. 2(a)).

The same pattern was true for the response classification (P_{Res}), where the controls differed significantly from the exposed fish on day 1 ($\chi^2 = 9.456$, d.f. = 2, $P = 0.009$) (Scheffe: $P < 0.01$). There were no differences between the TBTO-3 and TBTO-9 fish on day 1 (Scheffe: $P = 0.4$). On day 5 there were no differences between the groups ($\chi^2 = 0.353$, d.f. = 2, $P = 0.838$) (Fig. 2(b)).

Regarding recovery time (P_{Rec}) no significant temporal differences were found between the exposure groups for any of the days (Fig. 2(c)).

Finally, on day 1 of the experiment the TBTO-9 individuals showed a longer latency time than the control individuals and the TBTO-3 individuals ($\chi^2 = 21.349$, d.f. = 2, $P = 0.000$) (Scheffe: $P < 0.01$). There were no differences between TBTO-3 and TBTO-9 fish (Scheffe: $P = 0.5$). In the last day the difference between the
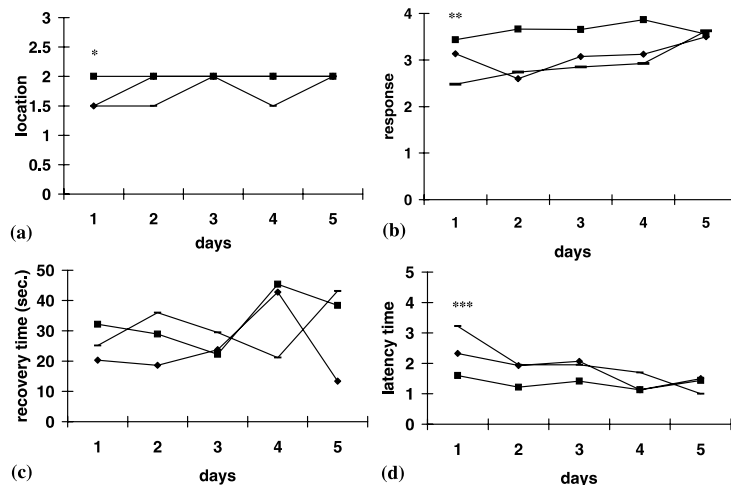

Fig. 2. Temporal changes in behaviour within 0.0 ppb (■), 3.0 ppb (◆) and 9.0 ppb (-) during five days following termination of TBTO exposure (day 1), (a) P_{Loc}, (b) P_{Res}, (c) P_{Rec}, (d) P_{Lat} (see Fig. 1 for parameter definitions).

groups disappeared ($\chi^2 = 0.981$, d.f. $= 2$, $P = 0.612$) (Fig. 2(d)).

4. Discussion

The main target in this investigation was to evaluate the usefulness of behavior in detecting effects on fish caused by contamination, and to see whether there were any differences between exposed and control fish.

A significant alteration in antipredator behavior was observed after only four days of sublethal exposure. This indicates that alteration in behavior is sensitive to chemical stress and may be used as an early warning indicator to exposure (Smith and Logan, 1997).

Due to the high mortality among the TBTO-27 fish, this group was exchanged with TBTO-3 in the middle of the experiment. It may be possible that the presented results from the TBTO-3 fish will be affected by the fact that the amounts of TBTO-3 individuals were lower than the other groups.

4.1. Response frequency

In nature, sticklebacks show variability in antipredator response, depending on factors such as predation risk (Huntingford et al., 1994), experience (Giles and Huntingford, 1984) and hunger (Milinski, 1993). Such variability were controlled for in this experiment, as the fish were derived from the same habitat, they were of approximately the same size, sex and nutritional state.

Some studies have investigated alterations in antipredator behavior caused by pollution (e.g. Sullivan et al., 1978; Brown et al., 1985; Little et al., 1993). A common conclusion is that prey exposed to chemicals are more vulnerable to capture than unexposed prey (Smith and Logan, 1997).

Adult threespine sticklebacks prefer to stay in open water, and when fish predators are rare or absent, sticklebacks avoid vegetation where macroinvertebrate predation might occur (Huntingford et al., 1994). The unexposed and low exposed individuals in our experiment preferred open water, whereas at the bottom of the aquarium there were more highly exposed individuals (Fig. 1(a)). Earlier studies indicate that minnows (*Phoxinus phoxinus*) exposed to 4.26–9.0 ppb TBTO (Fent and Meier, 1992) and rainbow trout (*Oncorhynchus mykiss*) exposed to 2.0 ppb TBTO (Triebskorn et al., 1994) showed less co-ordinated swimming behavior and sometimes rested motionless on the bottom of the aquarium.

We demonstrated that among the individuals who showed a reduced ability to escape from the dummy attack, there were more exposed than control fish (Fig. 1(b)). This deterioration in behavior is believed to be a result of one or more of the physiological effects caused

by TBTO (Triebskorn et al., 1994), such as inhibition of the mitochondrial ATP synthase complex, leading to diminished ATP production (Aldridge, 1976), deteriorated optical perception (Wester and Canton, 1987; Fent and Meier, 1992), or other effects on the nervous system (Krigman and Silverman, 1984; Schweinfurth, 1985). The same physiological effects may explain the reduced ability to respond immediately after the dummy attack as shown by the high exposure group (Fig. 1(d)). As ATP is the main energy source for muscle contraction, reduced levels may cause increased latency time (Brown et al., 1985). The small response delay of 0.5 s may be sufficient time for a predator to be successful in its attempt to capture the fish.

In this experiment we showed that among individuals with recovery time of 10 s or more, there were more controls than exposed fish. This finding is in contrary to what is expected, since it is previously documented that TBTO exposed rainbow trout are more stressed than control fish (Triebskorn et al., 1994), and that stressed fish usually show longer recovery time than not stressed fish (Pottinger et al., 1999). The measurement of stress in the study of Triebskorn et al. (1994) did not include any additional stress stimulation such as the heron bill stimulation in our experiment. The reason to our finding may be that this additional stress stimulation causes the control fish to be more stressed than the exposed individuals.

4.2. Temporal changes in response

Our results demonstrated that the control individuals showed a temporally constant response to the heron dummy and no habituation was observed (Fig. 2). Sticklebacks may habituate to predation if the predator pretends to attack regularly over time, but in heavily predated areas habitation will rarely occur (Huntingford and Coulter, 1989).

The first day after exposure to TBTO was terminated, there were significant differences between the groups with respect to several of the behavioral parameters (Fig. 2). The between group differences were, however, not detectable on day 5 after TBTO exposure. Thus, the observed effects of TBTO on the behavioral parameters studied herein seem to be reversible when exposure is terminated. This reversal of the effects is most likely linked to the fact that fish are capable of metabolizing and excreting TBTO (Bryan and Gibbs, 1991), although no attempt was made to measure possible metabolism of TBTO by sticklebacks in this study. When TBTO was no longer added to the aquaria, the excretion rate exceeded the uptake rate, causing the body concentration to decrease below the effect level. This reversibility of TBTO effects has previously been noted in other studies (e.g. Martin et al., 1989; Zucker et al., 1992). It is, however, important to note that

recovery is dependent on both exposure concentration and duration of exposure (Zucker et al., 1992).

Behavior is an ecologically relevant tool for detecting pollution in the environment since it reflects the consequence of physiological and biochemical alterations caused by contamination (Peakall, 1996). The presented short time study of behavioral effects caused by TBTO in sticklebacks shows that it is possible to use behavior as a tool to detect pollution. Behavior may be used in monitoring programs in study areas where it is possible to find relevant control sites. In addition behavior prevents unnecessary destruction and suffering of animals.

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Paper II

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Post-exposure effects of DDE and butyl benzyl phthalate on feeding
behavior in threespine stickleback

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Postexposure effects of DDE and butylbenzylphthalate on feeding behavior in threespine stickleback

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Abstract

In a laboratory experiment we documented effects of sublethal concentrations of p,p'-2,2-bis(p-chlorophenyl)-1,1-dichloroethylene (DDE) and butylbenzylphthalate (BBP) on feeding behavior in threespine stickleback *Gasterosteus aculeatus*. The fish were exposed for 31 days to either BBP (10 or 100 μg/L) or DDE (5 or 50 μg/L) or to a mixture of BBP and DDE in the corresponding concentrations. Five weeks after exposure termination, we showed that fish that had been exposed to the higher concentrations of DDE and/or BBP initiated feeding more often than control fish. The latency time to feeding (ranging from 0.25 to 5.0 min) differed between control fish and fish exposed to mixtures of DDE and BBP. This experiment shows that feeding behavior may be used as a suitable behavioral variable in the detection of effects of pollutants even long time after the termination of exposure.
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Keywords: Threespine stickleback; *Gasterosteus aculeatus*; BBP; DDE; Phthalate; Behavior; Feeding

1. Introduction

Feeding behavior, such as food consumption (Ema et al., 1991; Piersma et al., 1995; MacRury and Johnson, 1999), tactics of prey capture and feeding motivation (Little et al., 1990), and handling time and ingestion time of prey (Sandheinrich and Atchison, 1990), have been used to some extent in ecotoxicological studies. Feeding behavior is an ecologically relevant indicator of pollution since food consumption influences survival and reproduction of the animal (Little et al., 1993; Jamet, 1995).

2,2-Bis(p-chlorophenyl)-1,1-dichloroethylene (DDE) is a metabolic product of the insecticide 1,1,1-trichloro-2,2-bis(chlorophenyl)ethane (DDT). It is very persistent against biodegradation, and it is well documented that DDE is bioaccumulated in several species, including fish (MacEachen et al., 2000; Weisbrod et al., 2001). In most industrial countries the use of DDT is restricted, and with some exceptions where water

concentrations of 0.015 μg/L DDE (Albanis et al., 1998) and 0.13 μg/L DDE (Fernández et al., 2000) are measured, DDE is often not detectable in water (Tyler et al., 1998). However, due to its effectiveness and low-cost production, the insecticide is still in use in some developing countries, where water concentrations of DDT may reach 1–10 μg/L (Tyler et al., 1998). Due to its persistence the chemical is still found in Norwegian fish, where DDE levels of 100–1000 ng/g have been documented (Goksøyr et al., 1998). Effects of DDE include reproductive disorders (Guillette et al., 1994; Donohoe and Curtis, 1996, Peakall, 1996; Gray, 1998), and effects on growth, feeding, and schooling behavior in fish (Besch et al., 1977; MacRury and Johnson, 1999).

Butylbenzylphthalate (BBP) is another chemical that is suggested to cause reproductive disorders, although to a lesser extent than DDE (Jobling et al., 1995; Piersma et al., 1995; Gray, 1998). BBP is mainly used as a plasticizer in the production of, e.g., vinyl floors, toys, and synthetic leather, and concentrations of 0.1–1.6 μg/g have been measured in different food items that were wrapped up in packaging materials made of plastic (Page and Lacroix, 1995). In Norway total phthalate concentrations [dimethylphthalate (DMP), di-*n*-ethylphthalate (DEP), di-*n*-butylphthalate (DBP),

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BBP, bis(2-ethylhexyl)phthalate (DEHP), and di-*n*-octylphthalate] of 0.8–200 µg/L were detected in municipal wastewaters 16 months after an accidental spill from a Norwegian PVC factory in 1997 (NGI, 1997). From the same accident phthalate concentrations in the range of 2.4–5800 mg/kg were measured in different lake sediments surrounding the factory (NGI, 1997). European water concentrations of BBP are normally not detectable, but 0.7–2.95 µg/L BBP are examples of recently measured levels (Fromme et al., 2002). Since BBP is produced in large quantities (Tyler et al., 1998), and since the phthalate is not chemically bound to the matrix and thus migrates from the plastic into the environment (Staples et al., 1997), concern regarding the environmental toxicity of BBP is increasing. In most vertebrates BBP is metabolized to monobutylphthalate, monobenzylphthalate, hippuric acid, phthalic acid, benzoic acid, and an ω-oxidized metabolite. Some of these metabolites are suggested to be toxic (Ema et al., 1995; Nativelle et al., 1999; Parkerton and Konkel, 2000).

In nature it is likely that individuals will be exposed to a mixture of chemicals (Tyler et al., 1998), and in municipal wastewater DDT/DDE and phthalate esters often occur simultaneously (Mayer et al., 1972; Soto et al., 1997; Jobling et al., 1998).

The aim of this study was to investigate whether feeding behavior is suitable in detecting effects of sublethal concentrations of DDE and/or BBP. Since effects of chemical mixtures cannot be quantified from the respective individual chemicals (Soto et al., 1997) the effects caused by a mixture of DDE and BBP were also of interest. Postexposure effects of treatment to *p,p'*-DDE and/or BBP on feeding behavior in threespine stickleback were investigated because of the persistent properties of DDE and to some extent BBP. Postexposure effects are, in this experiment, defined as effects 5 weeks after termination of a 1-month exposure period to the mentioned chemicals.

2. Methods

2.1. Fish maintenance

Threespine sticklebacks of approximately equal size ($N = 280$; average weight 1.83 ± 0.21 g) were sampled with Plexiglas fry traps (Dolmen, 1982) in September 1998 in the lake Myrdalsvatnet, Hordaland county, in the southwestern part of Norway (lat. 60°18′ N, long. 5°23′ E). The fish were transported by air cargo to the laboratory at the Norwegian Institute for Water research (NIVA) in Oslo, Norway, where they were placed in 80-L glass aquaria (approximately 50 individuals per aquaria) with continuous water flow (pH 6.8 ± 0.1). To disinfect the fish against ectoparasites and external infections they were treated with formalin (4‰) for 10 min. Prior to and during the exposure and during the behavioral experiment the temperature and photoperiod corresponded to the natural pattern for the season and location. The fish were daily fed with frozen commercial mosquito larvae. During the exposure period the fish were fed ad lib, while the feeding regime was more standardized in the period of the feeding experiment, as explained later. Throughout the experiment the fish did not enter breeding state.

2.2. Exposure

To minimize contaminants from the wild the exposure of the fish started in April 1999, 7 months after the arrival to the laboratory. The sticklebacks were transferred from the 80-L maintenance aquaria to 14 glass exposure aquaria (40 L) with a stationary water system. There were 20 fish per exposure aquarium. The contaminants were administered via the water, as 50% (20 L) of the water was daily exchanged and replaced with water contaminated with DDE and/or BBP.

Six groups of fish ($N = 240$) exposed to *p,p'*-DDE and/or BBP and a seventh control group ($N = 40$) were defined. The exposure continued for 31 consecutive days. Each of the exposure groups consisted of two replicates. The six exposure groups were as follows: $2 \times Low_{DDE}$ (5 µg/L DDE, $N = 40$), $2 \times High_{DDE}$ (50 µg/L DDE, $N = 40$), $2 \times Low_{BBP}$ (10 µg/L BBP, $N = 40$), $2 \times High_{BBP}$ (100 µg/L BBP, $N = 40$), $2 \times Low_{DDE}High_{BBP}$ (5 µg/L DDE + 100 µg/L BBP, $N = 40$), and $2 \times High_{DDE}High_{BBP}$ (50 µg/L DDE + 100 µg/L BBP, $N = 40$).

The exposure solutions were prepared from two stock solutions: one DDE stock solution (5 g/L acetone) and one BBP stock solution (10 g/L acetone). The DDE exposure concentrations of 5 or 50 µg/L were obtained by adding DDE stock solution into the daily exchanging water. Likewise the BBP exposure concentrations of 10 or 100 µg/L were obtained by adding BBP stock solution into the daily exchanging water. The mixture groups were obtained by mixing stock solutions into the water. The control group was given the same treatment as the exposed groups but was exposed only to acetone (2.0 mL). After termination of the 31-day exposure period the fish remained in the exposure aquaria and continuous water flow (0.83 L/h) was restored.

Water plants (*Myriophyllum* sp. and *Hygrohypnum* sp.) were introduced into each aquarium to prevent stress.

2.3. Feeding behavior

The feeding behavior experiment was conducted in June 1999, i.e., 5 weeks after termination of the BBP/DDE exposure. From the six exposure groups a total of

192 [N(exposed) = 152, N(control) = 40] noninjured and nondiseased sticklebacks were selected for the behavioral experiment.

The fish were distributed as follows: $2 \times \text{Low}_{DDE}$ (5 μg/L DDE, $N = 39$), $2 \times \text{High}_{DDE}$ (50 μg/L DDE, $N = 20$), $2 \times \text{Low}_{BBP}$ (10 μg/L BBP, $N = 34$), $2 \times \text{High}_{BBP}$ (100 μg/L BBP, $N = 22$), $2 \times \text{Low}_{DDE}\text{High}_{BBP}$ (5 μg/L DDE + 100 μg/L BBP, $N = 19$), and $2 \times \text{High}_{DDE}\text{High}_{BBP}$ (50 μg/L DDE + 100 μg/L BBP, $N = 18$).

Prior to each trial in the feeding behavior experiment one exposed and one control fish were randomly selected and transferred from their home aquaria to the test glass aquarium ($41 \times 20 \times 29 \text{ cm}^3$) in where the three equally sized vertical compartments were separated with two removable transparent Plexiglas plates. The test fish pair that constituted a trial was placed in each of the outer compartments. The comparison of competing individuals is in accordance with previous studies (e.g., Milinski, 1982; Gill and Hart, 1996).

Dominance hierarchy according to size within the pair was a minor problem since all fish were of approximately the same size (Rowland, 1989; Olivera and Almada, 1996). Even though in situations where the size differed between the two individuals, a new random sampling was done.

The fish were given an acclimation period of 5 min, after which they seemed to be normalized. This has previously been shown to be an appropriate acclimation period (Ranta and Lindström, 1990). A food item (frozen commercially mosquito larvae as a $0.5 \times 0.5 \times 0.5 \text{ cm}^3$ cube) was introduced into the central compartment, halfway between the two fish, and the two removable Plexiglas plates were simultaneously and slowly raised.

The feeding behavior of the two fish was videotaped for 5 min for later analyses. The video camera was installed 1 m in front of the test aquarium, and the operator left the room immediately after starting the video recording. Using the video recordings, the fish that initiated feeding, and the latency time from the start of each trial to when the control and exposed fish respectively started to feed, were quantified. A total of 152 trials were conducted. Between each recording all remaining food was removed.

The exposed fish were used only once, and after each trial they were transferred to a separate aquarium. The control fish were reused after 4 days. All fish were starved for 12 h prior to the experiment in order to standardize conditions.

2.4. Analysis of DDE and BBP

After the feeding behavior experiment 15 controls, 17 Low$_{BBP}$, 15 Low$_{DDE}$, 15 High$_{BBP}$, 15 High$_{DDE}$, 15 Low$_{DDE}$High$_{BBP}$, and 20 High$_{DDE}$High$_{BBP}$ individuals

Table 1
Concentrations of DDE and BBP in the experimental fish

Treatment group	BBP conc. (ng/g) ww	DDE conc. (ng/g) ww	Amount of fish homogenized and analyzed
Control	<100	74	15
Low$_{BBP}$	<100	99	17
Low$_{DDE}$	<100	2000	15
High$_{BBP}$	NM	NM	
High$_{DDE}$	NM	NM	
Low$_{DDE}$High$_{BBP}$	<100	2000	15
High$_{DDE}$High$_{BBP}$	<100	19,000	20

Note: NM = not measured. Prior to analyses minimums of 15 fish were pooled in order to obtain enough material for the procedure. The fish were analyzed immediately after termination of feeding behavioral experiments. Detection limit (BBP) = 100 ng/g; Low$_{BBP}$ = 10 μg/L BBP, Low$_{DDE}$ = 5 μg/L DDE, High$_{BBP}$ = 100 μg/L BBP, High$_{DDE}$ = 50 μg/L DDE, Low$_{DDE}$High$_{BBP}$ = 5 μg/L DDE + 100 μg/L BBP and High$_{DDE}$High$_{BBP}$ = 50 μg/L DDE + 100 μg/L BBP.

(Table 1) were killed and stored ($-20\,^{\circ}\text{C}$) for later analyses. The Norwegian Institute for Water Research in Oslo, Norway, conducted the analyses of concentrations of DDE and BBP in the fish.

To obtain sufficient material for analyses, each sample contained a minimum of 15 fish that were pooled and homogenized (Table 1).

For analyses of DDE the samples were extracted twice using cyclohexane/acetone and an ultrasonication probe. The cyclohexane extract was isolated by adding NaCl solution. The organic extract was evaporated to dryness and the fat content was determined gravimetrically. Approximately 100 mg of the resulting lipid sample was dissolved in dichloromethane, internal standards were added (PCB-53 and PCB-204), and the sample was cleaned using size-exclusion chromatography (HPLC/GCP) and concentrated sulfuric acid. DDE was then analyzed using a gas chromatograph (Hewlett Packard Model 5890 Series II) equipped with a splitless autoinjector, a DB-5 capillary column (60 m, i.d. = 0.25 mm, film thickness = 0.25 μm), and an electron capture detector.

Before BBP analyses the extraction was cleaned on an ALOX column according to EPA Method 606 (EPA, 1984). Diallyl phthalate was added to the extraction as a recovery standard, while phenantrene was added as an internal standard. The extraction was analyzed using a gas chromatograph (GC) (Hewlett Packard) connected to an HP Model 5970 MSD instrument. The GC was equipped with an on-column injector and a capillary column type DB-5 (length = 60 m, i.d. = 0.25 mm, film thickness = 0.25 μm). BBP was identified and quantified according to retention time and mass peak signals.

2.5. Statistics

All data were statistically treated using the software program SPSS (version 9.0). Averages are expressed as medians with interquartile ranges, and differences in median were tested using a Mann–Whitney U-test. Frequency deviations from 50% were tested with a binomial test. Significance were defined when $P < 0.05$.

In 15 trials neither the control nor the exposed fish within a pair consumed the food item. These trials distributions were $High_{BBP} = 3$, $High_{DDE} = 2$, $Low_{DDE} = 6$, and $Low_{DDE}High_{BBP} = 4$ and were excluded from the statistical analyses. In trials where one of the pair did not eat, the latency time for that individual was defined as 5 min. The National Animal Research Authority approved the experiment.

3. Results

Some mortality among the exposed fish (Low_{DDE} [$N = 1$ (2.5%)], $High_{DDE}$ [$N = 20$ (50%)], Low_{BBP} [$N = 6$ (15%)], $High_{BBP}$ [$N = 18$ (45%)], $Low_{DDE}High_{BBP}$ [$N = 21$ (52.5%)], $High_{DDE}High_{BBP}$ [$N = 22$ (55%)]) was recorded in the beginning of the exposure period.

The analyzed concentrations of BBP and DDE in fish tissue are shown in Table 1. The concentrations of BBP were below the detection limit (i.e., < 100 ng/g ww) in all groups. The $High_{BBP}$ and $High_{DDE}$ fish were not analyzed because this material was lost due to a fatal error at the laboratory (NIVA). However, the high concentrations of DDE and BBP are both indirectly analyzed in the groups that were exposed to a mixture of DDE and BBP ($Low_{DDE}High_{BBP}$ and $High_{DDE}High_{BBP}$).

3.1. Initiating feeding behavior

Fish exposed to high concentrations of BBP ($High_{BBP}$) and DDE ($High_{DDE}$) initiated feeding significantly more often than the control fish (binomial test, $High_{BBP}$: $P = 0.004$; $High_{DDE}$: $P = 0.03$). Furthermore, fish exposed to a mixture of high concentration of BBP and a high concentration of DDE ($High_{DDE}High_{BBP}$) initiated feeding in all trials (binomial test, $P = 0.00$).

In the experiment where fish were exposed to low concentrations of BBP (Low_{BBP}) and DDE (Low_{DDE}), there were no differences between the controls and exposed fish with respect to feeding initiation (binomial test, Low_{BBP}: $P = 0.9$; Low_{DDE}: $P = 0.7$). Finally, control fish initiated feeding significantly more often than the fish exposed to a mixture of a low concentration of DDE and a high concentration of BBP ($Low_{DDE}High_{BBP}$) (binomial test, $P = 0.007$) (Fig. 1).
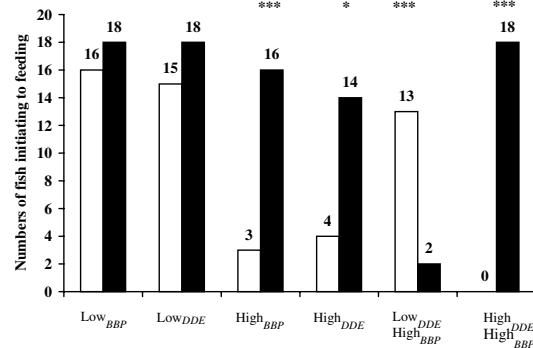

Fig. 1. The amount of control (white) and exposed (black) fish that initiated to feed. In each trial one exposed and one control fish competed for one item of frozen mosquito larvae. There were differences between control and exposed fish in feeding initiation for the $High_{BBP}$ (binomial test: $P = 0.004$), $High_{DDE}$ (binomial test: $P = 0.03$), $Low_{DDE}High_{BBP}$ (binomial test: $P = 0.007$), and $High_{DDE}High_{BBP}$. The labels of the treatment groups are as follows: $Low_{BBP} = 10$ μg/L BBP, $Low_{DDE} = 5$ μg/L DDE, $High_{BBP} = 100$ μg/L BBP, $High_{DDE} = 50$ μg/L DDE, $Low_{DDE}High_{BBP} = 5$ μg/L DDE + 100 μg/L BBP and $High_{DDE}High_{BBP} = 50$ μg/L DDE + 100 μg/L BBP.

3.2. Latency time to feeding

Fish exposed to $Low_{DDE}High_{BBP}$ started to feed significantly later than the controls (two-tailed Mann–Whitney U-test, $U = 46$, $N = 15$, $P = 0.004$), while the fish exposed to $High_{DDE}High_{BBP}$ started to feed significantly sooner than control fish (two-tailed Mann–Whitney U-test, $U = 1.0$, $N = 18$, $P < 0.001$). In the experiment where the fish were exposed to $High_{BBP}$, $High_{DDE}$, Low_{BBP}, or Low_{DDE}, there were no significant differences between exposed and control fish with respect to when they started to feed (two-tailed Mann–Whitney U-test, $High_{BBP}$: $U = 131$, $N = 19$, $P = 0.15$; $High_{DDE}$: $U = 105$, $N = 18$, $P = 0.17$; Low_{BBP}: $U = 518$, $N = 34$, $P = 0.73$; Low_{DDE}: $U = 508$, $N = 33$, $P = 0.64$) (Table 2).

4. Discussion

In this laboratory study we investigated the effects on feeding behavior 5 weeks after termination of exposure to DDE or BBP or to a mixture of DDE and BBP. One aim was to investigate the long-lasting effects of these contaminants after eliminating the exposure source.

The individual motivation and competition success for food often depend on factors such as hunger (Gill and Hart, 1994), fish size (Gill and Hart, 1994), prey size (Gill and Hart, 1996), degree of parasite infection (Milinski, 1986), swimming speed ability (Milinski, 1982; Gill and Hart, 1996), and fish jaw morphology

Table 2
Latency time (minutes) (median with interquartile ranges) to feeding start for control and exposed individuals

	Low_{BBP}	Low_{DDE}	$High_{BBP}$	$High_{DDE}$	$Low_{DDE}High_{BBP}$	$High_{DDE}High_{BBP}$
Control	0.42 (0.28–2.55)	0.53 (0.26–2.11)	1.22 (0.41–4.37)	1.59 (1.00–4.11)	0.56 (0.29–2.31)	5 (5.00–5.00)
Exposed	0.38 (0.30–2.02)	0.46 (0.25–1.51)	0.41 (0.25–2.16)	1.09 (0.32–1.53)	3.47 (1.32–5.00)	0.27 (0.13–0.59)
Statistics	$P = 0.73$ $N = 34$	$P = 0.64$ $N = 33$	$P = 0.15$ $N = 19$	$P = 0.17$ $N = 18$	$P = 0.004$ $N = 15$	$P < 0.001$ $N = 18$

Note: In each trial one exposed and one control fish competed for one item of frozen mosquito larvae. The differences in median are tested with Mann–Whitney U-test. Significance is defined as $P < 0.05$. $Low_{BBP} = 10 \mu g/L$ BBP, $Low_{DDE} = 5 \mu g/L$ DDE, $High_{BBP} = 100 \mu g/L$ BBP, $High_{DDE} = 50 \mu g/L$ DDE, $Low_{DDE}High_{BBP} = 5 \mu g/L$ DDE + 100 $\mu g/L$ BBP and $High_{DDE}High_{BBP} = 50 \mu g/L$ DDE + 100 $\mu g/L$ BBP.

(Ibrahim and Huntingford, 1988; Gill and Hart, 1994). The individuals used in this experiment contained no parasites, and the state of hunger, fish and prey size, and fish morphology were standardized as far as possible. Additionally, throughout the experiment the fish did not enter the breeding state, thus minimizing behavioral sex differences.

In the beginning of the 31-day exposure period the mortality among some of the exposed fish was high. The mortality ceased after a few days. The cause of this mortality was, however, unknown. Even if the fish used in the behavioral experiment were not diseased and their behavior appeared to be normal, it cannot be ignored that the observed behavioral effects were results of permanent physiological or morphological alterations in the surviving fish.

The results showed that feeding behavior in threespine sticklebacks was affected after exposure to DDE and BBP. Fish exposed to high concentrations of the test chemicals ($High_{BBP}$, $High_{DDE}$, and $High_{DDE}High_{BBP}$) showed increased feeding motivation compared to control fish in that they caught the food item first. The opposite result was observed in the fish that were exposed to a mixture of a low concentration of DDE and a high concentration of BBP ($Low_{DDE}High_{BBP}$). There were no differences in feeding motivation between controls and fish exposed to the lower concentrations of BBP (Low_{BBP}) or DDE (Low_{DDE}).

Increased feeding motivation as a result of xenobiotics is in accordance with other studies. Examples include increased feeding in tropical fish after exposure to toxicants (Warren, 1971) and increased feeding motivation in largemouth bass Micropterus salkoides exposed to organochlorines (MacRury and Johnson, 1999). Also in rats increased feeding motivation was observed after exposure to BBP (Piersma et al., 1995).

Decreased feeding, as a consequence of pollution is also recorded (Sandheinrich and Atchinson, 1990). Other examples include decreased feeding in bluegill sunfish (Lepomis macrochirus) exposed to cadmium (Bryan et al., 1995) and in largemouth bass exposed to pentachlorophenol (Mathers et al., 1985).

We suggest that the increased feeding motivation among some of the exposed fish may be because these were hungrier than the control fish. Since the time lag between exposure termination and start of feeding behavioral experiment was 5 weeks, the hunger may be a result of long-lasting biochemical and physiological compensatory mechanisms to prevent toxic effects caused by sublethal concentrations of the toxicants (Selye, 1956; Beyers et al., 1999). This hypothesis is suggested even though it is emphasized that no attempts were done to quantify body reserves or bioenergetics. The individual consequence of spending more time on feeding will be less time to other essential activities, such as reproduction and predator avoidance (Huntingford et al., 1994). Also previous studies have shown that hungry sticklebacks are willing to feed closer to a predator, and thus take larger risks to obtain food, than satiated fish (Milinski, 1993). Increased feeding may then result in reduced survival rates.

The fish exposed to a mixture of a low concentration of DDE and a high concentration of BBP ($Low_{DDE}High_{BBP}$) were less motivated for feeding than the control individuals. This result is unexpected when comparing with the rest of the results, also because we did not subjectively observe any differences in condition between the $Low_{DDE}High_{BBP}$ fish and the fish with increased feeding motivation.

The $High_{BBP}$ and $High_{DDE}$ exposed fish initiated feeding, but the corresponding control fish started to feed immediately after (Table 2). This may indicate that the control fish also were motivated to feed, but not to the same degree as the exposed fish. The significantly shorter latency time to feeding for fish exposed to $High_{DDE}High_{BBP}$ compared to the controls may indicate that the hunger among these exposed individuals increased their motivation to feed compared to the controls. On the other hand, the significantly longer latency time to feeding for fish exposed to $Low_{DDE}High_{BBP}$ compared to their controls was unexpected when looking at the rest of the results. This group of fish behaved unexpected and there is no obvious interpretation of this finding.

1   In the present study we documented behavioral effects
of BBP even though the BBP concentrations in the fish
3 samples were under the analytical detection limit
(100 ng/g) (Table 1). Since the most likely deposit
5 organs are the liver and the kidneys, which have been
shown to increase in weight in rats after exposure to
7 BBP (Piersma et al., 2000), it is possible that the dilution
of the small organs when the whole fish was analyzed
9 caused the nondetectable concentration. It is also
possible that BBP caused long-lasting morphological
11 alterations in the fish that may have caused the observed
results, but no histological studies have been conducted
13 to confirm this hypothesis. Alternatively BBP may have
formed metabolites (Nativelle, 1999) that are suggested
15 to be toxic (Ema et al., 1995; Parkerton and Konkel,
2000). Last, it is possible that the detection limit in the
17 analytical procedure was too high. Further investiga-
tions are required to establish the cause of significant
19 behavioral effects in the absence of detectable BBP
concentrations in the fish tissue. The chemical analyses
21 further indicated that the tissue concentrations of DDE
increased as a function of exposure level and that DDE
23 bioaccumulated in the fish (MacEachen et al., 2000;
Weisbrod et al., 2001). DDE was also detected in the
25 control individuals. The reason for this is, however,
unknown.

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29 **5. Conclusion**

31   The present study suggests that feeding behavior is a
suitable variable in the detection of effects of DDE and/
33 or BBP even 5 weeks after exposure termination. The
persistent properties and significant biological effects of
35 DDE are well known but regarding BBP, many
scientists claim that the toxicity is of little importance
37 (Gledhill et al., 1980; Rhodes et al., 1995; Carr et al.
1997). The present study, supported by others (e.g.,
39 Jobling et al., 1995; Piersma et al, 1995), shows that BBP
can cause considerable effects in animals. By studying
41 effects of DDE and BBP on feeding behavior, an
ecologically relevant trait, it is possible to detect
43 consequences of these chemicals even after the source
of exposure is eliminated.

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47 **6. Uncited references**

49   Brevik et al. (1991)

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Paper III

Butyl benzyl phthalate affects shoaling behavior and bottom-dwelling

behavior in threespine stickleback

By

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Butyl Benzyl Phthalate Affects Shoaling Behavior and Bottom-Dwelling Behavior in Threespine Stickleback

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In this laboratory experiment, the effects on fish behavior caused by butyl benzyl phthalate (BBP) were of interest. We showed that shoaling behavior and bottom-dwelling behavior in threespine stickleback, *Gasterosteus aculeatus*, were altered as a result of exposure to 0.1 mg/L BBP. Threespine sticklebacks, collected from a freshwater population in central Norway, were exposed to BBP for 26 days. BBP was administered daily through the water. We found that exposed fish aggregated more into one single shoal than control fish. Further, the exposed fish spent more time at the bottom of the test aquarium than the control fish. From these results we conclude that the behavior traits aggregation and bottom-dwelling activity may be suitable and sensitive in detecting effects of BBP in threespine stickleback. © 2002 Elsevier Science (USA)

Key Words: Gasterosteus aculeatus; shoaling behavior; bottom-dwelling behavior; BBP; biomarker.

INTRODUCTION

Shoaling behavior and bottom-dwelling behavior of fish have to some extent been used as indicators of pollution (Weis and Weis, 1974; Besch *et al.*, 1977; Vogl *et al.*, 1999; OConnor *et al.*, 2000; Wibe *et al.*, 2001). Shoaling is defined as fish staying in a group for social reasons, where structure of the group is less important. Schooling behavior additionally refers to the structure of the group, where fish swim in a polarized manner (Pitcher and Parrish, 1993). One function of shoaling and schooling is to avoid attack from predators, since a predator will find it difficult to select one individual out of many (Pitcher and Parrish, 1993).

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To stay at the bottom may be a result of motionless resting (e.g., OConnor *et al.*, 2000) or it may be an indication of stress (Israeli-Weinstein and Kimmel, 1998). Even though bottom-dwelling has been investigated in several studies (e.g. Vogl *et al.*, 1999; Grillitsch *et al.*, 1999; OConnor *et al.*, 2000; Wibe *et al.*, 2001) the discussion of whether the behavior is a consequence of stress or resting may be infrequent.

Threespine sticklebacks, *Gasterosteus aculeatus*, often form shoals that in the breeding season consist of females alone or a mixture of females and nonreproductive males (Whoriskey and FitzGerald, 1994). Throughout the rest of the year shoals may consist of both males and females (Wootton, 1984). It has been shown that shoals often consist of individuals of approximately the same size. This increases the homogeneity of the shoals, which is considered an efficient antipredator adaptation (Ranta *et al.*, 1992; Peuhkuri *et al.*, 1997). Shoaling may also increase foraging success since each individual can spend more time foraging instead of looking for predators, and the probability of finding food will increase when more individuals are searching (Pitcher and Parrish, 1993; Peuhkuri *et al.*, 1997).

Butyl benzyl phthalate (BBP) is a phthalate ester that is mainly used to increase the flexibility and workability of plastic products such as toys, packaging materials, and vinyl floors. Phthalates are produced in large quantities. Additionally, since the compounds are not chemically bound to the polymer matrix and thus may migrate from the plastic and into the environment (e.g. Adams *et al.*, 1995), there is an increasing interest in the environmental fate and toxicology of phthalates. Significant concentrations of BBP and other phthalates are found in food samples and in food packaging materials (Page and Lacroix, 1995). BBP concentrations of 0.05–0.8 µg/g have been found in food items (Page and Lacroix, 1995), and in Norway, concentrations of 1.8 µg/L

have been detected in municipal waste waters (SFT, 1998). As a comparison, the acute toxicity of BBP in fish varies between 731 and 6470 ppb (Mayer et al., 1972; Adams et al., 1995).

BBP may be metabolized to mono butyl phthalate (MBuP), mono benzyl phthalate (MBeP), hippuric acid, phthalic acid, benzoic acid, and an ω-oxidized metabolite. Some of these metabolites are suggested to be toxic (Ema et al., 1995; Nativelle et al., 1999; Parkerton and Konkel, 2000). Observed effects of BBP include reproductive disorders in fish and rats, suggested as a result of possible estrogen-mimicking properties of the phthalate (Jobling et al., 1995; Sharpe et al., 1995). Furthermore, altered feeding behavior in rats (Ema et al., 1991) and threespine stickleback (own unpublished data) and developmental and terratogenic effects in rats (Ema et al., 1995) are reported.

The aim of this study was to examine the influence of BBP on shoaling and bottom-dwelling behavior in threespine sticklebacks. In two different experimental setups, the differences between BBP-exposed fish and control fish in their ability to form shoals and to show preference for individuals of the same size were investigated. Also the difference in bottom-dwelling behavior in control and BBP-exposed fish was examined.

METHODS

Fish Maintenance

Threespine sticklebacks of two distinct size classes, small ($N = 211$, average size = 4.54 $\pm$ 0.34 cm) and large ($N = 160$; average size = 5.63 $\pm$ 0.48), were captured with Plexiglas fry traps (Dolmen, 1982) in the small freshwater lake Kindsethtjønna, Sør-Trøndelag county, central Norway (63.5°25' N, 10°45' E), in October. To minimize external infections, the fish were disinfected with NaCl (100 g NaCl in 10 L of water) for 15 min before transportation in plastic containers to Brattøra Research Centre, Norwegian University of Science and Technology, Trondheim, Norway.

In the laboratory the fish were divided into eight groups: two control groups with small fish ($N = 53 + N = 53$), two control groups with large fish ($N = 38 + N = 39$), two BBP-exposed groups with small fish ($N = 52 + N = 53$), and two BBP-exposed groups with large fish ($N = 42 + N = 41$). The respective groups were distributed between eight aquaria (85 L). The aquaria were equipped with gravel, water plants, Elodea sp., and a stationary water system with air supply. Daily, 25% of the water in the aquaria was exchanged with de-

chlorinated tap water contaminated with BBP and acetone (exposure groups) or acetone only (control groups).

The fish were fed daily ad lib with frozen mosquito larvae. In the laboratory the temperature was kept at 5–7°C and the photoperiod was 9L:15Dh, which are equivalent to the natural conditions for the actual season and location.

Exposure

The exposure to BBP started 1 week after the fish had arrived at the laboratory. A BBP stock solution was prepared using acetone as solvent (10 g BBP/L acetone). A sample of the stock solution (0.85 mL) was daily added to the aquaria water. This gave an exposure concentration of approximately 0.1 mg/L as the total volume in each aquarium was 85 L. The exposure continued for 26 consecutive days in October and November. The control fish were given the same treatment as the exposed fish, but only an equivalent amount of acetone without BBP was added to the daily exchanging water.

Shoal Choice Experiment

Ten minutes prior to each trial in the shoal choice experiment, test fish were transferred from their home aquaria (85 L) to a test aquarium (38 L) (Fig. 1a). The test aquarium was equipped with a stationary water system and a thin layer of gravel to prevent stress among the fish. The test aquarium was divided into three compartments separated with two fixed glass walls. The transfer of the shoals from the 85-L aquarium to the smaller 38-L test aquarium did not result in detectable stress to either small or large fish. After a few minutes in the test aquarium the fish swam in the same manner as in the larger home aquaria.

In the shoal choice experiment, 30 trials with control individuals and 32 trials with BBP-exposed individuals were conducted. Each trial consisted of a test shoal of 15 large fish in compartment "A," a test shoal of 15 small fish in compartment "B," and one large focal fish, defined as the choosing fish in compartment "C" (Fig. 1a). It has previously been shown that large fish are more selective than small fish with respect to association with fish of corresponding size (Ranta et al., 1992). Therefore, to be able to detect a change in choice between control and exposed fish, the focal fish were of size similar to that of the fish in the large fish group. The size of large and small shoal fish in each trial did not overlap. The location of the small and large test shoals were
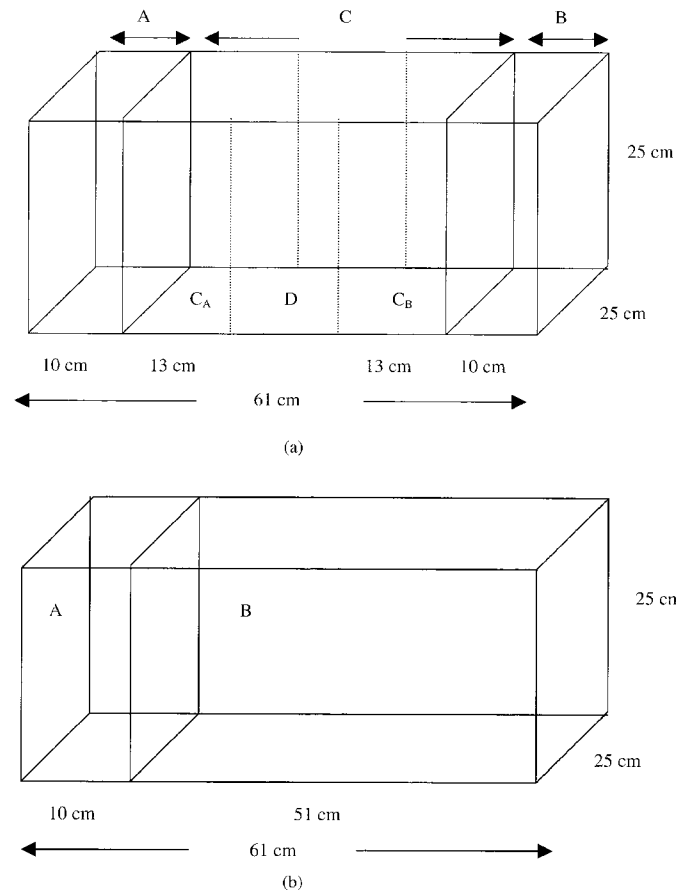
FIG 1. Experimental design. (a) Shoal choice experiment: A test shoal of 15 large and 15 small fish were placed in compartments "A" and "B," respectively. A focal fish was introduced into compartment "C" where it was allowed to choose between compartment "A" (the focal fish stayed in C_A) and "B" (the focal fish stayed in C_B). The solid lines represent permanent glass walls, while the dotted lines represent imaginable choice zones. A choice is defined as when the head of the focal fish crosses the dotted line. Compartment "D" is the neutral zone where no choice was recorded. (b) Size-assortative shoaling experiment: The aquarium was divided into two compartments, "A" and "B". A shoal containing 5 small and 5 large fish was placed into compartment "A." After the glass wall (solid line) was raised and the fish were allowed to swim in "A" and "B," the shoaling behavior was recorded.

randomized between the left and the right side of the test aquarium during the experiment.

After the acclimation period of 10 min (Ranta and Lindström, 1990) when the fish seemed to behave in their original manner, the time that the focal fish spent in front of either shoal, i.e., large fish (C_A) or small fish (C_B), was recorded. Each trial was video recorded for 10 min for further analyses. The video camera was placed 1 m in front of the test aquarium, and the operator left the room immediately after start of video recording. All fish were allowed to see each other during the entire trial, including the ac-

climation period of 10 min before the video recording started.

A choice was defined as when the head of the focal fish crossed the choice zone (dotted lines in Fig. 1a). The neutral zone ("D") was defined as no choice. A choice further required that the focal fish stayed in open water and not at the bottom. Time spent at the bottom was also recorded. Each focal fish was used only once, and one third of the fish in each test shoal were exchanged between each trial. To avoid reuse, the fish were transferred to a separate aquarium after each trial.

Size-Assortative Shoaling Experiment

The aim of the size-assortative shoaling experiment was to investigate whether there was a difference between BBP-exposed fish and control fish in their ability to segregate into size-assortative shoals. Ten minutes prior to each trial, five small and five large sticklebacks were transferred from their home aquaria to compartment "A" in the test aquarium (38 L) (Fig. 1b). The test aquarium consisted of two compartments ("A" and "B") separated by a removable glass wall. The test aquarium was further equipped with a stationary water system and a thin layer of gravel. In the size-assortative shoaling experiment, 30 trials with control individuals and 30 trials with BBP-exposed individuals were conducted. The size of the small and large fish in each trial did not overlap.

After the acclimation period of 10 min (Ranta and Lindström, 1990) the glass wall was carefully raised, and the distribution of the fish was recorded once every minute for 10 min, i.e., whether all 10 fish aggregated in one shoal or whether they were separated into two or more shoals. Two shoals were defined when two to eight individuals separated from the rest of the shoal. Individuals who were located less than one body length apart from each other belonged to the same shoal. Size-assortative shoals were formed when the size-mixed shoal divided into shoals consisting of individuals of the same size.

With the exception of 60% of the fish, each individual was only used once. The fish used twice were given a restoration time of 3 days between the first and the second time. It was considered important to avoid having the retested fish be put in a shoal with fish that they had already met. After each trial the tested fish were put in separate aquaria, to keep the tested and the not tested fish apart. The behavior was video recorded for later analyses using the same method as described for the shoal choice experiment.

After termination of the size-assortative shoaling experiment, the fish were killed. A random sample ($N_{small} = 40$, $N_{large} = 40$) was sex-determined by autopsy.

Chemical Analyses

After termination of the experiments most of the fish were stored ($-20°C$) before they were transported frozen with air cargo to the Norwegian Institute for Water Research in Oslo, Norway, for analyses of fish tissue BBP concentrations.

To obtain sufficient material for analyses several fish were pooled (adding up to 10 g) and homogen-

ized, before fat and fat-soluble pollutants were extracted with dichlormethane in an ultrasonic bath. The extraction was cleaned on an ALOX column according to EPA method 606 (EPA, 1984). Di-allyl phthalate was added to the extraction as a recovery standard, while phenantrene was added as an internal standard. The extraction was analyzed using a gas chromatography (GC), Hewlett Packard (HP) Model 5890 Series II, connected to a HP 5970 MSD instrument. The GC was equipped with an on-column injector and a capillary column type DB-5 (length 60 m, i.d. 0.25 mm, film thickness 0.25 μm). BBP was identified and quantified according to retention time and mass peak signals.

Statistics

For statistical analyses two-tailed tests were used. Means are presented as medians and interquartile ranges. Data were analyzed with the software SPSS (version 9.0), unless stated differently.

Whether a fish shoal segregated or not may be considered a binomial process. Thus, in order to test whether exposed fish differed from control fish in their probability of segregating from one to two shoals with time (1–10 min), a multiple logistic regression analysis was applied by using the GENMOD procedure (using a logit link function, SAS Institute Inc., 1996). The procedure fitted a generalized linear model to the data by the use of maximum likelihood techniques (e.g., McCullagh and Nelder, 1989). Accordingly, as explanatory variables, treatment (i.e., control or exposed) was included as a factor, whereas time (1–10 min) was included as a continuous covariate. To account for a correlation in time from 1 min to the next, another covariate, P_{t-1} was included in the model. This variable was calculated both for the control group and for the exposed group, as $P_{t-1} = A/N$, where A was the number of events (i.e., shoal segregation) in the previous time step, and, correspondingly, N was the number of trials ($N = 30$) in the previous time step.

RESULTS

Autopsy of a randomly selected sample of small and large fish showed that there was no bias in sex ratio between the two size groups that were used. Among the large fish ($N = 40$) there were 19 females and 21 males. Among the small fish ($N = 40$) the number of females and males were also 19 and 21, respectively.

TABLE 1

Average (Median with Interquartile Ranges) Amount of Time That the Control and Exposed Focal Fish Spent in Front of Shoals Containing Small or Large Fish, in the Neutral Zone, or at the Bottom

	Control[a]	Exposed[a]	Statistics
Time (min) in front of large fish (C_A)	2.29^{bcd} (0.46–7.61)	3.27^{ad} (0.00–5.40)	$U = 414$, $df = 61$, $P = 0.35$
Time (min) in front of small fish (C_B)	4.42^{cd} (1.19–7.45)	1.74^{cd} (0.00–4.96)	$U = 353$, $df = 61$, $P = 0.07$
Time (min) in neutral zone (D)	1.84^{b} (0.69–4.07)	0.48^{b} (0.29–0.99)	$U = 279$, $df = 61$, $P = 0.005$
Time (min) at the bottom	0^{a}(0.00–0.00)	1.03^{ac} (0.00–7.04)	$U = 243$, $df = 61$, $P < 0.001$

Note. Intergroup comparisons are tested with Mann–Whitney U test; intragroup multiple comparisons are tested with Kruskal–Wallis (Dunn).

[a]Comparable medians with different superscript letters are significantly different ($P < 0.05$).

Shoal Choice Experiment

Exposed focal fish spent more of the observed time at the bottom of the test aquarium than the control focal fish (Mann–Whitney U test: $U = 243$, $N = 62$, $P < 0.001$). Exposed focal fish also spent less of the observed time in the neutral zone (D) than control focal fish (Mann–Whitney U test: $U = 279$, $N = 62$, $P = 0.005$) (Table 1).

There were no differences between control and exposed focal fish in the time spent in front of either the shoal of large fish (C_A) or the shoal of small fish (C_B) (Fig. 1a) (time spent in $C_A + C_B/C_A + C_B + D$) (Mann–Whitney U test: $U = 392$, $N = 62$, $P = 0.21$) (Table 2).

There were no difference in time spent in C_A or C_B (Table 1) and time spent at random locations for either control focal fish or exposed focal fish (proportion of time expected by chance $= C_A + C_B/C_A + C_B + D = 0.73$ (Fig. 1a)) (exposed [one sample t test: $t = 0.317$, $N = 30$, $P = 0.8$], control [one sample t test: $t = 0.53$, $N = 30$, $P = 0.6$]) (Table 2).

The intragroup multiple comparison showed that exposed focal fish spent less of the observed time in the neutral zone than in C_A, C_B, or at the bottom (Kruskal–Wallis (Dunn): $\chi^2 = 18.5$, $N = 87$, $P = 0.0003$). The control focal fish spent less time in the neutral zone than in front of small fish ($P = 0.04$). There was no difference in their time spent in the neutral zone compared with their time

spent in front of large fish ($P = 1.00$). Furthermore, the control fish spent less of the observed time at the bottom compared to any other place (Kruskal–Wallis (Dunn): $\chi^2 = 32.91$, $N = 92$, $P < 0.01$) (Table 1).

Size-Assortative Shoaling Experiment

Exposed fish stayed in one shoal for a longer period of time than the control fish (Fig. 2) (Mann–Whitney U test: $U = 206$, $N = 60$, $P < 0.001$). Exposed fish also differed from control fish in their probability of segregating from one mixed shoal into two shoals with time (1–10 min) (Fig. 3) (GENMOD logistic regression, treatment: $N = 20$, $\chi^2 = 40.38$, $P < 0.001$; time: $N = 20$, $\chi^2 = 6.72$, $P = 0.01$).

Chemical Analyses

The analyses of BBP in fish homogenate showed that the concentrations in all exposed groups were under the detection limit (100 ng/g w.w.) for the instrument.

DISCUSSION

This study showed that shoaling behavior and bottom-dwelling behavior in threespine stickleback

TABLE 2

Differences between Controls and BBP-Exposed Fish in Average Observed Time in Front of Either Shoal with Small or Shoal with Large Fish

Proportion of observed time in C_A, C_B, or D	Control	Exposed	Statistics
$C_A + C_B/C_A + C_B + D$	0.82 (0.59–0.93)	0.92 (0.71–0.96)	$U = 392$, $df = 61$, $P = 0.21$
$(C_A + C_B/C_A + C_B + D)$	— see below		

	Expected proportion of time $(0.73)^{a}$
$(C_A + C_B/C_A + C_B + D)$	Control: One-sample t test: $t = 0.32$, $df = 29$, $P = 0.8$ Exposed: One-sample t test: $t = 0.53$, $df = 31$, $P = 0.6$

Note. There were no difference in time spent in C_A (time spent in front of large fish) or C_B (time spent in front of small fish) and time spent at random locations for either control focal fish or exposed focal fish. Expected proportion of time $(0.73) = (C_A + C_B/C_A + C_B + D)$ (D = neutral zone). Results are tested with Mann–Whitney U test (mean expressed as median with interquartile ranges) and one-sample t test. C_A, time in front of large fish; C_B, time in front of small fish; D, time in the neutral zone.

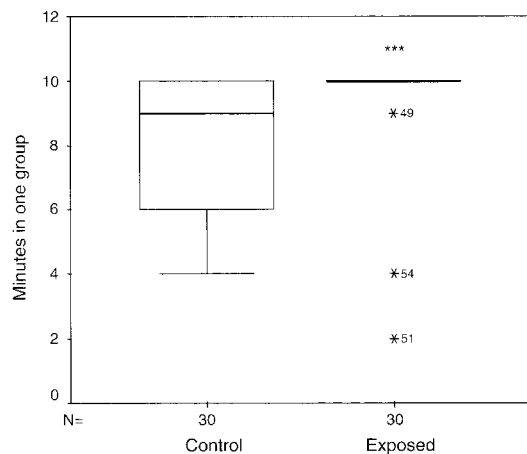[a]Expected proportion of time $(0.73) = (C_A + C_B/C_A + C_B + D)$ cm (Fig. 1a)

FIG 2. Shoaling behavior. The difference between control and exposed fish in average amount of minutes that they stayed in one group during one trial (10 min) (median with interquatile ranges) (Mann–Whitney U test: $U = 206$, $N = 60$, $P < 0.001$). Two shoals were formed when two to eight individuals separated from the focal shoal.

was affected by exposure to a sublethal concentration of BBP. Fish exposed to BBP aggregated significantly more than control fish. One explanation for this behavior is that BBP may act as a stressor that can cause aggregation. Clustering is a common response in sticklebacks that are exposed to stress (Wootton, 1984) and is probably an adaptive response to fright or general stress behavior (Ranta et al., 1992; Peuhkuri et al., 1997). Stress responses caused by pollution can result from physiological compensatory mechanisms that compensate for toxic effects of the pollutant (Beyers et al., 1999).

Aggregation has also been shown in guppies, Poecilia reticulata, which were exposed to octylphenol and 17β-estradiol for 4 weeks (Bayley et al., 1999). In contrast, dispersion behavior was observed in goldfish, Carassius auratus, and carp, Cyprinus carpio, exposed to dichlorodiphenyltrichloroethane (Weis and Weis, 1974; Besch et al., 1977) and carp exposed to mercury chloride, gasoline, and phenol (Besch et al., 1977). The differences in aggregation behavior may be due to chemical-specific effects, methodological differences between the studies, or biological differences between threespine stickleback, goldfish, and carp. The present experiment, which showed a difference between exposed and unexposed fish, indicates that aggregation behavior may be an appropriate indicator of pollution. But further standardized experiments are required to be able to compare results from different studies.

In this study, we also documented a difference between fish exposed to BBP and control fish in the probability of segregating from one to two shoals with time (1–10 min) (Fig. 3). At the start of each trial, it is plausible that both exposed and control fish aggregated as a stress response to the removal of the glass wall. After a short period without disturbances, the stress response among the control fish seemed to decrease and they started to form smaller shoals, while the exposed fish remained stressed and clustered for as long as we observed them (10 min).

We found that BBP-exposed sticklebacks spent significantly more of the observed time at the bottom of the test aquarium than control fish. The observations of the fish indicate that this may be a response to stress, as the exposed fish seemed to be more stressed than the control fish. Immediately after the start of the trial many of the exposed fish rushed to the bottom and stayed there throughout the observation period. The suggested explanation for this behavior is put forward even though no attempts were done to clarify whether the observed effects were a result of the direct toxic effect of BBP or the indirect sensitivity to stress induced by BBP. It is unlikely that the recorded bottom-dwelling behavior is a response of motionless resting as a result of high exposure, since no BBP was detected in the fish tissue when analyzed. Also, bottom-dwelling behavior as a likely result of motionless resting has previously been observed by the corresponding author (Wibe et al., 2001), as these fish behaved more motionlessly and weakly compared to those in the present study. Bottom-dwelling behavior has previously been used as an indicator of pollution (e.g.,
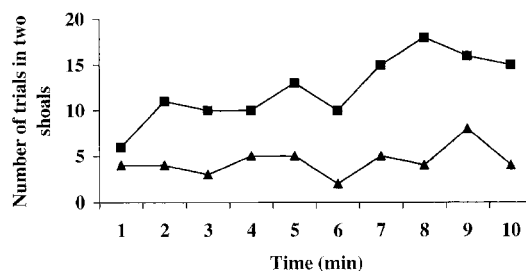

FIG 3. The segregation of exposed (▲) and control (■) fish with time (10 min). The Y axis shows the number of trials (N (control) = 30; N (exposed) = 30) where the fish were separated into two groups. Shoaling behavior was recorded every minute for 10 min. The exposed fish differed from control fish in their probability of dividing into two groups with time (GENMOD logistic regression: treatment, $P < 0.001$; time, $P = 0.01$).

Israeli-Weinstein and Kimmel, 1998; Vogl et al., 1999; OConnor et al., 2000).

The BBP-exposed focal fish spent significantly less time in the neutral zone than the control focal fish. They also spent less of the observed time in the neutral zone compared to any other of the measured locations (Table 1). An explanation for the short time spent in the neutral zone could be that the exposed fish preferred to stay with a shoal compared to staying alone. This explanation is also in accordance with the stress explanation and may be adaptive since sticklebacks under stress usually aggregate to a higher extent than individuals that are not stressed (Wootton, 1984). The intracomparison results also show that neither control nor exposed focal fish spent more time in front of either shoal. However, the control fish spent less time in the neutral zone than in front of small fish. This indicates that the results cannot be explained by behavioral differences between the shoal fish.

Even though the control fish segregated more often than the exposed fish, size-assortative shoaling behavior was not observed. This is in contrast to a previous study, where size-assortative shoaling in sticklebacks was observed within 6 min after a size-mixed shoal was released in an aquarium (Ranta and Lindström, 1990). Methodological differences may explain the different results as Ranta and Lindström (1990) used a larger test aquarium than that used in the present study. But it is also possible that size assortative shoaling is not an adaptive response in the population used in the present study since population differences in adaptive behavior may exist (Huntingford et al., 1994).

In this study, we observed significant behavioral effects of BBP even though the chemical analyses showed that BBP concentrations in the fish tissues were below the detection limit (100 ng/g) of the instrument. BBP is metabolized to MBuP, MBeP, hippuric acid, phthalic acid, benzoic acid, and an ω-oxidized metabolite of MBuP (Nativelle et al., 1999; Ema et al., 1995). MBuP has been suggested to be toxic, due to observed developmental abnormalities in rats (Ema et al., 1995). It has also been suggested that phthalate metabolites cause toxicity in fish (Parkerton and Konkel, 2000). Thus, it is possible that BBP metabolites, rather than BBP itself, cause the observed alterations in behavior in this study. Alternatively it is possible that BBP may produce permanent or prolonged biochemical or morphological effects; thus it would be interesting to clarify whether this may result in permanent or prolonged behavioral effects. Further studies are therefore required to establish the relation between

the specific BBP metabolites in fish tissue and their biological effects and whether BBP can cause permanent effects in these animals.

With respect to the use of shoaling behavior and bottom-dwelling behavior as indicators of pollution, bottom-dwelling behavior is easy to quantify and possible to standardize (Little and Finger, 1990). Since shoaling behavior may influence predator-prey interactions and thus survival, it is a variable of high ecological relevance (Little et al., 1993; Peakall, 1996). This study shows that both bottom-dwelling behavior and aggregation behavior are sensitive to exposure to BBP, but further investigations are required to be able to confirm whether these behavioral traits can serve as general indicators of pollution.

CONCLUSION

Changes in behavioral variables may be used as ecologically relevant indicators of pollution, since this will affect the individual fitness. In this study we showed that shoaling behavior and bottom-dwelling behavior were affected after exposure to a sublethal concentration of BBP. The findings also support that these behavioral traits are sensitive to pollution and that effects of contamination after relatively short times of exposure can be detected. Since both shoaling and bottom-dwelling behavior are ecologically relevant variables, sensitive to pollution, and methodologically easy to measure, we suggest that these variables may serve as significant effect biomarkers.

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Paper IV

Disruption of male reproductive behavior in threespine stickleback

Gasterosteus aculeatus exposed to 17β-oestradiol

By

Wibe, Å.E., Rosenqvist, G. and Jenssen, B.M.

Disruption of Male Reproductive Behavior in Threespine Stickleback *Gasterosteus aculeatus* Exposed to 17β-Estradiol

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In past years we have witnessed decreased reproductive capacity in many wildlife species due to exposure to chemicals with endocrine-disrupting properties. In this laboratory experiment male threespine sticklebacks *Gasterosteus aculeatus* exposed to 17β-estradiol (2.0 µg/g) dispersed in peanut oil, on days 1, 7, 14, and 21, showed impaired paternal care compared to control fish that were exposed to peanut oil only. There were no differences between the two groups in number of males that built nests or in courtship displays. However, exposed males started nest building significantly later than control males. This study suggests that some but not all essential traits of male reproductive behavior may be altered as a result of exposure to 17β-estradiol. To reveal harmful effects of chemicals with suggested reproductive-disrupting properties it is thus important to take a wide variety of variables related to reproductive behavior into consideration. © 2002 Elsevier Science (USA)

Key Words: reproductive behavior; Reproductive disorder; Threespine stickleback; *Gasterosteus aculeatus;* 17β-estradiol.

INTRODUCTION

For many years it has been known that some synthetic and natural substances can influence the reproductive system in animals (e.g., Eroschenko, 1981; Fry and Toone, 1981; and reviews by McLachlan, 2001; McLachlan *et al.*, 2001). Xenoestrogens can interfere with natural estrogens by binding to the physiological estrogen receptor and thus mimicing natural estrogen (Müller *et al.*, 1995). The affinity to the receptor is, however, less for most

xenoestrogens than for natural estrogen (Müller *et al.*, 1995). Xenoestrogens can also interfere with the synthesis or degradation of natural estrogen (Tyler *et al.*, 1998). The consequences of exposure to xenoestrogens may be reproductive disorders such as reduced fertility (Müller *et al.*, 1995), demasculinization or feminization (Müller *et al.*, 1995; Sumpter, 1995), reduced hatchability, reduced viability of offspring, impaired hormone activity, structural abnormalities of the reproductive tract, and/or altered adult sexual behavior (Fox, 1992; Guillette *et al.*, *1994*; Eskenazi and Kimmel, 1995; Sumpter *et al.*, *1996*; Kramer *et al.*, 1998). The above-mentioned effects have been reported in a number of animals (Colborn *et al.*, 1993) and even plants (Shore *et al.*, 1992).

That 17β-estradiol can act as an aquatic pollutant has been shown (Shore *et al.*, 1993; Brighty, 1996). 17β-Estradiol concentrations ranging from 2.7 to 48 ng/L have been measured in effluents in several localities in Europe (e.g., Brighty, 1996; Johnson *et al.*, 2000), whereas concentrations up to 141 ng/L have been reported from sewage treatment plants in Israel (Shore et al., 1993). It has been shown that a few nanograms per liter of natural estrogens can affect some variables of reproduction (Tyler *et al.*, 1998). Experimental studies have revealed several biochemical and physiological effects of 17β-estradiol, such as elevated production of the yolk protein vitellogenin in males (e.g., Sumpter and Jobling, 1995), reduced testicular growth (Madsen *et al.*, 1997; Panter *et al.*, 1998), and reduced egg production (Kramer *et al.*, 1998). There are, however, very few studies concerning the influence on reproductive behavior (Jones and Reynolds, 1997; Arcand-Hoy and Benson, 1998), with a few exceptions (e.g., Bayley *et al.*, 1999; Bjerselius *et al.*, 2001).

In the present study we used 17β-estradiol as a pollutant, but also as a model substance to study potential effects that xenoestrogens may have on

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reproductive behavior in male threespine stickle-backs. Reproductive success in male sticklebacks is determined by several factors, such as nest quality, number of eggs in the nest, paternal care, and the ability to obtain a female (Whoriskey and FitzGerald, 1994). Information about variables of the reproductive behavior that are impaired by estrogenic exposure is important in the hazard risk assessment of xenoestrogenic potentials.

The aim of this study was to provoke a behavioral effect of a sublethal concentration of 17β-estradiol and to investigate whether exposed male threespine sticklebacks were less able to perform optimal reproductive behavior than unexposed sticklebacks. Variables that were measured were nest building, courtship, and paternal care.

METHODS

Study Species

We used male threespine sticklebacks as a model species, because their reproductive physiology and behavior is well known and described (e.g., Wootton, 1984; Bell and Foster, 1994). The reproductive behavior in threespine stickleback is controlled by numerous hormones that constitute the hy-pothalamic–pituitary–gonadal axis (Arcand-Hoy and Benson, 1998). In the breeding season the transforma-tion of the male kidney into a glue-secreting organ, the male secondary sexual characters, and the male re-productive behavior are mainly regulated by the androgen 11-ketotestosterone (Borg, 1994; Guderly, 1994). The male builds a nest consisting of available algae and plants, into which he tries to attract females to lay their eggs. The male performs a char-acteristic zig-zag courtship dance to attract the female. The female will inspect the nest, and if she finds the nest and the male attractive enough, she enters the nest and lays her eggs. The male enters immediately after to fertilize the eggs. The male performs all the parental care, including fan-ning of the eggs and protection against predators.

Fish Maintenance

This experiment was conducted during May–July in 1997 and 1998. Fifty-four male threespine sticklebacks ($N_{1997} = 26$; $N_{1998} = 28$) with nuptial coloration (mean length 4.05 ± (SD) 0.65 cm) were collected using plexiglas fry traps (Dolmen, 1982) in the small lake Kindsethtjønna, Sør-Trøndelag county, central Norway (63.5°25′ N, 10°45′ E). To disinfect the fish against ectoparasites and external infections, they were treated with NaCl (10 g/L water) for 15 min before transportation in plastic containers (50 L) to Brattøra Research Centre, Norwegian University of Science and Technology, Trondheim. In the laboratory each individual was placed randomly and separately in glass aquaria (38 L) equipped with gravel, air stones, and a sta-tionary water system. Since it was not desirable that the males start nestbuilding before they were ex-posed to estradiol, the water plants (Elodea sp.) that were used as building material were not immersed until 1 day after the onset of the exposure. Water temperature (16.8°C ± 1.4) and photoperiod (18L:6D h) followed the natural pattern for the actual time and season both before exposure start and during the behavioral experiment. The fish were fed daily, alternately with dry food (Tetramin) and live Artemia.

In the experiment only healthy males were used. There was no significant differences in mortality between control (26%) and estradiol-exposed (33%) males during the experiment ($N = 54$, $\chi^2 = 0.36$, $P = 0.5$).

Exposure

The exposure to 17β-estradiol started 1 week after the animals arrived at the laboratory. In the expo-sure setup the fish were randomly divided into one exposure group ($N = 27$) and one control group ($N = 27$). In the exposure group male threespine sticklebacks were injected intraperitoneally with 17β-estradiol (Sigma; 2.0 μg/g body mass) dispersed in peanut oil (20 μl/g body mass). Since a behavioral effect was desirable, the exposure concentration was determined according to previous studies where comparable sublethal concentrations were shown to result in significant effects (e.g., Pankhurst et al., 1986; Ghosh et al., 1989; Madsen and Korsgaard, 1989; Washburn et al., 1993; Madsen et al., 1997). The control group received the same treatment as the exposure group except that they were exposed to peanut oil only. The dispersion was prepared using an ultrasonic bath (3 × 10 min). The fish were injec-ted on days 1 (1. exposure), 7 (2. exposure), 14 (3. exposure), and 21 (4. exposure), using a 23-gauge (0.6 × 25-mm) needle. In accordance with the National Animal Research Authority, the injection was conducted without anaesthesia as the anaesthe-sia treatment could inflict further stress on the fish in addition to the stress following exposure. During injection each fish was taken out of the home aquar-ium. Immediately after the injection each of the males was placed back in its respective aquaria

where shortly after arrival it resumed normal activities. All fish were starved for 12 h before injection to avoid interfering effects.

Behavioral Studies

Twenty-four hours after the first exposure to 17β-estradiol, a female that was ready to spawn was introduced to each male. The females were removed a few days after mating to avoid aggressive behavior toward the females. Aggressive behavior can disturb the males nesting and paternal behavior, as it is plausible that they spend more time on aggression than on reproductive behavior.

In the following 4 weeks each aquarium was manually observed for 5 minutes, three times every day. The observations were carried out in the morning, at noon, and in the afternoon. Three observers alternated in conducting the observations.

During each observation the following information was recorded: (1) presence or absence of a nest, (2) presence or absence of courtship behavior (including, e.g., zig-zag dancing and leading of the female to the nest; Wootton, 1984), and (3) presence or absence of paternal care (defined as fanning, glueing, and guarding of the nest; Wootton, 1984). Due to errors in the data sampling in 1997 results with regard to paternal care are available only for 1998.

The fungus *Saprolegnia parasitica* infected some of the fish. The majority of the infections broke out late in the experiment, and the infections were mostly too light to influence behavior. However, the difference between control and exposed fish with regard to fungus infection was recorded.

To avoid a subjective bias between exposed and control fish in data recording, the aquaria were labeled with blind numbers. The experiment was terminated 4 days after the fourth injection, and the fish were put to death.

Ethics

This experiment was approved by the National Animal Research Authority.

Statistics

To test for differences in frequency in exposed and control individuals, χ^2 or Fisher probability tests were used, depending on sample size. To test for differences in medians, Mann–Whitney U test was used. Nonparametric statistics were used when data were unevenly distributed. $P < 0.05$ was defined

TABLE 1
Behavioral Activities Observed in 1997 and 1998

Behavioral activity	Control	Exposed	Statistics
Nest present	19 ($N = 27$)	18 ($N = 27$)	$N = 54$, $\chi^2 = 0.09$, $P = 0.77$
Courtship	1.0 (1.0–2.0)	1.0 (0.0–1.0)	$N = 54$ $U = 76.5$, $P = 0.30$
Paternal care (1998)	16.5 (10.5–24.5)	2.0 (1.0–6.0)	$N = 28$ $U = 22.0$, $P = 0.003$

Note. "Nests present" is presented as number of males with nests, "courtship" is presented as median (interquartile ranges) of conducted courtships per male (e.g., zig-zag dance, leading of female) during the 4-week observation period, while "paternal care" is presented as median (interquartile ranges) of conducted paternal care activities per male (e.g., fanning, glueing, guarding) during the 4-week observation period. The data from 1997 and 1998 are presented in combination, with the exception of "paternal care," where only 1998 data are presented due to insufficient recordings in 1997. "Nest present" is tested with a χ^2 test, while "paternal care "and "courtship" are tested with a Mann–Whitney U-test. Significance is defined when $P < 0.05$.

as statistically significant. All statistics were tested with the program SPSS (version 9.0).

RESULTS

In 1997 the estradiol-exposed males were significantly more infected with *S. parasitica* than control males, as 58% of the 17β-estradiol-exposed males and 14% of the control individuals were infected (Fisher exact probability test, $N = 26$, $P = 0.03$). In 1998 no between-group differences were found, since 40% of the estradiol-exposed males and 38% of the control males were infected by fungus (Fisher exact probability test, $N = 28$, $P = 1.00$).

The proportion of nest building control males (70%) and nest building estradiol-exposed males (67%) did not differ ($N = 54$, $\chi^2 = 0.09$, $P = 0.77$) (Table 1). On the other hand, control males built their nests earlier than estradiol-exposed males. In the period between the first and the second exposure 39% of the estradiol-exposed males and 90% of the control males built nests. The rest of the nests were built between the second and the third exposures (Fisher exact probability test, $N = 37$, $P = 0.002$) (Fig. 1).

The exposed and control males showed different abilities to perform paternal care. In 1998 control males showed more paternal care per male than
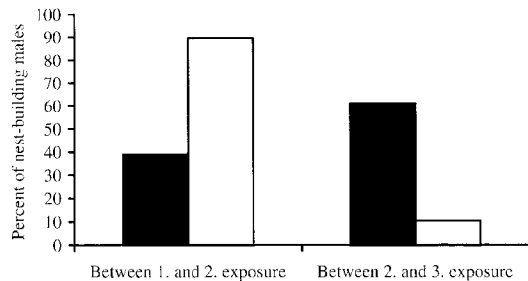
FIG. 1. Time of nest building in 1997 and 1998 for control (white) and 17β-estradiol-exposed (black) male sticklebacks. The fish were exposed once a week for 4 weeks. All nests were built either between 1. and 2. exposures or between 2. and 3. exposures. Estradiol-exposed males built their nests later than control males (Fisher exact probability test, $N = 37$, $P = 0.002$).

exposed males ($N = 28$, $U = 22.0$, $P = 0.003$) (Table 1).

The first observation of courtship display was done on the third day after the first exposure. There were no differences between control and exposed individuals with respect to recorded courtship displays per male during the entire experiment ($N = 54$, $U = 76.5$, $P = 0.30$) (Table 1).

DISCUSSION

Physiological effects of 17β-estradiol exposures in males are well documented (e.g. Colborn et al., 1993; Sumpter, 1995; Gray, 1998; Kinnberg et al., 2000), but there is little documentation on how these physiological alterations correspond to reproductive behavior (Arcand-Hoy and Benson, 1998; Bayley et al., 1999).

The present study shows that exposure to 17β-estradiol (2.0 μg/g body mass) alters reproductive behavior in male threespine stickleback. We showed that estradiol-exposed males started nest building later than control males (Fig. 1). We further found that exposed males devoted less time to paternal care than control males. These results suggest that threespine sticklebacks exposed to 17β-estradiol have a reduced ability to conduct characteristic male nesting behavior (nest building, fanning, glueing, and guarding). As suggested by Arcand-Hoy and Benson (1998), 17β-estradiol given to the male fish can disturb the hypothalamus–pituitary–gonadal axis in which several hormones, including androgens, normally regulate sexual development, sexual physiology, and sexual behavior. Since androgens, also in male sticklebacks regulate secondary sexual

characters and reproductive behavior (e.g., Guderley, 1994), a disruption of the androgen synthesis or the processes in which androgens participate can cause severe effects.

Impaired paternal care may cause reduced reproductive success among threespine stickleback (Whoriskey and FitzGerald, 1994). Delayed nest building can also result in impaired reproductive success, as males that nest late might get fewer or no females laying their eggs in the male nest (Mori, 1993). In a recent study, Barber et al., (2001) showed that early nestbuilding sticklebacks built nests of higher quality and had heavier kidneys due to glue production than did late nest building males. Since the transformation of the kidney into a glue-secreting organ is an androgen-dependent process (Guderly, 1994), exposure to 17β-estradiol may disturb and delay the transformation. Alterations in nesting behavior as a result of exposure to endocrine disruptors have also been found in other species, such as birds (Peakall, 1996; McCarthy and Secord, 1999).

In this experiment the number of males that built nests did not differ between control and exposed fish. This result is in contrast with a study on the wild cichlid Tilapia rendalli, where individuals exposed to the xenoestrogen endosulfan built fewer nests than unexposed fish (Douthwaite et al., 1981). However, since the cichlids were exposed to endosulfan from the surrounding environment, it is possible that the long exposure period prior to nest building impaired their ability to build nests. The threespine sticklebacks used in our experiment were exposed only once before they received nest building material. Since the concentration of 17β-estradiol increases with repeated injections (Pankhurst et al., 1986), the concentration was expected to increase as the exposure continued. It is therefore possible that a more dramatic effect on nest building behavior would have been observed if nest building material was introduced later in the experiment. When comparing results caused by 17β-estradiol and by other chemicals with suggested reproductive-disrupting properties, it is also important to be aware of the possibility that other non-estrogenic effects of the chemicals can cause the observed effects (reviews by, e.g., Tyler et al., 1998; McLachlan, 2001).

The males exposed to estradiol in this experiment did not differ from the control males with respect to time devoted to courtship behavior. This result contrasts with another study, where guppies (Poecilia reticulata) exposed to 17β-estradiol (10 μg/L continuous water flow) for 4 weeks showed impaired courtship behavior (Bayley et al., 1999). Also, in another study with guppies, the fish showed less courtship

behavior after exposure to the estrogenic pesticide lindane (1 µg/L) for 1 week (Schröder and Peters, 1988). However, the guppies in both mentioned experiments were exposed via the water. Thus the dissimilarities in methods could be an explanation for the different results. Also the major differences in male reproductive behavior in guppy and threespine stickleback, where the latter have all the paternal care, may explain the different results.

In the first experimental season the 17β-estradiol-exposed sticklebacks were more vulnerable to infection to *S. parasitica* than control fish. This is in accordance with previous studies, which report increased susceptibility to *S. parasitica* following exposure to chemicals (Cross and Willoughby, 1989; Carballo et al., 1995). It is possible that some effects of pollution on immune competence may be linked to hormone-disrupting effects. Indeed, others have also hypothesized that exposure to estradiol and other xenoestrogens result in immunosuppression or altered immune cellular function (Arcand-Hoy and Benson 1998; Fournier *et al.*, 2000). The immunotoxic effect of 17β-oestradiol is, however, complex and differs according to concentration and maturity of the animals (Fournier *et al.*, 2000). Immunosuppression can cause deleterious effects in individuals, such as infection with fungus. The absence of differences in fungus infection in 1998 in control and exposed fish can be the result of several factors, such as between-year differences in natural infection rate in the lake Kindsethtjønna, where the fish were caught 1 week prior to 17β-estradiol exposure.

In this study we used behavior as an indicator of pollution because behavior is the consequence of biochemical and physiological alterations, but also because changes in behavior as a result of pollution is believed to be an ecologically relevant indicator of pollution (Peakall, 1996). An alteration in reproductive behavior is believed to be of particular ecological relevance since this will have negative effects on reproductive success.

CONCLUSION

Many studies of reproductive disorders have been conducted since it became evident that certain chemicals and natural estradiols can act as reproductive hormone mimics and modifiers. Based upon the knowledge that the optimization of the reproductive success of an individual requires a diversity of successful reproductive activities, the present study suggests that not all variables of reproductive behavior are sensitive to exposure to 17β-estradiol. To obtain a complete evaluation of the effects of estradiols and xenoestrogens on reproductive behavior this study therefore demonstrates the importance of taking a wide variety of variables related to reproductive behavior into consideration.

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