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The Siamese fighting fish *(Betta splendens)* – An alternative fish species to use in evaluating the impact of endocrine disrupting chemicals with focus on aggressive performance

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PREFACE

The present study was conducted from September 2001 to April 2002, both as a degree project for a Master of Science degree in Biology at Uppsala University and as a Minor Field Study (MFS) for the Committee for Tropical Ecology, Uppsala University. It was partly conducted at the Department of Pathology, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, Ultuna (SLU) in Uppsala, Sweden, and at the Faculty of Veterinary Medicine, Chiang Mai University (FVM-CMU), Thailand. The study was financially supported by Sida (Swedish International Development cooperation Agency).

The study addresses the key question whether there are morphological changes in the testis of the Siamese fighting fish as a result of exposure to ethinylestradiol (EE_2). Furthermore if such exposure has impact on the willingness of the species to defend its territory. It also focuses on whether there are changes in the fish's willingness to defend its territory due to exposure of genestein or wastewater from a tofu factory in Thailand. A study by Maria Blomberg (in prep.) focuses on whether there are changes in colours and nesting ability of Siamese fighting fish when exposed to EE_2 and if there are changes in nesting ability and spawning capacity of the species when exposed to genestein or wastewater from a tofu factory in Thailand.

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INTRODUCTION

The worldwide structural change in human communities includes increasing industrial, agricultural and forestry development. This change often results in enhanced releases of chemicals into the environment. Degradation processes and new environmental conditions for the released substances could change their structural composition. Therefore environmental impacts are difficult to predict and prevent. Since aquatic ecosystems serve as a main sink for many industrial and municipal waste products, a considerable regional outlet of contaminants can be a big problem for the ecological balance, both in smaller limbic systems and in the sea. Many aquatic organisms are directly exposed to the different substances through their environment and even when emissions of these contaminants are stopped the effects may persist for a long time.

During recent years considerable attention has been given to the fact that chemical compounds can be biologically active by interfering with the endocrine system of animals. These substances, called endocrine disrupting chemicals (EDCs), include anthropogenic compounds as well as naturally occurring compounds produced by plants and fungi. EDCs are a growing problem in many aquatic ecosystems. The chemicals are known to cause sex changes and interference in reproduction in different animal species, e.g. feminization of male fish and alligators (Sumpter, 1995).

The worldwide production and use of soybean products have increased dramatically in the past centuries (Ensminger *et al.,* 1995; KeShun, 1997). Soybeans have a high content of different estrogen-like substances, especially the two isoflavones, genistein and daidzein (KeShun, 1997). It has been shown that these substances affect the endocrine system in fish, i.a. high plasma vitellogenin levels in cultured Siberian sturgeon (Pelissero *et al.,* 1991a). Industrial effluents originating from pulp and paper production have also been shown to contain EDCs with impact on the endocrine system in fish (Sandquist, 2001). Among the various industries in Thailand (forestry development and soybean refinement included) there is very limited knowledge about chemicals released into the environment.

It is difficult and expensive to perform chemical analysis on EDCs. Therefore it is important to develop non-expensive and reliable methods to disclose the presence of EDCs in the environment. The zebrafish *(Danio rerio)* is one key species that is promoted by OECD (Organization for Economic Cooperation and Development) as a candidate for evaluating EDCs. Core biomarkers for the zebrafish include vitellogenin and gonad histology. One alternative end-point that has been discussed for evaluations of EDCs is altered behaviour. The male Siamese fighting fish *(Betta splendens)* has a strong pronounced behaviour and is very territorial and defends its habitat (Simpson, 1968). The use of Siamese fighting fish as a test organism for evaluation of EDCs would be very suitable and interesting from regional perspectives in Thailand since the species is native in the country and a part of the natural aquatic ecosystem (Jaroensutasinee *et al.,* 2000).

AIMS OF THE STUDY

The aim of this study is to obtain basic knowledge of relevant methods that could be sensitive and accurate in order to reflect exposure to EDCs, e.g. estrogen-like substances, in the Siamese fighting fish. A further aim is to apply this knowledge by experimental exposure of the Siamese fighting fish to wastewater from a relevant industry and to study wild fish from a contaminated river.

Hypothesis

The fact that the Siamese fighting fish has very prominent male characteristics indicates that this species could be a suitable test organism for estrogen mimicking substances.

Exposure of the fish to estradiol-like substances may cause:

- *Changes in internal organs*
- *Decreases in ability and willingness to defend its territory*
- *A change in colours (Blomberg, M., in preparation)*
- *Reduced spawning capacity (Blomberg, M., in preparation)*
- *Changes in nesting ability (Blomberg, M., in preparation)*

BACKGROUND INFORMATION

The endocrine system

The internal system of chemical communication within animals is called the endocrine system. It controls many systems, including reproduction, metabolism, osmoregulation, embryonic development, digestion and growth. The endocrine system is complex and consists of hormones, ductless glands that produce and secrete these hormones into body fluids, and target cells that respond to the released hormones. Each hormone has a specific shape that is recognized by molecular receptors located on the surface of the target cells or inside them. Hence, hormones can be located everywhere inside the body, but only specific cells respond to them. Even at very low concentrations hormones are capable of regulating different systems. Minor changes in the concentration/proportion of one hormone can have a significant impact on and within the body. A given hormone can affect different target cells within an animal differently, or it may affect different species differently. (Campbell, 1996; Kardong, 1998)

Steroid hormones (including sex hormones) are lipid molecules produced from cholesterol. They enter their target cells by diffusion and a response is triggered (Fig. 1). Most non-steroid hormones (hormones derived from amino acids) attach to the cell surface and influence the activity within the target cell through second messengers of signal-transduction pathways. (Campbell, 1996; Kardong, 1998)

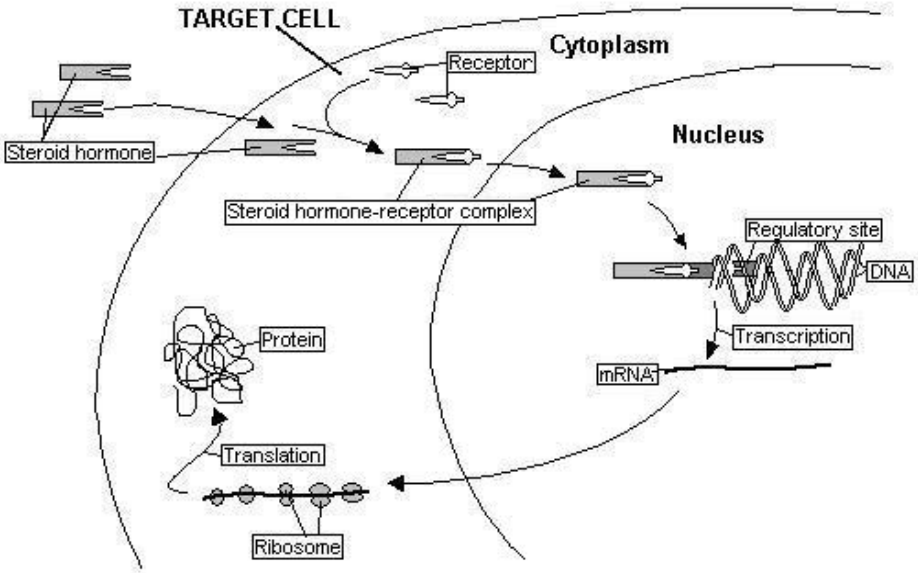

Fig. 1 Steroid hormone action in a target cell. The steroid hormone crosses the membrane of its target cell by diffusion and binds to a receptor protein inside the cytoplasm. This steroid hormone-receptor complex is transported into the nucleus where it attaches and binds to certain regulatory sites of the target cell genome. The binding to those sites along regions of the DNA-structure of the target cell can either induce or suppress the expression of specific genes.

Reproductive system and hormones

Hormones are very important in the reproductive system. They control primary and secondary sex characteristics (e.g. preparations of the ducts for receiving gametes, development of germ cells and external genitalia; respective sex drive, reproductive cycles, parental care and other

behaviours). The reproductive system also consists of gonads, gonadal products, gametes and the ducts that transport the gametes. (Campbell, 1996)

Three groups of gonadal steroids (sex hormones), i.e. androgens, estrogens and progestins, have major influence within the reproductive system. They all exist in both males and females but in different proportions. Estrogens stimulate the development and maintenance of the female reproductive system and secondary sex characteristics. Androgens stimulate the development and maintenance of the male reproductive system and secondary sex characteristics. Progestins are primarily involved in preparing and maintaining the uterus, which supports the growth and development of the embryo. The most important hormone in each group is testosterone, estradiol and progesterone, respectively (Fig. 2). (Kardong, 1998; Kalthoff, 1996)

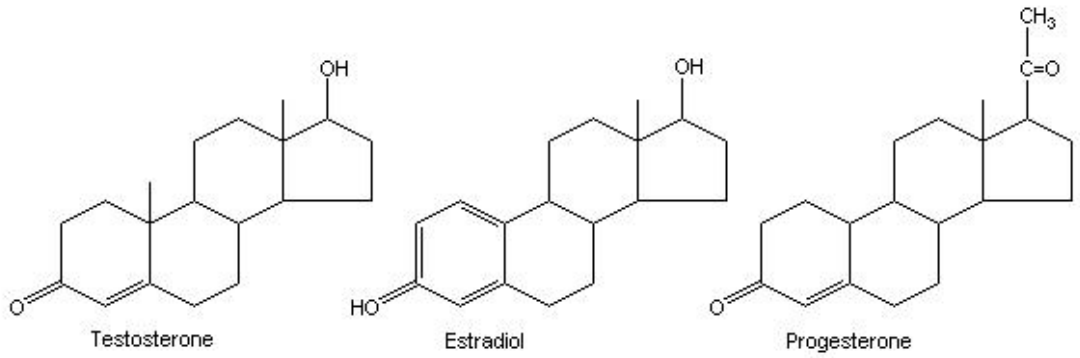

Fig. 2 The chemical structures of the most important sex hormones among androgens (testosterone), estrogens (estradiol) and progestins (progesterone) in vertebrates.

Gonadal steroid synthesis – testosterone

The synthesis of both estrogens and androgens is controlled by gonadotropins produced by the anterior pituitary gland. Those gonadotropins, called follicle stimulating hormone (FSH) and luteinizing hormone (LH) in mammals, are in turn controlled by the gonadotropin-releasing hormone (GnRH) from the hypothalamus (Fig. 3). FSH stimulates spermatogenesis in males and the maturation of follicles in the ovary of females. LH stimulates production of estrogens and androgens in both sexes, and ovulation in females. (Kalthoff, 1966; Klaasen, 1966) In fish, the gonadotropins are most commonly referred to as different gonadotropin hormones (GTHs) (Schulz *et al.*, 2001). The hormone system is regulated, maintained and controlled by negative feedback. When sufficient amounts of sex hormones are in the system, GnRH secreting neurons in the hypothalamus and endocrine cells in the pituitary are inhibited. Male individuals have a continuous secretion of GnRH and females have a more complex cyclic pattern of hormone release. (Kalthoff, 1966; Klaasen, 1966)

One important reproductive hormone in male vertebrates is testosterone. It is mainly produced by interstitial cells (leydig cells) located in the testis. The production of testosterone in leydig cells occurs under the influence of the gonadotropin hormone LH. In some fish (including the Siamese fighting fish) the leydig cells are situated between the seminiferous tubuli of the testis. (Campbell, 1966; Klaasen, 1966)

The gonadotropin hormone FSH affects the sertoli cells, located on the luminal side of the seminiferous epithelium (Fig. 4). Also the sertoli cells are sometimes capable of producing testosterone. In general, FSH together with testosterone plays an important role in spermatogenesis. The sertoli cells are responding to these hormones by producing a number of other hormones and proteins necessary for the development of sperms, including inhibin

and/or androgen-binding proteins. Nutrients, hormones and other molecules produced by the sertoli cells have to pass through the membranes of the cysts and, hence, those substances can be regulated between the cysts. Inhibin may aid in modulating FSH and it acts with negative feedback on the pituitary. Without inhibin or androgen-binding protein, the sertoli cells would probably synthesize estradiol instead of testosterone in response to FSH. (Campbell, 1966; Klaasen, 1966)

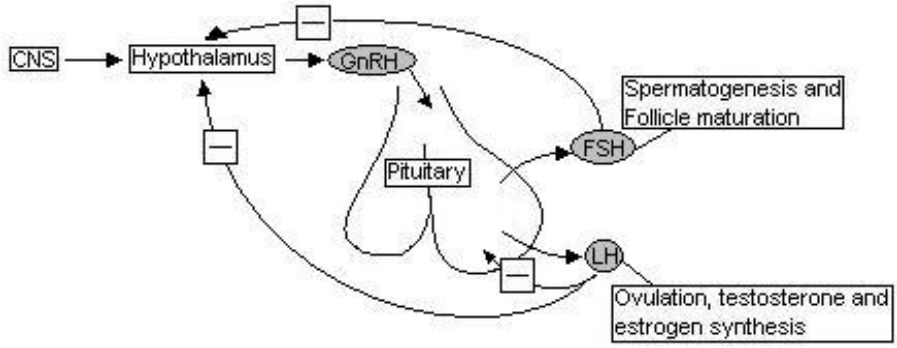

Fig. 3 The hypothalamic-pituitary-gonadal axis. Initially the central nervous system signals to the hypothalamus, which in response secretes gonadotropin-releasing hormone (GnRH). This hormone acts on the pituitary gland and stimulates the production of many different hormones, including gonadotropin-stimulating hormones such as follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH stimulates spermatogenesis in males and the maturation of follicles in the ovary of females. LH stimulates production of estrogens and androgens in both sexes, and ovulation in females. The appropriate levels of steroid sex hormones in the body are controlled and regulated by negative feedback, shown as - in the figure.

General morphology of testes and spermatogenesis in Siamese fighting fish

The morphology of testes differs between species, but the function is the same for all, i.e. to produce sperms (necessary for a successful reproduction) and hormones (to regulate primary and secondary sex characteristics). In most of the Osteichytes (Bony fish, which include the Siamese fighting fish), the testis is an elongated paired organ. Two general basic structures have been identified (Grier, 1981; Kardong, 1988). In one of them sperms are developed within compartments and in the other within tubuli (Kardong, 1988). These two testes types are divided into several subgroups. In this classification system, the Siamese fighting fish is of the tubular type (Fig. 4) (Kardong, 1988; Grier, 1981; Nagahama, 1988). Within this type the pipeline-like seminiferous tubuli run from the periphery of the testis to the opposite border, where they open into the vas deferens (sperm duct). Inside each seminiferous tubule there are cysts (follicles) in which spermatogenesis occurs. (Kardong, 1988)

During early spermatogenesis, primitive germ cells are found in the intratubular layer of connective tissue cells (somatic cells). As maturation proceeds, these somatic cells are called sertoli cells. The proliferation of these primitive germ cells (spermatogonia) by both mitotic and meiotic divisions results finally in cysts of mature sperms. The cysts bursts and the mature sperms enter the lumen of the tubule from where they are transported to the sperm duct. In sexually mature individuals, germ cells within one cyst undergo spermatogenesis simultaneously and the many cysts inside a tubule can be at different stages of differentiation (Fig. 4; cf. Fig 1.4). (Kardong, 1988)

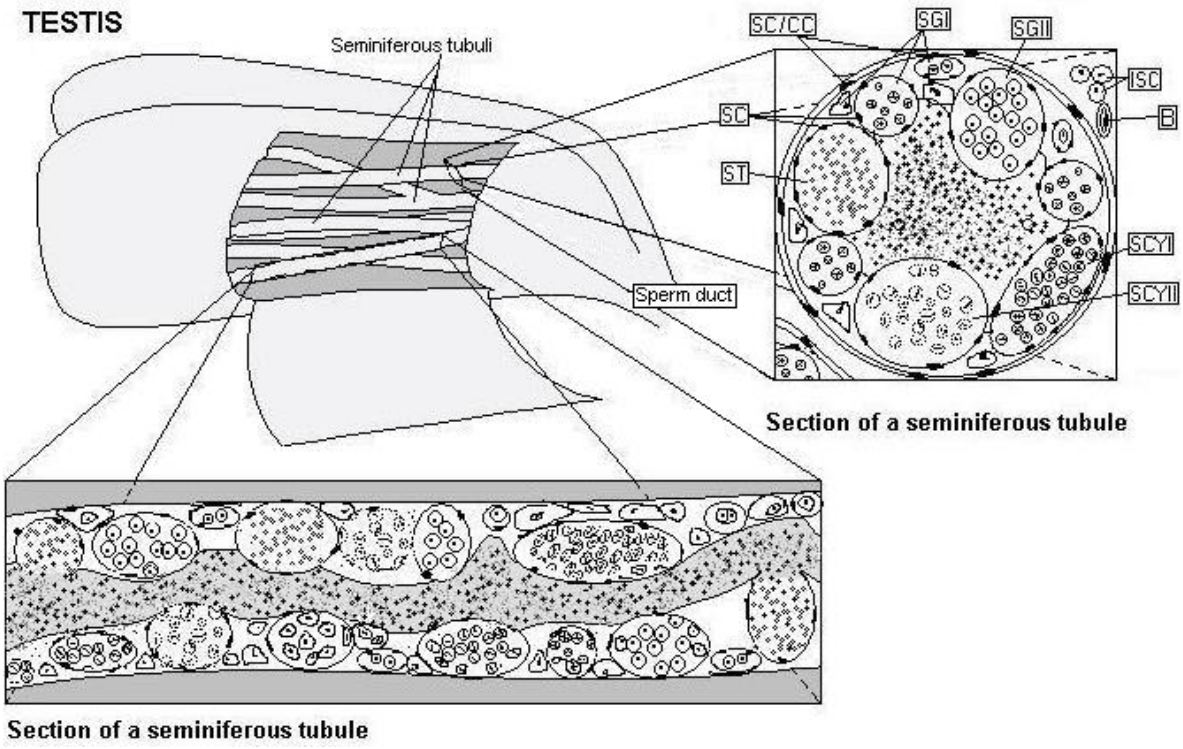

Fig. 4 The tubular testis type and the different stages of spermatogenesis. Cross sections of the seminiferous tubule show the different cell types within the testis. In each tubule, which is surrounded by connective tissue cells (CC), two different cell types are found: sertoli cells (SC) and sperms. Developing sperms are located within cysts. Initially there is a proliferation of a spermatogonium within the connective tissue cell (sertoli cell). As proliferation of these primary spermatogonia (SGI) proceeds, some connective tissue cells are differentiated into sertoli cells. This proliferation results in a nested clone (a cyst) containing immature sperms. The primary spermatogonia become secondary spermatogonia (SGII) that differentiate into primary spermatocytes (SCYI), secondary spermatocytes (SCYII), spermatids (ST), and finally spermatozoa (S) (mature sperms). Outside the tubule there are interstitional cells (ISC) and blood vessels (B). The nucleus of sertoli cells is enlarged in the figure and shown as a black dot at the epithelium of the cysts or tubuli.

Embryonic development of gonads

The vertebrate embryos are initially ambisexual and a non-differentiated gonad is present in the embryonic genital ridge (the same or adjacent tissues in the embryo from where both reproduction and urinary system arises). The gonadal differentiation takes place under influence or absence of a gene present on the Y chromosome. When this Y chromosome (with down stream effector and autosomal genes) is absent, the gonad will differentiate into a female gonad. When the Y chromosome is present the gonad will differentiate into a male gonad. (Campbell, 1996; Kardong, 1998; Kalthoff, 1996)

There are two ducts present in the embryo, the wolffian and müllerian. In females the müllerian ducts will develop into the oviduct of the reproductive system. The wolffian duct (also called archinephric or mesonephric duct) will function within the urinary system. In males, the müllerian ducts regress. Genes on the Y chromosome induce the secretion of a substance in the sertoli cells called the müllerian-inhibiting substance (MIS). MIS causes the regression of the müllerian ducts and the indifferent gonad develops into a testis. Testosterone secretion by leydig cells of the developing testis induces differentiation of the wolffian ducts into the internal structures of the reproductive tract. Therefore the male pathway of gonadal development is considered to be dependent of hormonal regulation whereas the female pathway is considered to be independent. (Kalthoff, 1996; Klaasen, 1996)

General mechanisms of endocrine disrupting chemicals (EDCs)

Xenobiotics (i.e. man-made or naturally occurring substances foreign to an organism) may interfere with animals and act as endocrine disruptors (Gulliette *et al.,* 1994; Klaasen, 1996; Sumpter, 1995). Basically an endocrine disruptor stimulates or inhibits processes in the endocrine system that may result in devastating consequences to organisms, its progeny or entire populations. The definition (1999) of EDCs by the European Commission is: An exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function.

EDCs can interfere with the endocrine system of animals acting as steroid agonists (It then mimics a hormone and is active in a target cell.), or antagonists (It only mimics the shape of hormones but does not elicit any response in the target cell.). EDCs can also interfere with the endocrine system through hormone synthesis, metabolism, excretion and transport of hormones, hormonal feedback systems or synthesis of hormone receptors. The results of this interference can be additive (adding the effects of individual chemicals), synergetic (having a greater effect of a mixture of chemicals than the sum of the chemicals), antiestrogenic (antagonistic estrogen) or antiandrogenic (antagonistic androgen). An endocrine disruptor may also interact with more than one steroid sensitive pathway, which makes responses to EDCs difficult to predict. The response to specific EDCs can also differ between different species.

The greatest risk to reproductive health caused by xenobiotics is during the embryonic, neonatal and pubertal periods, since the reproductive system's morphological and physiological development is then undergoing its maturation and is modulated by steroid hormones (Danzo, 1998). For example, during the differentiation of the reproductive system into male or female genitalia, there are several mechanisms that environmental steroid agonists/antagonists could interfere with. An exposure to antiandrogenic xenobiotics of a chromosomal male would influence the androgen-dependent differentiation of the wolffian derived structures and/or with the normal development of the male genitalia. Since estrogen receptors are present in the male genitalia tract, xenoestrogens could also interfere with these structures. Likewise, xenoandrogens could cause masculinisation in developing females since their reproductive tracts contain androgen receptors. (Klaasen, 1996; Danzo, 1998)

Testes inhibition morphology

Exogenous estrogens, deprivation of pituitary gonadotropin, malnutrition and deficiency of vitamins A, E or zinc induces inhibition of testis. Encountered lesions both in testis and epididymis include testicular atrophy (decrease in size of cells that have gained full development), necrosis (death of cells and tissues in living animals) of seminiferous tubuli, epididymal atrophy, spermatic granuloma (tissues with focal or continuous layer of granular vegetative growth that cover serosal surfaces of body cavities) and leydig cell hyperplasia (increase in the number of cells in a tissue) (Gopinath *et al.,* 1987).

Atrophy is the most frequently seen effect in testicular toxicity. Spermatids and spermatocytes are absent and the seminiferous tubuli consist mainly of sertoli cells. Initially the lumina of atrophic seminiferous tubuli often contain abnormal spermatids (giant multinucleated spermatids) and the seminiferous tubuli show vacuolated cells, reduced numbers of spermatids and occasional abnormal spermatids. In long standing and extreme cases the tubuli are lined only by a few sertoli cells and a reduced number of spermatids as well as prominent multinucleated giant spermatids. (Gopinath *et al.,* 1987)

Endocrine mimicking substances in soybeans

Oriental soy foods are part of the daily diet in many areas of our planet. The soybean plant (*Glycine max*) belongs to the legume family of plants and is the leading legume crop in the world (also the leading legume crop in the USA). Modern soy products, e.g. soy flour, textured soy protein, concentrates and isolates, have been used for several decades as functional ingredients by the food industry in industrialized countries. More traditional soyfoods, e.g. soymilk, sofu, tofu, miso and soy sauce, have been consumed by Asian populations for centuries and recently by industrialized nations. These products are now growing in popularity both in the United States and Europe because of specific health attributes ascribed to soyfoods and the easy transformation from an animal diet to a plant based one. (Ensminger *et al.,* 1995; KeShun, 1997)

From a nutrient perspective soybeans and soyfoods have primarily been identified to have high protein content. In more recent years clinicians and researchers have had another approach to soybeans. They have shown much interest in the potential role of soyfoods as preventors and treators in chronic diseases. For example, it has been shown that some substances in soybeans can inhibit the growth of cancer cells, lower the cholesterol levels in the body and inhibit bone resorption. The substances causing these improvements in human health are called isoflavones and belong to the flavonoid group (a group of aromatic compounds that includes many common pigments) of phytoestrogens (naturally occurring estrogens in plants and fungi). The most recent upurge and interest in soybeans and their products in western countries is almost entirely due to the large amounts of isoflavones present in soybeans. (KeShun, 1997)

The primary isoflavone in soybeans is genistein, (Fig. 5), and daidzein, and their respective ß-glycosides (sugar derivate that contain a non-sugar group bound to an oxygen or nitrogen atom and yields sugar on hydrolysis) genistin and daidzin. In non-fermented soy foods, such as tofu, the isoflavones appear mostly as ß-glycosides. In fermented soy products such as miso the aglycones (the non-sugar component of a glycoside molecule, i.e. genistein and daidzein) dominate. In general, there is always more of genistein in soybeans, but the ratio genistein: daidzein can vary greatly (1.2-2:1). Raw soybeans contain 2-4 mg of isoflavones and tofu products contain 4-8 mg of soybeans. During digestion, the microflora in organisms can convert the two isoflavones into other chemicals. Genistein is generally metabolized into the biologically inactive p-ethylphenol, and daidzein into several different products, e.g. equiol, dihydrodaidzein and O-desmethylangolensin (ODMA) (KeShun, 1997).

Fig. 5 The chemical structure of genestein, an isoflavone present in soybeans.

The ecotoxicological aspect of the global increase in the soy industry and soyfoods is that phytoestrogens might end up in the environment and affect the fauna. Isoflavones have an extremely limited distribution in nature and soybeans can be considered as the only natural dietary source of these isoflavones (KeShun, 1997). It has been shown that oral administration of soybeans induces vitellogenesis (egg yolk protein in female fish) in male fish (Pelissero *et*

al., 1991a). Both genistein and daidzein have estrogenic activity, but both are less potent than 17 ß-estradiol (the naturally occurring estrogen in vertebrates) (Pelissero *et al.,* 1991b).

Processing of soybeans can alter the characteristics of the soyprotein products. These treatments can involve the use of enzymes, solvents, heat, fractionation and pH adjustments, or a combination of these treatments. Some isoflavones are lost when going from soybeans to soyflour to soyisolates. In certain stages of making soyprotein concentrates isoflavone loss is particularly high. The usual method includes an aqueous alcohol wash of soy flakes, which includes a loss of isoflavones and other phytochemicals. (Ensminger *et al.,* 1995; KeShun, 1997)

The Siamese fighting fish

The Siamese fighting fish *(Betta splendens)* is a member of the Labyrinth fish family (Belonitiidae). *Betta splendens* is Latin and means the brilliant warrior, a very suitable name indeed, since males of the colourful species perform an elaborate aggressive display when provoked (Fig. 6). Among the various Belonitiidae, the Siamese fighting fish, with its commercially bred variation in fin shapes and colours, has its geographical origin in Southeast Asia. The word Siamese heritages from Siam, the former name of Thailand and the fish is very well known in the country.

Fig. 6 The Siamese fighting fish (*Betta splendens*) displaying aggressively with raised fins and operculae. Photo: A. Foberg

Wild living fighting fish are in general smaller with a length of 2.5-3 cm compared to the domesticated ones which have a length of 3-5 cm (Jaroensutasinee *et al.,* 2000; Simpson, 1968). The wild type has dull brownish to green body colour as well as short fins (Fig. 2.1.). During male display, blue-green rays appear particularly on the caudal fin and on some fish there are also a number of red, green or blue dots scattered in rows on the body. The female bodies have diffuse transversal stripes, which appear more distinctly during mating. Submissive fish, both males and females, become pale in colour and two dark longitudinal lines appear on the middle of the body. (Simpson, 1968)

The domesticated fish have been bred through centuries and there are fish of almost any colour, combinations of colours, fin length and shape of fins. During male threat display, individuals can have a slightly more intense colour and they are sometimes showing a paler colour during submissive behaviour. Females are in general more brownish to white and less colourful than males, but the breeding has also produced females with colours similar to male colours.

The species' natural habitat is quiet shallow freshwater ponds with muddy bottoms or flooded rice paddy fields (Jaroensutasinee *et al.,* 2000). They can breathe oxygen from the surface (due to their labyrinth organ), which enables the species to survive in low oxygen waters. During mating, male individuals build a bubblenest at the surface, court females in an aggressive reproductional behaviour and care for the developing eggs and newly hatched larval fish. The male fish is strongly defending its territory near the water column that is centred in the bubblenest. This aggressive display includes gill cover erection, fin spread, biting and tail beating towards encounters of same species. The display is very easily triggered and has a part in the species' wide use in laboratory studies of signalling and aggressive interactions (Oliviera *et al.,* 1998). The fry is sexually mature after approximately three months and the lifetime of one individual is between 2 and 4 years (Terciera, 1978).

STUDY 1

- Exposure to Ethinylestradiol, EE_2 -

MATERIAL AND METHODS

Experimental animals

Ten male Siamese fighting fish of different colour and size were bought from a local fish shop in Uppsala, Sweden. Each individual was identified and given a number. Due to the strong male characteristics of the species (Simpson, 1968), the individual fish were divided into matched pairs. This division was based on the fish colour, temperament and size. One individual in each pair was randomly selected for exposure to ethinylestradiol, EE_2, (10 ng/L) and the other was used as control (unexposed). (Table 1.1)

The temperament of individual males was classified by observations of the fish behaviour in connection with provocation by different objects such as pencils, erasers, mirrors, and models of fish. The behaviour was defined in two categories: Aggressive and Non-Aggressive. Aggressive individuals were interested in the object, had raised operculae (gill cover) and were making aggressive displays towards it. Non-Aggressive fish were submissive, had not raised operculae and sometimes tried to escape by swimming away from the object. Colours of the fish were defined by observations and sizes were measured from photos taken of the fish. The length was measured from the tip of the upper jaw to the caudal peduncle (beginning of the caudal fin).

Table 1.1 Data of the Siamese fighting fish used in this study. Individual fish number, exposure to ethinylestradiol (EE_2), the fish colour, temperament, body length, body height and matched fish in same pair. Colours in parentheses are colour of individual stripes of the dorsal and/or caudal fin.

Fish no.	Exposure to EE_2 (ng/L)	Colour	Temperament	Length (cm) ± 0.1	Height (cm) ± 0.1	Paired with fish no.
1	10	Blue	Aggressive	4.6	1.4	4
2	0	Red	Non-Aggressive	4.4	1.2	10
3	10	Blue	Non-Aggressive	4.0	1.3	9
4	0	Blue	Aggressive	4.3	1.6	1
5	0	Red (green)	Aggressive	4.2	1.3	7
6	0	Blue (purple)	Non-Aggressive	4.3	1.2	-
7	10	Red (green)	Aggressive	3.9	1.2	5
8	10	Blue (purple)	Aggressive	4.2	1.3	-
9	0	Blue	Non-Aggressive	4.1	1.2	3
10	10	Red (blue)	Non-Aggressive	4.0	1.2	2

Exposure conditions and water changes

Standardized water was used throughout the experiments. It was based on deionised water with following salts added; $CaCl_2$ x $2H_2O$ (117.6 mg/L), $MgSO_4$ x $7H_2O$ (49.3 mg/L), $NaHCO_3$ (25,9 mg/L) and KCl (2.3 mg/L).

A stock solution of EE_2 (250 mg/L) was prepared by solving EE_2 (Sigma) in absolute ethanol. It was stored dark and cold.

Fish No. 1, 3, 7, 8 and 10 were exposed to EE_2 (10 ng/L) for 40 days and fish No. 2, 4, 5, 6 and 9 were used as controls (unexposed).

Water was changed two to three times a week during the first month. Later the water was changed once a week. At water changes half the volume (0.5 L) in each jar containing a single fish was replaced with standardized water. Immediately thereafter EE_2 was added to the water of exposed fish, this to obtain a nominal concentration of 10 ng/L.

Laboratory environment

The room used for laboratory work had a temperature between 26 and 28°C. A timer controlled the light cycle of ten hours light. Each Siamese fighting fish was placed in new glass jars, melded in one piece (10 x 15 cm^2 x 15 cm) and filled with 1 L of water. The jars were placed in a row on a table. Fish in the same exposure group were placed next to each other and the two exposure groups were separated by a space of 5 cm. To prevent evaporation, two glass plates were placed on top of the jars, covering one exposure group each. At every side of each jar, brown non-transparent pieces of carton paper were placed to prevent unwanted stimuli from laboratory staff or neighbouring fishes. The fish were fed once to twice a day.

Territorial behaviour

Individual fish were provoked and observed in different behavioural tests. A few of these were performed before the exposure period of 40 days, but most of the tests were performed repeatedly during the entire exposure period.

Observation of temperament

The behaviour of individual fish was observed when provoked by a mirror. The behaviour was classified into two temperamental categories: Aggressive and Non-Aggressive, (Table 1.2). Four tests, two performed before and two performed at the end of the exposure period were used to investigate differences in the temperament between and within two groups of individuals, e.g. Siamese fighting fish exposed to EE_2 (10 ng/L) for 30 days and fish used as controls (unexposed).

Table 1.2 Definitions of temperament and behavioural displays

Aggressive	The fish is close to the mirror, has raised operculae and makes aggressive displays towards the mirror image.
Non-Aggressive	The fish is far away from the mirror, has non-raised operculae and sometimes tries to escape by swimming into the wall at the opposite side from the mirror image.
Towards	The fish swims towards the conspecific and has raised operculae.
Away	The fish swims away from the conspecific and has non-raised operculae.
Backwards	The fish faces the conspecific, has raised operculae and slowly swims backwards, but is still close to its mirror image.
Forward – back again	The fish quickly swims towards the conspecific as if there was a chicken race. The operculae are raised and when close to the mirror image, the fish quickly turns and swims back from where he started with either raised or non-raised operculae.
Circle	The fish quickly swims close to at least three aquarium edges. This circle can be of different radius and the operculae of the fish can be down or erected. Some fish swim the circle in a way that more resembles the symbol of the number eight.
Escape	The fish swims away from the conspecific as in panic, trying to find a way out of the test jar at the opposite side of the aquarium.
Air Gulp	The fish is motionless and close to the surface, slowly taking air.
Stare	The fish is motionless still with raised operculae, staring at the conspecific, sometimes very close to the mirror image and sometimes further away.
Head	The fish attacks the conspecific with its head and makes a swing with the head into the mirror image.
Tail Beat	The fish attacks the conspecific with its body by making a swing with the caudal fin, which hits the mirror image.
Cringing	The fish is cringing with its body and fins, showing its side to the conspecific. The fins and operculae are fully raised.
Side	The fish is standing still with raised operculae, showing his side to the conspecific.
Nothing	The fish has been paralyzed and is frozen in same position for a long time, often in the middle or at the far end of the aquarium. The operculae are down.

Observation of displays towards a mirror

Behaviour of individual fish was observed in mirror tests. A mirror (7 x 12 cm^2) was placed at one of the short sides of the jar containing the fish being tested. When the mirror was upright and the fish saw its mirror image (a conspecific) the clock was started. The frequency of 13 behaviours was observed during 2 min.. The observed behavioural displays were called Towards, Away, Backwards, Forward - back again, Circle, Escape, Air Gulp, Stare, Head, Tail Beat, Cringing, Side and Nothing, (Table 1.2). Three tests performed at the end of the exposure period were used to investigate differences in behavioural displays between fish exposed to EE_2 (10 ng/L) for 30 days and control fish (unexposed).

Morphology of testes

Preparation of sections

The fish used in the experiments were immobilised and put to death after the exposure period of 40 days. Testes were dissected and fixed in 1.5 % paraformaldehyde and 1.5 % glutaraldehyde (1:1). After approximately two weeks the specimens were postfixated in 1 % OsO_4 for one hour. After dehydration in graded series of ethanol the tissues were embedded in Agar 100 resin. Sections (1 μm) were cut and stained with toluidine blue and examined by light microscopy.

Stereological investigation of testes

Five randomly chosen regions from a randomly chosen section of each testis of the Siamese fighting fish were selected for a stereological investigation. Each region was photographed (LEICA DC 100) at a magnification of 40 X. A transparent grid divided into 2 cm^2 was randomly placed over each photograph. Intersections between different cell types of the testes overlapping the grid were counted. This was done three times for each photograph. Counted parameters were spermatogonia, primary and secondary spermatocytes, spermatids, mature spermatozoa, lumen, basalmembrane of tubuli, interstitial cells, blood vessels and others. Due to sampling errors, only eight testes (from fish no. 3, 4, 5, 6, 7, 8, 9 and 10) were possible to analyse.

Statistical analyses

G-tests were used to investigate differences in temperament between and within the two groups of Siamese fighting fish. Critical value ($p \leq 0.05$) for these G-tests is when $G_{adj} \geq 3.84$.

Differences in the species' observed behavioural display towards a mirror due to exposure of EE_2 were analyzed with t-tests (two samples, similar variance). T-tests were also made on the total number of displays and on displays similar to each other. Critical value ($p \leq 0.05$) is when $P \leq 0.05$. Multivariate analyses, (Principal Components Analysis) were made to reveal possible hidden behavioural differences between the two groups of Siamese fighting fish matched into pairs.

T-tests (two samples, similar variance) were made to investigate differences in number of cell structures in testes and in luminal space of the testis. T-tests were also made on immature spermatozoa (spermatogonia, primary and secondary spermatocytes, and spermatids), organelles inside lumen (spermatids and lumen) and organelles outside the tubuli (basalmembrane of tubuli, interstitial cells and blood vessels). Critical value ($p \leq 0.05$) is when $P \leq 0.05$.

RESULTS

Temperament

There are no differences in Aggressive or Non-Aggressive temperament between or within the Siamese fighting fish used in this study, i.e. five Siamese fighting fish exposed to EE_2 (10 ng/L) for 30 days and five control fish (unexposed). However, when looking at differences in temperament over time within the exposed group, the value of G_{adj} is high.

G-test performed on Siamese fighting fish before and after they were exposed to EE_2 or used as controls (unexposed) showed no difference in temperament between the two groups (G_{adj} = 0.26 and 0.75, resp.). G-tests performed within the control group and within the exposed group over time, showed no difference in temperamental change (G_{adj} = 0.20 and 3.19, resp.).

Displays towards a mirror

Differences in behavioural displays towards a mirror between Siamese fighting fish exposed to EE_2 (10 ng/L) for 30 days and control fish (unexposed) can be seen in the display called Towards (P = 0.026). Exposed fish are performing this display in higher frequency than control fish. Other displays showed no difference between the groups. (Fig. 1.1)

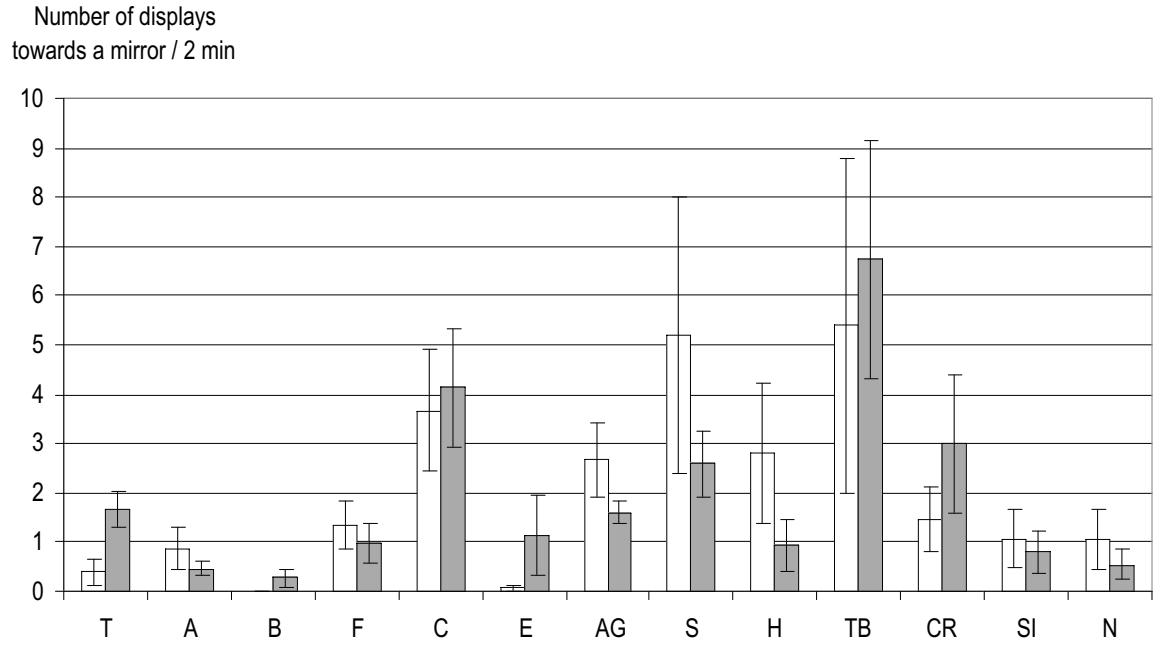

Fig. 1.1 Behavioural displays towards a mirror of two groups of Siamese fighting fish, i.e. fish exposed to ethinylestradiol, EE_2, 10 ng/L for 30 days (grey columns) and fish used as controls (unexposed) (white columns). Individuals were observed in mirror tests performed at the end of the exposure period. Numbers of displays towards the mirror during 2 minutes were counted. The data used were based on individual average displays from the three tests. T = Towards, A = Away, B = Backwards, F = Forward – Back again, C = Circle, E = Escape, AG = Air Gulp, S = Stare, H = Head, TB = Tail Beat, CR = Cringing, SI = Side, and N = Nothing

Multivariate analysis made on eight fish matched into four pairs, in which one was exposed to EE_2 (10 ng/L) for 30 days and one was used as control (unexposed), showed a clear grouping, both of exposed and unexposed fish as well as of fish of same colours, (Fig. 1.2). Multivariate analyses were also made on some of the behavioural displays observed. Those showed a grouping of exposed fish and fish of similar colour for the behaviours Towards, Backwards and Stare; Cringing and Side; and Air Gulp, Tail Beat and Escape. There was a grouping of

exposed fish for the behaviours Head and Tail Beat; and Air Gulp and total number of displays. There was also a grouping of fish with similar colour for the displays Head and Tail Beat; and Away and Escape.

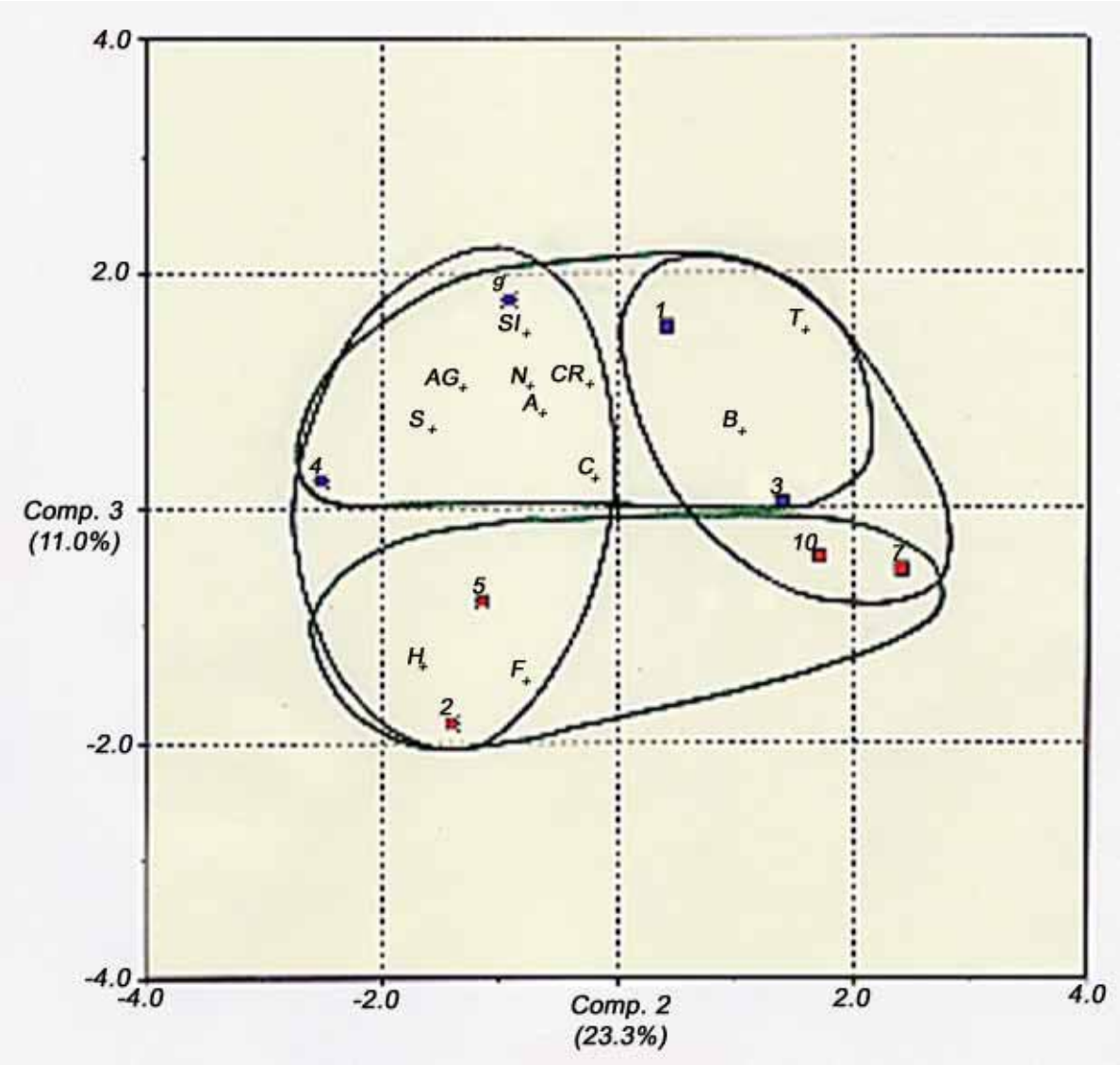

Fig. 1.2 Ordination diagram of behavioural display of Siamese fighting fish exposed to ethinylestradiol, EE_2, (10 ng/L) for 30 days or used as control (unexposed). Rings = control fish (fish no. 2, 4, 5 and 9), squares = exposed fish (fish no. 1, 3, 7 and 10), blue symbols = blue fish (fish no. 1, 3, 4 and 9), and red symbols = red fish (fish no. 2, 5, 7 and 10). Different behaviours observed; T = Towards, A = Away, B = Backwards, F = Forward – back again, C = Circle, E = Escape, AG = Air Gulp, S = Stare, H = Head, TB = Tail Beat, CR = Cringing, S = Side, N = Nothing. Comp. 2 and comp. 3 on the axes stands for the second and third principal components, i.e. the multivariate information from the dataset used, which is by the computer analyzed to explain the second respective third biggest variation within this combined data.

Stereological investigation of the testis

There is a difference in the amounts of primary spermatocytes (SCYII), and immature spermatozoa (SG, SCYI, SCYII and ST), between Siamese fighting fish exposed to EE_2 (10ng/L) for 40 days and fish used as controls (unexposed), ($P = 0.004$ and $P = 0.05$, resp.). There are no differences in amounts of other cell structures in testes between the two groups investigated (Fig 1.3).

The cell structures in testes of exposed fish were observed to be more commonly disorganized compared to cell structures in testes of unexposed fish. (Fig 1.4 and 1.5)

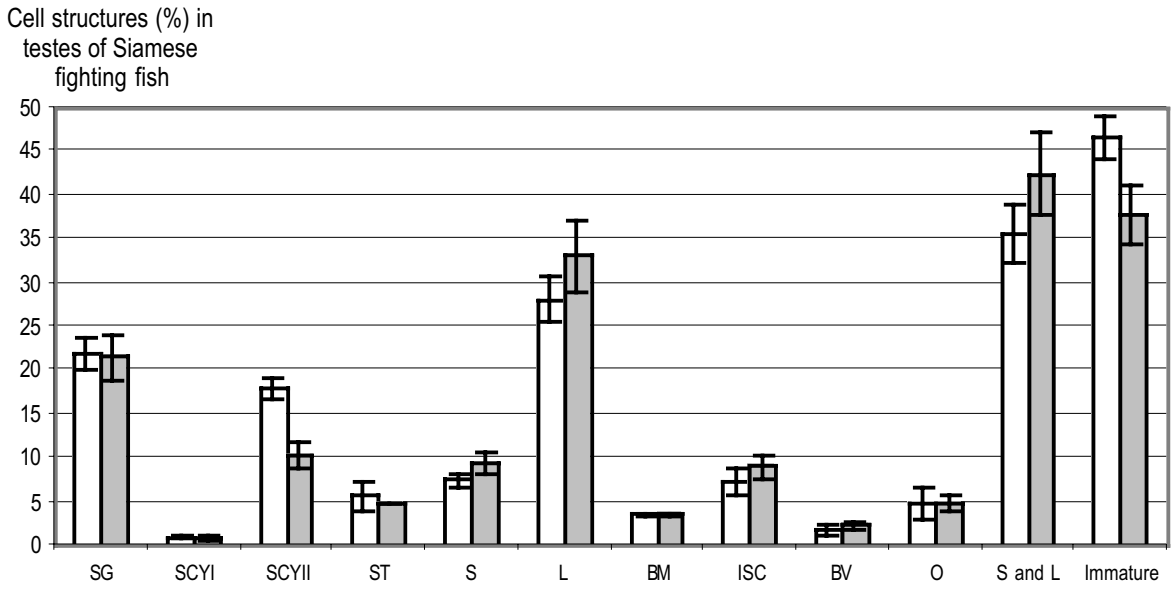

Fig 1.3 Cell structures in testes of Siamese fighting fish exposed to ethinylestradiol, EE_2, (10 ng/L) for 40 days (grey columns) and fish used as controls (unexposed) (white columns). Individual testes were investigated with stereological methods. SG = spermatogonia, SCYI = primary spermatocytes, SCYII = secondary spermatocytes, ST = spermatids, S = spermatozoa, L = lumen, BM = basal membrane of tubule, ISC = interstitional cells, BV = blood vessels, O = others, and Immature = Immature spermatozoa, i.e. spermatogonia, primary and secondary spermatocytes, and spermatids.

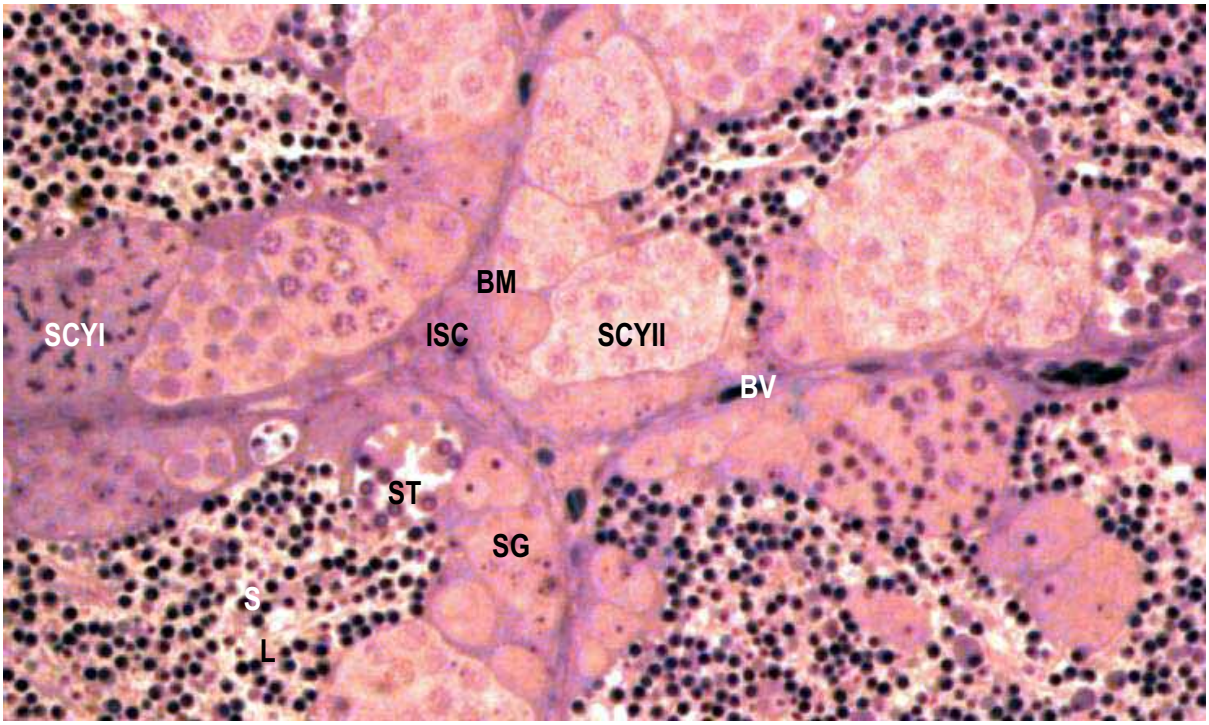

Fig. 1.4 Testis structure of Siamese fighting fish (unexposed). A clear differentiation of cell structures can be seen. Structures counted in the stereological investigation are marked; SG = Primary and secondary spermatogonia, SCYI primary spermatocytes, SCYII secondary spermatocytes, ST = spermatids, S = mature spermatozoa, L = lumen, BM = basal membrane of tubule, ISC = interstitial cells, and BV = blood vessels.

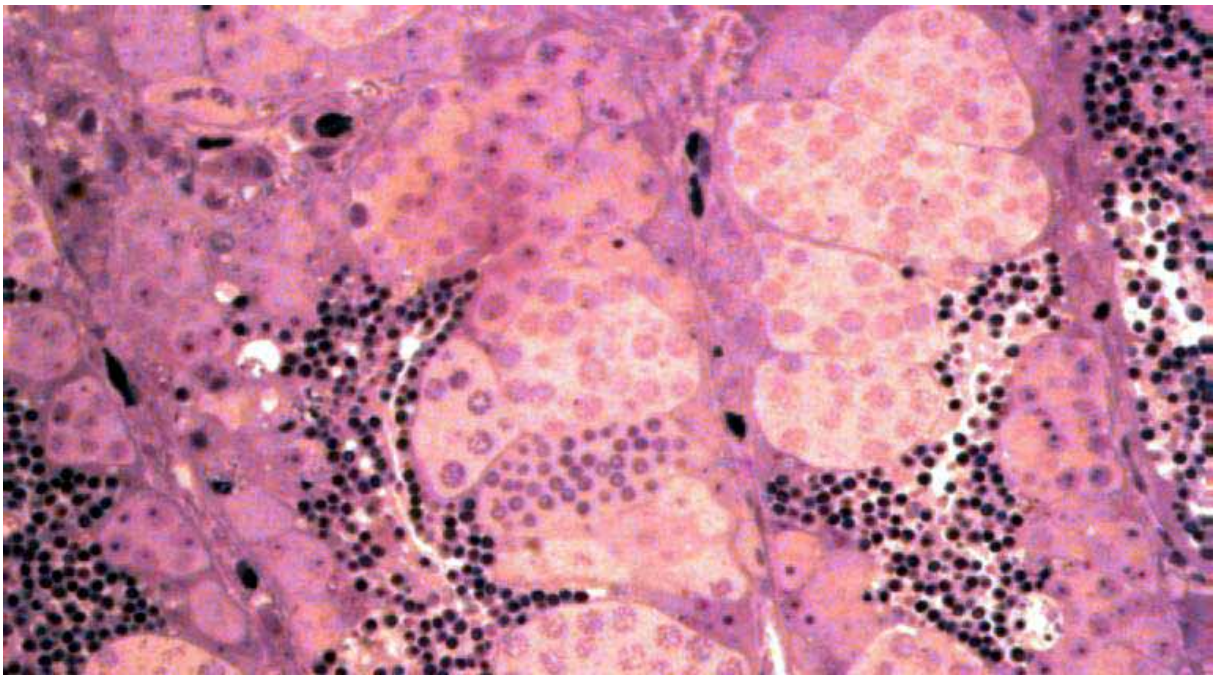

Fig 1.5 Testis of Siamese fighting fish exposed to ethinylestradiol, EE_2, (10 ng/L) for 40 days. Diffuse structures can be seen in the upper left corner.

DISCUSSION

This study shows that the testis morphology differs between unexposed and exposed fish. In addition to large numbers of immature spermatozoa (especially the primary spermatocytes) in the testes of unexposed fish, the testes of these fish seemed to have more distinct differences between the cell-types (cf. Fig. 1.4 and 1.5) The testes of exposed fish had on the contrary diffuse differences between the different cell-types. Some sections from exposed fish seemed also to have more vacuoles, a swollen basalmembrane, reduced lumen and more immature sperms. Other sections seemed to be unaffected. This high irregularity in the structures of the testes can be explained both by the fish being of different age (and hence responses different to the amount and length of exposure to EE_2) or simply by the fact that the exposure time for a clear morphology change in testis due to EE_2 is longer then the one used. The effects might just have started to appear. The concentration used is also a possible explanation. It is known that a lower concentration of exogenous estrogen could have a different effect on organelles than higher amounts. This dose dependent response was experienced by Chang *et al*. (1995), showing that 17-ß-estradiol either stimulates spermatogenesis or the sex reversal in black porgy (*Acanthopargus schlegeli)*. There is also the possibility of EE_2 acting in multiple pathways within the Siamese fighting fish. Known changes in the testis due to xenoestrogens are testicular atrophy, necrosis of seminiferous tubule and leydig cell hyperplasia (Gopinath, 1987). This could be seen in some of the sections, but not in all. The lower amount of primary spermatocytes among exposed fish indicates that a regression of these structures occurred.

One problem encountered when analyzing the collected behavioural data was that the Siamese fighting fish almost continuously were provoked with aggressive stimuli. On the other hand those many tests performed on the fish gave an opportunity to learn more about its behaviour and also to find out better ways of understanding how social interactions in the species are expressed. However, these many tests and provocations affected the behaviour of the fish. Several studies made on social interactions in *Betta splendens* indicate this phenomenon. It

has been shown that the previous social experience of an individual fish may have an effect on the strength of its aggressive display, and that the frequency in the response tends to increase with isolation and age as well as when the fish is facing a highly responsive fish. (Bronstein, 1984; Miley *et al.,* 1977; Halepin *et al.,* 1992; Halepin *et al.,* 1994; Woodward-Cain *et al.,* 1980) In some tests, fish used in this study had an abnormal increase in aggressive displays, i.e. Tail Beat and Head. When reaching this hyper-aggressive state, the individual was performing an aggressive display towards things that did not trigger any aggressive behaviour when shown to the fish in a normal condition. This aggressive state only occurred after the fish had been "primed" by several tests following each other and it was probably caused as a combination of the continuous aggressive stimulation and isolation of the fish. To minimize the risk of using data from hyper-aggressive fish, the data analyzed were from tests performed after the fish had rested for at least two days. However this time might have been too short. The memory of fish could be longer, but not certainly. According to Bronstein *et al.,* 1989, the priming and retention of agonistic motivation seems to be almost instant, but the memory of the species is not mentioned.

By using a mirror instead of live interactions, the fish was always exposed to another fish (its mirror image) of the same size, thus eliminating a response correlated to the size of the opponent. It is likely that a much bigger encounter induces a submissive display whereas a smaller one triggers an aggressive behavioural response. Why go into a fight if you most likely will loose? The mirror also enabled elimination of possible injuries caused from fights as well as maintaining most of the characteristics of a live interaction. But when using a mirror each individual is exposed to itself, and since they all look different, including different colour, it is possible that fish might respond differently. Woodward Cain *et al.,* (1980) showed that the fighting fish tend to attack aggressive opponents more than non-aggressive ones, and that the fish aggress when the individual is threatened or when the physical space is limited. This means that when a fish is facing a mirror image, it can trigger itself into a stronger display compared to a fish that is more careful in its approach to the mirror encounter. The test jars used in the tests were the same as the ones the fish were kept in. Hence, this small space might also contribute to the aggressive displays. The memory of the fish is also an issue that is reflecting the outcome of the fights. The fights end when the mirror is taken away and the fish might believe he was the cause of the fight ending. The winner has gained a better self esteem, and might be rougher in the following fights. Another problem when using a mirror is that the encounter is exactly mimicking the individual fish display. When facing live encounters, the fish displays every other turn. What the mimicked behaviour might do to the behavioural response of the fish is not fully known.

There was one difference in the display called Towards between the two exposure groups. A very high variance in the data might contribute to the insignificant results in the other observed displays. Only a few fish displayed the parameter Towards and at low frequency. Therefore it had much lower variance, which might contribute to the statistical significance. Another explanation is that the exposed fish had difficulties to understand what they were facing. It took some time for the fish to figure out that it really was an intruder, and hence they approached the mirror slowly and many times. The control fish (and hyper aggressive fish) understood immediately that it was an intruder and made aggressive displays towards it (with the difference that hyper-aggressive fish thought everything was intruders). The smaller frequency of the display Towards could also be a first sign of a change in behaviour due to exposure of EE_2. Other behavioural changes might first be seen after a longer exposure period as mentioned above, or if the fish should have been exposed during its neonatal or pubertal periods in life.

The temperamental test is the only test looking at the same exposure group over time. In behavioural studies it could be more accurate to have the same individuals before a test as controls, and then redo the test with some parameter changed. On the other hand, that could mean some changes in the behaviour due to the time between the two tests. To have two different exposure groups could reflect individual differences rather than the parameter investigated. In the temperamental test, both changes within the exposure groups over time as well as changes between the groups were studied. However, the result indicates that there might be a tendency to less aggressiveness due to exposure of EE_2 since those fish seemed to have become less aggressive over time, although it was statistically not significant.

Highly interesting is the multivariate analysis. It shows that exposed fish have behaviours that are similar to each other and that the control fish does not behave in same way. It also shows that fish of blue colour have similar behaviours, different from red coloured fish. There is of course a small chance that this is due to individual differences since only eight fish were analyzed.

In a future study it should be wise to let the fishes rest from aggressive stimuli before any tests are made. This rest combined with showing non-aggressive stimuli to the fishes would reduce the risk to trigger the fish into a hyper-aggressive response. A good idea is to have one side of the jars free and not to isolate the fish completely. The fish will naturally be exposed to stimuli caused by laboratory staff and the laboratory environment itself. At the same time the possibility of the laboratory staff affecting the fish negatively during the tests is reduced when the fish are used to their presence. It would also be wise to investigate the individual natural behaviour of the fish before any exposure as well as to use bigger test jars that would enable individuals to naturally avoid the mirror image.

STUDY 2

- Exposure to Genestein and Wastewater from a Tofu Factory -

A Minor Field Study

MATERIAL AND METHODS

Experimental animals

Domesticated Siamese fighting fish

30 male Siamese fighting fish of various size and colour were bought from a local fish store in Chiang Mai, Thailand. (Table 2.1) The temperament of individual fish was based on observations of the behaviour of the fish in connection with provocation to a pencil, each other and a mirror. The temperament was classified into three categories: Aggressive, Passive and Non-Aggressive. Aggressive individuals were interested in the object, had raised operculae and were making aggressive displays towards it. Passive individuals were insecure and did not know how to behave, some periods they were aggressive and other periods submissive to the stimuli. The Non-Aggressive fish were submissive, had down folded operculae and sometimes tried to escape by swimming away from the object. Length and weight were measured after the exposure period and final tests. The length was measured from the tip of the upper jaw to the caudal peduncle. The colour of the fish was based on observations.

Fish with similar temperament were randomly divided into three groups: fish going to be exposed to wastewater from a tofu factory (exposure group), the chemical genistein (positive control) and fish not going to be exposed (negative control). No consideration was taken to the fish colour or size. After dividing the fish into these groups, individuals were given a number for identification.

Wild-type Siamese fighting fish

Newly caught wild-type Siamese fighting fish, 5 males and 5 females, were bought from a local fish shop in Chiang Mai, Thailand. The natural habitat for these fish was in a river close to a village which was located closely to the Maejo University, approximately 15 km north of Chiang Mai city.

The fish had a length of 2.9 ± 0.3cm. Males and females were of similar phenotype. They had short fins and a brownish colour. Females showed a ventrally (white egg spot at the belly) when ready for spawning. Wide longitudinal dark stripes appeared on the female bodies when they found a partner. Males interested in females built big bubblenests. When carefully provoked with a mirror, the males showed an intense change in colour. The fish darkened and as its fins were raised, green-turquoise bright stripes between the rays of the dorsal and caudal fins appeared within a second.

During the entire experimental period the wild-type fish were very afraid and stressed. They often showed the characteristic submissive behaviour: bleaching in colour, fins close to its body, the appearance of two dark longitudinal lines on the body and lying motionless at the bottom of the jar. (Fig. 2.1)

Fig. 2.1 One of the male wild-type Siamese fighting fish acting submissive, trying to escape the camera. Photo: A. Foberg

The fish were easily infected by fungus. Two of the males died in fungal infections, although they were treated with antibiotics. Four other fish died due to stress. No wild-type fish were investigated further since it was impossible to perform any accurate tests.

Table 2.1 Characteristics of Siamese fighting fish not going to be exposed (negative control) (fish no. 1-10), fish going to be exposed to wastewater (5 %) from a tofu factory in Thailand (exposure group) (fish no. 11-20) and fish going to be exposed to genistein (100 μg/L) (positive control) (fish no. 21-30).

Fish No.	Length (cm) ± 0,2 cm	Weight (g) ± 0,001g	Temperament	Colour
1	4,3	1,98	Aggressive	Blue body and fins, darker blue colour on the head, a reddish touch at the stomach area, dark red pelvic fins with white tips.
2	4,1	1,90	Aggressive	Turquoise blue body that has a soft green shimmering tone, turquoise fins.
3	4,1	1,82	Aggressive	Red body, head and fins, green sparkly spots at the body, some green rays in the fins.
4	4,0	1,87	Aggressive	Turquoise green body and fins, a shimmering tone of red on the stomach area, dark red pelvic fins.
5	3,6	1,26	Aggressive	Purple red body and head, more reddish fins.
6	4,3	1,87	Passive	Blue green body, dark green head, blue turquoise fins with a brown colour at the area close to the body, brown pelvic fins.
7	4,0	1,79	Passive	Dark blue body with a greyer tone at the head. Redder colour at the stomach area, red pelvic fins.
8	4,1	1,74	Passive	Dark purple blue black body and fins, some reddish stripes at the anal and caudal fin.
9	4,2	1,99	Non-Aggressive	Red body and fins.
10	3,7	1,53	Non-Aggressive	Orange red body and fins, purple rays in the dorsal, anal and caudal fins, some black spots on the stomach area.
11	4,3	2,09	Aggressive	Turquoise blue green body and head, some red rays at the fins.
12	4,3	2,08	Aggressive	Dark blue black body and head, blue fins with rays of red colour.
13	4,5	2,04	Aggressive	Dark blue body, head and fins, some red stripes at the caudal fin.
14	4,0	1,96	Aggressive	Dark blue body and head, red stripes at the otherwise blue fins, dark red spots on the stomach area.
15	4,0	1,59	Aggressive	Black blue body and head, red purple and green spots on the caudal fin.
16	4,2	1,81	Passive	Black blue and steel grey body and head, a touch of dark red on the stomach area and also on the caudal fin.
17	4,1	1,91	Passive	Black blue body and fins.
18	4,0	1,56	Passive	Red orange body, head and fins, purple touch at the back area.
19	4,2	2,07	Non-Aggressive	Turquoise green blue body and head, turquoise fins except the pelvic fins that are red in colour.
20	4,0	1,63	Non-Aggressive	White brown body with brownish head, shimmering spots of green and blue on the body area.
21	4,4	1,99	Aggressive	Dark blue purple body, head and fins, reddish shimmering colour at the stomach area, turquoise green rays in the fins.
22	3,9	1,56	Aggressive	Dark red and brown body, head and fins, a purple touch on the fins.
23	4,2	1,87	Aggressive	Dark red body and fins, brighter red dorsal and caudal fin, some shimmering blue spots on the stomach area.
24	4,1	1,77	Aggressive	Red body, head and fins, some blue shimmering spots at the back, blue rays at the dorsal fin, green rays at the caudal fin.
25	4,0	1,47	Aggressive	Dark blue body and head, dark blue fins with a dark red touch, at the end of the caudal fin there is a transparent blue black area.
26	4,1	1,72	Passive	Dark blue body and head, dark blue turquoise caudal fin, dark red brown pelvic fins.
27	3,5	1,31	Passive	Red orange body, head and fins.
28	4,1	1,57	Passive	Purple body and head, dark purple fins with a touch of blue.
29	3,9	1,65	Non-Aggressive	Red body with a touch of orange, red head and fins, some green rays in the fins.
30	4,3	1,91	Non-Aggressive	Dark blue body and black blue head, dark blue fins with a dark red tone, a transparent area at the end of the dorsal and caudal fin.

Exposure conditions

During 21 days fish Nos. 1-10 were not exposed (negative control), fish No. 11-20 were exposed to wastewater (5 %) from a tofu factory in Thailand (exposure group) and fish No. 21-30 were exposed to genistein (100 µg/mL) (positive control).

Laboratory water

Laboratory water was used throughout the study. It was taken directly from the taps in the Aquatic Laboratory at the Faculty of Veterinarian Medicine, Chiang Mai University. The water had its origin from a University pond and was pumped into the building. Before entering the taps it was cleaned in a small cleaning facility. First the water went through a high pressure water pump into a filter turbid part (A.T.C. anthracite). It continued through a trap odor part (Calgon CAL Carbon) which contained 30 L of water. Filters of 5 and 1 micron as well as a bacterial filter of 0.3 micron were excluding organic particles. As a final step, the water was sterilized by ultraviolet radiation (39 watt). The water pH was between 6.5 and 7.5.

Wastewater from a tofu factory

The wastewater collected from the tofu factory was mixed, poured into plastic bags (2-5 L) and immediately frozen. The mixed water showed high amounts of nitrate and nitrite. At every water change some of it was diluted with tap water from the aqua laboratory to a concentration of 5 %. After the experiments, the wastewater was chemically analyzed at the Faculty of Veterinarian Medicine, Chiang Mai University. This analysis showed that the wastewater contained; DO = 18 ppm, pH = 4.55, Nitrite = 0.22 ppm, Nitrate = 6 ppm, Ammonia = 0.001 ppm, Alkalinity ($CaCo_3$) = 160 ppm, BOD7 = 14 ppm.

Collection site

The tofu factory was situated in the north east of Chiang Mai, about 40 km from Chiang Mai city. The collection site was directly after that the wastewater had passed through a cleaning facility and before entering its recipient, a small river leading to a pond, (Fig. 2.2). In the process, soybeans were used as the only raw material. Some salts were also added. The factory was using about 600 kg of soy beans a day, but it could differ somewhat depending on the market and the soybean quality. Each day about 500kg of tofu products were produced.

Fig. 2.2 Photograph of the recipient. Photo: A. Foberg

Genestein water

A stock solution of genistein (200 mg/mL) (Sigma) was prepared in 30 % ethanol. It was stored dark and cold. At every water change some of the stock solution was diluted with laboratory water to obtain a nominal concentration of 100 µg/mL.

Estimation of exposure

The estimation of the exposure concentration of genistein was based on a worst case scenario (Blomberg, M., in prep.) Each day approximately 600 kg soy beans were entering the factory. From this amount 500 kg tofu was produced. Genistein from at least 100 kg (600 kg - 500 kg)

soy beans could enter the wastewater each day. The water flow was measured to 0.23 L/s at a pipe (6 cm ∅) entering the cleaning facility and 0.015 L/s at a pipe (4 cm∅) leaving the cleaning facility. The low water flow in this last pipe was reflecting the low production during nights. The factory was estimated to produce tofu about ten hours a day. That means a water flow of 8250 L each day (0,23 L/s · 10 hours · 3600 s = 8250 L/day). The concentration of soybean products leaving the factory is 12 /L (100 kg soybeans each day / 8250 L water flow each day = 12 g/L soybeans). In soybeans there is approximately 8 µg genistein/g. Hence, in this worst case scenario, the concentration of genistein leaving the factory is 100 µg/L (12 g/L soybean products · 8 µg genistein/g soybean ≈ 100 µg/L).

Water changes

Half the water volume in each jar, i.e. 0.25 L, was changed every third day. The water was pumped out from the jars by hand pumps. Laboratory water was added to unexposed fish and fish exposed to genistein. Immediately thereafter, genistein was added directly to the fish in the positive control group, this to obtain a nominal concentration of 100 µg/L. Frozen wastewater (2-3 L) was taken to thaw, diluted with laboratory water (5 %) and added to the fish in the exposure group.

Laboratory environment

Each fish was placed in newly made aquaria (10 x 10 x 15 cm^3) and put in a single row. Three sides of each aquarium were covered with pieces of brown cartons to avoid visual contact between the individuals. The carton-walls were partly painted in four different colours: red, blue, green and black. These painted areas were of different style, size and motives such as dots, lines, squares and rings. The carton pieces were moved or turned about twice a week. The third side of every jar was facing an open area where students and staff passed by about 3-5 days a week. The temperature inside the laboratory followed the season. The average temperatures at the beginning of the study were approximately 25 °C during days and 10-15 °C at nights. At the end of the experiments the average temperatures were 35-40 °C during days and 20-25 °C at nights. The sunrise was about 6 a.m. and sunset around 7 p.m. The fish were fed twice a day. Every day faeces were taken out with a group specific pipette. The fish was acclimatized for one week before the experiments started.

Observation of behaviour towards a mirror

The behaviour of individual fish was observed in connection with provocation with a mirror. The fish was exposed to a mirror for 10 minutes and four displays called Time Close, Tail Beat, Air Gulp and Escape were counted (Table 2.2). Two mirror tests were performed before the exposure period of 21 days (day 1 and 3) and two mirror tests were performed at the end of the exposure period (days 21 and 23).

Table 2.2 Definition of displays

Time Close	The time the fish (the fish eye) is within 8 cm distance to the mirror.
Tail Beat	Rapid movements of the fish tail towards the mirror image. The beat must be clear and distinct in order to be counted.
Air Gulp	The fish takes air from the surface independently of its location in the aquarium.
Escape	The fish tries to swim out of the aquarium, away from the mirror image in a panic-like behaviour.

Experimental setup

Nine test aquaria (15 x 25 x 15 cm^3) were used. Under each aquarium (8 cm from one short side) green synthetic threads were placed. These threads were marker lines for the parameter Time Close. Each side of the test aquaria were isolated by thick carton paper in order to minimize movements and other visual stimuli from the surroundings. Water from the laboratory taps was added to a height of 10-12 cm in each aquarium. The fish being tested was acclimatized in the test aquarium for one to two hours before the initiation of the test. Two stopwatches were used to measure time, one for measuring the total experimental time of 10 min and one for measuring Time Close. Before every test, the water in each test aquarium was changed. Same aquaria were only used for fish within same exposure group. When the fish entered the 8 cm marked area, a mirror (15 x 20 cm^2) was placed in front of the fish and the test was initiated.

Statistical analysis

Multivariate analysis (Principal Components Analysis) was made to reveal possible hidden behavioural differences between the three groups of Siamese fighting fish.

RESULTS

The two multivariate analyses made on behavioural data from 30 Siamese fighting fish show no correlation between exposed fish and behaviour. However, when comparing the two analyses, red fish (fish no. 5, 10, 17, 18 and 27) seem to have an altered behaviour. They are displaying more Escape and Air Gulp after the exposure. (Fig. 2.3 and 2.4)

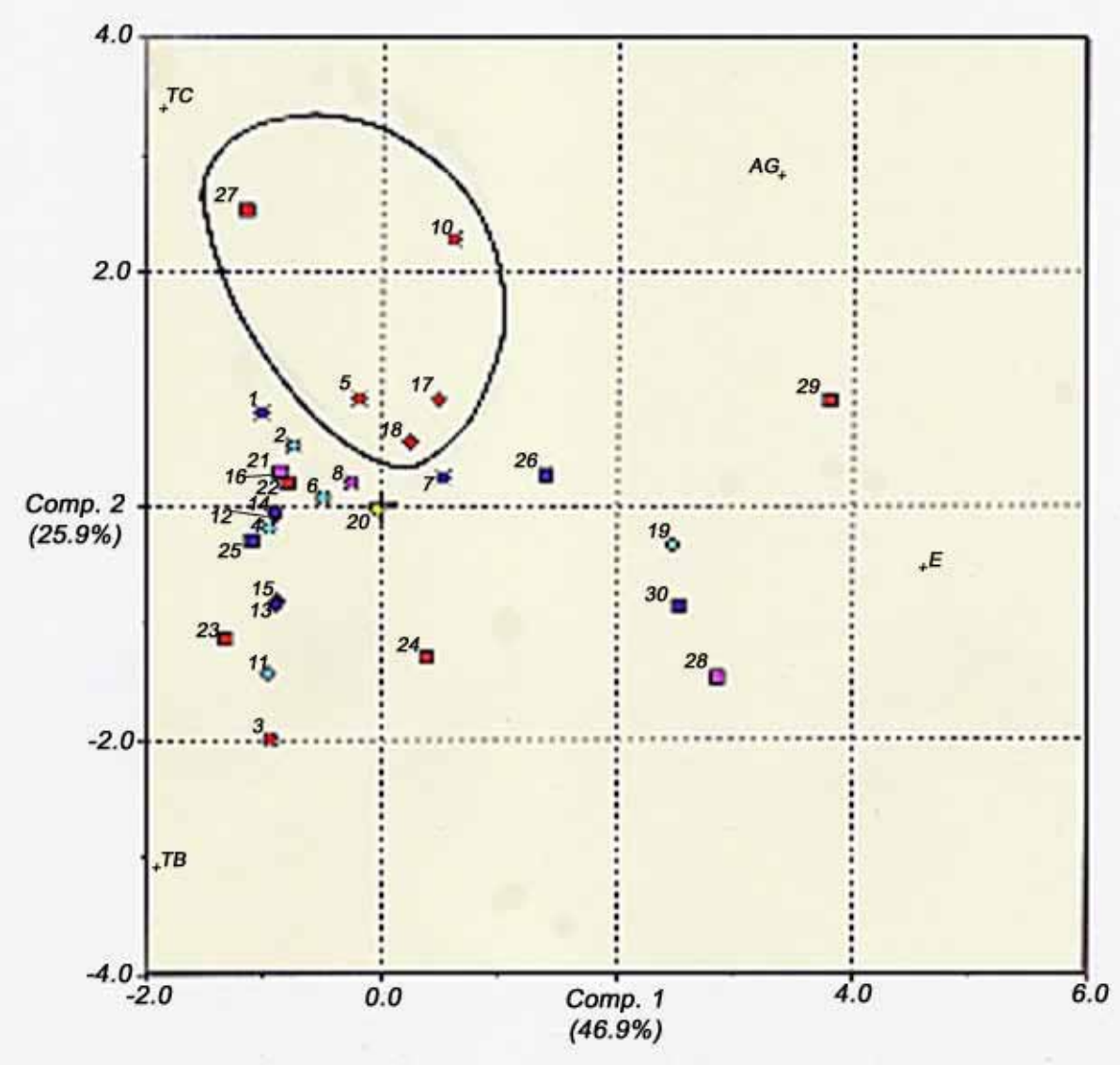

Fig. 2.3 Ordination diagram of Siamese fighting fish before an exposure to exogenous estrogen. Four displays were observed: Escape = E, Air Gulp = AG, Tail Beat = TB and Time Close = TC, for three groups of fish, i.e. fish going to be used as negative control (unexposed) (fish no. 1-10) = Rings, fish going to be exposed to wastewater (5 %) from a tofu factory (exposure group) (fish no. 11-20) = Squares, and fish going to be exposed to genistein (100 µg/L) (positive control) (fish no. 21-30) = Diamonds. Numbers next to the symbols are individual fish numbers and the colour of these symbols matches the colour of the fish. The marked fish will change their behaviours into more submissive ones (compare fig. 2.2) Comp. 1 and comp. 2 on the axes stand for the first and second principal components, i.e. the multivariate information from the dataset used, which is by the computer analyzed to explain the first and second biggest variation within this combined data.

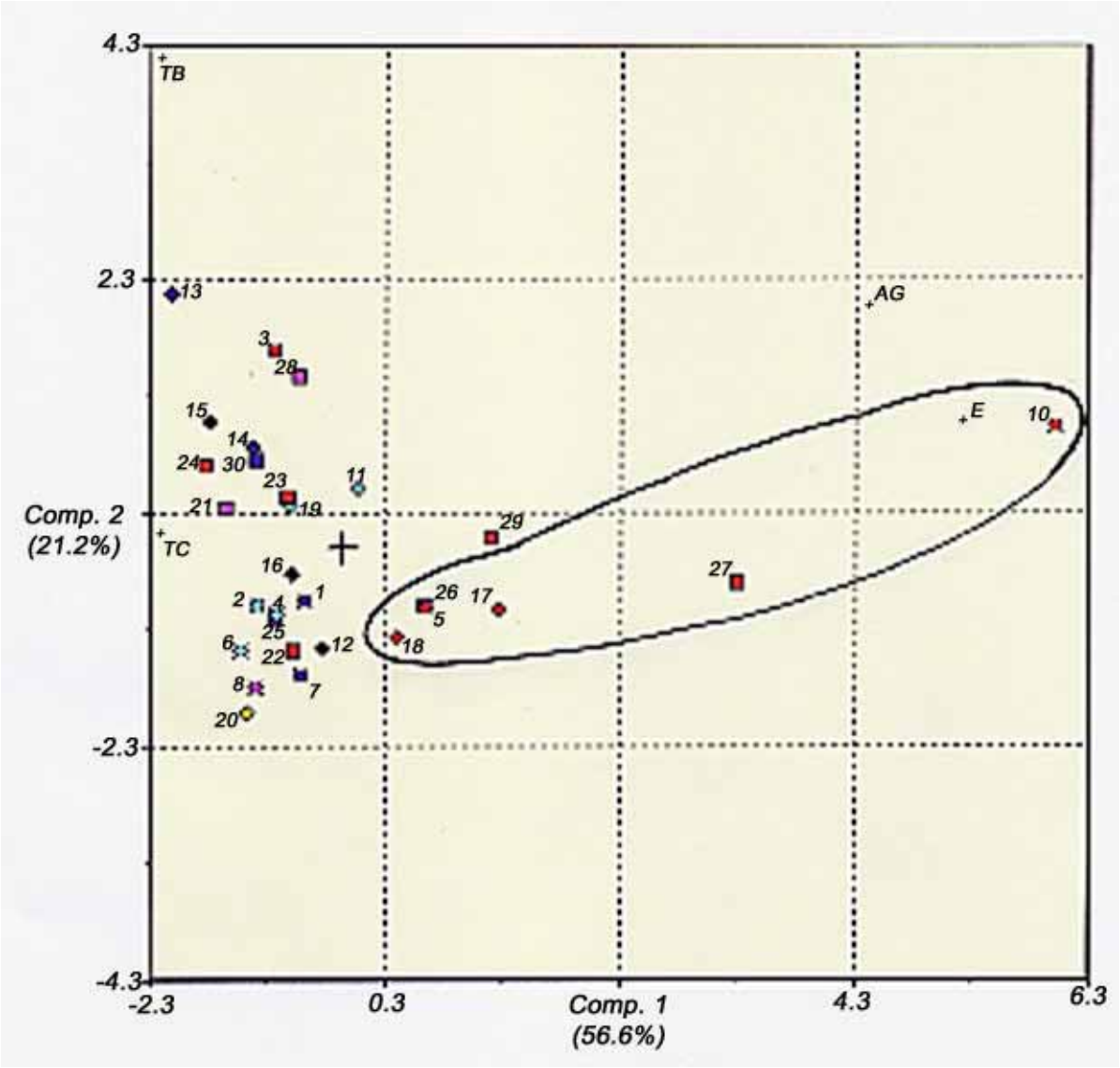

Fig. 2.4 Ordination diagram of Siamese fighting fish after an exposure to exogenous estrogen for 21 days. Four displays were observed: Escape = E, Air Gulp = AG, Tail Beat = TB and Time Close = TC, for three groups of fish, i.e. fish used as negative control (unexposed) (fish no. 1-10) = Rings, fish exposed to wastewater (5 %) from a tofu factory (exposure group) (fish no. 11-20) = Squares, and fish exposed to genistein (100 μg/L) (positive control) (fish no. 21-30) = Diamonds. Numbers next to the symbols are individual fish numbers and the colour of these symbols matches the colour of the fish. The marked fish have changed their behaviours into more submissive ones (compare Fig. 2.1) Comp. 1 and comp. 2 on the axes stand for the first and second principal components, i.e. the multivariate information from the dataset used, which is by the computer analyzed to explain the first and second biggest variation within this combined data.

DISCUSSION

The results from the multivariate analyses show no positive correlation between different behaviours and exposure of Siamese fighting fish to exogenous estrogens, i.e. genistein and wastewater from a tofu factory. However, some of the red fish have a more altered behaviour towards the displays Air Gulp and Escape compared to other fish. These results in combination with the results obtained from the first study in this thesis (Study 1 – Exposure to ethinylestradiol, EE_2) indicate that the colour of the fish has influence on the fish behaviour. The question to be asked is if this is due to different genetics of the fish of different colours,

or rather if this is due to different responses elicited by the mirror image, showing the fish its own colour?

One possible explanation for the absence of correlation between exposure groups and altered behaviour in the ordination diagrams is that the four displays investigated did not contain sufficient individual behavioural information. The idea was that Air Gulp should indicate if the individuals were stressed; hence need to take extra oxygen from the surface. Escape should indicate a clear submissive behaviour and Tail Beat a clear aggressive behaviour. The parameter Time Close should indicate fish acting territorial (including individuals that were territorial, but that did not display Tail Beat in high frequency). These few observed displays in combination with a variety of fish colours might have contributed to the unclear grouping of fish after the exposure.

The fish used in this study were not divided into groups based on colours. This was based on the theory that even though the fish responses differently to different colours, it would not necessarily mean that an exposure to exogenous estrogens would have a different effect on behaviour of individual fish. For example, fish of different colours should all show a less aggressive behaviour compared to their behaviour before an exposure. Therefore no consideration was taken to the fish colour before it was divided into matched exposure groups based on temperament. Theoretically it should be the temperament of individual fish that in highest grade determines the basic behaviour of the fish. Besides that, each temperamental category contained fish of every colour. From this point of view, the temperamental classification was correlated to colour since it was based on observations from the fish behaviour when it faced itself (its mirror image). In addition there were no colour-related behaviours observed among the fish. Also the size of the fish should not be of concern either because it was facing an opponent of the same size as itself.

However, it seems as the assumption that colour is not important when looking at changes in the behaviour of the fish due to an exposure to exogenous estrogens, was a mistake. Locally breeders in Thailand explained that red Siamese fighting fish in general are more aggressive than other fish, and that wild-type fish have the roughest aggressive display among the species. This may indicate that genes encoding for the aggressive display of the Siamese fighting fish are in connection to genes encoding for the colour of the species? Weiss *et al.* (1979), showed that gonadoectomized male Siamese fighting fish maintained their aggressive behaviour. On the contrary, many animals, including us humans, often become calmer after a successful castration. According to Weiss *et al.* the maintenance of the aggressive behaviour of *Betta splendens* is interpreted to mean that the adult aggressive behaviour in the species may not be under the control of gonadal hormones, and that the pituitary gonadotropins and internal glands are possible explanations of this maintained aggressiveness. However, a short recovery period of two weeks was used before testing the aggressiveness of the fish a second time. It is possible that it requires a longer period of time to observe behavioural effects from gonadoectomized animals.

Other possible explanations for the unclear change in behaviours are that the exposure time might have been too short and that the phytoestrogens genestein and daidzein do not have the same estrogenic potency as EE_2. In addition, the time used in this study was even shorter than the one used when the species was exposed to EE_2. The fact that genestein is less potent compared to EE_2, indicates that it should take longer time to see behavioural changes when fish are exposed to genestein compared to EE_2. Also, it was necessary to dilute the wastewater from the tofu factory. This means a lower dose of phytoestrogens in the water, which also might have contributed to the unclear behavioural change of the fish.

Another explanation for this unclear change is that these phytoestrogens do not affect the fish through the same chemical pathways as EE_2, and hence the aggressive display is not affected in the same way. This theory agrees with the study of Weiss *et al.* (1979). Female Siamese fighting fish perform similar aggressive displays as males when not ready for spawning (or sometimes towards other females). This might indicate that the aggressive behaviour is not only a male secondary sex characteristic. Among other animals, aggressive behaviours are considered as secondary sex characteristics. This is often combined with higher concentrations of testosterone in the blood. But not all aggressive behaviours are secondary sex characteristics. Maybe aggressive displays towards individuals of the same sex within the Siamese fighting fish are not secondary sex characteristics, and hence are not affected by the less potent chemical genestein? It is possible that EE_2 is potent enough and also able to affect the species through different mechanisms and hormonal pathways, which caused the altered behaviour in the first study.

There were also no assurances that the fish used in this study had not been exposed to hormones during its breeding. It is commercially more profit in male fish, since they are used more and are more beautiful, hence more expensive. To make genotypic females into phenotypic males is not unusual.

The fact that it was impossible to investigate the behaviour of wild-type fish indicates that behavioural studies on wild-type fish need to be conducted in other experimental setups. It is possible that a non-behavioural investigation of the fish will show effects of exposure of environmental estrogens more effective. The wild-type fish responded to the mirror in a very careful way. It seemed as if the fish became insecure when the mirror image displayed the same behaviour as the fish. In fights towards other individuals, the fish displays every other turn, and when this did not occur, the fish got confused.

It is also possible that there are clear changes in the reproductional behaviour of the fish, building of bubble nests and a change in the colour due to exposure of xenoestrogens. This is investigated by Blomberg M., (in prep.).

In a future study it should be wise to use fish of similar colour and size (age), and to do a closer investigation of the fish behaviour before an exposure. By using the tools of multivariate analysis in an early phase, fish of similar behaviour can easily be selected for further investigations. These individuals of similar behaviour could be divided into exposure groups and the effects of an exposure might more easily be seen. A longer exposure period should also be used. Tests can be performed every other week and the effect of an altered behaviour due to exposure can be followed through time. During this time it is important to stimulate the fish in order to prevent them from becoming hyper-aggressive.

CONCLUSION OF THE TWO STUDIES

The Siamese fighting fish is a promising candidate for evaluating endocrine disrupting chemicals. The species' pronounced behaviour, survival capacity in low oxygen water, the fact that they are small, easily available and relatively cheap, makes it an easy tool to use both in environmental monitoring programmes and in laboratory studies. Furthermore, the fish have been studied for a long time which makes them more valuable since there is past experience.

For future investigations, the fish to be used should be of the same age and colour. It should not have been bred for its fighting ability, which can influence the results in a behavioural study. When investigating aggressiveness, the experimental tanks should not be too small and contain a place for the fish to hide in order to have a non-forcing fight. If possible, fish with similar behaviour should be chosen and then divided into exposure groups. In that way the fish investigated would be more similar, and changes due to an exposure would be more easily detected. This could be done efficiently by using the tools of multivariate analysis. If this is no possibility, previous experiments have shown that the behavioural parameters studied, easily can be counted and measured with a high accuracy during the same period of time, this without making a video recording of the fish performance. When recording the parameters used in this study, it is easy to get an indication the temperament of the fish, i.e. Aggressive, Passive or Non-Aggressive.

Aggressive interactions between individuals of the same species might contain information on relative aspects such as fighting ability, condition and motivation that not could be gained from the signals alone. Apparently it is more useful to include all displays performed by the fish in multivariate analysis.

The advantage of using a mirror is that the fish will encounter a fish of a similar size and reduction of possible fish injuries in a fight. If fish of similar colour is used, eventually problems and questions correlated to fish colour are eliminated.

EPILOGUE

The first time I heard of endocrine disrupting chemicals (EDCs) released into the environment and the effects these chemicals have on wildlife fauna, I got really scared and upset. Yet another destructive consequence to life itself caused by us humans! It is unfair to every living organism and most of all unfair to coming generations, both our own as well as that of other animals. Nowadays, when more is known about environmental effects caused by EDCs there is still a growing discharge of these contaminants into the environment. Why do we have to continue with this when it is not only affecting animal reproduction and ecosystems negatively, but in the long run also our own future world?

Economics and money are still and have always been the major driving force for modern man. To make profit is unfortunately what counts. I realize it is difficult to stop using and producing products that are already established on the market, particularly those products which make human life easier to live. To exchange these products into less harmful ones is often an expensive and long-term project, which in many people's eyes is more than difficult to accomplish; especially when the alternatives have less function or profit. Sadly not so many ordinary people know about environmental problems caused by EDCs. Maybe if more people knew about this, the politic and economic views would change?

At the end of my education (Biology, Ecotoxicology at Uppsala University,) when the opportunity for me to apply for an MFS-scholarship occurred, I saw a great chance to learn more about EDCs as well as to perform a minor research with a friend in a foreign country. The MFS-scholarship resulted in two papers, one conducted by me and one (in preparation) conducted by my collaborator Maria Blomberg. These two studies are the first studies that provide baseline information regarding suitability of the Siamese fighting fish as a new candidate for evaluations of EDCs. Besides contributing to my personal knowledge and interest that I have gained by doing this research, I also hope this paper will give at least a few more people an awareness of some of the various and unnatural effects in animal reproduction connected to EDCs.

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Sincerely yours,

Anna Foberg

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