No indications of socially induced changes in brain aromatase activity in guppy (*Poecilia reticulata*) males

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Abstract

Aromatase is the enzyme that catalyzes the conversion of androgens into estrogens. It’s a member of P450 cytochrome family and is encoded by the CYP19-gene. The enzyme aromatase has an important role in regulating physiological and behavioral sexual mechanisms. This includes for instance activation, motivation and maintenance of the reproductive behaviors. The sexual behavior is affected by a complex series of events that requires the connection of endogenous hormonal and neurochemical changes with social interactions, especially between the opposite sexes. The aim of the present study was to examine how social interactions effect the aromatase expression and activity in the guppy brain. Guppy males were introduced into four different social conditions: Isolated, all male conditions, heterospecific (with zebrafish females) and conspecific female guppies. The focal males were kept under these conditions for two respectively four days. The sexual behavior, of each of the focal males was recorded daily during 10 minutes. The males with the guppy females showed, in contrast to the males in the other groups, a high frequency of reproductive behaviors. The brains of the focal males were collected and the brain aromatase activity was measured using tritiated water assay. I have also tried to analyze the gene-expression of aromatase with RT-PCR. However I was unable to analyze the results with the RT-PCR, because of possible primer-dimerization. Due to the limited time schedule, we were not able to solve the problem. ANOVA performed on the aromatase activity, revealed no significant difference between the different treatment groups. The variance was highest in the zebrafish category and lowest in the isolated males. There was no significant correlation between the mean number of reproductive behaviors and the aromatase activity in males that were together with guppy females. The results do not support the hypothesis that social interactions can affect the brain aromatase activity in guppy males.
Introduction

The enzyme aromatase, (P450_{arom}), is a member of the P450 cytochrome family complex and encoded by the CYP19-gene. The enzyme is also known as estrogen synthase as it catalyzes the conversion of testosterone (T) into 17β-estradiol (E$_2$) and androstenedione into estrone (Lephart 1996, Balthazart and Ball 1998). The aromatization of androgens to estrogens takes place in the endoplasmic reticulum and is classified as several-functions of oxidase reactions (McPhaul et.al 1988, Lephart 1996). A central function of aromatization is to play a very important and limiting role by testosterone in the control of many behavioral and physiological processes connected to reproduction. It has been shown to be important during activation and motivation of copulation, sexual partner preference, maintenance of masculinization of sexual behavior in males, steroid-hormone feedback on secretion of gonadotropic steroid hormone levels in the blood plasma and CNS-development (e.g. MacLusky and Naftolin 1981, Bakker et.al 1996, Bakker et.al 2002, Balthazart and Ball 1998, Balthazart et.al 2006, Dessi-Fulgheri 1982, Hallgren et.al 2006, Lephart 1996, Pierman et.al 2006, Steel and Hutchinson 1987, Todd et.al 2005). In all vertebrates mating behavior is composed of a complex series of events. Behavioral responses require the capability to connect endogenous hormonal and neurochemical changes with social interactions (Marsh et.al 2006). The most essential interactions among animals are between conspecifics. These social signals are often sexual in nature and have deep effects on neural function and behavioral profiles (Dessi-Fulgheri 1982, Marsh et.al 2006). It is a challenge in science to understand the mechanisms behind behavioral adaptations to changing social conditions (Marsh et.al 2006).

In some vertebrates there is more than one gene encoding for P450_{arom}, which is the case in the teleost fishes zebrafish (Danio rerio) (Kishida et.al 2001) and goldfish (Carrassius auratus). Goldfish has two CYP19 genes, which codes for brain P450_{arom} and ovary P450_{arom}, respectively. The gene products are not that equivalent; in fact there is more sequence identity when comparing goldfish ovarian cDNA with catfish (Ictalurus punctatus) ovarian cDNA, than comparing ovary and brain cDNA in the goldfish (Callard and Tchoudakova 1997). However only one gene encoding for P450_{arom} has been found in humans and two major mRNA derived from it by two polyadenylation signals (Means et.al 1989). In pigs, the two isoforms of P450_{arom}, have been suggested to be mediated through alternative splicing (Corbin et.al 1995).

Aromatase and its gene CYP19 have been studies in many vertebrates such as teleost fishes and rodents. It is clearly established that conversion of testosterone to estrogen in the brain plays a very important role in the sexual behavior of male rodents (Bakker et.al 1996, Bakker et.al 2002). Yet in
other species like rabbits, oestradiol has no functional importance in activating copulation behavior in the males. In fact testosterone was the most effective and irreplaceable steroid to activate male sexual behavior, in the castrated male rabbits (Ågmo and Södersten 1975). Authors in other studies implied that in guinea pigs and nonhuman primates conversion of testosterone to estradiol is not important or required to induce male reproductive behavior (discussed in two review articles Roselli and Resko 1993, Balthazart et.al 2004).

In many animals reproductive behavior is depended on appropriate circulating hormone levels (MacLusky and Naftolin 1981). If the production of hormones are depleted, i.e. by gonadectomy or through “hormonal” inhibition direct or indirectly (by aromatase inhibitor) in the CNS, the behavior will disappear or decrease (MacLusky and Naftolin 1981, Steel and Hutchinson 1987, Roselli and Resko 1993, Bakker et.al 1996, Pierman et.al 2006, Hallgren et.al 2006), and can be restored by replacement of hormone therapy or as in the case with aromatase inhibitor be recovered by itself (MacLusky and Naftolin 1981, Steel and Hutchinson 1987).

Several studies have shown in various kinds of vertebrates, that certain parts of the brain express the formation of estrogen through aromatization. Aromatase is often expressed in the reproductive control centers of the brain, such as specific parts of the telencephalon, hypothalamus, preoptic area, optic tectum and the pituitary (Pasmanik and Callard 1985, Callard and Tchoudakova 1997, Gelinas and Callard 1997, Todd et.al 2005). Aromatase has also shown to be expressed in the olfactory bulbs, of mice (Bakker et.al 2002) and goldfish (Gelinas and Callard 1997).

The way the gonadal steroids exert their effects on developing CNS is not yet fully understood. In many species estrogen is either a circulating hormone or locally active metabolite of circulating androgens (MacLusky and Naftolin 1981). Having only that as consideration makes it harder to approach the issue: How social interactions affect the sexual behavior, and how it affects the aromatase-levels? Aromatase levels are often measured indirectly by messangerRNA-concentration (by RT-PCR, reverse transcriptase polymerase chain reaction) (Means et.al 1989, Corbin et.al 1995, Callard and Tchoudakova 1997, Kishida et.al 2001,) or directly by enzyme activity measurements (Dessi-Fulgheri 1982, Pasmanik and Callard 1985, McPhaul et.al 1988, Beyer et.al 1994, Corbin et.al 1995, Hallgren 2006). The aromatase activity can be increased by the exposure to testosterone or to estrogens, but this effect is connected through an increase of transcription of mRNA for the enzyme, and takes hours to days to become effective, as demonstrated in the Japanese quail (Coturnix japonica) (Balthazart et.al 2004). However the aromatase activity can be modulated more rapidly.
than the regulation of its concentration, within minutes by phosphorylation processes (two review articles Balthazart et al. 2004, Balthazart et al. 2006).

A study made on sex-changing fishes bluebanded gobies (Lythrypnus dalli) showed that social interactions generate dramatic changes in the behavior and neuroendocrine activity in the fish. By removing the dominant male from a social group, the dominate female’s behavior and morphology was modified dramatically and the female’s sexual phenotype changed to a male. The brain aromatase activity in the dominate female was altered and became related to the brain aromatase activity in the males, that was a lower brain aromatase activity than all the others, (Black et al. 2005). Several studies on aromatase function in rodents have been done on mice with their CYP19-gene knockout, (ArKO-mice) (Bakker et al. 2002, Pierman et al. 2006) and the results increases the validity of the statement that aromatase has a crucial effect on sexual behavior. Many studies have shown that olfactory cues, pheromones, plays an outermost impact on the sexual activation, partner preference etc in most animals, and is not surprisingly disturbed in ArKO-mice (Bakker et al. 1996, Pierman et al. 2006). These studies on rodents suggest a connection between pheromone detection and brain aromatase. The resultants referred to these studies (on the bluebanded gobies and ArKO-mice) were the basis for this study.

Teleost fish brains have 100-1000 times higher aromatase activity compare to mammals and other vertebrates, and is therefore a good animal model to study the regulation, the enzyme (enzyme complex) and its role in regulating androgen-dependent responses (Pasmanik and Callard 1985). However, not many studies have been performed on aromatase and its importance in teleost fish reproductive behavior. Hallgren and collaborators 2006 showed in a study with guppies (Poecilia reticulata) that inhibition of the aromatase reduced two male specific sexual behaviors. The authors discussed the possibility that pheromones are involved in the behaviors studied.

The aim of this study was to find out if social interactions can affect the aromatase expression and activity in the brain of the guppy male. Guppy males were introduced to four different environmental conditions (treatment groups): Isolated, all male conditions, heterospecific group (zebrafish females) and the last category is with the opposite sex, (female conspecifics). In the present study I wanted to answer the question: Can sexual interactions influence the activity and mRNA production of aromatase in the male guppy brain? The hypothesis was that sexually active males placed with guppy females should show different (higher or lower) brain aromatase activity and expression compared to males in the other three categories.
Material and Methods

Presentation of the experiment animal

Guppies are small *Poeciliid* fishes and are sexually dimorphic tropical fishes native to streams and rivers in northeastern South America, and are found in small streams running through lowland and mountain rain forest. The males are smaller than the females and brightly colored with a long caudal fin (*Endler and Houde 1995*). The guppy is a viviparous fish (that gives birth to living offsprings). The male inseminate the female with his extended gonopodium, a modified anal-fin shaped like a rod and is the male reproductive organ that transport sperms into the female (*Clark and Aronson 1951*). The guppy female gives birth 28 days after being inseminated. When a female has ovulated she becomes receptive for mating during a period of 3 to 5 days (*Liley 1968*).

There is some genetic variation between populations caused by natural selection, like genetic differences in the production of drosopterin, the brightly red color in the body ornament (*Endler and Houde 1995, Grether et.al 2005*). Multiple sexual ornaments in male guppies, used for attracting females could contribute the maintenance of the high levels of polymorphism in the guppy populations, (Blows et.al 2003). Genetic variations of the MHC (major histocompatibility complex) have been found in two wild populations of Trinidadian guppies and these were caused by selection due to parasite infection (*Oosterhout et.al 2006*). The guppy has been a model for studies of evolutionary ecology and sexual selection for many years, (*Alexander et.al 2006*).

When a pair of guppies is placed in an aquarium, the male may ignore the female at first but more often he will persistently follow and court her, and may with the tip of his gonopodium stick at or near her genital opening (*Clark and Aronson 1951*). The male perform different distinct sexual behaviors.

In the gonopodium swing behavior, the gonopodium is coordinated with a forward motion of one of the pelvic fins and are swung laterally and forward, in a bending motion until the anterior part is directed ahead towards the upper area. As the swing develops to its extreme form the male’s body becomes arched in the vertical plane, and may twist into a sigmoid shape in the horizontal plane. The “gonopodium swing” is not necessary for insemination and is occasionally seen in isolated males. The swinging is often preformed by highly sexual active males who may have been swinging before their introduction to a female (*Clark and Aronson 1951, Liley 1966*).
The courting behavior (sigmoid display) is usually displayed at a visual distance from the female. The male arches its body either in the form of a “C”- or “S”-like shape (sigmoid shape), with the tail bent strongly to one side. The caudal fin is either fully open or closed and this position may last in a couple of seconds during which time the male appears tense, and its body trembles (Clark and Aronson 1951, Liley 1966, Farr 1980).

The male can attempt insemination by swimming alongside the female and brings his gonopodium forward and slightly to the side and thrust at the genital aperture of the female in a quick jabbing movement. The “gonopodium thrust” is usually performed from a position behind, below and slightly to the side of the female. The gonopodium may or may not make a brief contact with the female’s genital area, in most cases it is difficult to detect if the gondopodium touch the female (Clark and Aronson 1951, Liley 1966).

During most part of the courtship, the female swims away from the male when he approaches her. On some occasions when the male approaches in a “sigmoid display” the female may suddenly become passive almost stationary at a position near the bottom and copulation generally follows (Clark and Aronson 1951). Body ornaments in males, like bright color patterns and tail-size are preferable in precopulatory mate-choice in females (Endler and Houde 1995, Blows et.al 2003, Pilastro et.al 2004).

The male’s courting display (sigmoid display) frequency does not differ if the female is receptive or not. On the other hand non-receptive females, like pregnant females, do induce higher rates of closed display (when the fins are closed) and gonopodial thrusting (rape attempts) from the males. Receptive females and virgins, receive more open displays (with open fins). Males with less previous copulation success perform more closed court ship display than open displays (Farr 1980).

**Experiments**

The guppies were ordered from the aquarium fish wholesaler Simontorp Säteri AB. The breeding conditions were unknown.

The fishes were kept in a room with 12 hours light-dark cycles (“night” 7 p.m. - 7a.m.) and placed in 2.7 L aquarium (length 21.2cm, height 13.25cm and breadth 13.25cm), with 2.6 L water. Before the water was added to the aquaria it was aerated to get high oxygen content and adjusted to a temperature between 20°-26°C. Ektozon-salt was added to the water, to prevent infections as fungi and parasites. The concentration of the remedy was 1dl Ektozon-salt per 100 liter water (0.1%).
Aquarium sand and plants like duckweed (*Lemna minor*) and Java moss (*Vesicularia dubyana*) were placed in each aquarium. Before the fishes and the plants were put into the aquaria, the sand was inoculated with gravel taken from an “old” aquarium with guppies. This procedure should improve the development of nitrifying bacteria, *Nitrosomonas* and *Nitrobacter*, preventing the occurrence of high concentrations of toxic nitrogen metabolites, ammonia and nitrite. Lids were made for all the aquaria with mosquito-net.

**Experiment design 1 – mRNA**

**Experimental groups**

Twelve experiment guppy males were divided into four different treatment categories which gave three replicates of the same treatment (Fig.1). In the first category one male was placed alone in an aquarium (isolated). In the second treatment one male, the focal individual was placed together with two other guppy males (male group). To distinguish the experiment male (focal) from the other two males, a drawing was made of the experiment fish’s characteristic features and color patterns (Fig.2). In the third treatment group one male guppy was placed with two females of zebrafish (*Danio rerio*) (a heterospecific group). In the fourth treatment category one male was placed with two female guppies (female group).

**Treatment categories**

1. **Isolated male group**

2. **Focal male with two other guppy males, male group**

3. **Male guppy with two zebrafish females, heterospecific group**

4. **Male guppy with two female guppies, female group**

Fig.1. The arrangements of the social groups during both experiment part 1 and 2.
The rectangles and lines to the right show the way aquaria and screens, respectively, were arranged.
To make sure that individual experiment fishes in each category were not affected by the surrounding aquaria from other treatment groups, “walls” of Styrofoam-plates and plastic-paper packages were placed to separate the treatment groups from each other. However, the fishes in the same category had an outlook of the other aquaria in the same treatment group, except the treatment category with isolated males that were totally isolated from all the fishes.

Once a day around 2 p.m., a mating behavior observation-study was made on the experiment focal fishes, during 10min each. The observation study started with a different category of treatment every day. Three kinds of reproductive behaviors, *sigmoid display*, *gonopodium swings* and *gonopodium thrusts* were noted on a protocol. The fishes were fed once a day directly after the observation study, with aquarium-fish fodder, Sera Flora dry flake-fodder.

The water evaporated from the aquaria was replaced on the second day of the experiment. On the third day all non-focal fishes in all groups were removed and exchanged by new of the same kind.

The focal fishes were held in the aquaria for two respective four days. On the third respective fifth day all the experiment fishes were anesthetized in 0.5% 2-phenoxyethanol and decapitated. Brains were dissected and kept in individual tubes with RNaLater(Ambion) and stored in approximately -70°C. Both experiments were repeated once, total 48 brains were dissected.
**Messanger RNA**

**RNA-extraction**

The samples were stored in the freezer for approximately 3.5 months and thawed at room temperature for use. The brains were individually homogenized in RNAwis regant(Ambion). Total RNA was extracted from each homogenates according to RNAwis protocol and resuspended in 20µl RNase-free water. 8µl was further used for DNase-treatment. To be sure that the RNA was not contaminated an additional extraction-purification step was performed. During the extraction-purification, the samples were transferred to RNase-free tubes an extra time after the chloroform treatment to minimize chloroform and phenol remaining. Instead of isopropanol that was stated in the RNAwis protocol for pelleting the RNA, NaAc was used and precipitated for more than 5 hours. RNA was again dissolved in 20 µl RNase-free water. Full length of cDNA was obtained subsequently according to the Superscript III manual. All the cDNA samples were diluted 9 times in autoclaved H₂O for use in RT-PCR-analyses.

**Primer design**

The reference gene was β-actin and the gene of interest was CYP19B gene, codes for P450 aromatase in the guppy.

The forward primer in β-actin was, 5´-AATTGGGATGATATGGAGAA-3´ and the reverse primer was 5´-ACGCTTCTCTTTGATGTAC-3´. The forward and reverse primer of CYP19B was 5´-GCACATTGCTCGGTGTTGGAT-3´ respective 5´-TGGATTTTCATAAGCAGCTCC-3´, (Hallgren 2007).

**Real-time PCR**

Real-time PCR analyses were executed on Bio-Rad iCycler, MyiQ single color Real Time PCR Detection system with SYBR green fluorescent. The cDNA samples were run in optically clear 8-strip RT-PCR tubes (ABI Prism). Each sample was in a reaction contain 1x IQ SYBR green super mix, 0,5µM of reverse and forward primer, ddH₂O and 5µl of diluted cDNA from RT-reaction. Each reaction was in triplicates and the cycling parameters were: 95°C for 3min, followed by 65cycles of 95°C for 20sec, 55°C for 20sec and 72°C for 30sec, and ended with a 10min elongation period at 72°C. The melt curve analyzes was made in the end of the amplification phase, with 80 repeats of 10sec starting-point at 53°C as the temperature increases by 0,5°C for each repeat, (Hallgren 2007).
Experiment design 2 –Aromatase activity

Experimental groups
Two four-day experiments were arranged in the same way as stated before. The fishes were purchased from the same wholesaler, Simontorp Säteri AB. The only differences compared to the first experiments were that the mating behavior observations were performed once a day around 11 a.m. and feeding was done directly afterwards. No Ektozon were put into the water.

On the fifth day of each experiment all the focal fishes were anesthetized individually in 0,5% 2-phenoxyethanol and decapitated. Each brain was immediately dissected and homogenized in separate tubes in a 125µl mixture of 4/5 phosphate-buffer and 1/5-glycerol. The brain solution was subsequently transferred to individual cryotubes with screw lids and placed in liquid nitrogen. The collected brains were afterwards removed from the nitrogen and stored in -70°C or -140°C freezers. Total 24 brains were dissected.

Aromatase activity
The samples were rapidly thawed and put on ice. Before 80µl of each homogenate was transferred in individual 16x100mm round bottom glasstubes, 30µl ~2mM NADPH and 185µl buffer (phosphate, succorose and glycerol-solution pH 7,4) were put in the tubes. To start the reaction 5µl 146nM Androstendione (A-9630-Sigma) and 3H-Androstenedione (Net926-Perkins Elmer) was pipette to one glass tube at the time. The time was set at the exact moment each tube were put in the shake-water bath at 30°C, and the reaction was terminated with chloroform and 1700µl ddH2O after exactly 60min. The tubes were centrifuged and 1000µl of the hydrophilic phase were additionally filtered in 500µl active carbon-solution (5% w/v). 500µl of the supernatant was than mixed with 10ml Ultima Gold in scint-tubes. The production of titrated-water was measured on a Scint-counter.

Protein concentration
Protein concentration for each sample was analyzed according to Quick Start Bradford Reagent Assay (Bio-Rad) on Bio Rad 550 Microplate reader. The total protein concentration of individual samples was measured in µl/ml and was recalculated to µg/µl and multiplied by 80, because the volume of the homogenates of the aromatase-activity measurements was 80 µl. To get the aromatase concentration in each sample, the mean-value of the enzyme-measurements in DPM (disintegrations per minute, measures radioactivity the number of atoms in a given quantity of radioactive material, decay per minute) were divided by total protein concentration values.
Aromatase activity = \frac{\text{Mean-value of DPM}}{\text{Total protein in µg/µl x 80}}

Statistics
The behavior data were analyzed with Kruskal-Wallis non-parametric analyses of variance in case of significance differences followed by Dunn’s multiple comparison test. The enzyme activity data were analyzed with one way ANOVA. Paired comparisons were done with Wilcoxon's matched paired test. All calculations were done with Graph Pad Prism software (GraphPad Software Inc., San Diego, CA, USA). The level of significance was set at \( P<0.05 \).
Results

Experiment part 1

Mortality during the treatments of part 1

All the fishes survived during the whole treatment of two four-day experiment and two two-day experiment. Total 48 brains were dissected.

Behavior

The mean number of reproductive behaviors in the males was as expected much higher in the groups with females present compared to the activity, if any, in the other two groups with more than one fish present, that is the all male groups and the groups with a guppy male together with zebra fish (Fig. 3 - 4). A few gonopodium swings was observed in the all male groups and in the zebra fish groups, but almost no reproductive behaviors were observed among the isolated males. A pattern of sexual behavior was observed in males in the female category. Some of the individuals performed more gonopodium thrusts and less sigmoid display and others more sigmoid displays and less thrusts (Fig. 5). No clear pattern could be interpreted in the number of performed gonopodium swings by the males in the female group, when compared with the other reproductive behaviors (sigmoid display and gonopodium thrust). The reproductive status of the guppy females, whether they were virgins, pregnant, receptive or non-receptive were unknown and not examined. The frequency of behavior acts showed a non-significant decrease in two days experiment (Fig. 6-7) but there was, however, a statistical significant decrease from day 1 to 2 in the four day experiment (Fig. 8-9). The decrease was significant in the frequency of gonopodium swinging and thrust (Fig. 9).

Experiment 1 - Two days observations

![Graph showing the number of reproductive behaviors per 10 min (Mean ± SD) for different groups.](image)

**Fig. 3.** The number of behavior acts of focal males in different social groups. The non parametric variance analysis Kruskal Wallis test was followed by Dunn’s multiple comparisons test. *** = p < 0.001.
Experiment 1 - Four days observations

Fig.4. The number of behavior acts of focal males in different social groups. The non parametric variance analysis Kruskal Wallis test was followed by Dunn’s multiple comparisons test. ** = \( p < 0.01 \); *** = \( p < 0.001 \).

Fig.5. The behavior in the six different males in the female group, studied during two days in experiment part 1.
Fig. 6. The mean number of display, swing and thrust of males kept with two females during two days in experiment part 1. No significant differences between days were observed.

Fig. 7. The total mean number of behavior acts of males together with two females during two days in experiment part 1. No significant differences between days were observed.
Fig. 8. The mean number of display, swing and thrust of males kept with two females during four days in experiment part 1. No significant differences between days were observed. Wilcoxon’s match paired test was used to compare within behaviors between days. * = p < 0.05; (†) = 0.05 < p < 0.10.

Fig. 9. The total mean number of behavior acts in males together with two females during four days in experiment part 1. No significant differences between days were observed. Wilcoxon’s match paired test was used to compare within behaviors between days. * = p < 0.05; (†) = 0.05 < p < 0.10.
Brain aromatase expression

Great efforts were taken trying to make the RT-PCR technique to function, but no usable results were generated. RNA from the different male brains are still stored at -70 °C and will be analyzed later on when the technique works again. But the new future analyses do not fit in to the time schedule of the present work.

Suspecting that it was primer-dimerization, attempts were made to solve the problem. This work was preformed by Stefan Hallgren. New fresh primers were purchased, different annealing temperatures from 53°C to 61°C in the RT-PCR-program were tested and finally the primers efficiencies were tested by making a dilution-series on Stefan’s cDNA samples with 10x dilution each step.

Experiment part 2

Mortality during the treatments of part 2

There was no mortality during this experiment either; total 24 brains were dissected, from the two four-day experiments.

Behavior

As stated earlier the mean number of reproductive behaviors was much higher in the treatment category with female guppies present (Fig. 10). In contrast to the experiment part 1 there were some reproductive behaviors recorded in the all male groups. This can have been due to that some of the males had a light appearance with a color pattern almost beige. They were also bigger in body size compare to the others. Those males were seen courted by the others. A few gonopodium swings were observed in the heterospecific group (zebra fish females), but none in the isolated male-groups. Also during experiment part 2, a pattern of performed sexual behaviors could be seen by males in female group. Some of the males performed more sigmoid and others performed more thrust. However, the number of performed gonopodium swings, showed no correlated pattern to the other two behaviors (data not shown). The reproductive condition of the females (as mentioned earlier in experiment part 1) was unknown and not examined in this case either. Also in this experiment the number of swinging significantly decreased from day 1 to day 2, but the frequency of all acts together did not change (Fig.11-12).
Fig. 10. The number of behavior acts of focal males in different social groups. The non-parametric variance analysis Kruskal Wallis test was followed by Dunn’s multiple comparisons test. ** = p < 0.01; *** = p < 0.001.

Fig. 11. The mean number of display, swing and thrust of males kept with two females during four days in experiment part 2. No significant differences between days were observed. Wilcoxon’s match paired test was used to compare within behaviors between days. * = p < 0.05; (*) = 0.05 < p < 0.10.
Fig. 12. The total mean number of behavior acts in males together with two females during four days in experiment part 2. No significant differences between days were observed. Wilcoxon’s match paired test was used to compare within behaviors between days. * = p < 0.05; (*) = 0.05 < p < 0.10. No significant differences between days was observed.

Brain aromatase activity
The ANOVA revealed no significant differences between the treatments (Fig. 13). There was, however, a close to significant differences in variance (Bartlett’s test gave P = 0.08; Barlett’s statistics = 6.765) among the treatments. The variance was highest in the zebra fish group and lowest in the isolated males (Fig. 13). An F-test \((F = (SD_{zebra}^2/SD_{isol}^2))\) gave a significant difference in variance between these two groups \((P= 0.02)\). There was no significant correlation between the mean number of reproductive behaviors and the aromatase activity in males that were together with females (Fig. 14).
Fig. 13. The brain aromatase activity in males from different social groups. The ANOVA revealed no significant differences.

Fig. 14. The aromatase activity against the total number of reproductive acts. NS = > 0.05. Pearson r = 0.33 NS
Discussion

The aim of the present study was to test the hypothesis that the social environment can influence the expression and/or the activity of P450 aromatase in the brain of guppy males. The males were either isolated or placed in all male groups or in groups with only females. Some males were kept together with zebrafish females. There are few studies on the influence of the social environment on the brain aromatase activity in fish, but only in sex changing species (e.g. Black et al. 2005). It is not known if social interaction can affect brain aromatase also in fish that do not demonstrate sex change. The significance of the social environment and pheromones on the brain aromatase has however been demonstrated in rats (Dessi-Fulgheri 1982). Hallgren and collaborators (2006), studied the effects on the male reproductive behaviors by aromatase inhibition, suggested that female guppy pheromones might be of importance. The design in the present experiment should have revealed differences between males from the different groups, if the female presence had an influence. The aromatase activity measurements gave, however, no significant differences. We were unlucky with the RT-PCR and could not get any good results though great efforts to solve the problem. If there had been an effect by the social environment on the brain aromatase the effects should probably have been observed in activity rather than in the expression. Perhaps even though we could have been able to solve the problem with the RT-PCR, two and four days of social interactions may not have been long enough to see changes in the aromatase mRNA expression in the guppy brain. According to Balthazart and collaborators (2004) it takes hours to days to observe changes in the aromatase mRNA expression in the Japanese quail (Coturnix japonica). The aromatase activity can, however, change more rapidly (review Balthazart et al. 2004, Balthazart et al. 2006) and it should have been possible to observe differences. The heterospecific treatment group (zebrafish category) showed the highest aromatase activity, but not significantly different than the activity in the other groups. The ANOVA displayed no significant differences between all the treatment groups in the experiment. The variance in aromatase activity between the individuals in the zebrafish category was higher than in the other groups which was also revealed by close to significant difference (P=0.08; Bartlett’s test for equal variances) in variance among the groups. There was a significant difference in variance between the isolated group and the males from the zebrafish group. This difference in variance might reflect a difference in aromatase activity due to the group composition. Further, no significant correlation was observed between the total number of reproductive behaviors and the aromatase activities in the males with females. The lowest activity was seen in the isolated male group. During the observation study, vigorous swimming, up and down and from side to side was seen in the isolated males. This activity, which seems to be a form of escape behavior, could be seen in females courted persistently, or like in these cases total isolated individuals (cf. Liley 1966). Maybe as
mentioned earlier (in experiment part 1, mRNA expression) experiment part 2 (aromatase activity measurements) needs also to be arranged or prepared in a different way to give more manifest results (on a molecular basis).

The cDNA samples were run on the RT-PCR as stated earlier. Because of the short time schedule, there was not enough time to correct the problem with what is believed to be primer-dimerization. Since primers are present at high concentrations, weak interactions can arise between them, and are noticed as non-specific products (Brownie et al. 1997). Primer dimers are “often” observed in PCR and are not derived from template DNA. They can complicate experimental analysis, for example primer dimers might “mask” the true result in multiplex PCRs, differential display, amplicon (amplified pieces of DNA) cloning and in quantitative PCR methods, (Brownie et al. 1997). Brownies and collaborators produced primer-dimers from a variety of primers, trying to display and solve the problem with the appearance of primer-dimers. What they came up with was that there is no single mechanism for the formation of primer-dimer. They demonstrated a method for general suppression of primer-dimer formation that uses a sequence of additional nucleotides (a Tail) at the 5´ ends of amplimers (PCR primers).

In both experiments (part 1 and 2), males showed a clear sexual activity towards female guppies. The reproductive state of the females, whether they were virgins, pregnant, receptive or non-receptive was unknown and not examined. A clear pattern of sexual behavior was observed in the males with females. Some of these individuals performed more gonopodium thrusts and others more sigmoid displays. Farr suggested in his study from 1980, that a closed courting display performed by the male guppy should lower the female’s rejection of him. A male has several options to choose from, depending on the female’s behavior response. If she is unreceptive, he could try to rape her (perform gonopodium thrust) or simply move to another female. If she shows receptivity he could change his tactics to open sigmoid display (receptive females respond to open display from the males) and therefore increase his chances of mating (Farr 1980). According to Farr’s studies, the females in this study should than be more receptive if the male performs more sigmoid display and less gonopodium thrust or the opposite (unreceptive) if the male performs more thrusts and less displays. The differences in behavior between the males in the present study could have been due to differences in the receptivity of the females. It is known that the receptivity of guppy females coincides with the peak of estrogen production for several days after parturition and emission of a pheromone (Liley 1972 referred to in Stacey and Sorensen 2005).
Why than, does the total number of courtship behaviors by male guppies (the chasing, following, watching etc (Liley 1966)), not decrease towards unreceptive females? Farr (1980) suggested that in normal population of guppies, with several hundred individuals makes it hard for the male to distinguish chemical signals from receptive and non-receptive females from long distances. Receptive female cues are probably recognized from a close range, where the conflicts of other females’ cues are minimized.

No clear pattern could be interpreted in the number of performed gonopodium swings by the males in the female group and their frequency of sigmoid display or gonopodium thrust, which is a high frequency of either of these two behaviors, did not give a high frequency of gonopodium swing. Clark and Aronson (1951) have observed that males with a high frequency of gonopodium swings copulated more often than males with a lower frequency.

There was some sexual activity between the males in the all male groups during experiment part 2 but the sexual activity was low between the males in the experiment part 1. The fishes purchased for experiment part 2 were ordered during the autumn 2007 from another batch of fish than to the experiment part 1. In both cases, as stated earlier, the fishes were originally kept under unknown breeding conditions. It is known that behavior interactions during the early social environment, shapes the mating behaviors and sexual preference in guppy males (Poecilia reticulata). Homosexual behaviors are induced in all male conditions and the preference for males over females continues in mixed sex groups (Field and Waite 2004). There are also indications of that the olfactory sex preference is shaped by the social environment (Christian Sommer, unpubl. results). The higher sexual activity in experiment part 2 could, have however been due to that some of the males had female like appearances. They had a big bulge belly, were light almost beige in color, bigger in body size compared to the other males and caudal-fin was shorter (more female-shaped). The other males courted these males that had a female appearance. No such “female-males” were in the groups with low sexual activity.

**Conclusion**

In conclusion the present study can not support the hypothesis that the brain aromatase expression or activity can be influenced by the social environment though significant differences in behavior between the males from different social groups. The aromatase activity was not significant different between the males from different treatments and in the study of the aromatase mRNA expression the method did not work properly. According to the focal male behavior in the different groups, that
is high frequency of courting of females, low with males and nothing towards heterospecific females, the arrangement of social groups worked well.

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