Pheromonal Mediated Behaviour and Endocrine Responses in Salmonids

The impact of Cypermethrin, Copper, and Glyphosate

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Abstract

The effects of cypermethrin, copper and glyphosate on the endocrine system and subsequent response to female pheromones were investigated in mature male brown trout (Salmo trutta) parr. Responses measured were the amount of strippable milt, blood plasma levels of both an androgen (11-ketotestosterone (11-KT)) and a progestin (17α,20β-dihydroxy-4-pregnen-3-one (17,20b-P)), and behavioural changes. This was done in a two phased investigation where parr were exposed to one of the following via ambient water: 1) 0.1 or 1.0 mg L⁻¹ cypermethrin, 2) 10 or 100 mg L⁻¹ copper (Cu²⁺), or 3) 150 mg L⁻¹ glyphosate for a 96 hour period. Phase one was a priming experiment exposing parr to a treatment followed by priming with PGF₂α or ovarian fluid (OVF). Atlantic salmon (Salmo salar) parr were, also exposed to glyphosate during phase I. The second phase was centered on behavioural observations. Exposed parr were placed in a 35,000 L stream aquarium together with two ovulated females and four anadromous males. After the experiments a blood sample was taken, milt volumes measured and testes weighed. The plasma was analyzed for 11-KT and 17,20b-P concentrations using radioimmunoassay (RIA).

Results from phase I-priming: 1.0 mg L⁻¹ cypermethrin exposure lowered 17,20b-P and 11-KT; Copper exposure lowered milt volumes; glyphosate exposure lowered 11-KT in salmon and raised 17,20b-P in trout. Results from phase II-behaviour: 1.0 mg L⁻¹ cypermethrin exposure lowered 11-KT, milt and spawning behaviour; copper exposure lowered spawning behaviour and raised 11-KT; Glyphosate exposure lowered 11KT; continuous cypermethrin exposure raised 17,20b-P, 11-KT and gave a tendency towards increased aggression. It is concluded that low concentration exposure to the compounds examined can induce negative effects on male salmonid endocrine systems, either through a disruption in the olfactory system or through a direct effect.

Keywords: Cypermethrin, copper, glyphosate, behaviour, hormones, pheromones, olfaction, 11-ketotestosterone (11-KT), 17α, 20β-dihydroxy-4-pregnen-3-one (17, 20β-P), reproduction

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To Henrik, Rafael and Gabriel, with you everything is possible
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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Abbreviations

11-KT 11-ketotestosterone
17,20β-P 17α,20β-dihydroxy-4-pregnen-3-one
OVF ovarian fluid
RIA radioimmunoassay
EPSPS 5-enolpyruvylshikimate-3-phosphate synthase
StAR steroidogenic acute regulatory
AChE acetyl cholinesterase
EOG electro-olfactogram
GnRH gonadotrophin releasing hormone
FSH follicle stimulating hormone
LH lutenizing hormone
PGF$_{2α}$ prostaglandin F$_{2α}$
GSI gonado-somatic index
Introduction

Background
Pesticides in the environment
Increasing concern over environmental contaminants and their potential impact on ecosystems has led to an increasing amount of investigation into xenobiotic affect on specific biological systems. Far too often the definition of toxicity or toxic effect is centered upon acute toxicity and death of the organism. Herein toxicity or toxic effect will indicate any measurable adverse affect caused by a foreign chemical. Only with this clarification can viable safety limits be set. Numerous investigations concerning the impact of pesticides on the environment are based on acute toxicity in non-target organisms. There is a lack of information about what happens at sub-lethal concentrations. Low level, continuous exposure is what most organisms will experience over their lifetimes. During this work I examined the effect of three leading pesticides (cypermethrin- insecticide, copper sulfate- fungicide, and glyphosate-herbicide) on the olfactory mediated endocrine response of mature male parr, of both brown trout (*Salmo trutta* L.) and Atlantic salmon (*Salmo salar* L). The trout were exposed to each pesticide during separate investigations and the salmon were exposed only to glyphosate.

Cypermethrin
Cypermethrin (Fig. 1) is a pyrethroid that was first synthesized in the early 1970’s. It is used for pest control in forestry, agriculture and, more specifically, for treating salmonids for sea lice (*Lepeophtheirus salmonis* *Copepoda: C.*) (Ernst et al., 2001). Cypermethrin is present in surface waters on a worldwide scale, entering the systems from run-off, dispersal or direct application. A Welsh monitoring program focusing on the environmental impact of the sheep dipping process (cypermethrin is the main ingredient in sheep dip) showed that 20% of analyzed sites exceeded the maximum allowable concentration of cypermethrin; and that concentrations throughout the UK ranged from 0.078 to 2.8 μg L⁻¹ and in the US from 0.0046 to 0.048μg L⁻¹ (House et al., 1997; Environment Agency, 1998, 2003; Pfeuffer, 1999). It is currently accepted that sub-lethal levels of cypermethrin are harmful to aquatic wildlife (Nemcsök et al., 1999; Burridge et al., 2000; Gowland et al., 2002; Friberg-Jensen et al., 2003; and Lu et al., 2004).
In a study using a human androgen receptor mediated luciferase reporter gene assay in CV-1 African green monkey kidney cells, it was reported that 0.1mM cypermethrin acts as an anti-androgen by suppressing the luciferase expression (Sun et al., 2006). Furthermore, negative effects on the male mouse pup reproductive system were reported after their mothers were fed cypermethrin during lactation (Wang et al., 2010). The authors described a decrease in the weight of and malformations in the testes, a reduction in mRNA and protein levels of testicular cytochrome P450scc, i.e. the cholesterol side chain cleavage enzyme. Correspondingly, the level of serum and testicular testosterone at weaning was significantly decreased in males whose mothers were exposed to cypermethrin during lactation. In adulthood, P450scc and serum testosterone levels had restored to control level. However, the decreased testicular weight and histological changes were irreversible.

A similar in vivo study exposing pre-spawning stage freshwater catfish (Heteropneustes fossilis) to 1.0, 0.1, and 0.01 mg L⁻¹ cypermethrin reported alterations in the male reproductive system (Singh and Singh, 2008). The histological development of the testes was negatively effected along with a decrease in the gonado- somatic index (GSI), plasma levels of estradiol-17beta (E2) and 11-ketotestosterone (11-KT). The effect on the motility of sperm and the duration of that motility were dose dependent with the exposure to 1.0 mg L⁻¹ cypermethrin being lethal. The results indicated that cypermethrin exposure may cause an adverse effect in the reproductive system by affecting the hypothalamo-hypophyseal-gonadal axis.

An exposure to 4 ng L⁻¹ cypermethrin was sufficient to destroy the olfactory mediated response of mature male Atlantic salmon parr to female priming pheromones. This was measured by a reduction in both the plasma hormone levels and in the amount of milt produced. These reductions were correlated
to loss of cell function in the olfactory rosette using an electro-physiological technique (electro-olfactogram, EOG). Fertilization rates also decreased with an exposure of 0.1 μg L⁻¹ cypermethrin added at fertilization causing a reduction in the number of eggs fertilized (Moore and Waring, 2001). During this thesis work the effects of cypermethrin exposure on the olfactory system and the parr’s ability to detect pheromones were investigated.

Copper
Copper (Cu²⁺) is an essential metal ion important for the functioning of various proteins in fish (i.e. enzymes cytochrome oxidase, uricase, tyrosinase, superoxide dismutase, amine oxidase, lysyl oxidase). However, copper ion can be toxic in aquatic systems at low concentrations (De Oliveira-Filho et al., 2004). It is a known pollutant coming from various sources including industry, urban waste, mining, and agriculture. A large effort has been made to reduce the amount of copper released into the environment; sadly it is still contaminating many natural waters. Copper levels in natural water ways has been found to ranged from 0.04 μg L⁻¹ to 294 μg L⁻¹ with an extreme value of 20 mg L⁻¹ having been reported (WHO, 1998; Goodyear and McNiel, 1999; Neal and Robson, 2000; Mansour and Sidky, 2002; An and Kampbell, 2003; Baresel et al., 2006)

Brown et al. (1982) described harmful effects of copper on fish below levels acutely toxic to the organisms. For example, salmonid fish demonstrate behaviour avoidance at concentrations of 5 μg L⁻¹ Cu²⁺ or less. This discovery was further confirmed by several research groups working on Cu²⁺ affects in the olfactory system in various fish species. It was demonstrated that low concentrations of copper effects the olfactory system by destroying epithelial cells, and inhibiting the ability to detect and respond to odors (Beyers and Farmer, 2001; Baldwin et al., 2003; Sandhal et al., 2004). More specifically, a 20% decline in olfactory mediated behavioural response to odors was observed after juvenile coho salmon (Oncorhynchus kisutch) were exposed to 4.4 μg L⁻¹ for seven days (Sandhal et al., 2004). More recently, Sandhal et al. (2007) reported a difference in the ability of coho salmon to detect an odorant or respond to the presence of a predator after exposure to copper levels as low as 2.0 μg L⁻¹ for three hours. At higher concentrations (up to 20 μg L⁻¹) they reported a near abolishment of the salmon’s ability to detect a predator, implying that as copper concentrations increase, the effect on the olfactory system will increase correspondingly. This will continue until the olfactory system is no longer able to process and initiate odor-regulated behavioural responses. The African cichlid Tilapia mariae was exposed to 100, 40, 20 μg L⁻¹ Cu²⁺ during 96 H by Bettini et al. (2006). They examined the regeneration of cells in the olfactory epithelium using light microscopy and reported extensive damage in all cell types in fish exposed
to 100 μg L⁻¹ Cu²⁺. The cilia and microvilli in the olfactory rosette were completely missing and were regenerated only after 10 days recovery.

Glyphosate

Glyphosate (Fig. 2) is a non-selective, broad-spectrum systemic herbicide and is the most commonly used herbicide active ingredient in the world (Woodburn, 2000). Its herbicidal mechanism of action is to competitively inhibit 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme in the pathway of the aromatic amino acid biosynthesis linking primary and secondary metabolism in plants and bacteria (Carlisle and Trevors 1988; Lydon and Duke, 1989; Mohamed et al., 2005). A regional survey from the USGS Toxic Substances Hydrology Program (2000) of streams in the Midwest United States reported that 21 of the 52 streams sampled had detectable levels of glyphosate (max: 8.7 μg L⁻¹ mean: 0.02 μg L⁻¹) (Scribner et al., 2003). A second survey conducted in the U.S. reported levels as high as 328 μg L⁻¹ in stream water (Battaglin et al. 2008); and in Ontario stream water had levels as high as 40.8 μg L⁻¹ (Struger et al. 2008).

Figure 2. Structural formula of Glyphosate

It seems as glyphosate exposure can cause various toxic effects in vertebrates (Giesy et al., 2000). There is evidence to suggest that vertebrates may suffer from negative affects to both the reproductive and endocrine system. Mammalian cytochrome P450 aromatase activity was inhibited by an exposure to glyphosate, along with inhibition of aromatase gene expression in the human placental JEG13 cell line, (Richard et al., 2005). It was also reported that Roundup® was found to be at least two-fold more toxic than its active ingredient, glyphosate. This observation lead to a follow up study examining the endocrine disruption of Roundup Bioforce®, or glyphosate alone on human embryonic 293 and placental-derived JEG3 cells in vitro (Benachour et al., 2006). The authors reported an inhibition of aromatase activity after exposure to Roundup® (210 μM glyphosate) for 24 H. Furthermore, it was also reported that glyphosate acts as a partial inhibitor on microsomal aromatase (1–2 %, i.e., with 21–42 mM
glyphosate). All effects were dose and time dependent, i.e. an exposure of 0.01% Roundup® for 24 H provoked a reduction of estrogen production in embryonic 293 cells, but after 72 H exposure this dose of Roundup® became toxic. Likewise, a more recent study using human liver HepG2 cells in vitro examined the effect of an exposure to either glyphosate alone or glyphosate in commercial formulation and reported evidence of cytotoxicity, genotoxicity, anti-estrogenic, and anti-androgenic effects (Gasniera et al. 2009). The disruptions were more dependent on the formulation than on the glyphosate concentration itself. For the current study it important to mention that an exposure to 10 mg L⁻¹ glyphosate for 24 H had anti-androgenic effects on liver cells.

A study using the mouse MA-10 Leydig tumor cell line reported a reduction in progesterone production after an exposure to 25μg L⁻¹ Roundup® (Walsh 2000). This reduction was accompanied by a reduced steroidogenic acute regulatory (STAR) protein expression. An in vivo study giving an oral dose of Roundup Transorb® (5, 50, 250 mg kg⁻¹) to male prepubescence Wistar rats revealed reduced testosterone production and abnormal morphological development in the testis (i.e. the epithelium height decreased and the luminal diameter increased in the seminiferous tubules) in a dose dependent manner (Romano et al. 2009). The authors suggest that this formulation of glyphosate is an endocrine disruptor as an exposure during puberty caused disturbances in reproductive development. Similarly, an exposure to Roundup® (5 of 100 mg Kg⁻¹) influenced the reproductive system in the male mallard duck (Anas platyrhynchos) (Oliveira et al. 2007). Alterations in the structure of the testis and epidydimal region along with reductions of the plasma levels of testosterone and estradiol were reported. A pervious study on white New Zeeland rabbits (Oryctolagus cuniculus) showed that a sub-lethal, oral dose of glyphosate adversely affected the male the reproductive system (Yousef et al. 1995). The authors reported a decrease in semen volume, spermatozoa concentration, and sperm motility with a subsequent increase in the percentage of abnormal sperm.

In fish glyphosate, exposed as Roundup®, has been shown to inhibit acetylcholine esterase (AChE) activity (Glusczak et al. 2006; 2007). The earlier study examined the affect of Roundup® (calculated concentration of glyphosate) 20, 10, 6, and 3, 0 mg l⁻¹ (control), for 96 H on the fish piava (Leporinus obtusidens) (Glusczak et al. 2006). The latter study repeated this investigation with silver catfish (Rhamdia quelen;) and exposures to Roundup® (AI glyphosate) 0 (control), 0.4 or 0.2 mg L⁻¹ for 96 H (Glusczak et al. 2007). Both investigations displayed similar results. The authors reported that every group exposed to any tested glyphosate formulated concentration had significantly decreased AChE activity in brain tissue, while no difference was noted in the muscle tissue AChE activity.
Tierney et al. (2006, 2007) described effects of both glyphosate and Roundup® on the olfactory system in juvenile coho salmon (*Oncorhynchus kisutch*). During the first study it was demonstrated by EOG that a 30 min exposure to glyphosate was sufficient to impair detection of amino acid L-serine by cells in the olfactory rosette. Individuals exposed to 100 μg L⁻¹ glyphosate did not return to “normal” after a 60 minute recovery period. The follow up study demonstrated that an exposure to 100μg L⁻¹ glyphosate in Roundup® formulation for 20 min significantly reduced the EOG readings evoked from L-histidine stimulation.

The model species
Atlantic salmon and brown trout, begin life in fresh water rivers, mature at around two years of age, migrate out to sea and then return to their natal streams to spawn. They both have a parr, morphologically juvenile and sexually mature, male form in addition to the anadromous male. The parr can mature in the stream as early as one year of age and stay there for an undefined period with the possibility of migrating to sea at a later time (Hutchings and Meyers, 1994; Fleming, 1996).

Life strategies of the parr
The parr stay in the river after maturing and act as “sneakers” during spawning, attempting to fertilize eggs (Jones 1959; Thomaz 1997). The parr life history has been described by Thorpe et al. (1998) and Elliot (1994). The two male phenotypes differ from each other in morphological, demographical and ecological characteristics (Baglinière and Maisse 1999). In fact, in a genetic survey using brown trout microsatellites no differences were found between males that matured in the streams and those that migrated out to sea (Charles et al 2005). It was suggested that there is no genetic reason as to why some males stay in streams and some migrate after two years.

The parr male is capable of fertilizing eggs and contributing to the genetic viability of natural populations. This becomes more important in small rivers and with diminishing returning male populations. In both the salmon and the trout the reproductive behaviour of the parr is an important factor to the overall success of the species.

The habitat requirements for these two species can often overlap. However, the salmon is better adapted for faster water currents than the trout (Armstrong et al. 2003). During the observations made throughout this thesis work, the trout parr were better suited for the stream tank as the
salmon parr were more likely to be found hiding amongst the rock bottom in the aquarium. The salmon parr were mostly immobile while the trout parr were active.

Mature Male Trout Parr

In trout populations, usually one returning male is responsible for fertilizing the majority of the eggs in the redd, with a fertilization rate between 60 and 100% (Garcia-Vazquez et al. 2001). Multiple paternity within the same redd is usually the result of another anadromous male fertilizing the remaining eggs. It is suggested that in the presence of returning males the trout parr males are mostly injured and killed (Freyhof and Kottelat, 2008) and this could be the reason for a lower success rate than that seen in the salmon. There is a lack in data examining the ability of the trout parr to fertilize eggs in the absence of adult males.

Mature Male Salmon Parr

It has been established that the Atlantic salmon parr is able to stimulate digging females and to fertilize eggs (Thomaz et al. 1997). As the returning wild population dwindles the parr male will become a key factor in maintaining viable populations. The salmon parr could be responsible for an average of 40% of fertilizations (Vladić et al. 2002; Fleming, 1996). Garcia-Vazquez et al. (2001) reported a fertilization rate of 65% while other groups have reported a 20% fertilization rate (Jordan and Yougson 1992). The main difference between these two rates is the location, with the parr having higher fertilization rates in southern Europe. The parr male contribution to fertilization increases as the returning males become over matured or exhausted (Moran et al. 1996). The parr are even capable of substituting for absent anadromous males (Martinez et al. 2000).

Reproduction

Brown trout and Atlantic salmon have similar reproductive behaviours that are vital to spawning. At the onset of activation of the reproductive systems both males and females migrate back to their natal streams (Hara et al. 1965). The females are the first to enter the spawning grounds. Most females prefer the sediment grain size of 10-70mm (Reiser and Wesche 1977). After finding a suitable area, she begins digging the redd. This is done by turning on her side and using powerful beats with the tail and body to hit the gravel creating an indentation. While digging she will assess the size of crevasse by sliding her pelvic fin between the gravel, checking whether there is space for the eggs to fall between the stones. Usually, at least one
male has begun courting the female during the digging. He will do this by coming alongside the female and quivering with his body. The dominant male usually distinguishes himself by aggressively defending his position nearest to the female. When the female is ready to release eggs, she enters the redd and bends her body so that her vent is close to the gravel and her head is up. The dominant male then comes beside her and both fish quiver their bodies, mouths gaping until eggs and milt are released. The eggs fall in the crevasses, and the female moves upstream and begins to cover the eggs by using the same whole body technique used to dig the redd. After spawning, it is possible for the Atlantic salmon and brown trout to again change their osmoregulation from fresh water back to sea water and return to the oceans; however, many die after spawning (Elliot 1994; Booth et al. 1997).

Sexual maturation is characterized by the onset of spermatogenesis in males (Miura and Miura, 2003) and vitellogenesis in females (Patino and Sullivan, 2002). The male reproductive traits are described here as the male fish were in focus in this thesis work. The basic reproductive cycle of male teleost fish begins with regressed gonads being stimulated by environmental cues such as changing photoperiod, decreasing water temperature, and changing water quality (Campbell et al 2003). These environmental changes lead to activation of the hypothalamus, which secretes gonadotrophin releasing hormone (GnRH). This process is regulated by the brain-pituitary-gonad axis (Fig 3). Secretions from the pituitary gland are regulated by seasonality, meaning that the activation and maturation of the gonads is also seasonal (Lin and Peter 1996). As the sex steroid level increases by gonadal production, changes in secondary sexual characteristics will occur, gametes will develop and mature, behaviour changes and courtship sequences will become apparent and subsequent spawning. This cycle takes several months; gonadal maturation commences in the autumn, spawning is in the winter, and fry hatch in the late winter or early spring.

The GnRH activates the pituitary to secrete gonadotrophins (follicle stimulating hormone FSH and lutenizing hormone LH) (Fig. 3). This gland receives direct stimulation from various parts of the central nervous system, including the preoptic region, mediobasal hypothalamus, the olfactory system and tegmentum of the midbrain (Peter et al., 1990; Anglade et al., 1993). The physiological functions of gonadotrophins have been studied in detail in salmonids (Swanson, et al. 1989; Prat et al. 1996; Gomez et al., 1999). The levels of FSH and LH are not constant throughout the reproductive cycle. In immature males the levels of FSH are low and will increase at the beginning of spermatogenesis, while levels of LH strongly increase during spermiation. Stimulation of steroidogenesis and spermatogonial proliferation is suspected to be linked to FSH, as FSH is
already detectable in the plasma of immature fish (Swanson, et al. 1989; Mayer et al. 1990). The primary function of FSH is a regulatory role during early stages of gonadal development and gametogenesis while LH is involved in the final maturation of the gamete (Fostier et al. 1983; Tyler et al. 1997).

Sex steroids can influence gonadotrophin synthesis and release through positive or negative feedback, because they either directly affect the gonadotroph cells in the pituitary or indirectly via the GnRH system in the brain (fig. 3). This feedback system is dependent on the maturation of the fish (Larsen and Swanson, 1997). In mature male Atlantic salmon parr, however, gonadectomy (the surgical removal of the gonads) causes a reduction in the blood plasma levels of FSH and LH, suggesting that the steroids have a stimulatory effect on gonadotrophin synthesis (Borg et al., 1998).

Figure 3. Overview of the teleost hypothalamic-pituitary-gonadal axis. Testicular cells are illustrated. The regulation of the system is indicated through positive stimulation and negative feedback. See the text for more detail. GnRH, gonadotrophin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone.
Endocrine response

Increasing levels of sex hormones in the blood plasma indicate an activated endocrine system. Gametogenesis, the development of secondary sexual characters, and reproductive behaviour are all regulated by the presence of sex steroids (Borg, 1994). In the current study both 11-KT and 17,20β-P were investigated along with milt production and reproductive behaviours. In male fish, 11-KT is considered to be the main androgen responsible for gonadal development, spermatogenesis, secondary sexual characteristics, and spawning behaviour (Borg, 1994) and 17,20β-P is suspected to be vital in spermiation and contribute to spawning behaviour (Fostier et al., 1983; Kime 1993; Mayer et al. 1994; Kubokawa et al 2001). In salmonid males, plasma levels of 11-KT are highest during the pre-spawning period and decline at spawning, while 17,20β-P plasma levels increase dramatically just before the final spawning event (Mayer et al., 1990; Amer et al., 2001).

The levels of 11-KT and 17,20β-P in blood plasma vary with the season and maturation of the individual. These two hormones normally demonstrate a negative correlation with each other, so that when there are high levels of 11-KT there are low levels of 17,20β-P. For example, as the males begin to migrate upstream, the level of 11-KT increases while the level of 17,20β-P remains low (Onumal et al. 2005). Correspondingly, during final maturation of the sperm cells and spawning events the level of 11-KT lowers whiles the level of 17,20β-P increases.

During gonad maturation, the plasma level of 11-KT will be high as it is responsible for regulating the process. When the gonads have matured the level of 11-KT will be down regulated, as it is no longer needed. Gonad maturation precedes maturation of the sperm cell, which is regulated by 17,20β-P. As the gametes mature 17,20β-P is up regulated, causing an increase in the blood plasma. Regulation of both 11-KT and 17,20β-P are also influenced by the salmonid male’s ability to detect the female priming pheromone (Olsén and Liley, 1993; Moore and Waring et al. 1996).

Olfaction

The hydrodynamic structure of this system allows for water to freely enter and exit the olfactory rosette, meaning that the only way for a fish to block a substance from entering the olfactory rosette is through behavioural avoidance. (see review by Cox, 2008). A functioning olfactory system is vital to the survival and overall success of salmonid individuals.

In teleost, the olfactory system is crucial for spawning synchronization, mediating both endocrine responses (priming effect) and behavioural
responses (releaser effect) (Vermeirssen, 1997; review by Stacey and Sorensen, 2002). Several studies have outlined the direct correlation between the detection of pheromones by a functioning olfactory system and the activation of the endocrine system. (e.g. Essington and Sorensen, 1996; Yambe et al., 1999; Olsén and Liley, 1993; Olsén et al.2000; Yambe and Yamazaki, 2000; Ploing et al., 2001; LaBerge and Hara, 2003). Furthermore, the sensitivity to these pheromones has a seasonal variation, as the parr are only physiologically primed by olfactory detection of pheromones during the spawning season (Moore and Waring, 1996; Hamdani et al. 2008). Interestingly, the sensitivity of the olfactory system to pheromones also increases with increasing levels of hormones in male fish as seen during sexual maturation (Belanger et al. 2010).

In some salmonids, the female releases both a priming pheromone, (prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$)), and a releasing pheromone (molecule unknown) that induces both hormonal changes and attraction to females in the males (Scott et al. 1994; Moore and Waring, 1996; Waring et al., 1996; Olsén et al., 1998, 2002; Moore et al., 2002). Male brown tout parr with an experimentally induced anosmia (blocked nasal passage with dental silicone) had lower sex hormone levels, were less aggressive, and courted the female fewer times than control males, indicating the important role of olfaction in the male reproductive system (Olsén et al., 1998).

**Aims**

The main objective of this study was to investigate if exposure to cypermethrin (I, VI), copper (II), or glyphosate (III) has an affect on olfactory mediated reproductive responses in the parr. The main components of the study were responses quantified by the amount of milt produced and blood plasma levels of both 11-KT and 17,20$\beta$-P as well as behavioural changes. The specific aims were: (i) Is the level of 11-KT and 17,20$\beta$-P circulating in the plasma or milt volume effected? (ii) Do any of these exposures change the reproductive behaviour of the parr? (iii) If behavioural changes do occur, will they correlate to endocrine responses i.e. altered sex hormone levels and level of aggression?
Methods

Experimental Animals
Anadromous mature male and female brown trout were captured in the river Dalälven in the autumn of the year of study. They had returned to their natal river after two to three years in the Baltic Sea. These fish were kept at the National Board of Fisheries Research Station, Älvkarleby, and females were checked once a week to ensure ovulation.

The spermiating male brown trout parr and Atlantic salmon parr were two-year-old hatchery reared individuals randomly sampled from the station stock supplies. The parr were fed granulated food pellets (ca. 1% of the body mass per day) (Aller Aqua 500; Christiansfeld, Denmark). No food was given during exposures. The mass, total length and gonado-somatic index \(\text{GSI}= (\text{gonad mass} + \text{stripped milt mass}) / \text{mass of fish x 100}\) for each group was measured after each experiment.

Pesticide Exposure
Mature male parr were randomly selected from the stock tank and placed in clean, static, aerated treatment tanks (400 L volume). The water was changed every 24 H by emptying approximately 75% of the water before fresh water was allowed to vigorously flow through the tank for at least ten minutes. After this flushing period, the water was returned to the assigned volume, turned off, and the designated treatment was reapplied. The water was continuously aerated and the temperature averaged 3°C. The fish density was ca. 1 g L\(^{-1}\) water, which is near the recommended maximum for semi-static exposures (renewal tests in Welsh et al. 2008)

The control group was treated with either ethanol solvent or water only, as the experiment required. The treatment groups were exposed to one of the following treatments: 1) 0.1 or 1.0 \(\mu\text{g L}^{-1}\) cypermethrin diluted in ethanol; 2) 10 or 100 \(\mu\text{g L}^{-1}\) Cu\(^{2+}\) from copper sulfate diluted in water, or 3) 150 \(\mu\text{g L}^{-1}\) glyphosate diluted in water. All chemicals were purchased from Sigma-Aldrich (Stockholm, Sweden): cypermethrin (\(\text{C}_{22}\text{H}_{19}\text{Cl}_{2}\text{NO}_{3}\)) 98% pure, copper as CuSO\(_4\) 98% pure and glyphosate as glyphosate acid (N-(phosphonomethyl)-glycin, \(\text{C}_3\text{H}_8\text{NO}_5\text{P}\)) 99% pure.
Pesticide Analysis
Throughout the exposure period, water samples were taken from the holding tanks. These samples were collected before and immediately after change of water, as well as midway through the exposure period. The samples containing cypermethrin and glyphosate had a 10 ml portion of the preservative dichloromethane added to the water, creating a final volume of 1 L. They were kept at +7°C until they could be taken to the environmental laboratory for chemical quantification. No preservative was added to the copper samples; a 1 L sample was collected and kept at +7°C until processing. For all pesticides the predicted concentrations were confirmed by the quantification. The actual concentrations are given in papers I-IV.

The water of Dalälven River supplying the hatchery is analyzed several times per year (Department of Environmental assessment, University of Agriculture Uppsala, Sweden) and is reported in paper II (Jaensson and Olsén 2010).

Sampling of Fish
After each experiment, the parr were anaesthetized (0.05% 2-phenoxyethanol), weighed, total length measured, and stripped of milt. The milt was weighed, a blood sample was taken and the gonads removed and weighed to calculate GSI. The blood sample was drawn from a caudal vessel, and centrifuged; the plasma was decanted and frozen. The plasma was later analyzed for 11-KT and 17,20β-P using radioimmunoassay (RIA). The RIA procedure for analyzing 11-KT and 17,20β-P and cross-reactivities of the antiserum have been described by Scott et al. (17,20β-P in Scott et al. 1982, and 11-KT in Scott et al. 1984). The adult females and adult males were weighed and killed.

Experiments
Priming Experimental Design
Before priming with female odors (a mixture of ovarian fluid and urine ((OVF), (Olsén et al., 2000) or PGF$_2$α (Moore et al., 2002)) the male parr were exposed for 96 H to the pesticide under investigation. Three groups of parr were studied; pesticide exposed +OVF (“pesticide” exposed), control +OVF (control), and water control –OVF (water control). On the third day, milt was stripped and the parr were allowed to recover for 24 H in the same exposure concentrations. This enabled a clearer measurement of differences in increased milt volumes. For the glyphosate experiments, the parr were
stripped of milt on day one and then allowed to recover for the remaining exposure period. The milt quality was investigated during the glyphosate studies; all of the sperm used in the fertilization study should have matured during the exposure. For the recovery period, the male parr were replaced in the treatment tanks. After the 96 H exposure the appropriate priming treatment was then applied for 5 H. The estimated concentrations of PGF$_{2\alpha}$ was between $10^{-8}$ and $10^{-7}$ M, whether the sample was pure PGF$_{2\alpha}$ or PGF$_{2\alpha}$ in the OVF solution (Moore et al., 2002). After priming, 2-3 fish were anaesthetized with 0.05% 2-phenoxyethanol. Blood and milt from all fish were sampled within ca. 20 minutes after removal from treatment tanks, alternating between exposed and control fish.

**Fertilization Test**

The fertilization ability of Atlantic salmon parr sperm was examined after glyphosate exposure. The eggs (200 from four different females kept in OVF) used during this experiment were collected at the hatchery immediately after they were harvested from ovulating females. They were transported to the laboratory in 0.5 L fertilization cups where they were kept cool until being used. A milt sample was taken from each parr. The initial milt was collected in a capillary tube and centrifuged, and the amount of spermatozoa was recorded. The volume of milt needed for each male to have the same percentage of spermatozoa in the fertilization cup was calculated. The milt sample from each parr was then added to an assigned fertilization cup holding twenty eggs. Stream water was added to the cup to activate the sperm. Each sample was allowed to incubate for three minutes. After that time the eggs were rinsed and each group of twenty eggs placed in a hatchery tray. The hatchery tray system was created so that river water was freely flowing over the eggs. The eggs were allowed to mature, and it was noted how many of them hatched.

**Behavioural Experimental Design**

**Stream tank**

Behaviour experiments were performed in a stream aquarium (oval; 35 000 L) located in the Stream Water Ecology laboratory at the Swedish National Board of Fisheries research station, Älvdalen, Sweden. Aerated groundwater was used and has a higher content of minerals than the river water (ca: 70 mg L$^{-1}$; alkalinity: 24.8 mg L$^{-1}$; total hardness: 10.5 oH; conductivity: 449 μS cm$^{-1}$; pH: 7.5; Uppsala Water Laboratory, ISO/IEC 17025). Water was supplied continuously, and the temperature was 7°C. A turbine was located at the beginning of each long side of the tank to create a circular flow. The current just down stream from each turbine was 9-10 cm s$^{-1}$ (measured with Novu...
Stream Flow). Further downstream, the current varied between 3-8 cm s⁻¹, depending on the bottom structure and the depth. The bottom of each long side was covered with a thick layer of gravel. The photoperiod was set on a twelve-hour cycle. Further details about the stream tank, including a drawing and technical information is given in Olsén et al. (1998).

Experimental design
The chemical exposures were conducted in the same manner as described above. Each experimental group consisted of eight brown trout parr from each treatment (total of 16 parr), four anadromous males, and two ovulated females. On the third day of exposure during the copper and cypermethrin experiments, all the parr were anaesthetized, tagged with a numbered disc, and stripped of milt. Parr were allowed to recuperate for 24 H under the same exposure conditions. During the glyphosate experiments, the parr were stripped and tagged on day one, and the recuperation was over the remaining exposure period.

Approximately 12 H before the beginning of the behaviour observations, four anadromous brown trout males and two ovulating females were added to opposite sides of the tank. Nets were used to keep the males and females separated during the acclimatization period. Early morning on day four (96 H), the eight parr of each treatment were added to the stream tank and the nets removed. Fish were allowed to acclimatize for at least one hour before behaviour observations began.

For the continuous cypermethrin exposure experiments the stream aquarium was static as the treatment (either cypermethrin or ethanol) was added directly to the tank. The first half of the week was dedicated to the control experiment, with sixteen brown trout parr being exposed in the same way as described. After each experiment the aquarium was emptied and ground water continuously flowed (5000 L H⁻¹) through it during 48 hours. The internal circulation rate remained the same as in the other experiments. This was the only time that any chemical was added to the stream tank and the stream tank held in a static state. This experiment was the only one where sixteen control parr and sixteen exposed parr were observed in separate groups.

Observation of behaviour
Each individual parr was observed for six minutes (“focal parr”) – with courting behaviour and interactions with other males recorded. Courting involved the male approaching the digging female and quivering his body,
touching the caudal part of her body, or the anal fin area. Non-reproductive behaviours were also recorded, including acts of aggression (frequency of displays, biting, chasing) that were either initiated or received; time holding low, when the individual was lying on the substrate; time holding high, when the individual was off of the bottom and holding his position in the water column; time holding at surface, when the individual was held at the surface of the water; and time cruising; when the fish swam freely in the water column upstream or downstream. For a detailed table describing all behaviours, see paper II.

The 6 minute observations were repeated eight times for each fish (six times on day 1, and twice on day 2) periodically during the 24 H duration. Focal parr were studied in random order, and the two observers followed each fish the same number of times. All data for each fish were pooled to one observation. This gave eight observations for each treatment per trial. After each trial the stream tank was emptied of water and all fish were removed. The adult males and females were replaced by new fish.

Statistics

One-way ANOVA was used when more than two treatment groups were compared, and in case of significance the Newman-Keuls multiple comparisons post-test was applied. A two-tailed unpaired t-test was used in cases of comparisons between two groups. In case of differences in variance, data were log-transformed ($\log_{10}$). Correlations were calculated according to Spearman calculations. The program GraphPad Prism™ (Graph Pad Inc., California) was used for both statistics and graphs.
Table 1. Overview of results: (-) indicates exposed had lower amount or frequency; (+) indicates exposed had higher amount or frequency; (0) indicates the exposed had no difference in amount or frequency. All of the differences are significant unless indicated. (Exposure concentration) 150t indicating trout or 150s indicating salmon. Cyper cont is cypermethrin continuous exposure.

<table>
<thead>
<tr>
<th>Variables</th>
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<tr>
<td>Priming</td>
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<tr>
<td>Milt</td>
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<tr>
<td>17,20βP</td>
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<tr>
<td>11-KT</td>
<td>-(1.0)</td>
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<tr>
<td>Behaviour</td>
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<tr>
<td>Milt</td>
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<td>11-KT</td>
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<td>Courting</td>
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<tr>
<td>Aggression</td>
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Results and Discussion

The effect of exposure on the levels of strippable milt, and plasma levels of 11-KT and 17,20β-P

Phase I-Priming
The parr males pre-exposed to 1.0 μg L⁻¹ cypermethrin and then primed with OVF had significantly lower levels of 17,20β-P (Fig.4a $F_{(2,21)}=21.30^{***}$) and 11-KT in the blood plasma than did the control parr  (Fig.4b $F_{(2,21)}=4.12^{*}$) (Paper I).

The control parr produced almost twice as much milt as either of the copper treated groups (10 μg L⁻¹ and 100μg L⁻¹) after being primed with female pheromones (prostaglandin F₂α) (Fig. 4f $F_{(2,43)}=24.35^{***}$ ) (Paper II).

In the glyphosate study the exposed Atlantic salmon parr had significantly less 11-KT when compared to controls after the OVF priming period (Fig. 4k $F_{(2,45)}=5.12^{**}$) (unpublished data).

In the fertilization study using only Atlantic salmon exposed to glyphosate, there was no difference in the percentage of eggs that hatched into fry when comparing the exposed group to the control group, as nearly all of the eggs hatched. The amount of milt calculated to have an equal percentage of spermatozoa from each male used to fertilize eggs was not significantly different (unpublished data).

Phase II-Behaviour
The cypermethrin behavioural portion of the current project demonstrated that the exposed parr had lower levels of 11-KT in the blood (Fig. 5b $F(2,45)=5.18^{**}$) and lower amount of strippable milt (Fig. 5c $F(2,45)=3.82^{*}$) (paper I). The response to cypermethrin seemed to be dose dependent, as the parr treated with 0.1 μg L⁻¹ cypermethrin had no significant differences in any variable compared with the control males, although a trend towards lower milt volumes was seen when compared with the control parr (Fig. 5c $F(2,45)=3.82^{*}$) (Paper I). The parr exposed 0.1 μg L⁻¹ continuous cypermethrin (Paper IV) had significantly higher plasma
levels of 11-KT (Fig. 5e p=0.026) and 17,20β-P (Fig. 5d p=0.011) than control parr after behaviour observations implying that the parr in paper I could have been recovering. Continuous cypermethrin 0.1 μg L\(^{-1}\) exposure (paper IV) did not have an effect on the amount of strippable milt.

Parr exposed to 100 μg L\(^{-1}\) copper had significantly higher plasma concentrations of 11-KT (Fig. 5h p=0.014) than did the control fish, but no differences were seen in 17,20β-P levels, nor in the amount of strippable milt (Paper II).

Significantly lower amounts of 11-KT were seen in the blood plasma of the glyphosate-exposed groups in the brown trout parr after the behavioural experiments (Fig. 5k p=0.041) (Paper III). There was no difference in the amount of strippable milt or 17,20β-P plasma levels.
Figure 4
Figure 4.
Figure 4.
Figure 4. Amounts of sex hormones in the blood plasma and strippable milt in the priming experiments reported in papers I-IV. Columns represent means and the vertical bars standard deviations. “Water” or “water control” control group without priming exposure and only exposed to water. “Control” Primed control, control group that has been primed by female pheromones with pr exposure to either water or solvent. “1.0 Cyp + OVF” group exposed to 1.0 μg L⁻¹ Cypermethrin prior to priming. “10 Copper” group exposed to 10 μg L⁻¹ Copper prior to priming. “100 Copper” group exposed to 100 μg L⁻¹ Copper prior to priming. “150 Gly” group exposed to 150 μg L⁻¹ Glyphosate prior to priming. *p<0.05; **p<0.01; ***p<0.001. Comparisons between three treatments: One-way ANOVA, followed by Newman-Keuls multiple comparisons test; comparisons between two treatments: unpaired t test.
Figure 5.
Figure 5.
Figure 5.
Figure 5. Amounts of sex hormones in blood plasma and strippable milt after behavioural experiments reported in papers I-IV. Columns represent means and the vertical bars standard deviations. “Control” group exposed to either water or ethanol. “1.0 Cyp” group exposed to 1.0 μg L⁻¹ Cypermethrin. “0.1 Cyp” group exposed to 0.1 μg L⁻¹ Cypermethrin. “100” group exposed to 100 μg L⁻¹ copper. “150 Gly” group exposed to 150 μg L⁻¹ Glyphosate. *p<0.05; **p<0.01. Comparisons between three treatments: One-way ANOVA, followed by Newman-Keuls multiple comparisons test; comparisons between two treatments: unpaired t test.
Effects of exposure on the parr reproductive behaviour

Parr exposed to 1.0 \( \mu \text{g L}^{-1} \) cypermethrin had a lower number of courting events and spent less time near the female (Table 1) while parr treated with 0.1 \( \mu \text{g L}^{-1} \) cypermethrin demonstrated no significant differences in any variable compared with the control males (Paper I). Parr that were exposed continuously to 0.1\( \mu \text{g L}^{-1} \) cypermethrin had tendencies to be more aggressive (paper IV).

Copper exposed parr spent less time near the female and committed fewer courting acts compared to controls (paper II).

Behavioural Correlations

The parr exposed continuously to 0.1 \( \mu \text{g L}^{-1} \) cypermethrin had a positive correlation between the number of aggressive acts and the amount of time spent near the female (p=0.0016 \( r^2=0.74 \)). Similarly, these parr had a positive correlation in the number of courting acts performed and the amount of time spent near the female (p=0.045 \( r^2=0.26 \)), (Paper IV). There were no correlations found in these control parr.

The control parr tested during the cypermethrin investigation in paper I had a positive correlation between the number of courting events and the amount of time spent near a female (p < 0.0001; \( r^2 = 0.67 \)). There was also a positive correlation in the control group between 17,20\( \beta \)-P plasma concentration and the amount of time spent near a female (p < 0.017; \( r^2 = 0.24 \)). Likewise, in the control group, there was a significant positive correlation between the 17,20\( \beta \)-P plasma concentration and the number of courting events (p < 0.0022; \( r^2 = 0.35 \)). There were no correlations found in the exposed parr.

Effects of exposure on olfactory mediated endocrine response and behavioural response

From the results of the current study it appears that exposure to cypermethrin, copper or glyphosate may interfere with the endocrine system in mature male parr. The absence of behavioural responses from the copper (\( \text{Cu}^{2+} \) 100 \( \mu \text{g L}^{-1} \)) and cypermethrin (1.0 \( \mu \text{g L}^{-1} \)) exposed parr is in accordance with findings from several other research groups that have exposed reproductively mature fish to treatments inhibiting the olfactory system (Tierney et al. 2007; Jaensson et al. 2007; Moore and Waring, 2001; Winberg et al. 1992). It is possible that these two pesticides are directly affecting the olfactory system.
Cypermethrin
In a previous study (Moore and Waring, 2001), it was shown that exposure to cypermethrin inhibited the ability of male parr to mount a priming response to female pheromones. Cypermethrin directly affected the ability of the parr to smell the pheromonal compound PGF$_{2\alpha}$. These observations suggest that the altered behaviour of cypermethrin exposed parr is due to an inability to detect pheromones.

Cypermethrin acts upon the central nervous system by causing sodium ion channels to stay open longer than normal (Vijverberg and van den Bercken, 1990; Soderlund et al. 2002). This interference causes an extended depolarization of the nerve membrane, allowing for repetitive impulse activity (Narahashi, 1985; Vijverberg and De Weille, 1985) contributing to an increased release of neurotransmitters. It has been reported that fish are more sensitive to this affect on the voltage gated sodium ion channel than other vertebrates (Eells et al., 1993). It is possible that the difference seen in the first cypermethrin study (loss of behaviours and endocrine response in 1.0 μg L$^{-1}$ exposure group) could have been caused by loss of function in the neurological system in the olfactory rosette. In the second cypermethrin study, continuous low concentration exposure (0.1 μg L$^{-1}$) seemed to cause the nerve cell to become oversensitive. The continuously exposed parr had higher sex hormone levels and similar milt production compared to controls. The 0.1 μg L$^{-1}$ cypermethrin exposed parr in paper I had similar levels of sex hormones and a trend for lower milt production when compared to controls. This could be a demonstration of hormesis as the parr may experience different effects at continuous lower concentration exposure (0.1 μg L$^{-1}$) than effects at the higher concentration (1.0 μg L$^{-1}$) cypermethrin (paper I, IV).

Copper sulphate
Beyers and Farmer (2001) described how copper affected the reaction to predator stimulus, along with quantifying the presence or absence of olfactory receptors in the epithelium of the Colorado pike minnow. There were two different durations of exposure; 24 H with a range of six different copper concentrations (from <10 μg L$^{-1}$ to 266 μg L$^{-1}$), and 96 H with five different copper concentrations (ranging from <10 μg L$^{-1}$ to 120 μg L$^{-1}$). The fright response was reduced during both incubation periods. Damage to receptor cells in the olfactory rosette was reported. It was concluded that the reduction in behavioural response was due to copper exposure damaging the olfactory receptor cells. The copper exposure used during the current study corresponds with those presented by Beyers and Farmer (2001). It is possible that copper exposure damage the olfactory cells in the brown trout parr causing a reduced response to female pheromones.
When comparing copper exposed parr in the priming experiment with controls, the control primed group had nearly double the amount of strippable milt as the exposed primed group. Copper is actively transported, similar to amino acid and protein transportation having the same entry path as Na+ (Sloman, 2002). Copper can be taken up by the fish through the diet and even across the gills (Grosell and Wood, 2002). This means that copper could be affecting several systems in the fish. The only significant difference seen during the copper priming experiment was in the amount of strippable milt collected (the controls having almost twice the amount of either treated group). As there was no difference in the amount of 11-KT or 17,20β-P in the blood plasma, the effect could be directly on the testicles themselves. It is possible that the exposed fish in the behavioural study had sufficient time to recover or the effects were buffered by other compounds in the water, as there was no difference seen in strippable milt when comparing those groups.

**Glyphosate**

The results from the current study indicate that glyphosate may have an effect on 11-KT concentrations in the blood plasma of salmonids. Tierney et al (2006, 2007) has described the effects of both glyphosate and Roundup® on the olfactory system in salmonids. In 2006 they reported that a 10 min exposure to 1 mg L⁻¹ glyphosate alone was sufficient to significantly reduce the EOG reading detecting L-serine. The next year they demonstrated that 10⁻⁷ M L-histidine evoked EOG readings were significantly lower after an exposure for 20 min. to 100μg L⁻¹ Roundup® (AI glyphosate). However, the results from the current study do not demonstrate a strong affect on the olfactory system. Instead glyphosate may directly act upon the endocrine system indicated by a change in 11-KT production.
Conclusions

It appears the synthesis of 11-KT in the salmonid parr is affected by pesticide interference with the olfactory and endocrine system. Production of 11-KT was significantly altered by exposure to cypermethrin and glyphosate, both during priming and behavioural studies. Cypermethrin appears to decrease reproductive behavioural responses as well as sex hormone level implying that there may be an effect in the olfactory system. The effects described from copper exposure seem to be from mechanisms acting upon both the olfactory system and the endocrine system as there was a reduction in spawning behaviour and milt production, but not in sex hormones. However, glyphosate did not cause observable behavioural changes and only lowered 11-KT indicating that any effects were acting directly upon the endocrine system and not via olfactory regulation.

Our results demonstrate that there is a possibility of environmentally relevant pesticide concentrations being capable of suppressing the brown trout and Atlantic salmon male endocrine response, including spawning behaviour, to female pheromones. Detected levels in natural waterways of cypermethrin can range from 0.0046 to 2.8 μg L⁻¹ (House et al., 1997; Environment Agency, 1998, 2003; Pfeuffer, 1999) of Cu²⁺ can range from 0.04 μg L⁻¹ to 294 μg L⁻¹ (WHO 1998; Goodyear and McNiel, 1999; Neal and Robson, 2000; Mansour and Sidky, 2002; An and Kampbell, 2003; Baresel et al. 2006) and glyphosate can range from 328 μg L⁻¹ to 0.02 μg L⁻¹ (Scribner, et al. 2003; Battaglin et al. 2008; Struger et al. 2008). The levels of exposure in the current study are, therefore environmentally relevant (cypermethrin 1.0 and 0.1 μg L⁻¹, Cu²⁺ 100 and 10 μg L⁻¹ and glyphosate 150 μg L⁻¹). Investigating the effects of relevant levels of exposure is imperative to understanding what is happening in the environment. It is seldom that these animals actually come in contact with acute levels of pesticides. However, their environments are inundated with low levels of pesticides, and more research will need to be done using environmentally relevant exposures.
Sammanfattning på svenska


Varje experiment var uppdelat i två delar. Under första delen testades hanarnas hormonella respons till lukter från lekmogna honor efter att de exponerats för respektive bekämpningsmedel. Antingen användes ovarietska eller hormonet prostaglandin F$_{2a}$ som visat sig utgöra eller vara en viktig del av det feromon som örinhonors avgar för att stimulera hanar
hormonellt. Efter fem timmars exponering till honlukterna avbröts experimentet. Hanarna bedövades med 2-fenoxyetanol varefter ett blodprov togs och mängden tillgänglig sperma mättes. Fiskarna avlivades, längd och vikt mättes och testiklarna vägdes. Under andra delen av experimentet placerades pesticidexponerade hanar och kontrollhanar, som inte blivit exponerade för bekämpningsmedlet, i ett ovalt 35 000 L strömakvarium tillsammans med två lekmogna honor och fyra stora hanar som återkommit från havet för lek. Småhanarnas beteende registrerades individuellt under ett antal perioder på 6 min varandra under 24 timmar då det var ljus. Även beteendet hos honorna registrerades när de börjat gräva lekgropar och beteendet hos uppvaktande hanar och vilka som deltog i en eventuell lek noterades. Efter experimentet fångades samtliga fiskar och småhanarna bedövades varefter de mättes och blodprov togs. Experimenten visade som förväntat att cypermetrin och kopparsulfat hade negativa effekter, dels på hanarnas hormonella respons på honferomonerna men även deras beteende i strömakvariet var stört.

Hanar som exponerats för 1 μg L⁻¹ cypermetrin hade signifikant lägre blodplasmahalter av könshormonerna 11-ketotestosteron (11-KT) och 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) jämfört med kontrollerna som enbart blivit exponerade för pesticidens lösningsmedel etanol. I strömakvariet upptäcktes hanar som exponerats för 1 μg L⁻¹ cypermetrin inå uppvaktningsbeteenden och var signifikant kortare totaltid med honorna än kontrollerna. Dessa hanar hade även signifikant lägre blodplasmahalter av 11-KT och mängd tillgänglig sperma. Inga signifikant skillnader i beteende eller hormonnivåer jämfört kontrollerna observerades efter exponering till 0.1 μg L⁻¹ cypermetrin, men skillnader fanns när de två exponeringskoncentrationerna jämfördes. Hanar som exponerade till den lägre koncentrationen hade signifikant högre uppvaktningsfrekvens och var längre tid med honorna. Dessutom hade hanarna som exponerats för den lägre koncentrationen signifikant högre blodplasmakoncentration av 11-KT. För att undersöka om det eventuellt förekom en återhämtning hos de hanar som exponerats för den lägre koncentrationen gjordes ytterligare en studie där cypermetrin efter fyra dygn exponering till 0.1 μg L⁻¹ även tillsattes under beteendexperimentet. Resultaten från denna studie gav oväntade resultat. De hanar som exponerats för cypermetrin hade högre plasmahalter av både 11-KT och 17,20β-P jämfört med kontrollerna, dessutom hade de en tendens till att vara mera aggressiva.

I försöken med kopparsulfat erhölls resultat som visade på störningar. Efter exponering till 10 eller 100 μg L⁻¹ Cu erhölls efter exponering till honferomon signifikant lägre spermamängder jämfört med kontrollerna, men inga hormonella skillnader observerades. I beteendestudien var exponerade hanar (enbart 100 μg L⁻¹ Cu testades) signifikant kortare tid med honorna,
upvisade lägre frekvens uppvaktningsbeteenden men hade signifikant högra plasmanivåer av 11-KT. Exponerade simmade signifikant kortare tid uppströms.

Exponeringar till ogräsbekämpningsmedlet glyfosat gav inte lika drastiska effekter som de övriga två bekämpningsmedlen. Inga signifikanta beteendeskilnader mellan exponerade och kontroller kunde observeras, men fiskar exponerade för glyfosat (150 μg L⁻¹ testades) upvisade signifikant lägre blodplasmanivå av 11-KT. Efter exponeringen till honferomoner uppvisade de exponerade fiskarna signifikant högre blodplasmahalter av 17,20β-P jämfört med bägge kontrollerna. Resultaten med glyfosat tyder på att molekylen kan ha direkt endokrinstörande effekt snarare än effekt via störningar på feromondetektion.
Acknowledgments

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To Henrik, Rafael, and Gabriel, the three of you are my whole world and you have created the best of who I am.

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References


Prat, F., Sumpter, J. and Tyler, C. 1996. Validation of radioimmunoassays for two salmongonadotrophins (GTH I and GTH II) and their plasma concentrations throughout the reproductive cycle in male and female rainbow trout (Oncorhynchus mykiss). Biol. Reprod. 54: 1375-1382.


priming pheromone which enhances plasma levels of sex steroid and gonadotrophin II in males. J. Fish Biol. 44: 131-147.


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