Journal of FISH BIOLOGY
Published for the Fisheries Society of the British Isles

Journal of Fish Biology (2010) **76**, 800–817
doi:10.1111/j.1095-8649.2009.02519.x, available online at www.interscience.wiley.com

Effects of copper on olfactory-mediated endocrine responses and reproductive behaviour in mature male brown trout *Salmo trutta* parr to conspecific females

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(Received 17 December 2008, Accepted 4 November 2009)

In the present study, the effects of copper ($CuSO_4$) on the ability of mature male brown trout *Salmo trutta* parr to detect and react both physiologically and behaviourally to female pheromones were studied. The study was composed of two parts. In the first experiment, priming effects of the female pheromone prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were evaluated by determining the amount of milt produced and the blood plasma levels of 11-ketotestosterone (11-KT) and 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) after the $PGF_{2\alpha}$ exposure. In the second experiment, male parr were placed in a large stream tank together with a group of adult males and ovulated females and their individual behaviours were recorded. In the priming experiment, the amount of expressible milt was significantly lower, less than half, in groups exposed during 4 days to 10 or 100 μg l^{-1} copper compared with control parr only exposed to water. No significant differences were observed in plasma levels of 11-KT and 17, 20β-P. During the behavioural experiment, exposed parr spent less time with the female and had a lower number of courting events. Blood plasma levels of 11-KT were, however, significantly higher in the group exposed to 100 μg l^{-1} copper compared with the control group. Furthermore, the exposed group spent significantly less time swimming upstream than did the control group. The present study demonstrates that exposure to copper affects reproductive behaviours and endocrinology of *S. trutta* male parr.

Key words: hormones; olfaction; pheromones; pollutants; reproduction.

INTRODUCTION

Copper is a known pollutant from industry, urban waste, mining and agriculture. It enters streams and waterways by way of storm-water run off, overspray and direct deposition. Non-anthropogenic activities such as mineral leaching also contribute to copper contamination of waterways. Due to local activities and natural mineral composition, copper levels in contaminated waterways have been found to vary between a few μg l^{-1} to *c*. 100 μg l^{-1} and levels as high as just under 300 μg l^{-1} (Goodyear & McNiel, 1999; Neal & Robson, 2000; Olsvik *et al*., 2000; Mansour & Sidky, 2002; Harper *et al*., 2009; Heier *et al*., 2009). Interestingly, salmonids

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including brown trout *Salmo trutta* L. can be found in contaminated areas despite their high sensitivity (Harper *et al*., 2009). Although much has been done to reduce the amount of copper being released into the environment, it is still contaminating many natural waters. In fact the problems with copper contamination are increasing in certain areas. For instance, in Norway, the release of copper into the environment has increased by 40% during the period 1995–2006. This increase in copper release is caused by impregnation of fish-farming nets and seines plus increased use of anti-fouling agents (Norwegian Pollution Control Authority; www.miljostatus.no). The impregnation of nets has contributed to 50% of the increase.

It has been shown over several decades that copper is harmful to fishes (Brown *et al*., 1982). Several research groups have demonstrated that total concentrations of copper <100 μg l^{-1} can cause disruption in the olfactory system in several different species of fishes, both marine and fresh water (Brown *et al*., 1982; Beyers & Farmer, 2001; Baldwin *et al*., 2003; Sandhal *et al*., 2004). Moran *et al*. (1991) demonstrated that copper exposure caused neurons in the olfactory epithelium of *S. trutta* to deteriorate. The olfactory system is vital to the life cycle of the fishes. It is used to alert fishes about the presence of food, predators and potential mates (Hara, 1986). In a review paper by Stacey & Sorensen (2006), the importance of olfaction in the synchronization of spawning is described, both in terms of behavioural and physiological responses.

Salmonids avoid low concentrations of copper (≤ 5 μg l^{-1} Cu; Brown *et al*., 1982), which can block upstream swimming during spawning migration (Saunders & Sprague, 1967). Sandhal *et al*. (2007) demonstrated that a 20% reduction in olfactory response after juvenile coho salmon *Oncorhynchus kisutch* (Walbaum) were exposed to 4·4 μg l^{-1} for 7 days. The authors demonstrated a significant difference in the ability to respond to the presence of a predator after exposure for 3 h to copper levels as low as 2·0 μg l^{-1} (Sandhal *et al*., 2007). At higher concentrations, up to 20 μg l^{-1}, the ability to detect a predator was nearly abolished and fish were less likely to demonstrate predator avoidance behaviour. The authors imply that as the amount of copper increases the effect on the olfactory system will equally increase until the olfactory system is no longer able to process odour regulated behavioural responses. Bettini *et al*. (2006) exposed the African cichlid *Tilapia mariae* Boulenger to 20, 40 and 100 μg l^{-1} Cu during 4 days. They reported that the olfactory epithelium was extensively damaged in the group exposed to 100 μg l^{-1}. The damage was observed using light microscopy and was reported to include all cell types. Cilia and microvilli in the olfactory rosette were completely destroyed. The authors studied the recovery of the olfactory receptor cells after being exposed to 20 μg l^{-1} copper and found that after 10 days in clean water the olfactory epithelium no longer appeared different from the epithelium in control fish.

In most salmonid males, the endocrine system is stimulated by olfactory cues in connection with spawning. There have been several studies on the relationship between the olfactory system and the regulation of the endocrine system (Olsén & Liley, 1993; Olsén *et al*., 2000; Yambe & Yamazaki, 2000; LaBerge & Hara, 2003). Both Atlantic salmon *Salmo salar* L. and *S. trutta* males detect a priming pheromone, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and a releasing pheromone, identity unknown, which are released from females. Together these pheromones create hormonal changes and attraction to females, respectively (Waring *et al*., 1996; Olsén *et al*., 1998, 2002; Moore *et al*., 2002). It was then demonstrated that priming molecules are present

both in ovarian fluid and urine of ovulated females but only urine was attractive singularly (Scott *et al*., 1994; Olsén *et al*., 2001, 2002). The ability of males to detect both pheromones by way of olfaction is probably vital to reproduction. Olsén *et al*. (1998) examined effects of anosmia (loss of the sense of smell) on male *S. trutta* parr's behaviour and sex hormone blood plasma levels when placed together with ovulated nest digging females and adult anadromous males. They reported that parr with blocked nasal passages had significantly lower hormone levels, less volumes of milt, were less aggressive and courted females less than control males. This lack of reproductive response has been seen in other species. In representative cyprinids, cichlidae, salmonids and gobbies, reproductively mature male fishes having blocked nasal passages did not demonstrate the same response, behaviourally or physiologically, as fishes with functioning olfactory systems (Van den Hurk & Lambert, 1983; Kitamura *et al*., 1994; Olsén *et al*., 1998; Miranda *et al*., 2005; Belanger *et al*., 2007).

When the females ovulate, they release pheromones that the males detect by way of the olfactory system. Upon this detection, the male endocrine system is activated increasing the amounts of various hormones in the blood system. As the endocrine system is activated, the amount of 17α, 20β-dihydroxy-4-pregnen-3-one (17,20β-P) and 11-ketotestosterone (11 KT) will increase. 17,20β-P is important for the final maturation of eggs and spermatozoa and 11-KT is the major androgen in fishes (Stacey & Sorensen, 2006).

When these levels increase, males begin to demonstrate spawning behaviours and the amount of expressible milt increases. During the current study, the effects of copper on olfactory-mediated endocrine responses to a female pheromone and on behaviours in mature male *S. trutta* parr when placed with spawning adult individuals were examined. This was accomplished by way of two different experiments, priming and behavioural. The aim of the priming experiments was to test whether copper could affect the endocrine responses to the female pheromone, whereas the aim of the stream-tank behavioural experiment was to test the hypothesis that males exposed to copper are not as interested in the females as control males due to disruptions in the olfactory system. If copper is causing olfactory disruptions, then a decrease in the amounts of expressible milt and of 11KT and 17,20β-p in the blood should be seen. Also, the fish exposed to copper should not demonstrate the same level of reproductive behaviour as control fish.

MATERIALS AND METHODS

FISH

Anadromous mature male and female *S. trutta* were captured from the River Dalälven (60° 31′ N; 17° 26′ E) in the autumn of 2004. They had returned to their home river after 2 to 3 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. For the current study, the mean ± S.D. mass (M) of a sub-sample of adult males was 3·4 ± 1·6 kg (n = 12) and females weighed 3·4 ± 0·8 kg (n = 8). The experiments were performed in November (2004) during the natural spawning period of the River Dalälven *S. trutta* population.

The spermiating male *S. trutta* parr were 2 year-old hatchery-reared individuals randomly sampled from the station stock supplies. These males mature as parr, stay in the river and act as sneakers to fertilize some eggs when the adult fish spawn. Mature parr are also present in

populations of wild fish. Fish that mature as parr may migrate to sea later in their life cycle. Mature parr are the main subject in the current study. They are used in preference to mature males for several reasons. There are many parr available at the hatchery and the number of mature males returning to the river fluctuates every year. When planning experiments, it is vital to be able to rely on a certain number of individuals. Also, the anadromous mature males are important to the repopulation work at the hatchery while the parr are not. Many published studies use mature parr and therefore there is a large amount of material with which to compare the present results.

Fish were fed granulated food pellets (*c*. 1% of M per day) Aller Aqua 500 (www.alleraqua.com) but no food was given during the copper and water exposures. The M, total length (L_T) and gonado-somatic index (I_G) calculated from: $I_G = 100\ (M_G + M_{SM})M^{-1}$, where M_G, gonad mass, and M_{SM}, stripped milt mass, for each group were measured after each experiment (Tables I and II). There were no significant differences in size or I_G between the males of different treatment groups (Table I; one-way ANOVA, $P > 0{\cdot}05$; Table II, t-test $P > 0{\cdot}05$).

EXPOSURES TO COPPER

Mature male parr were randomly selected from the stock tank and placed in clean, static, aerated, treatment fibreglass tanks (400 l water; 10 × 10 dm bottom area). The fish density was *c*. 1 g l^{-1} water, which is close to the recommended maximum for semi-static exposures (renewal tests in Welsh *et al*., 2008). Half the top of each tank was covered with a lid and the other half with plastic netting. The tanks had no substratum. Control groups were treated with water and treatment groups were exposed to 10 or 100 μg l^{-1} copper diluted in water. The copper was purchased from Sigma as $CuSO_4$ (98% pure; www.sigmaaldric.com). Water was changed every 24 h for the entire 4 day exposure period and treatment reapplied. The procedure was the same as in a previous study (Jaensson *et al*., 2007). The copper concentration (total) was analysed in one water sample during exposure and found to be 7 μg l^{-1}, close to the nominal concentration 10 μg l^{-1} (SGAB Analytica, accrediated by Swedac no. 1087; www.infomine.com). Approximately 75% of the water was emptied before fresh stream water was allowed to flow vigorously through the tank for at least 10 min. After this flushing period, the water level was returned to the assigned level volume, the water supply turned off

TABLE I. Treatments of male *Salmo trutta* parr (exposure to copper diluted in water), mean ± S.D. mass (M), total length (L_T) and gonad-somatic index (I_G) of parr used in pheromone prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) experiments

Treatment with Cu	Days exposed	Priming solution	Hours primed	Number of fish	M (g)	L_T (mm)	I_G
Control (0 μg l^{-1})	4	$PGF_{2\alpha}$	5	20	98 ± 39	220 ± 24	3·51 ± 1·00
10 μg l^{-1}	4	$PGF_{2\alpha}$	5	10	102 ± 25	209 ± 31	3·08 ± 0·76
100 μg l^{-1}	4	$PGF_{2\alpha}$	5	20	116 ± 41	229 ± 19	3·11 ± 0·69

TABLE II. Treatments of male *Salmo trutta* parr (exposure to copper diluted in water) mean ± S.D. mass (M), total length (L_T) and gonad-somatic index (I_G) of parr used in behavioural (stream tank) experiments

Treatment with Cu	Days exposed	Number of fish	M (g)	L_T (mm)	I_G
Control (0 μg l^{-1})	4	32	100 ± 36	219 ± 21	3·24 ± 0·65
100 μg l^{-1}	4	31	99 ± 43	225 ± 22	3·12 ± 0·87

and either dissolved copper or water only added to create the needed concentration. The tanks were continuously aerated and the temperature averaged 5° C. The stream water entered the system directly from the hydroelectric dam upstream of the hatchery. Windows on the walls of the building secured a natural dark–light cycle (60° 34′ N). The water chemistry of the River Dalälven water supplying the hatchery is analysed several times a year (Department of Environmental Assessment, University of Agriculture Uppsala, Sweden; www.ma.SLU.se). It has a low concentration of copper and zinc, *c*. 1 and 10 μg l^{-1}, respectively. The ionic content of the stream water is low. The calcium and chloride concentrations have been measured to *c*. 0·25 meq l^{-1} (5 mg l^{-1}) and 0·05 meq l^{-1} (2 mg l^{-1}), respectively. The water has a pH of 7 (6·8–7·4) and the mean conductivity is *c*. 20 μS cm^{-1}. One sample of river water (24 November 2004) was collected and analysed (see Table III).

SAMPLES TAKEN AFTER EXPERIMENTS

After each experiment, parr were anaesthetized (0·05% 2-phenoxyethanol), weighed, measured, stripped of milt, milt weighed, blood taken and gonads removed and weighed to calculate I_G. Females and adult males were weighed and killed by a blow on the head. Blood was drawn from the caudal vessels. Immediately after collection, blood samples were centrifuged and plasma was decanted and frozen. Plasma was later analysed for content of 11KT and 17,20β-P by specific radioimmunoassay (RIA) as described previously (Moore *et al*., 2002).

TABLE III. The chemical composition of the Dalälven River water used during exposures and ground water used in the stream tank. Analyses done by Uppsala Water Laboratory (ISO/IEC 17025)

Chemical variable	River water	Ground water
Turbidity (FNU)	1·9	<0·1
Colour (mg l^{-1} Pt)	63·0	7·5
Chemical oxygen demand, COD-MN	10·0	<1·0
Conductivity (mS m^{-1}, 25° C)	4·5	44·9
pH	7·1	7·5
Alkalinity	12	248
Total hardness (°dH)*	<1·0	10·5
Calcium (mg l^{-1})	5·0	70·1
Magnesium (mg l^{-1})	0·7	3·1
Sodium (mg l^{-1})	2·3	8·3
Potassium (mg l^{-1})	0·6	2·0
Manganese (μg l^{-1})	22·9	<5·0
Ammonium (mg l^{-1})	<0·04	<0·04
Nitrite (mg l^{-1})	0·006	<0·007
Nitrate (mg l^{-1})	<5·0	1·2
Phosphate (mg l^{-1})	<0·04	<0·05
Fluoride (mg l^{-1})	<0·20	0·43
Chloride (mg l^{-1})	<4·0	16·0
Sulphate (mg l^{-1})	3·7	16·0
Iron (mg l^{-1})	0·30	<0·02
Copper (μg l^{-1})	<20	<20

* 1 °dH = 10 mg l^{-1} CaO.

PRIMING EXPERIMENTS

Priming experimental design

The same fibreglass tanks as described above were used during this study. $PGF_{2\alpha}$ (Sigma) was dissolved and diluted in ethanol before priming. It has been previously shown that *S. trutta* males exposed to $PGF_{2\alpha}$ increases their volumes of milt and sex steroid hormone levels (Moore *et al*., 2002). The copper-exposed group (five fish in each tank) was exposed to 10 or 100 $\mu g\ l^{-1}$ copper for 4 days before being exposed to the priming solution. The same procedure was used with five control fish. In total, there were 20 control fish and 30 copper-exposed fish. The number of tanks used in the study was restricted and, to limit the time at the hatchery, the experimental groups were composed of more than one fish at the time. On the third day of treatment, both controls and copper-exposed fish were stripped of milt (Moore *et al*., 2002) and allowed to recover for 24 h after water change with new solutions. The idea was that differences in priming responses with increased milt volumes between treatments should be clearer if the fish were stripped of milt (they were set to zero). After the 24 h recovery period, the parr were exposed to $PGF_{2\alpha}$. The prostaglandin and treatment were added after the water in the tank was changed in the same way as stated before. The priming exposure was for a 5 h period. The concentration of $PGF_{2\alpha}$ was chosen based on previous studies with *S. trutta* (Moore *et al*., 2002) and was estimated to be 10^{-8} M. After the priming, two to three fish were anaesthetized singularly in 0·05% 2-phenoxyethanol. Blood and milt from all fish were sampled within *c*. 20 min, and alternated between exposed and control fish.

BEHAVIOURAL EXPERIMENTS

Stream tank

Behaviour experiments were performed in a stream tank (oval; 35 000 l) located in the Stream Water Ecology Laboratory at the Swedish National Board of Fisheries Research Station, Älvkarleby. Aerated groundwater was used to eliminate outside influences and, as the water was not turbid, to aid with visual observations. The ground water has a higher content of minerals than the river water (*c*. 70 mg l^{-1}; alkalinity, 24·8 mg l^{-1}; total hardness, 10·5 °dH, 1 °dH = 10 mg CaO l^{-1}; conductivity, 449 $\mu S\ cm^{-1}$; pH 7·5; COD-MN, <1·0 mg l^{-1}; Uppsala Water Laboratory, ISO/IEC 17025; www.uppsala.se) (Table III). Water was supplied continuously and the temperature was set at 7° C. A turbine was located at the beginning of each long side of the tank to create a circular flow. The current just downstream from each turbine was 9–10 cm s^{-1} (measured with Novu Stream Flow). Further downstream, the current varied between 3 and 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 12L:12D cycle. Further information about the stream tank, including a drawing and technical information, is given by Olsén *et al*. (1998).

Experimental design

Exposure of male parr began under the same 4 day exposure to water or to 100 $\mu g\ l^{-1}$ copper dissolved in water as explained above. Each experimental group consisted of eight parr from each treatment (total of 16 parr), four anadromous males and two reproductively mature females. On the third day of exposure, 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. Approximately 12 h before the beginning of the behaviour observations, four anadromous males and two reproductively mature 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 4 (96 h), the eight parr of each treatment were added to the stream tank and the nets were removed. Fish were allowed to acclimatize for at least 1 h before behaviour observations began throughout the next 24 h.

Observations of behaviour

Each individual parr was observed for 6 min (focal parr), with courting behaviour and interactions with other males being recorded. Courting involved the male coming close to the digging female and quivering with his entire body. It also involved touching the caudal lateral part of her body or, more frequently, the anal-fin area. Non-reproductive behaviour was also recorded. This involved: acts of aggression (frequency of displays, biting and chasing) that were either initiated or received; time holding low, when the individual was lying on the substratum; time holding high, when the individual was off of the bottom and holding his position in the water column towards the current; time cruising, when the fish swam freely in the water column upstream or downstream. The upstream and downstream swimming were recorded separately. When a female started to dig a nest she was continuously followed by one of the observers. The female behaviour (frequency of probing, digging and spawning) and the behaviour of the different males, both small and adult, close to her were recorded. The behaviours recorded are summarised in Table IV, and for further information see Olsén *et al.* (1998).

The 6 min observations were repeated eight times for each fish (six times day 1 and twice day 2) during the light period of the experiment and focal parr were studied in random order

TABLE IV. The individual behaviours of male *Salmo trutta* parr recorded (for further details, see Olsén *et al.*, 1998)

Circumstances	Behaviour recorded	Description
Reproduction	Courting (frequency of bouts of quivering)	Male attending a nest digging female and quivers close to her
Reproduction	Time close to female	Male is present close to the female in or just outside her nest
Reproduction	Spawning	A female and at least one male are releasing their gametes with their bodies in parallel against the current just over the gravel
Aggression	Lateral and frontal display (frequency)	Includes erection of fins and flexing of the vertebrate column such that the head is above or below the horizontal mid-body axis, and flaring of the operculum opening. All or some of these components can be observed together
Aggression	Bite (frequency)	Snapping movement against another fish
Aggression	Attack and chase (frequency)	A fast approach towards another fish often followed by a bite. At least two attacks in a row against the same fleeing fish
General activity	Holding low (duration, s)	Fish laying motionless on the bottom
General activity	Holding high (duration, s)	Fish holding position in the water current
General activity	Cruising upstream (duration, s)	Free swimming upstream without any aggressions or reproductive behaviour
General activity	Cruising downstream (duration, s)	Free swimming downstream
Reproduction	Female probing (frequency)	Moving her erected anal fin into crevices of the bottom substratum
Reproduction	Female digging (frequency)	Female use her lateral side of the body and the caudal fin to make a depression in the bottom gravel

and the two observers followed each fish the same number of times. The two observers were 'calibrated' concerning the behaviours recorded. All data for each fish were pooled to one observation. This gave eight observations for each treatment per trial.

After a trial the stream tank was emptied of water and all fish were removed. New fish replaced the adult males and females. At no time was copper added to the stream tank.

As a limited number of adult fish was available, experimental groups had to include both control and exposed fish.

STATISTICS

One-way ANOVA was used when more that two treatment groups were compared and in case of significance this was followed by Newman–Keuls multiple comparisons post-test. A two-tailed unpaired *t*-test was used in cases of comparisons between two groups. In case of differences in variance, data were $\log_{10}$ transformed. Correlations were calculated according to Spearman. The programme GraphPad Prism™ (Graph Pad Inc.; www.graphpad.com) was used for both statistics and graphics.

RESULTS

PRIMING

One-way ANOVA revealed significant differences in milt volumes between the treatments [d.f. = 2,23,. $P < 0{\cdot}001$; Fig. 1(a)]. Newman–Keuls multiple comparisons test showed that the control group had nearly twice as much milt as either the 10 μg l^{-1} group ($P < 0{\cdot}001$) or the 100 μg l^{-1} group [$P < 0{\cdot}001$; Fig. 1(a)]. There was no significant difference seen between any of the groups in the blood plasma levels of 17,20β-P or 11KT [Fig. 1(b), (c)].

STREAM TANK

There was a significantly higher amount of 11KT in blood plasma of copper-exposed fish than in control fish ($P < 0{\cdot}01$), but no differences in concentrations of 17,20β-P in the plasma or amounts of milt were observed (Fig. 2). Control fish spent significantly more time in close proximity to the female ($P < 0{\cdot}05$) and they courted the female more than exposed fish ($P < 0{\cdot}05$; Fig. 3). Although each group of four anadromous males and two reproductively mature females spawned at least once during the observation periods, no parr male participated in a spawning event.

Of the non-prespawning behaviours, swimming upstream showed a difference between the exposed and non-exposed fish. The control fish spent significantly more time swimming upstream ($P < 0{\cdot}05$; Fig. 4). There were no significant correlations between hormone levels and behaviours.

DISCUSSION

The results in this study demonstrate the possibility that copper concentrations found in certain environments (Lauren & McDonald, 1986; McDonald & Wood, 1993; Olsvik *et al.*, 2000; Hansen *et al.*, 2006*a*, *b*; Heier *et al.*, 2009) are able to disturb male *S. trutta* courting behaviour and possibly disrupt spawning. Interesting though the high sensitivity of *S. trutta* and other salmonids to heavy metal pollutants

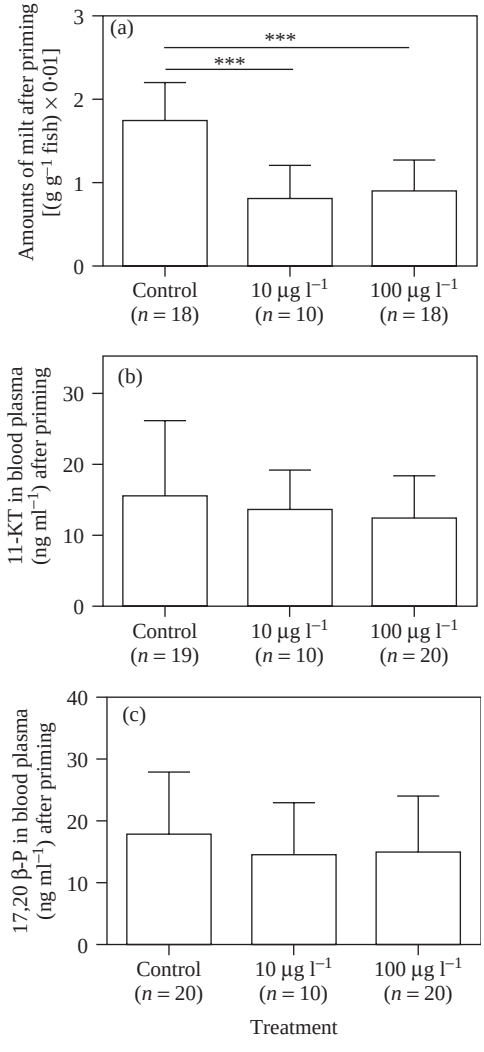

FIG. 1. The amounts of (a) strippable milt and the blood plasma concentrations of (b) 11-ketotestosterone (11-KT) and (c) 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) measured by radioimmunoassay in *Salmo trutta* parr after 5 h priming with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). 'Control' represents parr ($n = 20$ individuals) pre-exposed for 4 days to water only; '10 µg l^{-1}' represents parr ($n = 10$) pre-exposed for 4 days to 10 µg l^{-1} copper and '100 µg l^{-1}' represents parr ($n = 20$) pre-exposed for 4 days to 100 µg l^{-1} copper. Values are means + S.D. $^{***}P < 0{\cdot}001$ (one-way ANOVA, followed by Newman–Keuls multiple comparisons test).

(Linde *et al.*, 1998; Birge *et al.*, 2000) populations can be found in polluted rivers (Hansen *et al.*, 2006*a*) and in fact the fishes choose to use cold-water polluted parts of streams over less polluted but warmer areas (Harper *et al.*, 2009).

During the priming experiment, control parr produced more milt than either copper-exposed group after being exposed to female pheromones (prostaglandin $F_{2\alpha}$). During behavioural studies in the stream tank, control parr demonstrated more

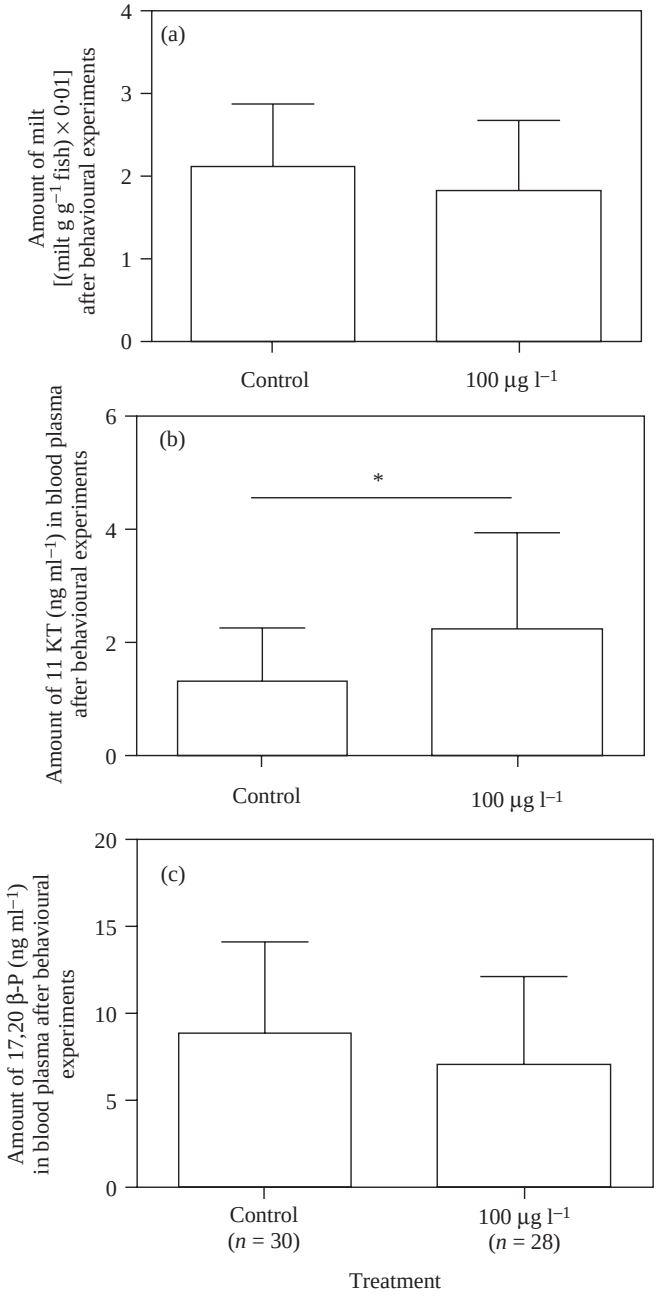

FIG. 2. The amounts of (a) strippable milt and the blood plasma concentrations of (b) 11-ketotestosterone (11 KT) and (c) 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) measured by radioimmunoassay and in *Salmo trutta* parr after 24 h in the stream tank. Values are means + S.D. 'Control' represents parr ($n = 30$) pre-exposed for 4 days to water only; '100 µg l^{-1}' represents parr ($n = 28$) pre-exposed for 4 days to 100 µg l^{-1} copper. $^{*}P < 0{\cdot}05$ (unpaired t-test).

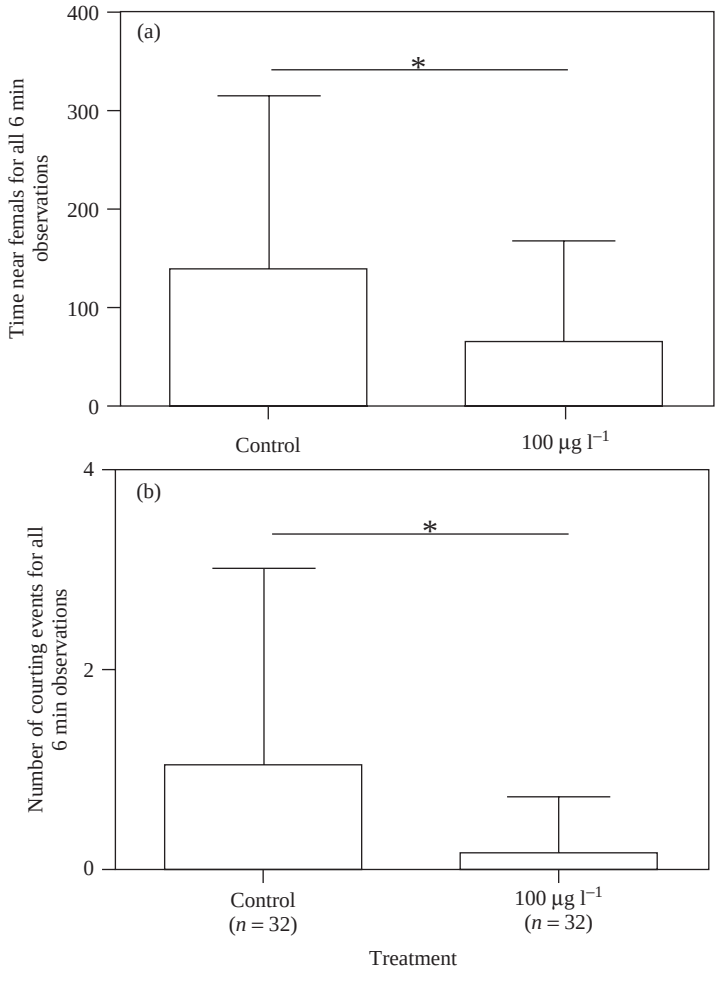

FIG. 3. (a) Reproductive behaviour data for male *Salmo trutta* parr from the 24 h stream tank 6 min observations: (a) time near female and (b) number of courting events. 'Control' represents 32 male *S. trutta* parr pre-exposed to water only. '100 µg l^{-1}' represents 32 parr pre-exposed for 4 days to 100 µg l^{-1} copper. The values are means + S.D. $^{*}P < 0{\cdot}05$ (unpaired *t*-test).

prespawning behaviours (close proximity to the female and number of courting events) than exposed fish. Among the other behavioural variables studied, the time of upstream swimming was reduced in exposed parr. There were some similarities with the results in the current study and those in Olsén *et al.* (1998) with anosmic and intact male *S. trutta* parr. Those similarities are that intact male parr spent more time in close proximity to the female, completed more courting events and had higher levels of expressible milt than anosmic parr when placed with a female.

Copper-exposed male parr *S. trutta* in the present study had reduced milt volumes after exposure to $PGF_{2\alpha}$ during the priming experiment but not after the exposure to mature females in the stream tank. There are some possible explanations to the different results. In the stream tank not only olfactory signals are present but also

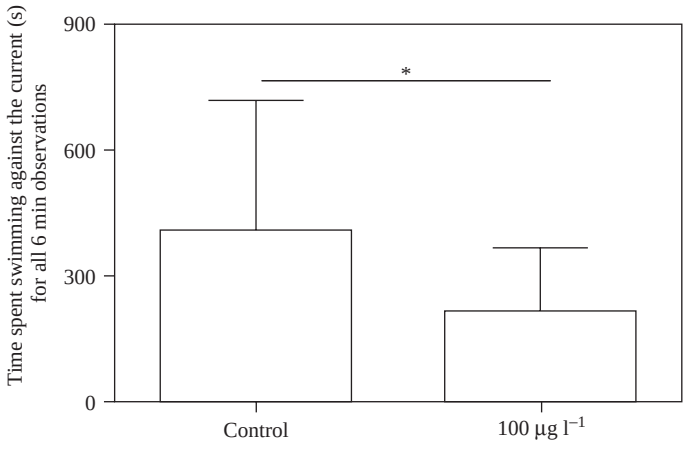

FIG. 4. The amount of time the male parr spent swimming upstream (total time recorded for each parr was 2880 s: 8 × 6 min × 60 s) during the 24 h stream tank experiment (see Fig. 3).

visual and sound cues can be detected. Sound from nest digging females can have endocrine priming effects in mature males (Moore & Waring, 1999) and there are also possible effects of female appearance in connection to the prespawning activities (Thompson *et al*., 2004). The female becomes darker in colour during nest digging and close to spawning she is almost black. It is also known that rainbow trout *Oncorhynchus mykiss* (Walbaum) males can spawn with models (Newcombe & Hartman, 1980) and both visual and vibrational stimuli are important in the spawning of kokanee salmon *Oncorhynchus nerka* (Walbaum) males (Satou *et al*., 1987), even though female odours are important to male behaviour and sex hormone status (Liley *et al*., 1993; Olsén & Liley, 1993). According to results, the copper treatment had greater effect on parr behaviour than the parr endocrine system. This may indicate that an intact olfactory sense is important to courting behaviour but information from other senses can also induce endocrine priming. It is interesting that exposed male *S. trutta* parr in the stream tank had higher blood plasma levels of 11-KT than the controls, but no differences were observed in frequency of aggressive acts. Previous studies have shown positive correlation between aggression and blood plasma androgen levels (Olsén *et al*., 1998). Sex hormone levels in plasma of the parr in the stream tank were much lower than in the priming experiment both in the exposed and the controls, *c*. 10 times lower of 11KT and half the concentration of 17,20β-P, a difference that probably was caused by a difference in social environment. Large aggressive males were fighting for access to females and small males too close to digging females and nests were chased a way. A great deal of motivation was probably needed to get close to the digging female and at the same time take the risk of being hurt (Broberg *et al*., 2000). These findings indicate that it is rather difficult to directly compare the results from the priming and the stream-tank experiment. The aim of the priming experiments was to test whether copper could affect the endocrine responses to the female pheromone, which was the case.

The reduced upstream swimming may also have been caused by the exposed males' difficulty detecting ovulated female attractants. *Oncorhynchus mykiss* males

have been shown to swim upstream (positive rheotaxis) to spawning fish or to ovulating females (Newcombe & Hartman, 1973; Emanuel & Dodson, 1979; Olsén & Liley, 1993) and *S. salar* male parr have been shown to be attracted to ovulating females urine odours (Olsén *et al*., 2001). Copper–zinc mining pollutants have also been shown to block upstream movement of *S. salar* during their spawning migration (Saunders & Sprague, 1967). It is known that the olfactory sense is important to salmonids to recognize and navigate to their home stream (Smith, 1985; Døving & Stabell, 2003). There may be additional causes behind the differences in swimming behaviour. In a previous study with zebrafish *Danio rerio* (Hamilton), it was shown that embryos exposed to copper ($\geq$20 μg l^{-1}) had fewer neuromasts in the lateral line and that after a 3 day exposure to 50 μg l^{-1} copper the cell damage did not recover (Linbo *et al*., 2005). In the present study, any disturbance of the swimming behaviour ability was not observable except that control fish were more frequently actively swimming upstream than copper-exposed fish. Furthermore, Baker & Montgomery (2001) found that after cadmium exposure in the ambient water banded kokopu *Galaxias fasciatus* Gray could not orientate in a water current and had less attraction to adult pheromones when compared with control fish. Sloman & Wilson (2006) recently reviewed effects of chemical contaminants on fish behaviour and the senses involved in these behaviours. Copper could also have influenced the swimming performance in the parr, for instance by impairment of oxygen uptake and decreased metabolism (Waiwood & Beamish, 1978; Beaumont *et al*., 1995, 2000).

Loss of behavioural responses in copper-exposed parr observed in this study agreed with findings described by several research groups (Van den Hurk & Lambert, 1983; Liley *et al*., 1993; Kitamura *et al*., 1994; Olsén *et al*., 1998; Belanger *et al*., 2004; Miranda *et al*., 2005; Jaensson *et al*., 2007). In all of these studies, reproductively mature fishes lost the ability to behaviourally respond to opposite sex pheromones after treatments causing anosmia in the treated males. The blocking of the olfactory sense in the present study might not have been permanent and the olfactory sense of males could have recovered. Electrophysiological studies with *O. mykiss* have shown recovery of olfactory bulbar responses to amino acid stimulation after short-time exposures (*e.g*. 30 min) to *c*. 10 μg l^{-1} (0·15 μM) copper, but a *c*. 70% inhibition after 30 min exposure to 100 μg l^{-1} was still present after rinsing of the olfactory epithelium with clean water for 40 min (Brown *et al*., 1982).

There are other more recent studies that suggest damage of the olfactory receptor cells by copper. In a study by Hansen *et al*. (1999*a*), *O. mykiss* and Chinook salmon *Onchorhynchus tshawytscha* (Walbaum) were exposed to copper. The number of olfactory receptor cells was significantly decreased after 4 h exposure to 25 μg l^{-1}. The authors observed also with electroencephalogram (EEG) abolishment of the olfactory bulb responses to 10^{-3} M L-serine in *O. tshawytscha* and *O. mykiss* after 1 h exposure to $\geq$50 μg l^{-1} and $\geq$200 μg l^{-1}, respectively.

In a study by Beyers & Farmer (2001), the effects of copper exposure on the Colorado pike minnow *Ptychocheilus lucius* Girard olfactory system was investigated by way of observing the alarm reaction to predator stimulus odour and by quantifying the presence or absence of olfactory receptor cells in the epithelium after exposure. Fish were exposed to one of six different concentrations ranging from $<$10 to 266 μg l^{-1} copper for 24 h or to five concentrations ranging from $<$10 to 120 μg l^{-1} copper for 96 h. During the behavioural portion of this study, fright reaction responses, the authors reported that there was a decline in olfactory function after the 24 and 96 h

exposures. During scanning electron microscopy, it was observed that the group exposed to 60 μg l^{-1} copper for 96 h had no olfactory receptor cells. The fish, however, were able to regenerate these cells after a 14 day recovery period. Furthermore, Hansen *et al*. (1999*b*) described changes in behavioural avoidance of copper seen in *O. tshawytscha* and *O. mykiss*. Individuals from both species were able to detect copper at low levels (*O. tshawytscha*, 0·7 μg l^{-1} and *O. mykiss*, 1·6 μg l^{-1}). When the level of copper increased, however, behavioural avoidance was abolished (*O. tshawytscha*, ≥44 μg l^{-1} and *O. mykiss*, ≥180 μg l^{-1}) indicating that copper exposure could lead to sensory impairment in both species.

When considering copper toxicity, it is important to separate the mode of entry to the receptor organism. Previous studies with salmonids have demonstrated that bicarbonate and the organic content of the water influence the toxicity of copper to the olfactory receptors. Winberg *et al*. (1992) demonstrated in an EOG study with *S. salar* that with increasing bicarbonate concentrations in the water the copper toxicity decreased. The authors stated that the concentration of free Cu^{2+} is important for the olfactory toxicity, but the Cu^{2+} activity also has some influence (Bjerselius *et al*., 1993). In a recent study, Scholz *et al*. (2008) demonstrated that the organic content of the water is even more important to the toxicity of copper compared with the bicarbonate content and water hardness. During the time period of the current study, and previously, the level of total organic carbon (TOC) in the River Dalälven averaged 6 mg l^{-1} (Swedish University of Agricultural Sciences; http://info1.ma slu.se/; Pettersson *et al*., 1997) and during the present experiments, the chemical oxygen demand (COD-MN; the amount of organic carbon that can be oxidized) was measured once to 10 mg l^{-1}. Due to the fact that TOC is equivalent to dissolved organic carbon (DOC), it may be inferred that the effect of copper on the *S. trutta* olfactory system was considerably reduced. Scholz *et al*. (2008) reported that olfaction in juvenile *O. kisutch* was not inhibited by an exposure to 20 μg l^{-1} copper with a DOC of 6 mg l^{-1}. The level of TOC in the river water, the water used during the 4 day exposure period, could have been buffering the olfactory system against degradation by copper exposure. In the Scholz *et al*. (2008) study, however, the exposure time was 30 min, where as in the current study the exposure period was 4 days and with higher copper exposures, there could be different dynamics controlling the amount of bioavailable copper at higher concentrations and for longer exposure times.

Despite these possible reversible acting processes, pre-exposed males still demonstrated fewer prespawning behaviours compared with control fish. In summary, despite olfactory systems that were probably recovering from copper exposure, copper-exposed mature male parr behaved differently when compared with intact individuals when placed in a stressful situation with adult males competing for access to ovulated nest digging females and exposed to the aggression of large males. It is not known, however, if this may lead to disturbed spawning and lower fitness. Future experiments will help answer these questions, and a more detailed study provides the results of disturbed behaviour and endocrine status caused by copper exposure on spawning success in *S. trutta* and other salmonid species.

This study was supported by FORMAS (the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning; to K.H.O.; dnr. 21.0/2003-0376) and the Foundation for Baltic and Eastern European Studies (Östersjöstiftelsen; to K.H.O.; dnr. 87/03). The ethical committee of Animal Research approved this study (dnr. S155-03). Gratitude is

given to the staff at the Fisheries Research Station, Älvkarleby and B. Ragnarsson for their never-ending help, assistance and advice.

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