



Free and conjugated androgen and progestin levels in the serum of stellate sturgeon (*Acipenser stellatus* Pallas) males treated with female coelomic fluid

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Summary

Based on the supposition that female coelomic fluid contents have a certain chemical influence on sturgeons, blood serum concentrations of free and conjugated testosterone (T), 11-ketotestosterone (11KT), 17,20 β ,21-trihydroxy-4-pregnen-3-one (20 β S), 17,20 β -dihydroxy-4-pregnen-3-one (DHP), 11-desoxycortisol (S) and free progesterone (P₄) have been measured by radioimmunoassay (RIA) and enzyme-linked immuno-sorbent assay (ELISA) in stellate sturgeon (*Acipenser stellatus* Pallas) males treated with female coelomic fluid (CF); CF steroid levels have also been analyzed. After treatment a significant elevation of free 20 β S and glucuronidated DHP and S levels and the decrease of free T and KT levels have been observed. The obtained data support the idea that the components of CF may play some part in pheromonal effects for sturgeon reproduction.

Introduction

It is well recognized that sex steroids play an important role in hormonal control of the reproduction in the animal kingdom, including fish (Kime, 1993; Barannikova et al., 2002). Fish use reproductive steroids as endogenous signals between reproductive tract and brain and as exogenous signals (pheromones) for gamete maturation synchronization and for spawning interactions among conspecifics. Sex steroids and their metabolites, as well as prostaglandins, have been demonstrated to have pheromonal effects at least in some Teleostei (see review by Stacey and Sorensen, 2002; Sorensen et al., 2004; Burnard et al., 2008).

One of the best known pheromonal systems in teleosts is described for goldfish (*Carassius auratus*) which releases pre- and post-ovulatory pheromones (Kobayashi et al., 2002; Sorensen and Stacey, 2004). The main pre-ovulatory pheromones are androstendione, maturation inducing steroid 17,20 β -dihydroxy-4-pregnen-3-one (DHP) and its sulphated metabolite, the main postovulatory pheromones are prostaglandin F_{2 α} and a metabolite 15-keto-prostaglandin F_{2 α} . Coelomic fluid (CF) has a pheromonal function including behavioral reactions in the spawning fish (Stacey and Goetz, 1982; Kasumyan, 1993; Scott and Vermeirssen, 1994). The amount of CF (or ovarian fluid or peritoneal fluid) which is released together with the eggs is about 10–30% of the total egg volume in the Salmonidae (Lahnsteiner and Weismann, 1999), and about 15–20% – in the Acipenseridae (Ginsburg and Dettlaff, 1969). As the amount of ovarian fluid is high it may surround the eggs during spawning. In brown trout (*Salmo trutta* f. *fario*), ovarian fluid in comparison to water,

prolongs the duration of sperm motility for >5 min and the fertilizability of eggs for >10 min independent from the quality of the egg batch from which the ovarian fluid derived; these stabilizing effects are related to the inorganic composition of ovarian fluid (Lahnsteiner, 2002).

The aim of this investigation was to analyze free and conjugated (sulphated and glucuronidated) testosterone (T), 11-ketotestosterone (11KT), 17,20 β ,21-trihydroxy-4-pregnen-3-one (20 β S), 17,20 β -dihydroxy-4-pregnen-3-one (DHP), 11-desoxycortisol (S) and free progesterone (P₄) levels in the CF and in the serum in stellate sturgeon (*Acipenser stellatus* Pallas) males treated with female CF in order to proof that the CF components may have a role in pheromonal communications in sturgeons.

Material and methods

Animals

Stellate sturgeon (*Acipenser stellatus* Pallas) wild breeders were captured from the Volga River delta during May–June 2005 and transported (over 18–20 h) to the Zitnenskiy Sturgeon Hatchery (Volga River delta) using a special transport loading boat.

After delivery to the Sturgeon Hatchery stellate sturgeon males (n = 7, 4.8 ± 0.17 kg body weight) were placed in the indoor 12.5 m²-tank (1 m deep) using a flow of river water (about 0.05 m³ min⁻¹) and held 6 days. Water temperature was 21.2–22°C during holding period in the tanks and during the experiment.

After delivery stellate sturgeon females were kept in the outdoor special holding pond (1000 m², 1.2–1.7 m deep; usually the loading density in the pond is one female per 10 m²) using a flow of river water (about 0.5 m³ min⁻¹). Water temperature was 15.5–17.2°C during the holding period in the ponds. After 10 days of the pond holding period four females (n = 4, 7.1 ± 1.16 kg body weight) were maintained in the indoor 12.5 m²-tank (1 m deep; flow of river water was about 0.05 m³ min⁻¹) and treated by LH-RH-A (desGly¹⁰-[D-Ala⁶]-Pro⁹-NH₂-Et-LH-RH) to induce final maturation (FM). The temperature was 20.4–21.4°C during this period. Dissolved oxygen was 8.1–8.3 mg l⁻¹ during at experiment and the pH ranged from 7.4 to 7.6.

Experimental design

The water level in the tank with males was decreased from 1 to 0.14 m during experiment. CF (0.7 L) was collected from ovulated females 24 h before the experiment and stored

refrigerated at 4–6°C. The CF was diluted in the river water (1 : 10) before experiment and small portions (500 ml) of this mixture were added 2 min interval for 0.5 h into water of tank containing males.

Sampling procedure

Four blood samples (1.5 ml) were taken repeatedly from the caudal vein of males using a syringe: (i) 1–1.5 h before the treatment; (ii) 1 h after the treatment; (iii) 3 h after the treatment; (iv) 6 h after the treatment.

Blood and coelomic fluid samples were taken from the females after ovulation. Blood samples were held until separation of the serum. Then the samples were collected, frozen and stored at –20°C until assayed for steroids.

Steroid assays

Concentrations (ng ml⁻¹) of free, sulphated and glucuronidated testosterone (T), 11-ketotestosterone (11KT), 17,20 β ,21-trihydroxy-4-pregnen-3-one (20 β S), 17,20 β -dihydroxy-4-pregnen-3-one (DHP) and 11-desoxycortisol (S) were measured by radioimmunoassay (RIA) (Canario and Scott, 1989; Scott and Canario, 1992). Concentrations (ng ml⁻¹) of free progesterone (P₄) and T, 11KT in female serum were measured by enzyme linked immuno-sorbent assay (ELISA) (Semenkova et al., 2002). The standards and unknown serum and CF were analyzed in duplicates.

Gamete quality evaluation procedure

Egg quality was defined at gastrulation as a percentage of normal embryos (Ginsburg and Dettlaff, 1969).

Statistical analysis

The data were analysed using *t*-Student test and Pearson moment product correlations. All data are presented as the mean and standard error of the mean (SEM) and the level of significance established at 5%.

Results

Composition of coelomic fluid in comparison to blood serum in the females

Three of four females matured and their fertilized eggs yielded 83.7 ± 2.9% of normal embryos. Free and conjugated forms

of sex steroids were detected in CF (Table 1). There were no significant differences between absolute concentrations of free and conjugated sex steroids in CF. There were positive correlations between free and conjugated T levels as well as between free and conjugated S levels, and between free and sulphated DHP levels.

The conjugated T levels were similar in blood serum and CF as well as free P₄ levels. But free serum T levels were higher than free coelomic fluid T levels ($P < 0.01$) as well as serum 11KT levels ($P < 0.05$).

Males

Free 20 β S levels significantly increased 1 h ($P < 0.05$) and 3 h ($P < 0.01$) after the treatment and decreased at the end of experiment and its conjugated forms did not significantly change (Fig. 1).

Free DHP and S levels did not significantly change during experiment (Figs 2 and 3). Glucuronidated DHP ($P < 0.01$) and S ($P < 0.05$) levels were elevated 1 h after the treatment.

Free 11KT levels decreased 1 h after the treatment ($P < 0.05$) (Fig. 4). Free and conjugated T serum levels did not change significantly during experiment (Fig. 5). A slight rise of swimming activity in males as a behavioural reaction to the CF of females was observed.

Discussion

In some studies it was found that CF can improve the fertilization rates (Ginsburg and Dettlaff, 1969) and has a pheromonal function in the behaviour of the spawning fish including sturgeon (Kasumyan, 1993; Scott and Vermeirssen, 1994). We detected the free and conjugated forms of sex steroids in CF in stellate sturgeon. In some teleost, gonadal steroids have important roles as pheromones, but although a wide variety of chemicals have been proposed to function as pheromones, only for certain gonadal steroids and prostaglandins have the pattern of release, olfactory detection, and biological responsiveness have been characterized (Sorensen and Stacey, 2004).

The present study demonstrates that the free androgen levels were lower in the CF than in the blood serum. The results are in good agreement with our previous observations in sturgeon. According to our earlier data in Russian sturgeon (*Acipenser gueldenstaedtii*), androgen levels were lower in the CF than in the blood serum (Bayunova et al., 2003). In stellate sturgeon, T and cortisol levels were also significantly lower in the CF

Table 1

The levels (ng ml⁻¹) of free and conjugated (sulphated and glucuronidated) testosterone (T), 11-ketotestosterone (11KT), 17,20 β ,21-trihydroxy-4-pregnen-3-one (20 β S), 17,20 β -dihydroxy-4-pregnen-3-one (DHP), 11-desoxycortisol (S) and progesterone (P₄) in the stellate sturgeon female serum and coelomic fluid. Data present mean and standard error of the mean (SE). The serum and coelomic fluid unknown samples have been analyzed in duplicates; number of examined fish was 3 ($n = 3$). Differences are significant for T and 11KT coelomic fluid levels in comparison to the serum levels: * $P < 0.05$; ** $P < 0.01$. Correlation coefficients (r) were high positive between free and conjugated form for T, DHP and S

Steroid	Free		Sulphate		Correlation coefficient, r	Glucuronide		Correlation coefficient, r
	Serum	Coelomic fluid	Serum	Coelomic fluid		Serum	Coelomic fluid	
T	111.9 ± 43.63	8.9 ± 3.50**	2.5 ± 0.94	3.0 ± 0.54	0.789; $P < 0.05$	1.7 ± 0.28	3.2 ± 0.58	0.908; $P < 0.01$
11KT	9.5 ± 2.34	2.3 ± 0.99*	–	1.37 ± 0.35	–	–	1.4 ± 0.22	–
P ₄	0.49 ± 0.12	0.53 ± 0.1	–	–	–	–	–	–
20 β S	–	0.22 ± 0.09	–	0.33 ± 0.15	–	–	0.18 ± 0.01	–
DHP	–	0.32 ± 0.13	–	0.45 ± 0.11	0.869; $P < 0.01$	–	0.39 ± 0.17	–
S	–	0.17 ± 0.03	–	0.18 ± 0.07	0.677; $P < 0.05$	–	0.17 ± 0.04	0.859; $P < 0.05$

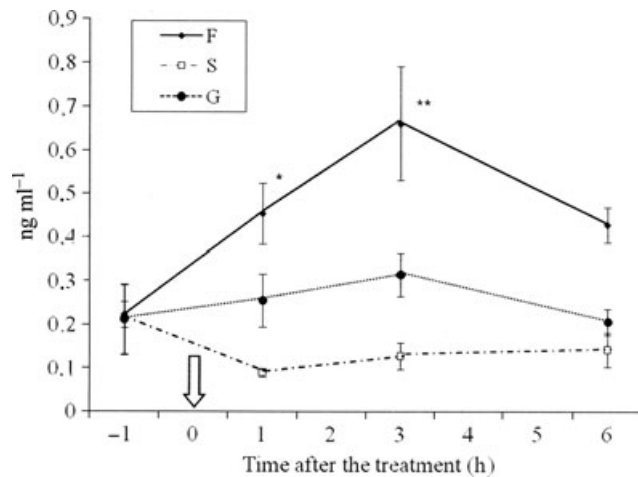


Fig. 1. Serum profiles of free (F) 17,20 β ,21-trihydroxy-4-pregnen-3-one (20 β S) and its conjugated forms (S- sulphate; G – glucuronide) in stellate sturgeon males after exposure to female coelomic fluid adding 2 min interval for 0.5 h to tank water. Data present mean and standard error of the mean (SE). The serum unknown samples have been analyzed in duplicates; number of examined fish was 7 (n = 7). Differences are significant in comparison to the initial levels: *P < 0.05; **P < 0.01. Arrow indicates the time of the treatment

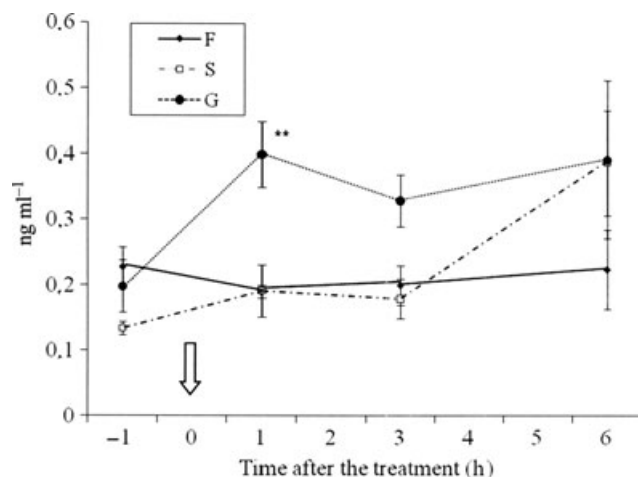


Fig. 2. Serum profiles of free (F) 17,20 β -dihydroxy-4-pregnen-3-one (DHP) and its conjugated forms (S- sulphate; G – glucuronide) in stellate sturgeon males after exposure to female coelomic fluid adding 2 min interval for 0.5 h to tank water. Data present mean and standard error of the mean (SE). The serum unknown samples have been analyzed in duplicates; number of examined fish was 7 (n = 7). Differences are significant in comparison with the initial levels: **P < 0.01. Arrow indicates the time of the treatment

than in the blood serum; conversely, P₄ levels were high in CF than in the blood (Barannikova et al., 2002). In our experiment, P₄ levels as well as sulphated and glucuronidated T levels are similar in serum and CF. We observed no significant differences between free and conjugated sex steroids levels in CF but there were positive correlations between free T, DHP, S levels and conjugated forms of these steroids in CF. The observations suggest that the certain levels of sex steroids in CF may be required to maintain ovulated eggs in a viable state. Similar levels of free and conjugated gonadal steroids we observed early in CF in stellate sturgeon females. According to the previous observations, the serum levels of free T and its sulphate peaked 10–13 h after LH-RH-A treatment and decreased during ovulation, and so did free serum DHP levels; free 20 β S and S serum levels significantly elevated 7–10 h after

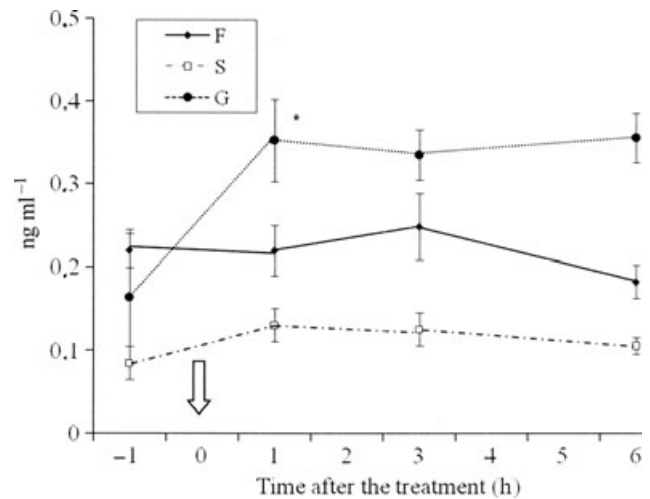


Fig. 3. Serum profiles of free (F) 11-desoxycortisol (S) and its conjugated forms (S- sulphate; G – glucuronide) in stellate sturgeon males after exposure to female coelomic fluid adding 2 min interval for 0.5 h to tank water. Data present mean and standard error of the mean (SE). The serum unknown samples have been analyzed in duplicates; number of examined fish was 7 (n = 7). Differences are significant in comparison with the initial levels: *P < 0.05. Arrow indicates the time of the treatment

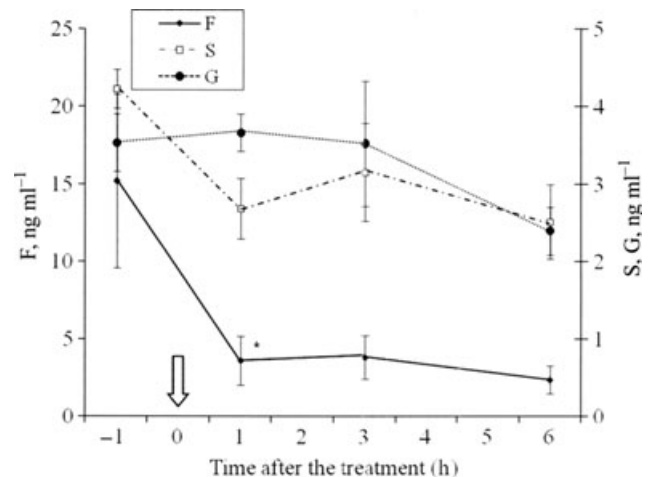


Fig. 4. Serum profiles of free (F) 11-ketotestosterone (11KT) and its conjugated forms (S- sulphate; G – glucuronide) in stellate sturgeon males after exposure to female coelomic fluid adding 2 min interval for 0.5 h to tank water. Data present mean and standard error of the mean (SE). The serum unknown samples have been analyzed in duplicates; number of examined fish was 7 (n = 7). Differences are significant in comparison with the initial levels: *P < 0.05. Arrow indicates the time of the treatment

the treatment, as well as their sulphates (Bayunova et al., 2008a). Taken together the data suggest that these forms of sex steroids could be part of a chemical communication system related to reproductive behaviour in sturgeons. In the European eel (*Anguilla anguilla*), sexually maturing breeders were able to stimulate spermatogenesis of immature males; release into water of free and conjugated forms of sex steroids by spermiating males and pre-ovulatory and ovulating females have induced high electro-olfactograms in neighbouring immature males (Huertas et al., 2006).

The observations by Kasumyan (1993) on the male swimming activity in the Russian sturgeon (*Acipenser gueldenstaedtii*) as a behavioural reaction to the female releaser post-ovulatory pheromone of CF have been visually supported by our

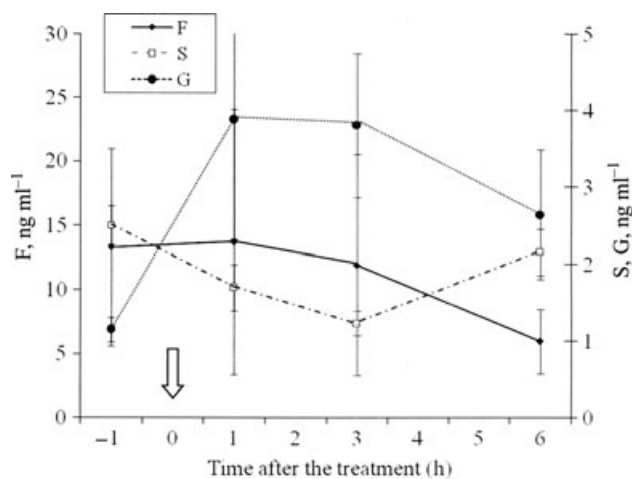


Fig. 5. Serum profiles of free (F) testosterone (T) and its conjugated forms (S- sulphate; G – glucuronide) in stellate sturgeon males after exposure to female coelomic fluid adding 2 min interval for 0.5 h to tank water. Data present mean and standard error of the mean (SE). The serum unknown samples have been analyzed in duplicates; number of examined fish was 7 ($n = 7$). Arrow indicates the time of the treatment

experiment of the same kind. In the American eel (*Anguilla rostrata*), odours released by sexually mature females have been suggested to serve as short-range attractants that affect the spawning behaviour of males (Briand et al., 2002). In the Eurasian ruffe (*Gymnocephalus cernuus*), ovulatory female undergoing oocyte maturation release a cue that strongly stimulates locomotor and sexual activity of male conspecifics and pheromone production was associated with elevated plasma levels of $20\beta\text{S}$ in maturing females (Sorensen et al., 2004).

Our data have demonstrated the change of free and conjugated sex steroid levels as a response of males to the female CF treatment. Free $20\beta\text{S}$ levels are significantly increased after the treatment and decreased at the end of the experiment; glucuronidated DHP and S levels are elevated after the treatment; free 11KT and T levels are decreased after the treatment. The research has already shown that fish excrete steroid metabolites (including pheromones) into water via urine, gills and bile (see review by Scott and Ellis, 2007); and it is recognized that CF is also the source of post-ovulatory pheromones in fish (Stacey and Goetz, 1982; Kasumyan, 1993). Greenwood et al. (2001) have observed a strong correlation between the amounts of steroids (free, sulphated and glucuronidated DHP) in blood and water after hormonal stimulation of FM in common dentex (*Dentex dentex*). Pavlidis et al. (2004) observed that the water concentrations of some free and conjugated steroids were related to the number of males and females (sex ratio) in the tank for common dentex. Free and glucuronidated DHP concentrations were elevated in plasma and released into water by female and male tench (*Tinca tinca*) after hormonal stimulation of FM and the steroids were detected by the male olfactory epithelium (Pinillos et al., 2002). According to our previous experiment, free and conjugated sex steroid levels (especially free $20\beta\text{S}$ and conjugated DHP) were elevated in blood serum and urine at spermiation being induced by LH-RH-A in stellate sturgeon males (Bayunova et al., 2008b). Summing up, the steroids may play pheromonal role related to the reproduction in fish, including sturgeon.

In conclusion, the rise of free and conjugated sex steroid levels in stellate sturgeon male blood serum and the elevation

of their speed activity after CF treatment suggest that the components of CF including free and conjugated forms of sex steroids can play significant role in sturgeon pheromonal communication. Therefore, the further studies are of great importance to identify the chemical nature of potential pheromones in sturgeons.

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