

Effect of 11-ketotestosterone on sperm production by
male spotted wolffish (*Anarhichas minor*)

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*M.Sc thesis in
International Fisheries Management*

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*Dedicated to my beloved sister
late Sasikala Subramaniam who lost her life
due to the civil war in Sri Lanka,
but living in memory of all my family members*

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Abstract

Three experiments were performed during spring 2004, to develop a method to elevate the blood plasma level of 11-ketotestosterone (11-KT) in male brood stock of spotted wolffish *Anarhichas minor*, and to study the effects of 11-KT on sperm production. Intraperitoneal implantation of silastic capsules containing 11-KT - coconut butter mixture and 11-KT - sesame oil mixture and intramuscular injection of 11-KT – propylene glycol mixture were tested. Blood plasma 11-KT levels were quantified by radioimmunoassay. Low and medium doses (*c.* 0.09 mg/kg and *c.* 0.18 mg/kg) of 11-KT-coconut butter mixture administered in silastic capsules did not elevate the plasma 11-KT levels. High dose (*c.* 0.37 mg/kg) of 11-KT – coconut butter mixture gave an increase (6.48 ± 1.89 ng/ml) of about 3 times pretreatment level (1.83 ± 0.36 ng/ml) for one week. 11-KT- sesame oil mixture (1 mg/kg dose) elevated the plasma 11-KT levels (22.58 ± 4.69 ng/ml) compared to the controls (14.98 ± 3.45 ng/ml) for one week. Silastic capsule implants did not maintain the elevation more than one week irrespective of dose or vehicle (coconut butter, sesame oil). Intramuscular injection of 11-KT dissolved in propylene glycol (1mg/kg) elevated plasma 11-KT (99.16 ± 56.04 ng/ml) to about 12 times the level of the controls (7.44 ± 2.04 ng/ml), and was identified as a more effective method than silastic capsule implantation. Sperm production was studied by examining milt volume, spermatocrit, milt pH and motility of sperm. 11-KT treatment with intramuscular injection significantly increased milt volume. Mean milt volumes in treatment and control groups were 14.26 ± 4.2 ml and 4.73 ± 2.8 ml respectively. 11-KT treatment had no significant effects on spermatocrit, which was very low (2 -4 %) in both treatment and control fish. Total sperm cell volume in milt was significantly high in treatment group. There were no significant differences in milt pH and sperm motility between 11-KT treatment and control groups. The results indicate that 11-KT induces the spermatogenesis but does not influence the motility of sperm.

Introduction

The spotted wolffish (*Anarhichas minor*) is a demersal marine fish found on both sides of the Atlantic Ocean and in the Barents Sea. It inhabits offshore waters over soft bottoms, often with boulders. The fish has thick grayish brown skin with many distinct spots on body and dorsal fin. It is a benthophage, feeding mainly on echinoderms, but also crustaceans, mollusks and fishes. The species belongs to the order Zoarciformes, suborder Zoarcoidei, and family Anarhichadidae of Perciformes (Anderson, 1994).

Recently wolffish has been identified as a promising candidate for cold water aquaculture (Tilseth, 1990) and studies have been carried out on its captive breeding. The reasons to consider wolffish as a culturable species are the high quality of their flesh, high growth rate in captivity, a non – aggressive behaviour, and few disease problems (reviewed by Foss *et al.*, 2004). An additional advantage is hatching of well-developed large fry, ready to be fed on formulated food (Falk-Petersen *et al.*, 1999). Experiments with on-growing of common wolffish (*Anarhichas lupus*) and spotted wolffish have shown high survival and fast growth to market size (Moksness, 1994; Pavlov, 1995; Falk-Petersen *et al.*, 1999).

Major constraint to the development of spotted wolffish aquaculture is low production of viable eggs and offspring. In the wild, males and females form pairs during summer or autumn. Prior to spawning, the male has a well-developed urogenital papilla which acts as a copulative organ. The genital pore of the female is opened just after egg ovulation. During internal insemination described by Johannessen *et al.* (1993), a small quantity of sperm is introduced into the genital pore of the female. Fertilization occurs during the next several hours. Fertilized eggs form an egg mass and it is protected by the male, apparently during the whole incubation period, lasting for more than 5 to 6 months (Ringø and Lorentsen, 1987).

In captive conditions, males kept together with females rarely show normal spawning behaviour and females released ovulated unfertilized eggs into water (Johannessen *et al.*, 1993; Pavlov and Moksness, 1994). Captive males produce small volumes of sperm during the spawning season (Falk-Petersen and Hansen, 1991; Pavlov *et al.*, 1997; Tveiten and Johnsen, 1999; Kime and Tveiten, 2002), which means that many male broodstock fish are needed. Therefore, methods for induction of sperm production might be beneficial for culture of this species.

Sperms are produced from the germ cells of testis via a series of developmental changes collectively known as spermatogenesis. The testis is composed of interstitial and lobular compartments. The interstitium between lobules consists of Leydig cells, fibroblasts and blood and lymph vessels. Leydig cells possess ultrastructural features commonly found in steroid producing cells such as agranular endoplasmic reticulum and mitochondria with tubular cristae. These suggest that Leydig cells produce steroid hormones (Nagahama, 1994).

Spermatogenesis involves an initial proliferation of spermatogonia, through repeated mitotic divisions, and these group to form primary spermatocytes. Primary spermatocytes then undergo meiosis to form secondary spermatocytes. Division of secondary spermatocytes results in the production of spermatids, which then undergo metamorphosis to the motile spermatozoa (Jobling, 1995).

Hormones regulate spermatogenesis mainly by the hypothalamic- pituitary-gonadal axis. Release of the glycoprotein hormone gonadotropin (GTH) from the pituitary is regulated by the inhibitory tone generated by dopamine and the stimulatory effects of GTH releasing hormone released from the hypothalamus (reviewed by Peter and Yu, 1997). Spermatogenesis is regulated by two distinct GTHs, follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Nagahama, 1994, 1997). FSH is present during the early stage of reproductive development and LH during the maturational phase (Swanson, 1991).

Steroid hormones are stored in very limited quantities at their sites of production (gonads and internal tissues), and are synthesized as needed. Cholesterol, a 27-carbon compound is the basic precursor for all the steroid hormones. The gonadal steroids, the androgens and oestrogens have a variety of reproductive functions including the induction of production of vitellogenin in the liver, the development of secondary sexual characters and the initiation of a number of behavioural changes related to spawning activities (Jobling, 1995).

In addition to the common androgen testosterone (T), 11-oxygenated androgens, especially 11-ketotestosterone (11-KT) are present in teleost fishes (reviewed by Borg, 1994). 11-KT was first demonstrated in plasma of post spawned male sockeye salmon *Oncorhynchus nerka* (Idler *et al.*, 1960). 11-KT is found in higher levels in the plasma of males than in females, whereas this is not the case for T. 11-oxygenated androgens are generally more effective than T in stimulating the development of male secondary sexual characters, reproductive behaviour and spermatogenesis.

During breeding season, the blood levels of several hormones show remarkable changes in male teleosts. GTH secretion induces an increase in the production of 11-KT, T and $17\alpha, 20\beta$ -dihydroxy-4-pregnen-3-one ($17,20\beta$ -P) which subsequently seems to affect milt quality (Schultz and Miura, 2002).

The effect of T on spermatogenesis has been investigated and both stimulatory and inhibitory effects have been found (reviewed by Borg, 1994). 11-KT injections were effective in increasing milt volume in goldfish *Carassius auratus* (Yamazaki and Donaldson, 1969). 11-KT was found to stimulate spermatogenesis in platyfish *Xiphophorus maculatus* (Schreibman *et al.*, 1986) and in channel catfish *Ictalurus punctatus* (Gannam and Lovell, 1991) and it induced all stages of spermatogenesis and stimulated the Sertoli cells in testis of Japanese eel, *Anguilla japonica*, *in vitro* (Miura *et al.*, 1991a). Kobayashi *et al.* (1991) observed spermatogenesis in regenerating gonadal tissue in incompletely ovariectomized goldfish implanted with silastic capsules filled with 11-KT.

Cavaco *et al.* (2002) studied the effect of combined treatment of T and 11-KT in African catfish, *Clarius gariepinus* and concluded T inhibits and 11-KT induces spermatogenesis. Their results showed that 11-KT but not T, stimulated spermatogenesis whereas T but not 11-KT, accelerated pituitary gonadotropin development. Cochran (1992) on the other hand found no stimulatory effect of 11-KT on spermatogenesis on regressed testis of the mummichog, *Fundulus heteroclitus*, *in vitro*.

Information about the endocrine regulation of spermatogenesis and spermiation in wolffish is scanty. Plasma concentration of $17,20\beta$ -P and its derivatives were low in spermiating male common wolffish (< 2.0 ng/ml) (Tveiten *et al.*, 2000). Examination of limited number of male common wolffish kept at ambient temperature, revealed the presence of T (up to 2.5 ng/ml) and 11-KT (up to 6.0 ng/ml) in plasma, indicating that these steroids are synthesised. Peak T and 11-KT concentrations seem to occur in September (Tveiten, unpublished data) which is close to the breeding season of wolffish. This indicates that T and 11-KT may be involved in spermatogenesis. The present study focused on the effect of 11-KT on sperm production of spotted wolffish. Sperm production was assessed by milt volume, spermatocrit, milt pH and motility.

Several methods of steroid administration are available. The most common method is implantation of encapsulated steroid mixed with cocoa butter (Lee *et al.*, 1986; Pottinger *et al.*, 1996; Cavaco *et al.*, 2002; Yamaguchi *et al.*, 2003). The use of silicone capsules containing steroid hormone was proposed by Dzuik and Cook (1966). These capsules release steroid hormones slowly over time so frequent administration is not necessary. Later, silicone capsules filled with steroids dissolved in arachis oil were developed; the release rate of steroid from these capsules could be controlled by varying the concentration of the steroids (Cohen and Milligan, 1993; Milligan and Cohen, 1994).

Silicone capsules containing crystalline steroids or a suspension of steroid in oil (sesame oil, castor oil, etc.) have been used to study the physiological roles of steroid hormones on reproductive events in a number of teleost species including goldfish, (Kobayashi *et al.*, 1991), African catfish, (Cavaco *et al.*, 1998), Atlantic croaker,

Micropogonias undulatus (Khan *et al.*, 1999) and salmonids (Borg *et al.*, 1998; Antonopoulou *et al.*, 1999). Method of 11-KT delivery to male spotted wolfish has not been reported previously. Therefore three methods (implantation of open silastic tubes with 11-KT - coconut butter mixture, implantation of silastic capsules containing 11-KT – sesame oil mixture and intra muscular injection of 11-KT – propylene glycol mixture) were tested in this study.

Objectives of this study were,

- Develop a method of 11-KT delivery to maintain high plasma 11-KT levels in treated groups over a period of time.
- Study the effect of 11KT treatment on total milt volume, spermatocrit, sperm motility and milt pH.

Materials and Methods

Experiment 1.

Experiment 1 was conducted at the Aquaculture Research Station of the University of Tromsø (70° N 19° E) over four weeks (in February 2004) to study the efficacy of 11-KT treatment with sialstic tubes containing 11-KT- coconut butter mixture. Nine mature (six year old) male spotted wolffish, identified using ultra sound scanning (Falco 100; Pie Medicals, The Netherlands) and by observing their genital openings, were tagged (Combi 2000[®]; Aas, Norway) through base of dorsal fin and were held in rectangular tank (1 m x 3 m x 40 cm) with water depth of 35 cm. The tank was supplied with seawater (32-34 ‰ salinity) at a rate which typically gave *c.* 90 % O₂ saturation in the outlet water. Fish were kept at ambient temperature and natural photoperiod, and were fed in excess with pellet food (DAN EX 2553; Skretting, Norway: pellet size 13 mm) using an automatic disk feeder which was switched on during the light period. Mean water temperature during the experiment was $3.6 \pm 0.22^{\circ}\text{C}$. Initial body weights (in g) and lengths (in cm) were measured for all fish. Condition factor was calculated as $(W/L^3) \times 100$, where W is body weight in g and L is length in cm (Table 2.1).

11-KT (Sigma Aldrich, USA) implants were prepared according to the method described by Lee *et al.* (1986), with slight modification. Briefly, solidified coconut oil was heated to about 45° C (The oil coagulates at about 20° C). Crystalline 11-KT was dissolved in ethanol and mixed with coconut oil in 1:1 ratio. Then the ethanol was evaporated with N₂ gas at 45° C water bath. The coconut butter - 11-KT solution (12.5 mg/ml) was sucked up in to a long silicone tube (inner diameter -1.588mm; outer diameter - 2.381mm; Degania Silicone Ltd.) using a syringe. The long tube containing solution was placed in a refrigerator until the solution became solid. Then the tube was cut into 25 mm pieces so that each capsule contained *c.*0.625 mg 11-KT in 50 µl solution. The implants were kept in a refrigerator until the implantation.

The nine fish in the tank were grouped as group A, B and C. One silastic capsule was implanted to each of group A fish (0.09 ± 0.02 mg 11-KT/kg body weight). Group B fish were implanted with 2 silastic capsules (0.18 ± 0.01 mg 11-KT/kg body weight). Group C fish were implanted with 4 silastic capsule (0.37 ± 0.07 mg 11-KT/kg body weight). All implantations were done into the body cavity with specially modified implanting tube. Skin on the anterior abdomen was pricked with scalpel and the tip of the implanter was inserted at the site of the prick. After evacuating the silastic capsules in to the intra peritoneal cavity, implanter was removed and gentle pressure was given to the insertion site to reseal the prick. The concentrations of 11-KT were selected based on the findings from previous but unpublished experiments (Tveiten; personal communication).

Table 2.1. Length (cm), weight (g) and condition factor of the fish in experiment 1.
(mean \pm SE is given for each group)

Fish length (cm)	Body weight (g)	Condition factor.
Group A		
73.5	6830	1.72
71.8	5420	1.46
72	7680	2.06
72.43 ± 0.93	6643.33 ± 1141.5	1.75 ± 0.30
Group B		
74	6550	1.62
68	7150	2.27
76	7150	1.63
72.67 ± 4.16	6950 ± 346.41	1.84 ± 0.38
Group C		
75	8300	1.97
68	6560	2.09
70.5	5480	1.56
71.17 ± 3.55	6780 ± 1422.8	1.87 ± 0.27

Blood sampling

Throughout the experiment 5% benzocain (50 ppm) was used as the anesthetic agent. At one week intervals, blood (2 ml) was withdrawn from the caudal vasculature using vacutainer tubes (13 x 75 mm) containing 30 U.S.P units Li - heparin (Becton, USA). The samples were held on ice until centrifugation at 2700 g for 10 minutes at 0 to 2° C. Plasma was then separated, transferred to eppendorf tubes and stored at -80° C until steroid extraction and analysis.

Measurement of plasma concentration of 11-KT

Radioimmunoassay (RIA) was performed to measure the plasma level of 11-KT, according to the procedure described by Schulz (1984, 1985). Briefly the theory is as follows: In RIA the antigen (11-KT) in a sample will compete with a known amount of radioactive labeled (³H) antigen for the binding sites of a limited amount of antiserum. When the system has reached equilibrium the free antigen (non labeled and labeled) is separated from the antiserum bound antigen, and the amount of radioactive labeled antigen is measured in the latter fraction (antiserum bound antigen) using a scintillation counter (β -emitter). The amount of antiserum bound radioactive labeled antigen is inversely proportional to the concentration of antigen in the sample. By using a number of dilutions of a standard solution containing a known amount of antigen one can make a standard curve, and this standard curve is further used to read the amount of antigen in the sample.

Tracer (³H labeled 11-KT)

³H labeled 11-KT (Amersham international, UK) was diluted with RIA-buffer (1liter: 3.87 g NaH₂PO₄+H₂O, 21.84 g Na₂HPO₄+ 12 H₂O, 9.00 g NaCl, 1.00 g Gelatin, 1 ml Na-azid, 5% solution and distilled water) until about 10 000 cpm/50 μ l.

Antiserum (AS)

Antiserum (raised in rabbit - NZW) diluted as 1:9 in RIA-buffer and stored at -80° C was thawed and diluted further to 30000 times before use.

Standard curve

Standard curve consists of 11-KT solutions in RIA buffer of different concentrations of non radio active 11-KT. The stock solution (100 µg/ml) was diluted 50 times to get the 2µg/ml stock. It was further diluted 50 times to get the 2000 pg/50 µl. The other concentrations of standard curve (1000, 500, 250, 125, 62.5, 31.25, 15.625, and 7.8125 pg; all per 50 µl) were obtained by serial dilution (1:1) of this solution.

Dextran-coated charcoal solution (DCC)

One gram activated charcoal and 0.10 g of dextran T 70 were dissolved in 100 ml RIA-buffer and the solution was stirred using magnetic stirrer at low speed, for 1 hour at 4° C. The DCC was stirred for 10 – 15 minutes every time before use. 11-KT that is not bound with antiserum is absorbed by DCC so that bound 11-KT will be separated.

Extraction

Non conjugated steroids were extracted from 0.3 ml plasma samples using 4ml diethyl ether. The mixture was shaken for 4 minutes, the water phase was frozen in liquid nitrogen, and the ether phase was separated and then evaporated on a water bath at 45° C under N₂ gas.

Assay

Samples were defrosted at room temperature and mixed well. Different solutions were pipetted out according to the scheme in Table 2.2., mixed well, covered with aluminium foil and incubated overnight at 4° C. Every sample had a duplicate. Six hundred µl RIA buffer and 50 µl tracer were directly added to each counting tube and left over night at 4° C for the total count

Table 2.2. Pipetting scheme for RIA (all volumes in μl)

	Buffer	Standard	Sample	[³H]	Antiserum
TB	100	-	-	50	200
NSB	300	-	-	50	-
Std. S1-S9	50	50	-	50	200
Sample 1-29	-	-	100	50	200

(TB : total bound; NSB: non specific binding; Std. 1-9: standard curve concentrations from 1 to 9; Sample 1-29: samples tested in one assay.)

After 15 – 16 hours samples were placed on ice and 300 μl DCC was added, left for 5 minutes and centrifuged for 5 minutes (4200 g at 4^o C). Supernatants were decanted in to counting tubes and 7 ml of ultima gold XR scintillation fluid (Perkin-Elmer, USA) was added and shaken well. Each tube was counted for 5 minutes on liquid scintillation analyzer (Tri-Carb 2900 TR; PerkinElmer)

Cross binding

Cross binding of other steroid with 11-KT antiserum was estimated with the help of following equation;

$X = (A-B)/C \times 100$, where X is the cross reactivity, A is the measured concentration of the 11-KT in sample, B is the known amount of 11-KT and C is the concentration of cross reacting steroid.

The 11-KT antiserum gave 3.06% cross binding with testosterone, -0.06 % with androstenidione, 0.88% with adrenosterone, 2.28% with 11- β -hydroxytestosterone, 1.06% with 5 α -dihydrotestosterone, 0.23% with 5 β -dihydrotestosterone, -0.65% with 5 α -androstane-3 β -17 β -diol, 0.09% with 17,20 β -P, 0.02% with estradiol, -0.81% with 17-hydroxyprogesterone, -0.21% with progesterone, -0.75% with cortisol, -0.10% with 11 β -hydroxyandrostenedion and -0.37% with pregnenolon.

The detection limits for the assays were 0.2 ng/ml. Validation of the 11-KT assay for wolffish is given by Tveiten *et al.* (2000).

Experiment 2.

Five year old mature male spotted wolffish maintained at ambient temperature and simulated natural photoperiod at the Aquaculture Research Station of the University of Tromsø (70° N 19° E) were used for experiments 2 and 3. For 18 months prior to the start of the experiments 2 and 3, the fish had been exposed to simulated natural photoperiod which was 6 months advanced from natural. That means during the spring, the fish were experiencing autumn (decreasing day length) as shown in Figure 2.1.

Experiment 2 was conducted over four weeks (from week 18 to week 22) to study the efficacy of 11-KT treatment with sealed silastic tubes containing 11-KT-sesame oil mixture. Male fish identification, tagging and feeding were similar to experiment 1. Ten fish of treatment group and ten fish of control group were kept in separate tanks (1 m x 1 m x 60 cm) with water depth of 35 cm. Fish were weighed in g and measured in cm (Table 2.3). Mean water temperature during the experiment was $5.1 \pm 0.42^\circ \text{C}$.

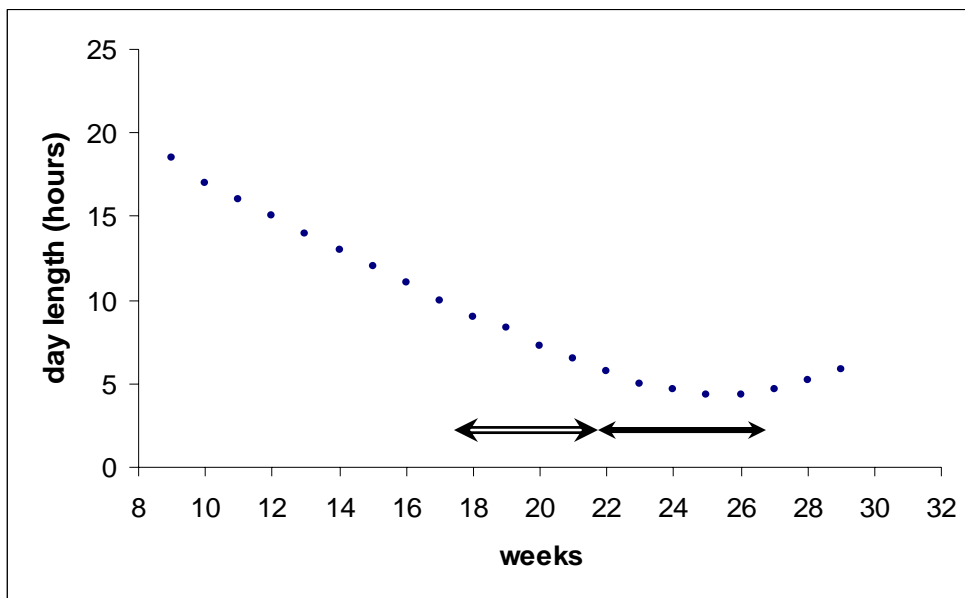


Figure 2.1. Simulated photoperiod advanced from natural during spring 2004. The second experiment was conducted from week 18 (L9 : D15) to week 22 (L6 : D18) as indicated with \longleftrightarrow . The third experiment was conducted from week 22 (L6 : D18) to week 27 (L5 : D19) as indicated with \longleftrightarrow .

11-KT implants were prepared with sesame oil. Crystalline 11-KT was dissolved in ethanol and mixed with sesame oil (1:1). The sesame oil- 11-KT solution was kept in 45°C water bath and homogenized with sonication. Ethanol was evaporated with N₂ gas. Silicone tubes were cut in to 32 mm pieces. One end was sealed (4 mm) with silicone jelly. Fifty µl solution was filled in to 24mm of the tube and the other end was sealed with silicone jelly. One tube for each kilo of fish was implanted (dosage 1mg 11-KT/kg body weight). The dose used was *c.*3 x higher than the highest dose used in experiment 1. Implants for the control group were prepared without 11-KT. Weekly blood sampling and measurement of 11-KT in the plasma were performed as in experiment 1.

Table 2.3.Initial length (cm), weight (g) and condition factor of the fish in experiment 2. (mean ± SE is given for each group)

Treatment fish			Control group fish		
Weight (g)	Length (cm)	Condition factor	Weight (g)	Length (cm)	Condition factor
4533	64.6	1.68	3534 [‡]	65.8 [‡]	1.24 [‡]
4511	68.0	1.43	4560	66.3	1.56
4222	65.7	1.48	4589	67.0	1.52
4908 [‡]	67.2 [‡]	1.61 [‡]	5310	70.4	1.52
3568	61.4	1.54	4495	64.8	1.65
3630	66.0	1.26	3835	60.6	1.72
4699	67.4	1.53	4872	71.1	1.35
4463	66.6	1.51	4965 [‡]	72.3 [‡]	1.31 [‡]
4726	66.6	1.59	4228	67.9	1.35
4989	71.4	1.37	4020 [‡]	64.5 [‡]	1.49 [‡]
4424.9±488.22	66.49±2.54	1.50±0.12	4440.8±544.36	67.07±3.52	1.47±0.15

[‡] Fish with extreme low plasma 11-KT level [‡] Fish with extreme high plasma 11-KT level

Experiment 3.

Experiment 3 was conducted over 5 weeks (from week 22 to week 27) to study the efficacy of 11-KT treatment with intramuscular injection of 11-KT dissolved in propylene glycol, and to study the effect of 11-KT on sperm production. Ten male fish of treatment group and ten male fish of control group were kept in separate tanks (1 m x 1 m x 60 cm) with water depth of 35 cm. Length (cm), weight (g) and condition factor of fish are given in table 2.4. Male fish identification, tagging and feeding were similar to experiment 1. Mean water temperature during the experiment was $6.4 \pm 0.51^{\circ}\text{C}$

Table 2.4. Mean weight, length and condition factor of fish (n=10) in experiment 3.

	Control	Treatment	P >
Mean weight (g)	4291.60 \pm 561.29	4298.60 \pm 512.10	0.9
Mean length(cm)	67.07 \pm 3.52	66.49 \pm 2.54	0.6
Mean condition factor	1.42 \pm 0.11	1.46 \pm 0.13	0.5

Crystalline 11-KT was dissolved in ethanol and mixed with propylene glycol (1:1). Ethanol was evaporated with N₂ gas in 45^o C water bath. Intramuscular injection (micro lance, 0.6 x 25 mm; Bectone, USA) of 11-KT dissolved in propylene glycol was given (dorsal musculature) to fish in the treatment group (dose – 2 mg 11-KT/kg body weight). After 3 and 4 weeks of first injection, 2nd and 3rd injections were given with half dose (1mg 11-KT/kg body weight – calculated in relation to initial body weight). Controls were treated similarly but without 11-KT.

Weekly blood sampling and measurement of 11-KT in the plasma were performed as in experiment 1. Blood samples were taken before injection given in weeks 3 and 4. Terminal measurements were taken 5 weeks after the first injection.

Total milt volume

All fish were manually stripped to collect milt. The pointed end of disposable pasteur pipettes (3 ml. Costelloe, Ireland) were removed and these pipettes were used for milt collection. Open ends of these pipettes were placed over the genital openings. Milt was collected by gentle abdominal massage following emptying of the urinary bladder, and placed on ice until measurements were taken. Total milt volume was measured in ml.

Sperm activity

Immediately after collection of milt, activity of sperm was recorded on a subjective relative scale from 0 to 3 by observation under a compound light microscope (400 X magnifications). Vibrating sperms were considered non motile. The 0 to 3 scale was divided into percentage of motile cells: 0 - Non motile sperms; 1- less than 50% of motile cells; 2 – about 50% of motile cells; 3- more than 50% of motile cells.

Milt pH

pH of milt was recorded using pH paper indicator (Merck, Germany).

Spermatocrit

Sperm density was recorded using a spermatocrit index. Milt was drawn into glass microhaematocrit capillary tubes (Modulohm, Denmark; 75 x 1.2 mm). One end of the tube was then sealed with clay and the tubes were centrifuged for 10 minutes at 4500g. Sperm cells pack down in the capillary tube forming an opaque layer while seminal fluid remains as a clear layer in the top of the tube. Lengths of cell layer and the fluid layer were measured with Vernier caliper. Spermatocrit was defined as the ratio of packed cell layer length to the total length of cell layer and fluid layer in the tube times 100.

Terminal sampling.

Some fish did not release any fluid during stripping, whereas others released fluid (probably urine) which did not contain sperm cells when viewed under the microscope.

These two types of fish were slaughtered and dissected to confirm sex and to assess maturity stage.

Statistical analysis

Student t-tests assuming equal variance were performed to compare the differences in plasma 11-KT levels between pre and post treatment and between control and treatment groups. t – tests were also performed to compare the differences in milt volume, spermatocrit, milt pH and subjective activity of sperm between control and treated groups in experiment 3. Possible correlations between the above parameters and body weight, length and condition of fish were tested. Box plots were made using Statplus (Berk and Cary, 2004). The effect of treatment on sperm production was analysed by Hotelling's t-test with response variables milt volume, milt pH and spermatocrit. The chosen test is a multivariate extension of student t-test which is more powerful than the univariate test when analyzing correlated response variables (Hair *et al.*, 1998). A probability level of $P < 0.05$ was considered significant in all tests. Values are given as mean \pm standard error.

Results

Experiment 1.

There were no significant differences ($P>0.5$) in body weight, length and condition factor between groups of fish in experiment 1 (Table 2.1). Encapsulated 11-KT-coconut butter mixture was not effective in maintaining high plasma concentrations of 11-KT. There were no significant differences in the plasma levels of 11-KT in the low dose (group A) fish over the four weeks (Figure 3.1). Medium dose (Group B) fish showed significant difference ($P<0.05$) in all four weeks compared to the pre-implant level of 11-KT, but the elevation after one week of implantation (which was the highest of all four weeks) was only 3 times (4.38 ± 0.88 ng/ml) higher than the pre-implant level (1.32 ± 0.62 ng/ml).

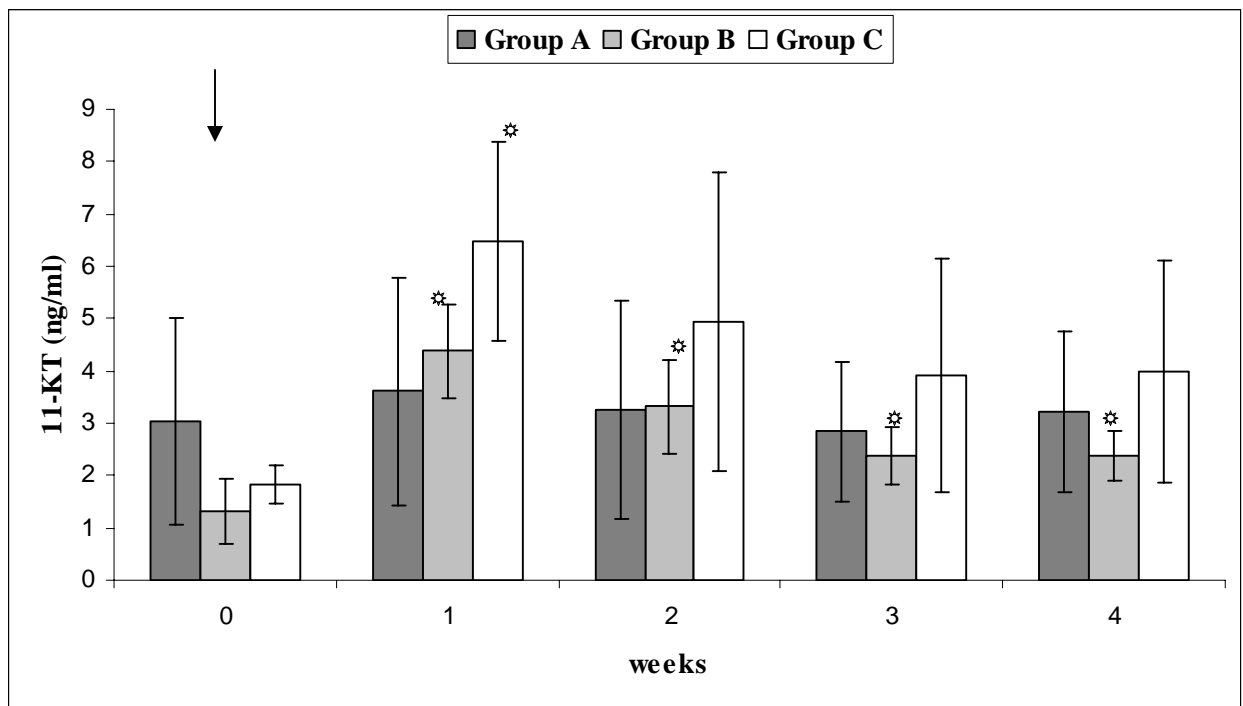


Figure 3.1: Weekly mean ($n=3$) plasma levels of 11-KT in experiment 1. Group A (0.09 ± 0.02 mg 11-KT/kg body weight), Group B (0.18 ± 0.01 mg 11-KT/kg body weight) and Group C (0.37 ± 0.07 mg/kg body weight) indicate three different dosages. The initial mean levels just before the implantation are indicated in week 0. Arrow indicates the implantation.

* Indicates significant difference from the respective pre-implant (week 0) levels.

In high dose fish (Group C) plasma level of 11-KT was significantly ($P < 0.05$) elevated (6.48 ± 1.89 ng/ml) from the pre-implant level (1.83 ± 0.36) only after one week of implantation. Other weeks were not significant ($P > 0.2$) from the pre-implant level. Levels of 11-KT were not significantly different amongst treatment groups (low, medium and high) for any of the four sampling weeks. Therefore dose differences did not result in any differences in the elevation of plasma 11-KT.

Experiment 2.

There were no significant differences in weight, length and condition factor of fish between control and treatment groups (Table 2.3). Sealed silastic tubes with 11-KT - sesame oil mixture were not effective in maintaining high plasma level of 11-KT (Figure 3.2).

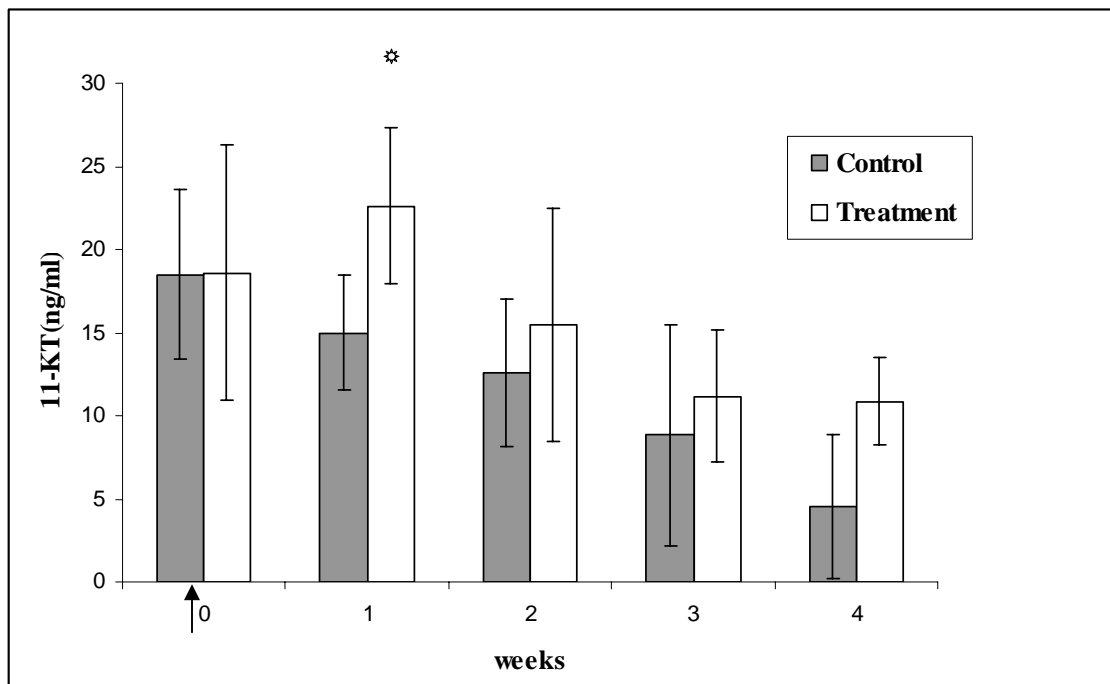


Figure.3.2. Plasma levels of 11-KT in fish (n=9) implanted with sealed silicone tubes with 11-KT- sesame oil mixture and in the control (n=7). Arrow indicates the week of implantation.

* Indicates the significant different from the control level.

One fish in the treatment group and 2 fish in control group which had extreme high level of 11-KT were eliminated as outliers (shown as † in table 2.3). Another fish in the control group that showed extreme low plasma 11-KT levels (shown as † in table 2.3) was also eliminated. The initial (prior to implantation) mean (n=9) level of 11-KT in the treatment group was 18.59 ± 7.70 ng/ml. After 1, 2, 3 and 4 weeks of implantation the mean levels of 11-KT were 22.58 ± 4.69 ng/ml, 15.42 ± 7.01 ng/ml, 11.15 ± 3.98 ng/ml and 10.85 ± 2.62 ng/ml respectively.

There were no significant differences between pre-implants and post-implants for any of the four weeks post – implantation. The difference between control and treatment was significant after one week of implantation where the mean control plasma 11-KT was 14.98 ± 3.45 ng/ml and mean treatment plasma level of 11-KT was 22.58 ± 4.69 ng/ml. After 2, 3 and 4 weeks of implantation, controls and treatments did not show any significant differences.

Experiment 3.

11-KT delivery

There were no significant differences in initial weight, length and condition factor of fish between control and treatment group in experiment 3 (Table2.4). There were no significant correlations between 11-KT levels and final weights or lengths or the condition factor of fish in either control or treatment group (table 3.1). Consequently data within treatment and control group were pooled for comparison.

Figure 3.3 shows the levels of 11-KT in the intramuscular injection treatment and respective controls. The initial dosage (2 mg 11-KT/kg of fish) was doubled from the previous silastic capsular treatment. The pre-injection mean level (13.10 ± 9.25 ng/ml) was increased about 15 times (202.44 ± 108.32 ng/ml) after one week of treatment, but plasma concentration of 11-KT dropped after 2 and 3 weeks (10.92 ± 3.14 ng/ml and 9.55 ± 1.88 ng/ml respectively). Plasma 11-KT level of the treatment group in week 1 was significantly higher than the pre-treatment level and the controls in week 1. There were no differences in

the 11-KT levels of treatment group in week 2 and 3 compared to the pre-treatment level and their respective control.

Based on the findings from weeks 0 to 3, additional injections were given to the treatment group. On week 3 and 4, 2nd and 3rd injections of half dosage (1mg 11-KT/kg body weight) were given. The week 4 and 5 levels in the injected fish were 99.16 ± 56.04 ng/ml, and 103 ± 33.51 ng/ml (Figure 3.3). Mean 11-KT level in the treatment group was significantly higher in weeks 4 and 5 compared to the respective controls and the pre-treatment level.

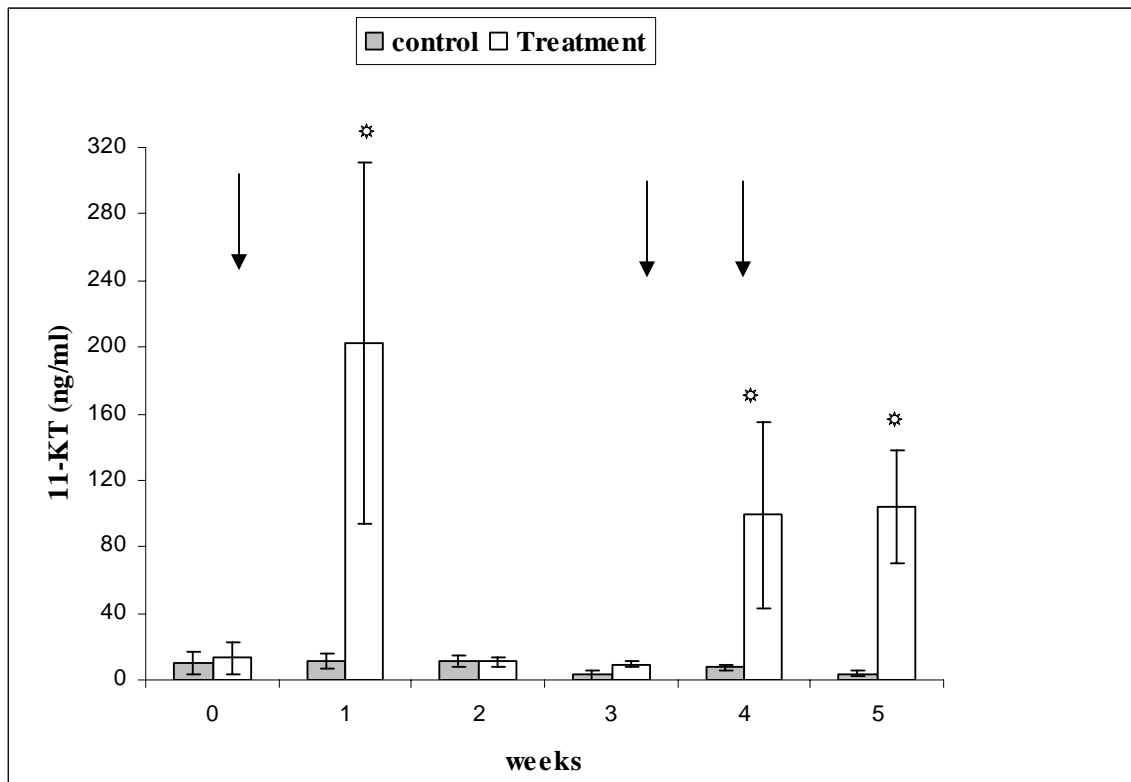


Figure.3.3: Mean (n=9) plasma levels of 11-KT in fish treated with intramuscular injection of 11-KT dissolved in propylene glycol and its control (n=8) (arrows indicates the 1st, 2nd and 3rd injections at week 0, 3 and 4 respectively). Week 0 shows the initial pre-injected mean levels just before the 1st injection.

* Indicates significant difference between pre-treatment levels (at week 0) and also between their respective control level.

Table 3.1 summarizes the results of the terminal measurements of milt volume, milt pH, spermatocrit and sperm cell volume. Two fish in the control group (marked as * in the table) did not show any sperm cells under light microscopy and were identified as immature fish. These fish were eliminated from the analysis. One fish in the treated group (marked as † in the table) showed extremely high milt volume and spermatocrit. It was found as an outlier during analysis and was eliminated from the statistical analysis.

Table 3.1. Summary of the terminal measurements of milt volume, milt pH, spermatocrit, total sperm cell volume and mean 11-KT levels in blood plasma (week 4 and 5) of treated and control group in respect to the condition of fish.

Weight (g)	Length (cm)	Condition of fish	Mean 11-KT(weeks 4 & 5) in blood (ng/ml)	Milt volume (ml)	milt pH	Sperm atocrit (%)	Total sperm cell volume (ml)
11-KT treated group fish							
4338.5	64.6	1.61	100.78	14.3	6.5	3	0.42
4271	68	1.36	123.36	13.1	6	2	0.26
4070	65.7	1.44	51.24	23.1	6.5	3.7	0.85
4796.5	67.2	1.58	130.49	14.3	6.5	1.7	0.24
3427	61.4	1.48	78.62	9.6	6.7	1.7	0.16
3439	66	1.20	79.37	12.2	6.5	1.2	0.14
4555	67.4	1.49	107.18	13.9	6.6	1.3	0.18
4656	66.6	1.58	60.24	18.2	7	2.1	0.38
4626	66.6	1.57	182.39	9.7	7.1	3.4	0.32
4807 [†]	71.4 [†]	1.32 [†]	185.9 [†]	32.2 [†]	7.1 [†]	10.4 [†]	3.34 [†]
Control group fish							
3436.5	65.8	1.21	2.07	2.4	7.1	5.8	0.14
4267	66.3	1.46	0.82	4.1	6.5	3.8	0.15
4435	67	1.47	0.65	3.4	6.5	3.1	0.10
5019	70.4	1.44	0.70	4.1	6.5	1.6	0.06
4254	64.8	1.56	1.16	3.1	6.5	2.6	0.08
3454.5	60.6	1.55	1.84	11.5	6.5	3.2	0.36
4776	71.1	1.33	1.88	5	6.8	2.2	0.11
5026	72.3	1.33	5.49	4.3	7	4.1	0.17
4228*	67.9*	1.35*	7.84*	*	*	*	*
4020*	64.5*	1.49*	0.39*	*	*	*	*

[†] outlier

* immature fish

Milt volume

There were no correlations between milt volume and condition factor of fish or between milt volume and body weight or between milt volume and length of fish, neither in the control group nor in the treatment group. Milt volume in the control group (4.73 ± 2.8 ml) was about 3 times lower than that in the treatment group (14.26 ± 4.2 ml) (Figure 3.4). T-test reveals a significant difference ($P < 0.00001$).

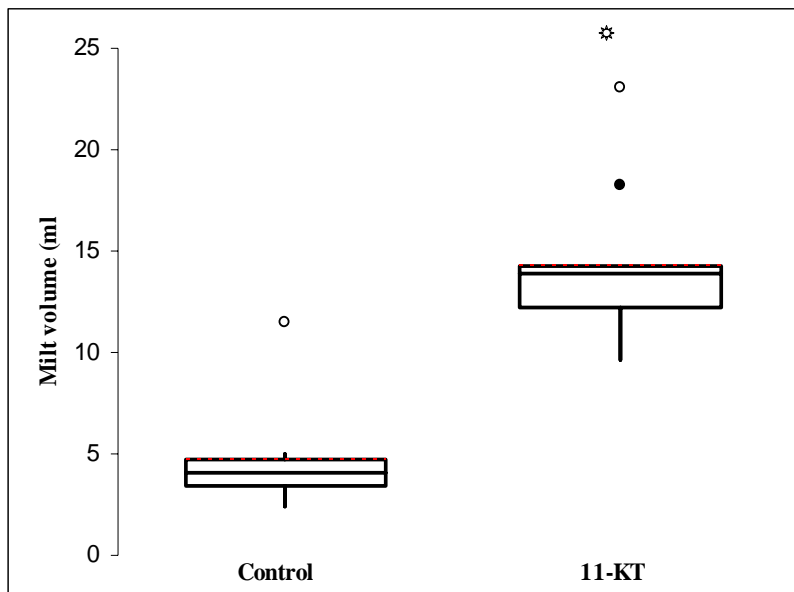


Figure 3.4. Mean milt volume in control (n=8) and 11-KT treatment groups (n=9). Box represents 50% of the values. Whiskers indicate the minimum and maximum values. Straight line crossing the box indicates the median and the dashed line indicates the mean. Circles outside the box indicate the outliers. * Indicates significance difference

Spermatocrit

There was no significant correlation for spermatocrit with body weight, length of fish or condition factor of fish. 11-KT treatment did not show any significant effect on spermatocrit (Figure 3.5). Average spermatocrit for the control group was $3.3 (\pm 1.29)$ % whereas that of treatment group was $2.33 (\pm 0.91)$ %. (t- test, $P > 0.07$).

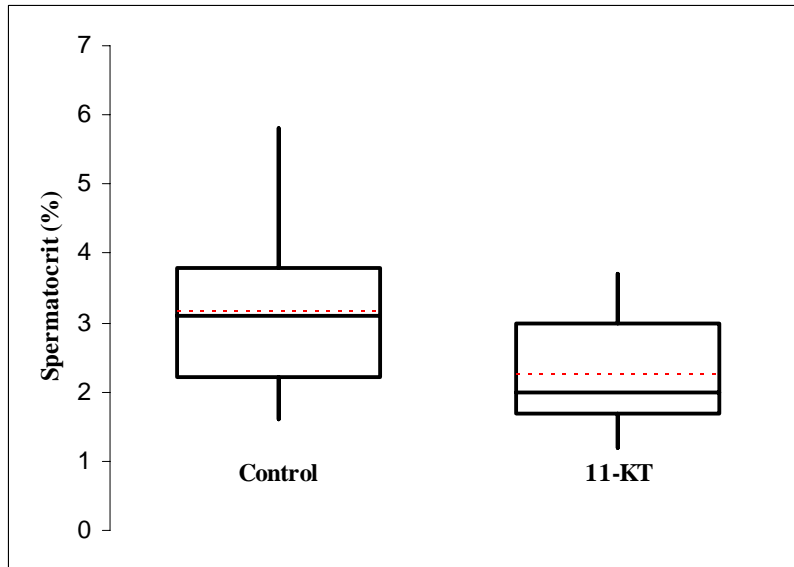


Figure 3.5. Mean spermatocrit of control (n= 8) and treatment (n=9) groups. Box represents 50% of the values. Whiskers indicate the minimum and maximum values. Straight line crossing the box indicates the median and the dashed line indicates the mean.

Total sperm cell volume

Multiplying spermatocrit values by respective milt volumes gives the total sperm cell volume. Total sperm cell volume in treatment group is significantly ($P < 0.04$) higher than in the control group (Figure 3.6). Average sperm cell volume of control group was 0.15 (± 0.09) ml and that of treatment group was 0.33 (± 0.21) ml.

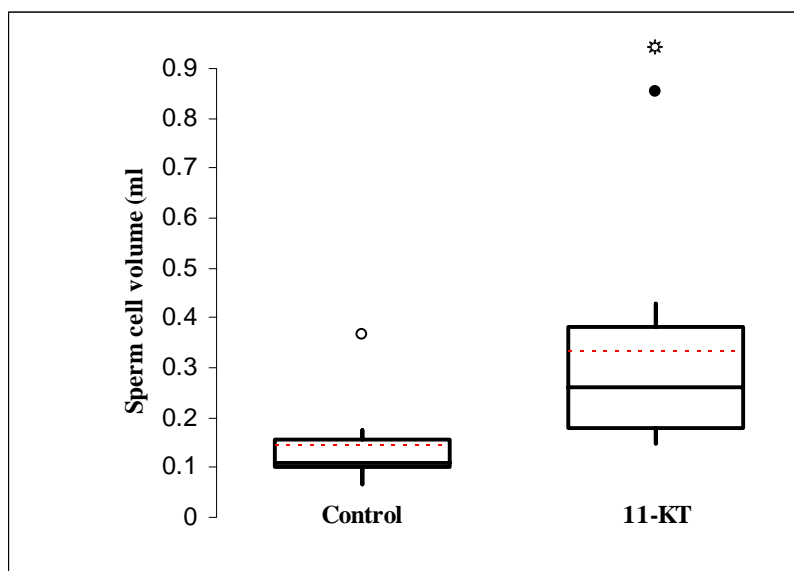


Figure 3.6. Mean sperm cell volumes of control (n=8) and treatment (n=9) group. Box represents 50% of the values. Whiskers indicate the minimum and maximum values. Straight line crossing the box indicates the median and the dashed line indicates the mean. Circles outside the box indicate the outliers
* significant difference.

Milt pH

11-KT treatment did not significantly influence milt pH (Figure 3.7). Average pH of the milt of control group was $6.67 (\pm 0.25)$ and the average pH of the milt of treated group was $6.60 (\pm 0.32)$. (t-test, $P > 0.6$).

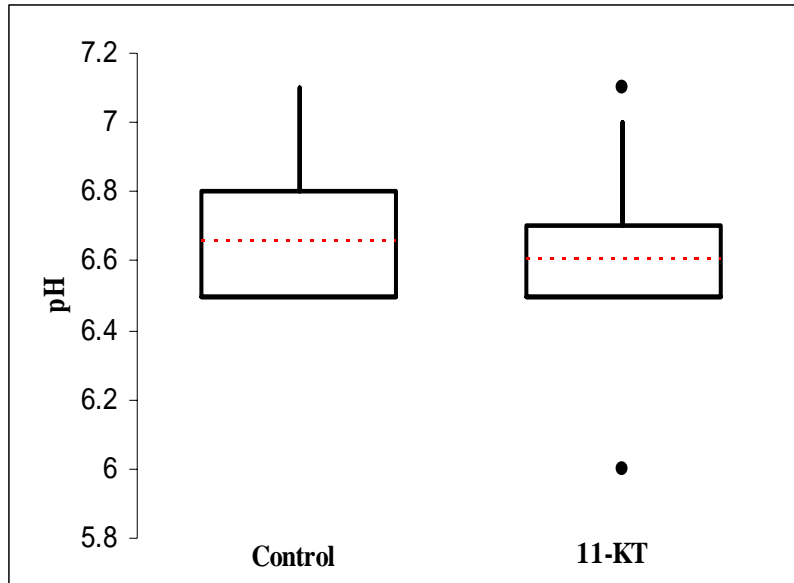


Figure 3.7. Mean pH of milt of control and treated groups. Box represents 50% of the values. Whiskers indicate the minimum and maximum values. Straight line crossing the box indicates the median and the dashed line indicates the mean. Circles outside the box indicates the outliers

Subjective activity of sperm

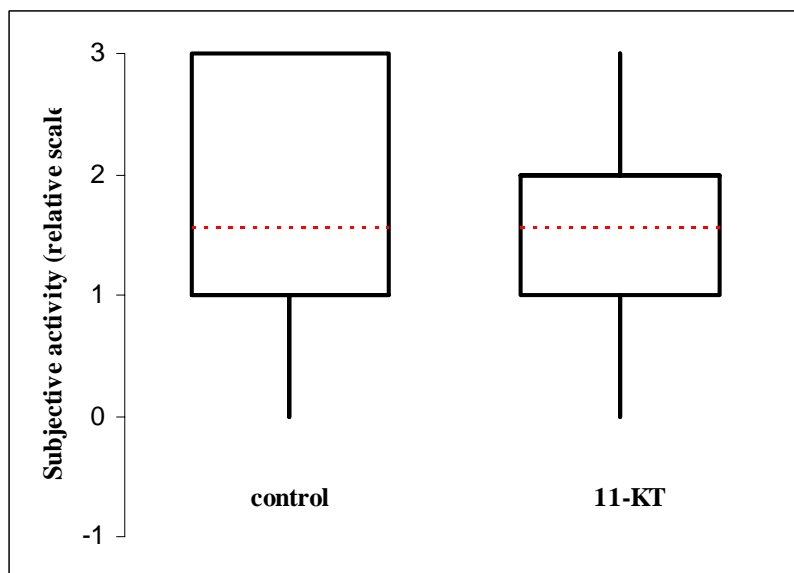


Figure 3.8. Subjective activity of sperm in control and treated group. Relative scale from 0 to 3 indicates non active, low, medium and high activity. Box represents 50% of the values. Whiskers indicate the minimum and maximum values. Dashed line indicates the mean.

Subjective activity of wolfish sperms, as observed under light microscopy did not show any significant difference between control and treatment groups (Figure 3.8). On the other hand activity tended to increase with increasing milt pH (Figure 3.9).

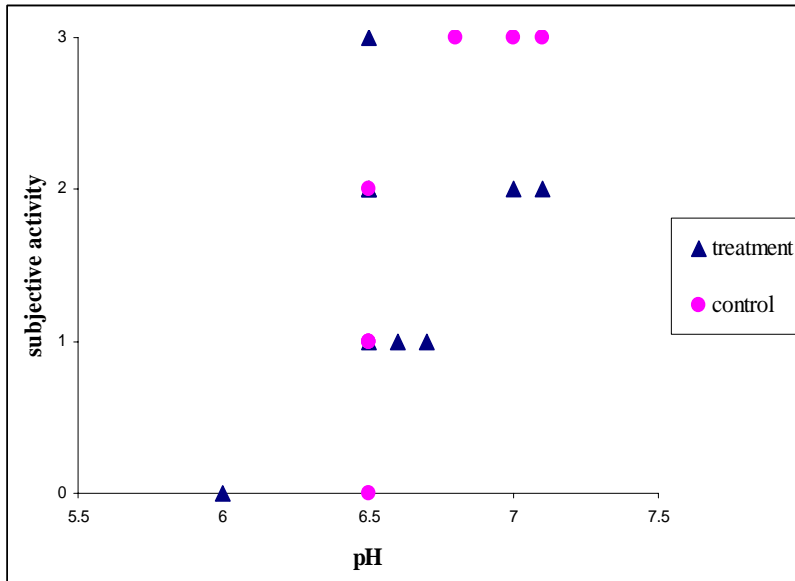


Figure3.9. Subjective activity of sperm related to milt pH.

Hotelling's t-test

Hotelling's t test enables us examine overall reproductive performance, treating dependent variables as covariates. Milt volume milt pH and spermatocrit were used as dependent variables. 11-KT treatment had a significant effect on sperm production as shown by the composite responses of milt volume, milt pH and spermatocrit (hotelling's t-test, $P < 0.0006$).

Discussion

Experiment 1.

Results of experiment 1 show that silastic tubes containing 11-KT-coconut butter mixture did not elevate plasma 11-KT to levels substantially above the pretreatment concentration (Figure 3.1). Previous studies indicate that the release rates of steroids from silicone capsules into the body fluids and subsequent steroid profiles are dependent on steroid hormone structure. Dzuik and Cook (1966) described that the rate of passage of less polar steroids, such as progesterone, androstenedione, and testosterone through a silicone capsule was higher than that of more polar steroids such as estrogens. Plasma levels of E2 in goldfish implanted with capsules filled with 200 µg of E2 in sesame oil were less than plasma 11-KT or testosterone levels in fish implanted with 200 µg of these steroids (Sohn *et al.*, 1998). These findings were taken as justification for the attempt of silastic capsular implantation in the present study.

It was hypothesized that the silastic tubes containing 11-KT dissolved in coconut butter slowly release the 11-KT into the blood stream over a long period of time. Coconut butter was used for convenience; it is solid below 15° C, but was heated to 45° C to make the solution of 11-KT- coconut butter mixture, it was not necessary to seal the silicone tubes as the mixture solidified again at low temperature.

It has been reported that the release of steroids from silicone capsules could be readily controlled by the concentration of steroids dissolved in oil. Release of progesterone dissolved in arachis oil from silicone capsules was linearly correlated with the internal concentration of this steroid (Cohen and Milligan, 1993; Milligan and Cohen, 1994). Therefore three dosages were tested and elevations were expected to be low, medium and high relative to pretreatment levels.

The low dosage did not result in elevation of plasma 11-KT, but medium and high doses elevated 11-KT about 3 times above the pretreated levels. These levels were reduced

after one week, so the failure to maintain elevated levels in all three dosages suggests that 11-KT treatment with silastic tubes containing 11-KT-coconut butter mixture was ineffective.

Diffusion of steroid hormones through silicone membranes is influenced by solubility of the hormone in body fluid (Dzuik and Cook, 1966) and temperature (Lessman and Habibi, 1987). Temperature during the experiment 1 was below 4° C and 11-KT-coconut butter mixture was solid in this temperature. Therefore, a possible reason for the failure of steroid release may be the solid stage mixture in the implants.

Another possible reason for failure is the low relative concentration of 11-KT in the implants. Studies in which encapsulated 11-KT has succeeded have been carried out on small fish such as common carp, *Cyprinus carpio* of *c.*23 g body weight (Consten *et al.*, 2002) and juvenile African catfish of *c.*25g (Cavoca *et al.*, 2001), and high doses (*c.*30µg/g body weight) were applied. This is about 90 times higher than the dose in experiment 1. In the above studies ethanol solvent was removed at room temperature over night. Steroids are stable until about 45°C, so removal of ethanol at 45°C water bath would not be expected to lead to destruction of the steroid.

Experiment 2.

Since the highest dose in experiment 1 did not maintain the elevation, dosage was increased 3 times from the highest dose of experiment 1. In order to keep the mixture as liquid, sesame oil instead of coconut butter was tried as the vehicle in experiment 2. Sesame oil -11-KT mixture formed a precipitate and to dissolve it, mixture was kept in 45°C water bath and sonicated. Sesame oil is not solidified at the experiment temperature range so the capsules were sealed. It was expected that the sesame oil would slowly release the 11-KT over a long period of time. Nevertheless the results for the steroid profile were similar to those obtained with the high dose in experiment 1. That is, in week 1 significant elevation was found and no differences were found for other weeks. There were no

significant differences between pre treatment level and post treatment levels. This reveals that the encapsulated 11-KT- sesame oil treatment had limited effect at the dosage applied.

Main reasons for the failure of elevation may be the sealing of capsules and low relative concentration. Another possible reason is the immediate metabolism of 11-KT. 11-KT metabolism is varied in different fish in different stages. High plasma T levels have been reported to inhibit the 11-KT action and to induce the conversion of 11-KT in to other substances which may not be detected by 11-KT assay (Cavoca *et al.*, 1999). That is, release rate might be lower than 11-KT clearance rate in blood.

Lee *et al.* (1986) suggested that implantation of silicone capsules into the dorsal musculature is effective. But Crim (1985) found that the same dosage capsules were equally effective via intraperitoneal implantation. Therefore in the experiment 3 intramuscular injection was applied instead of the trial of intramuscular implantation of silastic capsule.

Experiment 3.

11-KT delivery.

Results of experiment 3 reveal that although intramuscular injection of 11-KT dissolved in propylene glycol is effective in elevating plasma 11-KT levels significantly, it is not effective in maintaining such levels (Figure 3.3). Therefore weekly injection was needed to maintain plasma 11-KT levels elevated over a period of time. Crim (1985) pointed out that the choice of methodology for delivering steroids to elevate and maintain high plasma levels, depends primarily upon economic and convenience considerations while endeavoring to minimize trauma. From this point of view intramuscular injection was chosen as a better method to deliver the 11-KT, but repeated injection is expensive, which is a draw back of this method.

Milt volume.

Spermatogenesis in fish is thought to be mediated through the actions of 11-KT produced by Leydig cells (Nagahama, 1994). In organ culture, 11-KT can induce all stages of spermatogenesis including spermatogonia proliferation, meiotic division and spermiogenesis (Miura *et al.*, 1991a). In most teleost fish studied, testis growth and development coincide with increased plasma levels of 11-KT, and to a lesser extent, T (Scott *et al.*, 1980; Borg, 1994). *In vivo* and *in vitro* studies show that 11-KT is most effective as a direct stimulator for spermatogenesis (Miura *et al.*, 1991a, 1991b; Borg, 1994; Cavaco *et al.*, 1998; Weltzien, *et al.*, 2002), while T is most effective as a stimulator of hypothalamic and pituitary (Schreibman *et al.*, 1986) activity, leading to further activation of the testis. Therefore, it was expected that 11-KT would induce spermatogenesis in spotted wolffish, and this would be reflected in milt volume and spermatocrit.

Results of experiment 3 show that 11-KT treatment increases milt volume about 3 times compared to the control fishes (Figure 3.4). This clearly indicates that 11-KT induces spermatogenesis. Location of urinary bladder in wolffish makes it difficult to get milt without contamination of urine. Since spotted wolffish does not release a 'thick creamy' milt but pale semitransparent milt (Kime and Tveiten, 2002), it was difficult to differentiate urine and milt. Therefore more attention was paid during emptying the urinary bladder.

It has been reported that spotted wolffish releases about 0.5 to 6 ml strippable milt (Kime and Tveiten, 2002; Falk-Peterson and Hansen, 2003). Mean milt volume (4.73 ± 2.8 ml) in the control group of present study is in agreement with those studies.

Increase in plasma 11-KT in association with increased milt volume have been found in goldfish (Kobayashi *et al.*, 1991), carp (Takashima *et al.*, 1984) and black porgy, *Acanthopagrus schlegelii* (Chang *et al.*, 1991). There is also evidence of correlation

between plasma androgen levels and high milt production in white sucker, *Catostomus commersoni* (Scott *et al.*, 1984), rainbow trout (Liley *et al.*, 1986), mummichog (Cochran, 1992) and snapper, *Pagrus auratus* (Carragher and Punkhurst, 1993). In most of the above studies, 11-KT caused a marked cytological activation of Sertoli cells, but not Leydig cells, suggesting that the action of 11-KT on spermatogenesis is mediated through the action of Sertoli cells. Mechanism by which 11-KT exerts its effects on wolffish spermatogenesis needs to be studied.

Sperm density

No difference in the spermatocrit between control and treatment (Figure.3.5) reveals that 11-KT treatment has no effect on sperm density. Possible reason for this is very low sperm density in wolffish milt. It has been reported that in both common wolffish (Johanessen *et al.*, 1993) and spotted wolffish (Kime and Tveiten, 2002), sperm density is very low (spermatocrit ranges between 0.5 – 5.5%). Control and treatment groups in the present study show the spermatocrit within this range. But the total sperm cell volume which is proportional to the milt volume was significantly high in treatment group (Figure.3.6). This reveals that 11-KT induces the proliferation of spermatogonia during spermatogenesis.

Milt pH and motility of sperm

No significant difference ($P>0.6$) in the milt pH in control (6.67 ± 0.25) and treated (6.60 ± 0.32) group and no significant difference ($P>0.7$) in the subjective activity of sperms in control (1.75 ± 1.16) and treated (1.55 ± 0.88) groups in the present study reveal that 11-KT does not influence the final maturation and motility of sperm.

Generally in teleosts, spermatozoa within the testicular lobules are immotile and may lack fertilization capacity. The development from non-functional gametes to mature spermatozoa fully capable of vigorous motility and fertilization is referred as sperm maturation or capacitation or spermiation (Schultz and Miura, 2002). In number of teleost

species, plasma levels of T and 11-KT are high during the later stage of spermatogenesis and rapidly decline after the onset of spermiation (Ueda *et al.*, 1983; Scott and Baynes 1982; Swanson, 1991; Miura *et al.*, 1991b). *In vitro* incubation studies on 11-KT production by testicular fragments at different stages of development have revealed that the capacity of the fragments to produce 11-KT is high during spermatogenesis but declines after the onset of spermiation (Sakai *et al.*, 1989).

In salmonids and eel, sperm maturation is induced by the high pH (about 8) of seminal plasma in the sperm duct (Schultz and Miura, 2002). Miura *et al.* (1991b, 1992) suggested that 17,20 β -P is related to the regulation of sperm maturation. 17,20 β -P is produced in response to GTH stimulation in testis during the breeding season and mediates its effect through an increase in seminal plasma pH which in turn increases sperm cyclic adenosine monophosphate (cAMP). Therefore increase in the seminal fluid pH reveals the final maturation and higher motility of sperm. During maturation stage pH of sperm duct fluid in fish is often found to increase from 7.4 to 7.5-8.5 (reviewed by Jobling, 1995).

In wolffish fertilization is probably internal (Keats *et al.*, 1985; Johannesen *et al.*, 1993; Pavlov, 1994; Pavlov and Moksness, 1994) (although some evidence and questions have been put forwarded by Kime and Tveiten (2002) against it). Therefore wolffish releases motile sperms unlike most other species, where an osmotic shock usually induces sperm motility. Wolffish sperm remains active for more than 24 hours, and the pH ranging from 5 to 9 had little effect on motility; motility at pH 4.5 was low (Kime and Tveiten, 2002). This evidence supports the present results of pH and motility. That is spotted wolffish sperm is motile at pH 6.5 to 7. 11-KT treatment did not influence the pH or motility.

Results of the pooled control and treatment fish show an increasing trend of motility with increasing milt pH (Figure 3.9). This requires further clarification of individual plasma level of 17,20 β -P which was not quantified in this study. King and Young (2001) reported that 11-KT levels in blood and seminal plasma tended to be negatively correlated with 17,20 β -P levels in Atlantic salmon *Salmo salar*.

Plasma levels of 17,20 β -P in wolffish have been reported as low ranging about 2ng/ml (Tveiten *et al.*, 2000). Studies on the plasma levels of 17,20 β -P after elevation of 11-KT and the effects of 17,20 β -P on motility of wolffish sperm are needed. Fertilization success depends on both number of sperm cells and its motility (Lahnsteiner *et al.*, 1998). Present study indicates that 11-KT can be a tool for inducing spermatogenesis however, studies on the induction of sperm motility are needed for a successful production of viable offspring in captivity.

Conclusion.

Intramuscular injection of 11-KT dissolved in propylene glycol is more effective method than sialstic capsular implantation method to deliver 11-KT in order to elevate the blood plasma 11-KT levels in wolffish.

11-KT treatment to mature male wolffish significantly increased the milt volume and sperm cell volume while treatment has no effects on sperm density, milt pH and sperm motility.

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