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Note

Influence of an exogenous androgen, testosterone propionate on milt quality and short-term preservation of milt of Asian seabass *Lates Calcarifer*

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ABSTRACT

Asian Seabass *Lates calcarifer* is widely used for pond and cage culture practice in the Kingdom of Saudi Arabia. A study was carried out to understand the influence of an exogenous androgen, Testosterone propionate (TP) on milt quality of *L. calcarifer*. Intramuscular injection of TP at dose 1.0 ml kg^{-1} was given to mature male fishes grown in a brood stock pond. A control was also maintained without giving hormone injection. After 36 h of injection, milt was collected and milt quality was assessed for colour, sperm cell concentration, motility and viability of spermatozoa. Short-term preservation of milt was carried out in oxygen and marine teleost Ringer's medium at 4°C for 120 h. No significant change was observed in colour and pH of milt, but nature of milt changed from thick to watery due to hormone injection. Enhanced milt volume (14%) and sperm cell concentration (4%) was noticed in fishes from the treatment group 36 h post-injection, when compared to control. Sperm motility duration and percentage motility were high in treatment fishes than control. Milt preserved in oxygen medium showed high sperm motility and viability when compared to Ringer's medium. Intramuscular injection of TP at 1.0 ml kg^{-1} improved milt volume and quality and therefore can be used for induced breeding of Asian Seabass.

Keywords: Asian Seabass, Hormone, Intramuscular injection, Milt quality, Ringer's medium, Sperm motility, Testosterone propionate

The use of synthetic hormones for induced breeding in fish has been a long-standing trend (Rottmann *et al*., 1991; Seifi *et al*., 2011). Androgenic hormone treatment become a vital technique for sperm management and for increasing juvenile production in hatcheries (Fauvel *et al.*, 2012). Pituitary gland hormone, Human chorionic gonadotropin, Luteinizing hormone releasing hormone and 17α-Methyl testosterone are the commonly used hormones for induced breeding in aquaculture sector (Garcia *et al*., 2002; Seifi *et al*., 2011). These hormones play significant role in gonad maturation, milt production and spermiation. Another widely used hormone is gonadotropin-releasing hormone (GnRHa), which stimulates pituitary gland and releases gonadotropic hormone (GTH) for inducing milt production in fishes (Linhart and Billard, 1994; Lim *et al*., 2004; Vermierssen *et al*., 2004; Basavaraj and Hedge, 2005). Testosterone propionate (TP) is a synthetic androgen, which promotes growth rate and gonad maturation in tilapia (Sambhu and Jayaprakas, 2002). Studies show that TP has stimulatory effect on spermatogenesis in squirrel, rabbit and rat (Cheng and Casida, 1949; Jezek *et al*., 1993).

In fish hatchery sector, milt preservation is an important technique and serving as store house for selective breeding, hybrid breeding and commercial seed production of cultivable species (Agarwal *et al.*, 2004; Adams *et al*., 2008; Sonia *et al*., 2017). In addition, use of germplasm preservation is expanded to certain specific areas such as creation of self-fertilisation inbred lines, production of triploid-tetraploids and possible creation of mutant lines (Dong *et al*., 2005; Yang and Tiersch, 2009; Lawson, 2016). Short-term preservation of milt is a simple procedure involving the maintenance of fresh semen for few hours or even several days and is highly useful in farms and in hatcheries (Tirpan *et al*., 2016). It will be useful in transporting semen collected from different places to the hatcheries or from one hatchery to another. The techniques used for short-term preservation are meant to reduce the metabolic activity of the cells in order to extend their life span (Baynes and Scott, 1987). Several investigators have reported that oxygenated undiluted semen stored in the refrigerator at 4°C showed enhanced sperm motility and viability (Bart *et al.,* 1998; Tsai and Lin, 2012).

The Asian seabass *Lates calcarifer* is used for cage and pond culture practices (Philipose *et al*., 2013; Kumar *et al*., 2016, 2017; Sambhu, 2019). Hatchery production of juveniles and availability of seeds are the major concern of fish farmers. Considering the importance of milt preservation in fish breeding, a study was conducted to assess the effect of the exogenous androgen, Testosterone propionate (TP) on milt quality and short-term storage of

spermatozoa of Asian seabass, an ideal candidate species for coastal aquaculture.

Mature male fishes of *L. calcarifer* were collected from the brood stock rearing ponds of the Fish Farm, Faculty of Marine Science, King Abdulaziz University, Jeddah. Five fish (2.2±0.24 kg) were stocked in a maturation tank (4000 l) containing seawater (salinity: 40 ppt). After recording their individual weight, intramuscular injection of TP at 1.0 ml kg^{-1} was administered near the base of the first dorsal fin. Five fish administered with physiological saline (Na Cl-0.9%) and maintained in separate tank, served as control. Fishes were fed twice with a pellet feed (crude protein: 40%) at 8:00 and 17:00 hrs and 20% of water from the experimental tanks were exchanged daily. Water quality parameters such as dissolved oxygen, pH, temperature and salinity were analysed daily at 10.00 hrs following standard analytical procedures (APHA, 1995).

Milt collection

Genital area of fish was wiped with clean cotton/ tissue paper and gentle pressure was applied on the abdominal sides to get rid of the feacal matter and urine. Extreme care was taken to avoid contamination of milt with blood, urine or mucus. Milt was collected from the control and treatment fishes before (0 h) and after injection (36 h). A graduated pipet was used to collect milt and it was transferred immediately to a polythene bag kept in an ice box for analysis of milt quality and for short-term storage study.

Milt quality was assessed for colour, volume, pH, sperm cell concentration, motility and viability of spermatozoa. Colour and physical appearance of milt was recorded at the time of stripping. Milt volume was calculated as the total quantum of milt drawn in a graduated pipette on a single stripping. Sperm cell concentration of the milt was assessed using Neubar-hemocytometer (Sukcharoen *et al*., 1994). Milt was diluted with saline (NaCl 0.9%) solution and spermatozoa were counted under a compound microscope at 400x magnification and expressed as number of spermatozoa per microlitre of milt. Duration of motility (time period during which spermatozoa show active forward movement) was studied under the activating media (seawater). One drop of milt was taken on a glass slide and mixed with activating media (dilution ratio 1:400). Slide was then observed under a pre-focused microscope (450x). Duration of motility was recorded from the time of mixing up of milt with activating media to the time up to which at least 20% of spermatozoa exhibit active forward movement. Motility percentage was calculated as: Motility (%) = Number of motile spermatozoa/Total number of spermatozoa x 100. Motility score was recorded based on an arbitrary scoring system developed by Agarwal (2011) and was measured as; 0 - 1% (Immotile, Score-I), 1<25% (motility score II), 25<50% (motility score-III), 50<75% (motility score IV) and 75<100% (motility score V).

Sperm viability was determined using Eosin-Nigrosine dye exclusion method (Chao *et al.,* 1987). One drop of milt was mixed with two drops of 10% nigrosine and one drop of 5% Eosin. It was then thoroughly mixed and a thin uniform smear was prepared on a glass slide. The slides were air dried and observed under a microscope. Dead spermatozoa appeared pink or red in colour, and live ones in grey or ash colour. From each field, live, dead and total spermatozoa were enumerated as; Viability (%) = Number of live spermatozoa/Total number of spermatozoa x 100.

Short-term preservation of milt was carried out at 4°C in a refrigerator. Two types of preservation methods were employed as follows:

*Method 1- Storage of milt in an oxygen atmosphere***:** Objective of the method was to evaluate the role of oxygen on milt storage. For this, 0.25 ml milt was taken in a petri dish and placed in an inflated polythene bag (1 l capacity), filled with oxygen and tied on the top. Extreme care was taken to avoid falling of condensed water on the samples. Three samples each were kept at 4°C in the refrigerator. At every 24 h intervals, samples were carefully taken out with a capillary tube, thawed at 27°C and analysed for sperm motility and viability. Each time when the samples were drawn, bags were filled with pure oxygen and then tied.

Method 2 - Storage of milt with the extender, Marine teleost Ringer: The experiment was aimed to check whether milt can be stored with the extender, marine teleost Ringer (Stoss, 1983). For this, 0.25 ml milt was mixed with 0.75 ml of the extender (dilution ratio 1:3) and was taken in a 15 ml screw capped polypropylene storage vial. The samples (3 nos.) were stored at 4°C in the refrigerator and sperm motility and viability were examined at 0, 24, 48, 72, 96 and 120 h of storage.

t-test was employed to compare the mean value of semen quality between control and treatment fishes using Microsoft Excel 2000.

Data on water quality parameters recorded in control and treatment tanks during the experimental period is depicted in Fig. 1. No significant difference ($p>0.01$) was observed among the recorded water quality parameters between treatment and control groups. The details of milt quality parameters before and after hormone injection are presented in Table 1. Colour of the milt (white) was not affected, but appearance changed from thick to watery due

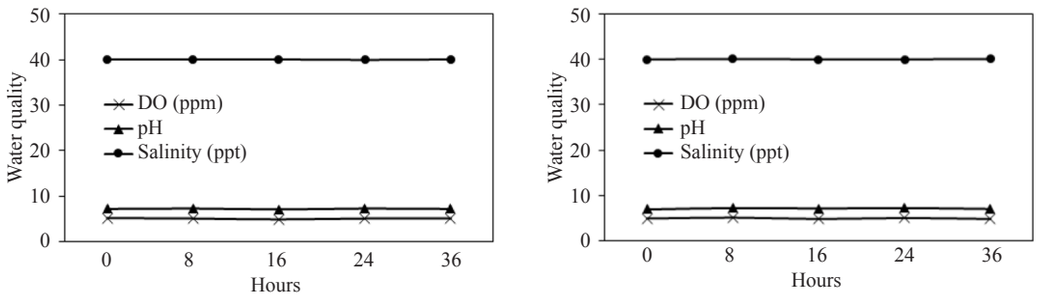

Fig. 1. Water quality parameters recorded during the study period (a) Control and (b) Treatment

Table 1. Data on milt quality of Asian seabass before and after hormone injection

Parameters	0 h			36 h		
	Control mean	Treatment mean	p(T<=t)	Control mean	Treatment mean	p(T<=t)
Milt volume (ml)	1.3	1.4	0.3239	0.8	1.6	0.0042
pH	7.1	7.2	0.1778	7.1	7.1	0.0042
Sperm concentration ($x10^6$ ml^{-1})	35.5	35.9	0.8329	35.7	37.3	0.3797
Motility duration (s)	172	170	0.0993	161	174	0.0006
Sperm motility (%)	90.3	91	0.4539	88.3	93.3	0.0195
Viability (%)	92.7	91.8	0.1424	92.4	92.1	0.8652
Motility score	V	V	-	V	V	-

p(T<=t) two-tail @ alpha 0.05

to hormone injection. Compared to control, milt volume increased by 14% and sperm cell concentration by 4% in hormone treated fish. Hormone injection did not show considerable change in pH value of milt. Sperm motility duration and percentage motility were high in treatment fishes than control. However, sperm viability and motility score were not influenced by hormone injection.

Results of short-term preservation (120 h) of milt in oxygen medium and Ringer's medium at 4°C is shown in Fig. 2. Sperm motility duration, motility percentage and viability of both control and treatment were found to be lowered after 120 h of storage. Significant difference in sperm motility and viability was not observed (p>0.01) between control and treatment milt stored in oxygen medium (Table 2), whereas, significant difference was noticed in motility and viability between control and treatment milt stored in Ringer's medium (Fig. 2).

Brood stock management plays significant role in captive breeding of Asian seabass (Palmer *et al*., 1993). Hatchery sector currently focuses on developing techniques for the short-term preservation of gametes for successful production of juveniles for large-scale aquaculture production (Hoga *et al*., 2018; Haque *et al*. 2021). In induced breeding, use of hormone treatment is a widely accepted practice to stimulate milt availability of farm raised brood stock fishes (Garcia, 1993: King

Table 2. t-Test analysis for sperm motility and viability between control and treatment after 120 h of storage in oxygen medium

Parameters	Sperm motility (%)		Sperm viability (%)	
	Control	Treatment	Control	Treatment
Mean	51	52.4	49.6	50.8
Variance	5	2.3	1.3	1.7
Observations	5	5	5	5
Pearson correlation	-0.58977		-0.23544	
Hypothesized mean difference	0		0	
df	4		4	
t Stat	-0.93127		-1.39497	
p(T<=t) one-tail	0.202216		0.117748	
t Critical one-tail	2.131847		2.131847	
P(T<=t) two-tail	0.404433		0.235496	
t Critical two-tail	2.776445		2.776445	

p(T<=t) two-tail @ alpha 0.05

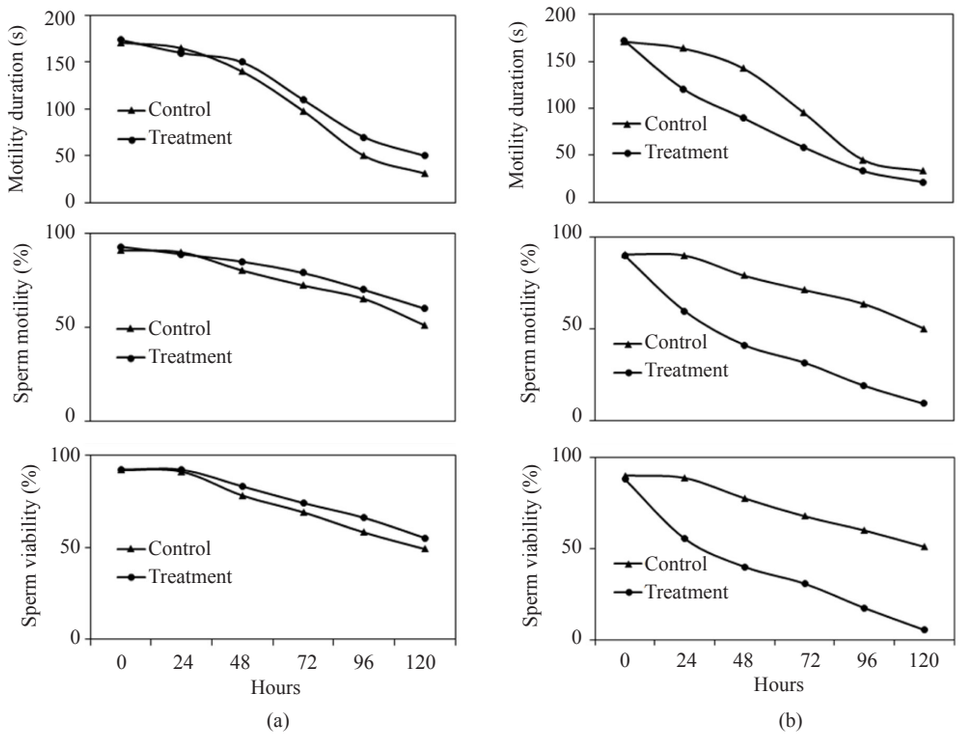

Fig. 2. Variation in motility and viability of sperm stored in (a) Oxygen and (b) Ringer's solution, at 4^0C

and Young, 2001; Zohar and Mylonas, 2001; Garcia *et al*. 2002). Results of the present study show that when testosterone propionate at 1.0 ml kg^{-1} was given intra-muscularly, it enhanced milt quality of Asian Seabass, *L. calcarifer*. Further studies are required to optimise the dose of hormone required for gonad stimulation and also to test its fertilisation capacity with spawned eggs.

Milt of control fish was found to be thick and viscous, whereas it appears as thin and watery in hormone-injected (treatment) fish. Similar hydrated nature of milt was observed in rabbitfish, *Siganus guttatus* when biweekly injection of Luteinizing Hormone Releasing Hormone (LHRH) was applied (Garcia, 1993). Milt quality was improved when a combination of LHRH and 17α-methyl testosterone was injected in *L. calcarifer* (Garcia *et al*., 2002). According to them, the milt production response and the hydrated nature of the milt to exogenous hormones was due to the synergistic effect of hormonal therapy in inducing the production of seminal plasma (Garcia *et al*., 2002). Therefore, it is inferred that the hydrated nature of milt observed in the present study may be due to the stimulatory effect of testosterone propionate on seminal plasma secretion.

Studies have shown that hormone injection resulted in enhanced sperm cell concentration, milt volume, sperm motility and seminal plasma secretion in brood stock of several species of marine fishes (Linhart and Billard, 1994; Mylonas *et al*., 1995, 1997; Sorbera *et al*., 1996; Clearwater and Crim, 1998). Testosterone and other androgens serve as precursor of plasma ketotestosterone synthesis and impart stimulatory effect on gonad ripening and milt production in teleost fishes (Khan *et al.*, 1999; Schulz and Goos, 1999; Zanuy *et al.*, 1999). The enhanced milt volume (14%) and sperm cell concentration (4%) observed in the hormone injected fishes could be attributed to the influence of the exogenous testosterone administered.

Major problems which affect short-term storage of milt are temperature, gaseous exchange, microbial attack and desiccation (Fauvel *et al*, 1998; Pereira 1999; Fauvel *et al*, 2012). Reports show that anaerobic storage of sperm at 4°C resulted in low motility and aerobic storage (in oxygen) at the same temperature showed high fertility rate (94%) after 216 h (Saad *et al.,* 1988; Chereguini *et al.,* 1997; Gwo, 2010). The enhanced, sperm motility; motility duration and viability recorded in the present study can be correlated to the presence of oxygen in the medium as reported above.

Results of the present study show that administration of the exogenous androgen, testosterone propionate at 1.0 ml kg^{-1} improved milt quality of Asian seabass. Short-term storage of milt for 120 h at 4^oC in oxygen atmosphere showed high sperm viability, motility and motility duration than that of the milt stored in Ringer's

medium. The findings of the study has great potential for application in the hatchery production of Asian seabass, *L. calcarifer*.

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