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Short communication

Tracking gender factors in fish surface mucus: temporal patterns in individual Koi (*Cyprinus carpio*)

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The androgen 11-ketotestosterone (11-KT) is recognized as a major sex steroid responsible for testicular development in fish, with peak values generally at the height of the spawning season (Barry et al., 1990; Chang and Chen, 1990; Borg, 1994; Cuisset et al., 1994; Cyr et al., 1998). Vitellogenin (Vtg), a calcium-binding phospholipoglycoprotein (85–200 kDa subunit molecular weights), is a conserved molecule in teleosts, and is normally undetectable or detectable in small quantities in biological fluids of immature females and males, but peaks during oogenesis in females (Copeland and Thomas, 1988; Mommsen and Walsh, 1988; Goodwin et al., 1992; Wiegand, 1996). Previously, we reported that autologous blood serum, muscle tissue or surface mucus of Koi (*Cyprinus carpio* L.), and muscle tissue or surface mucus of a number of wild marine teleost species, may be used for the determination of 11-KT (Schultz et al., 2005). The use of surface mucus as a source of Vtg has been reported by a number of laboratories (Gordon et al., 1984; Kishida et al., 1992; Moncaut et al., 2003).

In preliminary studies of gender-related factors in surface mucus reported here, a method for minimally invasive sampling was developed with polyurethane sponges for facile collection and preservation. Then, for 8 months, monthly levels of both 11-KT and Vtg-derived yolk proteins (VDYP) in the surface mucus were monitored in individual fish, using Koi maintained in aquaculture as the experimental model.

Thirty maturing or sexually mature Koi were purchased from a commercial aquaculture operation (Summerland Tropical Fish Farms, Homestead, FL). The average total length (TL) was 27.0 cm and the average weight was 331.7 g. The fish were held at the University of Miami, Rosenstiel School of Marine and Atmospheric Science Experimental Hatchery located on Virginia Key, FL. Twenty of the 30 (1–20) fishes were killed, staged microscopically for gender (Barbieri et al., 1994; Lowerre-Barbieri et al., 1996), and the blood serums of nine (nos 1–9; eight females and one male) were used to determine the cross-reactivity of a caprine antiserum developed for blue marlin VDYP (Tan et al., 2006). This information was necessary for both practical and economic reasons, because an enzyme-linked immunosorbent assay (ELISA) for VDYP was developed and is routinely used to test biopsied tissue specimens of blue marlin in our laboratory (Tan et al., 2006). Each of the nine sera was run in 8–18% SDS-PAGE under reducing conditions, followed by a Western blot (Laemmi, 1970; Towbin et al., 1979). Fig. 1 shows the blots of 3 of the sera, and 1 sample of surface mucus, using gravid females (F1 and F2) and one adult male (M1). The method generated a reduced heterogeneous molecule composed of two apparent subunits (1 and 2) of the female Koi, with masses of *c*. 125 and 105 kDa, respectively. The male serum had little or no activity. Two protein bands were also present in the surface mucus of F2 with molecular masses of *c*. 105 and 90 kDa, possibly the result of partial degradation of a single heterogeneous molecule. Pre-immune goat serum was non-reactive. Of greatest significance: the goat antiserum, which reacted with blue marlin VDYP, cross-reacted with Koi VDYP, in both serum and surface mucus of females. No reactivity was observed in additional Western blots with serums from three post-oviparous female Koi (nos 7–9). To demonstrate the interchangeability of reagents, carp yolk protein Vtg used for the standard curve of the carp sandwich ELISA (Biosense Laboratories AS, Bergen, Norway) could be substituted for blue marlin VDYP standards in the ELISA for blue marlin (Tan et al., 2006). Therefore, the Koi VDYP were quantified by the ELISA described for blue marlin VDYP.

The 11 remaining killed Koi (nos 10–20) were used to develop a minimally invasive collection method for surface mucus to assay VDYP and 11-KT. The latter was purchased from Cayman Chemical Co. (Ann Arbor, MI) as an ELISA kit and used as described by the manufacturer. We had previously published a study in which 11-KT was assayed in Koi surface mucus scraped from the lateral side of each fish with a sterile, rounded stainless steel spatula (Schultz et al., 2005).

In this study, for comparative purposes, the surface mucus was collected by two methods: (i) with a sterile, rounded stainless steel spatula on one side of an individual fish, and immediately after; (ii) with a polyurethane sponge on the opposite side of the same fish, using light pressure. The non-scented product, termed 'Buf-Puf gentle facial sponge' (3M, St Louis, MN) was cut into four equal pieces (*c*. 2.5-cm wide, 2.5-cm long, 2.5-cm thick), and one piece was used per fish. A detailed description of the size of the surface area of the skin being wiped, the direction (frontal-caudal or caudal-frontal), and the pressure applied to the sponge is not of primary importance as long as the surface mucus extracted from the sponge contains at least one or more mg ml^{-1} of protein as determined with a protein assay kit (e.g. Bio-Rad Laboratories,

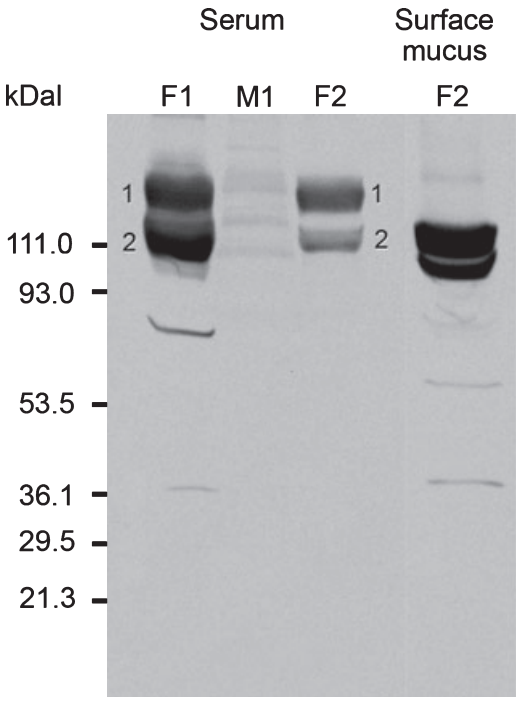

Fig. 1. Serums from two gravid female Koi (*Cyprinus carpio*) F1, F2 and one adult male (M1) run in 8–18% gradient SDS-PAGE followed by a Western blot. The procedure was repeated with surface mucus collected from F2. The two females were sexually staged microscopically as ripe, stage 5, and the male was classified as regressing, stages 2–3. Gels were probed with a polyclonal caprine antiserum raised against blue marlin vitellogenin-derived yolk proteins. Reference standard molecular weights (kDa) shown on the left. Numbers 1 and 2 are the reactive Koi proteins of vitellogenin. The caprine pre-immune serum was non-reactive with the serums of the three Koi

Hercules, CA). The concentration of protein in the surface mucus of adult Koi had to be diluted to achieve a concentration of 1 mg ml^{-1}. However, it is advisable to first quantify the protein in surface mucus of other species of fish under investigation because of high variability in protein concentration (D. R. Schultz and N. Perez, unpublished observations). The values for mucus extractions from all the Koi reported previously (Schultz et al., 2005) as well as in these experiments are reported with the same common denominator, namely, mg of total soluble protein (TSP).

Spatula-collected samples were scraped into glass tubes and two drops of a protease inhibitor cocktail were added (see below) and processed for the gender factors. The sponge with absorbed surface mucus was placed into the cylinder of an empty 25 ml syringe with forceps, and 1 ml of ice-cold Tris-buffered saline, pH 7.5, was added to the cylinder. The barrel of the syringe was used to compress the sponge and collect the fluid into a glass centrifuge tube. The fluid was centrifuged (15 000 ***g***, 15 min, 4°C) and to the supernatant fluid was added two drops of a protease inhibitor cocktail (16 μg ml^{-1} benzamidine HCl, 10 μg ml^{-1} phenanthroline, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} pepstatin A, and 1 mM phenylmethyl sulfonyl fluoride (BD PharMingen, San Diego, CA). The sample was then frozen and stored at −80°C.

In Table 1, 11-KT in the surface mucus was quantified by ELISA using 11 fish (nos 10–20; seven males and four females) to compare the two methods of collection. The values pg 11-KT mg^{-1} TSP from the right and left side of each of the staged Koi were similar enough with both techniques to justify use of either method, but collection with sponges was less stressful for live fish (unpublished data), and therefore was selected for the experiments to follow. The collection of surface mucus with sponges and assay of the second gender factor VDYP was also shown to be feasible (see results below). Sponges soaked in Tris-buffered saline containing the protease inhibitor cocktail, but no surface mucus and treated as described above as a legitimate sample (0 control), resulted in 0 values in the ELISAs for both gender factors. Therefore, sponges were used to collect the surface mucus each month for 8 months from individual Koi maintained in aquaculture.

The 10 viable Koi (nos 21–30) were maintained in an outdoor-holding facility covered with 50% shade cloth to reduce solar heating and exclude potential predators. Each fish was identified by its unique color patterns and markings. The circular fiberglass holding tanks were 6.1-m wide × 1-m deep (29 200 L fresh de-chlorinated tapwater) and continuously aerated to maintain oxygen saturation. Floating feed (Zeilger Co., Gardenes, PA, 38:12:4) was provided at a rate of 3–5% of body weight day^{-1}. Monthly, the water level was lowered and the Koi were individually netted, weighed (Mettler 1600; Fisher Scientific, Pittsburgh, PA), measured for TL, and the surface mucus collected from all fish within minutes as described with polyurethane sponges. The fish were handled with sterile, latex gloves and no apparent injury to the fish occurred after the measurements and mucus collection with re-introduction to the aquaculture tank. Gender of the 10 fish was not known to the investigators at the time of measurements and sample collection, and was determined at the end of the trial via insertion of a catheter into the anal pore to obtain gonadal tissue. A dissecting microscope was used to view the gonadal samples and to identify each fish as male or female (Barbieri et al., 1994; Lowerre-Barbieri et al., 1996; Grier, 2002).

Table 1
Comparative ratios of 11-ketotestosterone (pg 11-KT mg^{-1} total soluble protein) extracted from surface mucus of seven male and four female Koi (*Cyprinus carpio*), using two different methods of collection on the same fish: spatula (right lateral side), polyurethane sponge (left lateral side)

	Surface mucus collection (pg 11-KT mg^{-1} total soluble protein)			
Fish no.	Spatula right	Sponge left	Sex	Stage/classification
10	68	90	M	Regressing, states 2–3
11	90	134	M	Mid-maturation, stage 3
12	65	88	M	Mid-maturation, stage 3
13	238	225	M	Late maturation, stage 3
14	79	77	M	Mid-maturation, stage 3
15	92	130	M	Regression, early maturation
16	170	338	M	Regressed, stage 2
17	18	28	F	Ripe, stages 4–5
18	24	37	F	Ripe, stage 5
19	1.2	8	F	Ripe, state 5
20	16	42	F	Ripe, stages 4–5

Samples of surface mucus were collected monthly and stored at −80°C until final collection. After 8 months, all samples were thawed and assayed at the same time so that the reagents and conditions for the ELISAs were the same. Each sample was divided into three equal volumes: the first was extracted with diethyl ether and prepared for assay of 11-KT by ELISA as described in Schultz et al. (2005). For the second, the assay for TSP was carried out, and for the third, 0.5 ml was assayed for VDYP by ELISA as described by Tan et al. (2006). The results were recorded as pg 11-KT mg^{-1} TSP and ng VDYP mg^{-1} TSP.

In Fig. 2, four of the fish were females (nos 21, 24, 26 and 30) and three were males (nos 22, 23 and 28) based on the peak values of VDYP/TSP or 11-KT/TSP in March, respectively. The genders of the remaining three fish were revealed in June (two females, nos 25 and 29) (one male, no. 27). The data from

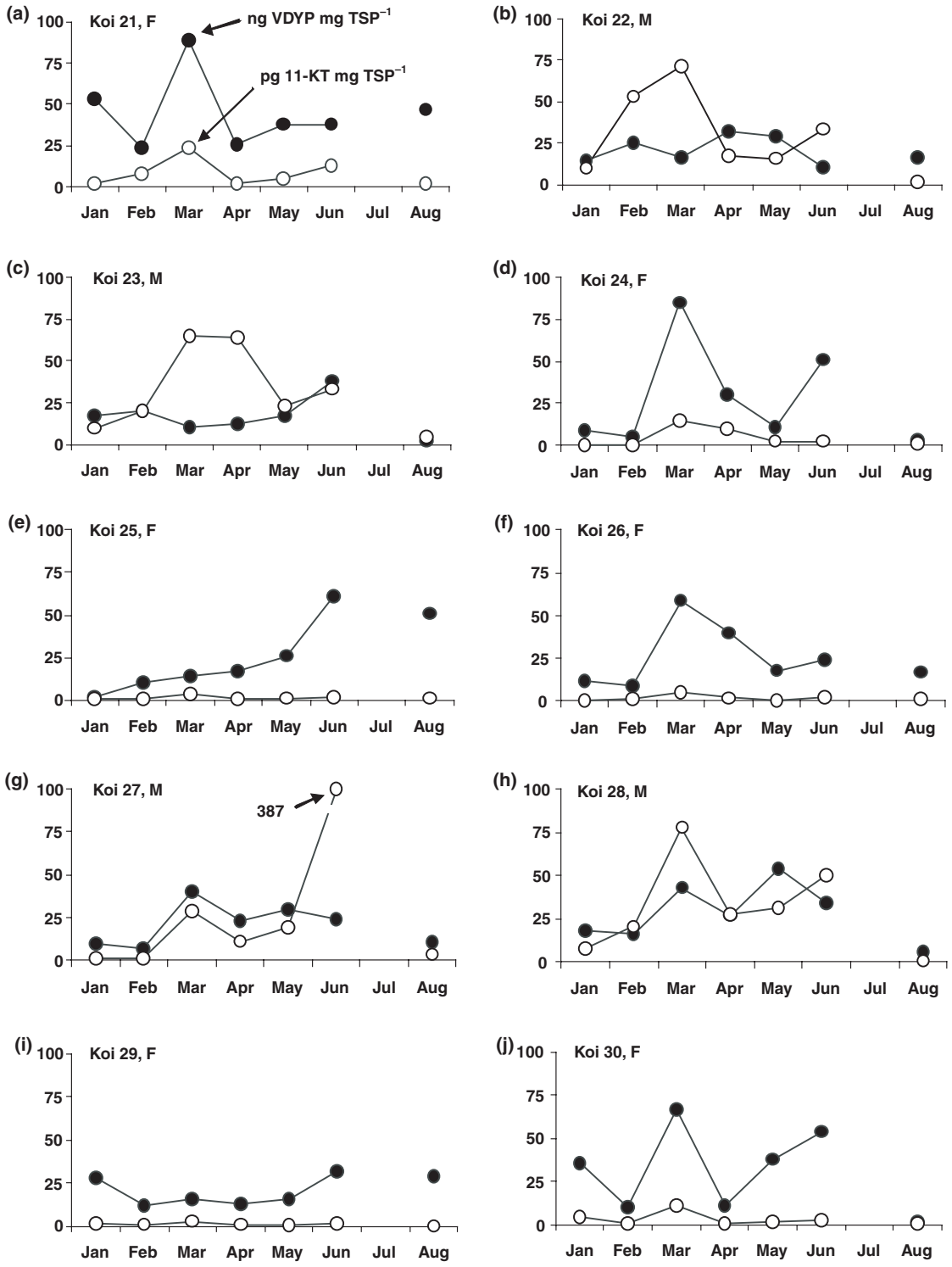

Fig. 2. Monthly collection with polyurethane sponges and assay of surface mucus for gender factors, recorded as pg 11-ketotestosterone/mg total soluble protein (TSP) (○–○) and ng vitellogenin-derived yolk proteins/mg TSP (●-●) from the same adult Koi maintained 8 months in an aquaculture facility. Sex of each fish (male, M; female, F) was determined only after 8 months from peak values of the gender factors. After termination of the experiment, the sex was verified by catheterization and examination of gonad tissue microscopically

the ELISAs in Fig. 2 which predicted the gender based on peak values of 11-KT and VDYP exactly matched the genders determined from gonad examination of each fish after termination of the experiment. Numerous eggs and 2–3 cm healthy young fish were observed in the same aquaculture tank containing the experimental fish, indicating an environment favorable for spawning behavior and fertilization of eggs.

The two notable findings from these preliminary experiments with Koi maintained in aquaculture for 8 months are: (i) collection of surface mucus with polyurethane sponges and storage of the absorbed fluid at −80°C for future assays of 11-KT and VDYP is a facile, accurate and non-invasive method for discriminating between sexually mature male and female fish. A limitation is that little or no activity is present in mature fish outside of the breeding season, as well as immature males and females, but this problem exists with any study of gender which depends on immunoassays of sex steroid hormones and Vtg in biological fluids (Schultz et al., 2005). In other unpublished experiments, the sponges containing absorbed surface mucus have either been frozen directly and extracted later, or immediately extracted and the fluid frozen, with equal success. (ii) Multiple samples of surface mucus from the same fish may be collected over prolonged periods of time with no apparent injury.

Figure 2 also shows that VDYP were detected in males and 11-KT was detected in females. It is known that male and immature teleosts possess the Vtg gene; Kime et al. (1999) reported that under normal conditions they do not synthesize enough estrogen to induce its expression. However, a number of investigators have reported Vtg in male fish under normal conditions (Copeland and Thomas, 1988; Ding et al., 1989; Sumpter, 1991; Goodwin et al., 1992; Goodbred et al., 1996). The presence of 11-KT in female fish has been documented in numerous earlier studies, and in some species the sex steroid may have several functions in addition to its role as an androgen (Leatherland et al., 1982; Slater et al., 1994; Cuisset et al., 1995; Lokman et al., 1998; Schultz et al., 2005). The use of polyurethane sponges for the minimally invasive collection of surface mucus to assay gender factors may have utility in aquaculture or other captive fish research applications where knowledge of gender, sex ratios and/or reproductive readiness is important to quantify. The methodology described here for surface mucus collection and assays for Koi has also been used successfully for both large (e.g. billfishes) and small (e.g. tilapia, fathead minnow) species of fish (D. R. Schultz, unpublished experiments).

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