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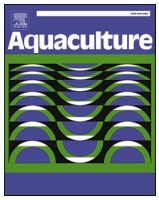

Using skin mucus for the identification of ovulation biomarkers in North American Atlantic salmon (*Salmo salar*)

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ABSTRACT

In Maine, North American Atlantic salmon production has experienced a 20% decrease in eye-up eggs, the point of embryo development where the black eye spot is visible. The maternal endocrine environment is suggested to be an important factor for ovulation and viable embryos but needs to be further investigated. Combining new sampling matrices (skin mucus) and advanced technologies (liquid chromatography tandem mass spectrometry (LC-MS/MS)) will advance the knowledge of the endocrine status in reproductively mature North American Atlantic salmon. The objectives of this study were to 1) identify mucus as a viable matrix to measure steroid hormones, 2) quantify steroid hormone profile of North American Atlantic salmon during ovulation and 3) compare hormone profiles of salmon with high and low eye-up rates. Mucus and plasma were collected from sexually mature female North American Atlantic salmon (n = 30). A group of females were sampled pre-ovulation (n = 9) and a separate group post-ovulation (n = 21) then analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Fish collected post-ovulation were slotted into groups based on eye-up rate, fish with ≥70% eye-up rate (high eye-up, n = 5) and fish with ≤70% eye up rate (low eye-up, n = 16). In mucus allopregnanolone (p = 0.01), 11-deoxycorticosterone (p = 0.05) and 11-deoxycortisol (p = 0.01) were significantly higher after ovulation. In plasma, allopregnanolone was significantly lower after ovulation (p = 0.01 and 17α, 20β-hydroxyprogesterone was significantly elevated (p = 0.05) after ovulation. Fish with a low eye-up rate had elevated mucus concentrations of corticosterone (p = 0.02). While fish with a high eye-up rate had elevated plasma testosterone concentrations (p = 0.04). Circulating androstenedione (r = 0.46, p = 0.03) and testosterone (r = 0.47, p = 0.03) were significantly correlated with eye-up rate. Mucosal concentrations of steroid hormone correlated with circulating concentrations of related steroids and reflect changes in reproductive physiology. The significant changes in mucosal and circulating steroid concentrations of the 5α reduced pregnane, allopregnanolone suggests a role of 5α-reduced pregnanes during ovulation in North American Atlantic salmon. To our knowledge this is the first time any 5α-reduced pregnane has been reported in teleosts. Mucosal concentrations of precursor glucocorticoids significantly elevated after ovulation combined with significant correlation with hydroxylated pregnanes suggests a dual mechanism needed for ovulation between glucocorticoids and pregnanes. Additionally, glucocorticoids and androgens appear to influence the viability of fertilized eggs highlighting the need for further research into the role's multiple hormones play in both oocyte maturation and embryo viability.

1. Introduction

As the fastest growing form of food production in the world, marine aquaculture and specifically production of North American Atlantic salmon in the United States contributes to seafood supply, supports commercial fisheries, and local job growth. With the growth in aquaculture, producers are focused on production efficiency to ensure the biggest economic return. However, one aspect of aquaculture that is often overlooked is broodstock management which consists of rearing fish to reproductive maturity, actively spawning these broodstock and

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then rearing the offspring for new broodstock. The USDA-ARS National Cold Water Marine Aquaculture Center in Franklin, ME developed a North American Atlantic salmon broodstock program that has been in place since 2007 (Wolters et al., 2009; Zhao and Wright, 1985). However, the facility has experienced a decrease in eye-up rates, a developmental stage used to determine if an embryo will hatch. When the selective breeding program began in 2008, eye up rates averaged 84 ± 1.5% and as of 2018, the eye-up rate was 56 ± 2.5% (unpublished internal data from the National Cold Water Marine Aquaculture Center). Other major producers of North American Atlantic salmon have also observed a decrease in eye up rates over this same period (Thayer and Hamlin, 2016). Many paths of study have been take to determine how to produce quality eggs [Reviewed by (Bobe and Labbe, 2010)]. These study indicate that multiple factors are required, for example; appropriate maternal nutrition (Eskelinen, 1989; Izquierdo et al., 2001; Noori et al., 2019), water quality (Haffray et al., 1995; Ketola et al., 1988), water temperature (King et al., 2003), photoperiod (Bonnet et al., 2007) and appropriate maternal endocrine environment (Hwang et al., 1992; Mingist et al., 2007; Sopinka et al., 2017; Srivastava and Brown, 1993; Taylor et al., 2016) which has yet to be fully characterized.

The maternal endocrine environment has been determined as an important factor for viable embryos (Eriksen et al., 2006; King et al., 2003; Srivastava and Brown, 1993; Thayer and Hamlin, 2016). However, there are still gaps in the body of knowledge of the maternal endocrine pathways in North American Atlantic salmon which lead to the production of viable eggs. Steroid hormone pathways which are essential for the development of oocytes differ depending on two distinct time periods: vitellogenesis and oocyte maturation. Vitellogenesis is the incorporation of vitellogenin proteins by the oocyte and their processing into yolk proteins (Lubzens et al., 2010). Vitellogenic Atlantic salmon have elevated circulating estradiol (E_2) concentrations which induce the production of vitellogens from the liver (Oppenberntsen et al., 1994). At the termination of vitellogenesis and the beginning of oocyte maturation there is a documented decrease in E_2 concentrations and an increase in circulating 17α, 20β-hydroxyprogesterone (17,20 OHP) concentrations (Nagahama, 1997; Nagahama and Yamashita, 2008). This hydroxylated progesterone metabolite is often referred to as a maturation inducing substance (MIS) or maturation inducing hormone (MIH) which binds to oocyte membrane-specific progesterone receptors to activate the dissolution of the germinal vesicle and reinitiate meiosis (Fostier and Jalabert, 1986).

In addition to oocyte maturation, maternal hormones are necessary for oocyte viability after ovulation and can potentially dictate embryo survival. Steroid hormones have been detected in teleost ovarian follicles, eggs, and embryos. Concentrations of E_2, testosterone, 11-ketotestosterone, cortisol and progesterone have been measured in unfertilized and fertilized eggs (Dejesus and Hirano, 1992; Thayer and Hamlin, 2016). While oocytes and embryos were thought to be passive recipients or responders of maternal steroids, literature shows these tissues synthesize, metabolize and excrete hormones (Bobe and Labbe, 2010; Dejesus and Hirano, 1992; Li et al., 2012a; Moore and Johnston, 2008; Sopinka et al., 2017; Zhao and Wright, 1985). Progesterone and cortisol receptors have been detected on oocytes and fertilized embryos and studies suggest that maternally derived steroid hormones are essential for the development of the embryo (Fent et al., 2018; Li et al., 2012a; Li et al., 2012b). The importance of these maternally derived steroid hormones for embryo viability suggests a need for fully understanding the maternal hormone pathways at the time of ovulation. While steroid hormones of the Atlantic salmon endocrine profile have been measured, the complexity of endocrine pathways require a more in-depth analysis to impart a complete understanding of fish fertility and oocyte quality.

There are several limitations in the measurement of fish steroids, including time, cost, and the level of invasiveness relative to sampling, e.g., blood or tissue sampling which immediately affects the fish endocrine profile reviewed by (Popovic et al., 2012). Serum, plasma, or tissues have historically been used to measure steroid hormone concentrations in fish. Not only are these matrices difficult to collect but handling fish during collection induces a significant endocrine stress response which can disrupt the resting endocrine profile. Fish skin mucus, a complex material that contains a broad range of small molecules, including steroid hormones, is relatively easy to collect and a non-lethal alternative to historic matrices. Skin mucus has been used to measure cortisol as a biomarker of stress (Bertotto et al., 2010) and exposure to endocrine disrupting compounds (Ekman et al., 2015) but has yet to be used to measure steroid hormones involved in reproductive function. The use of skin mucus as a matrix to measure fish hormones will enable aquaculture producers and scientists to measure hormones of fish populations in a non-lethal manner which would reduce stress, time, and materials.

The study of fish steroidal pathways has traditionally been performed by focusing on one steroid hormone at a time using immunoassay technologies. As immunoassays are limited to one steroid hormone per assay, multiple assays and potentially multiple extractions are required to develop a full endocrine profile. In addition, immunoassays indirectly measure steroid hormones through antibody binding, which is prone to cross-reactivity and can inaccurately quantify the target compound. The issues with cross-reactivity and lack of breadth of immunoassays contribute to a shift in steroid hormone measurement techniques. Mass spectrometry (MS) methods have become advantageous because they are direct detection methods employing highly resolved unique masses and fragmentation patterns. Specifically, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a versatile analytical technique for steroid hormone measurement, generating qualitative, semi-quantitative and quantitative data based on the atomic and molecular structure.

The objectives of this study were to 1) determine whether mucus could be used as a viable matrix to measure steroid hormones, 2) quantify steroid hormone profiles during ovulation of North American Atlantic salmon and 3) compare hormone profiles of salmon with high eye-up rates (≥ 70%) to salmon that have lower eye-up rates (≤ 70%).

2. Materials

2.1. Sample collection

All animal experiments comply with the National Research Council's Guide for the Care and Use of Laboratory Animals (Alvarez and Pardo, 1997). NA Atlantic salmon were reared at the USDA-ARS National Cold Water Marine Aquaculture Center (NCWMAC Franklin, ME, USA), a recirculation-based aquaculture facility designed to follow strict biosecurity standards. Reproductively mature Atlantic salmon were held in two 46 m^3 tanks exposed to natural light conditions, with a flow rate of 4470 L/min, a make-up rate of 0.011 gal/min, and stocked at a maximum density of 40 kg/m^3. Salmon were housed in a high salinity brackish well water (~ 15 ppt) from 2 to 3 years of age. In August of 2019 at approximately 3.5 years of age, broodstock were transferred into fresh well water with an average salinity of 0.51 ± 0.09 ppt. Additional water quality parameters, monitored daily, were as follows: temperature (12.75 ± 0.49 °C), dissolved oxygen (DO, 70–120% saturation), pH (7.84 ± 0.12), total ammonia nitrogen (0.26 ± 0.11 mg/L), unionized ammonia (0.005 ± 0.002), nitrite (0.02 ± 0.01 mg/L), nitrate (0.61 ± 0.04 mg/L), CO_2 (2.25 ± 0.48 ppm), alkalinity (87 ± 8.14 ppm). All fish were feed 2 to 4 times/ day on a commercially prepared diet with a pellet size of 6.0 mm to 12.0 mm.

In early fall 2019, broodstock, identified as likely to become sexually mature, were separated by sex. Maturity likelihood was based on vent swelling, body confirmation and color. All identified females exhibited abdominal swelling, suggesting final oocyte maturation. Female fish were then checked for "ripeness" every Monday and Wednesday starting on the third week of October. A female was defined as "ripe" if her abdomen was extremely soft, her vent was extended, and eggs could easily be expressed through manual palpation. Samples were collected from all female fish both "ripe" (post ovulation) and "unripe" (pre

ovulation). If females were identified as "ripe" they would be spawned the next day. "Unripe" fish would be returned to a holding tank. "Ripe" fish were anesthetized in tricaine methane-sulfonate (MS-222, Syndel, Ferndale, WA, USA) at a concentration of 100 mg/L for 15 min and removed from the holding tank for sampling and spawning. Each fish had a passive integrated transponder (PIT) tag number which was recorded before sampling for identification. To collect mucus, the fish were placed on their side and skin mucus was absorbed on to two strips of pre-weighed 2.0 cm × 0.5 cm strips of glass microfiber filter paper (type GF/A, 90 mm diameter) per fish. The glass fiber filter paper was placed on the side of the fish using forceps, then slid down the length of the fish, avoiding the gills and vent. The filter paper was removed with forceps, placed in a 2.0 mL cryovial and flash frozen in liquid nitrogen. Unused filter paper strips were dipped in the holding tank at time of collection, placed in a 2.0 mL cryovial and frozen along with the mucus samples to serve as a blank. Blood was then collected from the caudal vein with a 22-gauge needle and heparinized vacutainer blood and centrifuged at 2000 X g for 15 min at 4 °C to separate plasma. Approximately 2 mL of the plasma was transferred to a cryotube and stored at −80 °C until analysis. All samples were shipped in a liquid nitrogen vapor Dewar to NIST Charleston and stored at −80 °C until extraction.

Female fish were strip spawned following serum and mucus collections. Females were wiped with towels to remove water from the vent. Fish were then gently massaged from the abdominal area in one motion from the dorsal area of the fish to the vent to strip the eggs into a bucket. One cup of eggs (~ 1000 eggs) was removed from the collection bucket and placed in a 1-quart container for fertilization. Freshly stripped milt was added to the eggs along with a sperm activator, mixed and incubated for 5 min. After fertilization, eggs were then disinfected with a 50

Table 1
Ionization of compounds with APCI ionization. The quantifying product ion was selected based on relative abundance and is bolded in the table below. Retention time (RT, min) Collision energy (CE,*V*), and declustering potential (DP,*V*) are presented for each compound.

Compound	Abbrv.	RT	Precursor ion (*m/z*)	Product ion (*m/z*)	CE (V)	DP (V)
Pregnenolone	P_5	12.7	299.2	**105.0**	54	68
			299.2	131.0	52	68
Progesterone	P_4	11.8	315.2	**97.1**	34	90
			315.2	109.1	33	90
17α-hydroxyprogesterone	17OHP	10.6	331.3	**97.1**	29	138
			331.3	109.0	31	138
17α-hydroxyprogesterone $^{13}C_3$		10.6	334.6	**100.1**	31	138
20α-hydroxyprogesterone	20OHP	11.1	317.3	**109.1**	37	41
			317.3	97.1	36	43
17α,20β-hydroxyprogesterone	17,20OHP	10.8	333.1	**97.1**	32	102
			333.1	109.0	34	86
5α-dihydroprogesterone	5α DHP	13.1	317.2	**119.1**	44	133
			317.2	105.1	55	133
5α-dihydroprogesterone $^{13}C_3$		13.1	322.2	**124.1**	55	133
Allopregnanolone	AP	13.7	301.2	**105.0**	59	86
			301.2	93.0	51	22
Allopregnanolone-d_5		13.7	307.2	**119.0**	51	22
20α-dihydroprogesterone	20α DHP	12.9	323.0	**91.1**	76	34
			323.0	105.1	48	34
3β,20α-dihydroprogesterone	3β, 20α DHP	12.4	285.1	**81.0**	37	93
			285.1	119.1	44	101
3β,20β-dihydroprogesterone	3β, 20β DHP	13.6	285.2	**81.0**	69	127
			285.2	95.0	42	81
11-deoxycorticosterone	DOC	10.3	331.2	**97.0**	26	97
			331.2	109.2	32	98
11-deoxycorticosterone $^{13}C_3$		10.3	33.4	**100.0**	32	98
11-deoxycortisol		9.5	347.2	**97.1**	40	94
			347.2	109.1	44	94
11-deoxycortisol$^{13}C_3$		9.5	350.2	**100.0**	44	94
Corticosterone		9.3	347.2	**121.0**	31	90
			347.2	105.2	35	90
Cortisol		8.2	363.2	**121.1**	34	60
			363.2	267.2	31	60
Cortisol$^{13}C_3$		8.2	366.1	**124.0**	31	60
Cortisone		7.5	361.2	**163.1**	35	60
			361.2	121.0	46	60
Cortisone$^{13}C_3$		7.5	364.0	**166.0**	46	60
Dehydroepiandrosterone	DHEA	10.7	271.2	**91.0**	63	114
			271.2	105.1	57	133
Androstenedione	A_4	10.0	287.3	**97.1**	29	100
			287.3	109.0	23	100
Androstenedione$^{13}C_3$		10.0	290.6	**100.2**	23	100
11-oxoAndrostenedione	11OXOA_4	7.6	303.2	**121.1**	35	91
			303.2	244.1	47	78
19-norAndrostenedione	19norA_4	9.4	273.1	**109.0**	30	109
			273.1	83.1	35	107
Testosterone	Test	10.4	289.1	**97.1**	36	188
			289.1	109.0	35	188
Testosterone$^{13}C_3$		10.4	292.6	**112.2**	35	188
5α-dihydrotestosterone	5α DHT	11.3	291.3	**91.1**	35	59
			291.3	215.1	56	63
11-ketotestosterone	11KT	8.4	303.2	**121.1**	38	91
			303.2	105.1	48	91
11-ketotestosterone $^{13}C_3$			306.2	**121.0**	48	91

ppm Iodine solution for 30 min and water hardened for one hour before moving into the incubator. Fertilized eggs were kept at <8 °C, a salinity of 0 ppt, a pH of 6.6–6.8 and 70–120% dissolved oxygen saturation. At 250 degree days eggs were checked for eyes and if apparent eggs were shocked. To shock eggs were placed a one gallon bucket half full of system water and then dumped into a second one-gallon bucket from three feet high. This is repeated 3 times and the eggs were returned to the incubator. After 24 h eggs were placed in a Van Gaalen fish egg sorter (VMG Industries, CO, USA) which uses modulated infrared light incorporated in an electronic scanning device to detect dead eggs and accurately separate them from live eyed eggs. The counters then provide totals of both live and dead eggs sorted. Half a cup of live eggs (~ 450) was then placed into a trough system to hatch. The trough system had the same water quality parameters as the incubation. The eye-up rate is calculated as follows:

$$[\text{Live eggs}/(\text{Live eggs} + \text{dead eggs})]^{*}100$$

2.2. Standards

Standards for the steroid hormones measured in this study (See Table 1 and Fig. 1) include androstenedione (A_4), testosterone, 17α-hydroxyprogesterone (17OHP), progesterone (P_4), corticosterone, cortisone, cortisol (Sigma Aldrich, St. Louis, MO; ≥ 98% purity), dehydroepiandrosterone (DHEA), 5α -dihydroprogesterone (5αDHP), allopregnanolone (AP), 11-deoxycorticosterone, and 11-deoxycortisol pregnenolone, 3β,20α-dihydroxy-dihydroprogesterone (βαDHP), 3b,20B-dihydroxy-dihydroprogesterone (ββDHP), 20α-hydroxy-dihydroprogesterone (20αDHP), 17α, 20β hydroxyprogesterone (17,20OHP), 11 ketotestosterone (11KT) and 11-oxoAndrostenedione (11oxoA_4) (Steraloids (Newport, RI; ≥98% purity)). All steroid hormones measured were paired with internal standards that which had the same or very similar chemical composition for quantification (testosterone-$^{13}C_3$, androstenedione-$^{13}C_3$, 17-hydroxyprogesterone-$^{13}C_3$, and cortisol-$^{13}C_3$ (Cerilliant; Round Rock, TX, USA; 99.99% purity), 11 ketotestosterone -d3, 5α-dihydroprogesterone $^{13}C_3$, allopregnanolone d_4 (Isosciences; Amblar, PA, USA) progesterone-$^{13}C_3$ (Cambridge Isotopes; Tewksbury, MA, USA; 98% purity)) (Table 2).

2.3. Sample preparation

In preparation for LC-MS/MS analysis, salmon mucus samples were subjected to a protein precipitation before analysis. The filter paper used for mucus absorption was weighed before mucus was absorbed from the fish and then after absorption to calculate the amount of mucus collected as a wet weight. For extraction 100 μL of the 1 ng/μL internal standard mix was added gravimetrically to each tube along with 500 μL of 70% methanol (Optima LC/MS Grade, Fisher scientific, Hampton, NH, USA) in water. Samples were vortexed for 30 s and the resulting mixture was transferred to a 2 mL tube with ceramic beads. The original sample tube was then washed with 500 μL of 70% methanol and added to the bead tube. This step was repeated one additional time for a total of 1.5 mL. Samples were kept on ice and transferred to the Omni bead ruptor elite (Kennesaw, GA, USA) where they were shaken twice for 1 min at 60 mph. The mixture was transferred to 16 mm × 100 mm glass tubes and an additional 2 mL of 70% methanol was added and placed at 4 °C overnight. Samples were then centrifuged at 5000 xg for 10 min, and the supernatant was transferred to a new 16 mm × 100 mm glass tube and dried to completion under N_2 at 45 °C in a Biotage TurboVap LV (Salem, NH, USA).

Plasma samples were processed using a liquid/liquid method to extract steroid hormones. Adapted from the method (Legacki et al., 2016), 500 μL of plasma was added to 16 × 100 mm screw top tubes followed by 100 μL of the 1 ng/μL internal standard mix. Methyl-*tert* buytl ether (2.5 mL) was added to all samples, placed on a rocker for 15 min and centrifuged at 5000 *g* for 10 min. The supernatant was

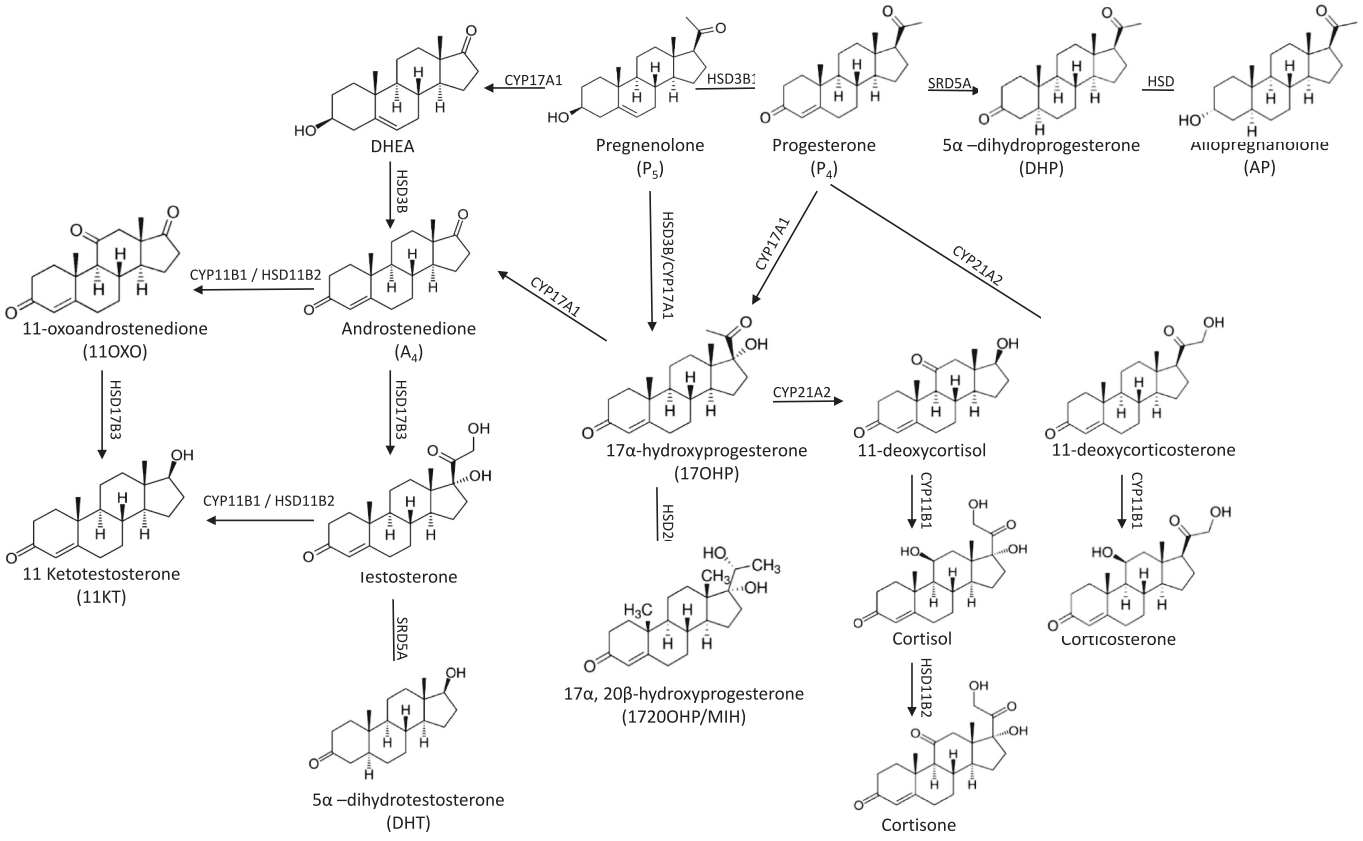

Fig. 1. A schematic representation of spawning female Atlantic salmon endocrine pathways at the time of ovulation

Table 2
Parameters for method validation include: internal standard used for normalization, slope of the line and R^2 used for linearity and quantification, Extraction efficiency (%EE), Matrix effect (% ME), limit of detection (LOD, ng/g mucus), limit of quantitation (LOQ, ng/g mucus), and inter assay coefficient of variation (%CV) for all compounds detected.

Compound	Internal standard	R^2	Extraction efficiency (% EE)	Matrix effect (% ME)	LOD (ng/g mucus)	LOQ (ng /g mucus)	% CV
P_5	Progesterone $^{13}C_3$	0.999	87	-6	0.1	0.1	4.7
P_4	Progesterone $^{13}C_3$	0.999	86	4	0.1	0.1	2.9
17OHP	17α-hydroxyprogesterone $^{13}C_3$	0.999	89	3	0.1	0.1	4.6
17,20OHP	17α-hydroxyprogesterone $^{13}C_3$	0.999	72	-11	0.1	0.1	4.1
20OHP	17α-hydroxyprogesterone $^{13}C_3$	0.999	83	7	0.1	0.1	3.9
5αDHP	5α-dihydroprogesterone $^{13}C_5$	0.999	70	3	0.1	0.5	4.6
3αDHP	Allopregnanolone-d_5	0.999	78	6	0.1	0.5	6.4
20αDHP	5α-dihydroprogesterone $^{13}C_5$	0.999	96	-5	0.1	0.5	9.2
αβDHP	5α-dihydroprogesterone $^{13}C_5$	0.999	85	4	0.1	0.5	8
ββDHP	5α-dihydroprogesterone $^{13}C_5$	0.999	86	2	0.1	0.5	7.5
11-deoxycorticosterone	11-deoxycorticosterone $^{13}C_3$	0.999	92	-2	0.1	0.5	8.1
11-deoxycortisol	11-deoxycortisol $^{13}C_3$	0.999	93	-2	0.1	0.1	5.4
Corticosterone	Cortisol $^{13}C_3$	0.999	85	-11	0.1	0.5	6.3
Cortisol	Cortisol $^{13}C_3$	0.999	88	6	0.1	0.1	2.9
Cortisone	Cortisone $^{13}C_3$	0.999	81	1	0.1	0.1	5.1
DHEA	Androstenedione $^{13}C_3$	0.999	79	-4	0.1	0.5	5.8
A_4	Androstenedione $^{13}C_3$	0.999	73	7	0.1	0.1	3.4
11oxoA_4	Androstenedione $^{13}C_3$	0.999	76	1	0.1	0.5	7.4
19norA_4	Androstenedione $^{13}C_3$	0.999	86	4	0.1	0.1	7.2
Testosterone	Testosterone $^{13}C_3$	0.999	84	1	0.1	0.1	6.4
11KT	11 Ketotestosterone -d_3	0.999	84	5	0.1	0.1	2.3
5αDHT	5α-dihydroprogesterone $^{13}C_5$	0.999	89	1	0.1	0.5	3.5

transferred to another 16 × 100 mm glass tube and dried to completion, as described above for the mucus extractions. The dried sample was reconstituted with 200 μL 50% methanol (volume fraction in water) and transferred to an autosampler vial with an insert for analysis.

To better represent matrix extracts, calibrants were prepared with charcoal stripped fish plasma and processed along with samples. Dextran-coated charcoal (final concentrations 0.25%), 0.25 M sucrose, 1.5 mM $MgCl_2$ and 10 mM HEPES solution adjusted to a pH of 7.4 were incubated overnight at 4 °C, centrifuged (500 xg for 10 min) to pellet charcoal. The supernatant was decanted, and the charcoal pellet was mixed with an equivalent volume of fish plasma. The plasma/charcoal mixture was then vortexed and incubated for 12 h. at 4 °C before use. A seven-point calibration curve (0.1 ng/mL, 0.5 ng/mL, 1.0 ng/mL, 2.5 ng/mL, 5 ng/mL, 10 ng/mL, 50 ng/mL) composed of all native standards listed in Table 1 with the addition of 100 μL of all isotopically labeled standards at approximately 1 ng/μL (internal standards; Table 1). The seven-point calibration curve was run at the beginning and end of each sample set to account for any method drift. The two calibrations curves were averaged, and quantitation of compounds was determined by a non-weighted linear regression analysis of the ratio of analyte area to the ratio of the area of designated internal standard. All calibration curves were expected to be linear ($R^2 \geq 0.99$) and inter assay accuracy was determined by spiking a blank matrix sample (filter paper or plasma), extracting them, and including two with every run (Table 2). The limit of detection (LOD), defined by the lowest calibrator and limit of quantitation (LOQ), defined as 3 times the limit of detection, were calculated for each compound (Table 2).

To test for matrix and extraction effects, a set of frozen unused filter paper tubes and empty tubes were spiked with 100 μL of a 1 ng/μL standard mix and 100 μL of the 1 ng/μL internal standard mix and extracted. Extraction efficiency (%EE) was assessed by spiking two sets of empty tubes with standard mix and comparing peak areas of the set that were extracted with protein precipitation to those that were not extracted. Another measure of recovery in matrix, measured as matrix effect (%ME) was assessed by spiking tubes with filter paper and comparing peak areas from tubes spiked and then extracted without filter paper. A set of quality control (QC) samples, which were comprised filter paper spiked with 20 μL of the 1 ng/μL standard calibration mix for a concentration of 20 ng/mL were run with each analysis. The data from the QC samples between each analysis was used to calculate the %CV for each compound.

2.4. Instrument method

A volume of 5 μL from both mucus and plasma extractions were injected with an Agilent Infinity II (Agilent, Santa Clara, CA,) liquid chromatography system onto an ACE Excel 2 C18 column (5.0 μm, 100 mm × 2.1 mm) for compound separation. A gradient with two mobile phases (water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B)) was delivered at 0.4 mL/min. The gradient used was 0.0 to 3.0 min at 40% B, 3.0 to 7.8 min at 40% to 75% B, 7.8 to 12.0 min at 83% B, 12.0 to 15.0 min at 100% B, 15.0 to 16.0 min at 100% B, 16.0 to 16.1 min at 40% B, 16.1 to 21.0 min at 40% B. Mass spectral analysis was run with a SCIEX tandem mass spectrometer (AB Sciex, Framingham, MA, USA) and ionization achieved utilized an atmospheric-pressure chemical ionization (APCI) source in positive mode. Source conditions are as follows: Cone temperature (TEM) was 500 °C, Nebulizer Current (NC) was 5 μA, Curtain Gar (CUR) was 35 psi, Collision Gas (CAD) was 7 psi, Ion source gas (GS1) was 40 psi, Entrance potential (EP) was 5 V and Collision Cell Exit Potential (CXP) was 10 V. Resolution of Q1 and Q3 were both ser at 0.7 *m/z*. Detection and quantitation of all steroid hormones were accomplished using multiple reaction monitoring (MRM) with a minimum of two transitions per compound.

2.5. Statistical analysis

Differences in steroid concentrations of pre- ovulation ($n = 9$) and post- ovulation ($n = 21$) salmon were assessed using the standard least squares fit model in JMP (Cary, NC). Correlations were determined using multivariate analysis (JMP) and $p \leq 0.05$ was considered significant for all analyses. Salmon which ovulated were further divided into fish that had a high eye-up rate (≥70%, $n = 5$) and a low eye-up rate (≤70%, $n = 16$). The division at 70% was based on industry standards as ≥70% eye-up rate is considered the lowest ideal rate of success. Due to the low eye-up rate for the year fish were sampled there were only four fish with an eye-up rate ≥ 70% collected for this analysis (Fig. S1). For comparison of steroid concentrations each group was given a nominal designation and were also assessed using the standard least squares fit

model in JMP. For correlations between eye-up % and steroid concentrations a multivariate analysis was used (JMP).

3. Results

An LC-MS/MS method was developed to measure 11 pregnanes, 5 glucocorticoids, and 6 androgens in salmon mucus and plasma. There was no observed, direct correlation between mucosal and circulating steroid concentrations of the same compound. However, pre-ovulation circulating pregnenolone was inversely correlated with mucosal 17,20OHP ($r = -0.83$, $p = 0.01$, Fig. 2A) and mucosal cortisol ($r = -0.75$, $p = 0.03$, Fig. 2A). Post-ovulation circulating pregnenolone was positively correlated with two mucosal glucocorticoids [cortisol ($r = 0.62$, p = 0.01), cortisone ($r = 0.60$, p = 0.01) Fig. 2B] and two mucosal androgens [DHEA ($r = 0.82$, $p = 0.05$), DHT ($r = 0.89$, p = 0.05) Fig. 2C].

3.1. Mucus

Of the 22 compounds measured in salmon mucus, 6 pregnanes, all 5 glucocorticoids and all 6 androgens were detected (Fig. 1). The highest measured mucosal concentrations are of 5αDHP metabolite, AP both pre- (15.21 ± 4.99 ng/ g mucus) and post-ovulation (53.57 ± 22.37 ng/ g mucus, p = 0.01). The other pregnanes detected were P_5, P_4, the hydroxylated pregnanes (17OHP, 17,20OHP), and 5α-reduced pregnanes (5αDHP, βαDHP, ββ DHP). The 5α reduced pregnane, 5αDHP, was detectable pre-ovulation (2.96 ± 1.32 ng/g mucus) and undetectable (<LOQ) post-ovulation. Both metabolites of 5αDHP, βαDHP and ββ DHP, were not significantly different between pre- and post-ovulation, and were detected in the post-ovulation stage while 5αDHP was not. None of the other mucosal pregnanes were significantly different between the two stages. All glucocorticoids were detected in salmon mucus with cortisone had the highest concentrations, both pre-(11.16 ± 0.66 ng/ g mucus) and post- (16.15 ± 2.49 ng/ g mucus, $p = 0.05$) ovulation. The glucocorticoid precursors, 11 deoxycorticosterone (0.74 ± 0.09 ng / g mucus vs 3.65 ± 1.27 ng / g mucus, $p = 0.05$) and 11 deoxycortisol (0.77 ± 0.18 ng / g mucus vs 3.18 ± 1.46 ng / g mucus, $p = 0.01$) were both significantly higher post-ovulation when compared with pre-ovulation concentrations (Fig. 3). Six androgens were detected in the post-ovulation mucus samples, but two androgens (5αDHT and testosterone) weren't detected pre-ovulation. However post-ovulation 5αDHT had the highest concentration among the androgens measured (9.25 ± 1.78 ng/ g mucus, p = 0.05) and testosterone was detected at a concentration of 1.82 ± 0.8 ng/ g mucus. The rest of the androgens were detectable at both stages and no significant differences seen in DHEA, A_4, or 11oxoA_4 concentrations between the two stages.

Correlations between steroids measured in mucus varied depending on stage of ovulation. Pre-ovulation mucosal P_5 is inversely correlated with 17,20OHP ($r = -0.89$, $p = 0.04$) and progesterone is inversely correlated with both 17OHP ($r = -0.99$, $p = 0.02$) and 17,20OHP (r = −0.99, $p = 0.03$) whereas post-ovulation no correlation is observed (Table 3). Mucosal cortisol is significantly correlated with 17OHP ($r = 0.67$, p = 0.03), 17,20OHP ($r = 0.61$, $p = 0.05$), 11-deoxycortisol ($r = 0.93$, $p = 0.001$) and βαDHP ($r = 0.98$, p = 0.02) pre- ovulation. This pattern is no longer significant post-ovulation. Pre-ovulation cortisone is significantly correlated with 17,20OHP ($r = 0.78$, p = 0.01), 11-deoxycortisol ($r = 0.79$, p = 0.02) and βαDHP ($r = 0.97$, p = 0.03) and these correlations are no longer significant post-ovulation. However, post-ovulation cortisone is significantly correlated with ββDHP ($r = 0.83$, p = 0.04), and 5αDHT ($r = 0.87$, p = 0.05). After ovulation, 11 deoxycorticosterone is significantly correlated with 11 deoxycortisol (r = 0.87, $p = 0.05$) and testosterone is inversely correlated with 5αDHT (r = −0.99, $p = 0.02$) (Table 3).

3.2. Plasma

The steroid profile detected in plasma from all sampled salmon contained four detectable pregnanes (P_5, 17OHP,17,20OHP and AP), 4 glucocorticoids (11-deoxycortisol, corticosterone, cortisol, and cortisone) and 5 androgens (DHEA, A_4, 19norA_4, testosterone and 11KT) (Fig. 4). Of these compounds, cortisol was the most abundant steroid both pre- (181.51 ± 23.22 ng/mL plasma and post-(195.35 ± 15.06 ng/ mL plasma, Fig. 4, $p = 0.04$) ovulation. Overall, there were fewer circulating steroids measured in the plasma (13) than those measured in mucus (18). Inverse of what was seen in mucus, plasma AP was significantly higher pre-ovulation (5.14 ± 2.67 ng/ mL plasma) when compared to concentrations post-ovulation (1.28 ± 0.2 ng/ mL plasma, $p = 0.001$, Fig. 4). A significant difference in 17,20OHP concentrations post-ovulation (44.09 ± 5.0 ng/mL plasma) is observed as compared with pre-ovulation (29.23 ± 4.9 ng/ mL plasma, p = 0.05, Fig. 4). Other steroids measured were not significantly different between the two stages.

Plasma steroid hormones had a pattern of correlation post-ovulation compared to pre-ovulation which is opposite of what was seen in mucus steroid hormones (Table 4). Two plasma glucocorticoids, corticosterone and cortisone, were significantly correlated with P_5 [corticosterone ($r = 0.73$, $p \leq 0.01$), cortisone ($r = 0.74$, p ≤ 0.01), Table 4] post-ovulation but not pre-ovulation. Post- ovulation the pregnane, AP, was inversely correlated with corticosterone ($r = -0.67$, p = 0.001) and cortisone (r = −0.67, p = 0.001) but not pre-ovulation. However, corticosterone and cortisone were significantly correlated with 11-deoxycortisol both pre- and post-ovulation (Table 4). The androgen, DHEA, was correlated with 17,20OHP at both stages [pre-ovulation ($r = 0.67$, p = 0.05), post-ovulation ($r = 0.52$, $p = 0.01$)] and is correlated with P_5 ($r = 0.51$, $p = 0.02$) post-ovulation. Pre-ovulation 17OHP was not correlated with its metabolite 17,20OHP but is correlated post-ovulation (r = 0.51, p =

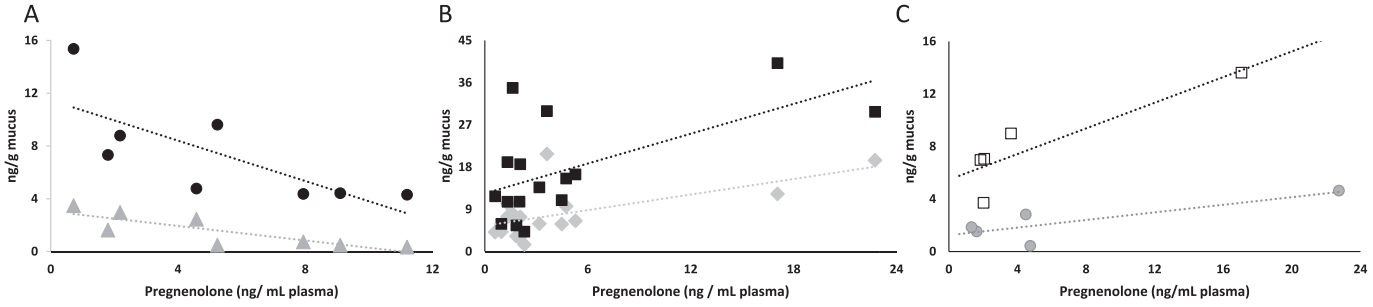

Fig. 2. A) Pre-ovulation there was an inverse correlation between plasma pregnenolone and mucosal cortisol (black circles, r2 = −0.747, p = 0.03) and mucosal 17,20 OHP (gray triangles, r2 = −0.8314, p = 0.01). B) Post-ovulation, there was a significant correlation between circulating plasma pregnenolone and mucosal cortisone (black squares, r2 = 0.6037, $p = 0.01$) and mucosal cortisol (gray diamonds, r2 = 0.623, p = 0.01). C) Post-ovulation, there was a significant correlation between circulating plasma pregnenolone and mucosal 5αDHT (white squares, r2 = 0.8862, $p = 0.05$) and mucosal DHEA (gray circles, r2 = 0.8197, p = 0.05).

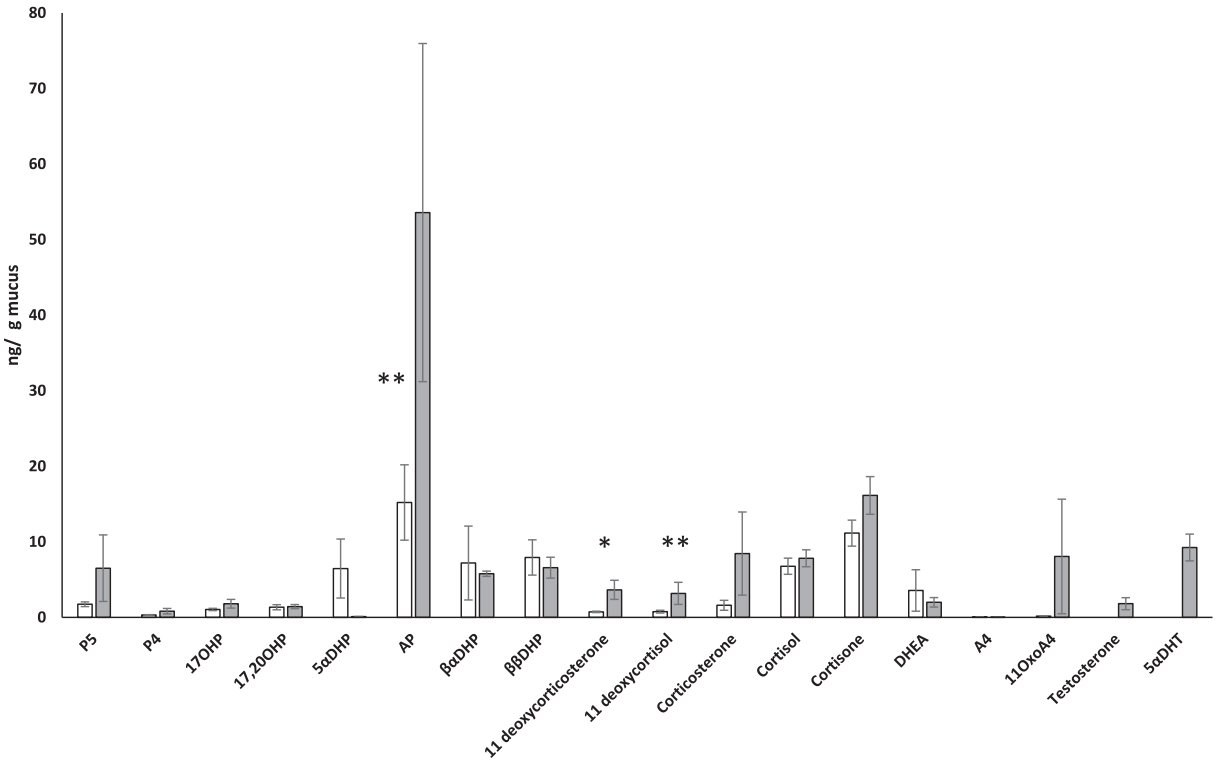

Fig. 3. Mucosal steroid hormone profile of female Atlantic salmon pre ovulation ($n = 9$, white bars) and post ovulation ($n = 20$, gray bars). Allopregnanolone (AP), 11 deoxycorticosterone, and 11 deoxycortisol had significantly higher concentrations after ovulation. $^{**}p \leq 0.01$, $^{*}p \leq 0.05$.

Table 3
Correlation matrix of mucosal steroid hormones; Pregnenolone (P_5), Progesterone (P_4), 17α-hydroxyprogesterone (17OHP),17α, 20β-hydroxyprogesterone (17,20OHP), Cortisol, Cortisone, 11 deoxycortisol, 11 deoxycorticosterone, 3β, 20α-dihydroprogesterone (βα-DHP), 3β, 20β-dihydroprogesterone (ββDHP), Testosterone and 5α-dihydrotestosterone (5αDHT). R values are presented below ** ≤ 0.01, * ≤ 0.05.

Variable	By variable	Before ovulation	After ovulation
P5	17,20 OHP	−0.89*	−0.12
P4	17OHP	−0.99*	0.64
P4	17,20 OHP	−0.99*	−1.00
Cortisol	17OHP	0.67*	−0.20
Cortisol	17,20 OHP	0.61*	−0.24
Cortisol	11 deoxycortisol	0.93**	−0.20
Cortisol	βαDHP	0.98*	0.91
Cortisone	17,20 OHP	0.78**	0.01
Cortisone	11 deoxycortisol	0.79*	−0.30
Cortisone	βαDHP	0.97*	0.98
Cortisone	ββDHP	0.59	0.83*
Cortisone	5αDHT	N/A	0.81*
11 deoxycorticosterone	11 deoxycortisol	N/A	0.87*
Testosterone	5αDHT	N/A	−0.99*

0.02) as well as A_4 ($r = 0.51$, $p = 0.02$). Post-ovulation the pregnane 17,20OHP was inversely correlated with the androgen 11KT ($r = -0.98$, $p = 0.02$). Post-ovulation cortisol was inversely correlated with an androgen, 19norA$_4$ ($r = -0.99$, $p = 0.02$) and significantly correlated with A_4 ($r = 0.78$, $p = 0.04$) pre-ovulation. Both cortisol and A_4 were significantly correlated with testosterone both pre- and post-ovulation (Table 4).

3.3. Eye-up rate

The average eye-up rate for the low eye-up group (40.88 ± 5.0% eye-up ($n = 16$)) was significantly lower than the average eye-up rate for the high eye-up group, 82.66 ± 4.25% eye-up ($n = 5$, $p = 0.001$). The low number of fish in the high eye-up group is less than ideal to draw strong conclusions, but the data set was large enough to apply statistical analysis and provide foundational steroid information for reproductive fitness in female salmon.

Mucosal corticosterone was significantly higher in the low eye-up group (11.31 ± 7.39 ng/g mucus) than the high eye-up group (0.78 ± 0.3 ng/ g mucus, $p = 0.02$, Fig. 5A). Mucosal progesterone was only detectable in fish with a low eye-up rate (0.47 ± 0.1 ng / g mucus). However, the hydroxypregnanes and AP were detectable in both groups but, were only quantifiable in one sample (same fish) and detectable in the other samples but were under the LOQ in high eye-up fish. These single samples were not included in statistical analysis and were graphed without error bars as a result. The mucosal 5α-reduced metabolite 3β,20β-DHP was also detected in the low eye-up group (6.56 ± 1.69 ng / g mucus) and not detectable in the high eye-up group. Fish with high eye-up rates had significantly more circulating cortisol (239.66 ± 31.25 ng / mL plasma vs 181.50 ± 16.18 ng / mL plasma, Fig. 5B). There was also significantly more circulating testosterone in the high eye-up group (2.11 ± 0.4 ng / mL plasma) compared with the low eye-up group (1.05 ± 0.09 ng / mL plasma, $p = 0.04$). Plasma hydroxypregnanes (17OHP and 17,20OHP) were not significantly different in hormone concentrations between the two eye-up groups but AP was quantifiable in fish with a low eye-up rate (1.28 ± 0.23 ng / mL plasma) and not detectable in fish with a high eye- up rate. Two plasma androgens (A_4 and testosterone) were significantly correlated with eye-up rate, A_4 ($r = 0.46$, $p = 0.03$, Fig. 6A) and testosterone ($r = 0.46$, $p = 0.03$, Fig. 6B).

4. Discussion

The decrease in eye-up rates of North American Atlantic salmon over the last few years is concerning and needs to be addressed. To our knowledge this study represents the most comprehensive analysis of North American Atlantic salmon reproductive endocrine system and introduced a new matrix for the measurement of fish steroid hormones.

External mucus is continuously secreted by goblet cells in the

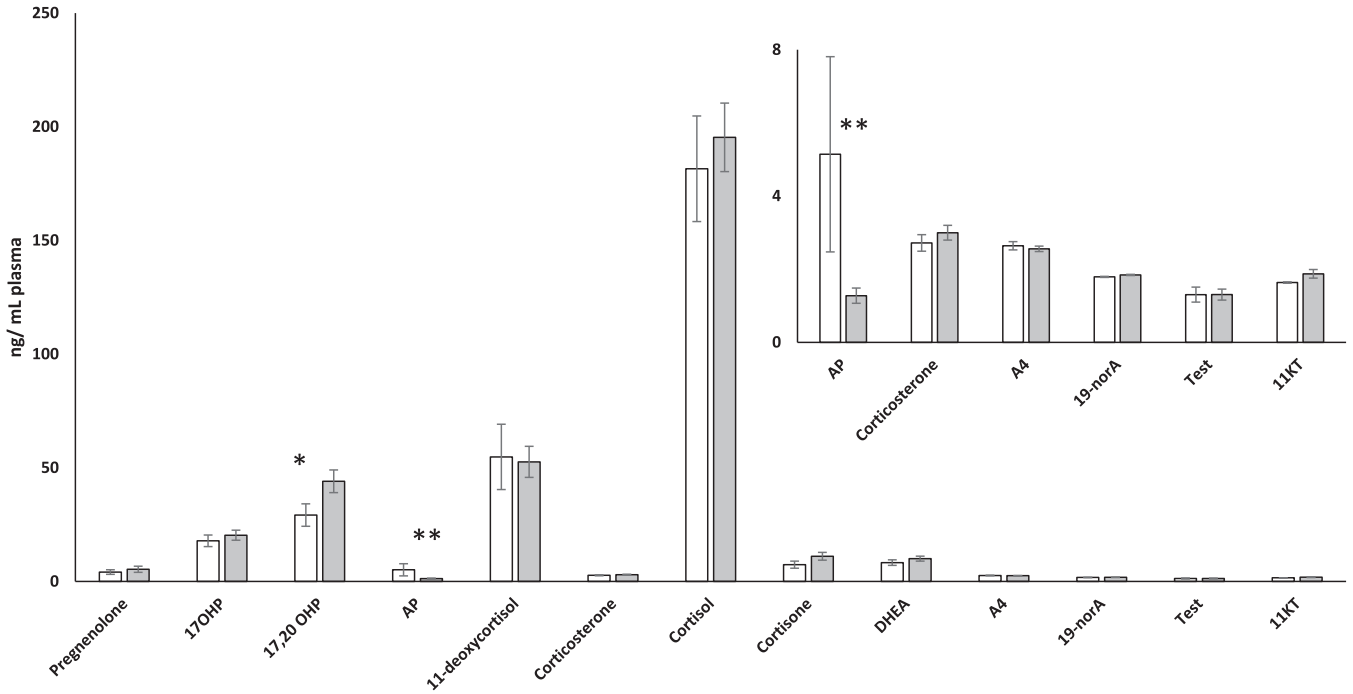

Fig. 4. Plasma steroid hormone profile of female Atlantic salmon pre ovulation (n = 9, white bars) and post ovulation (n = 20, gray bars). 17α,20β-hydroxyprogesterone (17,20 OHP) had significantly higher concentrations after ovulation and Allopregnanolone (AP) had significantly higher concentrations before ovulation. Inset is a more detailed view of steroid hormones with lower concentrations. **p ≤ 0.01, * p ≤ 0.05.

Table 4
Correlation matrix of plasma steroid hormones; Pregnenolone (P_5), 17α-hydroxyprogesterone (17OHP),17α, 20β-hydroxyprogesterone (17,20OHP), Allopregnanolone (AP), 11 deoxycortisol, Corticosterone, Cortisol, Cortisone, Androstenedione (A_4), 19norAndrostenedione (19norA$_4$), and Testosterone r values are presented below ** ≤ 0.01, * ≤ 0.05.

Variable	By variable	Before ovulation	After ovulation
P5	Corticosterone	0.43	0.73**
P5	Cortisone	0.45	0.74**
P5	DHEA	0.01	0.51*
17OHP	17,20OHP	0.55	0.51*
17OHP	A4	0.67	0.51*
17,20OHP	DHEA	0.67*	0.52*
17,20OHP	11KT	N/A	−0.98*
AP	Corticosterone	−0.87	−0.67*
AP	Cortisone	−0.81	−0.67*
11-deoxycortisol	Corticosterone	0.88**	0.56**
11-deoxycortisol	Cortisone	0.84**	0.48*
Cortisol	19norA4	0.83	−0.99*
Cortisol	A4	0.78*	0.34
Cortisol	Testosterone	0.71*	0.45*
A4	Testosterone	0.92**	0.87**

epidermis, easily collected, and can be a valuable source of information for the reproductive status of fish. Metabolites such as glucose, lactate, protein, and steroid hormones in skin mucus have already been studied to judge their suitability for determination of physiology (De Mercado et al., 2018; Fernandez-Alacid et al., 2018; Schultz et al., 2005). Steroid hormones measured in skin mucus have historically been studied using immunoassays but with the adaptation of LC-MS/MS methodology, 18 different steroid hormones were identified simultaneously in reproductively active salmon. While some compounds measured were significantly different depending on reproductive status or eye-up rate, mucus steroids measured did not correlate directly with circulating plasma steroids. This contradicts a study showing a direct relationship with circulating plasma and mucus cortisol (Carbajal et al., 2019); where cortisol was measured with an enzyme immunoassay and was reported to have a high % of cross reactivity with multiple steroid hormones that were found in the mucus, specifically cortisone (15.7%), 11-deoxycortisol (15%) and corticosterone (4.81%). This cross reactivity could account for discrepancy of direct correlation between this study and the one above, as using LC-MS/MS technology allows for accurate identification of steroids and removes potential cross reactivity. Additionally, the study above uses juvenile rainbow trout (*Oncorhynchus mykiss*) and it is very possible that fish at different physiological status have differing hormonal concentrations in both plasma and mucus which can change the correlation between steroid in these matrices.

Steroidogenic enzymes in the salmon skin may contribute to differences detected between circulating and mucosal steroid hormones. Extra-glandular steroidogenesis has been described in human skin (Hannen et al., 2011; Slominski et al., 2013) but to our knowledge, steroidogenic enzymes have not been reported in either fish skin or mucus. As the first line of defense, the skin mucus of fish contains numerous immune cells which protect from environmental pathogens and disease. Additionally, human immune cells have functional steroid hormone receptors which are capable of metabolizing hormones, (Chakraborty et al., 2021). Localized steroidogenesis in the skin or mucus could also account for the lack of correlation between circulating and mucosal hormones. However, mucosal concentrations of steroid hormone did correlate with circulating concentrations of related steroids. Circulating P_5 was correlated with mucosal glucocorticoids, pregnanes and androgens at both stages of ovulation. As P_5 is the initial precursor for all steroidogenesis this correlation suggests that P_5 could be transferred from the plasma circulation to support mucosal steroidogenesis. Mucosal steroid hormone patterns do reflect changes in reproductive physiology suggesting that mucus can be an appropriate matrix to non-lethally monitor ovulation through endocrine biomarkers.

Multiple measurable androgens were quantifiable in Atlantic salmon plasma and mucus samples, before and after ovulation. In plasma samples the androgens detected were DHEA, A_4, 19norA$_4$, testosterone and 11KT. The androgens measured in mucus were similar except that 11KT was not observed and both 11OxoA$_4$ and 5αDHT were observed.

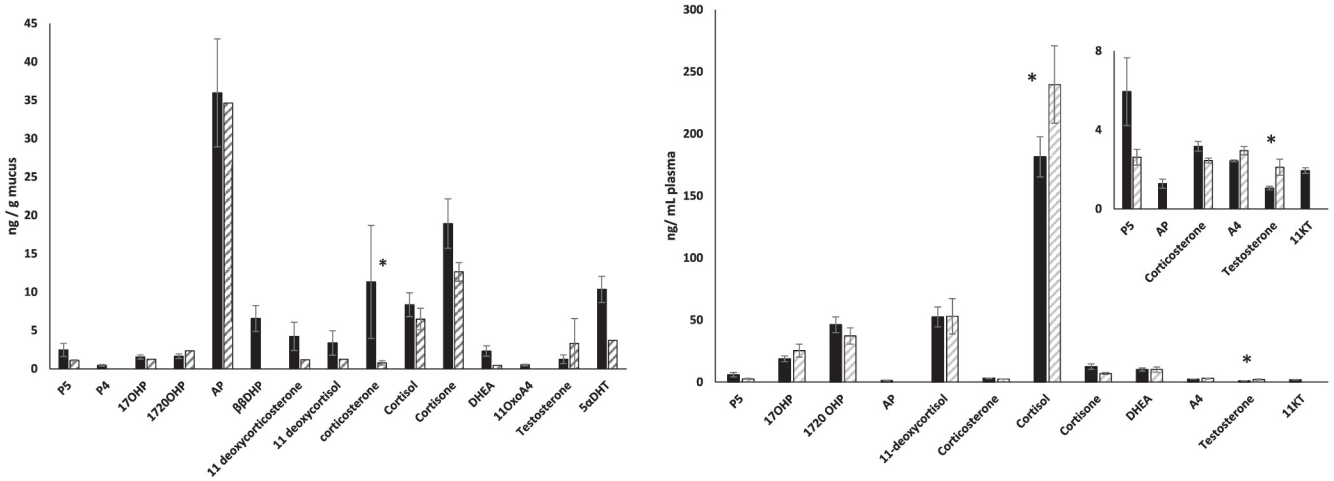

Fig. 5. A) Mucus steroid hormone profile of female Atlantic salmon with <70% eye-up ($n = 16$, black bars) and > 70% eye-up ($n = 4$, black ticked bars). Corticosterone was significantly higher in fish with <70% eye-up. B) Plasma steroid hormone profile of female Atlantic salmon with <70% eye-up (n = 16, black bars) and > 70% eye-up (n = 4, black ticked bars). Cortisol and testosterone were significantly higher in fish with a < 70% eye-up rate **p ≤ 0.01, * p ≤ 0.05.

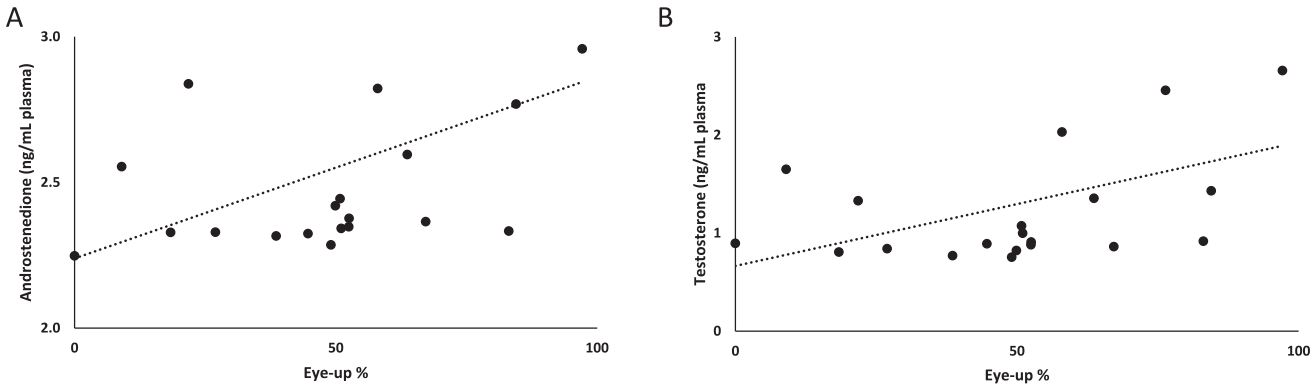

Fig. 6. A) There is a significant correlation between the eye-up % and two plasma androgens. A) Androstenedione (r2 = 0.46, $p = 0.03$) and B) Testosterone (r2 = 0.47, p = 0.03).

Androgens have been implicated in preparing the oocyte for ovulation in salmonoids. Land locked Atlantic salmon have a surge in plasma testosterone following the start of germinal vesicle migration (So et al., 1985) and cultured mature Atlantic salmon follicles treated with purified chum salmon gonadotropin produced both testosterone and A_4 which increased in concentration over time (Wright and Zhao, 1988; Zhao and Wright, 1985). Wright and Zhao (1988) suggest testosterone initiates germinal vesicle migration followed by 17,20OHP which induces germinal vesicle dispersion (Wright and Zhao, 1988). While plasma androgens were not significantly different between pre- and post-ovulation, mucosal androgens, testosterone and 5αDHT, were only detected post-ovulation. The 5αDHT concentrations measured in the mucus samples, and not the plasma, could also indicate a possibility that the enzyme responsible for 5αDHT production, 5α-reductase, may be present in Atlantic salmon skin. The 5α reduced testosterone, 5αDHT, has previously been measured in salmon bile (Truscott, 1983) but it has not been measured in salmon skin. However, incubation of trout skin with testosterone produced significant amounts of 5αDHT (Hay et al., 1976) and 5α-reductase has also been identified in human skin (Luuthe et al., 1994). Additionally, the concentrations of 5αDHT and testosterone were inversely correlated in the mucus post-ovulation, suggesting that metabolism of testosterone by skin 5α-reductase is quite rapid and causes a reduction in the testosterone substrate. Plasma androgen concentrations, testosterone and A_4 were positively correlated with eye-up rates. These results differ from findings in a previous study on the Northeast Atlantic salmon (Thayer and Hamlin, 2016) which did not detect a correlation between eye-up rates and testosterone but rather found a correlation between eye-up rates and 11KT. However, the 11KT was measured with an immunoassay which may have had cross reactivity with A_4 and other circulating androgens. However, our study supports the positive correlation between androgens and eye-up rate and introduces the need for more investigation into androgens and oocyte quality.

A surprising number of 5α-reduced pregnanes were observed in both plasma and mucus samples. Aa far as we are aware this is the first time 5α reduced pregnanes have been measured in Atlantic salmon. Mucus AP had the highest concentration of mucus pregnanes measured in any sample and was significantly greater post-ovulation compared to pre-ovulation. Conversely, plasma AP was elevated pre-ovulation similar to 5αDHP concentrations which were only measurable pre-ovulation. It is possible that 5α reductase activity increases in the mucus after ovulation and 5αDHP is metabolized directly into AP and therefore not measurable in the mucus. While 5α-reduced androgens (5αDHT) have been measured in circulating teleost plasma, 5αDHP or its metabolites have not been studied. The teleost brain exhibits 5α- reductase activity, (reviewed by (Diotel et al., 2011) and high levels of reductase activity have been measured in the goldfish, toadfish and zebrafish (Pasmanik and Callard, 1983, 1985, 1988; Pasmanik et al., 1988). In these studies, goldfish had levels of 5α-reductase equal to mammals and show increased pituitary activity at time of reproductive inactivity in both

male and female fish (Pasmanik and Callard, 1988). The function of 5α pregnanes in teleosts is not known and the significant changes in both mucus and plasma concentrations inform a need for future work.

The steroid hormone profile measured in plasma samples compared to mucus samples were anchored by three pregnanes; P_5, 17OHP and 17,20OHP. There was no circulating progesterone measured, but the role of P_4 in Atlantic salmon ovulation may be an intermediate in the biosynthesis of more active steroids, specifically 17OHP and 17,20OHP. In support of this hypothesis, the Atlantic salmon progesterone receptor has the highest affinity for 17,20OHP and very low affinity for P_4 (Chen et al., 2011) suggesting that P_4 has less bioactivity than other more important pregnanes. There were low levels of progesterone measured in the mucus which could be a result of plasma excretion into the mucus or may indicate that salmon skin is synthesizing progesterone as a precursor to other steroids. The enzyme responsible for progesterone production, 3β-hydroxy dehydrogenase (3βHSD) is in the skin of humans, and rodents but has not been identified in fish skin, [reviewed by (Simard et al., 2005)]. Concentrations of hydroxylated P_4, 17OHP, were not significantly different between stages in either plasma or mucus matrices. However, it could be that the function of 17OHP as in other teleosts, is to regulate 17,20OHP production by upregulating steroidogenic enzymes responsible for 17,20OHP production (Hasegawa et al., 2022). Of these three pregnanes in the plasma profile, 17,20OHP had the highest concentration in both the pre-ovulation and post-ovulation stages, followed by 17OHP and pregnenolone. These concentrations agree with previous publications which observed that 17,20OHP was the highest circulating steroid hormone in teleost fish during late oocyte maturity and ovulation [reviewed by (El Mohajer et al., 2022; Nagahama, 1997; Nagahama and Yamashita, 2008)]. The hormone 17,20OHP is commonly called the maturation-inducing hormone (MIH) and is responsible for oocyte maturation and the initial induction of ovulation (Knight and Van der Kraak, 2015; Tokumoto et al., 2011). Specifically, 17,20OHP is thought to be a follicular mediator of gonadotropin released from the pituitary and responsible for germinal vesicle breakdown (Sangalang and Freeman, 1988). Both circulating 17,20OHP and mucosal 17,20OHP were correlated with mucus corticosterone and mucus cortisone suggesting, again, interactions between glucocorticoids and pregnanes during ovulation and germinal vesical breakdown. The preovulatory follicle has the ability to synthesis 17,20OHP but it has also been shown that the interrenal tissues can also synthesis 17,20OHP (Sangalang and Freeman, 1988). Combined with the observed glucocorticoid correlations, the possibility of interrenal gland synthesis of steroid hormones responsible for ovulation and possible quality oocytes is likely. 5αDHT was significantly correlated with 17,20OHP and cortisone, both were elevated after ovulation similar to land-locked Atlantic salmon which had measurable levels of circulating 17,20OHP and testosterone through the final stages of oocyte maturation and both hormones, along with 5αDHT effected oocyte maturity (So et al., 1985).

Of the glucocorticoids that were measured all were observed in plasma samples except for 11-deoxycorticosterone. No significant differences were seen among any of the circulating glucocorticoid compounds measured either before or after ovulation in plasma. However, there was a significant increase in two mucosal glucocorticoids, 11 deoxycorticosterone and 11 deoxycortisol, precursors to corticosterone and cortisol respectively. Cortisol had the highest concentration of all circulating glucocorticoids measured which correlates with many reports on cortisol concentrations in Atlantic salmon and many other teleost species [reviewed by (Eriksen et al., 2006; Mommsen et al., 1999)]. The differences seen between glucocorticoid levels in plasma and mucus could be due to the sensitivity of glucocorticoids to stressful stimuli. Glucocorticoids increase within minutes after a stressful stimulus including being removed from a tank and placed in a small tub for spawning (Lai et al., 2021). The dramatic increase in glucocorticoids could mask any changes that occur during ovulation. The measured increase in glucocorticoids at the time of ovulation correlates with studies on other teleosts. For example, in catfish, a combination of luteinizing hormone (LH) and 11 deoxycorticosterone were potent inducers of ovulation (Sundararaj and Goswami, 1969) and in vivo human chorionic gonadotropin (hCG) injections induced oocyte maturation along with plasma cortisol and corticosterone increases (Mishra and Joy, 2006). In carp, circulating cortisol was stimulated along with 17,20OHP during induced ovulation with pituitary extract (Kime and Dolben, 1985). Ovulation can also be induced by treatments with corticosteroids (cortisol,11-deoxycortisol and DOC) in Ayu (Hirose and Ishida, 1974), yellow perch (Goetz and Theofan, 1979)), rainbow trout (Milla et al., 2006) and Eurasian perch (El Mohajer et al., 2021). It is possible that corticosteroids may play a larger role in Atlantic salmon ovulation than previously thought giving glucocorticoids an additional physiological role.

Glucocorticoids may also play a role in the embryo development of Atlantic salmon. Circulating cortisol concentrations were significantly higher in the high eye-up group when compared with those in the low eye-up group. In contrast, mucus concentrations of corticosterone were significantly higher in the low eye up group compared with the high eye-up group. While these compounds are both glucocorticoids they are metabolized through different pathways; corticosterone being derived directly from progesterone via 11 deoxycorticosterone and cortisol derived from the 17α hydroxyprogesterone pathway. A second pathway for the biosynthesis of cortisol was described in the European eel, and focused on the bypassing of progesterone through 17α-hydroxypregnenolone, to 17α-hydroxyprogesterone and then to cortisol (Sandor, 1969). This pathway is identical to the synthesis of cortisol in the mammalian adrenal gland (Payne and Hales, 2004). As no progesterone was detected in the plasma of the sampled Atlantic salmon it is possible that this alternative pathway is utilized at the time of ovulation (Refer to Fig. 1 for clarification). It may be possible that the expression of key enzymes in glucocorticoid pathways during ovulation may shift to support quality oocytes with elevated cortisol levels. Cortisol induces oocyte hydration in vitro in rainbow trout (Milla et al., 2006) and injections of high doses of cortisol promoted ovarian tissue hydration associated with an increase of sodium content in ayu (*Plecoglossus altivelis*) (Hirose and Ishida, 1974). Cortisol seems to play a crucial role in the hydration and ovulation of mature follicles in a majority of fish [reviewed by (Faught and Vijayan, 2018)]. Regulation of glucocorticoids during oogenesis may be essential for quality oocyte development and further investigation into the role glucocorticoids play in the production of quality Atlantic salmon oocytes may provide a critical biomarker for broodstock.

Reproductive studies in fish are fundamental for the ever-growing aquaculture industry by improving broodstock programs through increased egg production and enhanced viability of those eggs. By defining the endocrine pathways which support the production of oocyte development, a map to understanding natural steroidal cycles to identify disruption becomes more evident. Changes in both mucosal and plasma steroid hormones support how dynamic the endocrine system is during ovulation in Atlantic salmon, a vital time point for any broodstock program. This study validates the use of skin mucus for the measurement of steroid hormones with LC-MS/MS. This study has also detailed the most comprehensive analysis of steroid hormones during spawning in North American Atlantic salmon as well as detecting significant differences in some glucocorticoid, pregnane and androgen concentrations at different time point during spawning. While more work must be done on how these endocrine fluctuations affect the quality of oocytes and their ability to develop, there is now a more detailed pathway to support the study of reproductive endocrinology in Atlantic salmon aquaculture.

NIST disclaimer

Certain commercial equipment, instruments, or materials are identified in this paper in order to specify the experimental procedure adequately. Such identification is not intended to imply

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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