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Determination of cortisol in lake sturgeon (*Acipenser fulvescens*) eggs by liquid chromatography tandem mass spectrometry

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ABSTRACT

Quantifying cortisol concentrations in fish eggs is important to understand the effects of environmental conditions on maternal physiological condition and on egg provisioning and quality. Data are particularly relevant to studies of the ecology of threatened species such as lake sturgeon (*Aciperser fulvescens*) as well as assessments of larval physical and behavioral phenotypes, fish health and caviar quality in sturgeon aquaculture. This study focuses on development of bioanalytical methods for high sensitivity and robust determination of cortisol in sturgeon eggs. Sample preparation was optimized after investigating protein precipitation and liquid-liquid extraction techniques. Ethyl acetate was found to be the most efficient solvent (recovery parameter) and also provided the best sample clean up (matrix effect parameter). The method was determined to be linear for cortisol concentrations between 0.025 and 100 ng/mL. The limits of detection and quantification were 0.025 and 0.1 ng/mL respectively. Intra- and inter-day performances of the method were validated at three concentrations (0.25; 10 and 100 ng/mL). The method was applied to field-collected samples for the determination of endogenous cortisol in lake sturgeon eggs. Cortisol was detected in all egg samples and statistical analysis showed significant differences between fertilized and non-fertilized eggs.

1. Introduction

Female fish provisioning takes place for species with no post ovulatory parental care. Essential products are provisioned in the egg (mRNAs, lipids, cortisol, etc.) and support offspring development. The elucidation of mechanisms involved in fish provisioning is essential to fish management in aquaculture as well as for environmental protection plans. In particular, high levels of cortisol may drastically impact development during early life stages. As highlighted by Sopinka et al., links between egg composition, maternal stress and offspring quality need to be established [1]. Environmental stressors experienced by females influence physiological and endocrinological processes associated with egg provisioning, with implications for offspring phenotype, survival, and population-level recruitment [2].

Although the link between offspring quality and maternal parameters (stress or egg composition) could be different among fish species, the lake sturgeon (*Acipenser fulvescens*) represents a challenging model. First, the lake sturgeon is a regionally-listed threatened species and a priority for conservation and management in Canada and the United States [3]. Significant efforts are being made to repopulate North American fresh waters with lake sturgeon. Second, sturgeon take over 20 years to attain reproductive maturity [4], making their populations especially vulnerable to rapid environmental change and other stressors. Repopulation strategies involve collecting eggs and sperm from spawning adults, rearing larvae in hatcheries, and releasing hatchery-produced juveniles to bolster local populations. Thus, a better understanding of sturgeon physiology will ultimately lead to improvements in hatchery techniques and management efforts. Elevated maternal cortisol stimulated by environmental perturbation has been shown to be reflected in offspring cortisol levels, which affects the ability of offspring to survive stressful environmental conditions through triggering physical and behavioral phenotypes suited to high-risk environments [5]. Fish species are specifically impacted by maternal stress via egg provisioning. In salmon and sticklebacks, females subjected to chronic stress during oogenesis produced eggs with significantly higher egg cortisol concentrations [6,7]. Stress in fishes, associated with increased maternal cortisol levels, is known

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to alter reproductive timing and has negative effects on fecundity, egg size, gamete quality, and offspring survival [8–10]. For example, in coho salmon, experimentally elevated egg cortisol has been shown to increase offspring boldness under predation threat [11], suggesting that egg cortisol concentrations can have downstream ecologically relevant effects on offspring behavior and survival [12].

Measuring cortisol concentrations in lake sturgeon eggs could therefore indirectly estimate the levels of chronic stress the parental female has experienced, enabling a new method to infer effects of environmental and ecological factors on this threatened species. Since sturgeon are large and long-lived, administering chronic stress treatments to adults in captivity is generally not feasible, and observing transgenerational effects of stress is difficult. Therefore, collecting eggs from spawning females for cortisol analysis is therefore a more convenient method for quantifying female stress.

The understanding of sturgeon physiology is also receiving strong interest in the aquaculture industry [13]. In fact, assessments of sturgeon stress are needed to examine the health of cultured sturgeon and to ensure quality of eggs (caviar) produced. Caviar quality is influenced by the aquaculture techniques and environmental conditions experienced by female sturgeon [14]. Plasma cortisol levels have been used to evaluate welfare of farmed sturgeon [15], but egg cortisol concentrations have not been previously examined as a way to indicate maternal stress in cultured sturgeon. Using harvested eggs to assess chronic stress levels of cultured sturgeon simplifies the process by eliminating the need for blood samples from adults, whose plasma cortisol levels vary with time of day and handling technique [16]. This method also extends to understanding the effect of aquaculture conditions not only on females, but also on how stress is transmitted to the eggs. Therefore, a method for assessing how conditions affect female sturgeon stress levels and how these effects influence eggs is not only relevant to support quality assessment in the caviar industry but also to study how environmental and ecological factors influence stress levels of adults, and downstream impacts on eggs and offspring.

Cortisol has been quantified in fish eggs using radio immunoassay (RIA) or enzyme linked immunoassay (ELISA). Tintos et al. reported the use of ELISA for the determination of cortisol in teleost fish reporting a limit of quantification of 0.3 ng/mL [17]. Simontacchi et al. reported the use of RIA for the analysis of cortisol in sturgeon eggs and larvae [18]. Although LC–MSMS techniques have been reported for the analysis of cortisol in human urine or saliva [19,20], there is no method for the analysis of cortisol in sturgeon eggs. In this study, liquid-liquid extraction and protein precipitation sample preparation techniques were tested for an optimal extraction of cortisol from a high lipid matrix (sturgeon eggs). Ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MSMS) was then implemented to allow the high sensitivity determination of cortisol. Finally, the method was thoroughly validated and applied to determine cortisol levels in sturgeon eggs.

2. Material and method

2.1. Chemicals

Acetonitrile (ACN), ammonium acetate, cortisol, cortisol-d_4 (Fig. 1), diethyl ether (Et_2O), methyl *tert*-butylether (MTBE), ethyl acetate (AcOEt) and formic acid were purchased from Sigma-aldrich (Sigma-Alrich MO, USA).

2.2. Sample preparation

Method development and validation experiments were carried out on lake sturgeon eggs homogenate (1 g of eggs per mL of phosphate buffer; 50 mM saline) to minimized matrix contribution to inter-sample variations. Due to the large number of samples to process, solid phase extraction (SPE) methods [21,22] were not considered and only protein precipitation (PPT) [19] and liquid-liquid extraction (LLE) [20] were evaluated in this study. Extraction methods were evaluated for cortisol-d_4 to eliminate the error due to the contribution of endogenous cortisol. Each data point was made of five replicates spiked at 5 ng/mL. Recovery and matrix effects were used to evaluate the extraction and clean-up performances respectively. Cortisol-d_4 (10 ng) was spiked in 0.5 mL of sturgeon eggs homogenate and vortexed.

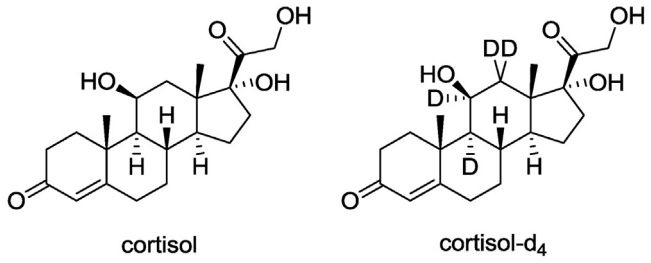

Fig. 1. Chemical structures of cortisol and cortisol-d_4.

For protein precipitation, ice cold acetonitrile (1 mL) was added to each sample, vortexed and shaken for 10 min. After an incubation at 4 °C for 20 min, samples were centrifuged (9000*g*, 10 min, 4 °C), and the supernatant was withdrawn and freeze-dried. Residues were reconstituted in 1 mL of a methanol:water (1:1) solution, centrifuged, transferred to an autosampler vial and stored at −18 °C until LC–MSMS analysis.

For liquid-liquid extraction, three solvents were evaluated (MTBE, AcOEt and Et_2O). Samples were vigorously mixed with 1.4 mL of solvent and shaken for 10 min. Samples were then centrifuged (9000*g*, 10 min, 4 °C) and frozen at −18 °C. Once the aqueous and fat layers were frozen, the organic layer was withdrawn and transferred to a new tube to be evaporated under vacuum. Samples extracted with Et_2O were dried at 40° Celsius under streams of nitrogen. Residues were reconstituted in1 mL of a methanol:water (1:1) solution, centrifuged, transferred to an autosampler vial and stored at −18 °C until LC–MSMS analysis.

2.3. Liquid chromatography tandem mass spectrometry

Cortisol concentrations were determined by the use of a Waters Xevo TQ-S mass spectrometer (Waters, Millford, MA, USA). Chromatographic separation was achieved by an H-Class UPLC system with a Waters BEH C18 column (2.1 × 100 mm, 1.7 μm particle size). Oven temperature was regulated at 30 °C. The solvents A and B were water (10 mM ammonium acetate) and acetonitrile, respectively. Flow rate was kept constant at 0.17 mL/min and the following gradient was applied (time in min; % of A): (initial; 82), (2; 60), (3; 50), (6; 10), (6.1; 1), (7; 1), (7.01; 82), (10; 82). The parameters of electrospray in the positive ion mode were: capillary voltage 3 kV, desolvation gas flow 800 L/h, cone gas flow 150 L/h, source temperature 150 °C and desolvation temperature 600 °C. Cone voltage and collision energies were optimized with Quantoptimize software from Waters. The optimum MSMS conditions including, cone voltages, collision energies, parent ions and daughter ions are summarized. Data were acquired with MassLynx 4.1 and processed for calibration and quantification of the analyses with TargetLynx software from Waters.

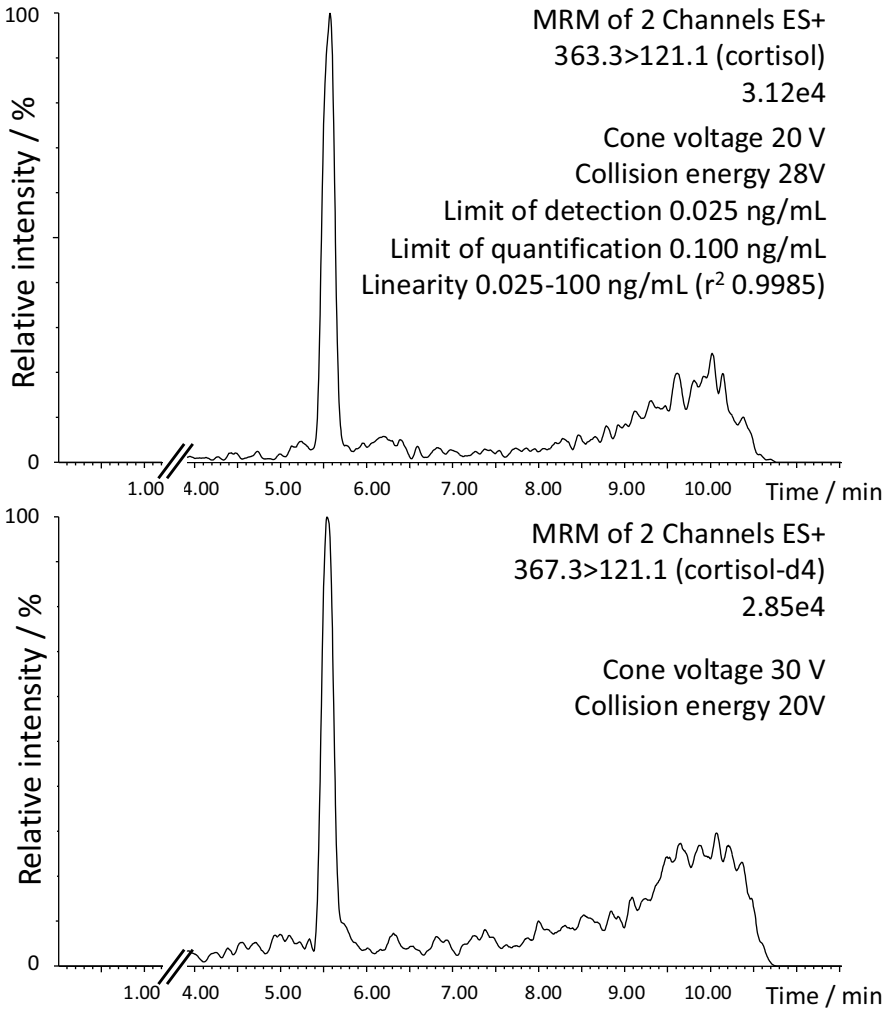

Fig. 2. Ion chromatogram of endogenous cortisol (top) and cortisol-d_4 (bottom) in lake sturgeon egg. Cortisol was estimated to 200 pg/g and cortisol-d_4 spiked at 10 ng/mL.

2.4. Method validation

Matrix effect and recovery were determined with five replicates at 10 ng/mL (Cortisol-d_4) in sturgeon eggs. Accuracy and precision parameters were evaluated at three concentrations as advised by the FDA guidance for bioanalytical method development [23]. Matrix effect were expressed by the ratio of the signal area in eggs spiked post extraction matrix subtracted with the area and blank eggs on the signal area in standard solution [24]. Matrix effect inferior or superior to 100% will indicate signal suppression or enhancement respectively. Recovery was expressed by the ratio of the signal area in eggs spiked pre extraction on the signal area in eggs spiked post extraction. Matrix effect and recovery parameters do not necessarily need to be close to 100%, but values determined for the target (cortisol) and the internal standard (cortisol-d_4) must be close [25].

The limit of detection (LOD) was determined by a signal to noise ratio (S/N) superior or equal to three and the limit of quantification (LOQ) for an S/N superior or equal to ten. Method linearity range was estimated using the calibration curve built by the plot of the relative standard signal area versus internal standard signal in function of the spiked concentration of standard. The $1/\chi$ least square regression of the calibration curve assessment method of linearity is represented by the coefficient of correlation (r^2).

The concentrations chosen for quality control were set according to FDA guidance for bioanalytical validation [23]. The low quality control (LQC; 0.25 ng/mL) does not exceed three times the LLOQ, the medium quality control (MQC; 10 ng/mL) is around the middle of the linear range and the high quality control (HQC; 100 ng/mL) is at the highest concentration of the method linearity range.

Intra-day accuracy and precision were obtained by the average of 15 replicates per concentrations while inter-day parameters were determined by the comparison of five replicates per concentrations over five consecutive days. Concentrations in the blank sample were subtracted from the QC samples and used to calculate accuracy and precision parameters. Precision was determined by the standard deviation within replicates while accuracy was assessed by the ratio of the determined concentration to the spiked concentration. Accuracy must be within the range of 85–115% for MQC and HQC while 80–120% range is tolerated for LQC. Precision must be lower than 15% for MQC and HQC while a limit of 20% is set for LQC.

2.5. Sample collection

Eggs and sperm were collected from lake sturgeon spawning in the Upper Black River in Onaway, Michigan, USA (IACUC SOP 03/14-039-99). From four females, samples of unfertilized eggs were stored after snap-freezing in liquid nitrogen. Additional eggs from these same four females were fertilized using sperm from two males (IACUC SOP 03/14-037-99), and incubated at 18 °C. Samples

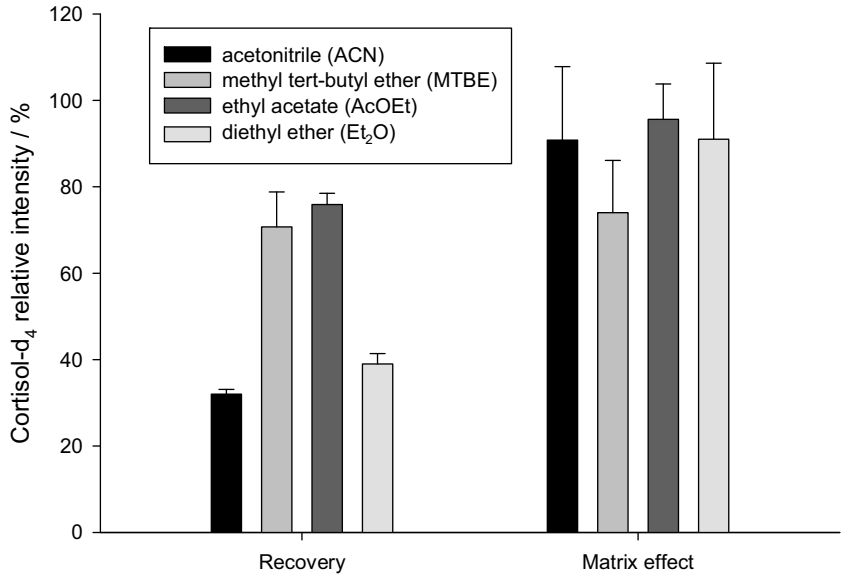

Fig. 3. Matrix effect and recovery parameters comparison for cortisol extraction from sturgeon egg matrix by protein precipitation (ACN) or liquid-liquid extraction (MTBE, AcOEt and Et_2O).

Table 1
Monitoring of cortisol and cortisol-d_4 stability over five consecutive days. Signal areas were normalized to the highest signal area over the five days.

	Day 1	Day 2	Day 3	Day 4	Day 5
cortisol	(100.0 ± 7.3)	(90.9 ± 6.8)	(97.5 ± 6.1)	(90.6 ± 4.6)	(90.7 ± 5.3)
cortisol-d4	(94.1 ± 5.5)	(87.9 ± 12.4)	(100.0 ± 5.1)	(95.6 ± 4.8)	(96.8 ± 9.7)

of these fertilized eggs were taken halfway through the incubation period, past the neurulation stage, at 36 Cumulative Temperature Units (CTUs) calculated to be 9 days post-fertilization and preserved by snap-freezing in liquid nitrogen (IACUC 05/16-056-00). The sample set included three samples of unfertilized eggs from each of the four females and six samples of fertilized eggs from each of these females at 10 °C.

3. Results and discussion

3.1. Liquid chromatography tandem mass spectrometry

UPLC–MSMS has become the method of choice for the analysis of steroids in biological matrices [26–28]. Although often reported for sensitivity enhancement, chemical derivatization is not mandatory for upstream liquid chromatography separation whereas gas chromatography does require chemical derivatization [29–34]. Electrospray (ESI) is presently the most used ionization technique for the LC–MS analysis of steroids [35]. Nevertheless, alternatives such as atmospheric pressure chemical ionization [36] and photo ionization [37] (APCI and APPI) are available for the ionization of steroids but show low response to ESI. Ionization and tandem mass spectrometry parameters were individually optimized for cortisol and cortisol-d_4. Using the optimized transitions 363 > 121 (cortisol) and 367 > 121 (cortisol-d_4) limits of detection and quantification of 0.025 and 0.1 ng/mL respectively were achieved in standard solution. Similar MSMS transitions have been reported in the literature. For example, Jonsson et al. reported LOQ of 0.5 ng/mL [19] while Antonelli et al. reported LOD of 0.07 ng/mL [38]. Despite the reported sensitivity (0.3 ng/mL in teleost fish plasma), ELISA determination of cortisol involves extensive sample preparation and therefore is ill-suited to use for processing large numbers of samples [17]. The sensitivity determined for our assay is therefore competitive to existing methods and is shown to be effective for the determination of cortisol in sturgeon eggs. Therefore, only ESI was evaluated and used.

Liquid chromatography separation of steroids has been widely reported in the literature and is usually achieved by reversed phase chromatography [27]. Several column dimensions (1.0 × 50; 2.1 × 100 and 1.0 × 50 mm BEH C18 particle size 1.7 μm) and solvent additives (formic acid, ammonium acetate or both) were evaluated. The Waters BEH C18 2.1 × 100 mm column and a gradient between water (ammonium acetate 10 mM) and acetonitrile were found to give the sharpest peak and higher signal to noise ratio (data not shown) and was therefore selected for the analysis of cortisol in sturgeon eggs (Fig. 2).

3.2. Sample preparation

Extraction of cortisol from eggs has been reported in the literature. Early methods dealing with RIA determination of cortisol in teleost eggs reported the liquid-liquid extraction using ethyl ether [39]. For example, Jesus et al. used ethyl ether to extract egg homogenates in PBS [40]. This method was also applied on gilthead sea bream, *Sparus aurata* for the determination of cortisol in larvae [41]. Mingist et al. preferred CCl_4 to optimize removal of lipids, which are known to interfere with RIA [8] while Auperin et al. used ethyl acetate and cyclohexane to achieve the LLE of cortisol from juvenile rainbow trout (*Oncorhynchus mykiss) plasma* [42]. Zubair et al. implemented SPE upstream of RIA [43]. In this study, we evaluated protein precipitation and liquid-liquid extraction techniques. Fig. 3 shows the comparison of matrix effect and recovery parameters using different solvent or protein precipitation. Acetonitrile resulted in the lowest recovery (32%) and the

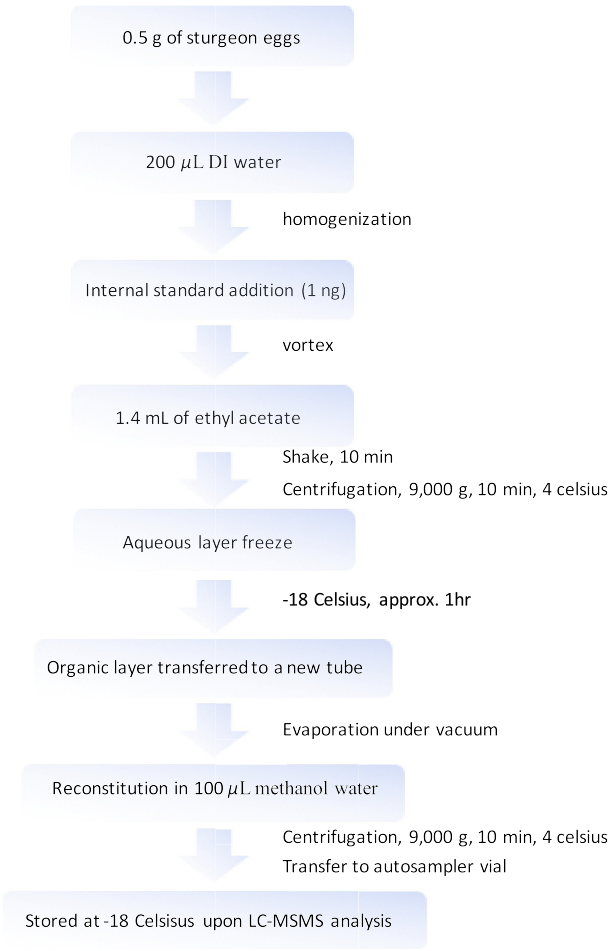

Fig. 4. Sample preparation procedure.

extracted samples ended with a milky texture confirming the high lipid content. Although acetonitrile has been used for cortisol sample preparation (PPT), but the utility has only been examined on samples of saliva which contains a limited amount of lipids [19]. High lipid contents of the sample is likely to lead to chromatography column and MS source pollution if larger numbers of samples have to be run. Three solvents were evaluated for LLE, namely diethyl ether, MTBE and AcOEt. MTBE and AcOEt have been shown to provide the best recoveries, respectively 71 and 76% (see Fig. 3), while Et_2O has been shown to provide relatively low recovery when compared to the other solvents. With respect to sample clean up, matrix effect determination was used to compare and select the optimal LLE solvent. The extraction of cortisol using MTBE led to an ion suppression phenomena of 26% (matrix effect of 74%). Therefore, AcOEt was selected for further method development as it has shown satisfactory matrix effect (96%) and a higher recovery (Table 1).

3.3. Method validation

Matrix effect and recovery parameters were validated at 10 ng/mL in sturgeon egg homogenates. Recovery was determined at (76.2 ± 1.5) % and (75.9 ± 2.6) % for cortisol and cortisol-d_4 respectively. These values are in the order of magnitude of LLE of steroids [35]. The similarity between the recovery of cortisol and the internal standard suggests that cortisol-d_4 is an appropriate reference for cortisol extraction and therefore a relevant model for the optimization of cortisol extraction (Fig. 3). It should also be noted that no interferences were observed between cortisol and cortisol-d_4 tandem mass spectrometry signals. Matrix effect parameters were determined at (95.6 ± 8.2) % and (93.4 ± 7.1) % for cortisol and cortisol-d_4 respectively. These values suggest a slight ion suppression phenomenon but no significant difference to standard solution (normalized at 100%). Following the example of recovery, matrix effect values were determined to be similar for cortisol and cortisol-d_4 indicating that cortisol-d_4 is an effective internal standard for the determination of cortisol by liquid chromatography tandem mass spectrometry (Fig. 4).

Stability was assessed over five consecutive days (samples stored at 4° Celsius away from light) and the signal of cortisol decreased by about 10%. Signal intensity was normalized to the highest signal over five days. No significant differences were observed between cortisol and cortisol-d_4 change throughout day by day monitoring. Retention time inter- and intraday was also monitored retention times were (5.44 ± 0.01; mean ± standard deviation) min and (5.43 ± 0.01) min for cortisol and cortisol-d_4 intraday (n = 15). Retention times were (5.44 ± 0.02) min and (5.43 ± 0.01) min for cortisol and cortisol-d_4 interday (five days; n = 25).

Accuracy and precision were validated over five consecutive days (n = 15, intraday; n = 25, interday; Table 2). Intra- and interday precision showed conform values (<2.1%) for MQC and HQC. LQC precision was measured at higher values of 3.7 and 5.5% for intra- and interday, respectively. However, these values stayed far below the limit of 20% set by the FDA guidance for bioanalytical method development. The accuracy parameter was also determined within the allowed range. Intraday accuracy was 91.3 and 93.2% while interday accuracy was 95.0 and 90.6% for MQC and HQC, respectively, which is in compliance with the allowed range between 85 and 115%. LQC accuracy was evaluated at 105.3 and 111.9% for intra and interday respectively, in compliance with the allowed range of 80 to 120%.

3.4. Application

Applicability of the validated method was further determined through measurement of endogenous cortisol in sturgeon egg and embryo samples. Samples were divided into two groups (fertilized and non-fertilized eggs). Cortisol was measured at 39.2 ± 11.7 pg/g (mean ± standard deviation; n = 24) in fertilized eggs while non-fertilized eggs were determined to have a cortisol concentration of 543.4 ± 194.4 pg/g (mean ± standard deviation; n = 12). Statistical analysis (Student *t*-test) showed the two groups differed significantly in cortisol levels ($p < 0.01$). These results demonstrate that

Table 2
Intra- and interday accuracy and precision parameters for the determination of cortisol in spiked sturgeon eggs homogenate.

		spiked (ng/mL)	measured (ng/mL)	accuracy (%)	precision (%)
Intraday	LQC	0.25	(0.26 ± 0.01)	105.3	3.7
	MQC	10	(9.13 ± 0.08)	91.3	0.8
	HQC	100	(93.17 ± 0.53)	93.2	0.5
Interday	LQC	0.25	(0.28 ± 0.01)	111.9	5.5
	MQC	10	(9.50 ± 2.11)	95.0	2.1
	HQC	100	(90.56 ± 0.75)	90.6	0.8

the method is sensitive and reliable to determine cortisol in sturgeon eggs.

Yolk size still comprised a large proportion of the egg for the fertilized eggs as well as for the unfertilized eggs. Therefore, yolk size and associated lipid levels were similar in both groups and unlikely to be a major contributor to the differences in cortisol levels between fertilized and unfertilized eggs. Moreover, these results are consistent with observations on egg cortisol in several other fish species. Paitz et al. found a significant difference in cortisol levels between stickleback eggs at the unfertilized stage and three days post fertilization [44]. Cortisol has also been shown to decline soon after fertilization in zebrafish [45], rainbow trout [46], and other teleosts [39].

4. Conclusion

A sensitive, selective and robust method has been developed and optimized to determine cortisol concentrations in lake sturgeon eggs and embryos. Ethyl acetate was shown to be the best extraction method in terms of recovery and matrix effect, and therefore selected for further method development. The matrix effect, recovery, stability, retention time repeatability, interferences, accuracy and precision (intra- and interday) demonstrate that the method performances satisfy criteria set forth by FDA. This method will be useful for studying sturgeon physiology, development, aquaculture and conservation.

Conflict of interest

Authors declare no conflict of interest.

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