Journal of Visualized Experiments

Cortisol Extraction from Sturgeon Fin and Jawbone Matrices

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59961R2
Full Title:	Cortisol Extraction from Sturgeon Fin and Jawbone Matrices
Keywords:	ELISA assay; Fin cortisol; Jawbone cortisol; Stress; Sturgeon; Washing solvents
Corresponding Author:	Jalil Ghassemi Nejad Konkuk University Seoul, KOREA, REPUBLIC OF
Corresponding Author's Institution:	Konkuk University
Corresponding Author E-Mail:	jalilgh@konkuk.ac.kr
Order of Authors:	Jalil Ghassemi Nejad
	Mohammad Ataallahi
	Mohammad Hassan Salmanzadeh
	Kyung-Taek Park
	Hong-Gu Lee
	Alireza Shoae
	Atieh Rahimi
	Kyung-Il Sung
	Kyu-Hyun Park
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Chuncheon, Gangwon, South Korea

TITLE:

Cortisol Extraction from Sturgeon Fin and Jawbone Matrices

AUTHORS & AFFILIATIONS:

Jalil Ghassemi Nejad [1,2], Mohammad Ataallahi[1], Mohammad H Salmanzadeh[3], Kyung T Park[3], Hong G Lee[2,4], Alireza Shoae[5], Atieh Rahimi[6], Kyung I Sung[1, *] and Kyu H Park[1, *]

Jalil Ghassemi Nejad: jalilgh@konkuk.ac.kr
Mohammad Ataallahi: ataallahim@kangwon.ac.kr
Mohammad H Salmanzadeh: mh_salmanzadeh@yahoo.com
Kyung-Taek Park: kyungtaekster@gmail.com
Hong-Gu Lee: hglee66@konkuk.ac.kr
Alireza Shoae: ashoae@yahoo.com
Atieh Rahimi: atiehrahimi.um@gmail.com
Kyung-Il Sung: kisung@kangwon.ac.kr
Kyu-Hyun Park: kpark74@kangwon.ac.kr

[1]College of Animal Life Sciences, Kangwon National University, Chuncheon, Gangwon, Republic of Korea
[2]Team of An Educational Program for Specialists in Global Animal Science, Brain Korea 21 Plus Project, Konkuk University, Seoul, Republic of Korea
[3]Persian Gesture, No.1/167, 3rd Majd, Sanabad-Mashad, Khorasan Razavi, Iran
[4]Department of Animal Science and Technology, College of Animal Bioscience and Technology, Konkuk University, Seoul, Republic of Korea
[5]Department of Animal Science, Animal physiology, AgResearch, Hamilton, New Zealand
[6]Faculty of Agriculture, Animal Science Department, Ferdowsi University of Mashhad, Mashhad, Iran

CORRESPONDING AUTHOR:

Kyu H Park
kpark74@kangwon.ac.kr

Kyung l Sung
kisung@kangwon.ac.kr

KEYWORDS:

ELISA assay, Fin cortisol, Jawbone cortisol, Stress, Sturgeon, Washing solvents

SUMMARY:

In this study, we present a protocol for cortisol extraction from the fin and jawbone of sturgeon species. Fin and jawbone cortisol levels were further examined by comparing two washing solvents followed by ELISA assays. This study piloted the feasibility of jawbone cortisol as a novel stress indicator.

ABSTRACT:
The aims of this study were to develop a technique for the extraction of cortisol from sturgeon fins using two washing solvents (water and isopropanol) and quantify any differences in fin cortisol levels among three main sturgeon species. Fins were harvested from 19 sacrificed sturgeons including seven beluga (*Huso huso*), seven Siberian (*Acipenser baerii*), and five sevruga (*A. stellatus*). The sturgeons were raised in Iranian farms for 2 years (2017–2018), and cortisol extraction analysis was conducted in South Korea (January–February 2019). Jawbones from five *H. huso* were also used for cortisol extraction. Data were analyzed using the general linear model (GLM) procedure in the SAS environment. The intra- and inter-assay coefficients of variation were 14.15 and 7.70, respectively. Briefly, the cortisol extraction technique involved washing the samples (300 ± 10 mg) with 3 mL of solvent (ultrapure water and isopropanol) twice, rotation at 1,800 rpm for 2.5 min, air-drying the washed samples at room temperature (22–28 °C) for 7 days, further drying the samples using a bead beater at 50 Hz for 32 min and grinding them into powder, applying 1.5 mL methanol to the dried powder (75 ± 5 mg), and slow rotation (40 rpm) for 18 h at room temperature with continuous mixing. Following extraction, samples were centrifuged (9,500 x *g* for 10 min), and 1 mL supernatant was transferred into a new microcentrifuge tube (1.5 mL), incubated at 38 °C to evaporate the methanol, and analyzed via enzyme-linked immunosorbent assay (ELISA). No differences were observed in fin cortisol levels among species or in fin and jawbone cortisol levels between washing solvents. The results of this study demonstrate that the sturgeon jawbone matrix is a promising alternative stress indicator to solid matrices.

INTRODUCTION:
Cortisol is a reliable indicator of animal stress. Cortisol extraction provides a valid framework for researchers to monitor stress levels and general patterns in stressors. For example, previous studies have conducted methodological validation of hair cortisol measurements using various methods in humans[1,2], monkeys[3,4], cattle[5], sheep[6], and goldfish[7,8]. In fish species, cortisol measurements in matrices such as scales, skin mucus, feces, and blood[9] have been shown to provide information on fish health. When blood sampling is problematic or scales are lacking, alternative matrices for cortisol extraction are needed. In fish, alternative matrices can include the jawbone, a hard tissue similar to the human tooth[10].

The development of new matrices and validated techniques to determine fish stress levels is of particular interest to the caviar industry, where sturgeon can experience prolonged exposure to environmental stress factors[11]. The sex of sturgeon cannot be determined before 2 years of age, and sturgeon do not have scales. Because cortisol gradually accumulates in solid matrices during the growth stage[2,7,12], long-term cortisol accumulation data from hard matrices such as fins and jawbones could provide insight into stress levels at different growth stages. In contrast, blood cortisol levels provide a snapshot of stress levels at the time of death and cannot accurately represent stress during long-term rearing conditions[13,14]. With increasing competition in the caviar market, new approaches to improve stress conditions for the production of healthier eggs among sturgeon species during long-term rearing (8–12 years or longer) are an increasingly important area of research. Due to the high cost of sturgeon, harvested samples are extremely costly ($8,000–15,000 per mature fish depending on species

and growth stage), a limiting factor for research projects. However, the development of an appropriate technique for cortisol extraction from sturgeon fins and jawbones could be usefully applied both to fish farming systems and in wild fish to improve the quality and harvest of sturgeon eggs for both consumption and conservation.

As well as providing reliable results[6], the selection of an appropriate cortisol extraction technique is of critical importance to ensure that other compounds present in the matrix during sample preparation do not confound the output, which might lead to inconsistent results. It is equally important to determine whether fin and jawbone cortisol levels are influenced by hormone levels in the surrounding water. Heimbürge et al.[15] suggested that a number of factors may influence cortisol levels including age, sex, pregnancy, season, color[12], and body region from which cortisol is extracted[16]. However, little information is available on the effects of washing solvents on cortisol extraction in fish body matrices[8], and none on these effects in sturgeon, except for sturgeon eggs[17].

Although analyzing baseline cortisol levels from the fins and jawbones of sturgeon requires that the fish be euthanized, this approach does not entail the invasive techniques required for blood sampling in live sturgeon. Fin and jawbone samples are easily collected, and extraction from these tissues can be performed rapidly. Similarly, hormone extraction and analysis are straightforward and require little specialized equipment.

In this study, we present a new and easily applied technique for the extraction, washing, and determination of cortisol from fish fins and jawbones, with the aim of determining whether cortisol levels measured from these matrices can be reliably used as stress indicators. The advantages of this technique include an easy and non-invasive[8] approach, less data variation, and reliable output[1,6,8,17]; the technique is applicable to fish species without scales such as sturgeon. The technique requires slaughter of the fish, selection of appropriate washing solvents[2,4], proper grinding of samples[3,5], professional enzyme-linked immunosorbent assay (ELISA) application[5,7], and extensive knowledge of the incorporation of cortisol sources into solid matrices[6].

We applied two different washing solvents (ultrapure water and isopropanol) to obtain basal cortisol levels in fins from three sturgeon species: beluga (*Huso huso*), Siberian (*Acipenser baerii*), and sevruga (*A. stellatus*), under standard environmental conditions for each species. Jawbones of *H. huso* were also used to evaluate stress in sturgeon. This is the first study to measure cortisol levels in sturgeon jawbones. The results of this study will provide comparative cortisol data for sturgeon species in the early growth stage (~1 year) prior to sex determination.

PROTOCOL:

The following experimental procedures and methods were approved by the Animal Welfare and Ethics Authority of Kangwon National University, Chuncheon, Republic of Korea.

1. Fin collection

1.1. Capture the sturgeon gently using a net to minimize injury and stress.

1.2. Rinse the fish carefully with fresh water and then wipe the body surface with an absorbent towel prior to euthanasia.

1.3. Hit the head of the fish using a plastic hammer such that the fish is stunned or loses consciousness. Remove the head using a knife.

1.4. Measure the body weight (g) and length (cm).

1.5. After euthanasia, collect fin samples by cutting as close as the body as possible using sterilized surgical scissors.

NOTE: Individual, non-recycled absorbent towels must be used for each fish. Descriptive statistics for the species used in this study were as follows: beluga sturgeon (*H. huso*): age = 18 ± 2.1 months, body weight = 2,700 ± 300 g, and body length = 55 ± 5 cm; Siberian sturgeon (*A. baerii*): age = 9.6 ± 2.4 months, body weight = 1,750 ± 250 g, and body length = 45 ± 5 cm; sevruga sturgeon (*A. stellatus*): age = 14 ± 1.3 months, body weight = 1,000 ± 100 g, and body length = 65 ± 5 cm.

2. Fin preparation for cortisol extraction

2.1. Place the fin samples (one sample per tissue: ~3 g) on laboratory weighing paper (107 mm × 210 mm) and dry at room temperature for a few days until dry.

2.2. Wrap samples in sheets of aluminum foil, place in labeled plastic bags, and transfer to the laboratory.

2.3. Store samples in a refrigerator for further use, including washing, cortisol extraction, drying, and ELISA analysis (**Figure 2**).

3. Fin cortisol analysis

3.1. Calibrate the digital analytical scale (accuracy: 0.0001) and weigh out 300 ± 10 mg samples with weighing paper on the scale pan.

3.2. Wash the samples.

3.2.1. Transfer each sample into a 15 L conical polypropylene tube. Add 3 mL of isopropanol to each tube using a 5,000 µL single-channel pipette.

3.2.2. Rotate the tubes at 1,800 rpm for 2.5 min to wash out cortisol and remove any potential

external contamination. Repeat this procedure twice.

3.2.3. Air-dry the washed samples at room temperature (22–28 °C) for 7 days.

3.2.4. Repeat the washing procedure using ultrapure water as the washing agent.

3.3. Extract the jawbone from the body tissue using bone-cutting forceps. Apply steps 1.5–3.2.4 to the jawbone samples.

3.4. Weigh out (75 ± 5 mg) dried fin or jawbone samples and grind using a bead beater at 50 Hz for 32 min.

3.4.1. Deliver 1.5 mL of methanol into each tube containing powdered fin or jawbone using a 1000 µL pipette. Place the samples on a tube rotator at slow rotation (40 rpm) for 18 h at room temperature to extract cortisol with continuous mixing.

3.5. Following cortisol extraction, centrifuge the samples at 9,500 x *g* for 10 min at room temperature. After centrifugation, collect the top organic layer containing cortisol (1 mL) from each sample and place it into a separate 1.5 mL microcentrifuge tube.

3.5.1. Dry the samples by incubation at 38 °C to evaporate the methanol. Keep the extracted cortisol samples under a fume hood overnight to allow methanol to dissipate.

NOTE: The cortisol-containing layer is usually yellowish in color.

4. **Fin cortisol detection**

4.1. Thaw the dried fin or jawbone samples at room temperature for 1.5 h prior to using the ELISA kit.

4.2. Add 400 µL of phosphate buffer, vortex, and centrifuge at 1,500 x *g* for 15 min.

4.3. Run each sample (25 µL) in duplicate to improve assay accuracy and reliability. Remove any data outside the standard curve as outliers.

4.4. Set a microplate reader to 450 nm, then set to µg dL^{-1} and read the optical density of the plate.

4.4.1. Use the microplate software with a four-parameter non-linear regression curve fit. Convert the cortisol levels of the samples obtained from the software into pg mg^{-1} using the following equation:

F = 10,000E (A/B) (C/D),

where F = the final value of the fin cortisol level in (pg mg^{-1}), E = the volume (mL) of the assay buffer used to reconstitute the dried extract, A = the concentration (μg dL^{-1}) provided by the assay output, B = the weight (mg) of the fin subjected to extraction, C = the volume (mL) of methanol added to the powdered fin, and D = the volume (mL) of methanol recovered from the extract and subsequently dried down[3].

5. Statistical analysis

5.1. Divide each sample into two sub-samples prior to the washing procedure and then run in duplicate during the ELISA kit assay (2 × 2 = 4 observations per sample) to improve the power of the test and reliability of the results.

5.2. Compare the effects of the two washing solvents and their interactions by applying the general linear model (GLM) procedure in the SAS software environment to the measurement data[18].

5.3. Test differences between means using Tukey's test at a significance level of $p < 0.05$. Accept $0.05 < p < 0.10$ as evidence of a tendency rather than as a significant difference.

REPRESENTATIVE RESULTS:

The presented fin cortisol extraction technique was developed and confirmed in this study using three sturgeon species. Cortisol levels obtained using ultrapure water and isopropanol as washing solvents were compared (**Figure 2**). Cortisol from *H. huso* jawbones was examined to determine whether sturgeon jawbones might be used as an alternative matrix to fins. The effects of washing solvent, sturgeon species, and their interaction are shown in **Table 1**. Cortisol levels tended to be higher in fin samples washed with isopropanol than in those washed with water ($p = 0.089$). There were no significant differences in fin cortisol levels ($p = 0.525$) among sturgeon species. There was no significant interaction between washing solvents and sturgeon species ($p = 0.947$). Washing solvent had no significant effect on cortisol level in *H. huso* sturgeon ($p = 0.45$) (**Table 2**). Intra-assay and inter-assay coefficients of variation were 14.15 and 7.70, respectively. The data showed high similarity among fins of the three sturgeon species (**Table 1**) and in *H. huso* jawbones (**Table 2**). We did not investigate correlations between cortisol levels in jawbones and those in fins of different sturgeon species because we obtained jawbone samples only from *H. huso*. These relationships should be explored in a future study.

FIGURE AND TABLE CAPTIONS:

Figure 1. (**A**) Photograph of *Huso huso* sturgeon (10 years old). (**B**) Morphological characteristics of sturgeon.

Figure 2. Infographic of fin cortisol analysis[5–6] performed in the laboratory. All photographs presented in the infographic abstract were taken in the laboratory.

Table 1. Fin cortisol levels in three sturgeon species obtained using two different washing

solvents.

Table 2. Jawbone cortisol levels in beluga sturgeon (*Huso huso*) using two different washing solvents.

DISCUSSION:

Sturgeon is sometimes called a "living fossil" because it has exhibited few adaptations throughout past millennia. The sturgeon genus *Acipenser* contains 27 species that produce caviar; however, three species (beluga, baerii, and sevruga) produce most of the global caviar supply. Sturgeon are vulnerable to over-fishing and interference in their natural habitat and are therefore more critically endangered than any other group of species. Sturgeon belong to the oldest group of living vertebrates, which has existed for 150 million years. *Acipenser* species mature and grow slowly; some (e.g., *H. huso*) can live for 100 years and exceed 2,000 kg in weight. Sturgeon are cartilaginous fish without scales, and are characterized by five rows of large, bony plates called scutes and tactile barbels located at the front of the mouth (**Figure 1**). Physiological differences between these species and other fish include decreased plasma (corticosteroid) responses to environmental stressors. Our fin cortisol measurements provide evidence that the sturgeon jawbone accumulates cortisol in proportion to circulating concentrations.

Fish display numerous responses to physical, chemical, and perceived stressors. These reactions are well known as adaptive mechanisms that allow the fish to cope with environmental disturbances and maintain a homeostatic state. If a stressor is sufficiently prolonged or serious that the fish is unable to regain homeostasis using its natural responses, then the fish may experience adverse effects, endangering its overall health and/or life[19]. The sex of sturgeon can be determined from around 2 years of age. Therefore, to determine whether cortisol levels and sturgeon sex are correlated, it is necessary to document long-term cortisol accumulation in fins and jawbones (as a new approach and alternative matrix) of sturgeon. This study is the first to report fin and jawbone cortisol levels in sturgeon.

The role of a cortisol wash solvent is to remove external cortisol sources from the skin mucus[9]. Aerts et al.[14] used distilled water to remove external cortisol contamination from fish skin; in previous studies[2,5,12,17], we compared the effects of using isopropanol and water as a solvent to examine hair cortisol content. The effects of wash solvent can vary among samples due to differences in the properties of sturgeon eggs[13], skin[15], fins, and jawbones. Brossa[7] reported that cortisol level in scales of goldfish (*Carassius auratus*) remained constant when isopropanol was used as the solvent regardless of the number of washes, whereas cortisol levels varied when water was used. Our results showed that washing solvent had no effect on jaw cortisol levels. Differences between these studies include the number of washes, shaking vs. vortexing, isopropanol purity, and importantly, sensitivity or resistance of scales/skin to external liquid penetration. Ghassemi Nejad et al.[20] demonstrated that the application of different assays such as RIA and ELISA can lead to differences in output. Steroids are more soluble in lower molecular mass alcohols (e.g., methanol) than in alcohols of higher molecular weight such as isopropanol[4]. Methanol extraction denatures protein by breaking non-covalent bonds, thus allowing hair

cortisol release. Methanol also modifies hormone structure by breaking non-covalent bonds, resulting in the release of cortisol from tissues. To effectively homogenize sturgeon fins and jawbones prior to methanol extraction, a bead beater can be used to efficiently break down the tissue structure. This procedure requires time to completely grind fin and jawbone samples; therefore, the process must be repeated to ensure complete pulverization and homogenization prior to cortisol extraction. Slow rotation for 18 h permits the gradual removal of cortisol by washing.

As suggested in previous studies of mammal hair[4,5,6], external or internal sources of cortisol content in fins and jaws, other than blood, should not be neglected. Although this study was not specifically designed to investigate how cortisol diffuses from blood to fins or jaws, it highlights the need to expand our knowledge of this process to better interpret fluctuations in cortisol from these matrices. The properties of fins and jawbones differ from those of scales and skin. Bussy et al.[17] quantified cortisol levels in lake sturgeon (*A. fulvescens*) eggs to investigate environmental effects on maternal physiological condition and egg quality. They used methyl tert-butylether (MTBE), ethyl acetate (AcOEt) MTBE, and diethyl ether (Et_2O) as washing solvents and concluded that ethyl acetate was the best extraction solvent in terms of recovery and matrix effect. In the present study, isopropanol removed greater amounts of external cortisol from skin mucus during washing, leading to a slight overestimation of cortisol from sturgeon fins, which must be carefully considered when interpreting extraction results. It is possible that isopropanol was able to wash out the skin of the fin, as has been reported in a previous study[7]. Isopropanol is known to penetrate hair follicles and fish scales[4,7]. The results of the present study indicate that the choice of solvent had no significant effect on cortisol levels, suggesting that cortisol extraction may be more difficult in some parts of the fin than in others using ultrapure water; in such cases, isopropanol may be used as an alternative.

This study demonstrated the applicability of the jawbone as a novel matrix for the reliable indication of stress in sturgeon. *H. huso* jawbone cortisol values were similar to those extracted from fins of the same species; future studies should confirm this result among different species, ages, and sexes via correlation analysis. A low number of fish was used in the current study due to the high cost of sturgeon; we attempted to overcome this limitation by testing each sample twice and also duplicating methanol extraction for the ELISA. Using quadruple multiplication could increase the power of the test to cover the low number of samples.

We conclude that the type of washing solvent moderately affected cortisol extraction from fins, but not jaws, of sturgeon. Before generalizing the conclusion of this study and to validate these results, further research using different species and solvents should be conducted. The present work provides evidence that the sturgeon jawbone can be applied as an alternative matrix in future studies using cortisol as an index of stress in sturgeon. The suitability of ELISA for fin and jawbone cortisol measurement was also demonstrated in the present study. Future research should focus on two aspects: 1) determining the correlation between cortisol levels in jawbones and those in fins of sturgeon and 2) harvesting matrix samples for cortisol measurement from older fish and their caviar to determine long-term stress levels in different sturgeon species over the lifespan.

ACKNOWLEDGMENTS:

This work was conducted with the support of the Cooperative Research Program for Agriculture Science & Technology Development (Project title: Livestock productivity change analysis with climate change, Project No. PJ012771), Rural Development Administration, Republic of Korea. The authors gratefully acknowledge Persian Gesture CEO Mohammad Hassan Salmanzadeh and his team, who provided fish from the three sturgeon species examined in this study.

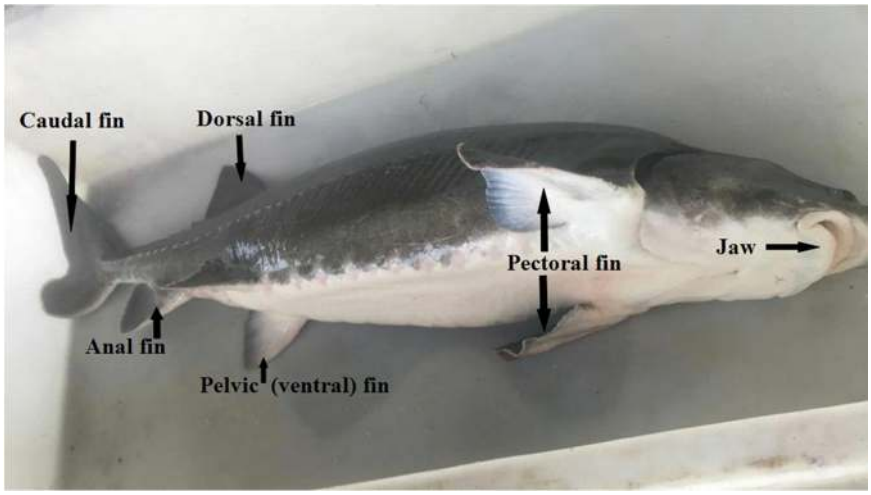
DISCLOSURES:

The authors have no conflicts of interest to disclose.

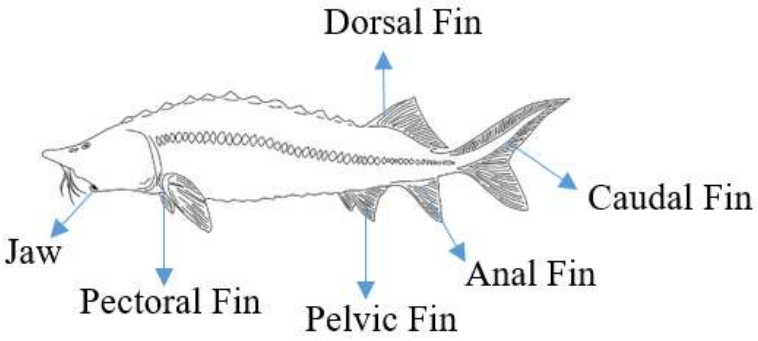
REFERENCES

1. Meyer, J. S., Novak, M. A. Hair cortisol: a novel biomarker of hypothalamic–pituitary–adrenocortical activity. *Endocrinology*. **153**, 4120–4127 (2012).
2. Ghassemi Nejad, J. et al. A cortisol study: facial hair and nails. *Journal of Steroids Hormonal Sciences*. **7**, 1–5 (2016).
3. Meyer, J. S., Novak, M. A., Hamel, A, Rosenberg, K. Extraction and analysis of cortisol from human and monkey hair. *Journal of Visualized Experiments*. **24**, e50882 (2014).
4. Davenport, M. D., Tiefenbacher, S., Lutz, C. K., Novak, M. A., Meyer, J. S. Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. *General and Comparative Endocrinology*. **147**, 255–261 (2006).
5. Ghassemi Nejad, J., Ataallahi, M., Park, K. H. Methodological validation of measuring Hanwoo hair cortisol concentration using bead beater and surgical scissors. *Journal of Animal Science and Technology*. **61**, 41–46 (2019a).
6. Ghassemi Nejad, J. et al. Wool cortisol is a better indicator of stress than blood cortisol in ewes exposed to heat stress and water restriction. *Animal*. **8**, 128–132 (2014).
7. Brossa, A. C. Cortisol in skin mucus and scales as a measure of fish stress and habitat quality. Ph.D. dissertation. Faculty of Veterinary Medicine, Universitat Autonoma de Barcelona, (2018).
8. Carbajal, A. et al. Cortisol detection in fish scales by enzyme immunoassay: biochemical and methodological validation. *Journal of Applied Ichthyology*. **34**, 1–4 (2018).
9. Bertotto, D. et al. Alternative matrices for cortisol measurement in fish. *Aquaculture Research*. **41**, 1261–1267 (2010).
10. Ghassemi Nejad, J., Jeong, C., Shahsavarani, H., Sung, K. I., Lee, J. Embedded dental cortisol content: a pilot study. *Endocrinology & Metabolic Syndrome*. **5**, 240 (2016).
11. Pankhurst, N. W. The endocrinology of stress in fish: an environmental perspective. *General and Comparative Endocrinology*. **170**, 265–275 (2011).
12. Ghassemi Nejad, J., Kim, B. W., Lee, B. H., Sung, K. I. Coat and hair color: hair cortisol and serotonin levels in lactating Holstein cows under heat stress conditions. *Animal Science Journal*. **88**, 190–194 (2017).
13. Baker, M. R., Gobush, K. S., Vynne, C. H. Review of factors influencing stress hormones in fish and wildlife. *Journal of Nature Conservation*. **21**, 309–318 (2013).
14. Aerts, J. et al. Scales tell a story on the stress history of fish. *PLoS One*. **10**, e0123411 (2015).

15. Heimbürge, S., Kanitz, E., Otten, W. The use of hair cortisol for the assessment of stress in animals. *General and Comparative Endocrinology*. **270**, 10–17 (2019).
16. Ghassemi Nejad, J. et al. Comparing hair cortisol concentrations from various body sites and serum cortisol in Holstein lactating cows and heifers during thermal comfort zone. *Journal of Veterinary Behavior: Clinical and Application Research*. **30**, 92–95 (2019b).
17. Bussy, U., Wassink, L., Scribner, K. T., Li, W. Determination of cortisol in lake sturgeon (*Acipenser fulvescens*) eggs by liquid chromatography tandem mass spectrometry. *Journal of Chromatography B*. **1040**, 162–168 (2017).
18. SAS. 1999. SAS/STAT User's Guide (Version 8.01 Edition). SAS Inst. Inc., Cary, NC, USA.
19. Barton, B. A. Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology*. **42**, 517–525 (2002).
20. Ghassemi Nejad, J. et al. Measuring hair and blood cortisol in sheep and dairy cattle using RIA and ELISA assay: a comparison. *Biological Rhythm Research*. Accepted (2019).



(A)



(B)

Figure 1.

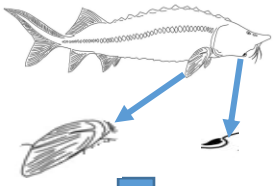
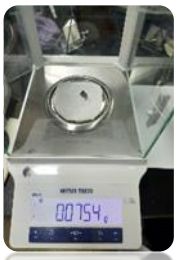
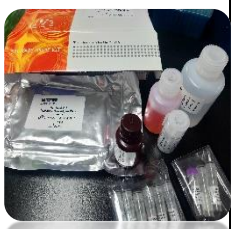
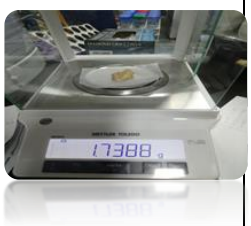
1. Fin and jaw collection	2. Sample preparation	3. Grinding	4. Cortisol extraction	5. Final kit analysis
1.1. Pectoral, fins and jaws were sampled (>2g) 	2.1. Weighing (~300 mg) & washing via isopropanol (3mL, twice, 2.5 min) and then drying (7d, 22-24°C) 	3.1. Sample weighing (75 mg) by a digital scale 	4.1. Application of methanol (1.5 mL) 	5.1. Salimetric ELISA kit 
1.2. Placed in aluminum foil & labeled 	2.2. Sample washing by ultrapure water  and then drying (7d, 22-24°C)	3.2. Samples (75 mg) were ground by a bead beater (32 min-50Hz) 	4.2. Rotation (40 rpm & 18 h) 	5.2. Reading ODs using Microplate reader at 450 nm 

Figure 2.

Table 1.

	Sturgeon species (SS)				Washing solvent (WS)			P-value		
	Huso huso	*Acipenser baerii*	*Acipenser stellatus*	SEM	Water	Isopropanol	SEM	SS	WS	SS×WS
Cortisol (pg mg-1)	3.46	2.85	3.34	0.41	2.86	3.69	0.33	0.5	0.08	0.95

Table 2.

	Washing solvent (WS)		SEM	P-value
	Water	Isopropanol		
Cortisol (pg mg-1)	1.11	1.43	0.31	0.45

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table of Materials.xlsx

Company	Catalog Number
Ansell	63754090
Baskoolnikoo	101 EM
Becton Dickinson Falcon	35-7550
BioTek	8041000
BrandTech	703459
Cleanwrap	30cm x100m
Daejung chemicals & Metals	5035-4400
Daejung chemicals & Metals	5558-4100
DLAB Scientific	824-222217777
Eppendorf Research Plus	M21518D
Eppendorf Research Plus	R25623C
Fisherbrand	09-898-12B
GeneReach Biotechnology Corp	tp0088
Hangzhou Miu Instrument	MU-E30-1044
Hyundai Micro	H20050
Kwang Dong Industrial	KD 901-22128175
Labo Gene	9.900.900.729
LMS	VTX-3000L
Lotte Aluminum	B0722X5FK5
Mettler Toledo	ME204
MDM	MDM-0110
Neptune Scientific	REF 2100.N
Pond H2O	Hoz135
Salimetrics	1-3002-4
Sankyo	26-188A
VITLAB	18A68756
Yuhan Kimberly	1707921546
Yuhan Kimberly	41117

Comments/Description
Disposal latex surgical gloves
Platform scale-electronic weighing 100kg
Serological pipette to deliver up to 24 mL
Micro plate reader with 450 nm and 490 to 492 nm reference filters
Reagent reservoirs
Zipper storage plastic bag
Isopropyl alcohol
Methyl alcohol
Tube rotator- MX-RL-Pro
Precision pipette to deliver 1.5 and 10 mL
Precision pipette to deliver 15 and 25 µL
Weighing paper (107 x 210 mm)
Bead beater, 50/60 Hz 2A
Plate rotator with orbit capable of 500 rpm
Disposable polypropylene tubes to hold at least 24 mL
Fume hood
Micro-centrifuge capable of 1500 x g
Mini vortex mixer
Lotte aluminum foil roll
Digital scale
Ultrapure water
Pipette tips
Large fish net
Salivary cortisol kit
Bone cutting forceps
Precision multichannel pipette to deliver 50 µL and 200 µL
Towel
Tissue paper (107 × 210)

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Comparing Two Washing Solvents Used in Extracting Cortisol from Fin and Jaw Matrices for Three Sturgeon Species
Author(s):	Jalil Ghassemi Nejad, Mohammad Ataallahi, Mohammad H Salmanzadeh, Kyung T Park, Hong G Lee, Alireza Shoae, Atieh Rahimi , Kyung I Sung, and Kyu H Park,

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Kyuhyun PARK
Department:	Animal Industry Convergence
Institution:	Kangwon National University
Title:	Assistant Professor
Signature:	 Date: March 07 / 2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Dear Editor,

The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see:

http://www.textcheck.com/certificate/K0Xk0D

Please find the revised version. We believe that the changes are now made our manuscript more readable and comply with the standards of the JoVE.

NOTE: As previously communicated, we need to film the protocol after acceptance to be happening in July (maximum end of July). It is because the expert technician who does the laboratory work and is a PhD student at Kangwon National University may not be available after July 2019. His presence is necessary for making the video and for the kit procedure filming. Please let us know the possibility that may affect our decision on our manuscript.

Thank you in advance for your understanding and for your kind attention to our query.

Editorial comments:

1. Please employ professional copy-editing services as the language in the manuscript is not publication grade. There are many phrases that severely affects the quality of the manuscript, particularly in the Introduction, Representative Results, and the Discussion.

The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see:

http://www.textcheck.com/certificate/K0Xk0D

2. Please shorten the title to the proposed: Extracting Cortisol from Fin and Jawbone Matrices of Sturgeon

Title is now changed as suggested.

3. Please shorten the Summary to be less than 50 words.

Summary is shortened to be less than 50 words (48).

4. Please shorten the Abstract to be less than 300 words.
Needful changes done! (284 words). We moved the first two sentences of the ABSTRACT to be the first sentences of the INTRODUCTION section as we thought that they are useful statements.

5. Many protocol details are still missing. Needful details are added to the protocol and as per below suggestions by the Editor.
1.2: Rinse with what? Dry with what?

Rinsed with fresh water. Dried with absorbent towel. The text is now amended accordingly.

1.4: How is euthanasia performed? How is the fin cut? With what?

The euthanasia performed by hitting the head of sturgeon using a plastic hammer in order to make the fish to be stunned or losing their consciousness and then they were slaughtered by cutting the head by knife. The fins were cut and removed completely as close as the body by using surgical scissors (~3 g).

2.1: Cut the fins again? To what parameters? Cut with what?
2.1 Deleted because of the repetition.

2.2: Dimensions of the paper tissue? Dimensions? How many samples per tissue? Dry for how long?

It was laboratory weighing paper (KIMTECH science) that is now addressed in the Table of Materials. Dimensions were 107 × 210 mm. Dried at room temperature for few days until drying. The text is now amended accordingly in blue.

3.1: Scale how?

At first the digital analytical scale was calibrated and used by placing the weighing paper on the pan and then measuring the weight of the fin tissues for 300 ± 10 mg prior to wash with solvents.

3.2: This step can be deleted. Deleted.

3.4: As what steps? The procedure cannot be the same as the fins are external and the jawbones are internal. More details are needed for the jawbone extraction.

Yes. We added information regarding the difference of cutting the jawbone to the text. The only procedure which were different from fin collection was the tools (double bone cutter) we used to cut the jawbones. The device (bone cutter) is now added to the Table of Materials). For the steps that were similar to the fin preparations, we mentioned only the steps numbering in order to avoid redundancy.

“ 3.4. Extract the jawbone. Apply the same procedure as above explained for the fin to the jawbone samples.

We mentioned the steps that were the same as fin (steps 1.5. to 3.2.4.)

Please move the equipment list from the protocol to the Table of Materials.

Moved.
4.1: For how long and at what temperature?

Thaw the dried fin and jawbone cortisol extraction samples at room temperature at minimum of 1.5 hours prior to the use of the kit (added to the text).
5. Please provide citations.

Citation is now provided.