Dynamics of endogenous and water cortisol release in Asian Seabass *Lates calcarifer* after acute stress in a farm scale recirculating aquaculture system

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Abstract

Stress in farmed fish is associated with poor feeding, slow growth, disease, and mortality. Therefore, it is essential to closely monitor the stress levels in fish to optimize farming practices which could then enhance productivity and welfare in aquaculture operations. Cortisol, a stress hormone that can be found in the blood, is a reliable biomarker for evaluating fish stress. As blood sampling is highly invasive, alternative cortisol sampling methods such as fin, mucus, and the

surrounding water which contains released cortisol, have been proposed as less invasive or non-invasive sampling methods. However, a comprehensive understanding of their temporal dynamics and associations with plasma cortisol levels is still lacking. In this study, we subjected *Lates calcarifer*, Asian sea bass within a farm-scale (3,000 L tank, 9,000 L system) high-flow rate (8,000 L/hour) Recirculating Aquaculture System (RAS) to an acute handling stress challenge specifically involving chasing and air exposure, and quantified cortisol dynamics both within different biological samples including blood, fin, and mucus and in tank water from multiple sampling points. We showed that handling stress induced an expected increase in plasma and mucosal cortisol, peaking at 4 hours and 24-48 hours, respectively, and that plasma and mucus cortisol were moderately correlated, especially during the stress period. Fin cortisol did not show consistent dynamics. Water cortisol similarly rose, but peaked within 40 minutes from the start of the stressor, in a pattern that was dependent on the site of sampling within the RAS system, likely due to RAS circulation dynamics. Our study is the first to examine the impact of stress on cortisol accumulation and release in Asian Sea bass in a farm-scale RAS, thus providing insights that complement existing research on the efficacy of fin, mucus, and water cortisol as stress indicators that could help optimize aquaculture productivity and welfare.

Keywords

Stress, Cortisol, Asian Seabass, Barramundi, Mucus, Recirculating Aquaculture System (RAS)

Introduction

Fish stress has negative effects on growth, survival, and meat quality, and is both a cause and consequence of disease (Bly et al., 1997; Daskalova, 2019; Snieszko, 1974). Pathogenic infections (Ellis et al., 2007; Triki et al., 2016) and environmental stressors such as poor water quality (Lupica and Turner, 2010; Mota et al., 2017a; Zarantoniello et al., 2021), handling (Ellis et al., 2004; Scott et al., 2001), noise (Mickle and Higgs, 2018), and overcrowding (Odhiambo et al., 2020; Pavlidis et al., 2013) commonly induce the stress response in fish, which is marked by the secretion of the stress hormone, cortisol. As such, cortisol has been utilized as a biological

indicator of fish stress levels in both laboratory and aquaculture settings (Martínez-Porchas et al., 2009; Sadoul and Geffroy, 2019; Tanaka et al., 2023). Unfortunately, conventional methods of obtaining cortisol from fish blood, or whole-body samples are invasive and themselves stress-inducing (Sadoul and Geffroy, 2019; Scott et al., 2001).

Measurements of cortisol concentrations from mucus and fin have been used as less-invasive methods for stress monitoring (Bertotto et al., 2009; De Mercado et al., 2018; Ghassemi Nejad et al., 2019; Simontacchi et al., 2008). Simontacchi et al (2008) evaluated plasma cortisol levels in European sea bass subjected to different pre-slaughter conditions. Cortisol levels in plasma were found to correlate with those detected in mucus, even though cortisol levels in mucus were substantially (up to 20 times) lower than in plasma. Bertotto et al (2010) investigated transportation stress in European sea bass and other fishes and similarly observed a good correlation between cortisol levels in plasma, mucus and fin samples.

Cortisol within the animal's bloodstream is also released into the surrounding water through passive diffusion (Sadoul and Geffroy, 2019), and water cortisol levels were previously shown to correlate with plasma cortisol levels (Fanouraki et al., 2008; Scott et al., 2001). Hence, regular measurement of cortisol in the water has been proposed as a non-invasive means of detecting elevated stress levels in farmed fish (Sadoul and Geffroy, 2019; Scott et al., 2001). This method might be particularly useful in land-based farms that use Recirculating Aquaculture Systems (RAS) technologies for high-intensity farming (Mota et al., 2017a).

A handful of previous studies have looked into the potential of water cortisol as a non-invasive stress marker. Fanouraki et al (2008) subjected European Seabass (*Dicentrarchus labrax*) to 5 minutes of chasing and a 1-1.5 minutes air exposure, within a 2 m^3 flow-through tank, at a water flow rate of 1 L/minute or 60 L/hour (Fanouraki et al., 2008). This resulted in increased plasma cortisol concentrations (2,184 nM or 791,612 ng/L) that peaked at 1-hour post stress. Water cortisol concentrations peaked at 4 hours post-stress reaching 7.2 ± 0.20 ng/L. In the same study, a confinement challenge was also conducted with two stocking densities (20 or 50 kg/m^3)

78 within 12 L buckets with supplied O_2 and a high water flow rate. The higher stocking density tank
79 was observed to have had a 2-3 fold higher water cortisol concentration throughout the 24-hour
80 experiment. In a different study, Scott et al (2001) showed that 90-second air exposure of rainbow
81 trout (*Oncorhynchus mykiss*) induced stress as measured from water cortisol (Scott et al., 2001).
82 The study was conducted in a 150 L flow-through tank, with a stocking density of 30 kg/m^3 at a
83 water flow rate of 2 L/minute or 120 L/hour. A progressive increase in water cortisol concentrations
84 was observed after single and repeated stress, peaking at 2 hours (25 ng/L) and 5 hours (100
85 ng/L) respectively, and gradually declining thereafter. The above studies used small volume, flow-
86 through setups not comparable to farm-scale conditions.

87 More recently, water cortisol experiments have been conducted in RAS systems, albeit in
88 relatively small setups. A study by Mota et al (2017) examined the effect of RAS flow rate on water
89 cortisol concentrations prior to and following an acute stressor (Mota et al., 2017b). Nile Tilapia
90 (*Oreochromis niloticus*) were raised at a stocking density of 67 kg/m^3 in 72 L tanks. After two
91 weeks at a flow rate of 675 L/kg feed/day, water exchange rates were adjusted to 150 L/kg
92 feed/day (LowRAS) or 1,500 L/kg feed/day (HighRAS) for 4 weeks, where LowRAS expectedly
93 led to an increase in water cortisol concentrations over 4 weeks. Cortisol concentration peaked
94 at 2 hours following an acute stressor of 60-second air exposure, increasing 30% in LowRAS from
95 5 ng/L to 7 ng/L, unlike the HighRAS condition, where a significant change was not observed.
96 Another study by the same authors investigated the water exchange rate and pH on circulating
97 cortisol in rainbow trout (*Oncorhynchus mykiss*) and the accumulation of cortisol in a RAS tank
98 over a 70-day experimental period, with a starting stocking density of around 10 kg/m^3. On day
99 35, plasma cortisol was significantly elevated at low pH (5.8) with a mean concentration of 24.4
100 +/- 9.5 ng/mL, and elevated water cortisol (2.5 ng/L per kg) was also observed at low water
101 exchange rates (480 L/kg feed/day). These studies suggest that the flow rate of a RAS tank may
102 significantly impact the ability to detect water cortisol, and a lower rate is essential for ensuring
103 the utility of water cortisol as an on-farm stress diagnostic tool.

The Asian sea bass (*Lates calcarifer*), also known as Barramundi, is a commercially important fish species widely distributed in the Indo-Pacific region (Islam et al., 2023). It holds a prominent position among the marine species farmed in Singapore because of its consumers' high demand, sustainability, economic benefits, palatable taste, and nutritional value (Glencross, 2006; Taylor, 2022). In aquaculture farming, Asian sea bass have a low feed conversion ratio, which means it requires less feed to produce one kilogram of fish as compared to other aquaculture species (Katersky and Carter, 2005). Additionally, it is commonly raised in RAS, which minimizes the environmental impact and reduces the risk of diseases and parasites, making it a sustainable fish to farm, especially in countries with limited land and water supplies. Overall, this high-value food fish generates revenue for fish farmers, as well as for local restaurants and markets (Keat, 2021). Similar to other farmed fish, Asian sea bass are often subjected to stress during various aquaculture practices, such as transportation, handling, and stocking. Therefore, stress detection in Asian sea bass is of significant interest to both the fisheries and aquaculture industries. However, few studies have been carried out on stress in Asian sea bass (Ardiansyah and Fotedar, 2016; Hong et al., 2021), and no quantification of cortisol accumulated or released into multiple tissue types or water has been reported.

Hence, in this study, we sought to characterize cortisol dynamics of Asian sea bass in response to stressors, particularly whether fin and mucus tissues may allow for accurate and less-invasive proxies of stress in this species. Furthermore, we investigated if water cortisol levels within a larger, farm-like RAS setting (9,000 L system, 8,000 L/hour flow rate) would be an accurate measure of fish stress within the system, and compared water cortisol concentrations across different sampling points in the RAS system.

Materials and Methods

Animal Husbandry and Experimental Setup

Asian sea bass (*Lates calcarifer*) were reared in a RAS system at the Aquaria of Republic Polytechnic, Singapore, and all experiments were approved by the Republic Polytechnic's

130 Institutional Animal Care and Use Committee (IACUC Protocol #2022/RP/00001). Chasing
131 experiments were carried out in a 9,000 L saltwater (30 ppt) system, which consisted of 2 circular
132 blue fiberglass tanks (each 3,000 L) containing the fish and 1 rectangular tank (3,000 L) as the
133 sump (Figure 1A-B). Water was circulated at 8,000 L/hour.

134 ***Experimental Fish***

135 Asian sea bass were obtained from two local commercial fish farms. Upon arrival, the juvenile
136 Asian sea bass (~30 g) were subjected to a two week quarantine period to facilitate adaptation
137 and address any diseases that the fish might have brought. Each batch of fish was placed directly
138 and quarantined in a single fiberglass RAS tank "Tank 1" (Figure 1A-B). Details of each batch of
139 fish used in the experiments can be found in Table 1.

140 **Table 1: Summary of Asian sea bass used in experiments**

Experiment Details			**At start of experiment**			
Biological Replicates	**Start Date**	**Fish Supplier**	**Number of fish**	**Average Weight (g)**	**Stocking density (system, kg/m^3)**	**Stocking density (tank, kg/m^3)**
Exp 1	7 Sep - 9 Sep 2022	Local Farm A	134	140.67	2.094	6.283
Exp 2	7 Dec - 9 Dec 2022	Local Farm B	446	92.36	4.575	13.731
Exp 3	1 Mar - 3 Mar 2023	Local Farm B (same batch as Exp 2)	432	165.82	7.959	23.878

141

142 During experimental periods, fish were fed three times a day (9:30 am, 12:30 pm, and 4:30 pm)
143 at a rate of about 10% body weight (BW) per day using a commercial fish diet (5 mm sinking
144 pellets; 46% crude protein, 10% crude fat, 5% crude fiber, 16% crude ash, 4.5% phosphorus, and
145 2.2% lactic acid). The amount of feed was also controlled accordingly due to the recirculating
146 aquaculture system filter carrying capacity and fish stocking densities.

147 Water quality parameters were measured daily by the YSI Pro Quattro probe (YSI, USA)
148 and the SpinTouch FX (Lamotte, USA). The 9,000 L RAS system that was used for chasing stress
149 experiments was circulating aerated saltwater (dissolved oxygen, 5.74 ± 4.03 mg/L; water
150 temperature, 27.58 ± 3.8 °C; salinity maintained at 30 ppt). Ammonia levels were controlled to
151 be below 1 ppm, nitrite concentrations were maintained below 1 ppm, nitrate levels were kept
152 below 100 ppm and pH was maintained below 8.0.

153 ***Handling Stress experiments***

154 Fish were starved and not fed during the evenings the day before experiments commenced. "Pre-
155 chase" samples were collected before any chasing stresses were induced on the fish. Fish were
156 subjected to a period of chasing with progressively longer chasing durations: 5 minutes ("Chase
157 (5 min)"), 10 minutes ("Chase (10 min)") and 15 minutes ("Chase (15 min)"), with a 5-minute
158 "rest" interval between each of the 3 chases. Fish were chased by two personnel using large nets
159 (Figure 1C), each in a repetitive "C" shape manner hence covering the entire breadth and depth
160 of the tank. At the end of each of the respective chases, a net full of fish was lifted in one stroke
161 and air exposed for 10 seconds. Following this, 5 fish from this net were collected for sampling.

162 Post-chase samples were collected at 30 minutes ("Post-chase (30 min)"), 1 hour ("Post-
163 chase (1 h)"), 2 hours ("Post-chase (2 h)"), 4 hours ("Post-chase (4 h)"), 24 hours ("Post-chase
164 (24 h)"), 48 hours ("Post-chase (48 h)"), from the end of the chasing period.

165 Hence, there were a total of 11 sampling time points for chasing experiments; where 5
166 random fish were sampled at each sampling time point (Figure 1D). At each sampling point, the
167 following samples and data were collected: water from various parts of the tank (see "Water
168 cortisol collection" section), fish body weight (total weight of the 5 fish), plasma, fin, and mucus
169 (See "Biological sample collection" section).

170 ***Biological sample collection***

171 After being netted, the fish were anesthetized in a bucket using Tricaine methanesulfonate MS-
172 222 (70 mg/L) and sodium bicarbonate (0.14 g/L) dissolved in fresh saltwater from the reservoir.

173 While still in the bucket, the fish were collectively weighed. The anesthesia took effect in
174 approximately 5 minutes, after which the fish were subjected to plasma, fin, and mucus collection,
175 as described in the following sections. On average, the entire process of collecting plasma, fin,
176 and mucus samples required approximately 5 minutes per fish.

177 ***Plasma collection***

178 Blood was extracted from the caudal artery/vein by inserting a 21G sterile needle at a 45° angle
179 until it reached the spine. Upon contact with the spine, the needle was withdrawn slightly, and
180 blood was extracted into a sterile 1.0 mL syringe. Approximately 0.5 mL of the collected blood
181 was subsequently injected into a heparin tube and inverted 5 times to thoroughly mix the blood
182 within the heparin tube and prevent coagulation. The blood in the heparin tube was then
183 centrifuged at a 45° angle, at 3,000g for 10 minutes to facilitate the separation of blood plasma.
184 The plasma was carefully pipetted into a cryovial, which was then stored in dry ice before being
185 transferred to a -20 °C freezer.

186 ***Mucus collection***

187 The fish were swabbed on their left flank, along the lateral line. This process was repeated 4 times
188 on each side of the swab. The swab was then placed into a 2 mL tube, where 1 mL of phosphate
189 buffer solution (PBS) was added to the sample swab. The samples were then stored in dry ice
190 before being transferred to a -20 °C freezer.

191 ***Fin collection***

192 A fin clip was collected by cutting about 1.5 cm of the top part of the caudal fin. Tweezers were
193 then used to pick up the fins to be placed into 2 mL labeled Eppendorf tubes. The Eppendorf
194 tubes with the fins were then filled with 1 mL of PBS and stored in dry ice before being transferred
195 to a -20 °C freezer. After handling each fish, both tweezers and scissors were sanitized with 70%
196 ethanol.

197 After sample collection, the fish were first placed in recovery saltwater buckets with no
198 tricaine to recover from the anesthesia. This measure was implemented to prevent the

resampling of the same fish at each sampling time and to avoid any potentially stressful effects caused by the reintroduction of the sampled fish. In the first experiment where water cortisol was only measured from “Tank 1 - Furthest from inlet”, the sampled fish were placed into “Tank 2” post-recovery from anesthesia. The latter was the other blue fiberglass tank connected to the entire recirculating system. In subsequent experiments, given that we were also sampling water from other parts of the system including Tank 2, the sampled fish were placed into a separate holding tank not connected to the circulating system and only returned at the end of the day after the “Post-chase 4 h” time point (Experiment 2), or in the case of Experiment 3, these fish were culled. We note that there were minimal changes in the system stocking densities caused by these differences (Supplementary Table 1), and as reported in the “Results” section, these minor variations did not appear to affect the overall trend of biological or water cortisol changes.

Water sample collection

In Experiment 1, tank water was only collected from a single sampling point “Tank 1 Furthest from Inlet”. In Experiments 2 and 3, tank water was additionally sampled from 6 other sampling points (Figure 1A). Besides 1 - “Furthest From Inlet Tank 1”, water was sampled from the 2 - “Central Pipe”, 3 - “Inlet Tank 1” , 4 - “Sump”, 5 - “Skimmer”, 6 - “UV” compartment and 7 - “Furthest From Inlet Tank 2”. Gloves were consistently worn during the collection of water cortisol samples to avoid potential contamination from human skin-derived cortisol. Water was collected using siphons, positioned at a height that was midway through the water column. These siphons were thoroughly rinsed with clean freshwater at each respective collection point. To ensure thorough flushing of the siphon at each sampling point, the tank water was allowed to flow through the siphon for at least 30 seconds before a 100 mL or 125 mL glass bottle was filled to the brim. These water samples were subsequently kept chilled in an ice box and later transferred to a 4°C fridge for storage. Water from the system that had been siphoned out but not collected in the glass bottles was returned into the system to maintain a relatively constant water volume within the system.

225 ***Cortisol extraction from fin and mucus samples***

226 Sample processing was performed according to protocols adapted from previous methods
227 (Ghassemi Nejad et al., 2019) with some modification. Mucus samples stored in a -20 °C freezer,
228 were thawed to room temperature before processing. The microcentrifuge tube containing the
229 swab with mucus was vortexed for 30 seconds, after which, the mucus was scrubbed from the
230 swab with the wall and rim of the tube repeatedly. Following this, the solution was filtered using a
231 0.22 µm Polyvinylidene fluoride (PVDF) syringe filter and stored at a 4 °C fridge for analysis within
232 a 24-hour timeframe.

233 Fin samples in PBS stored in a -20 °C freezer were thawed to room temperature before
234 processing. Each fin sample was washed twice with isopropanol and subsequently air-dried for
235 2-3 days. The dried fin sample was ground into powder by using a mortar and pestle, and the
236 weight of the ground powder was recorded. To facilitate cortisol extraction, 1.5 mL of methanol
237 was added to the ground powder and shaken over a 3-day period. After centrifugation at 9,500
238 rpm for 10 minutes, the resulting supernatant was collected and placed in an oven at 38 °C for
239 methanol evaporation. The extracted cortisol was reconstituted in 400 µL of PBS, filtered through
240 a 0.22 µm PVDF syringe filter, and stored at 4°C fridge for analysis within 24 hours or in a -20
241 °C freezer for long-term storage.

242 ***Cortisol quantification from biological samples (ELISA)***

243 The cortisol levels from the three biological substrates (plasma, fin and mucus) were quantified
244 using an enzyme-linked immunosorbent assay (ELISA) kit (Fish Cortisol ELISA, CUSABIO,
245 Houston, TX, USA). The ELISA is based on the competitive inhibition enzyme immunoassay
246 principle, involving the interaction between pre-coated cortisol and cortisol within the samples,
247 and has a cortisol detection range of 0.0023–10 ng/mL.

248 Plasma and aliquots of cortisol extracted from fins and mucus were subjected to analysis
249 following the protocol provided by the manufacturer. Briefly, all reagents and samples were
250 brought to room temperature prior to use. Plasma samples were diluted 100 times with the

251 provided sample diluent before testing. Extracted cortisol from fin and mucus samples were tested
252 as these were without further dilution. In each well, 50 µL of sample or standard was mixed with
253 50 µL of 1× antibody and incubated at 37 °C for 40 minutes. Each well was then washed three
254 times with the wash buffer and 100 µL of Horseradish peroxidase conjugated secondary antibody
255 (HRP conjugate) was added immediately. The plate was incubated in the dark at 37 °C for 30
256 minutes and subjected to five additional washes. Color development was initiated by adding 90
257 µL of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate to each well and incubating the plate at 37
258 °C for 20 minutes. The reaction was then stopped by adding 50 µL of stop solution. The plate was
259 gently tapped to ensure thorough mixing. The optical density of each well was determined within
260 5 minutes using a microplate reader, the Infinite M200 Spectrophotometer (Tecan Trading AG,
261 Switzerland) at a wavelength of 450 nm. The cortisol concentration in the sample is calculated
262 based on the standard curve, prepared by plotting the optical density at 450 nm of cortisol
263 standards (0 - 10 ng/mL) derived from serially diluted cortisol stock (10 ng/mL).

264 ***Cortisol extraction and quantification from water samples (HPLC)***

265 Cortisol from tank water samples was extracted through liquid-liquid extraction technique and
266 analyzed by using high-performance liquid chromatography (HPLC) (Ney et al., 2021; Viljoen et
267 al., 2019) with some modifications. For liquid-liquid extraction of cortisol from water samples,
268 equal volumes of dichloromethane and water samples were mixed together and the mixture was
269 left to stand for 10 minutes. The organic phase containing cortisol was decanted from the mixture,
270 and evaporated using a rotary evaporator (IKA RV 10, Germany). The resulting precipitate was
271 then reconstituted in a 50% methanol solution and filtered through a 0.22 µm PVDF filter.

272 The cortisol extracted from water samples was analyzed using HPLC (Shimadzu LC-
273 2050C 3D, Japan) coupled to a photodiode array (PDA) detector set at 245 nm. The HPLC was
274 performed by injecting 100 µL of the extracted water sample through a Shim-Pact GIST C18
275 normal-phase column (inner diameter 4.6mm, length 250 mm, particle size 5µm). An isocratic
276 flow of mobile phases (methanol and 10 mM ammonium formate in water in a 1:1 ratio) was

277 performed for 30 minutes. Using the HPLC conditions mentioned above, cortisol was eluted with
278 a retention time of 14.5 minutes. The HPLC is calibrated to cortisol standards ranging from 1 pM
279 to 10 μM in 50% methanol solution. All water samples and standards were spiked with 500 μL of
280 1 μM cortisol internal standard. Cortisol peak area from the standards was measured and plotted
281 against the cortisol standards concentration to build a standard curve. The cortisol concentration
282 in the water sample is calculated based on the standard curve according to the peak area in each
283 water sample. Each water sample was measured as duplicates.

284 ***Data analysis and Statistics***

285 Cortisol concentrations obtained from plasma, fin and mucus samples were calculated based on
286 a standard curve run on each plate and expressed in ng/L. Adjustments were made for dilution
287 factor (plasma), initial sample weight (100 mg of fin/1 mL of PBS) and mucus dissolved in 1 mL
288 PBS. In Figure 3, the water cortisol concentration was not standardized to biomass. However, a
289 standardization to system stocking density (kg/m^3) was performed in Supplementary Figures 3-4.

290 To compare cortisol concentrations across different time points, a 2-way ANOVA was
291 applied, followed by the Dunnett test with multiple comparison correction. To ascertain the
292 relationship between less-invasive measurements of mucus and fin cortisol and cortisol levels in
293 blood plasma, we conducted simple linear regression analysis. Using the Grubbs' test (Alpha =
294 0.0001) to identify outliers, we removed two fin cortisol (Experiment A, Pre-chase and Post-Chase
295 (24hr)) and one plasma cortisol (Experiment A, Chase (15min)) measurement in our analysis. In
296 the figures, asterisks are used to indicate statistical significance levels as follows *$p<0.05$,
297 **$p<0.01$, ***$p<0.001$, ****$p<0.0001$

298 **Results**

299 The acute handling stress experiment was repeated three times over a period of six months, as
300 described in Table 1. Given its large scale, we were unable to simultaneously conduct multiple
301 replicates, and instead conducted biological replicates sequentially over time. The origins, age,
302 weight, and stocking densities of the fish inevitably varied, which led to varying absolute cortisol

concentrations as quantified by ELISA analyses (Table 1, Supplementary Figure 1). However, handling stress and sampling protocols were kept consistent, and consistent behavioral changes were observed in each chasing experiment, where the fish exhibited rapid swimming, actively evaded the nets, and displayed signs of distress upon exposure to air. Instances of vomiting were observed, and foamy bubbles were observed on the surface of Tank 1, starting from around the "Chase (10 min)" period and increasing over time, which may reflect increased mucus secretion.

These behavioral changes coincided with a concurrent rise in plasma cortisol levels (Figure 2A), which significantly increased at the Chase (10 min), Post-chase (30 min, 1 hr, and 4 hr) time points ($p = 0.0183$ (Chase-10 min), $p = 0.0172$ (Post Chase-30 min), $p = 0.0066$ (Post Chase-1hr), $p < 0.0001$ (Post Chase-4hr)). Plasma cortisol returned to baseline by 24 hours post-chase. Similarly, we observed an increase in mucus cortisol post-stress (Figure 2B), albeit along a different time course. There appeared to be a gradual increase in mucus cortisol concentrations over time, which reached significance from 4 hours post-chase ($p = 0.0033$), and continued to increase and remain significantly elevated 24 and 48 hours post-chase ($p < 0.0001$ (Post Chase-24hr), $p<0.0001$ (Post Chase-48hr)). Notably, mucus cortisol concentrations were about 100 fold lower than plasma or fin cortisol concentrations (Figure 2A-C). Unlike plasma and mucus cortisol, no consistent changes in fin cortisol were observed.

We also correlated cortisol levels across the biological samples (fin, mucus, and plasma) collected from each individual fish. Plasma cortisol was most strongly correlated with mucus cortisol ($r = 0.42$, $p < 0.0001$, Figure 2D), whereas both fin and plasma ($r = 0.27$, $p = 0.0011$, Figure 2E), and fin and mucus cortisol ($r = 0.14$, $p = 0.1032$, Figure 2F), were only weakly correlated. Notably, the correlation between plasma and mucus cortisol was highest during the chasing period ($r = 0.65$, $p < 0.0001$) as compared to the pre-chase ($r = 0.33$, $p = 0.5375$) and post-chase ($r = 0.17$, $p = 0.0015$) periods (Figure 2G). In contrast, plasma cortisol was more strongly correlated with fin cortisol during the pre-chase period, albeit non-significant ($r = 0.40$, $p = 0.1552$), as compared to the chase ($r = 0.22$, 0.05003) and post-chase ($r = 0.29$, $p = 0.0058$) period (Figure

329 2H). Fin and mucus cortisol were most highly correlated in the pre-chase ($r = 0.51$, $p = 0.0643$)
330 and chase ($r = 0.22$, $p = 0.1434$) period as compared to post-chase ($r = 0.09$, $p = 0.4259$) period
331 (Figure 2I).

332 Next, we quantified cortisol from tank water sampled from 1 to 7 points in the RAS
333 indicated in Figure 1A. In Experiment 1, tank water was only collected from a single sampling
334 point "Tank 1 Furthest from Inlet", whereas in Experiments 2 and 3, tank water was additionally
335 sampled from 6 other sampling points to study the distribution of cortisol across various locations
336 within the RAS.

337 Due to the low concentrations of cortisol in water relative to biological samples, and the
338 higher potential for antibody interference in ELISA due to contaminants (e.g., salts and organic
339 material) in tank water, we opted to use HPLC analysis for more precise measurements of water
340 cortisol concentrations. Consistent with reports in existing literature, water cortisol concentrations
341 were in the range of ~10 to ~100 ng/L, about 1,000 fold lower than the concentrations observed
342 in plasma and fin samples, and 10-fold lower than mucus samples. In all sampling points within
343 the tank containing the fish (Tank 1), we observed a consistent trend of increased water cortisol
344 starting from the chasing period (Figure 3A-C). For the "Tank 1 Furthest from Inlet" sampling point,
345 there was a significant increase in water cortisol at the Chase (15 min, $p = 0.0197$) and Post-
346 chase (30 min) time points ($p = 0.0367$). Water cortisol concentrations were also significantly
347 elevated at the "Inlet of Tank 1) at the Chase (15 min) time point ($p = 0.0287$), with a similar peak
348 for the "Central pipe" albeit non-significant. The cortisol concentrations from the other RAS
349 compartments did not exhibit distinct variations, although water cortisol concentrations in the
350 "Sump" and "Skimmer" were on average higher during and after the chase (Figure 3D and 3E).
351 The "UV" compartment, on the other hand, did not show any trends (Figure 3F). Interestingly, in
352 Tank 2, which was not holding any fish, a similar trend of increased water cortisol, significant at
353 the Chase (10 min) time point, was observed (Figure 3G). This observation suggests the
354 possibility that cortisol released into the water from one tank may be distributed to other RAS

355 tanks within the system. Detailed cortisol concentration data from individual experiments, as
356 absolute values or normalized to system stocking density (since some fish were removed), can
357 be found in Supplementary Figures 2-4.

358 **Discussion**

359 ***Handling stress leads to an acute rise in plasma cortisol***

360 In this study, the effects of handling stress on Asian sea bass cortisol concentrations across tissue
361 types were assessed. Figure 4 shows the cortisol concentration ranges found in different tissue
362 types of Asian sea bass and the RAS tank water. Highest concentration of cortisol is observed in
363 plasma, which is very invasive. Less-invasive samples such as fin, mucus, and water have lower
364 cortisol concentration than plasma. Water samples, which are the least invasive, have the lowest
365 concentration range < 300 ng/L.

366 From this acute handling stress study, first, we confirmed that our manipulation indeed
367 triggered a significant increase in plasma cortisol starting from the Chase (10 min) time point,
368 which would be 20 minutes into the chasing period (Figure 2A). There continues to be a significant
369 increase in plasma cortisol up to 4 hours post-chase, and recovery at 24 and 48 hours. We note
370 that some of the chase and post-chase time points did not attain statistical significance, which
371 could be attributed to biological variability across the 3 replicates, influenced by the large number
372 of fish in the tanks and variations in fish age, genetic background, and size (Table 1). Despite
373 these differences, the overall time course of the rise in plasma cortisol is consistent with many
374 other studies (Fanouraki et al., 2008; Mota et al., 2017a; Scott et al., 2001).

375 ***Relationship of plasma to mucus and fin cortisol***

376 We also simultaneously sampled mucus and fin cortisol from individual fish, to investigate the
377 viability of these less-invasive sampling methods as reliable indicators of fish stress in aquaculture
378 settings. Our findings revealed that mucus cortisol was significantly increased following the stress-
379 inducing event as seen in post-stress (at the 4-hr time point). However, this increase followed a
380 slower time course compared to that of plasma cortisol. Specifically, mucus cortisol remained

381 significantly elevated even at 24 and 48-hours post-stress intervals, even when plasma cortisol
382 had already fully returned to baseline levels. While the biological basis of this is still not well-
383 understood, it is speculated that either the accumulation of plasma cortisol in the mucus, or the
384 reabsorption of cortisol from the water might account for these observed patterns. Despite the
385 differences in cortisol dynamics, we observed a reasonably strong correlation between plasma
386 and mucus cortisol (r = 0.65) during the chasing period. Similarly, one of the few other studies
387 reported the strongest correlation (r = 0.70) between mucus and plasma cortisol in rainbow trout
388 (*Oncorhynchus mykiss*) during the hours following confinement stress, relative to the control or
389 late phase (Carbajal et al., 2019). Our data thus suggests that mucus cortisol could be a useful,
390 less invasive way to sample fish stress in Asian Sea bass. In contrast, cortisol extracted from fin
391 did not display any consistent trend or relationship with plasma cortisol. While this does not
392 preclude its utility in estimating fish stress, it may be less robust across the varied conditions
393 across our experiments.

394 ***Water cortisol changes post-stress in the RAS system***

395 We also measured water cortisol within the fish holding tank (Tank 1) across all three acute stress
396 experiments, and observed a significant rise in water cortisol in the Chase (15 min) and post-
397 chase (30 min) periods, which correspond to 40 minutes up to 70 minutes (1 hr 10 mins) from the
398 initiation of the chasing period. Notably, while the rise in water cortisol paralleled the rise in plasma
399 cortisol, a declining trend was evident by the end of the chasing period. This was in contrast to
400 plasma (or even mucus) cortisol concentrations, which continued to rise.

401 In the latter two of the three experiments, we further measured water cortisol across
402 different parts of the tank and also different RAS compartments. The same trend was still
403 observed in other parts of Tank 1, where the peak in water cortisol still occurred at 40 minutes
404 from the commencement of the stress event (the “Chase (15 min)” time point)) and decreased
405 earlier than plasma cortisol. These results suggest that the dynamics of water cortisol in the RAS
406 system may be influenced by other factors, including flow rate, filtration (i.e. protein skimmer), or

407 binding to the plastic tank walls (since it is hydrophobic). Alternatively, cortisol could potentially
408 be reabsorbed by the fish, possibly into the mucosal layer.

409 We also observed the presence of water cortisol in other RAS compartments, such as the
410 protein skimmer and sump, indicating the distribution of water cortisol from Tank 1 to other
411 compartments. While water cortisol concentrations in the “Sump” and “Skimmer” were on average
412 higher during and after the chase, the increase was not significant, that could be due to the low
413 number of biological replicates and the varying absolute water cortisol concentrations across the
414 two replicates (Supplementary Figures 2-4). The exception was for the UV compartment, where
415 a change in water cortisol post-chase was not so apparent, either due to UV radiation-induced
416 degradation, or other factors. Interestingly, a rise in cortisol, significant at the Chase (10 min) time
417 point, was observed in Tank 2, which did not contain any fish. This result highlights that due to
418 water recirculation in RAS, other connected tanks may also be exposed to the cortisol released
419 from a stressed tank of fish. It would be interesting to further investigate the effects and
420 implications of water cortisol transmission to fish in connected RAS tanks.

421 Overall, water cortisol dynamics appeared to be more consistent with plasma cortisol
422 trends than mucus (or fin). We note that we used a more sensitive method (HPLC) to quantify
423 water cortisol than plasma, mucus, or fin cortisol. This was for two reasons, firstly, water cortisol
424 concentrations were significantly lower than in biological samples, and secondly, fish tank water
425 is a more complex matrix than biological samples, containing a multitude of additional salts and
426 organic materials that may interfere with antibody affinity binding in ELISA. More convenient and
427 sensitive methods of water cortisol detection would be valuable.

428 ***Caveats and limitations of our study***

429 In typical research studies, biological replicates would be conducted in parallel on a single batch
430 of fish, within smaller tank setups. In our study, we aimed to simulate the conditions of a farm-
431 scale setup, using 3,000 L tanks on a 9,000 L RAS system. Due to the scale of our experiments,
432 the limited number of such systems available, and the challenge of acclimatizing and cultivating

433 a large number of fish, we were constrained to conduct one biological replicate at a time, with our
434 experiments spread over a course of 6 months. We also were only able to measure water cortisol
435 from different RAS compartments in 2 of the 3 experiments.

436 As expected, the cortisol measurements obtained in this study exhibited variability across
437 samples and time points. Several factors contributed to this variability; firstly, the variations in the
438 ages, sizes, genetic background, and stocking densities of the fish tested (Table 1). Secondly,
439 despite maintaining a highly consistent chasing and air exposure stress protocol, the random
440 selection of the 5 fish sampled per time point (per experiment) likely contributed additional
441 variability due to the large numbers of fish present in the tank. We also acknowledge the slight
442 protocol differences between our experiments, resulting in small albeit minimal system stocking
443 density changes within and across experiments (Supplementary Table 1 and Methods). While
444 not ideal in an experimental setting, the upside of such variability is that any signal we have
445 obtained would likely be reproducible even with the normal diversity experienced in on-farm
446 settings. Across all biological replicates, we observed an increase in plasma, mucus, and water
447 cortisol levels, with relatively consistent dynamics. Importantly, our results further argue that
448 surveying water cortisol, which provides an average readout of the stress levels in the system,
449 might be a means of overcoming the variability from sampling individual fish. Further, given that
450 the stocking densities fell within the low- (6 kg/m^3) to medium-range (20 kg/m^3), it is possible that
451 more pronounced changes in cortisol might be observable at higher densities.

452 ***Conclusions***

453 Our results corroborate that in a farm-scale RAS setting across varying conditions, water cortisol
454 is a reliable measure of Asian sea bass stress and that different tissue types such as fin and
455 mucus may reflect stress over different dynamical timescales. These results are likely
456 generalizable to other fish species and tank setups, though it is important to note that the time
457 course and concentrations or rates of cortisol release may differ across species. The findings of
458 a significant correlation between mucus cortisol and plasma cortisol, especially during acute

stress exposure, highlight the potential utility of mucus cortisol as a non-invasive measure of fish stress. Future experiments could explore the application of these methods to other types of stressors (e.g. ammonia exposure, infections) or more chronic stressors such as high stocking density. Overall, our results suggest that continuous monitoring of water cortisol in aquaculture RAS setups may be a viable approach for detecting fluctuations in fish stress levels, allowing for the detection of anomalies in a timely manner.

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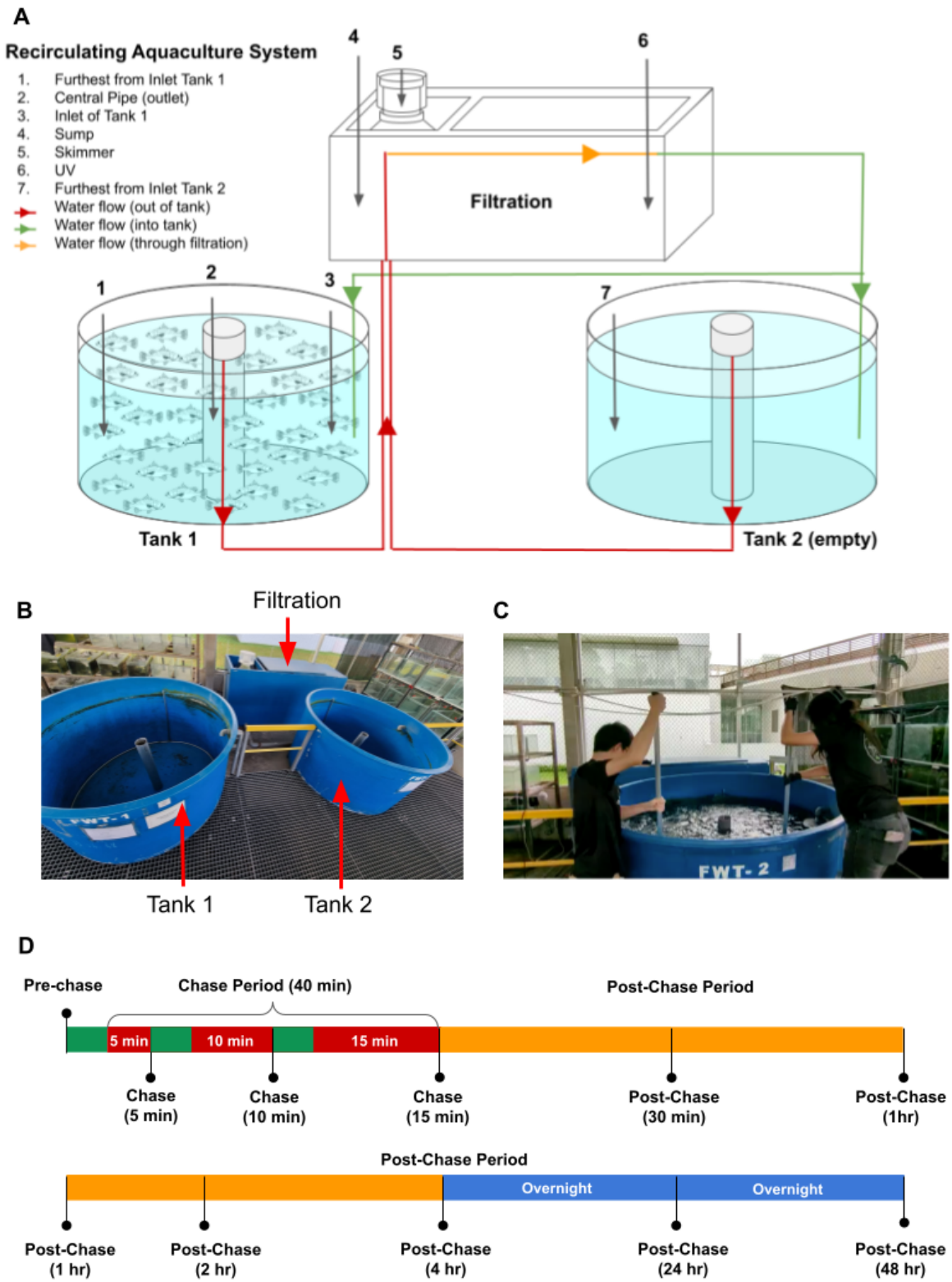
575 **Figures and Legends**

576 **Figure 1**

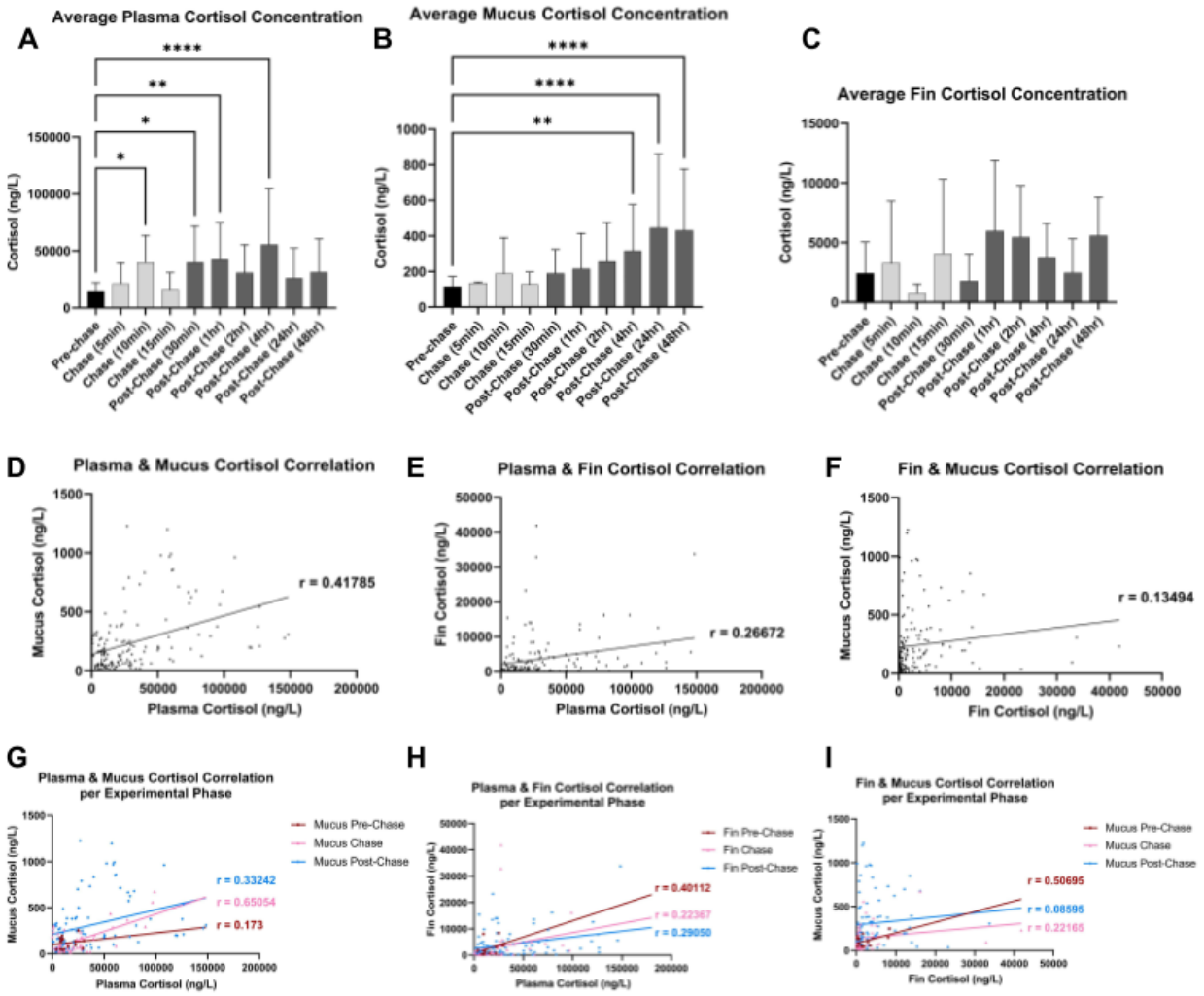


577

Figure 1: Schematic of experimental setup and design

(A) Design of the Recirculating Aquaculture System (RAS) used in our experiments, including water sample collection points.
(B) Image of the RAS
(C) Image showing how chasing was performed in Tank 1 using large nets in a C-shape manner. Air Exposure for as many fish as possible was performed for 10 s at the end of each chasing period (5 min, 10 min, 15 min) before 5 fish were sampled per time point.
(D) Experimental timeline from Pre-Chase to Post-Chase (48 hr). Black lines correspond to sampling time points. Red boxes represent progressively longer stress periods separated by 5-minute intervals (green boxes). Orange boxes represent the post-stress period on the same day, following which sampling was also done after overnight rest periods (blue boxes).

627 **Figure 2**



628

629 **Figure 2: Changes in plasma, mucus, and fin cortisol levels during and after a period of**
630 **chasing and air exposure stress**

631

632 (A) The average plasma cortisol concentrations of Asian sea bass across sampling points
633 (N = 5 fish per experiment, 15 fish per sampling point except for Chase (15min) where
634 one outlier was excluded) p = 0.0183 (Chase (10 min)), 0.0172 (Post-Chase (30 min)),
635 0.0066 (Post-Chase (1hr)), <0.0001 (Post-Chase (4hr))
636 (B) The average mucus cortisol concentrations of Asian sea bass across sampling points (N
637 = 5 fish per experiment, 15 fish per sampling point) p = 0.0033 (Post-Chase (4hr)),
638 <0.0001 (Post-Chase (24hr)), <0.0001 (Post-Chase (48hr))
639 (C) The average fin cortisol concentrations of Asian sea bass across sampling points (N = 5
640 fish per experiment, 15 fish per sampling point except for Pre-Chase and Post-Chase
641 (24hrs) where one outlier was excluded each)
642 (D) Correlations between plasma and mucus cortisol concentrations released from individual
643 fish across all time points (N = 148, r^2 = 0.1746, p <0.0001)
644 (E) Correlations between plasma and fin cortisol concentrations released from individual fish
645 across all time points (N = 147, r^2 = 0.07114, p = 0.0011)
646 (F) Correlations between mucus and fin cortisol concentrations released from individual fish

across all time points (N = 147, r^2 = 0.01821, p = 0.1032)

(G) Correlations between plasma and mucus cortisol concentrations released from individual fish per experimental phase (Pre-Chase: N = 15, r^2 = 0.02993, p = 0.5375. Chase: N = 45, R2 = 0.4232, p <0.0001. Post-Chase: N= 89, r^2 = 0.1105, p = 0.0015)

(H) Correlations between plasma and fin cortisol concentrations released from individual fish per experimental phase (Pre-Chase: N = 14, r^2 = 0.1609, p = 0.1552. Chase: N = 45, R2 = 0.05003, p = 0.1444. Post-Chase: N= 89, r^2 = 0.08439, p = 0.0058)

(I) Correlations between mucus and fin cortisol concentrations released from individual fish per experimental phase (Pre-Chase: N = 14, r^2 = 0.2570, p = 0.0643. Chase: N = 45, R2 = 0.04913, p = 0.1434. Post-Chase: N= 88, r^2 = 0.007388, p = 0.4259)

The asterisks (*) denote significant differences between means, with the corresponding significance levels: * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

Figure 3

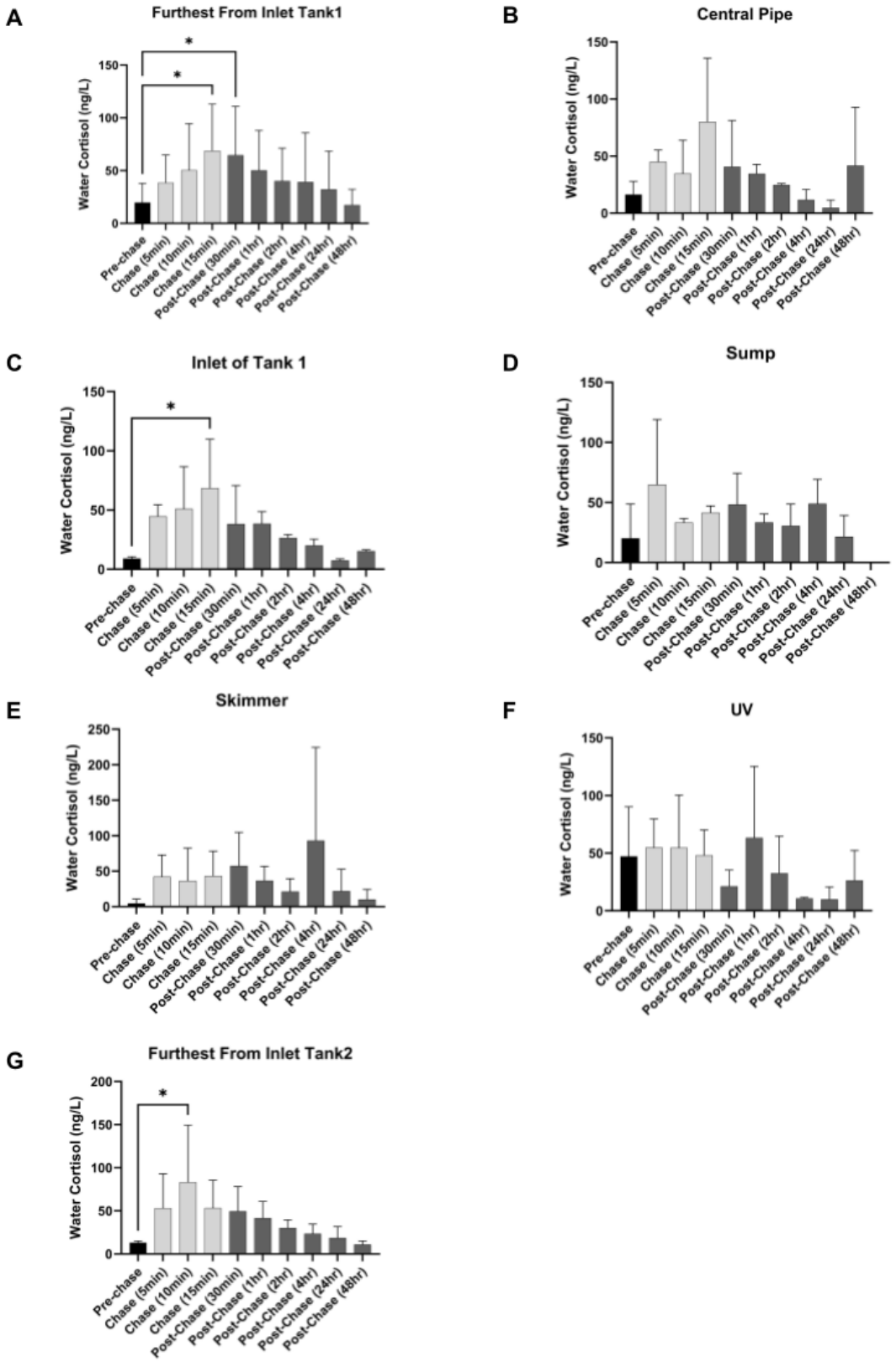

A
Furthest From Inlet Tank1
B
Central Pipe
C
Inlet of Tank 1
D
Sump
E
Skimmer
F
UV
G
Furthest From Inlet Tank2
Water Cortisol (ng/L)
Pre-chase
Chase (5min)
Chase (10min)
Chase (15min)
Post-Chase (30min)
Post-Chase (1hr)
Post-Chase (2hr)
Post-Chase (4hr)
Post-Chase (24hr)
Post-Chase (48hr)

Figure 3: Change in water cortisol across different sampling points in the RAS system during and after a period of chasing and air exposure stress

(A) Mean water cortisol concentrations (ng/L) at Furthest From Inlet Tank 1 (N = 3 per time point) p = 0.0197 (Chase (15 min)), 0.0367 (Post-Chase (30min))
(B) Mean water cortisol concentrations (ng/L) at Central Pipe (N = 2 per time point)
(C) Mean water cortisol concentrations (ng/L) at Inlet of Tank 1 (N = 2 per time point) p = 0.0287 (Chase (15 min))
(D) Mean water cortisol concentrations (ng/L) at Sump Tank (N = 2 per time point)
(E) Mean water cortisol concentrations (ng/L) at Skimmer (N = 2 per time point)
(F) Mean water cortisol concentrations (ng/L) at UV (N = 2 per time point)
(G) Mean water cortisol concentrations (ng/L) at Furthest From Inlet Tank 2 (N = 2 per time point) p = 0.0395 (Chase (10min))

Figure 4

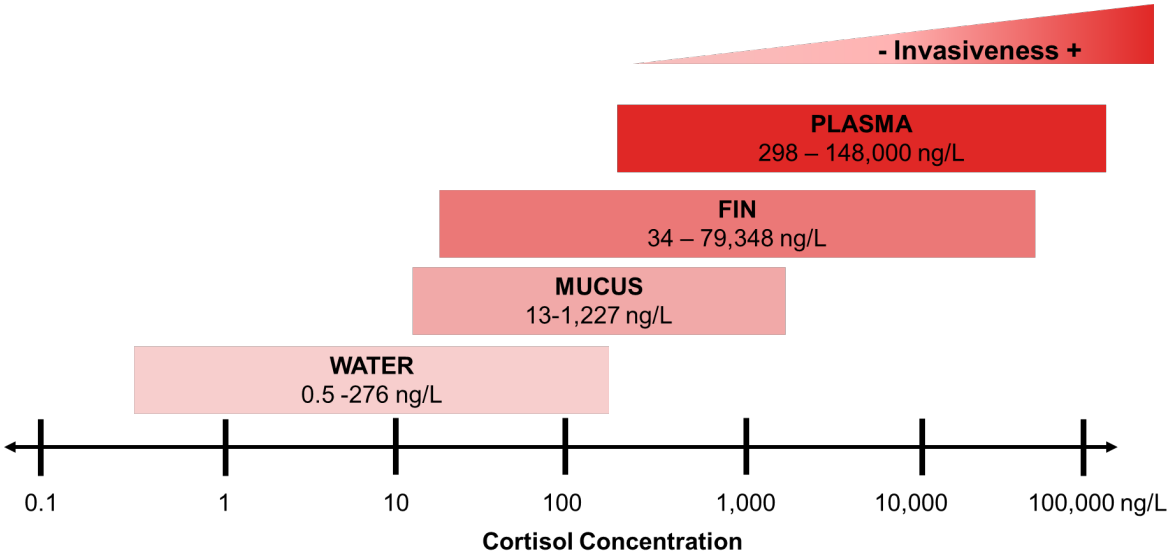


Figure 4: Cortisol concentrations across tissues and in water in Asian sea bass
Cortisol concentrations in this figure are derived from our handling stress experiments. The invasiveness level of each sampling method is indicated by the red color code. Note that the fin and mucus cortisol were normalized to the initial sample weight (100 mg of fin/1 mL of PBS) and mucus was dissolved in 1 mL PBS.