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

Marie Ruoyun Tan[1], Khin Moh Moh Aung[2], Nur Asinah binte Mohamed Salleh[2], Jolin Yean Ai Tan[3], Kai Xin Chua[3], Gaynah Javier Doblado[3], Kai Lin Chua[3], Valarie Tham[3], Jovian Jing Lin[3], Vindhya Chaganty[3], Dinah Mardhiana Yusoff[3], Shubha Vij[1,4], Xiaodi Su[2, 5], Laura Sutarlie[2]* & Caroline Lei Wee[3]*

[1] Republic Polytechnic, School of Applied Science, 9 Woodlands Ave 9, Singapore 738964

[2] Institute of Materials Research and Engineering (IMRE), Agency for Science, Technology and Research (A*STAR), 2 Fusionopolis Way, Innovis #08-03, Singapore 138634, Republic of Singapore

[3]Institute of Molecular and Cell Biology, Agency for Science, Technology and Research (A*STAR), 61 Biopolis Drive, Proteos, Singapore 138673, Republic of Singapore

[4]Tropical Futures Institute, James Cook University Singapore, 149 Sims Drive, 387380, Singapore

[5]Department of Chemistry, National University of Singapore, Block S8, Level 3, 3 Science Drive 3, Singapore 117543, Republic of Singapore

*Co-corresponding authors

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 1 hour 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 complementing 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, 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 (Snieszko, 1974; Bly, Quiniou and Clem, 1997; Daskalova, 2019). 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 (Scott, Pinillos and Ellis, 2001; Ellis *et al.*, 2004), noise (Mickle and Higgs, 2018), and overcrowding (Pavlidis *et al.*, 2013; Odhiambo *et al.*, 2020) 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, Martínez-Córdova and Ramos-Enriquez, 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 (Scott, Pinillos and Ellis, 2001; Sadoul and Geffroy, 2019).

Measurements of cortisol concentrations from mucus and fin have been used as less-invasive methods for stress monitoring (Simontacchi *et al.*, 2008; Bertotto *et al.*, 2009; De Mercado *et al.*, 2018; Ghassemi Nejad *et al.*, 2019). 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 (Scott, Pinillos and Ellis, 2001; Fanouraki *et al.*, 2008). Hence, regular measurement of cortisol in the water has been proposed as a non-invasive means of detecting elevated stress levels in farmed fish (Scott, Pinillos and Ellis, 2001; Sadoul and Geffroy, 2019). 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 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) within 12 L buckets with supplied O_2 and a high water flow rate. The higher stocking density tank was observed to have had a 2-3 fold higher water cortisol concentration throughout the 24-hour experiment. In a different study, Scott et al (2001) showed that 90-second air exposure of rainbow trout (*Oncorhynchus mykiss*) induced stress as measured from water cortisol (Scott, Pinillos and Ellis, 2001). The study was conducted in a 150 L flow-through tank, with a stocking density of 30 kg/m^3 at a water flow rate of 120 L/hour. A progressive increase in water cortisol concentrations was observed after single and repeated stress, peaking at 2 hours (25 ng/L) and 5 hours (100 ng/L) respectively, and gradually declining thereafter. The above studies used small volume, flow-through setups not comparable to farm-scale conditions.

More recently, water cortisol experiments have been conducted in RAS systems, albeit in relatively small setups. Mota et al (2017) examined the effect of RAS flow rate on water cortisol concentrations prior to and following an acute stressor (Mota *et al.*, 2017b). Nile Tilapia (*Oreochromis niloticus*) were raised at a stocking density of 67 kg/m^3 in 72 L tanks. After two weeks at a flow rate of 675 L/kg feed/day, water exchange rates were adjusted to 150 L/kg feed/day (LowRAS) or 1,500 L/kg feed/day (HighRAS) for 4 weeks, where LowRAS expectedly led to an increase in water cortisol concentrations over 4 weeks. Cortisol concentration peaked at 2 hours following an acute stressor of 60-second air exposure, increasing 30% in LowRAS from 5 ng/L to 7 ng/L, unlike the HighRAS condition, where a significant change was not observed. Another study by the same authors investigated the water exchange rate and pH on circulating cortisol in rainbow trout (*Oncorhynchus mykiss*) and the accumulation of cortisol in a RAS tank over a 70-day experimental period, with a starting stocking density of around 10 kg/m^3. On day 35, plasma cortisol was significantly elevated at low pH (5.8) with a mean concentration of 24.4 +/- 9.5 ng/mL, and elevated water cortisol (2.5 ng/L per kg) was also observed at low water

exchange rates (480 L/kg feed/day). These studies suggest that the flow rate of a RAS tank may significantly impact the ability to detect water cortisol, and a lower rate is essential for ensuring 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, high-flow 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 Institutional Animal Care and Use Committee (IACUC Protocol #2022/RP/00001). Chasing experiments were carried out in a 9,000 L saltwater (30 ppt) system, which consisted of 2 circular blue fiberglass tanks (each 3,000 L) containing the fish and 1 rectangular tank (3,000 L) as the sump (Figure 1A-B). Water was circulated at 8,000 L/hour.

Experimental Fish

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

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

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

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

Handling Stress experiments

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

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

Hence, there were a total of 11 sampling time points for chasing experiments; where 5 random fish were sampled at each sampling time point (Figure 1D). At each sampling point, the following samples and data were collected: water from various parts of the tank (see "Water

171 cortisol collection" section), fish body weight (total weight of the 5 fish), plasma, fin, and mucus
172 (See "Biological sample collection" section).

173 ***Biological sample collection***

174 After being netted, the fish were anesthetized in a bucket using Tricaine methanesulfonate MS-
175 222 (70 mg/L) and sodium bicarbonate (0.14 g/L) dissolved in fresh saltwater from the reservoir.
176 While still in the bucket, the fish were collectively weighed. The anesthesia took effect in
177 approximately 5 minutes, after which the fish were subjected to plasma, fin, and mucus collection,
178 as described in the following sections. On average, the entire process of collecting plasma, fin,
179 and mucus samples required approximately 5 minutes per fish.

180 ***Plasma collection***

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

189 ***Mucus collection***

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

194 ***Fin collection***

195 A fin clip was collected by cutting about 1.5 cm of the top part of the caudal fin. Tweezers were
196 then used to pick up the fins to be placed into 2 mL labeled Eppendorf tubes. The Eppendorf

197 tubes with the fins were then filled with 1 mL of PBS and stored in dry ice before being transferred
198 to a -20 °C freezer. After handling each fish, both tweezers and scissors were sanitized with 70%
199 ethanol.

200 After sample collection, the fish were first placed in recovery saltwater buckets with no
201 tricaine to recover from the anesthesia. This measure was implemented to prevent the resampling
202 of the same fish at each sampling time and to avoid any potentially stressful effects caused by
203 the reintroduction of the sampled fish. In the first experiment where water cortisol was only
204 measured from "Tank 1 - Furthest from inlet", the sampled fish were placed into "Tank 2" post-
205 recovery from anesthesia. The latter was the other blue fiberglass tank connected to the entire
206 recirculating system. In subsequent experiments, given that we were also sampling water from
207 other parts of the system including Tank 2, the sampled fish were placed into a separate holding
208 tank not connected to the circulating system and only returned at the end of the day after the
209 "Post-chase 4 h" time point (Experiment 2), or in the case of Experiment 3, these fish were culled.
210 We note that there were minimal changes in the system stocking densities caused by these
211 differences (Supplementary Table 1), and as reported in the "Results" section, these minor
212 variations did not appear to affect the overall trend of biological or water cortisol changes.

213 ***Water sample collection***

214 In Experiment 1, tank water was only collected from a single sampling point "Tank 1 Furthest from
215 Inlet". In Experiments 2 and 3, tank water was additionally sampled from 6 other sampling points
216 (Figure 1A). Besides 1 - "Furthest From Inlet Tank 1", water was sampled from the 2 - "Central
217 Pipe", 3 - "Inlet Tank 1" , 4 - "Sump", 5 - "Skimmer", 6 - "UV" compartment and 7 - "Furthest From
218 Inlet Tank 2". Gloves were consistently worn during the collection of water cortisol samples to
219 avoid potential contamination from human skin-derived cortisol. Water was collected using
220 siphons, positioned at a height that was midway through the water column. These siphons were
221 thoroughly rinsed with clean freshwater at each respective collection point. To ensure thorough
222 flushing of the siphon at each sampling point, the tank water was allowed to flow through the

223 siphon for at least 30 seconds before a 100 mL or 125 mL glass bottle was filled to the brim.
224 These water samples were subsequently kept chilled in an ice box and later transferred to a 4°C
225 fridge for storage. Water from the system that had been siphoned out but not collected in the
226 glass bottles was returned into the system to maintain a relatively constant water volume within
227 the system.

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

229 Sample processing was performed according to protocols adapted from previous methods
230 (Ghassemi Nejad *et al.*, 2019) with some modification. Mucus samples stored in a -20 °C freezer,
231 were thawed to room temperature before processing. The microcentrifuge tube containing the
232 swab with mucus was vortexed for 30 seconds, after which, the mucus was scrubbed from the
233 swab with the wall and rim of the tube repeatedly. Following this, the solution was filtered using a
234 0.22 µm Polyvinylidene fluoride (PVDF) syringe filter and stored at a 4 °C fridge for analysis within
235 a 24-hour timeframe.

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

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

246 The cortisol levels from the three biological substrates (plasma, fin and mucus) were quantified
247 using an enzyme-linked immunosorbent assay (ELISA) kit (Fish Cortisol ELISA, CUSABIO,
248 Houston, TX, USA). The ELISA is based on the competitive inhibition enzyme immunoassay

249 principle, involving the interaction between pre-coated cortisol and cortisol within the samples,
250 and has a cortisol detection range of 0.0023–10 ng/mL.

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

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

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

275 The cortisol extracted from water samples was analyzed using HPLC (Shimadzu LC-
276 2050C 3D, Japan) coupled to a photodiode array (PDA) detector set at 245 nm. The HPLC was
277 performed by injecting 100 µL of the extracted water sample through a Shim-Pact GIST C18
278 normal-phase column (inner diameter 4.6mm, length 250 mm, particle size 5 µm). An isocratic
279 flow of mobile phases (methanol and 10 mM ammonium formate in water in a 1:1 ratio) was
280 performed for 30 minutes. Using the HPLC conditions mentioned above, cortisol was eluted with
281 a retention time of 14.5 minutes. The HPLC is calibrated to cortisol standards ranging from 1 pM
282 to 10 µM in 50% methanol solution. All water samples and standards were spiked with 500 µL of
283 1 µM cortisol internal standard. Cortisol peak area from the standards was measured and plotted
284 against the cortisol standards concentration to build a standard curve. The cortisol concentration
285 in the water sample is calculated based on the standard curve according to the peak area in each
286 water sample. Each water sample was measured as duplicates.

287 ***Data analysis and Statistics***

288 Cortisol concentrations obtained from plasma, fin and mucus samples were calculated based on
289 a standard curve run on each plate and expressed in ng/L. Adjustments were made for dilution
290 factor (plasma), initial sample weight (100 mg of fin/1 mL of PBS) and mucus dissolved in 1 mL
291 PBS. In Figure 3, the water cortisol concentration was not standardized to biomass. However, a
292 standardization to system stocking density (kg/m^3) was performed in Supplementary Figures 4-5.

293 To compare cortisol concentrations across different time points, a 2-way ANOVA was
294 applied, followed by the Dunnett test with multiple comparison correction. To ascertain the
295 relationship between less-invasive measurements of mucus and fin cortisol and cortisol levels in
296 blood plasma, we conducted simple linear regression analysis. Using the Grubbs' test (Alpha =
297 0.0001) to identify outliers, we removed two fin cortisol (Experiment A, Pre-chase and Post-Chase
298 (24hr)) and one plasma cortisol (Experiment A, Chase (15min)) measurement in our analysis.

299 Since the biological replicates are conducted on batches of fish with different
300 characteristics and spanning six months, we also performed a boot-strapping based permutation

301 test to establish the statistical robustness of our conclusions (MATLAB, USA). For 10,000
302 iterations, we first randomly sampled an experiment (1, 2, or 3), and from this experiment then
303 randomly sampled (with replacement) 5 fish from each chase / post-chase time point. We then
304 compared the mean cortisol concentration of this group with the pre-chase control, which was
305 similarly randomly sampled. This allowed us to quantify the percentage of iterations in which the
306 cortisol concentration would be higher in each post-chase time point compared to the pre-chase
307 controls, providing a p-value for each time point. This permutation analysis was conducted for
308 each tissue type. In this analysis, we did not exclude any outliers. Using this same analysis we
309 also estimated the distribution of effect sizes (cohen's d) that would be observed (Supplementary
310 Figure 2). In the figures and tables, asterisks are used to indicate statistical significance levels as
311 follows *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

312 **Results**

313 The acute handling stress experiment was repeated three times over a period of six months, as
314 described in Table 1. Given its large scale, we were unable to simultaneously conduct multiple
315 replicates, and instead conducted biological replicates sequentially over time. The origins, age,
316 weight, and stocking densities of the fish inevitably varied, which led to varying absolute cortisol
317 concentrations as quantified by ELISA analyses (Table 1, Supplementary Figure 1). However,
318 handling stress and sampling protocols were kept consistent, and consistent behavioral changes
319 were observed in each chasing experiment, where the fish exhibited rapid swimming, actively
320 evaded the nets, and displayed signs of distress upon exposure to air. Instances of vomiting were
321 observed, and foamy bubbles were observed on the surface of the tank, starting from around the
322 "Chase (10 min)" period and increasing over time, which may reflect increased mucus secretion.

323 To quantify the effects of such handling stress on plasma, mucus, and fin cortisol across
324 these diverse conditions, we employed two statistical methods, a 2-way ANOVA (across
325 experiment and time point) as well as bootstrapping analysis. For the latter, we randomly sampled
326 with replacement data from fish within each experiment, as well as the experiment (1, 2, or 3)

327 from which the fish were sampled, for 10,000 iterations, to generate a bootstrapped distribution
328 of effect sizes (Supplementary Figure 2) as well as a p-value for each time point (Table 2)
329 demonstrating the probability of cortisol concentrations being higher at that time point relative to
330 control.

331 The results of both statistical analyses are tabulated in Table 2, and we only consider
332 results that were significant across both statistical tests. Under this stringent criterion, we report
333 a significant increase in plasma cortisol levels (Figure 2A), at the Chase (10 min), Post-chase (30
334 min and 1 hr) time points, with a complete return to baseline by 24 hours post-chase. In contrast,
335 mucus cortisol showed a slower time course (Figure 2B), with a gradual increase of mucus cortisol
336 over time, which only achieved significance at 24 and 48 hrs post-chase. Notably, mucus cortisol
337 concentrations were about 100 fold lower than plasma or fin cortisol concentrations (Figure 2A-
338 C). No significant values were obtained for fin cortisol (Figure 2C).

339
340 **Table 2: Summary of statistical analyses results using ANOVA and bootstrapping**
341 (dark gray = p<0.05 in both tests, light gray = p <0.05 in one test)

Time points	Plasma		Mucus		Fin	
	ANOVA	**Bootstrap**	**ANOVA**	**Bootstrap**	**ANOVA**	**Bootstrap**
Chase (5 min)	0.9737	0.3374	>0.9999	0.4147	0.9998	0.7656
Chase (10 min)	0.0183*	0.0115*	0.7128	0.6060	0.9673	0.5951
Chase (15 min)	>0.9999	0.1726	>0.9999	0.3560	0.9715	0.7236
Post-chase (30 min)	0.0172*	0.0194*	0.7181	0.2667	>0.9999	0.8522
Post-chase (1 hr)	0.0066**	0.0464*	0.3574	0.3423	0.4202	0.3211
Post-chase (2 hr)	0.2616	0.1431	0.0803	0.1634	0.5990	0.3574
Post-chase (4 hr)	<0.0001***	0.3089	0.0033**	0.0718	0.9930	0.4320
Post-chase (24 hr)	0.6323	0.3656	<0.0001***	0.0193*	>0.9999	0.8369
Post-chase (48 hr)	0.2317	0.1114	<0.0001***	0.0000***	0.5471	0.3100

342

343 Next, we correlated cortisol levels across the biological samples (fin, mucus, and plasma)
344 collected from each individual fish. Plasma cortisol was most strongly correlated with mucus
345 cortisol ($r = 0.42$, $p < 0.0001$, Figure 2D), whereas both fin and plasma ($r = 0.27$, $p = 0.0011$, Figure
346 2E), and fin and mucus cortisol ($r = 0.14$, $p = 0.1032$, Figure 2F), were only weakly correlated.
347 Notably, the correlation between plasma and mucus cortisol was highest during the chasing
348 period ($r = 0.65$, $p < 0.0001$) as compared to the pre-chase ($r = 0.33$, $p = 0.5375$) and post-chase
349 ($r = 0.17$, $p = 0.0015$) periods (Figure 2G). In contrast, plasma cortisol was more strongly
350 correlated with fin cortisol during the pre-chase period, albeit non-significant ($r = 0.40$, $p = 0.1552$),
351 as compared to the chase ($r = 0.22$, 0.05003) and post-chase ($r = 0.29$, $p = 0.0058$) period (Figure
352 2H). Fin and mucus cortisol were most highly correlated in the pre-chase ($r = 0.51$, $p = 0.0643$)
353 and chase ($r = 0.22$, $p = 0.1434$) period as compared to post-chase ($r = 0.09$, $p = 0.4259$) period
354 (Figure 2I).

355 The dynamics of water cortisol circulating in farm-scale RAS systems has not been
356 previously characterized, posing a practical challenge to implementing on-farm water cortisol
357 monitoring at scale. Hence, we sought to understand how water cortisol may be distributed in the
358 RAS system following an acute stressor. To this end, we quantified cortisol from tank water
359 sampled from 1 to 7 points in the RAS indicated in Figure 1A. In Experiment 1, tank water was
360 only collected from a single sampling point "Tank 1 Furthest from Inlet", whereas in Experiments
361 2 and 3, tank water was additionally sampled from 6 other sampling points to study the distribution
362 of cortisol across various locations within the RAS.

363 Due to the low concentrations of cortisol in water relative to biological samples, and the
364 higher potential for antibody interference in ELISA due to contaminants (e.g., salts and organic
365 material) in tank water, we opted to use HPLC analysis for more precise measurements of water
366 cortisol concentrations. Consistent with reports in existing literature, water cortisol concentrations
367 were in the range of ~10 to ~100 ng/L, about 1,000 fold lower than the concentrations observed
368 in plasma and fin samples, and 10-fold lower than mucus samples. In all sampling points within

369 the tank containing the fish (Tank 1), we observed a consistent trend of increased water cortisol
370 starting from the chasing period (Figure 3A-C). For the “Tank 1 Furthest from Inlet” sampling point,
371 there was a significant increase in water cortisol at the Chase (15 min, $p = 0.0197$) and Post-
372 chase (30 min) time points ($p = 0.0367$). Water cortisol concentrations were also significantly
373 elevated at the “Inlet of Tank 1) at the Chase (15 min) time point ($p = 0.0287$), with a similar peak
374 for the “Central pipe” albeit non-significant. The cortisol concentrations from the other RAS
375 compartments did not exhibit distinct variations, although water cortisol concentrations in the
376 “Sump” and “Skimmer” were on average higher during and after the chase (Figure 3D and 3E).
377 The “UV” compartment, on the other hand, did not show any trends (Figure 3F). Interestingly, in
378 Tank 2, which was not holding any fish, a similar trend of increased water cortisol, significant at
379 the Chase (10 min) time point, was observed (Figure 3G). This observation suggests the
380 possibility that cortisol released into the water from one tank may be distributed to other RAS
381 tanks within the system. Detailed cortisol concentration data from individual experiments, as
382 absolute values or normalized to system stocking density (since some fish were removed for
383 sampling), can be found in Supplementary Figures 3-5.

384 **Discussion**

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

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

392 From this acute handling stress study, first, we confirmed that our manipulation indeed
393 triggered a significant increase in plasma cortisol starting from the Chase (10 min) time point,
394 which would be 20 minutes into the chasing period (Figure 2A). There continues to be a significant

395 increase in plasma cortisol up to 1 hour post-chase, and recovery at 24 and 48 hours. We note
396 that some of the chase and post-chase time points did not attain statistical significance, which
397 could be attributed to biological variability across the 3 replicates, influenced by the large number
398 of fish in the tanks and variations in fish age, genetic background, and size (Table 1). Despite
399 these differences, the overall early detection of cortisol is consistent with previous such studies
400 where it has been shown that plasma cortisol typically peaks between 0.5-4 h post stress stimulus
401 in fish (Scott, Pinillos and Ellis, 2001; Fanouraki *et al.*, 2008; Scott *et al.*, 2008; Mota *et al.*, 2017a).
402 For instance, it peaks at 1-2 hr in carp and 2-4 hr in roach post-tag insertion (Lower *et al.*, 2005);
403 2 hr in gilthead seabream post-crowding stress (Guardiola, Cuesta and Esteban, 2016), 0.5 - 1
404 hr in rainbow trout (Ellis *et al.*, 2004) and 3 hr in Atlantic Salmon post air exposure stress (Ellis *et*
405 *al.*, 2007); and 4 hr post-injection with ACTH (adrenocorticotropic hormone) in Threadfin (Bshary
406 *et al.*, 2007). Interestingly, a study in European seabass recorded significantly high plasma
407 cortisol already at 0 hr post chasing and air exposure stress, peaking at 1 hr post-stress
408 (Fanouraki *et al.*, 2008), which coincides with the plasma cortisol trends in our Asian seabass
409 study.

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

411 We also simultaneously sampled mucus and fin cortisol from individual fish, to investigate the
412 viability of these less-invasive sampling methods as reliable indicators of fish stress in aquaculture
413 settings. Our findings revealed that mucus cortisol was significantly increased following the stress-
414 inducing event, becoming significantly elevated at the post-stress 24- and 48-hour time points,
415 even when plasma cortisol had already fully returned to baseline levels. However, this increase
416 followed a slower time course compared to that of plasma cortisol. Similarly as shown in Madaro
417 et al (2022), the rise in cortisol in mucus of Atlantic salmon after netting stress was delayed
418 compared to blood plasma (Madaro *et al.*, 2023). In their study, mucus levels remained
419 significantly elevated even at 300 min post-stress, which was the longest time point they
420 assessed. Guardiola et al (2016) also reported a sustained increase in mucus cortisol at 24 and

421 48-hours post-crowding stress, whereas plasma cortisol only showed a significant increase at 2
422 hours post-stress (Guardiola, Cuesta and Esteban, 2016).

423 While the biological basis of these differences is still not well-understood, it is speculated
424 that either the accumulation of plasma cortisol in the mucus, or the reabsorption of cortisol from
425 the water might account for these observed patterns. Despite the differences in cortisol dynamics,
426 we observed a reasonably strong correlation between plasma and mucus cortisol (r = 0.65) during
427 the chasing period. Similarly, one of the few other studies reported the strongest correlation (r =
428 0.70) between mucus and plasma cortisol in rainbow trout (*Oncorhynchus mykiss*) during the
429 hours following confinement stress, relative to the control or late phase (Carbajal *et al.*, 2019).
430 Our data thus suggests that mucus cortisol could be a useful, less invasive way to sample fish
431 stress in Asian Sea bass. In contrast, cortisol extracted from fin did not display any consistent
432 trend or relationship with plasma cortisol. While this does not preclude its utility in estimating fish
433 stress, it may be less robust across the varied conditions in our experiments.

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

435 We also measured water cortisol within the fish holding tank (Tank 1) across all three acute stress
436 experiments, and observed a significant rise in water cortisol in the Chase (15 min) and post-
437 chase (30 min) periods, which correspond to 40 minutes up to 70 minutes (1 hr 10 mins) from the
438 initiation of the chasing period. In comparison to other previous water cortisol studies, these water
439 cortisol changes were observable in a higher flow system (8000L / hr), again demonstrating the
440 feasibility of water cortisol monitoring in farm-scale settings (Scott, Pinillos and Ellis, 2001;
441 Fanouraki *et al.*, 2008; Mota *et al.*, 2017a, 2017b). Notably, the rise in water cortisol paralleled
442 the rise in plasma cortisol, except that plasma cortisol continued to be significantly elevated at 1-
443 hr post-chase.

444 In the latter two of the three experiments, we further measured water cortisol across
445 different parts of the tank and also different RAS compartments, to identify ideal sampling
446 locations for water cortisol for on-farm stress monitoring. The same trend was still observed in

447 other parts of Tank 1, where the peak in water cortisol still occurred at 40 minutes from the
448 commencement of the stress event (the “Chase (15 min)” time point)) and decreased earlier than
449 plasma cortisol. These results suggest that the dynamics of water cortisol in the RAS system may
450 be influenced by other factors, including flow rate, filtration (i.e. protein skimmer), or binding to
451 the plastic tank walls (since it is hydrophobic). Alternatively, cortisol could potentially be
452 reabsorbed by the fish, possibly into the mucosal layer.

453 We also observed the presence of water cortisol in other RAS compartments, such as the
454 protein skimmer and sump, indicating the distribution of water cortisol from Tank 1 to other
455 compartments. While water cortisol concentrations in the “Sump” and “Skimmer” were on average
456 higher during and after the chase, the increase was not significant, that could be due to the low
457 number of biological replicates and the varying absolute water cortisol concentrations across the
458 two replicates (Supplementary Figures 3-5). The exception was for the UV compartment, where
459 a change in water cortisol post-chase was not so apparent, either due to UV radiation-induced
460 degradation, or other factors. Interestingly, a rise in cortisol, significant at the Chase (10 min) time
461 point, was observed in Tank 2, which did not contain any fish. This result highlights that due to
462 water recirculation in RAS, other connected tanks may also be exposed to the cortisol released
463 from a stressed tank of fish. It would be interesting to further investigate the effects and
464 implications of water cortisol transmission to fish in connected RAS tanks.

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

472 ***Caveats and limitations of our study***

473 In typical research studies, biological replicates would be conducted in parallel on a single batch
474 of fish, within smaller tank setups. In our study, we aimed to simulate the conditions of a farm-
475 scale setup, using 3,000 L tanks on a 9,000 L RAS system. Due to the scale of our experiments,
476 the limited number of such systems available, and the challenge of acclimatizing and cultivating
477 a large number of fish, we were constrained to conduct one biological replicate at a time, with our
478 experiments spread over a course of 6 months. We also were only able to measure water cortisol
479 from different RAS compartments in 2 of the 3 experiments.

480 As expected, the cortisol measurements obtained in this study exhibited variability across
481 samples and time points. Several factors contributed to this variability; firstly, the variations in the
482 ages, sizes, genetic background, and stocking densities of the fish tested (Table 1). Secondly,
483 despite maintaining a highly consistent chasing and air exposure stress protocol, the random
484 selection of the 5 fish sampled per time point (per experiment) likely contributed additional
485 variability due to the large numbers of fish present in the tank.

486 While not ideal in an experimental setting, the upside of such variability is that any signal
487 we have obtained would likely be reproducible even with the normal diversity experienced in on-
488 farm settings. Across all biological replicates, we observed an increase in plasma, mucus, and
489 water cortisol levels, with relatively consistent dynamics. Importantly, our results further argue
490 that surveying water cortisol, which provides an average readout of the stress levels in the system,
491 might be a means of overcoming the variability from sampling individual fish. Further, given that
492 the stocking densities fell within the low- (6 kg/m^3) to medium-range (20 kg/m^3), it is possible that
493 more pronounced changes in cortisol might be observable at higher densities.

494 **Conclusion**s

495 Our results corroborate that in a farm-scale RAS setting across varying conditions, water cortisol
496 is a reliable measure of Asian sea bass stress and that different tissue types such as fin and
497 mucus may reflect stress over different dynamical timescales. These results are likely

generalizable to other fish species and tank setups, though it is important to note that the time course and concentrations or rates of cortisol release may differ across species. The findings of 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.

Acknowledgements

This research is supported by the A*STAR Agritech and Aquaculture Horizontal Programme Office (A2HTPO) seed grant (C211018003) awarded to Laura Sutarlie, Xiaodi Su, Caroline Lei Wee and collaborators in Republic Polytechnic and Aquaculture Innovation Center (AIC), and also the Confirma Programme awarded to Caroline Lei Wee by A*STAR. We would like to extend our gratitude to Ng Yi Long, for his valuable help and guidance in fish husbandry. We would also like to acknowledge Tan Kwang Meng and Joyce Koh for their assistance during the experiments. Additionally, we would like to also thank the teams of Republic Polytechnic Final Year Project students: Cung Nei Mawi Lian, Nur Adillah Bte Rahiam, Nur Iffah Dianah Bte Jumadi, Nurul Insyirah Binte Mohamed Arffah, Putri Nur Aisyah Bte Sazali, Yong Sai Kit, Cheryl Lim, Lee Jerry, Sammi Hong Wenxuan and Stahlmann Sarah Elizabeth. We also would like to acknowledge Dr. Lee Chee Wee, Dr. Saravanan Padmanabhan, and Dr. Diana Chan from AIC for discussions.

References

Ardiansyah and Fotedar, R. (2016) ‘Water quality, growth and stress responses of juvenile barramundi (Lates calcarifer Bloch), reared at four different densities in integrated recirculating aquaculture systems’, *Aquaculture* , 458, pp. 113–120. Available at: https://doi.org/10.1016/j.aquaculture.2016.03.001.

Bertotto, D. *et al.* (2009) ‘Alternative matrices for cortisol measurement in fish’, *Aquaculture research* [Preprint]. Available at: https://doi.org/10.1111/j.1365-2109.2009.02417.x.

Bly, J.E., Quiniou, S.M. and Clem, L.W. (1997) ‘Environmental effects on fish immune mechanisms’, *Developments in biological standardization*, 90, pp. 33–43. Available at: https://www.ncbi.nlm.nih.gov/pubmed/9270832.

Bshary, R. *et al.* (2007) ‘Do cleaning organisms reduce the stress response of client reef fish?’, *Frontiers in zoology*, 4, p. 21. Available at: https://doi.org/10.1186/1742-9994-4-21.

Carbajal, A. *et al.* (2019) ‘Comparative assessment of cortisol in plasma, skin mucus and scales as a measure of the hypothalamic-pituitary-interrenal axis activity in fish’, *Aquaculture* , 506, pp. 410–416. Available at: https://doi.org/10.1016/j.aquaculture.2019.04.005.

Daskalova, A. (2019) ‘Farmed fish welfare: stress, post-mortem muscle metabolism, and stress-related meat quality changes’, *International aquatic research*, 11(2), pp. 113–124. Available at: https://doi.org/10.1007/s40071-019-0230-0.

De Mercado, E. *et al.* (2018) ‘Skin mucous: A new approach to assess stress in rainbow trout’, *Aquaculture* , 484, pp. 90–97. Available at: https://doi.org/10.1016/j.aquaculture.2017.10.031.

Ellis, T. *et al.* (2004) ‘A non-invasive stress assay based upon measurement of free cortisol released into the water by rainbow trout’, *Journal of fish biology*, 65(5), pp. 1233–1252. Available at: https://doi.org/10.1111/j.0022-1112.2004.00499.x.

Ellis, T. *et al.* (2007) ‘Acute viral and bacterial infections elevate water cortisol concentrations in fish tanks’, *Aquaculture* , 272(1), pp. 707–716. Available at: https://doi.org/10.1016/j.aquaculture.2007.07.235.

Fanouraki, E. *et al.* (2008) ‘Water cortisol is a reliable indicator of stress in European sea bass, Dicentrarchus labrax’, *Behaviour*, 145(10), pp. 1267–1281. Available at: https://doi.org/10.1163/156853908785765818.

Ghassemi Nejad, J. *et al.* (2019) ‘Cortisol Extraction from Sturgeon Fin and Jawbone Matrices’, *Journal of visualized experiments: JoVE* [Preprint], (151). Available at: https://doi.org/10.3791/59961.

Glencross, B. (2006) ‘The nutritional management of barramundi, Lates calcarifer - a review’, *Aquaculture Nutrition*, 12(4), pp. 291–309. Available at: https://doi.org/10.1111/j.1365-2095.2006.00410.x.

Guardiola, F.A., Cuesta, A. and Esteban, M.Á. (2016) ‘Using skin mucus to evaluate stress in gilthead seabream (Sparus aurata L.)’, *Fish & shellfish immunology*, 59, pp. 323–330. Available at: https://doi.org/10.1016/j.fsi.2016.11.005.

Hong, J. *et al.* (2021) 'Effects of transporting stress on the immune responses of Asian seabass *Lates calcarifer* fry', *Aquaculture research*, 52(5), pp. 2182–2193. Available at: https://doi.org/10.1111/are.15071.

Islam, M.A. *et al.* (2023) 'Culture technique of seabass, Lates calcarifer in Asia: A review', 04(01), pp. 6–017. Available at: https://doi.org/10.53771/ijstra.2023.4.1.0174.

Katersky, R.S. and Carter, C.G. (2005) 'Growth efficiency of juvenile barramundi, Lates calcarifer, at high temperatures', *Aquaculture* , 250(3), pp. 775–780. Available at: https://doi.org/10.1016/j.aquaculture.2005.05.008.

Keat, L.E. (2021) *The innovation and transformation of Asean's agri-food industry*. Available at: https://www.businesstimes.com.sg/international/asean/innovation-and-transformation-aseans-agri-food-industry (Accessed: 19 April 2023).

Lower, N. *et al.* (2005) 'A non-invasive method to assess the impact of electronic tag insertion on stress levels in fishes', *Journal of fish biology*, 67(5), pp. 1202–1212. Available at: https://doi.org/10.1111/j.1095-8649.2005.00815.x.

Lupica, S.J. and Turner, J.W., Jr (2010) 'Noninvasive assessment of nitrate-induced stress in koi Cyprinus carpio L. by faecal cortisol measurement', *Aquaculture research*, 41(11), pp. 1622–1629. Available at: https://doi.org/10.1111/j.1365-2109.2010.02496.x.

Madaro, A. *et al.* (2023) 'Acute stress response on Atlantic salmon: a time-course study of the effects on plasma metabolites, mucus cortisol levels, and head kidney transcriptome profile', *Fish physiology and biochemistry*, 49(1), pp. 97–116. Available at: https://doi.org/10.1007/s10695-022-01163-4.

Martínez-Porchas, M., Martínez-Córdova, L.R. and Ramos-Enriquez, R. (2009) 'Cortisol and Glucose: Reliable indicators of fish stress?', *Pan-American journal of aquatic sciences*, 4(2), pp. 158–178. Available at: http://dx.doi.org/ (Accessed: 24 September 2021).

Mickle, M.F. and Higgs, D.M. (2018) 'Integrating techniques: a review of the effects of anthropogenic noise on freshwater fish', *Canadian journal of fisheries and aquatic sciences. Journal canadien des sciences halieutiques et aquatiques*, 75(9), pp. 1534–1541. Available at: https://doi.org/10.1139/cjfas-2017-0245.

Mota, V.C. *et al.* (2017a) 'Cortisol and testosterone accumulation in a low pH recirculating aquaculture system for rainbow trout (Oncorhynchus mykiss)', *Aquaculture research*, 48(7), pp. 3579–3588. Available at: https://doi.org/10.1111/are.13184.

Mota, V.C. *et al.* (2017b) 'Water cortisol and testosterone in Nile tilapia (Oreochromis niloticus) recirculating aquaculture systems', *Aquaculture* , 468, pp. 255–261. Available at: https://doi.org/10.1016/j.aquaculture.2016.10.023.

Ney, L.J. *et al.* (2021) 'Chloroform-based liquid-liquid extraction and LC-MS/MS quantification of endocannabinoids, cortisol and progesterone in human hair', *Journal of pharmaceutical and biomedical analysis*, 201, p. 114103. Available at: https://doi.org/10.1016/j.jpba.2021.114103.

Odhiambo, E. *et al.* (2020) 'Stocking Density Induced Stress on Plasma Cortisol and Whole Blood Glucose Concentration in Nile Tilapia Fish (Oreochromis niloticus) of Lake Victoria, Kenya', *International Journal of Zoology*, 2020. Available at:

https://doi.org/10.1155/2020/9395268.

Pavlidis, M. *et al.* (2013) 'Husbandry of zebrafish, Danio rerio, and the cortisol stress response', *Zebrafish*, 10(4), pp. 524–531. Available at: https://doi.org/10.1089/zeb.2012.0819.

Sadoul, B. and Geffroy, B. (2019) 'Measuring cortisol, the major stress hormone in fishes', *Journal of fish biology*, 94(4), pp. 540–555. Available at: https://doi.org/10.1111/jfb.13904.

Scott, A.P. *et al.* (2008) 'Non-Invasive Measurement of Steroids in Fish-Holding Water: Important Considerations When Applying the Procedure to Behaviour Studies', *Behaviour*, 145(10), pp. 1307–1328. Available at: http://www.jstor.org/stable/40296047.

Scott, A.P., Pinillos, M. and Ellis, T. (2001) 'Why measure steroids in fish plasma when you can measure them in water?', in *14th International Congress of Comparative Endocrinology*. Monduzzi Editore S.p.A. Available at: https://www.cefas.co.uk/publications/files/3402.pdf (Accessed: 24 September 2021).

Simontacchi, C. *et al.* (2008) 'Alternative stress indicators in sea bass Dicentrarchus labrax, L', *Journal of fish biology*, 72(3), pp. 747–752. Available at: https://doi.org/10.1111/j.1095-8649.2007.01717.x.

Snieszko, S.F. (1974) 'The effects of environmental stress on outbreaks of infectious diseases of fishes', *Journal of fish biology*, 6(2), pp. 197–208. Available at: https://doi.org/10.1111/j.1095-8649.1974.tb04537.x.

Tanaka, Y. *et al.* (2023) 'Cortisol Biosensors: From Sensing Principles to Applications', *World Scientific Annual Review of Functional Materials*, 01, p. 2330001. Available at: https://doi.org/10.1142/S2810922823300015.

Taylor, M. (2022) *What Are the Health Benefits of Barramundi?*, *WebMD*. Available at: https://www.webmd.com/diet/health-benefits-barramundi (Accessed: 19 April 2023).

Triki, Z. *et al.* (2016) 'Effects of short-term exposure to ectoparasites on fish cortisol and hematocrit levels', *Marine biology*, 163(9), p. 187. Available at: https://doi.org/10.1007/s00227-016-2959-y.

Viljoen, F.P. *et al.* (2019) 'An HPLC-DAD validated method for the detection and quantification of cortisol, corticosterone and melatonin in plasma samples of two different animal species', *Die Pharmazie*, 74(4), pp. 206–211. Available at: https://doi.org/10.1691/ph.2019.8225.

Zarantoniello, M. *et al.* (2021) 'Salinity, Temperature and Ammonia Acute Stress Response in Seabream (Sparus aurata) Juveniles: A Multidisciplinary Study', *Animals : an open access journal from MDPI*, 11(1). Available at: https://doi.org/10.3390/ani11010097.

Figures and Legends

Figure 1

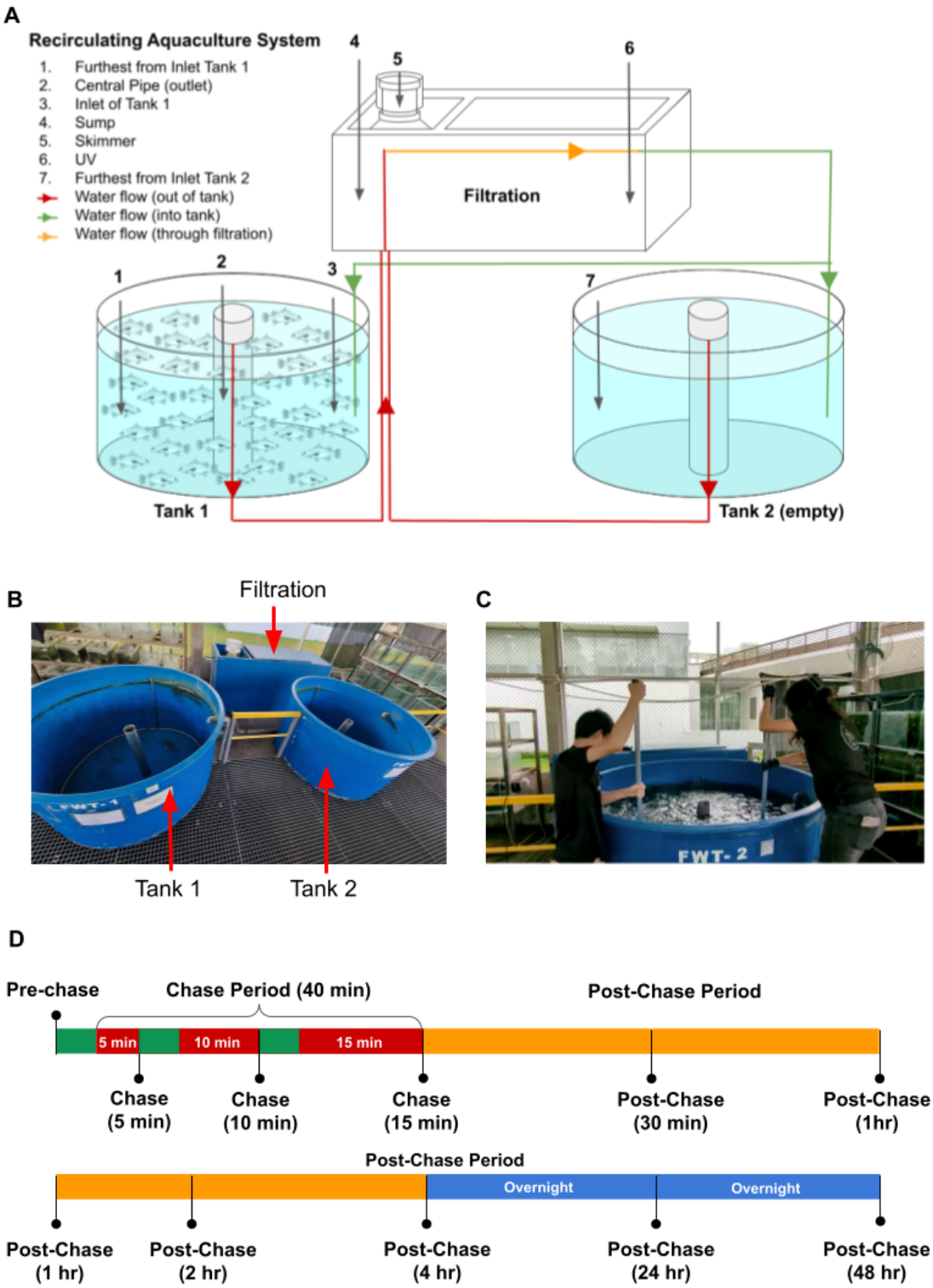
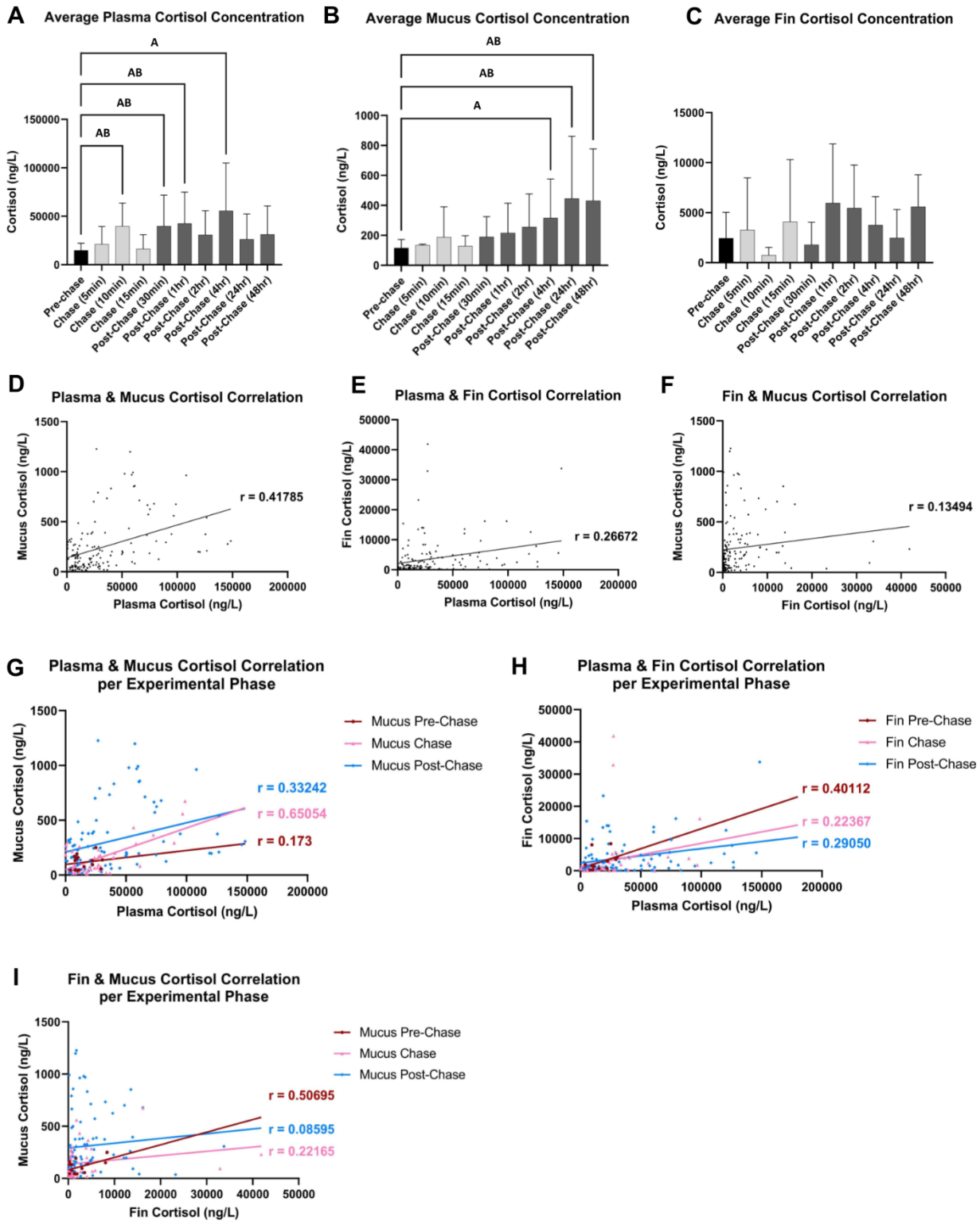


Figure 1: Schematic of experimental setup and design

(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).

692 **Figure 2**



693

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

(A) The average plasma cortisol concentrations of Asian sea bass across sampling points (N = 5 fish per experiment, 15 fish per sampling point except for Chase (15min) where one outlier was excluded). A indicates statistical significance with two-way ANOVA only, whereas AB indicates statistical significance with both ANOVA and Bootstrapping analysis. P-values are reported in Table 2.

(B) The average mucus cortisol concentrations of Asian sea bass across sampling points (N = 5 fish per experiment, 15 fish per sampling point). P-values in Table 2.

(C) The average fin cortisol concentrations of Asian sea bass across sampling points (N = 5 fish per experiment, 15 fish per sampling point except for Pre-Chase and Post-Chase (24hrs) where one outlier was excluded each). P-values in Table 2.

(D) Correlations between plasma and mucus cortisol concentrations released from individual fish across all time points (N = 148, R2 = 0.1746, p <0.0001)

(E) Correlations between plasma and fin cortisol concentrations released from individual fish across all time points (N = 147, R2 = 0.07114, p = 0.0011)

(F) Correlations between mucus and fin cortisol concentrations released from individual fish across all time points (N = 147, R2 = 0.01821, p = 0.1032)

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

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

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

Figure 3

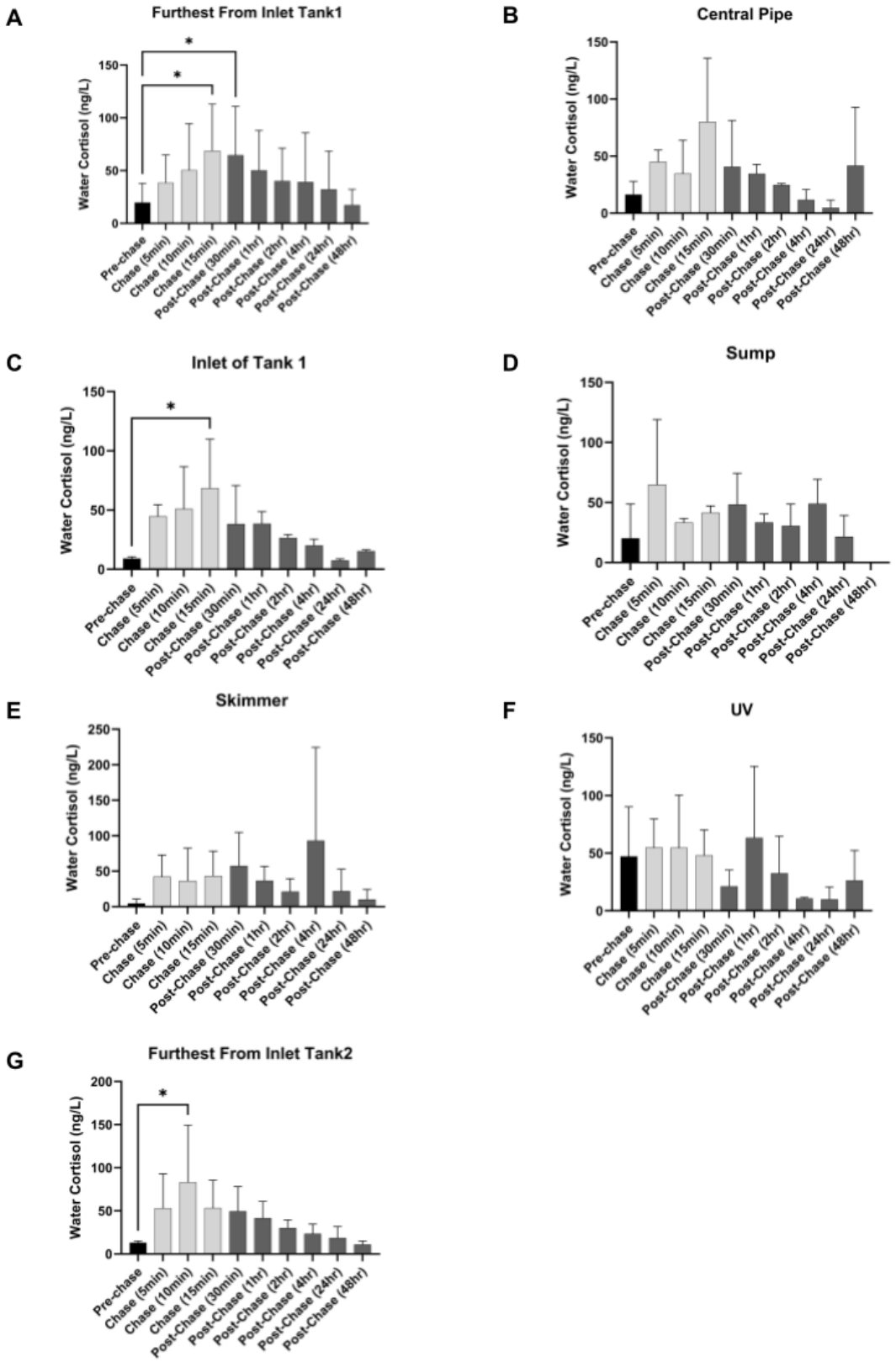

A
Furthest From Inlet Tank1
B
Central Pipe
C
Inlet of Tank 1
D
Sump
E
Skimmer
F
UV
G
Furthest From Inlet Tank2
Water Cortisol (ng/L)
Pre-chase
Chase (5min)
Chase (10min)
Chase (15min)
Post-Chase (30min)
Post-Chase (1hr)
Post-Chase (2hr)
Post-Chase (4hr)
Post-Chase (24hr)
Post-Chase (48hr)

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

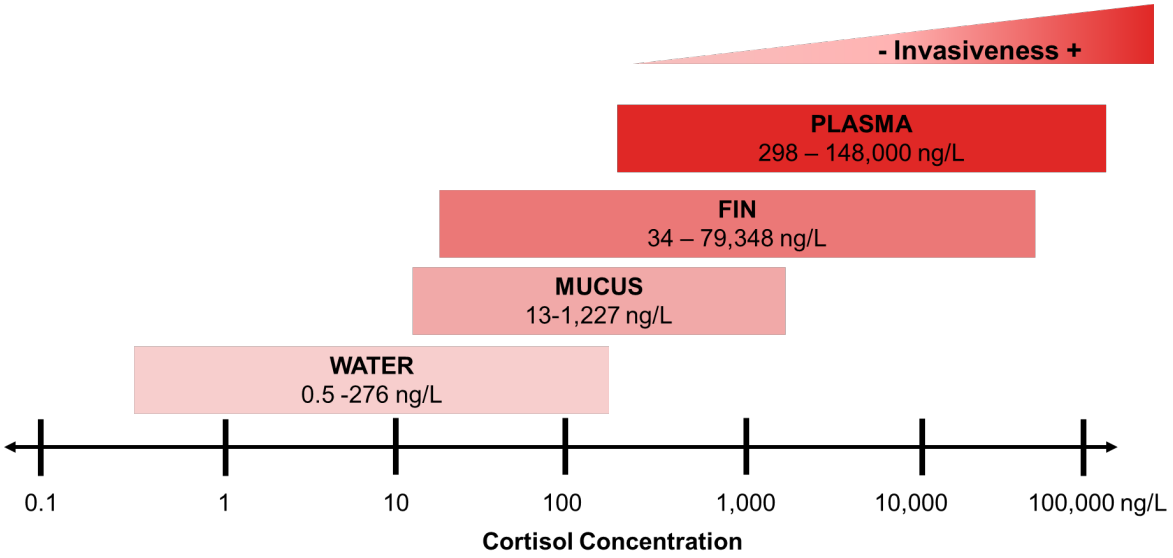


Figure 4: Cortisol concentrations across tissues and in water in Asian sea bass
Cortisol concentrations in this figure are derived from our handling stress experiments. The invasiveness level of each sampling method is indicated by the red color code. Note that the fin and mucus cortisol were normalized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