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Dynamics of endogenous and water cortisol release in Asian Sea bass *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 stress levels in fish to optimize farming practices, which could then enhance productivity and welfare in aquaculture operations. Cortisol, a circulating stress hormone, 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 that 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 (3000 L tank, 9000 L system) and high-flow rate (8000 L/h) Recirculating Aquaculture System (RAS) to an acute handling stress challenge involving chasing and air exposure, and quantified cortisol dynamics 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 h and 24–48 h, respectively, and that plasma and mucosal cortisol were moderately correlated, especially during the stress period. Fin cortisol did not show consistent dynamics. Water cortisol similarly rose, but peaked within 40 min 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.

1. 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 teleost 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;

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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., 2010; De Mercado et al., 2018; Ghassemi Nejad et al., 2019). Simontacchi et al. (2008) evaluated plasma cortisol levels in European sea bass (*Dicentrarchus labrax*) 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 (Bertotto et al., 2010).

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 sea bass to 5 min of chasing and a 1–1.5- min air exposure, within a 2 m^3 flow-through tank, at a water flow rate of 60 L/h (Fanouraki et al., 2008). This resulted in increased plasma cortisol concentrations (2184 nM or 791,612 ng/L) that peaked at 1 h post stress. Water cortisol concentrations peaked at 4 h 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-h experiment. In a different study, Scott et al. (2001) showed that 90-sec 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/h. A progressive increase in water cortisol concentrations was observed after single and repeated stress, peaking at 2 h (25 ng/L) and 5 h (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 also in relatively small setups. Mota et al. (2017b) 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 1500 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 h following an acute stressor of 60-sec 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 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 this region 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 accumulation or release into multiple tissue types or water has been reported.

Hence, in this study, we sought to characterize the cortisol dynamics of Asian sea bass in response to acute stress, particularly whether fin and mucus tissues may allow for accurate and less-invasive measurement of stress in this species. Furthermore, we investigated if water cortisol levels within a larger, farm-like, high-flow RAS setting (9000 L system, 8000 L/h 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.

2. Materials and methods

2.1. Animal husbandry and experimental setup

Asian sea bass 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). Experiments were carried out in a 9000 L saltwater (30 ppt) system, which consisted of two circular blue fiberglass tanks (each 3000 L) containing the fish and one rectangular tank (3000 L) as the sump (Fig. 1A-B). Water was circulated at 8000 L/h.

2.2. 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. Experiments were performed in a single fiberglass RAS tank "Tank 1" (Fig. 1A-B). Details of each batch of fish used in the experiments can be found in Table 1.

During experimental periods, fish were fed three times a day (9:30 am, 12:30 pm, and 4:30 pm) at about 10 % body weight (BW) per day using 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 based on 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 SpinTouch FX (Lamotte, USA). The 9000 L RAS system that was used for experiments circulated 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 maintained below 1 ppm, nitrite concentrations below 1 ppm, nitrate levels below 100 ppm, and pH between 7.5 and 8.0.

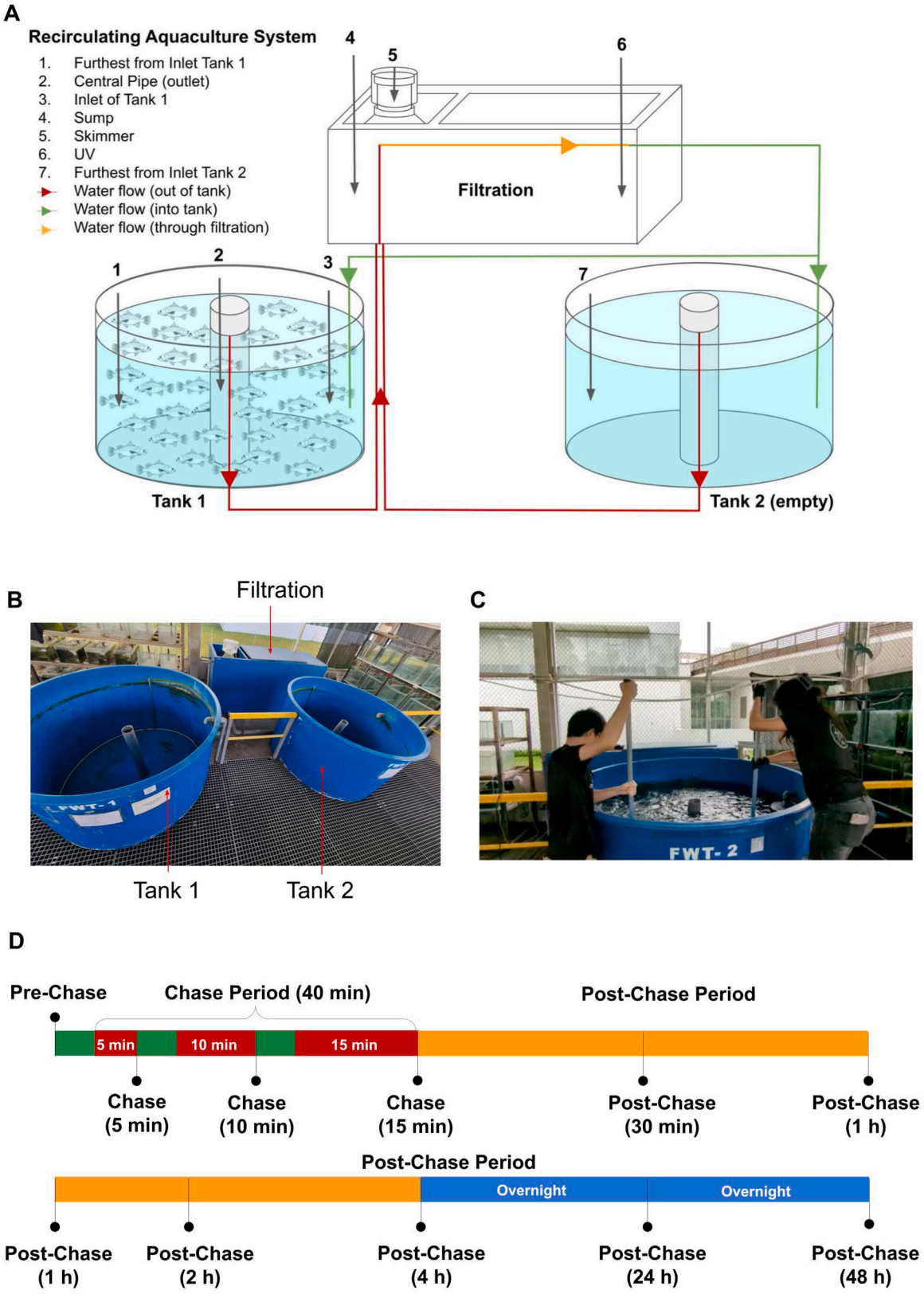


Fig. 1. Schematic of experimental setup and design. (A) Design of the Recirculating Aquaculture System (RAS) used in our experiments, including water sample collection points. (B) Image of the RAS. (C) Image showing how chasing was performed in Tank 1 using large nets in a C-shape manner. Air exposure for as many fish as possible was performed for 10 sec 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 h)". Black lines correspond to sampling time points. Red boxes represent progressively longer chasing periods separated by 5-min 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).

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 A	7 Sep - 9 Sep 2022	Local Farm A	134	140.67	2.094	6.283
Exp B	7 Dec - 9 Dec 2022	Local Farm B	446	92.36	4.575	13.731
Exp C	1 Mar - 3 Mar 2023	Local Farm B (same batch as Exp 2)	432	165.82	7.959	23.878

2.3. Handling stress experiments

Fish were not fed from the evening before experiments commenced. "Pre-Chase" samples were collected before any stress was induced on the fish. Fish were then subjected to a period of chasing with progressively longer chasing durations: 5 min ("Chase (5 min)"), 10 min ("Chase (10 min)") and 15 min ("Chase (15 min)"), with a 5-min "rest" interval between each of the three chases. Fish were chased by two personnel using large nets (Fig. 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 sec. Following this, five fish from this net were collected for sampling.

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

Hence, there were a total of 11 sampling time points for chasing experiments, where five random fish were sampled at each sampling time point (Fig. 1D). At each sampling point, the following samples and data were collected: water from various parts of the tank (see ***Water sample collection*** section), fish body weight (total weight of the five fish), plasma, fin, and mucus (see ***Biological sample collection*** section).

2.4. Biological sample collection

After being netted, the fish were anesthetized in a bucket containing Tricaine methanesulfonate MS-222 (70 mg/L) and sodium bicarbonate (0.14 g/L) dissolved in fresh saltwater. While still in the bucket, the fish were collectively weighed. The anesthesia took effect within 5 min, after which the fish were subjected to plasma, fin, and mucus collection, as described in the following sections. Sampling was performed in a factory line manner in the order of blood, fin, and then mucus. The entire process of collecting plasma, fin, and mucus samples required approximately 5 min per fish. Given the staggered sample collection procedure, all samples were collected from all fish within 5–10 min post-anesthesia.

2.5. Plasma collection

Blood was extracted from the caudal artery/vein by inserting a 21 G sterile needle at a 45° angle until it reached the spine. Upon contact with the spine, the needle was withdrawn slightly, and blood was extracted into a sterile 1.0 mL syringe. Approximately 0.5 mL of the collected blood was subsequently injected into a lithium heparin tube (Greiner MiniCollect 0.5 mL) and inverted five times to thoroughly mix the blood within the heparin tube and prevent coagulation. The blood in the heparin tube was then centrifuged at a 45° angle, at 3000 g for 10 min to facilitate the separation of blood plasma. The plasma was carefully pipetted into a cryovial, which was then stored in dry ice before being transferred to a −20 °C freezer.

2.6. Mucus collection

DNA buccal swabs (Isohelix, UK) were used for mucus collection. The fish were swabbed on their left flanks, starting from the front in the caudal direction, along the lateral line. This process was repeated four times on each side of the swab to ensure that the mucus was collected on the entire swab. The swab was then placed into a 2 mL tube, where 1 mL of phosphate buffered saline (PBS) was added to the sample swab. The samples were then stored in dry ice before being transferred to a −20 °C freezer.

2.7. Fin collection

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

After sample collection, the fish were first placed in recovery buckets containing salt water without 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 B), or in the case of Experiment C, 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.

2.8. Water sample collection

In Experiment A, tank water was only collected from a single sampling point "Tank 1 Furthest from Inlet". In Experiments B and C, tank water was additionally sampled from 6 other sampling points (Fig. 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 sec 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 into the glass bottles was returned into the system to maintain a relatively constant water volume within the system.

2.9. Cortisol extraction from fin and mucus samples

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

Fin samples in PBS stored in a −20 °C freezer were thawed to room temperature before processing. Each fin sample was washed twice with isopropanol and subsequently air-dried for 2–3 days. The dried fin sample was ground into powder by using a mortar and pestle, and the weight of the ground powder was recorded. To facilitate cortisol extraction, 1.5 mL of methanol was added to the ground powder and shaken over a 3-day period. After centrifugation at 9500 rpm for 10 min, the resulting supernatant was collected and placed in an oven at 38 °C for methanol evaporation. The extracted cortisol was reconstituted in 400 μL of PBS, filtered through a 0.22 μm PVDF syringe filter, and stored in a 4°C fridge for analysis within 24 h or in a −20 °C freezer for long-term storage.

2.10. Cortisol quantification from biological samples

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

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

2.11. Cortisol extraction and quantification from water samples

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

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

2.12. Total protein quantification from mucus samples

The total protein concentration in the mucus samples was quantified by using colorimetric Bradford assay (Kruger, 2009; Ernst and Zor, 2010), employing Coomassie Brilliant Blue dye that shifts its maximum absorption wavelength from 465 nm to 595 nm upon binding to proteins. Briefly, 100 μL of Bradford reagent was mixed with 50 μL of filtered mucus in PBS and incubated at room temperature for 15 min. The absorbance of the mixture at 595 nm was measured using a microplate reader, the Infinite M200 Spectrophotometer (Tecan Trading AG, Switzerland). The total protein concentration in the sample was calculated based on the standard curve, prepared by plotting the absorbance at 595 nm from protein standards (0.1–0.4 mg/mL) derived from serially diluted bovine serum albumin in PBS.

2.13. Data analysis and statistics

Cortisol concentrations obtained from plasma, fin and mucus samples were calculated based on a standard curve run on each plate and expressed in ng/L (ng/mg for mucus). Plasma cortisol concentrations were then adjusted for dilution factor, fin cortisol concentrations were adjusted by fin mass followed by volume of PBS solution added, and mucus cortisol concentrations were adjusted by total protein concentration (ng mucus cortisol / mg total protein). In Fig. 3, the water cortisol concentration was not standardized to biomass. However, a standardization to system stocking density (kg/m^3) was performed in Supplementary Figures 4–5.

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

Since the biological replicates were conducted on batches of fish with different characteristics and spanning six months, we also performed a boot-strapping based permutation test to establish the statistical robustness of our conclusions (MATLAB, USA). For 10,000 iterations, we first randomly sampled an experiment (A, B, or C), and from this experiment, then randomly sampled (with replacement) five fish from each chase / post-chase time point. We then compared the mean cortisol concentration of this group with the pre-chase control, which was similarly randomly sampled. This allowed us to quantify the percentage

of iterations in which the cortisol concentration would be higher in each post-chase time point compared to the pre-chase controls, providing a p-value for each time point. This permutation analysis was conducted for each tissue type. In this analysis, we did not exclude any outliers. Using this same analysis we also estimated the distribution of effect sizes (cohen's d) that would be observed (Supplementary Figure 2). In the figures and tables, asterisks are used to indicate statistical significance levels as follows *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3. Results

The acute handling stress experiments were repeated three times over a period of six months, as described in Table 1. Given its large scale, we were unable to simultaneously conduct multiple replicates, and instead conducted biological replicates sequentially over time. The origins, age, 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 the tank, starting from around the "Chase (10 min)" period and increasing over time, which may reflect increased mucus secretion.

To quantify the effects of such handling stress on plasma, mucus, and fin cortisol across these diverse conditions, we employed two statistical methods, a 2-way ANOVA (across experiments and time points) as well as bootstrapping analysis. For the latter, we randomly sampled with replacement data from fish within each experiment, as well as the experiment (A, B, or C) from which the fish were sampled, for 10,000 iterations, to generate a bootstrapped distribution of effect sizes (Supplementary Figure 2) as well as a p-value for each time point (Table 2) demonstrating the probability of cortisol concentrations being higher at that time point relative to control.

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

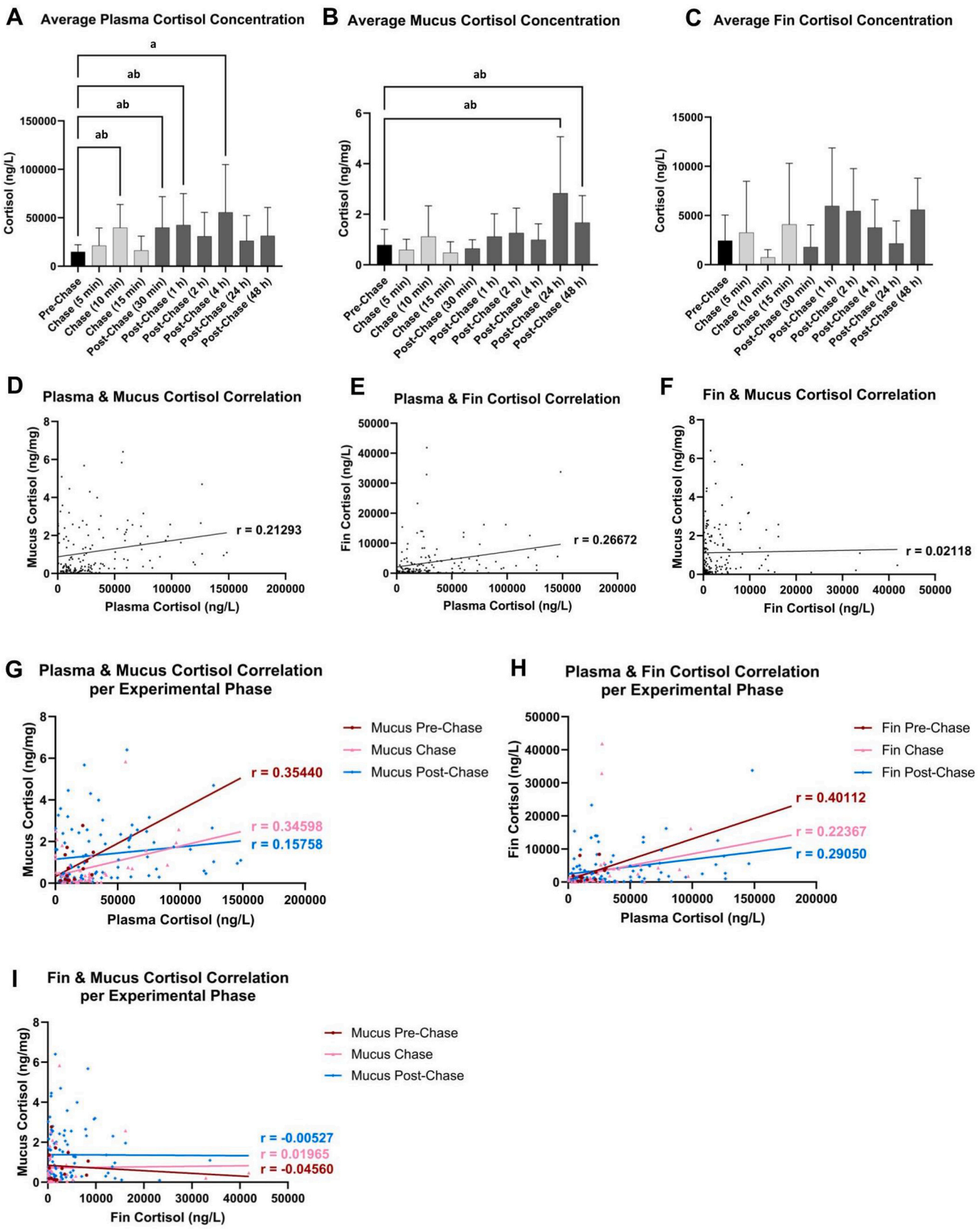
Next, we correlated cortisol levels across the biological samples (fin, mucus, and plasma) collected from each individual fish. Plasma cortisol was moderately correlated with mucus cortisol ($r = 0.21$, $p = 0.0091$, Fig. 2D) and fin cortisol ($r = 0.27$, $p = 0.0011$, Fig. 2E), whereas fin and mucus cortisol were weakly correlated ($r = 0.021$, $p = 0.10$, Fig. 2F). Notably, the correlation between plasma and mucus cortisol was highest and significant during the chasing period ($r = 0.35$, $p = 0.021$), similarly high albeit non-significant in the pre-chase period ($r = 0.35$, $p = 0.21$), and lower in the post-chase period ($r = 0.16$, $p = 0.14$) (Fig. 2G). In contrast, plasma cortisol was more strongly correlated with fin cortisol during the pre-chase period, albeit non-significantly ($r = 0.40$, $p = 0.16$), as compared to the chase ($r = 0.22$, 0.050) and post-chase periods ($r = 0.29$, $p = 0.0058$) (Fig. 2H). Fin and mucus cortisol were overall not significantly correlated across pre-chase ($r = -0.046$, $p = 0.88$), chase ($r = 0.020$, $p = 0.90$), and post-chase ($r = -0.0053$, $p = 0.96$) periods (Fig. 2I).

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

Due to the low concentrations of cortisol in water relative to biological samples, and the higher potential for antibody interference in ELISA due to contaminants (e.g., salts and organic material) in tank water, we opted to use HPLC analysis for more precise measurements of water cortisol concentrations. Consistent with reports in existing literature, water cortisol concentrations were in the range of ~10 to ~100 ng/L, about 1000-fold lower than the concentrations observed in plasma and fin samples, and 10-fold lower than mucus samples. In all sampling points within the tank containing the fish (Tank 1), we observed a consistent trend of increased water cortisol starting from the chasing period (Fig. 3A-C). For the "Tank 1 Furthest from Inlet" sampling point, there was a significant increase in water cortisol at the "Chase (15 min)" ($p = 0.0197$) and "Post-Chase (30 min)" time points ($p = 0.0367$). Water cortisol concentrations were also significantly elevated at the "Inlet of Tank 1" at the "Chase (15 min)" time point ($p = 0.0287$), with a similar peak for the "Central Pipe" albeit non-significant. The cortisol concentrations from the other RAS compartments did not exhibit distinct variations, although water cortisol concentrations in the "Sump" and "Skimmer" were on average higher during and after the chase (Figs. 3D and 3E). The "UV" compartment, on the other hand, did not show any trends (Fig. 3F). Interestingly, in Tank 2, which was not holding any fish, a similar trend of increased water cortisol, significant at the "Chase (10 min)" time point, was observed (Fig. 3G). This observation suggests the possibility that cortisol released into the water from one tank may be distributed to other RAS tanks within the system. Detailed cortisol concentration data from individual experiments, as absolute values or normalized to system stocking density (since some fish were removed for sampling), can be found in Supplementary Figures 3–5.

Table 2
Summary of statistical cortisol analysis results using ANOVA and bootstrapping.

Time Points	Plasma		Mucus		Fin	
	ANOVA	Bootstrap	ANOVA	Bootstrap	ANOVA	Bootstrap
Chase (5 min)	0.9737	0.3374	0.9944	0.5743	0.9988	0.7656
Chase (10 min)	0.0183*	0.0115*	0.8504	0.6377	0.9673	0.5951
Chase (15 min)	> 0.9999	0.1726	0.9020	0.9127	0.9715	0.7236
Post-Chase (30 min)	0.0172*	0.0194*	0.9994	0.4534	>0.9999	0.8522
Post-Chase (1 h)	0.0066**	0.0464*	0.8509	0.4421	0.4202	0.3211
Post-Chase (2 h)	0.2616	0.1431	0.5320	0.1454	0.5990	0.3574
Post-Chase (4 h)	<0.0001***	0.3089	0.9909	0.2406	0.9930	0.4320
Post-Chase (24 h)	0.6323	0.3656	<0.0001***	0.0489*	>0.9999	0.8369
Post-Chase (48 h)	0.2317	0.1114	0.0301*	0.0173*	0.5471	0.3100



(caption on next page)

Fig. 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 (15 min)" where one outlier was excluded). The letter "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 of individual fish across all time points (N = 149, $r^2 = 0.04534$, p = 0.0091). (E) Correlations between plasma and fin cortisol concentrations of individual fish across all time points (N = 148, $r^2 = 0.07114$, p = 0.0011). (F) Correlations between mucus and fin cortisol concentrations of individual fish across all time points (N = 149, $r^2 = 0.0004488$, p = 0.7983). (G) Correlations between plasma and mucus cortisol concentrations of individual fish per experimental phase (Pre-Chase: N = 15, $r^2 = 0.1256$, p = 0.2137. Chase: N = 44, $r^2 = 0.1197$, p = 0.0214. Post-Chase: N= 89, $r^2 = 0.02483$, p = 0.1403). (H) Correlations between plasma and fin cortisol concentrations of individual fish per experimental phase (Pre-Chase: N = 14, $r^2 = 0.1609$, p = 0.1552. Chase: N = 44, $r^2 = 0.05003$, p = 0.1444. Post-Chase: N= 89, $r^2 = 0.08439$, p = 0.0058). (I) Correlations between mucus and fin cortisol concentrations of individual fish per experimental phase (Pre-Chase: N = 14, $r^2 = 0.002079$, p = 0.8770. Chase: N = 45, $r^2 = 0.0003860$, p = 0.8981. Post-Chase: N= 88, $r^2 = 2.775\times10^{-005}$, p = 0.9609).

4. Discussion

4.1. Handling stress leads to an acute rise in plasma cortisol

In this study, the effects of handling stress on Asian sea bass cortisol concentrations across tissue types were assessed. Fig. 4 shows the cortisol concentration ranges found in different tissue types of Asian sea bass and the RAS tank water. Plasma sampling, a highly invasive method involving the drawing of blood, allowed for detection of the highest concentrations of cortisol. Fin sampling, which is also invasive to some extent, provided a wider range of cortisol concentrations spanning those of mucus and plasma. A less invasive method, mucus sampling, provided lower concentrations of cortisol compared to plasma sampling. Water samples, which are the least invasive, detected the lowest concentration of cortisol, with a range of < 300 ng/L.

From this acute handling stress study, first, we confirmed that our manipulation indeed triggered a significant increase in plasma cortisol starting from the "Chase (10 min)" time point, which would be 20 min into the chasing period (Fig. 2A). There continues to be a significant increase in plasma cortisol up to 1 h post-chase, and recovery at 24 and 48 h. We note that some of the chase and post-chase time points did not attain statistical significance, which could be attributed to biological variability across the three replicates, influenced by the large number of fish in the tanks and variations in fish age, genetic background, and size (Table 1). Despite these differences, the overall early detection of cortisol is consistent with previous such studies where it has been shown that plasma cortisol typically peaks between 0.5 and 4 h post-stress stimulus in fish like European sea bass, goldfish (*Carassius auratus*) and rainbow trout (Scott, Pinillos and Ellis, 2001; Fanouraki et al., 2008; Scott et al., 2008; Mota et al., 2017a). For instance, it peaks at 1–2 h in common carp (*Cyprinus carpio)* and 2–4 h in roach (*Rutilus rutilus*) post-tag insertion (Lower et al., 2005); 2 h in gilthead seabream (*Sparus aurata*) post-crowding stress (Guardiola, Cuesta and Esteban, 2016), 0.5–1 h in rainbow trout (Ellis et al., 2004) and 3 h in Atlantic Salmon *(Salmo salar)* post-air exposure stress (Ellis et al., 2007). Interestingly, a study in European seabass recorded significantly high plasma cortisol already at 0 h post-chasing and air exposure stress, peaking at 1 h post-stress (Fanouraki et al., 2008), which coincides with the plasma cortisol trends in our Asian seabass study.

4.2. Relationship of plasma to mucus and fin cortisol

We also simultaneously sampled mucus and fin cortisol from individual fish, to investigate the viability of these less-invasive sampling methods as reliable indicators of fish stress in aquaculture settings. Our findings revealed that mucus cortisol was increased following the stress-inducing event, becoming significantly elevated at the post-stress 24- and 48-h time points, even when plasma cortisol had already fully returned to baseline levels. Hence, this increase followed a slower time course compared to that of plasma cortisol. Similarly as shown in Madaro et al. (2022), the rise in cortisol in mucus of Atlantic salmon after netting stress was delayed as compared to blood plasma (Madaro et al., 2023). In their study, mucus levels remained significantly elevated even at 300 min post-stress, which was the longest time point they assessed. Guardiola et al. (2016) also reported a sustained increase in mucus cortisol at 24 and 48 h post-crowding stress, whereas plasma cortisol only showed a significant increase at 2 h post-stress (Guardiola, Cuesta and Esteban, 2016). While the biological basis of these time course differences is still not well understood, it is speculated that either the slow accumulation of plasma cortisol in the mucus, or the reabsorption of cortisol from the water might account for these observed patterns, though in the latter case, more evidence has been presented for sex steroid reuptake than for cortisol (Scott and Ellis, 2007; Sadoul and Geffroy, 2019).

Despite the differences in cortisol dynamics, we observed a moderate correlation between plasma and mucus cortisol overall (r = 0.21, p = 0.0091), especially during the chasing period (r = 0.35, p = 0.021). Our data thus suggests that mucus cortisol could be a useful, less invasive way to sample fish stress in Asian Sea bass; however, the correlations observed are weaker than those in other studies, possibly due to the acute nature of our experiments.

A number of other studies have also looked at the correlations between plasma and mucus cortisol across species. Fernandez-Alacid et al. (2019) reported strong correlations between plasma and mucus cortisol in Meagre (*Argyrosomus regius*) after hypoxia (r = 0.769) and netting stress (r = 0.765) (Fernández-Alacid et al., 2019). Bertotto et al. (2010) also reported strong correlations of plasma and mucus cortisol after 3-h transport stress in European sea bass (r = 0.63), common carp (r = 0.81), and rainbow trout (r = 0.79) (Bertotto et al., 2010). Fernandez-Montero et al. (2020) also reported correlations between plasma and mucus cortisol in greater amberjack (*Seriola dumerili*) after temperature (r = 0.22), stocking density/handling (r = 0.73) and fasting (r = 0.38) stress (Fernández-Montero et al., 2020). Carbajal et al. (2019) observed a stronger correlation (r = 0.70) between mucus and plasma cortisol in rainbow trout during the hours following confinement stress, relative to the control or late phase; in these experiments plasma cortisol was elevated past 24 h, suggesting severely stressful conditions (Carbajal et al., 2019).

Cortisol extracted from fin in our study did not significantly increase post-stress, but was overall also significantly correlated with plasma cortisol (r = 0.27, p = 0.0011). Hence, it could be a viable stress biomarker, albeit not under acute stress challenge. In fact, a related matrix, scale, has been proposed to be a better biomarker for chronic stress, showing slower but more sustained cortisol levels relative to plasma under longer-term and repeated stress conditions (Laberge, Yin-Liao and Bernier, 2019; Samaras et al., 2021). Fin, a calcified structure, shares similarities with scales and hence may be a better biomarker for chronic stress over longer time scales of days, weeks or months. Notably, Bertotto et al. (2010) showed that fin cortisol was correlated with plasma cortisol in common carp (r = 0.92) and rainbow trout (r = 0.88) after 3-h transport stress, suggesting stress accumulated over time scales such as hours may also be sufficient to induce fin cortisol accumulation (Bertotto et al., 2010).

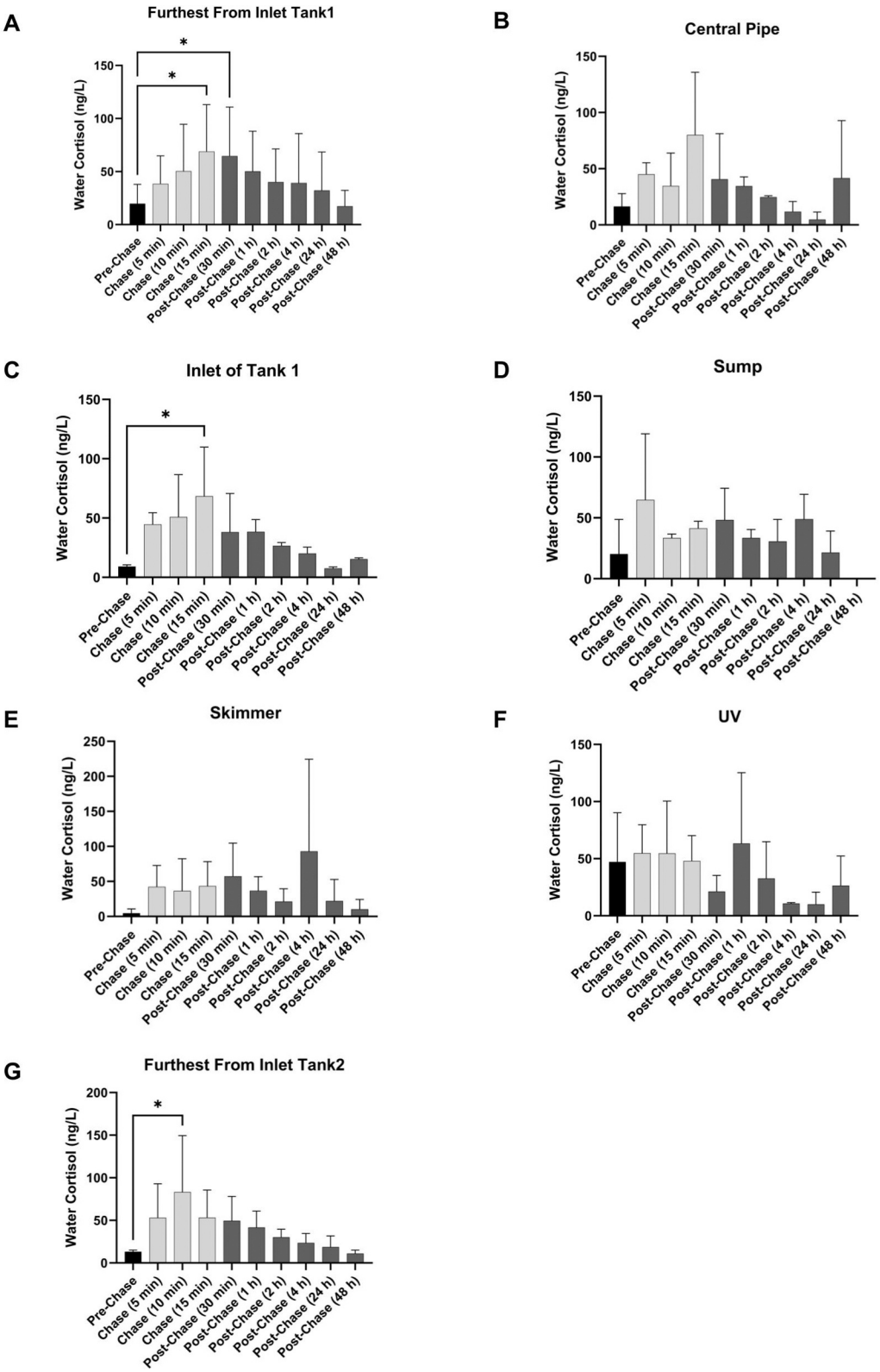


Fig. 3. Change in water cortisol across different sampling points in the RAS system during and after a period of chasing and air exposure stress. (A) Mean water cortisol concentrations (ng/L) at "Furthest From Inlet Tank 1" (N = 3 per time point), p = 0.0197 (Chase (15 min)), 0.0367 (Post-Chase (30 min)). (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" (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 (10 min)).

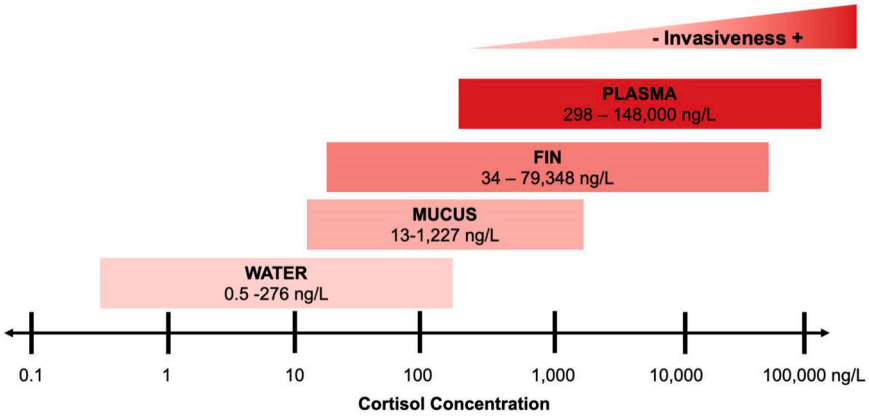


Fig. 4. Cortisol concentrations across tissues and in water in Asian sea bass. Cortisol concentrations in this figure are derived from our handling stress experiments. The invasiveness level of each sampling method is indicated by the red color code. Note that the fin cortisol were normalized to the initial sample weight (100 mg of fin/1 mL of PBS) and mucus was dissolved in 1 mL PBS. While we report mucus cortisol standardized to total protein in our results, absolute mucus cortisol concentration is provided here to allow for comparison across matrices.

4.3. Water cortisol changes post-stress in the RAS system

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

In the latter two of the three experiments, we further measured water cortisol across different parts of the tank and also different RAS compartments, to identify ideal sampling locations for water cortisol for on-farm stress monitoring. The same trend was still observed in other parts of Tank 1, where the peak in water cortisol still occurred at 40 min from the commencement of the stress event (the “Chase (15 min)” time point) and decreased earlier than plasma cortisol. Cortisol molecule stability in water is affected by temperature and chemical/ microbial activity in the water (Kelkar et al., 2023). In wastewater, for example, cortisol has a half-life of around 15 h and 4 h at 25℃ and 35℃, respectively (Kelkar et al., 2023). The half-life of cortisol is approximately 16 h in fish tank water at 12℃ (Sadoul and Geffroy, 2019). The fish tank water in this study was filtered salt water and had an average temperature of 27℃. Hence, the cortisol molecules in this water might be relatively stable in the first few hours of our experiments. The earlier decrease of water cortisol that we observed relative to plasma cortisol levels suggest that the dynamics of water cortisol in the RAS system may be influenced by other factors, including water circulation flow rate, filtration (i.e. protein skimmer), or adsorption on the plastic tank walls (since it is hydrophobic) (Scott and Ellis, 2007; Sadoul and Geffroy, 2019). Alternatively, cortisol could potentially be reabsorbed by the fish, possibly into the mucosal layer (Scott and Ellis, 2007; Sadoul and Geffroy, 2019).

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

Overall, water cortisol dynamics appeared to be more consistent with plasma cortisol trends than with mucus (or fin). We note that we used a more sensitive method (HPLC) to quantify water cortisol than for plasma, mucus, or fin cortisol. This was for two reasons, firstly, water cortisol concentrations were significantly lower than in biological samples, and secondly, fish tank water is a more complex matrix than biological samples, containing a multitude of additional salts (calcium carbonates, bicarbonates, nitrates, nitrites) and other organic materials (e.g, proteins, lipids from leftover feed or waste) that may potentially interfere with antibody affinity binding in ELISA (Chuang et al., 2005; Wang et al., 2013). Sample dilution may reduce matrix interference, though at the expense of further decreasing cortisol concentration. More convenient, robust, and sensitive methods of water cortisol detection would be valuable for future studies.

4.4. Caveats and limitations of our study

In typical research studies, biological replicates would be conducted in parallel on a single batch of fish, within smaller tank setups. In our study, we aimed to simulate the conditions of a farm-scale setup, using 3000 L tanks on a 9000 L RAS system. Due to the scale of our experiments, the limited number of such systems available, and the challenge of acclimatizing and cultivating a large number of fish, we were constrained to conduct one biological replicate at a time, with our experiments spread over a course of six months. We also were only able to measure water cortisol from different RAS compartments in two of the three experiments.

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

While not ideal in an experimental setting, the upside of such variability is that any signal we have obtained would likely be reproducible

even with the normal diversity experienced in on-farm settings. Across all biological replicates, we observed an increase in plasma, mucus, and water cortisol levels, with relatively consistent dynamics. Importantly, our results further argue that surveying water cortisol, which provides an average readout of the stress levels in the system, might be a means of overcoming the variability from sampling individual fish.

5. Conclusions

Our results corroborate that in a farm-scale RAS setting across varying conditions, water cortisol is a reliable measure of Asian sea bass stress and that different tissue types such as fin and 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, concentrations, and rates of cortisol released may differ across species. The findings of a significant correlation between mucus cortisol and plasma cortisol highlight the potential utility of mucus cortisol as a less-invasive measure of Asian sea bass 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.

CRediT authorship contribution statement

Marie Ruoyun Tan: Formal analysis, Methodology, Investigation, Writing - Original Draft, Writing - Review & Editing, Supervision, Project administration. **Khin Moh Moh Aung:** Formal analysis, Methodology, Investigation, Writing - Original Draft. **Nur Asinah binte Mohamed Salleh:** Formal analysis, Methodology, Investigation, Writing - Original Draft. **Jolin Tan:** Formal analysis, Methodology, Investigation, Writing - Original Draft. **Kai Xin Chua:** Formal analysis, Investigation, Writing - Original Draft, Visualization. **Gaynah Javier Doblado:** Investigation, Writing - Original Draft. **Kai Lin Chua:** Investigation. **Valarie Tham:** Investigation. **Jovian Jing Lin:** Investigation, Visualization. **Vindhya Chaganty:** Investigation. **Dinah Mardhiana Yusoff:** Investigation. **Shubha Vij:** Methodology, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **Xiaodi Su:** Conceptualization, Methodology, Formal analysis, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **Laura Sutarlie**: Conceptualization, Methodology, Formal analysis, Resources, Writing - Original Draft, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **Caroline Lei Wee:** Conceptualization, Methodology, Formal analysis, Resources, Writing - Original Draft, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2024.102223.

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