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Thermal stress response of juvenile milkfish (*Chanos chanos*) quantified by ontogenetic and regenerated scale cortisol

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ABSTRACT

Milkfish (*Chanos chanos*) is one of the most important aquaculture species in Asian countries. These teleost fish are traditionally cultured in outdoor-based systems and therefore have to cope with daily and/or seasonally changing environmental conditions. Temperature changes beyond the optimal range of a species are known to induce an endocrine stress response through activation of the hypothalamic-pituitary-interrenal (HPI) axis, hereby triggering the release of cortisol in order to regain homeostasis. The pertinent literature on milkfish lacks data on chronic stress, however, long-term elevations of cortisol are known to be detrimental for the fish performance. This study is the first on chronic stress in juvenile milkfish quantified by using ontogenetic (OG) and regenerated (RG) scale cortisol. We analyzed scale cortisol of juvenile milkfish, which were exposed to a gradual temperature increase of 1 °C per day in the range from 26 °C to 33 °C, followed by an exposure to constant 33 °C for 21 days. Significant higher RG scale cortisol concentrations were observed in juvenile milkfish exposed constantly to 33 °C, compared to milkfish kept at 26 °C. Even the gradual temperature increase from 26 °C to 33 °C over 7 days resulted into higher OG scale cortisol concentrations. Although milkfish showed reduced growth in the first 7 days at 33 °C, overall fish growth was positively affected by higher temperature. As anthropogenic activities affecting the climate are increasing, and taking into account the widespread use of outdoor aquaculture systems, which are prone to natural fluctuations, an increase in temperature is most likely to be considered a stressor in milkfish aquaculture, as indicated by our results. Hereby, the use of scale cortisol was shown to be effective to quantify even minute and gradual temperature changes, making it a powerful tool in optimizing aquaculture systems as well as in monitoring gradual climate changes in wild stock.

1. Introduction

Milkfish (*Chanos chanos*) is one of the most important aquaculture species in Asian countries (de Jesus-Ayson et al., 2010; FAO, 2017). In 2015, the annual global production of milkfish reached > 1.1 M tons with the Philippines, Indonesia and Taiwan as main producers (de Jesus-Ayson et al., 2010; FAO, 2017). The euryhaline nature of this teleost fish has led to a wide range of different culture systems integrated into fresh (e.g. lakes), brackish (e.g. ponds) as well as marine environments (e.g. coastal areas) (Bagarinao, 1994; de Jesus-Ayson et al., 2010). Milkfish are traditionally cultured in pond systems (usually brackish), but nowadays also cages or pen systems are widely used (fresh - marine). In a typical production cycle, wild-caught or in hatcheries reared fry (2–3 cm) are cultured in nursery ponds up to the required "fingerling" size (5–8 cm, 30–40 g), after which they are transported to grow-out ponds, pens or cages until harvest (20–40 cm, 400–700 g, depending on the market prize) (Bagarinao, 1994; de Jesus-Ayson et al., 2010).

The global intensification in aquaculture has led to an increasing threat for fish performance by a variety of multi-level stimuli (Ashley, 2007; Conte, 2004). Fish management procedures including, but not limited to stocking density (Lupatsch et al., 2010; Montero et al., 1999; Ruane et al., 2002; Schram et al., 2006), feeding strategy (Li et al., 2014; Sitjà-Bobadilla et al., 2005), sorting and transport (Acerete et al., 2004; Iversen et al., 2005), as well as environmental conditions, such as global temperature rise, can potentially cause stress and affect fish performance and welfare. Depending on the type, duration, severity, (un-) predictability, and (un)controllability of a stimulus or better of a set of stimuli, whole body performance (metabolism, growth), reproduction and immune system can be negatively affected (Barton,

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2002), resulting in a decline in production yield and fish welfare. Subsequently, a more sustainable aquaculture is pivotal.

Besides potential stressors due to the lack of optimization of one or more fish management related procedures, outdoor systems, as commonly encountered in milkfish aquaculture, are additionally prone to daily and seasonal environmental fluctuations. For decades, salinity and how changes in salinity affects milkfish adaptation (Lin et al., 2003; Tang et al., 2009; Yang et al., 2016), metabolism and growth (Alava, 1998; Chang et al., 2018; Swanson, 1998; Swanson, 1996) was the main parameter studied. Though milkfish are ectothermic and therefore strongly affected by temperature, comparatively few studies focused on the potential of temperature as a stressor in milkfish aquaculture. Water temperature in milkfish culture systems typically varies between 24 °C and 33 °C, with limits at 15 °C and 40 °C during cold snaps or heat waves, respectively, depending on the geographic region (Bagarinao, 1994; Martinez et al., 2006; Verceles et al., 2000). In this framework, the pertinent literature mainly focuses on sudden temperature drops during winter (Chang et al., 2018; Kang et al., 2015; Kuo and Hsieh, 2006). Hsieh et al. (2003), for instance, reported a hyperglycemic and hyperlactemic plasma response in milkfish exposed to a cold shock treatment (15 °C) as indicators for acute stress. At present, data on the effect of temperature increase on milkfish are scarce.

Fish faced with stressful stimuli launch an endocrine stress response through activation of the hypothalamic-sympathetic-chromaffin cell (HSC) axis and hypothalamic-pituitary-interrenal (HPI) axis in order to cope with the perturbed situation (Wendelaar Bonga, 1997). Catecholamines are released within seconds (via HSC axis), while glucocorticoids, cortisol or corticosterone depending on the species, are released within minutes (via HPI axis) into the plasma (Wendelaar Bonga, 1997; Barton, 2002). Across teleost fish species, cortisol is generally accepted as biomarker for stress (Demers and Bayne, 1997; Grutter and Pankhurst, 2000; Koakoski and Oliveira, 2012). In this physiological reaction to a stressor, distinction must be made between 'eustress', characterized by (mild) stressors with positive consequences (e.g. increased metabolic performance) and 'distress', characterized by (more severe) stressors with adverse effects, which can be adaptive or maladaptive (Schreck and Tort, 2016). Furthermore, one must distinguish between acute and chronic stressors as short elevations of cortisol, as seen in acute stress, are normally adaptive and can be temporary profitable for the fish. On the other hand, long-term elevations of cortisol are known to be detrimental as they result in a decreased performance (growth, reproduction), immunity and even survival (Barton, 2002; McEwen and Wingfield, 2003). As plasma cortisol solely provides a snapshot of HPI axis activity, other matrices, such as mucus, feces and water were considered, but found inadequate to capture chronic stress (Cook, 2012). Recently, scale cortisol, using ontogenetic as well as regenerated scales, was proven to reflect chronic stress in fish (Aerts et al., 2015).

In our study the effect of high water temperature on milkfish was tested: (i) a gradual increase of 1 °C a day from 26 °C to 33 °C, followed by (ii) a 21-day exposure of constant 33 °C, using ontogenetic and regenerated scale cortisol, respectively. This is to our knowledge the first study using scale cortisol to evaluate chronic stress in milkfish. We predicted that a minute and gradual increase as well as a constant high water temperature would potentially constitute chronic stressors for milkfish reflected by increased scale cortisol.

2. Material and methods

2.1. Experimental setup

Experiments were conducted in the Leibniz-Centre for Tropical Marine Research (ZMT) in Bremen (Germany) according to German guidelines and regulations regarding animal welfare (permission according to §11 section 1 clause 1, Tierschutzgesetz). Juvenile milkfish (4 months) were provided by the Brackish Water Aquaculture Development Center from Situbondo (Indonesia) and were transported to ZMT within 24 h. Upon arrival, all 256 fish were quarantined in a single recirculation system (RAS) of 2500 L. After six weeks (0.01% mortality, $N = 3$), 150 fish were randomly selected and equally divided in six experimental tanks and allowed to further acclimatize for four weeks. Each tank was integrated into an independent experimental RAS of 250 L, each equipped with a bio-filter, automatic temperature control system (Jemitec; TSic-506F, accuracy ± 0.1 °C), aeration and lightning (AquaPhoton, T5). Three weeks before experimental conditions were initialized six fish per tank were tagged by injecting a PIT tag (7 * 1.35 mm) into the dorsal muscle tissue close to the neurocranium. During both acclimatization periods, fish were kept under a 12 h light: 12 h dark cycle and fed daily (09:30 h & 16:30 h) at 5% BW with commercial pellets (Algae Pellets, Vitalis Aquatic Nutrition). Water quality parameters were set at 26.1 ± 0.1 °C, a salinity of 35.6 ± 0.3 PSU and 97.8 ± 3.1% oxygen saturation and monitored daily. In addition, ammonia, nitrite and nitrate were checked weekly by using water quality test kits (JBL, Germany).

Fish in the control (CTR) tanks (N = 3) were kept for 28 days (D-7 to D21) at 26 °C, while fish in the stress (HIGH) tanks (N = 3) were treated as follows: during the first 7 days (D-7 to D0) water temperature was gradually increased from 26 °C by 1 °C per day to 33 °C and subsequently kept constant at 33 °C for 21 days (D0 to D21) (Fig. 1). Due to logistical feasibility each sampling was carried out over two days. The tagged fish of each tank were sampled for scales on the onset of the experiment (i.e. at D-7), after 7 days of gradual temperature increase (i.e. at D0) and at the end of the experiment (i.e. at D21).

Fish were anesthetized (0.1%; *v*/v) and at D21 finally euthanized (1%; v/v) using 2-phenoxyethanol. At each sampling, fish weight (g) and total length (cm) were recorded and used to calculate the Fulton condition factor (FCF = W/L^3) per fish. In total 60 ontogenetic scales (OG) were sampled dorsal to the lateral line from the left flank of each tagged fish at D-7 and D0, respectively. At D21, 60 regenerated scales (RG) per fish were sampled from the body area used for OG sampling at D0. According to Aerts et al. (2015), the mucus was removed and scales were dried with soft tissue before being stored at 4 °C. After sampling, fish were allowed to fully recover (~5 min) before being re-introduced to the experimental tanks. At D7 and D14 additionally 8 non-tagged fish were taken out per tank for other studies. To prevent possible contamination of the water and samples with exogenous glucocorticoids (e.g. from hands), sampling as well as maintenance was done using gloves. Furthermore, to monitor possible contaminations or accumulations of glucocorticoids in fish tanks, water (50 mL) was sampled each day at 09:00 h from each tank for glucocorticoid profiling and stored at

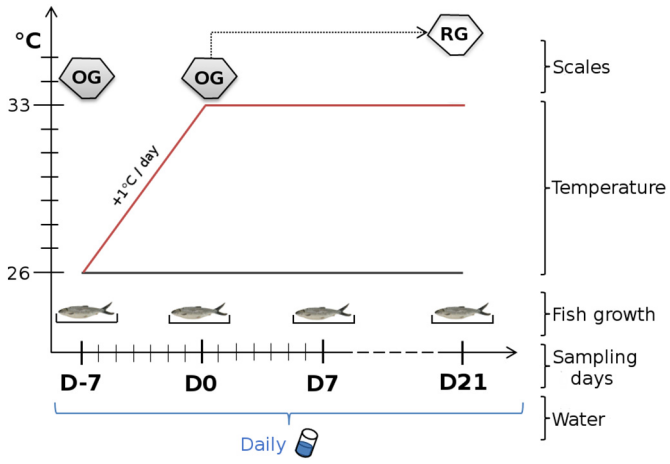

Fig. 1. Sampling design. Fish scale sampling comprised sampling of ontogenetic scales (OG) at D-7 and D0 as well as regenerated scales (RG) at D21. Water for glucocorticoid profiling was sampled daily from each tank. Fish growth was monitored throughout the entire experiment (D-7 – D21).

−20 °C.

2.2. Glucocorticoid analysis

Scales were cut and further homogenized using PowerBead tubes (ceramic 2.8 mm, Qiagen) and a bead ruptor (PowerLyzer 24, Qiagen). HPLC-gradient grade methanol was used as extraction solvent and purification was done using GracePure™ SPE C18-Max 500 mg/6 mL solid-phase extraction (SPE) columns. After resuspension, ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) was used to quantify scale cortisol and water glucocorticoid profile according to or modified from Aerts et al. (2015), respectively.

2.3. Statistical analysis

All parameters were modeled using a linear mixed model with the glimmix procedure in SAS 9.4 (SAS Institute Inc., Cary, NC) with treatment, day of sampling and their interaction (where appropriate) as fixed effect. Fish-ID was included as random effect to correct for repeated measure within the same fish. To achieve normality, the glucocorticoid concentrations were Log-transformed. The transformed data were assumed to be sufficiently normally distributed based on a graphical examination of the residuals (histogram and QQ-plot). In case of post-hoc pairwise comparisons between the treatments within a day of sampling, *P*-values were adjusted with a Tukey-Kramer adjustment.

3. Results

3.1. Fish growth was positively affected by a higher temperature

At the start of the experiment (D-7) fish length, weight and body condition factor were not significantly different between treatments (Table 1). For the total period of 28 days (D-7 to D21) a significant difference for length as well as weight was observed in both treatments (CTR $p < .05$; HIGH $p < .001$). Fish length and weight was significantly different between treatments after 7 days of temperature increase (D0 $p < .05$) and after 21 days of constant temperature (D21 $p < .01$), though not at D7.

Body condition factor was not significantly different in and between treatments and remained constant during the entire experimental period (Table 1).

3.2. Ontogenetic scale cortisol had increased after 7 days of gradual temperature increase

At the start of the experiment (D-7) as well as after 7 days (D0) OG cortisol was not significantly different in and between treatments. However, HIGH fish already showed a higher concentration (0.0020 ± 0.001 vs. 0.0014 ± 0.001 $\mu g\,kg^{-1}$, outliers excluded for calculated average) as well as a higher variability in OG cortisol after 7 days of gradual temperature increase (Fig. 2).

3.3. Regenerated scale cortisol had increased after 21 days of constant high temperature

RG cortisol was clearly higher in HIGH fish and differed significantly after 21 days of constant temperature from CTR fish ($p < .01$) (Fig. 3).

3.4. Glucocorticoids did not accumulate in tank water

Daily glucocorticoid profiling of tank water revealed that cortisol as well as other glucocorticoids were present in all tanks throughout the experiment, though in too low concentrations to be accurately quantified with UPLC-MS/MS ($< 0.003\,\mu g\,L^{-1}$).

4. Discussion

Ectothermic organisms, such as milkfish, are strongly affected by water temperature (Schulte, 2015). Temperature changes beyond the optimal range of a species are known to induce an endocrine stress response through activation of the HPI axis, hereby triggering the release of cortisol (for teleost fish) in order to regain homeostasis (Benítez-Dorta et al., 2017; Liu et al., 2016; Pribyl et al., 2016). In milkfish aquaculture, water temperature in outdoor systems, such as ponds, pens and cages, normally varies between 24 °C and 33 °C and sometimes even reaches upper limits of 40 °C (Bagarinao, 1994; Martinez et al., 2006; Verceles et al., 2000). Data about the physiological consequences for juvenile milkfish exposed to increasing temperature are scarce and when available these studies focused on maximal temperature limits, such as determining lethal temperatures for milkfish fry and fingerlings at 43 °C and 39 °C, respectively (Panikkar et al., 1953). Our aim was to investigate the response of juvenile milkfish exposed to conditions which constitute a realistic temperature range for outdoor milkfish aquaculture systems (26 °C - 33 °C), hereby avoiding a heat-shock, and examine whether juvenile milkfish would exhibit indications of chronic stress, reflected by increased scale cortisol (OG and RG). Subsequently, acclimatization and control temperature was set in accordance to the culture temperature of the Indonesian provider at 26 °C, while 33 °C was selected as high temperature.

4.1. Cortisol as biomarker for stress in milkfish

Overall, glucocorticoids such as cortisol, are measured using a variety of analytical methods ranging from radio- (RIA) or enzyme (EIA) immunoassays to chromatographic analysis (Cook, 2012). Immunoassays are common practice in most laboratories and are used for the determination of cortisol in a plethora of tissues, including but not limited to plasma, feces, water, etc. However, results obtained with

Table 1
Fish growth data. Significant differences between sampling days within each treatment are represented by different letters, differences between treatments by braces.

		Sampling day			
	Treatment	D-7	D0	D7	D21
Length (cm)	CTR	12.6 ± 0.4^{a}	12.7 ± 0.4^{ab}	12.9 ± 0.5^{ab}	13.1 ± 0.5^{b}
	HIGH	12.7 ± 0.5^{a}	13.1 ± 0.6^{b}	13.2 ± 0.7^{b}	13.6 ± 0.8^{b}
Weight (g)	CTR	17.4 ± 2.0^{a}	18.2 ± 2.3^{ab}	19.2 ± 2.6^{ab}	19.7 ± 2.9^{b}
	HIGH	18.1 ± 1.8^{a}	19.9 ± 2.2^{ab}	20.6 ± 2.5^{b}	22.5 ± 3.2^{c}
Condition factor	CTR	0.88 ± 0.05^{a}	0.89 ± 0.05^{a}	0.89 ± 0.04^{a}	0.88 ± 0.04^{a}
	HIGH	0.89 ± 0.06^{a}	0.87 ± 0.05^{a}	0.90 ± 0.08^{a}	0.90 ± 0.07^{a}

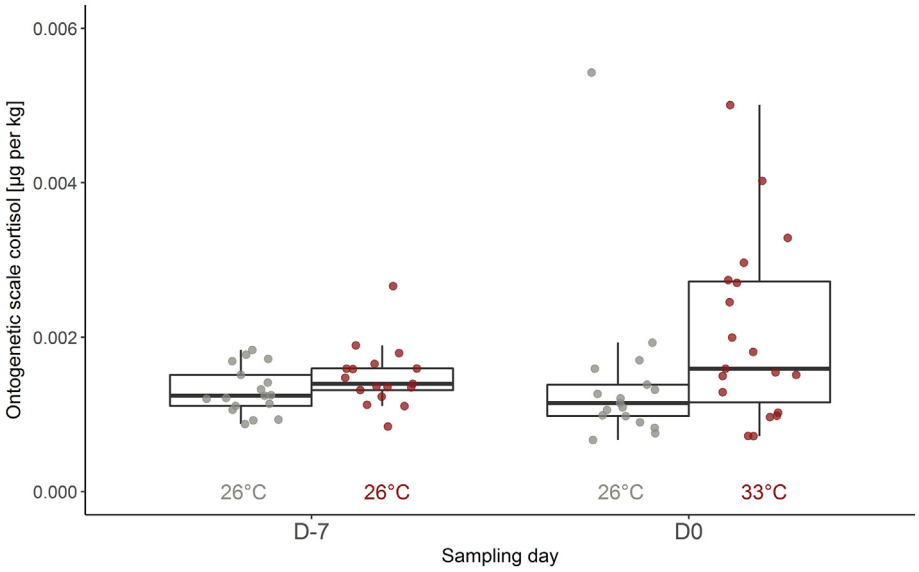

Fig. 2. OG Cortisol after 7 days of gradual temperature increase. CTR ($N = 18$) and HIGH (N = 18) fish are indicated by grey and red dots, respectively. Three values (one from HIGH at D-7; two from CTR, one each at D-7 and D0) were omitted from the plot for reasons of presentation. Fifty percent of the observations occur between the lower and upper edges of the box (the first and third quartiles) and the whiskers extend to the most extreme observation, which is no > 1.5 times the interquartile range from the box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

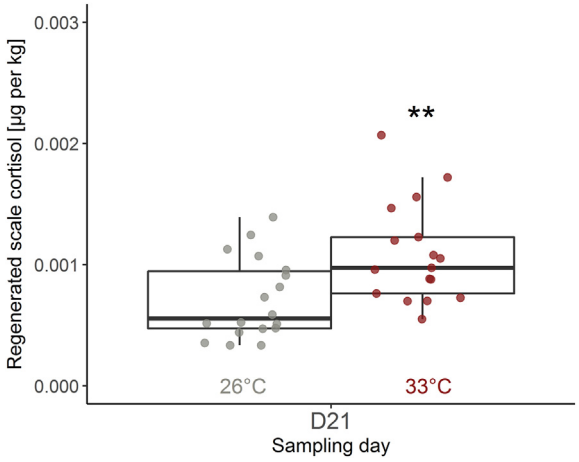

Fig. 3. RG cortisol after 21 days of constant temperature. CTR (N = 18) and HIGH (N = 18) fish are indicated by grey and red dots, respectively. ** = $p < .01$. Error bars in scatter/boxplots are defined as in Fig. 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

these antibody based techniques can be biased by cross-reactivity from other glucocorticoids as well as by compounds with similar physical-chemical properties (Lane, 2006; Murphy et al., 1981; Turpeinen et al., 1997). Surprisingly, only a few studies were found using plasma cortisol as stress indicator in milkfish (Kumar et al., 2016; Wei et al., 2017). Hwang et al. (1992) examined whole-body cortisol level of newly hatched fish larvae from different species and reported comparatively low cortisol levels for milkfish larvae. It should be noted, however, that whole body levels of cortisol are prone to be biased by a multiple of cortisol precursors as well as phase I metabolites making in-depth analytical validation pivotal. In addition, pooling of larvae can mask effects due to individual responses (coping style) as average cortisol values are quantified.

As plasma cortisol, the most commonly used biomarker for stress in the pertinent literature, merely provides a snapshot of HPI axis (re) activity, fish scales were chosen to quantify cortisol as scales provide a retrospective view on HPI axis (re)activity (Aerts et al., 2015). Scale cortisol was quantified by using UPLC-MS/MS in relation to the stress response induced by temperature increase over time. In addition, plasma cortisol values are easily biased by fish handling procedures and are pulsatile due to circadian rhythmicity. Fish scales are an adequate tissue to quantify cortisol over time as scales grow slowly but continuously with the fish (Schönbörner et al., 1979) which subsequently leads to a persistent incorporation of cortisol (Aerts et al., 2015). Furthermore, compared with other methods sampling of fish scales is a non-invasive sampling method and therefore provides the possibility to monitor stress levels of individual fish over time without large interferences. Hereby, distinction must be made between ontogenetic (OG) and regenerated (RG) scales, respectively. OG scales are formed in early life of the fish, while RG are scales grown back whenever the fish loses a scale. Both OG and RG scales provide a retrospective view on HPI axis (re)activity, experienced by the fish from onset of scale growth until sampling (Aerts et al., 2015). Hereby, RG cortisol provides a more detailed view as scale cortisol levels were 'reset' by pulling out the scale at the beginning of the trial hereby inducing scale regeneration (Aerts et al., 2015). Subsequently, RG cortisol levels represent solely cortisol produced within that specific time period without taking into account the level of cortisol that was already present in the scale before pulling out the scale. This is the first time that the use of scale cortisol, OG as well as RG cortisol, is demonstrated in milkfish, which is in line with the study from Aerts et al. (2015), who reported the effective use of OG and RG cortisol for carp.

4.2. Fish history and -management across groups did not influence the results

Handling during transport, tagging or capture can induce an acute stress response resulting in an increase in plasma cortisol (Acerete et al., 2004; Iversen et al., 2005; Robertson et al., 1988). However, fish usually show a quick recovery within hours or a few days (species-dependent) when handling procedures are not chronically repeated (Acerete et al., 2004; Barton and Peter, 1982; Robertson et al., 1988). In our study potential disturbances due to handling were kept as short as possible and fish were allowed to recover for at least 3 weeks after transport or tagging before experimental conditions were initialized. As scale cortisol provides a retrospective view on the HPI axis (re)activity (Aerts et al., 2015) and OG cortisol levels at onset of the experiment (D-7) were found to be similar for all fish (CTR/HIGH), indicating that fish were exposed to similar (stressful) conditions before the start of the experiment. In addition, all fish had a similar body condition factor and had grown throughout the experiment, as indicated by their increased length and weight. Sex-specific distinction was not possible.

In several studies waterborne steroids were shown to be (re-)uptaken by fish, hereby affecting plasma concentrations of the respective

hormones (Scott and Ellis, 2007; Maunder et al., 2007). During our entire experiment, levels of water cortisol as well as other glucocorticoids in the individual RAS were monitored and found to be present, though in too low concentrations to be accurately quantified with UPLC-MS/MS. Water glucocorticoid profiling confirmed that scale cortisol levels in this study were not influenced by an accumulation of water glucocorticoids over time due to insufficient water renewal, nor by anthropogenic contamination with exogenous glucocorticoids (e.g. from hands).

4.3. Seven days of gradual temperature increase resulted in higher OG cortisol levels

The response to a stressor depends, besides the type, on the duration, severity, (un)predictability, and (un)controllability of the applied stimulus (Schreck and Tort, 2016). A gradual increase of 1 °C per day for 7 consecutive days can be considered as a physical stimulus with mild severity, low unpredictability, and no controllability. A repeated or prolonged exposure to an acute stimulus can be seen as a potential chronic stressor (Sopinka et al., 2016) making the gradual increase over 7 days a potential chronic stressor.

After 7 days of temperature increase (D0), OG cortisol level of HIGH fish were found to be higher, though not significantly, than at the start of the experiment (D-7) and higher, though not significantly, compared to CTR fish, indicating a clear response of the HPI axis to the applied gradual temperature increase. OG cortisol levels in CTR fish remained similar as expected. Furthermore, within this 7 day period HIGH fish were shown to be significantly longer and heavier compared to CTR fish, confirming that a higher, though not too high, temperature has a positive effect on growth.

In general, this positive effect of higher temperature on growth is not surprising. Within the optimal temperature range of a species, increasing temperatures will lead to an increase in performance (biochemical reactions, metabolic rate, growth etc.) until reaching the peak of optimal temperature at which performance is maximized and subsequently decline (Huey and Kingsolver, 1989; Schulte, 2015). Fish confronted with chronic stress are known to show decreased growth as the available energy is used to regain physiological homeostasis (Wendelaar Bonga, 1997). Besides studies regarding the general temperature tolerance and the determination of lethal temperatures, studies about the physiological effects of higher or increasing temperatures on juvenile milkfish are scarce. Villaluz and Unggui (1983) reported the highest growth rate of milkfish fry at 29.5 °C; however, high fluctuations were recorded within these temperature treatments. Subsequently, it remains to be seen which temperature constitutes the borderline between a positive effect on metabolism and a starting negative stress induced effect in juvenile milkfish. Or in terms of HPI axis activity, which temperature induces a significant change in released cortisol, shifting the physiological response from eustress (anabolic) to distress (catabolic) (Gorissen and Flik, 2016). The high variability in OG cortisol at D0 indicates that HIGH fish definitively reacted to the applied temperature increase. Individual differences in physiological as well as behavioral response to stress are described for many species (Castanheira et al., 2013; Huntingford et al., 2010; Martins et al., 2011; Silva et al., 2010; van Raaij et al., 1996). Biro et al. (2010) reported that reef fish in Australia already exhibited individual differences in behavior when exposed to a minute diel temperature variation of < 3 °C. As 7 days of gradual temperature increase led to a higher though not significant difference in OG cortisol between as well as within the treatments, we can only hypothesize that this treatment was not long and/or severe and/or unpredictable enough to induce severe chronic stress. A prolonged duration for a few days and/or an increased daily temperature might have been enough to see clear differences in OG cortisol between and/or within treatments. This is an important topic for further research, as climatological changes caused by increasing anthropogenic activities, especially in coastal regions, lead to a gradual increase in water temperature affecting not only outdoor milkfish aquaculture systems.

4.4. Twenty-one days of constant high temperature resulted in higher RG cortisol levels

HIGH fish showed a clear response in HPI axis activity during 21 days exposure to constant 33 °C, indicated by significantly higher RG cortisol compared to CTR fish. Besides the primary stress response, indicated by this elevated cortisol level, changes in behavior can also be an indication for stress (Biro et al., 2010; Øverli et al., 2002; van Raaij et al., 1996). Effects on the whole-body performance (tertiary stress response), are seen in our study in the reduced growth of HIGH fish in the first 7 days at 33 °C (D7) as well as in the overall observations of animal care takers, whereby HIGH fish showed a more reactive behavior during routine work.

Taking into account that the applied temperature treatment concerns a commonly seen temperature range in outdoor milkfish aquaculture systems, the observed significant increase in RG cortisol and also, though not quantitative, response on whole-body performance is surprising. On the other hand, at D21 length and weight of HIGH fish was again significantly higher compared to CTR fish. This might be an indication that HIGH fish started to habituate to the treatment between D7 and D21. In terms of severity and unpredictability, the exposure to constant 33 °C is probably a comparatively mild stimulus and therefore it is possible that fish can habituate. Furthermore, the stress response of fish depends on the perception of the potential stressor and varies between species and even individuals of the same species (Barton, 2002; Cockrem, 2013). So, was the applied thermal stimulus intense enough to induce a significant stress response in our milkfish? A single increase of 1 °C will probably not be perceived as a severe stressor, but a persistent repetition consequently leading to a considerable increase in temperature, is more likely to be considered a stressor by the fish. On the other hand, if temperature remains constant, fish might habituate and no longer perceive these conditions as stressful. However, a more detailed view on the stress response, consisting of not only cortisol levels, but also of gene expression of pre-receptor regulating oxido-reductases, such as 11β-hydroxysteroid dehydrogenase type I and II, and glucocorticoid- and mineralocorticoid receptors, is needed to clearly point out a possible habituation process. A decrement of the HPI axis reactivity, for instance, could be a sign for habituation (Cyr and Romero, 2009). Since scale cortisol was measured in milkfish for the first time and the pertinent literature lacks information about scale regeneration in this species, we assumed a similar regeneration process as described by Ohira et al. (2007) reporting complete regeneration for goldfish scales within 14 to 28 days. During our experiment we noticed that scales were already regenerated after 14 days, therefore in future studies sampling of RG scales after 14 days should be considered to obtain an even more detailed view on the HPI axis activity.

5. Conclusion

As anthropogenic activities affecting the climate are increasing, and taking into account the widespread use of outdoor aquaculture systems, which are prone to natural fluctuations, an increase in temperature is most likely to be considered a stressor in milkfish aquaculture, as indicated by our results. Hereby, the use of scale cortisol was shown to be effective to quantify even minute and gradual temperature changes, making it a powerful tool in optimizing aquaculture systems as well as in monitoring gradual climate changes in wild stock.

Competing Interests

The authors have no competing interests.

Author contributions

Conceived and designed the experiments: IH, AK, AG and JA. Performed the experiments: IH. Analyzed the data: IH and JA. Contributed reagents/materials/analysis tools: AK, AG, and JA. Wrote the paper: IH and JA. Performed the scale and water glucocorticoid analyses: IH. Performed the statistical analyses: BA. Discussed the results and contributed to the final manuscript: IH, BA, AK, AG, and JA.

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