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State of thermal tolerance in an endangered himalayan fish *Tor putitora* revealed by expression modulation in environmental stress related genes

Amarjit Kaur[1,2,4], Shahnawaz Ali[1,4]✉, Onkar Singh Brraich[2], C. Siva[3] & Pramod Kumar Pandey[1]

Increasing temperature due to global warming in the Himalayan regions has severe implications for the survival of aquatic ectotherms. To study the thermal acclimation and heat tolerance of an endangered Himalayan fish species, *Tor putitora*, we examined tissue-specific mRNA expression patterns of heat-shock proteins (*HSP90β*; *HSP70*, *HSP60*, *HSP47*, *HSP30*, and *HSP20*), warm-temperature acclimation proteins (*WAP65-1*) and cyclin-dependent kinase inhibitor 1B (*CDKN1B*) genes in liver, brain, gill, kidney, muscle, and gonad tissues at the intervals of 10, 20, and 30 days during a high-temperature treatment (34.0 °C) for 30 days. All the tested genes have exhibited tissue-specific and time-dependent expression patterns. Heat shock proteins' differential expression and modulation across examined tissues indicate their role in long-term cellular adaptation, protection against the cytotoxic effect of hyperthermia, and species acclimation to higher temperatures. *WAP65-1* and *CDKN1B* expression in treatment groups suggests its involvement in maintaining homeostasis, long-term temperature acclimation, and thermotolerance during chronic thermal exposure. The response of studied genes under heat stress indicates their potential use as environmental stress biomarkers in this species. The present study elucidates molecular mechanisms regulating the thermal acclimation capacity and thermotolerance of *T. putitora* and its survival under future projections of widespread warming in the Himalayan region.

Keywords Heat shock protein, Thermal tolerance, Climate change, *Tor putitora*, *WAP65-1*, *CDKN1B*

The ambient water temperature is the most critical ecophysiological variable affecting the performance of ectotherms. Fishes are, thus, more sensitive and vulnerable to any change in the temperature that lies beyond their physiological limits[1]. Therefore, the adaptation to dynamic changes in the environmental temperature would determine their long-term survival and fitness[2]. The increasing temperature due to global climate change has altered the thermal regimes of the aquatic ecosystem and the recent climate change models predicted an increase in mean water temperatures from 1.4 to 3.1 °C the next 65 years, posing a profound threat to the fish[3,4]. Moreover, the threat severity is even higher in the Himalayan regions as the estimated rate of warming is three times greater than the global average[5].

Environmental stressors mostly lead to myriad physiological and cellular processes in fish through remarkable alteration in the transcript abundance of many genes[6]. The abilities to modulate expressions of different allozymes, genes, cell membrane modifications, or alterations to the intracellular environment that may or may not be reversible are some of the strategies of acclimation of thermal physiology observed across ectotherms[7]. In addition, the process of thermoregulation in fish enables them to adaptive shift in performance, thereby enhancing acclimation capacity at longer time scales[8]. Higher temperatures involve biological implications, including protein denaturation and misfolding as a cellular response to stress[9]. Incidentally, heat

[1]Molecular Genetics Lab, ICAR-Directorate of Coldwater Fisheries Research, Bhimtal, Nainital, Uttarakhand 263136, India. [2]Department of Zoology and Environmental Sciences, Punjabi University, Patiala, Punjab 147002, India. [3]ICAR-Central Institute of Brackishwater Aquaculture, Chennai, Tamil Nadu 600028, India. [4]Amarjit Kaur and Shahnawaz Ali contributed equally to this work. ✉email: alicife@gmail.com

shock response (HSR) is the most common endogenous mechanism in fish to minimize cellular damage in the event of both acute and chronic temperature changes[10]. Different types of stressors, including temperature, leads to the expressions of heat shock proteins (HSPs) in cells to maintain many critical cellular processes as typical characteristics of HSR in fish[11]. These HSPs, or molecular chaperones, are critical in the synthesis, transport, and folding of proteins[12]. Three major families of HSPs are classified based on their molecular weights viz., HSP90 (85–90 kDa), HSP70 (68–73 kDa), and low molecular weight (LMW) heat shock proteins (16–47 kDa)[10,13,14]. When exposed to proteotoxic stressors, the higher expression of a large number of HSP genes (e.g., *HSP90*, *HSP70*, *HSP60*, *HSP47*) ensure survival under stressful condition through stabilization of damaged protein and prevents protein aggregation, which otherwise lead to irreversible cell damage and ultimately to cell death[12,15]. Basu et al. extensively reviewed the role of different HSPs and their functional significance in fish[10]. In addition to HSPs, the transcript level of warm-temperature-acclimation-associated 65-kDa protein (*WAP65*), a plasma glycoprotein first identified in goldfish (*Carassius auratus*) which is homologous to hemopexin found in mammals, also markedly increases in accordance with ambient temperature elevation[16,17]. The role of *WAP65* in acclimation to warm temperatures has been investigated in several fish species in response to an increase in temperature[16,18–21]. Under chronic thermal stress conditions, cyclin-dependent kinase inhibitor 1B (*CDKN1B*), a gene mainly involved in cell cycle regulation and apoptosis, was found to be up-regulated as a cellular stress response in fish[22,23]. However, the magnitude of the expression of most of the genes involved in thermotolerance and acclimation correlates to the stress level experienced in the natural environment[10]. Further, most of these genes showed tissue-specific responses depending on the various molecular functions associated with each tissue type[24]. Additionally, fishes differ in their molecular responses to temperature variations depending on the level of environmental variability, prior acclimation, and degree of exposure, i.e., acute or chronic thermal stress[22,23,25,26]. Overall, the exposure to thermal stress in fish induces a set of multiple genes associated with HSR and warm temperature acclimation as a consequence of cellular stress responses that provide adaptive regulation to heat stress and higher plasticity to acclimatize to change in temperature[10,22,27]. Therefore, understanding the mechanism of tissue-specific molecular response to thermal stress provides necessary insight into thermal tolerance and acclimation in aquatic ectotherms, and it became more relevant under climate change scenarios[24,28].

Golden mahseer (*T. putitora*) is an 'endangered' cold water rheophilic cyprinid fish species of great ecological, food, and recreational value in the Indian Himalayan region and also found in Southeast Asian drainages[29–31]. It is a large-bodied potamodromous freshwater fish migrating mainly to feed and spawn in the hill stream. The water temperature of the feeding ground of golden mahseer has been recorded in the range of 14 to 22 °C, while the spawning and incubation temperate varies from 16 to 25 °C in river water[32]. The Himalayan region is highly vulnerable to climate warming, and it is predicted that the region may experience an increase in temperature that may be at least 0.3 °C more than the predicted average global warming of 1.5 °C[33,34]. Recently, it was reported that the breeding phenology of golden mahseer in natural water bodies appears to have undergone a transition over the last ten decades as an implication of regional climate change[35]. Therefore, we investigated the tissue-specific mRNA expressions of genes involved in thermal tolerance and warm temperature acclimation to study the implications of chronic thermal exposure to golden mahseer. The expressions of both high and low molecular weight HSP genes (*HSP90β*, *HSP70*, *HSP60*, *HSP47*, *HSP30*, *HSP20*), *WAP65-1*, and *CDKN1B* were measured during the time course of thermal stress. In addition, we also tried to elucidate the significant roles of *WAP65-1* and *CDKN1B* in warm temperature acclimation and thermotolerance in golden mahseer. We also presumed that *T. putitora* would show plasticity in their HSR during thermal exposure in a tissue-specific manner. The data presented in the current study would help elucidate the function of heat shock proteins and the adaption of golden mahseer under a thermally stressed environment.

Results

The relative fold change in mRNA levels of high (*HSP90β*, *HSP70*, and *HSP60*) and low (*HSP47*, *HSP30*, and *HSP20*) molecular weight HSP genes, *WAP65-1*, and *CDKN1B* were quantified in different tissues (liver, brain, gill, kidney, muscle, and gonad) of the fishes sampled at control (T_0) and during high-temperature exposure (T_{10} to T_{30}). We have also measured the length and weight of the fish in control and treatments at the start and at the time of sampling. There were no significant differences found in the total length, weight, or condition factor of the fish sampled at different time points (data not shown).

Effects of chronic thermal stress on mRNA expression of *HSP90β*, *HSP70* and *HSP60* genes

Under the chronic thermal exposure, the mRNA expressions of high molecular weight chaperones, *HSP90β* (Fig. 1A–F) and *HSP70* (Fig. 2A–F) were significantly and consistently upregulated across all examined tissues except gonad at T_{10} relative to control (T_0). Among all tissues, the liver showed the highest expression of *HSP90β* (Fig. 1A), whereas, the *HSP70* expression level was 10-fold higher in the brain ($p < 0.0001$), gill ($p < 0.001$), kidney ($p < 0.001$) and muscle ($p < 0.01$) tissues at T_{10} compared to the constitutive expression at control (T_0) (Fig. 2B–E). Further, we also observed that the expression level of *HSP90β* remained persistently upregulated in the liver and brain after an exposure of 20 (T_{20}) and 30 days (T_{30}) (Fig. 1A, B) and significantly downregulated in the kidney (Fig. 1D), while it returned to basal value in gill and muscle (Fig. 1C, E). However, mRNA expression of *HSP70* remained upregulated after an exposure of 30 days (T_{30}) in four tissues (brain, gill, kidney, and muscle) except the liver as compared to control (T_0) (Fig. 2A–E). In addition, a pronounced decreased level of transcript abundance of *HSP90β* (Fig. 1A–E) and *HSP70* (Fig. 2A–E) were measured at T_{30} compared to T_{10} across different tissues. In gonads, no significant differences in the mRNA expression of *HSP90β* and *HSP70* were measured between the different time points (Figs. 1F and 2F).

Measurement of mRNA expression of *HSP60* indicated a significant variation across the tissues during different time points of high-temperature exposure (T_{10} – T_{30}) (Fig. 3A – F). In liver, brain, and muscle tissues, it increased significantly at T_{10} and remained elevated across all tissues at T_{30}, with an apparent decrease at T_{20}

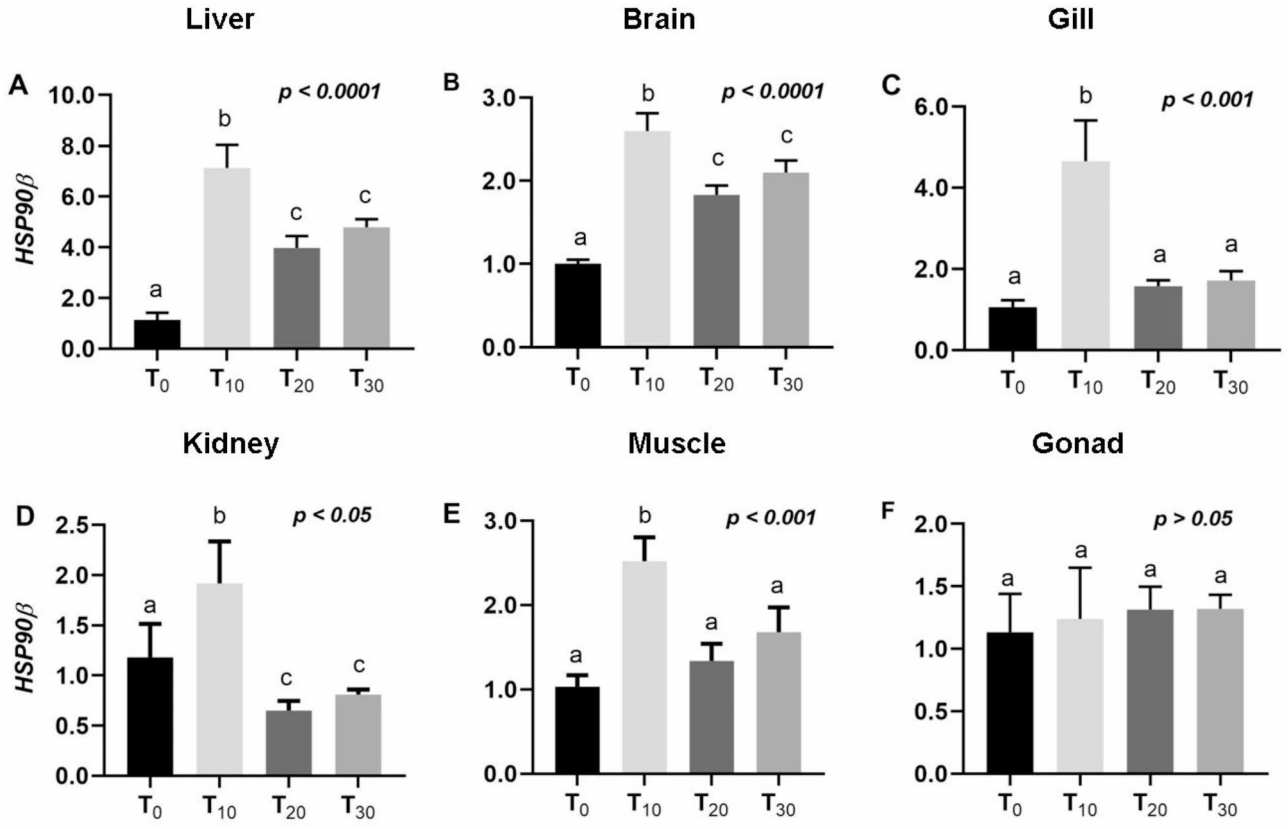

Fig. 1. Relative fold change of *HSP90β* (**A**–**F**) mRNAs in different tissues of golden mahseer examined in the control (T_0) and over the time-course (days) of high temperature exposure (T_{10}, T_{20}, and T_{30}). Results are presented as mean ± SEM ($n = 5$ fish per time point) and were analysed using one-way ANOVA, followed by Tukey's multiple comparison test. Differences were considered significant at $p < 0.05$. Different superscripts (a, b, c) above the bars indicate significant difference.

in liver and muscle (Fig. 3A, B, E). We also observed a consistently high abundance in the brain (Fig. 3B) and a linear increase in transcript abundance in gill tissue (Fig. 3C) during the high-temperature exposure (T_{10} – T_{30}). In addition, liver and muscle expression of *HSP60* showed distinct peaks at T_{10} and T_{30} with a more than 10-fold change ($p < 0.0001$) in hepatic tissue (Fig. 3A, E). Similar to *HSP90β* and *HSP70*, no significant differences observed in the transcript levels of *HSP60* between the different time points in gonads (Fig. 3F).

Effects of chronic thermal stress on mRNA expression of *HSP47*, *HSP30* and *HSP20* genes

The relative fold change in mRNA levels of LMW HSPs (*HSP47*, *HSP30*, and *HSP20*) in different tissues of the fishes sampled at control (T_0) and at different time points of high-temperature exposure (T_{10} – T_{30}) are shown in Figs. 4, 5, and 6. In the liver, brain, gill, and gonad tissues, the mRNA levels of *HSP47* were significantly upregulated at T_{10} relative to control (T_0) (Fig. 4A-C, F), and among all these tissues the highest expression was measured in the liver (12.65 fold, $p < 0.0001$) (Fig. 4A). We also observed consistently high transcript abundance in all examined tissues after an exposure of 30 days (T_{30}) (Fig. 4A-F). Further, in the kidney, a progressive increase in mRNA expression of *HSP47* was measured at different time intervals of thermal exposure (T_{10} – T_{30}) (Fig. 4D).

The *HSP30* gene expression demonstrated a similar pattern in brain and gill tissues, where a significant linear progression in transcript abundance was detected (Fig. 5B, C) during high-temperature exposure (T_{10} to T_{30}). Further, the highest mRNA expression of *HSP30* was measured in the gill tissue under thermal stress. It was remarkably upregulated (22.90-fold, $p = 0.02$) over a period of 10 days of exposure (T_{10}) relative to control (T_0) and reached the peak level (29.43-fold, $p = 0.02$) after an exposure of 30 days (T_{30}) (Fig. 5C). On the other hand, in kidney and gonad, the mRNA level of *HSP30* was significantly downregulated at the later period (T_{20} – T_{30}) of thermal exposure (Fig. 5D, F). The transcript level of *HSP20* showed a significant difference in brain, gill and gonad tissues with a bimodal peak in the brain at T_{10} and T_{30} (Fig. 6B, C, F). However, gill mRNA showed an increased expression level after a thermal exposure of 10 days (T_{10}), which remained upregulated during a later period (T_{20} – T_{30}) compared to control (T_0) (Fig. 6C). In the liver, the expression of *HSP20* displayed apparent fluctuation. However, the differences were statistically insignificant compared to T_0 (Fig. 6A).), whereas in the gonad, mRNA expression was significantly upregulated at T_{10} and returned to the constitutive expression value after 20 days (T_{20} – T_{30}) of thermal exposure (Fig. 6F). No significant differences in *HSP20* mRNA expression

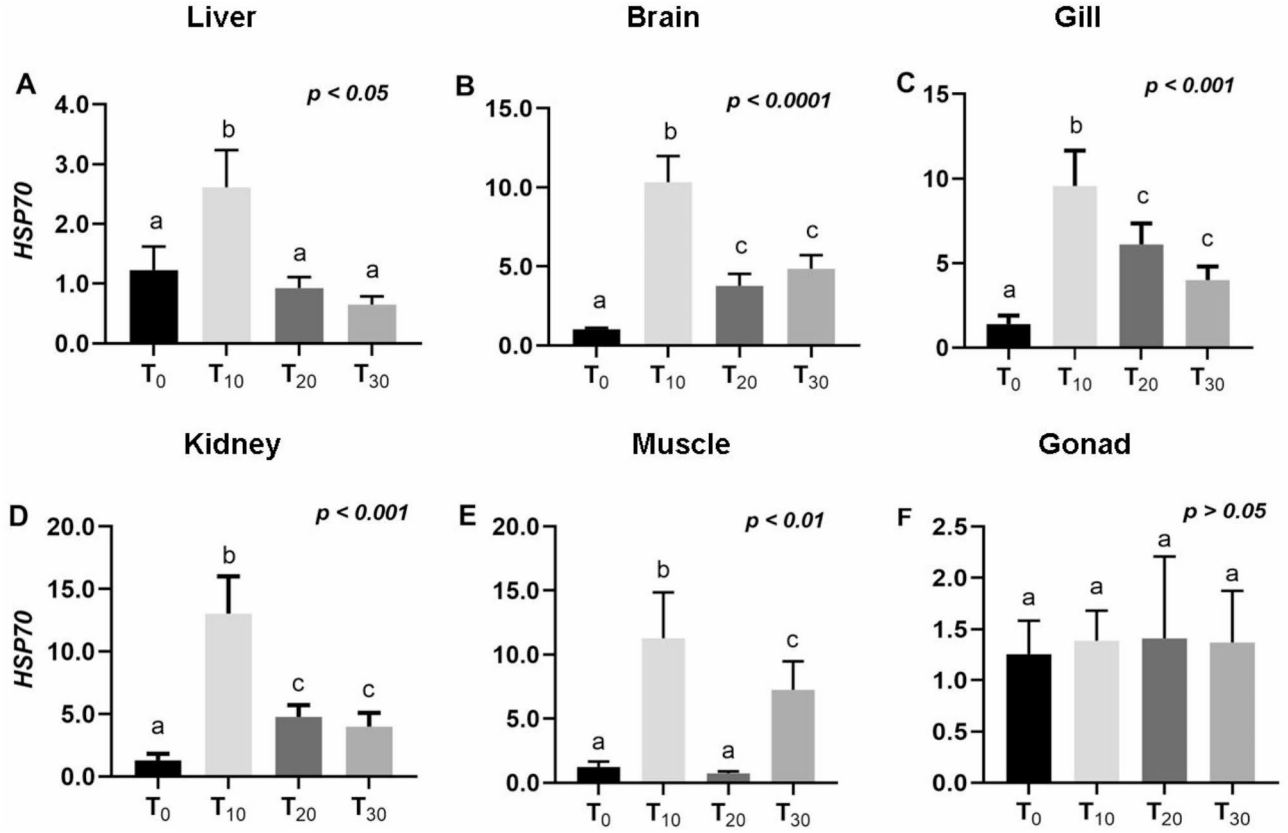

Fig. 2. Relative fold change of *HSP70* (**A**–**F**) mRNAs in different tissues of golden mahseer examined in the control (T_0) and over the time-course (days) of high temperature exposure (T_{10}, T_{20}, and T_{30}). Results are presented as mean ± SEM ($n = 5$ fish per time point) and were analysed using one-way ANOVA, followed by Tukey's multiple comparison test. Differences were considered significant at $p < 0.05$. Different superscripts (a, b, c) above the bars indicate significant difference.

were observed in the kidney and muscle tissues at different time points of high-temperature exposure (T_0 to T_{30}) (Fig. 6D, E).

Effects of chronic thermal stress on mRNA expression of *WAP65-1* and *CDKN1B* genes

The transcript of *WAP65-1* was widely distributed in golden mahseer tissues. However, the expression was the most abundant in the kidney (Fig. 7). Golden mahseer *WAP65-1* was then highly expressed in the liver and gill, and followed by the brain and gonad. However, the expression of *WAP65-1* was significantly ($p < 0.0001$) downregulated in muscle during the time course of heat exposure (T_{10} – T_{30}) (Fig. 7E). It was also noted that there was a linear increase in the transcript abundance in the liver, brain, and gill tissues (Fig. 7A – C) with the increasing time course of thermal exposure (T_{10} – T_{20}), and it remained persistently upregulated in the brain (T_{20} – T_{30}). However, the expression of *WAP65-1* returned to its constitutive level in all other tissues (liver, gill, kidney, and gonad) (Fig. 7). The mRNA expression levels of *CDKN1B* measured across different tissues of golden mahseer are depicted in Fig. 8. Among all examined tissues, only liver and brain showed a very low level of *CDKN1B* mRNA, and it was persistently high during the period of thermal exposure (Fig. 8A, B). The summary of the expression pattern of genes in different tissues at various time points of the thermal exposure is depicted in the Table 1.

Discussion

In the current study, the state of thermal tolerance and adaption were examined by tissue-specific mRNA expression of HSPs and genes related to thermal acclimation and thermotolerance (*WAP65-1* and *CDKN1B*) in *T. putitora* over long-term exposure to an elevated temperature (34 °C). The results showed that golden mahseer exhibits plasticity in its HSR by altering its expression of HSPs in response to the duration of thermal exposure and also shows considerable heterogeneous expression patterns during the time course of heat stress. The upregulation of both *HSP90β* and *HSP70* in all the examined tissues (except gonad) after a thermal exposure of 10 days (T_{10}) indicates heat shock response to short-term stress, while persistent upregulation of *HSP90β* in liver and brain as well as *HSP70* in brain, gill, kidney and muscle tissue on a long-term (20 and 30 days) exposure suggest acclimation towards heat shock and modulation in expression of HSPs in these tissues of golden mahseer[36]. Tissue-specific and time-dependent variations in the expression of the HSPs in response to thermal stress have previously been reported in teleost like striped snakehead (*Channa striatus*), pool barb

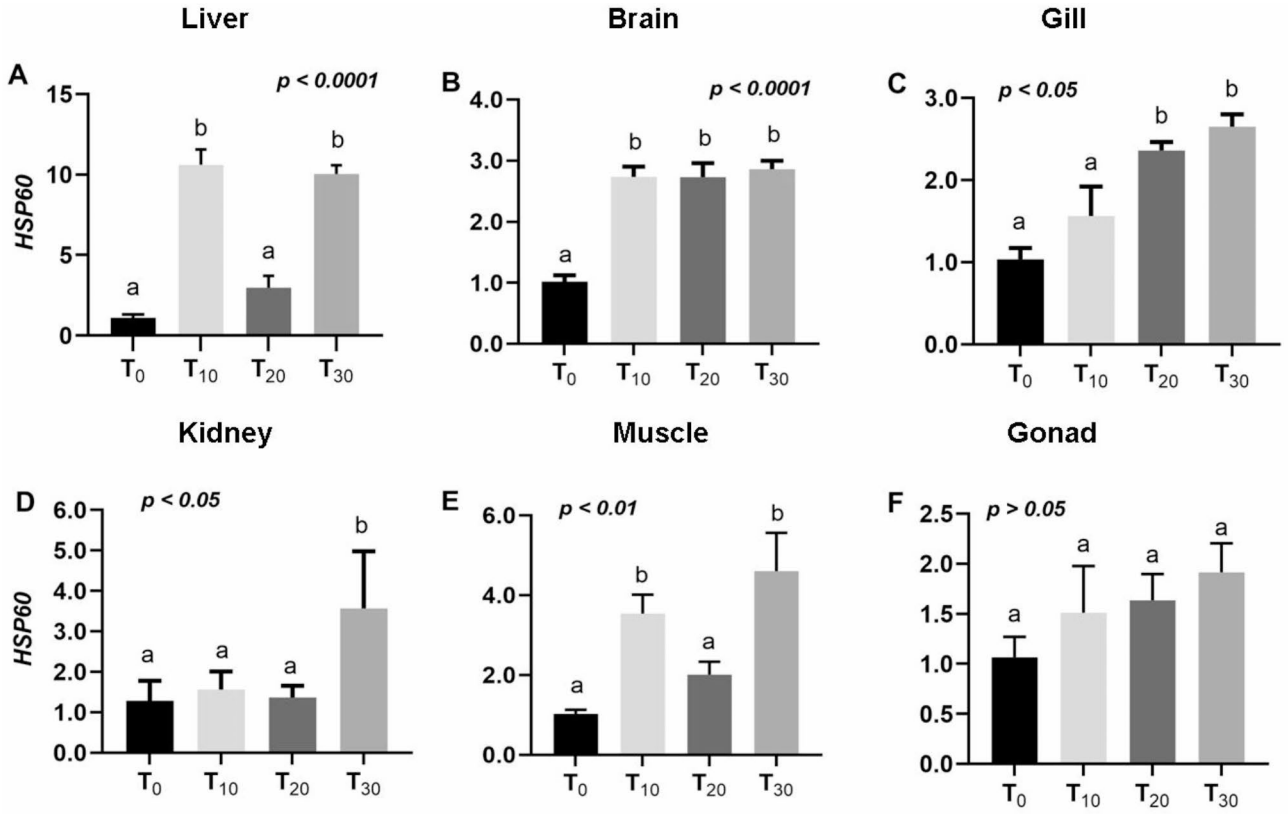

Fig. 3. Relative fold change of *HSP60* (**A**–**F**) mRNAs in different tissues of golden mahseer examined in the control (T_0) and over the time-course (days) of high temperature exposure (T_{10}, T_{20}, and T_{30}). Results are presented as mean ± SEM ($n = 5$ fish per time point) and were analysed using one-way ANOVA, followed by Tukey's multiple comparison test. Differences were considered significant at $p < 0.05$. Different superscripts (a, b, c) above the bars indicate significant difference.

(*Puntius sophore*) and spotted sea bass (*Lateolabrax maculatus*) and gill, liver, and muscle have been observed as primary tissues responding to heat stress in fish[36–41]. However, in our study, heat-induced upregulation in mRNA expression of *HSP90β* and *HSP70* was also observed in other examined tissues like the brain and kidney of golden mahseer, which indicates its sensitivity to elevated temperature, adaptation towards heat stress and role in organismal survival[37,42]. *HSP90* is known to be the most commonly expressed HSP and it plays an active role in maintaining several components of the cytoskeleton and steroid hormone receptors[43]. It was found upregulated in the fish residing in hot spring run-off and also during experimentally challenged to higher temperatures such as snow trout (*Schizothorax richardsonii*)[39,44,45]. Similarly, in the present study, the upregulation of *HSP90β* might be indicative of long-term stress adaptation as among two major cytoplasmic isoforms of *HSP90* (i.e., *HSP90α* and *HSP90β*), *HSP90β* is reported to be involved in long-term cellular adaptation[46]. The upregulation of *HSP70* under prolonged heat exposure in the present study indicates its role in the protection against the cytotoxic effect of hyperthermia and reduction in oxidative stress caused by continuously elevated temperature as well as preventing polypeptide aggregation and refolding of damaged proteins under heat stress[47–49]. The present finding also conforms with past studies that reported overexpression of *HSP70* in aquatic organisms to resist the pressure of rising temperatures[50]. It is also noted that the lack of introns in the heat-inducible form might have allowed efficient transcription, leading to strong induction of *HSP70* genes during heat shock[36,51]. Furthermore, the high mRNA expression pattern of *HSP70* in gill, kidney, and muscle tissue indicated that it might be correlated to immune and metabolic response under heat stress[49,52,53]. However, further investigation is required to confirm these tissues' physiological function and correlation under heat stress in this species.

In the current investigation, the highest mRNA expression of *HSP60* in the liver probably indicates its higher requirement in reducing the heat-induced protein damage in the hepatic tissue than other tissues of golden mahseer. In addition, the persistent high mRNA expression of *HSP60* under chronic thermal exposure (30 days) in the present study suggests that hepatic damage caused by temperature stress is not easily reversed. Moreover, the continuous synthesis of *HSP60* in different tissues (brain, gill, and muscle) also suggests its role in the acclimation and survival of golden mahseer (*T. putitora*) at higher temperature, as reported in the striped snakehead (*C. striatus*) under heat stress[38]. The higher expression of *HSP60* in gill tissue suggests an increase in resistance of gill cells against constant exposure to heat stress, and upregulation in renal tissue indicates that the kidney might have adapted to chronic heat stress, as reported in rainbow trout[54]. We found that during the high-temperature exposure (T_{10} –T_{30}) to golden mahseer, *HSP60* expression patterns varied among tissues,

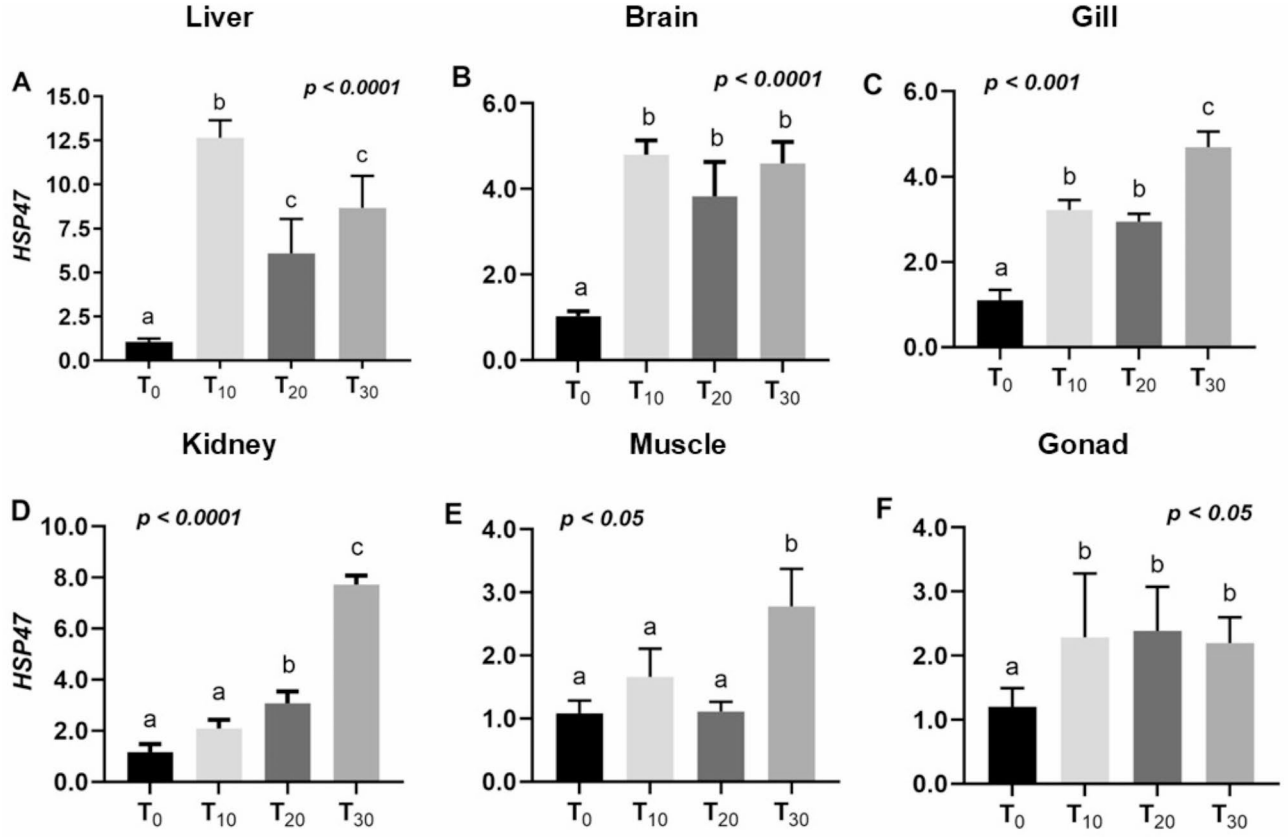

Fig. 4. Relative fold change of *HSP47* (**A**–**F**) mRNAs in different tissues of golden mahseer examined in the control (T_0) and over the time-course (days) of high temperature exposure (T_{10}, T_{20}, and T_{30}). Results are presented as mean ± SEM ($n = 5$ fish per time point) and were analysed using one-way ANOVA, followed by Tukey's multiple comparison test. Differences were considered significant at $p < 0.05$. Different superscripts (a, b, c) above the bars indicate significant difference.

where it showed remarkably high transcript level after ten days of exposure and then declined afterward but remained significantly high compared to control (T_0). This apparent modulation and constant upregulation of *HSP60* could be perceived as a danger signal of stressed or damaged cells[55,56] and species acclimation to higher experimental temperatures where continuous synthesis of *HSP60* is necessary for its survival[22,38]. In addition, higher expression of *HSP60* has also been seen as a potential early warning system for environmental changes[57].

Notably, in our study, no significant mRNA expression of *HSP90β*, *HSP70*, and *HSP60* was detected in golden mahseer gonads during heat stress (T_{10} –T_{30}). In contrast to the present investigation, Mahanty et al.., reported the downregulation of all three genes in pool barb (*P. sophore*) gonads under thermal stress[58]. The absence of significant changes in expression of *HSP90β*, *HSP70*, and *HSP60* in the gonads of *T. putitora* after chronic thermal exposure might be due to tissue specificity as heat shock response differs among tissues and also supports the hypothesis that certain tissue is more sensitive than others in regulating the thermal limits of an organism[37]. Nevertheless, due to their ubiquitous presence and higher expression in various cellular forms, *HSP90β*, *HSP70*, and *HSP60* genes have been identified as critical biomarkers responsible for the adaptive effect of thermal stress in fish[38,59,60].

LMW chaperones, *HSP47*, *HSP30*, and *HSP20*, showed varied expression patterns in different tissues of golden mahseer during thermal exposure. In the present study, the highest expression of *HSP47* (also known as *Serpin H1*) in the hepatic tissue of golden mahseer was in contrast to the earlier findings in zebrafish where it was upregulated in the brain but not in the liver or muscle after a heat shock response[61]. Similarly, no differential expression was detected in hepatic *HSP47* in goldfish (*C. auratus*) sampled from lakes having continuously high temperatures[62]. On the other hand, *HSP47* upregulation was reported in the liver of pool barb (*P. sophore*) sampled from a hot spring and after experimental heat shock treatment[58]. The increase in transcript level of *HSP47* has also been reported under chronic temperature stress in the gills, liver, kidney, and muscle tissues of salmonids[62,63] and Atlantic cod[64]. Based on the previous studies and present findings, it could be observed that the *HSP47* gene expression varies among teleost in a species-specific manner in response to environmental conditions and also shows tissue-specific expression pattern[37,65,66]. *HSP47* is a collagen-binding glycoprotein that is found in the endoplasmic reticulum (ER), and interestingly, it is the only stress protein in the ER induced by heat shock[67]. In our investigation, the upregulation of *HSP47* in different tissues of golden mahseer during the

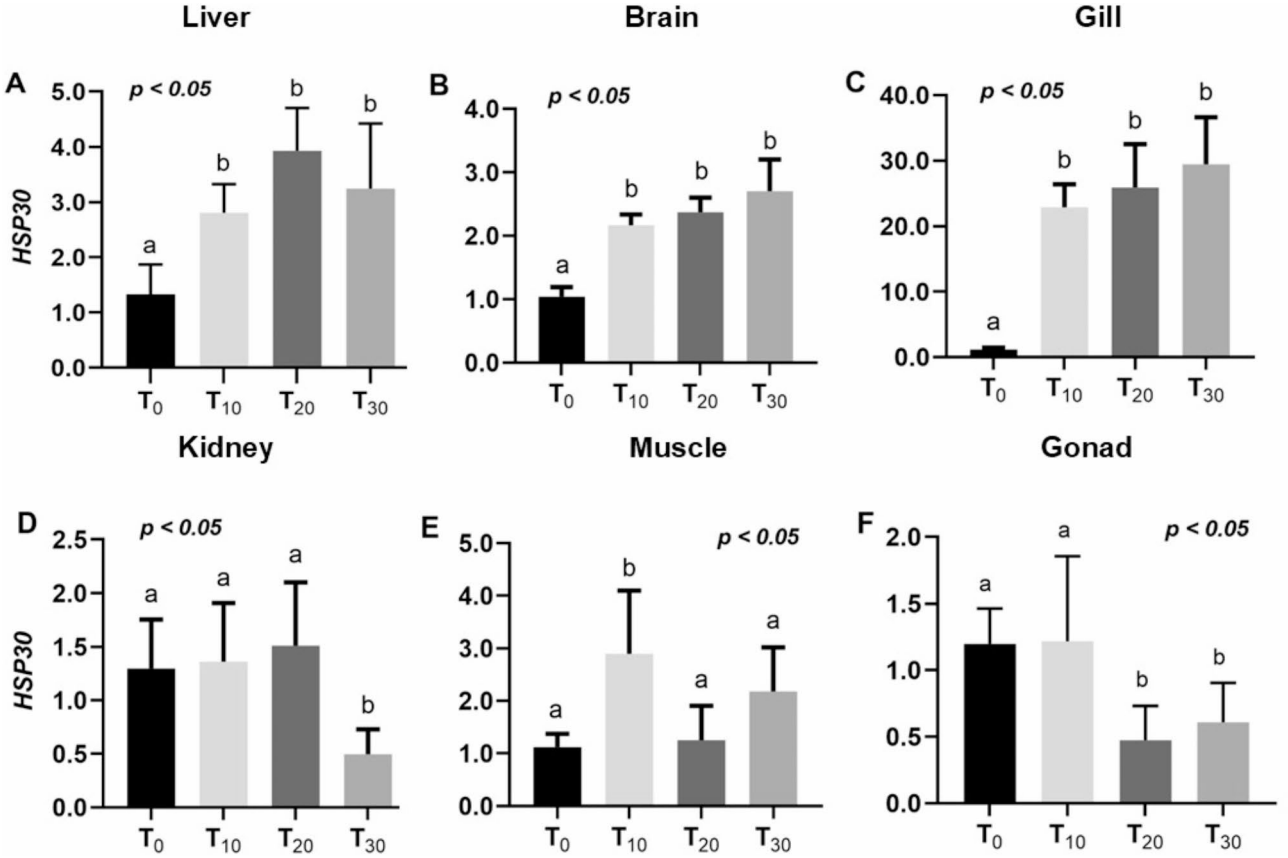

Fig. 5. Relative fold change of *HSP30* (**A**–**F**) mRNAs in different tissues of golden mahseer examined in the control (T_0) and over the time-course (days) of high temperature exposure (T_{10}, T_{20}, and T_{30}). Results are presented as mean ± SEM ($n = 5$ fish per time point) and were analysed using one-way ANOVA, followed by Tukey's multiple comparison test. Differences were considered significant at $p < 0.05$. Different superscripts (a, b, c) above the bars indicate significant difference.

time course of heat exposure suggests its involvement in the refolding of misfolded proteins and degradation of potentially toxic misfolded or aggregated proteins[67].

The differential expression of *HSP30*, and *HSP20*, a member of small heat shock protein family (sHSPs), were examined across different tissues of heat-stressed golden mahseer. The highest expression of both the genes in gill indicates its higher sensitivity than other tissues to a higher temperature as gill remained directly exposed to heat stress environment, therefore often selected as the target organ for analysis of HSP gene expression in adult fish[68]. Expression across different tissues also suggests its role in the prevention of proteins' irreversible aggregation, as these HSPs maintain a refolding conformation by binding to abnormal proteins produced due to heat stress[69,70]. Our results are consistent with previous findings that reported highest *HSP30* mRNA expression in the gill of Atlantic salmon (*Salmo salar*) rainbow trout (*Oncorhynchus mykiss*) under heat stress[71,72]. In addition, consistent high expression of *HSP30* in the heat-stressed liver also indicates its role in modulating the thermal acclimation in golden mahseer (*T. putitora*) as reported in goldfish (*C. auratus*) and gilt-head bream (*Sparus aurata*)[73,74]. A linear increase in *HSP30* mRNA expression in the brain tissue during the time course of high-temperature exposure suggests its potential role in protein homeostasis and thermotolerance in golden mahseer to heat stress as found in different teleost[72,75]. The downregulation of *HSP30* in the kidney and gonad after a high-temperature exposure of 30 days indicates a tissue-specific expression pattern of this heat shock protein in golden mahseer[37,65,72]. In contrast to present finding, *HSP20* was also detected in several other tissues of goldfish under heat stress[76]. Modulation of *HSP20* in brain and gonad tissues suggests thermotolerance and acclimation as well as its distinct sensitivity compared to the other organs in golden mahseer. The role of *HSP20* in thermotolerance and its regulation by heat stress has also been reported in disk abalone (*Haliotis discus discus*)[77]. Furthermore, specific substrates and other regulators besides the well-known heat shock protein factors (Hsfs) were found to influence the temporal and tissue-specific constitutive expression of sHSPs[78]. However, the information on the temperature regulation of *HSP20* in fish needs to be better understood[76]. Even though the liver and kidney are essential organs involved in immunity and metabolism, the absence of significant mRNA expression of *HSP20* in these tissues of heat-stressed golden mahseer would need further investigation[54].

In the present investigation, differential expression of *WAP65-1* was ubiquitously detected in all the examined tissues, with the highest expression being in the renal tissue of heat-stressed golden mahseer. *WAP65* has been found to be involved in the acclimation of fish to warm temperatures, and therefore ubiquitously detected in

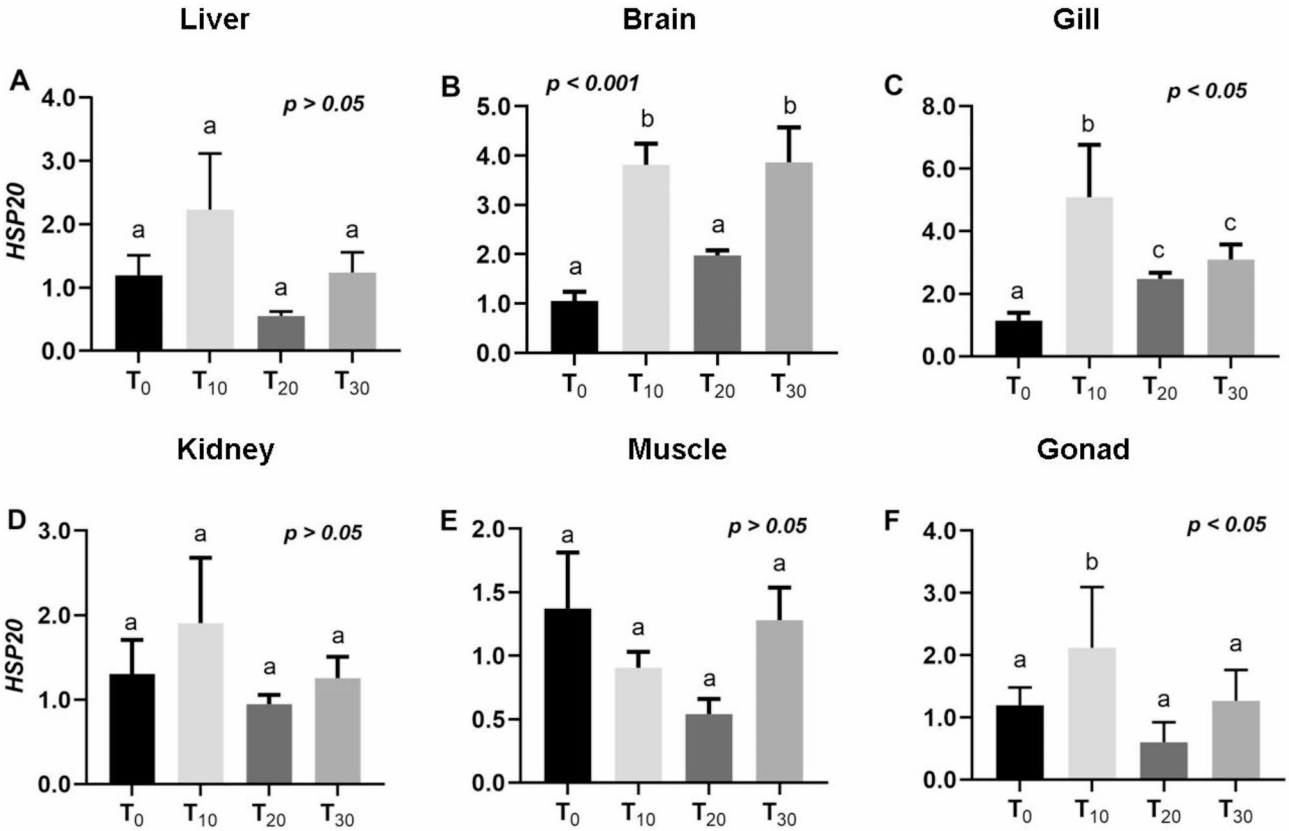

Fig. 6. Relative fold change of *HSP20* (**A**–**F**) mRNAs in different tissues of golden mahseer examined in the control (T_0) and over the time-course (days) of high temperature exposure (T_{10}, T_{20}, and T_{30}). Results are presented as mean ± SEM ($n = 5$ fish per time point) and were analysed using one-way ANOVA, followed by Tukey's multiple comparison test. Differences were considered significant at $p < 0.05$. Different superscripts (a, b, c) above the bars indicate significant difference.

most tissues with variable expression levels in several teleost[16,79–81]. However, in contrast to our findings, other studies have revealed predominantly high transcript abundance of *WAP65-1* in the hepatic tissue in *Plecoglossus altivelis*[81], *Scophthalmus maximus*[82], *Misgurnus mizolepis*[80], *Takifugu rubripes*[19], and *Oryza latipes*[83]. In *C. auratus* (10-fold) and *Cyprinus carpio* (8-fold) increase in mRNA expression of *WAP65-1* in hepatopancreas have been detected during the temperature acclimation from 10 to 30 °C[16,18]. In addition, tissue-specific contrasting expression patterns of *WAP65-1* have been reported in different fish species; for example, a low level of mRNA expression has been detected in the kidney of mud loach (*M. mizolepis*) after a heat treatment to 32 °C, while it did not express either in renal or brain tissues of European sea bass (*Dicentrarchus labrax*) at any time points of four weeks of thermal exposure[79,80]. The kidney being a primary hematopoietic organ in fishes, the highest expression of *WAP65-1* in renal tissues of golden mahseer in the present study indicates its probable role in maintaining homeostasis during chronic thermal exposure, as it is known that *WAP65-1* plays a crucial role in warm temperature acclimation, most likely by limiting the body's accumulation of free heme and free metals, thereby reducing toxicity[79,83,84]. Interestingly, we observed that the mRNA transcript level of *WAP65-1* returned to basal value after an exposure of 30 days in the liver, gill, kidney, and gonad tissues except the brain, while it was downregulated in the muscle tissue in the heat-exposed golden mahseer. Our findings in heat-shocked golden mahseer differ from the previous results obtained in seabass, where no expression of *WAP65* was detected in muscle tissue during the second and third week but again expressed after four weeks' post-acclimation[79]. This suggests that *WAP65-1* might express differentially in various organs in different organisms. Qualitatively speaking, our findings are in line with other previously published reports that showed *WAP65* expression in some or all of the examined tissues in different species[18,19,80,85]. Consequently, our finding supports the notion that *WAP65-1* express in a wide range of tissues in teleost in response to a warm temperature acclimation in a species-specific manner[86].

Nevertheless, our result indicates that *WAP65-1* plays an essential role in golden mahseer's long-term temperature acclimation process[81]. However, further elucidation of functions and pathways of *WAP65-1* will provide better understanding related to the association of this protein in the temperature-acclimation system of poikilotherms. Since *WAP65-1* gene expressed under stress condition in wide variety of golden mahseer tissue, this gene appears to be a potential biomarker for environmental monitoring and evaluation of fish quality under thermal regulation[79].

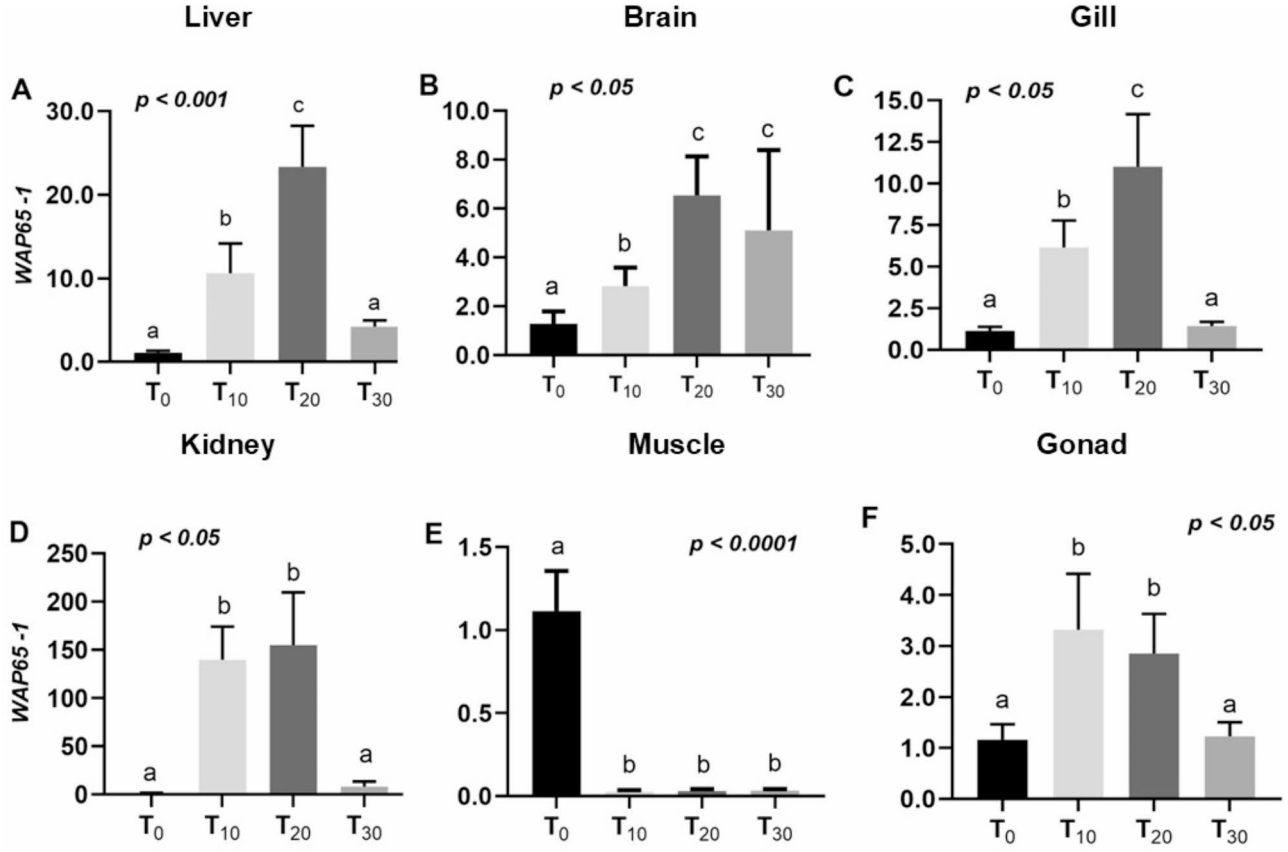

Fig. 7. Relative fold change of *WAP65-1* (**A**–**F**) mRNAs in different tissues of golden mahseer examined in the control (T_0) and over the time-course (days) of high temperature exposure (T_{10}, T_{20}, and T_{30}). Results are presented as mean ± SEM ($n = 5$ fish per time point) and were analysed using one-way ANOVA, followed by Tukey's multiple comparison test. Differences were considered significant at $p < 0.05$. Different superscripts (a, b, c) above the bars indicate significant difference.

Under chronic thermal stress, *CDKN1B* primarily regulates the cell division and cell cycle by downregulating the cell cycle, which was indicated by its upregulation[23,87]. A significantly high level of mRNA transcript of *CDKN1B* in hepatic and brain tissues of golden mahseer during high-temperature exposure suggests its role in controlling the cell cycle as a cellular stress response as found in eurythermal goby fish (*Gillichthys mirabilis*) where high transcript abundance of *CDKN1B* was detected in the gill tissue under the extreme thermal stress[22]. Furthermore, in the event of heat stress, blockage of the energy-demanding process of cell division is essentially required to facilitate the energy-driven process of cellular stress response like the HSPs function. In addition, it also helps prevent the cell from replicating before DNA repair can be effected where heat-induced damage to their DNA has incurred[23]. The high expression pattern of the *CDKN1B* gene in golden mahseer under heat stress indicates the acclimation capacity of this species to the thermal stress and the role of this gene in the prevention of cell damage and apoptosis as described in goby fish (*G. mirabilis*) and delta smelt (*Hypomesus transpacificus*)[22,87]. Although the involvement of the cyclin-dependent kinase (CDK) family of proteins in cell cycle regulation, proliferation, and development has been explored, its role in providing thermotolerance in different teleost is not completely understood and needs further investigation[87,88].

The present study demonstrated the acclimation capacity and thermotolerance in an endangered fish species, golden mahseer, exposed to high temperature and to our knowledge, this is the first account of extensive information on the tissue-specific response of environmental stress-related genes in a Himalayan fish species. In contrast to some cold climate fish like emerald rockcod (*Trematomus bernacchii*) which is unable to show a heat shock response, the molecular response particularly, HSPs induced by thermal stress in golden mahseer (*T. putitora*) suggests some form of plastic response that has fitness consequences and would be helpful in its survival in case of sustained alternation of the environment due to climate change[6,24,89]. In addition, the modulation of transcript abundance of HSPs, *WAP65-1*, and *CDKN1B* genes in golden mahseer also suggests their utility as critical biomarkers for the determination of adaptive change in this species under complex stress environments. However, further studies at the transcriptome and proteome levels are warranted to better understand the molecular mechanism underpinning the thermal tolerance and environmental adaptation to increasing temperature at species and population levels.

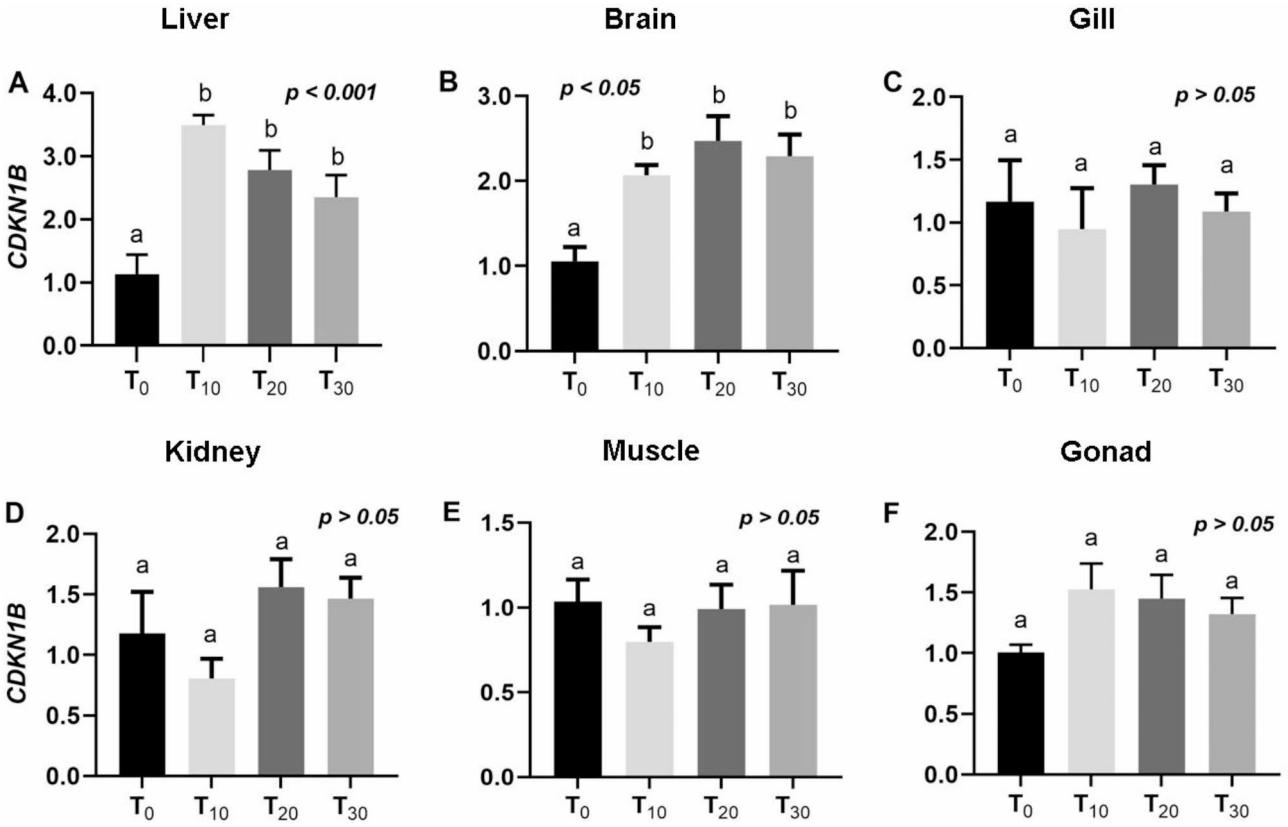

Fig. 8. Relative fold change of *CDKN1B* (**A**–**F**) mRNAs in different tissues of golden mahseer examined in the control (T_0) and over the time-course (days) of high temperature exposure (T_{10}, T_{20}, and T_{30}). Results are presented as mean ± SEM ($N = 5$ fish per time point) and were analyzed using one-way ANOVA, followed by Tukey's multiple comparison test. Differences were considered significant at $p < 0.05$. Different superscripts (a, b, c) above the bars indicate significant difference.

Methods

Experimental design and tissue collection

To examine the differential regulation of HSPs, *WAP65-1*, and *CDKN1B* genes in response to chronic thermal stress, golden mahseer were subjected to heat treatment. Healthy golden mahseer (*T. putitora*) of both sexes ($n = 200$) with average body weight of 24.94 ± 8.26 g and average length of 14.3 ± 1.70 cm was collected from the hatchery of ICAR-Directorate of Coldwater Fisheries Research, Bhimtal, and acclimatized for a period of six weeks in a 1000 L FRP tank at water temperature 25 ± 0.5 °C with optimum dissolved oxygen (6.5-7.0 mg/l) and pH (~7.7) to avoid any incidental mortality. Fishes were fed twice daily with a commercially available pelleted feed (crude protein – 40%; crude lipid – 15%) at the rate of 3% of body weight. For chronic thermal exposure, four glass aquaria, i.e., one as 'control' and the other three as 'treatment' tanks of 90 L water holding capacity, were set up, and each tank was stocked with $n = 20$ fishes randomly selected from the pre-acclimatized specimens. The temperature in the 'control' tank was maintained at 25.0 ± 0.5 °C. In contrast, the temperature in the treatment tanks was increased at the rate of 1.0 °C/day over nine days until it reached the targeted experimental temperature of 34.0 °C. Subsequently, a high temperature (34.0 ± 0.5 °C) was continuously maintained for 30 days in the treatment tanks (Fig. 9). The control and experimental temperature were selected based on the temperature profile of the natural occurrence of *T. putitora* and the future climate projection of widespread warming (~5–8 °C) in the Himalayan region[32,90]. The desired water temperature was maintained in the 'control' and 'treatment' tanks using stainless steel water heaters (300-W, Sobo, China), and thermostat (STC-1000, India). During the exposure period, each day, one-third of the aged water was replaced with pre-heated (for Control = 25.0 °C; Treatment = 34.0 °C) freshwater to avoid any major fluctuation in temperature. Optimum dissolved oxygen level (DO ≥ 7 ppm) was maintained using adequate aeration in the tank, and other water quality parameters such as pH (~7.7-8.0) and ammonia (< 0.05 ppm) were regularly monitored on a daily basis. Fishes were fed *ad libitum* with commercially available pelleted feed (crude protein- 40%; crude lipid-15%) during the experimental period. No abnormal behaviour, diseased symptom, or mortality was observed in experimental fishes during the exposure period.

For sampling, five individuals were collected randomly from the 'control' (T_0) and 'treatment' tanks over the time course of high-temperature exposure at the intervals of 10 days (T_{10}), 20 days (T_{20}), and 30 days (T_{30}) after achieving the targeted experimental temperature. Prior to sampling, the fishes were fasted for 24 h and then euthanized with an overdose of 100 mg/L tricaine methane sulfonate (MS-222, Sigma, St. Louis, MO).

Gene name	Thermal exposure period (days)	Tissue type					
		Liver	Brain	Gill	Kidney	Muscle	Gonad
HSP90β	10 days (T10)	↑	↑	↑	↑	↑	—
	20 days (T20)	↑	↑	—	↓	—	—
	30 days (T30)	↑	↑	—	↓	—	—
HSP70	10 days (T10)	↑	↑	↑	↑	↑	—
	20 days (T20)	—	↑	↑	↑	—	—
	30 days (T30)	—	↑	↑	↑	↑	—
HSP60	10 days (T10)	↑	↑	—	—	↑	—
	20 days (T20)	—	↑	↑	—	—	—
	30 days (T30)	↑	↑	↑	↑	↑	—
HSP47	10 days (T10)	↑	↑	↑	—	—	↑
	20 days (T20)	↑	↑	↑	↑	—	↑
	30 days (T30)	↑	↑	↑	↑	↑	↑
HSP30	10 days (T10)	↑	↑	↑	—	↑	—
	20 days (T20)	↑	↑	↑	—	—	↓
	30 days (T30)	↑	↑	↑	↓	—	↓
HSP20	10 days (T10)	—	↑	↑	—	—	↑
	20 days (T20)	—	—	↑	—	—	—
	30 days (T30)	—	↑	↑	—	—	—
WAP65-1	10 days (T10)	↑	↑	↑	↑	↓	↑
	20 days (T20)	↑	↑	↑	↑	↓	↑
	30 days (T30)	—	↑	—	—	↓	—
CDKN1B	10 days (T10)	↑	↑	—	—	—	—
	20 days (T20)	↑	↑	—	—	—	—
	30 days (T30)	↑	↑	—	—	—	—

Table 1. Summary of the expression pattern of genes in different tissues at various time points of the thermal exposure. '↑' upregulation; '↓' downregulation; '—' basal level of gene expression.

Subsequently, the weight (g) and length (cm) of individual fish were recorded, and a total of six tissues that include liver, brain, gill, kidney, muscle, and gonad, were collected rapidly in cryotubes and quickly frozen in liquid nitrogen and stored at -80 °C, until further use.

RNA extraction and cDNA synthesis

Total RNA was extracted from each tissue using RNeasy Plus Mini Kit (Qiagen, USA/New Delhi), following the manufacturer's instructions. RNA was further treated with the DNase I, RNase free kit (Thermo Fisher Scientific, USA) to remove the genomic DNA contamination. The concentration and quality of total RNA were determined by using NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) at 260 nm absorbance and agarose gel electrophoresis (1.3% MOPS/formaldehyde agarose gels) respectively. Total RNA (1 µg) from each tissue sample was reverse transcribed into complementary DNA (cDNA) using the Prime Script™ 1st strand cDNA synthesis kit (Clonetech Takara Bio, CA, USA) and random hexamer primers, as per the manufacturer's instructions. Finally, cDNA was stored at -20 °C until further use.

mRNA expression analysis: quantitative RT-PCR

The mRNA level of selected HSPs, *WAP65-1* and *CDKN1B* in control (T_0) and during high-temperature exposure (T_{10}, T_{20}, and T_{30}) were analysed in the six tissues that include liver, brain, gill, kidney, muscle, and gonad of individual fish ($N = 5$ biological replicates). Specific primers for each gene were designed using Primer-BLAST suite (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on the DNA sequences presented in GenBank (Table 2). The optimal annealing temperature of each primer ranged between 60 and 62 °C, and the amplicons size ranged between 82 and 158 bp. Specificity of gene amplification was confirmed by melt curve analysis and agarose gel electrophoresis. PCR efficiency was calculated based on the slope of a standard curve generated using two-fold serial dilutions of pooled cDNA. The efficiency was calculated as follows: $E\ (\%) = (10^{-1/\text{slope}} - 1)\ x100$[91]. The acceptable E value was defined as between 90 and 110%. The target mRNA expression levels were quantified using a CFX 96 Real-time PCR detection system (Bio-Rad, USA) using SYBR Premix Ex Taq II (Tli RNAseH Plus) (Takara, USA) and gene-specific primers following the manufacturer's instructions. The reactions were performed using 10 µL of SYBR Premix Ex Taq II qPCR Mix (2X), 5µL 25-fold diluted cDNA, 0.4 µM of each primer, and nuclease-free water to a final volume of 20 µl in two technical replicates for each of five independent biological experiments. The PCR was performed using the following program: 95 °C for 30s, followed by 40 cycles of 5 s at 95 °C, 10 s at 60 °C, and 10 s at 72 °C. Melting curves (from 65 to 95 °C, at a temperature gradient of 0.5 °C per 10 s) were systematically monitored to confirm the specificity of the amplification reaction at

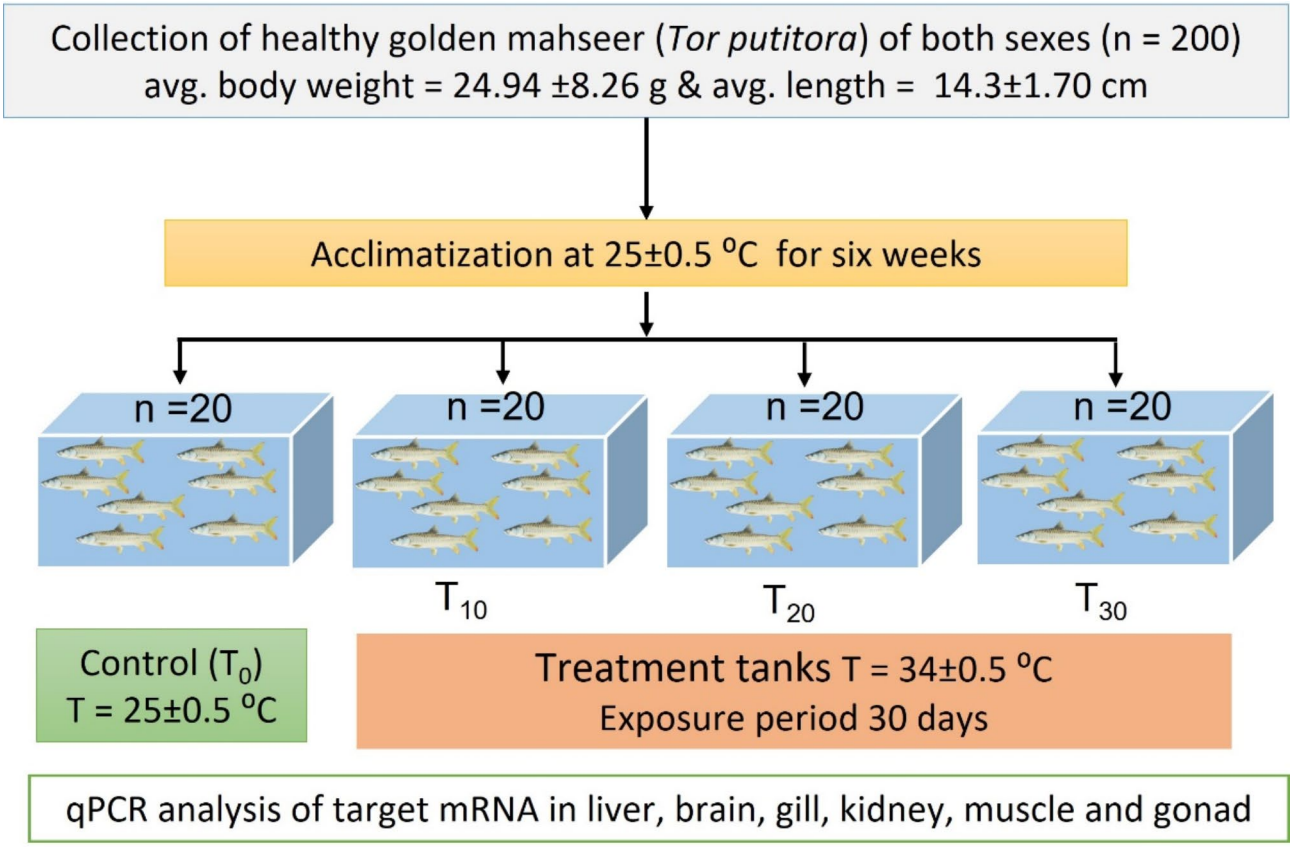

Fig. 9. Schematic representation of the experimental design and sampling points during the entire period of the experiment. T_0 sampling was done after the acclimation period of six weeks, whereas T_{10} (10 days), T_{20} (20 days), and T_{30} (30 days) represents the sampling after an exposure of high-temperature treatment (34.0 ± 0.5 °C).

Gene	Primer pairs (5′-3′)	Amplification efficiency (%)	GenBank accession no.
TpHSP90β	F: GGAGAAGAAGATGCCCTGGAAC R: GCTTGATGTTGACGACGCTCT	99	OQ928015.1
TpHSP70	F: ATCACCATCACCAACGACAAG R: TTTCCTTTCAGGTTCTCATCTTCC	100	OQ928014.1
TpHSP60	F: GCAGCCCTAAAGTCACCAAAG R: CCATCTCCAGCCTCCTCATT	104.6	OQ928013.1
TpHSP30	F: CGGCTTTGCTTTGACGCT R: CCCTTCCCATCCTCTTGCTT	103.3	OQ928011.1
TpHSP20	F: CGACTTGCCTCCTTCTCTTC R: GGTCCAACTTTCCCATTCCA	105.4	OQ928010.1
TpHSP47	F: TAAGGTCAGCGTGGAGGTCA R: ATGGCAGAGGCGTGGAATAC	104.8	OQ928012.1
TpCDKN1B	F: TCAAAGCAAACGCTCACACATC R: TCAACTTCAGCCCTCATCGG	99.7	XM_026238549
TpWAP65-1	F: AGAGCACTGATGACCACATTGA R: GAGAAGACTGCATCCACTTCAC	104.2	ON156522.1
TpTBP	F: TCGTCCCTCAGTTGCAGAAT R: CTGGCTCGAAGTGCTATCGT	99.60	OP377748.1
TpRPS18	F: GACGGAAGACGCAAAATCGC R: CACCACCCTCTCCACCTCATC	105.10	OP377749.1

Table 2. Primers and related information of the target gene and the candidate reference genes.

the end of the last amplification cycle. No-template control (NTC) reactions were also included on each plate. Following the MIQE guidelines, two reference genes namely ribosomal protein S18 (*RPS18)* and tata box binding protein (*TBP*) were used for the normalization of target gene expression data after comparatively evaluating the expression of seven commonly used reference genes[92]. The stability of reference genes was determined using the RefFinder (https://www.heartcure.com.au/reffinder/)[93]. Relative quantification of target mRNA expression was performed using the model described by Pfaffl after correcting for reaction efficiency[94].

Statistical analysis

All the data generated during the study were statistically analysed using the GraphPad Prism software (ver.10, La Jolla, CA, USA) and results are presented as means ± SD. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was applied to find the significant difference in target mRNA expression in all the tissues between the different time points of thermal exposure from the control. Differences were considered statistically significant when *P* value was less than 0.05.

Data availability

The gene sequence has been submitted to NCBI and is available against the GenBank accession number provided (Table 2).

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Author contributions
A.K. performed the laboratory work, draft manuscript preparation, data analysis. S.A. co-supervision, conceptualization, methodology, reviewing and editing. O.S.B. supervision, reviewing and editing. C. S. experimental design, data analysis. P.K.P. project resources. All authors have read and approved the final manuscript.

Declarations

Competing interests
The authors declare no competing interests.

Ethics declarations
For the care and use of animals during the study, all applicable international, national and institutional norms were followed. The protocols for fish maintenance, handling, and sacrifice were approved by the Institutional Animal Care and Use Committee (DCFR/IACUC/2020/3842-48).

Additional information
Correspondence and requests for materials should be addressed to S.A.