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Effects of thermal stress on the expression of glucocorticoid receptor complex linked genes in Senegalese sole (*Solea senegalensis*): Acute and adaptive stress responses.

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Abstract

The present study examined the short and mid-term effects of a rise in temperature from 18 ºC to 24 ºC on the expression of genes related to the stress response regulation in juveniles of Senegalese sole, *Solea senegalensis*. The animals were exposed to a temperature increase of 6 ºC, after 1 month of acclimation at 18 ºC. After this process, samples of different tissues were collected from a total of 96 fish at four sampling points: 1 hour, 24 hours, 3 days and 1 week. The transcript levels of a set of genes involved in the stress response such as glucocorticoid receptors 1 and 2, corticotrophin-releasing factor, corticotrophin-releasing factor binding proteins, proopiomelanocortin A and B, and cellular stress defense (heat shock protein 70, 90AA and 90AB) were quantified at these sampling points. Additionally, blood samples were also taken to measure the circulating plasma cortisol concentration.

Thermal stress induced by increasing temperature prompted an elevation of plasma cortisol levels in juvenile Senegalese sole after 1 h as a short-term response, and a consecutive increase after one week, as a mid-term response.. Senegalese sole seemed to respond positively in terms of adaptive mechanisms, with a rapid over-expression of *gr*s and *hsp*s in liver and brain, significantly higher after one hour post stress, denoting the fast and acute response of those tissues to a rapid change on temperature. The ratio *hsp90*/gr also increased 24 h after thermal shock, ratio proposed to be an adaptive mechanism to prevent proteosomal degradation of GR. As a mid-term response, the elevation of brain *crfbp* gene expression one week after thermal shock could be an adaptive mechanism of negative feedback on HPI axis

Taken together, these data suggested an initial up-regulation of the glucocorticoid receptor complex linked genes in response to a temperature increase in Senegalese sole, with heat shock protein 90 potentially being a regulatory factor for the glucocorticoid receptor in the presence of cortisol.

Abbreviations:

ACTH Adrenocorticotropic hormone

CRF Corticotrophin-releasing factor

CRFBP Corticotrophin-releasing factor binding proteins

CSR Cellular stress response

GR Glucocorticoid receptor

HPI Hypothalamus pituitary Interrenal

HSP Heat shock protein

POMC Proopiomelanocortin

1. Introduction

In fish as in other vertebrates, most biological processes including growth, reproduction and disease resistance are influenced by temperature, but a subacute or acute change of the optimal temperature range could eventually induce a thermal stress response modulating or compromising the normal function of these processes (Cossins et al., 1995). Such an allostatic load activates cellular stress response (CSR), which involves prevention and repair of macromolecular damage (McEwen and Wingfield, 2003), activation of molecular chaperones to refold proteins that have been denatured (Logan and Somero, 2011), initiation of proteolysis to remove proteins that cannot be rescued through activities of chaperones (Feder and Hofmann, 1999) or even apoptotic pathways if heat stress is severe (Kültz, 2005). The interaction between these mechanisms is complex, apoptosis being mediated in part by an increase of circulating cortisol levels (Bury et al., 1998; Laing et al., 2001), which would be enhanced by activation of the glucocorticoid receptor (GR) (Van der Salm et al., 2002). This mechanism is also regulated by the accumulation of Heat Shock Protein 70 (HSP70) that is related to a low GR protein content in cells (Boone et al., 2002). This up-regulation of *hsp70* can block apoptosis through the inhibition of several caspase proteins (Beere, 2004) and naturalize damaged proteins before initializing the apoptotic process.

The levels of cortisol are also regulated by a negative feedback on the Hypothalamus-Pituitary-Interrenal (HPI) axis activation. Hence, cortisol secretion can inhibit corticotrophin-releasing factor (CRF) transcription and also modulate the synthesis of CRF receptors that mediate CRF actions (Westphal and Seasholtz, 2006). Besides, increase of cortisol can modulate the CRF binding proteins (CRFBP) that block CRF (Flik et al. 2006). Cortisol is also involved in the synthesis and release of proopiomelanocortin (POMC) from the pituitary corticotrophs for adenocotricotropin hormone (ACTH) synthesis. The effect of stress on pituitary *pomc* mRNA levels varies according to the nature of the stressor stimulus (Aguilera, 1994). However, concentration of circulating cortisol after stress differs among and within species (Pottinger,

2010), and the effects of increasing cortisol within all those mechanisms differ consequently among species.

At the cellular level, the effects of cortisol are mediated by intracellular glucocorticoid receptors (GR), of the superfamily of nuclear receptors acting as ligand dependent transcription factors to control and regulate gene expression (Mommsen et al., 1999). Teleosts generally have two glucocorticoid receptor genes (GR1 and GR2) that are expressed in most organs (Bury and Sturm, 2007; Stolte et al., 2008). Depending on the teleost species, it has been suggested that each GR requires a different concentration of cortisol to initiate transcription, e.g., GR2 being 60-fold more sensitive than GR1 in rainbow trout (*Oncorhynchus mykiss*) (Prunet et al., 2006). In the cytosol, GRs are in an inactive form within a multi-protein complex along with several HSPs such as HSP70 and 90, whose functions include the assembly, functionality and transport of genetic resources (Pratt and Toft, 1997) and play an important role in the process of acquired thermo-tolerance (Fangue et al., 2006). HSP70 is essential in the assembly and maintenance of the GR heterocomplex (Pratt and Welsh, 1994), whereas HSP90 has been suggested to stabilize the GR heterocomplex against proteolytic degradation (Dundjerski et al., 2000). The two major isoforms of HSP90, HSP90AA and HSP90AB, are involved in cell proliferation and differentiation. HSP90AA has been associated with growth promotion, cell cycle regulation, and stress-induced cytoprotection and HSP90AB has been mainly associated with early embryonic development and long-term cell adaptation among other processes (reviewed in Sreedhar et al., 2004).

On the other hand, steroid receptors can bind hormones in the absence of HSPs, but there is considerable evidence that HSPs can increase the binding capacity of the steroid receptor, facilitate nuclear translocation of the receptor complex, and enhance the proteolytic half-life of the receptor complex (Pratt and Welsh, 1994; Czar et al., 1997). Analysis of hepatic tissue taken from hypercortisolemic rainbow trout demonstrated that levels of free HSP70 decreased after exposure to heat shock, whereas the amount of HSP70 bound to the GR increased in this tissue after the heat shock (Basu et al., 2003). Although HSPs have a relatively

short half-life, their levels remain elevated in the whole organism long after the stressor is finished, which indicates their role in long-term adaptation (Morimoto and Santoro, 1998) and homeostasis (Iwama et al., 1998).

Senegalese sole (*Solea senegalensis*) is a marine teleost that inhabits coastal and estuarine areas, which is subjected to wide changes in environmental temperature (from 13 to 28 °C; Dinis et al., 1999; Imsland et al., 2003; Vinagre et al., 2006), being large thermal variations also observed under farming conditions (Imsland et al., 2003). Juvenile Dover sole (*Sole sole*) are thermo-sensitive, thus capable of detecting temperature differences and behavioural thermoregulation (Schram et al., 2013). In this sense, it has been observed that increasing the rearing temperature up to 22 °C enhanced the growth of juvenile sole (Schram et al., 2013). On the other hand, elevated temperatures can have a negative influence on fish health and lead to decreased growth and increased mortality (Dominguez et al., 2004). To the authors' knowledge, the effects of temperature oscillations on the response capacity of this species in terms of expression the stress-related genes has not been evaluated The aim of this work was to determine up to what extent and how fast the stress response at central level and the feed-back mechanisms were involved after a thermal stress in the sole. Assessing the effects of temperature oscillations would be of interest in order to optimize farming conditions of this species without triggering a stress response in the fish.

2. Material and methods

2.1. Experimental fish and sample collection

The experiments were conducted in the facilities of the University of Las Palmas de Gran Canaria (ULPGC, Gran Canaria, Canary Islands, Spain), and all experimental conditions and sampling protocols were approved by the Animal Welfare and Bioethical Committee of the ULPGC (Ref 007/2012 CEBA ULPGC). One hundred and sixty eight Senegalese sole juveniles of 62.3 ± 21.3 g (mean ± SD) initial body weight obtained from a local farm (ADSA, Castillo del Romeral, Gran Canaria, Spain) were randomly distributed into 24 indoor plastic tanks

(60x40 cm) (7 fish per tank). Tanks were supplied with filtered seawater, at a temperature of 18 ˚C, and natural photoperiod (around 12L: 12D). Water dissolved oxygen values ranged 6.2 ± 0.7 g/l. Fish were manually fed with a commercial diet (Skretting Spain, Cojovar, Burgos, Spain) until apparent satiation for 5 weeks (twice daily, 6 days a week). After an acclimation period of 30 days, a heat shock was applied to half of the tanks (12 tanks) by increasing 6 ºC, from 18 to 24 ˚C in one hour, using individual electronic heaters in each tank, whereas the other half of the tanks was kept as a control at 18ºC. Fish from both heat treated and control tanks were sampled after 1 h, 24 h, 3 days and 1 week (triplicate tanks for each sampling point and each temperature).

All fish were sacrificed by immersion in an anesthetic overdose of clove oil. Blood from 4 fish per tank was collected in less than 4 minutes by caudal sinus puncture and stored into tubes previously treated with Lithium heparine. Blood was centrifuged at 800 x g during 10 min to obtain plasma samples that were stored at -80 ºC until analysis.

In addition, samples of 60 mg of intestine, liver, muscle, gills and brain were collected from four fish per tank (triplicate tanks for each sampling point at either 18 or 24 ºC). Samples were placed in RNA Later (Sigma-Aldrich, Sant Louis, MO, USA), stored at 4 ºC and finally frozen at -80 ºC until RNA extraction.

2.2. Stress indicators

2.2.1. Circulating plasma cortisol concentration

Plasma cortisol concentration was determined by radio-immunoassay using the trypsin–antitrypsin method as previously described for marine fish species (Rotllant et al., 2001), at the Department of Cell Biology, Physiology and Immunology, from Universitat Autònoma de Barcelona (Bellaterra, Spain).

2.2.2. Relative expression of stress-related genes

The expression of *gr1, gr2, hsp70, hsp90aa, hsp90ab,crf,crfbp, pomca* and *pomcb* genes was conducted using oligos previously described for this species (Infante et al., 2008; Manchado et al., 2008; Salas-Leiton et al., 2010, 2012; Benítez-Dorta et al., 2013), using qPCR (Table 1).

2.3. RNA extraction, cDNA synthesis and Quantitative real time (qPCR) analysis

One hundred milligrams of tissue (equal amount from 4 fishes per tank, approximately 25 mg per fish) were pooled (per type of tissue; n = 3) and total RNA extracted using 1 ml TRI Reagent (SIGMA-Aldrich, St. Louis, MO, US). Total RNA concentration, purity and quality were measured by spectrophotometry (NanoDrop 1000, Thermo Scientific Inc., USA) and by electrophoresis using 500 ng of total RNA in a 1% agarose gel. The reverse transcription (RT) reactions were carried out in 20 µl volume using iScriptTM cDNA Synthesis Kit (Bio-Rad Hercules, California, USA) containing 1 µg of total RNA. In addition the reverse transcription was carried out with a systematic negative control (NTC-non template control) containing no RNA. Additionally, negative controls containing no enzyme (RT-) were performed to later check for genomic DNA contamination. At the end of the RT reactions, all cDNA samples were kept at -20 ºC

All PCR reactions were performed in i-cycler thermocycler with optical module (Bio-Rad Hercules, California, USA) using 12.5 µl Brillant SYBR Green qPCR Master Mix (Bio-Rad Hercules, California, USA), 1µl of a 1:5 dilution of the cDNA and the amount previously optimized of each primer in a final volume of 25 µl. Cycling conditions consisted of denaturation and enzyme activation for 7 min at 95 °C, followed by 40 cycles at 95 °C for 15 seconds and 70 °C for 30 seconds. Each run was ended with a melting curve analysis resulting in a melting peak profile specific for the amplified target DNA. In addition amplifications were carried out with a systematic negative control (NTC) containing no cDNA. Each assay was performed in duplicate. Three housekeeping genes were tested (ubiquitin, elongation factor 1 α and glycerol phosphate dehydrogenase) and *ubiquitin* selected as housekeeping as being the most stable in the different tissues according to GeNorm (Vandesompele et al., 2002; Table 1).

192 The efficiency of the primers for each gene was previously evaluated to ensure that it was close
193 to 100%. The relative gene expression was estimated by the - method (Livak and
194 Schmittgen, 2001). Additionally, the HSP90/GR ratio was calculated by dividing the
195 normalized relative expression values of the two genes in each tissue and sampling point.

196 *2.4. Statistical analysis*

197 All data were tested for normality and homogeneity of variance. Samples were normally
198 distributed. Means and standard errors (SE) were calculated for each parameter measured.
199 Statistical analyses followed methods outlined by Sokal and Rohlf (1995). The effects of
200 temperature and time after temperature change were analyzed by Two-Way ANOVA, where
201 temperature and time after stress were established as fixed factors. Significant differences were
202 considered when $P<0.05$. A Student–Newman–Keuls (SNK) test was conducted for *post-hoc*
203 multiple comparisons. Analyses were performed using the SPSS Statistical Software System
204 v20.0 (SPSS, Chicago, IL, USA) and R (version 3.1.0).

205 **3. Results**

206 *3.1. Circulating plasma cortisol concentration*

207 Thermal stress induced a significant ($P<0.05$) increase of plasma cortisol concentration one hour
208 after the increase in temperature, with values of 32.2 ± 3.9 (mean ± SE) ng cortisol/ml plasma.
209 After this, plasma cortisol concentration returned to basal levels. However, 7 days after the heat
210 shock, a new significant ($P<0.05$) increase in plasma cortisol was found, with values ranging
211 around 23.2 ± 3.3 ng of cortisol/ ml of plasma (Fig. 1), showing cortisol evolution after thermal
212 stress a biphase-like response. No differences in cortisol levels were observed in unstressed fish.

213 *3.2. Expression of stress-related genes in liver*

214 In liver, the relative expression of *gr1*, *gr2* and *hsp70* increased ($P<0.05$) 1 hour after the
215 temperature increase, with a progressive decrease in values towards the end of the experimental
216 period for *gr1* and *hsp70*. *Hsp90aa* gene expression (Fig. 2) increased ($P<0.05$) within the first

24 hours after thermal stress, decreasing towards the end of the experimental period. For *hsp90ab* the highest (P<0.05) expression levels were observed 24 hours post stress, then decreasing until the end of the experimental period (Fig. 2). Two-way ANOVA did not show significant differences in all the evaluated genes regarding "temperature" whereas all genes except *hsp90aa* proved to be significantly regulated by factor "time" with an interaction between both factors regulating the expression of all stress-related genes.

3.3. Expression of stress-related genes in muscle

In muscle, no temperature effect was detected on the relative expression of *gr1* (Fig. 3). The relative expression of *gr2* increased (P<0.05) after three days post-stress (Fig. 3). Regarding HSPs, no effect was observed on *hsp70* expression (Fig 3), *hsp90aa* was up-regulated (P<0.05) one hour after heat shock, decreasing significantly (P<0.05) 24 h after thermal stress (Fig. 3), whereas *hsp90ab* increased (P<0.05) 1 week after heat shock (Fig. 3). No regulation was observed either by "temperature" or "time" for any evaluated gene, but the interaction between both factors for *gr2*, hsp90aa and *hsp90ab* was significant.

3.4. Expression of stress-related genes in intestine

Thermal stress induced an increase (P<0.05) in the relative expression of *gr1* and *gr2* after one week in the intestine (Fig. 4), although values obtained after 24h were significantly (P<0.05) higher when compared with fish held at 18 ºC. The increase of temperature had different effects on the relative expression of *hsp*s genes in the intestine. After 24 h there was a significant (P<0.05) increase of *hsp70* at 24 ºC (Fig. 4) and a significant (P<0.05) increase of *hsp90aa* after 1h post temperature increase (Fig 4). Recovery values were similar to those observed at 18ºC after 24h. *Hsp90ab* significantly (P<0.05) increased after three days of thermal stress, and remained significantly increased (P<0.05) after one week (Fig. 4). The factor "time" regulated all evaluated genes, whereas "temperature" only affected significantly *gr1* and *gr2*. Interaction between both factors showed effects on *gr1*, *gr2* and *hsp90aa*.

3.5. Expression of stress-related genes in gills

In gills, the relative expression of *gr1* increased (P<0.05) 24 h and one week after the start of the heat shock (Fig. 5). *Gr2* expression also increased (P<0.05) after one week of thermal stress (Fig. 5). Heat shock stress caused a significant increase of *hsp70* and *hsp90aa* expression after 24 h (Fig. 5), then recovering initial values after 3days of thermal stress. However, thermal stress induced a progressive increase (P<0.05) of *hsp90ab*, being values significantly (P<0.05) higher after one week than those obtained for fish held at 18 ºC (Fig. 5). The two-way ANOVA showed no regulation of "temperature" on any of the studied genes, but time regulated *hsp70* and *hsp90aa* whereas the interaction between the two factors affected *gr2* and *hsp90ab*.

3.6. Expression of stress-related genes in brain

The change of temperature induced an increase (P<0.05) of relative expression of brain *gr1* and *gr2* at 24 h, recovering the initial values after 2 days of acclimation at 24 °C (Fig. 6). Thermal stress had no effect on the expression of *hsp70* gene in brain, although higher values were observed 1 h after the heat shock (Fig. 6). However, the relative expression of *hsp90aa* reached a maximum value (P<0.05) 1 h after the start of the heat shock, followed by recovery of initial values after 24 h (Fig. 6). Besides, the change of temperature induced a significant increase in the expression of *hsp90ab* during the first 24 hours after heat shock being significantly higher at 1 and 24h (Fig. 6), then decreasing after 3days (Fig. 6). The individual effect of the factors "temperature" and "time" did not elicit transcriptional regulation on any evaluated *gr* or *hsp* according to the two-way ANOVA, while an interaction between these parameters regulated the expression of all these genes except for *hsp70*.

On the other hand, the increase of the temperature induced a significant increase (P<0.05) in the relative expression of *crfbp* (Fig. 7) 1 week after the beginning of the stress in brain. The increase in temperature induced a significant (P<0.05) up-regulation in the expression of brain *pomca* and *pomcb* after one week (Fig. 7), while the relative expression of *crf* remained unchanged (Fig. 7). An interaction between "temperature" and "time" existed for all of the

genes excepting for *crf*, with individual factors not exerting any regulation according to the two-way ANOVA.

3.7 hsp90/gr ratios

Expression ratios of *hsp90*/*gr* were calculated in each tissue and sampling point showing enhancement of ratios in brain after 1h of thermal stress and in liver and gills 24 h after the start of the thermal challenge (Table 2).

4. Discussion

After a stressful situation, increased circulating levels of cortisol as a short-term response produce alertness and induce a metabolic shift for providing energy to deal with the stressor and maintain homeostasis (Mommsen et al., 1991). At mid term, physiologic processes tend to adapt to compensate the stress with some limitations (Shreck et al., 2001). In the present study an increase of cortisol could be observed as a short-term response to the elevation of temperature, highlighting the role of plasma cortisol as a sensitive indicator of thermal stress. This increase has also been observed in juvenile Atlantic cod (*Gadus morhua L.*) exposed to an acute thermal challenge where plasma cortisol levels showed an exponential increase with temperature (Pérez-Casanova et al., 2012), being these results in agreement with several other studies in different teleost species (Wenderlaar-Bonga, 1997; Afonso et al., 2008; Kumar et al., 2015). However, after a week of thermal acclimation, a secondary peak in plasma cortisol was observed. Similarly, roach (*Rutilus rutilus*) subjected to confinement stress showed inability to return to basal plasmatic cortisol levels, being this effect more obvious when the temperature was 16 °C relative to fish held at 5 °C and observing a secondary peak 24 h after the initial disturbance (Pottinger et al., 1999). Although it was not clear in the precedent study it was hypothesized that the failure to return to a baseline may represent an effect of the stressor on the set point of baseline activity of the HPI axis, involving an homeostatic feedback mechanism that would maintain cortisol levels in the blood due to the effect of the stressor.

293 Temperature changes have been described to trigger alterations in the expression of both *gr*
294 (Fernandino et al., 2012) and *hsp* (Roberts et al., 2010) in fish. Increased levels of *hsp*s after a
295 temperature shock are indicative of stress (Roberts et al., 2010), and are directly related to an
296 increased thermo-tolerance after a rise of cortisol (Basu et al., 2002). Results obtained in the
297 present study agree with this *hsp* rise, although its expression seeming to be tissue – dependent.
298 Thus, the expression of *hsp90* was higher in muscle, brain and gills of Chinook salmon
299 (*Oncorhynchus tshawytscha*) following heat shock, when compared to liver, kidney and tail fin
300 tissues (Palmisano et al., 2000).

301 Stressful conditions have been shown to induce cortisol binding to GRs in fish (Prunet et al.,
302 2006). This alteration depends on the intensity of the stress, as cortisol may fail to bind to GR1
303 in non- or mild stressful conditions whereas both GR1 and GR2 may be mobilized in highly
304 stressful conditions (Bury et al., 2003; Prunet et al., 2006). Among different stressors,
305 temperature has been described to induce serious alterations in the GR-complex, both in
306 mammals (Matic et al., 1998) and fish (Fernandino et al., 2012). In this sense, Fernandino and
307 co-authors (2012) described an increased expression of *gr*s of pejerrey (*Odontesthes*
308 *bonariensis*) larvae held at different temperatures with larvae held at 29 ºC showing significant
309 increase in *gr1* expression when compared to larvae held at 17 ºC. This is in agreement with the
310 results obtained in the present experiment, when an increase of *gr* expression after temperature
311 increase was observed. Specifically, *gr1* expression increased in liver and brain in the first 24 h
312 after heat stress whereas mRNA levels in other tissues such as intestine increased 1week after
313 thermal stress, with no effect on muscle. The response of *gr1* and *gr2* seemed to be tissue
314 specific in Senegalese sole as proposed for other species (Teles et al., 2013; Greenwood et al.,
315 2003; Ducouret et al., 1995). On the other hand, in the present experiment after thermal stress,
316 *gr1* was more expressed than *gr2* in liver, intestine and gills, similarly to the results previously
317 found in Tilapia (*Oreochromis mossambibus*) (Aruna et al., 2012).

318 The activation of GR depends not only on the expression of *gr* gene, but also on the intracellular
319 HSP90/GR ratio (Kang et al., 1999). The binding of HSP90 allows GR to be competent for

ligand binding (Segnitz and Gehring, 1997), being the nuclear retention of GR attenuated by the over-expression of HSP90 (Tago et al., 2004). The increase of intracellular HSP90 levels results in an increased HSP90/GR ratio, mainly in the nucleus, which inhibits GR binding to its DNA response element (Kang et al., 1999). The positive modulation of the response amplitude to steroids is the result of an optimal HSP90/GR ratio, whereas abnormally low or high ratios will negatively interfere with the response of GR (Qian et al., 2001). An increase of the HSP90/GR ratio has been proposed in rainbow trout hepatocytes treated with cortisol and subjected to a heat shock as a modulator of the GR-dependent promoter activity (Sathiyaa et al., 2001). These changes favor tissue responsiveness to glucocorticoids and could further increase tissue receptiveness to glucocorticoid stimulation (Vijayan et al., 2003). This is in agreement with the results obtained in the present experiment, as an increased HSP90/GR ratio can be found after 24h of thermal stress in liver and gills, and 1h after thermal stress in brain, corresponding with the peak in plasma cortisol. Whether this elevation of HSP90/GR ratio is an adaptive mechanism remains unclear, but a preventive role on proteosomal degradation of GR has been proposed both for mammals (Segnitz and Gehring, 1997) and fish (Aluru and Vijayan, 2007).

The response of the GR complex to cortisol leads to different effects depending not only on the type of tissue, but also on the type of stressor and the evolution of the response to stress (Vegiopoulos and Herzig, 2007; Aruna et al., 2012). A specific *gr* response for each tissue throughout time after heat shock has been observed in the present study in terms of relative quantification. Similar over-expression has also been described in tilapia subjected to handling stress during the course of seawater acclimation and handing stress (Aruna et al., 2012). In the present experiment, 24 h after the onset of the heat shock, the expression of *crf* tended to be higher than in unstressed fish along with *gr*s in the brain, suggesting a possible role for GR controlling the feedback response through CRF in brain. Further experiments would be necessary in order to clarify the brain GR response against other type of stressors in Senegalese sole, not only regarding the feed-back mechanisms but also trying to identify specific responses in different areas of the brain in which these receptors are highly represented.

347 Another tissue directly involved in the adaptation of teleost to environmental stressors is the
348 gills (McCormick et al., 2008). The aerobic cost for protein synthesis in the gills is high, and
349 specially during stressful situations (Lyndon and Houlihan, 1998), including changes of
350 temperature (Lee et al., 2003). The expression of *gr1* appeared up-regulated 24 h after the heat
351 shock, perhaps due to the faster capability of gill GR1 to respond to stress than GR2 (Aruna et
352 al., 2012) and the critical role of the gills in cortisol-regulated functions such as osmoregulation.

353 As a short-term response to thermal stress, the liver *gr* expression increased during the first
354 hours, corresponding to the peak levels of plasmatic cortisol found in the present study.
355 Cortisol-mediated molecular changes in the gluconeogenic and protein catabolic pathways are
356 GR-activated in rainbow trout hepatocytes, suggesting a key role for GR-specific signaling in
357 this adaptive response (Aluru and Vijayan, 2007). The short-term response in the liver of
358 Senegalese sole could suggest an increase in liver metabolic activity to cope with the heat
359 induced stress, as animals need to increase their metabolism and energy supply (Mora and
360 Maya, 2006).

361 Intestinal *gr* expression increased after one week of thermal stress, corresponding to a new
362 increase of plasma cortisol. The observed results in intestine are in agreement with previous
363 results in other fish species such as Mozambique tilapia (*Oreochromis mossambicus*) subjected
364 to cortisol implantation (Takahashi et al., 2006), suggesting the importance of the *gr* up-
365 regulation as an adaptive mechanism to stressful situations in the intestinal tissue through
366 regulation of tissue differentiation, development and metabolism.

367 On the other hand, HSP90AB has been mainly associated to long-term cell adaptation (Sreedhar
368 et al., 2004). In the present experiment, as a mid-term response to thermal stress, *hsp90ab*
369 increased significantly in intestine, and also in muscle in agreement with results reported for
370 Chinook salmon (Palmisano et al., 2000). HSP90 has been proposed to play a reorganization
371 role in tissue temperature acclimation through its action on proteolytic destruction of denatured
372 enzyme isoforms or protein phosphorylation (Immamura et al., 1998). It would be interesting to

elucidate the role of these genes after long-term temperature acclimation in Senegalese sole, as this species is subjected to a wide range of temperature fluctuations even under semi-extensive or extensive culture (Arjona et al., 2010; Castro et al., 2012).

Interestingly, thermal stress induced some changes in brain one week after the start of the heat shock, finding elevation of *pomc* and c*rfbp* expression in Senegalese sole. A previous trial in this species, found an increase in *crf* expression in brain together with enhanced plasmatic cortisol levels with no alteration in *crfbp* when juvenile Senegalese sole were subjected to high density conditions (Wunderink et al., 2011). Differences in the regulation of both genes were attributed to an adaptive response to chronic stress, as feed-back regulation can attenuate plasma cortisol levels (Mommsen et al., 1999). In our case the inverse was observed with no alteration of *crf* expression, whereas *crfbp* levels were enhanced 7 days after the stress, which could be indicative of an adaptive response, given that a second peak in plasmatic cortisol was observed at day seven post-heat shock. Besides, CRFBP has also been reported as an inhibitor of the CRF-mediated ACTH release in pituitary mammal cells (Potter et al., 1991). Both stress and glucocorticoids can up-regulate *crfbp* mRNA expression, which in turn exerts a negative feedback on CRF actions (Westphal and Seasholtz, 2006; Huising et al., 2004). The thermal-induced increase of *crfbp* found in the present study could be indicating the activation of a negative feedback on the ACTH release in sole after one week of thermal stress as has previously been suggested for the same species (Salas-Leiton et al., 2012). It must be noted though that expression analysis was performed in whole brain tissue whereas CRF neurons are mostly present in the preoptic area in the hypothalamus (Ando et al., 1999) and thus differences in expression levels could expected if RNA from only the preoptic area would have been used.

On the other hand, a study in the closely related common sole (*Solea solea*) found a decrease of *pomca* mRNA levels in brain which has been considered an adaptive response of the fish to farm stocking density conditions (Palermo et al., 2008). In view of the results obtained in the current study, *pomc* elevation after one week (albeit not significant) together with an increase in plasma cortisol found in the present experiment could be indicating an inadequate adaptation of

Senegalese sole to the new thermal conditions. However, differences in expression could also be related to other functions of POMCs, as it is also post-transcriptionally processed into melanocortins involved in a wide range of physiological functions (Cone, 1999). For instance, POMCA1 and POMCB have been identified to play central anorexigenic roles in Atlantic salmon (Valen et al., 2011).

In summary, thermal stress induced by increasing temperature prompted an elevation of plasma cortisol levels in juvenile Senegalese sole after 1 h as a short-term response, and a consecutive increase after one week, as a mid-term response. Senegalese sole seemed to respond positively in terms of adaptive mechanisms, with a rapid over-expression of *gr*s and *hsp*s in liver and brain, significantly higher after one hour post stress, denoting the fast and acute response of those tissues to a rapid change on temperature. The ratio *hsp90*/*gr* also increased 24 h after thermal shock, ratio proposed to be an adaptive mechanism to prevent proteosomal degradation of GR. As a mid-term response, the elevation of brain *crfbp* gene expression one week after thermal shock could suggest a negative feedback mechanism of on HPI axis. Further experiments are required to elucidate how Senegalese sole responds to longer periods of acclimation to thermal increases.

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675 **Figure legends**

676

677 **Figure 1**. Circulating plasma cortisol levels (ng/ml) after temperature increase. Results of the
678 Two-way ANOVA did not show an effect of temperature or time on plasma cortisol levels
679 (P>0.05), whereas the interaction of temperature and time regulated plasma cortisol
680 concentration. Different letters within a temperature group denote significant (P<0.05)
681 differences. * denotes significant differences (P<0.05) between fish held at 18 ºC and 24 ºC for
682 a given time. N= 12.

683 **Figure 2.** Relative expression of *gr1, gr2, hsp70, hsp90aa* and *hsp90ab* in liver after heat
684 shock.. N= 3 (4 fish pooled per tank, triplicate tanks). Levels of expression are relative to the
685 control for each time sampling point. Two-way ANOVA analyses revealed a significant
686 (P<0.05) effect of time on *gr1, gr2, hsp70,* and *hsp90ab*. No effect of temperature as individual
687 factor was detected. However, interaction between time and temperature had a significant
688 (P<0.05) effect on all the genes evaluated. Different letters within a temperature group denote
689 significant (P<0.05) differences. * denotes significant differences (P<0.05) between fish held at
690 18 ºC and 24 ºC for a given time

691

692 **Figure 3.** Relative expression of *gr1, gr2, hsp70, hsp90aa* and *hsp90a* in muscle after heat
693 shock. N= 3 (4 fish pooled per tank, triplicate tanks). Levels of expression are relative to the
694 control for each time sampling point. Two-way ANOVA analyses revealed no effect of
695 temperature or time as individual factor on the studied genes. However, However, interaction
696 time and temperature had a significant (P<0.05) effect on *gr1, hsp 90aa* and *hsp90ab*. Different
697 letters within a temperature group denote significant (P<0.05) differences. * denotes significant
698 differences (P<0.05) between fish held at 18 ºC and 24 ºC for a given time.

699

Figure 4. Relative expression of *gr1, gr2, hsp70, hsp90aa* and *hsp90a* in intestine after heat shock. Levels of expression are relative to the control for each time sampling point. Two-way ANOVA analyses revealed a significant (P<0.05) effect of temperature on *gr1* and *gr2*. No effect of time as individual factor was detected. However, interaction time and temperature had a significant (P<0.05) effect on *gr1*, *gr2* and *hsp90aa*. Different letters within a temperature group denote significant (P<0.05) differences. * denotes significant differences (P<0.05) between fish held at 18 ºC and 24 ºC for a given time. N= 3 (4 fish pooled per tank, triplicate tanks)**.**

Figure 5. Relative expression of *gr1, gr2, hsp70, hsp90aa* and *hsp90ab* in gills after heat shock. Levels of expression are relative to the control for each time sampling point. Two-way ANOVA analyses revealed a significant (P<0.05) effect of time on *hsp70* and *hsp90aa*. No effect of temperature as individual factor was detected. However, interaction time and temperature had a significant (P<0.05) effect on *gr2* and *hsp90ab.* Different letters within a temperature group denote significant (P<0.05) differences. * denotes significant differences (P<0.05) between fish held at 18 ºC and 24 ºC for a given time. N= 3 (4 fish pooled per tank, triplicate tanks)**.**

Figure 6. Relative expression of *gr1, gr2, hsp70, hsp90aa* and *hsp90a*; (in brain after heat shock. Levels of expression are relative to the control for each time sampling point. Two-way ANOVA analyses revealed no effect of temperature or time as individual factor on the studied genes. However, a significant (P<0.05) interaction time and temperature was detected for *gr1*, *gr2*, *hsp90aa* and *hsp90ab.* Different letters within a temperature group denote significant (P<0.05) differences. * denotes significant differences (P<0.05) between fish held at 18 ºC and 24 ºC for a given time. N= 3 (4 fish pooled per tank, triplicate tanks)**.**

725

726 **Figure 7.** Relative expression of *crf*, *crfbp*, *pomca* and *pomcb*, in brain after heat shock. Levels
727 of expression are relative to the control for each time sampling point. Two-way ANOVA
728 analyses revealed no effect of temperature or time as individual factor on the studied genes.
729 However, a significant ($P<0.05$) interaction time and temperature was detected for *crfbp*, *pomca*
730 and *pomcb*. Different letters within a temperature group denote significant ($P<0.05$) differences.
731 * denotes significant differences ($P<0.05$) between fish held at 18 ºC and 24 ºC for a given time.
732 N= 3 (4 fish per tank, triplicate tanks)**.**

733

734

Table 1. Primers sequences used qPCR analysis

Target	Primer	Sequence 5´-3´	Amplicon (bp)	Acc. N.	Reference
gr1	F	CCTGCCGCTTCCACAAGTGTCTGATG	130	AB614369	Benitez-Dorta et al. 2013
	R	TTCAACTGGTGGAGGTGGCGGTGT			
gr2	F	TCAGCGTGGAGTTCCCGGAGATG	92	AB614370	Benitez-Dorta et al. 2013
	R	GGTGGAACAGCAGCGGCTTGATG			
hsp70	F	GCTATACCAGGGAGGGATGGAAGGAGGG	119	AB513855	Salas-Leiton et al., 2010
	R	CGACCTCCTCAATATTTGGGCCAGCA			
hsp90aa	F	GACCAAGCCTATCTGGACCCGCAAC	105	AB367526	Manchado et al., 2008
	R	TTGACAGCCAGGTGGTCCTCCCAGT			
hsp90ab	F	TCAGTTTGGTGTGGGTTTCTACTCGGCTTA	148	AB367527	Manchado et al., 2008
	R	GCCAAGGGGCTCACCTGTGTCG			
crf	F	CGGCGTCTATTACAAGGGAAAGTTGGGAAC	98	FR745427	Salas-Leiton et al., 2012
	R	TCGGACCTCCTCCCCCTCTCCAT			
crfbp	F	AGCTGCTGGGGGGCAATGGCATA	94	FR745428	Salas-Leiton et al., 2012
	R	CCAACCTTCATCTGGGCGAGTCCTCT			
pomca	F	CGGCCCATCACAGTCTACAGCTCCA	131	FR874846	Salas-Leiton et al., 2012
	R	TACGCGCCGTCCTTTTTCTCGTG			
pomcb	F	GGATGCGGCAAAAGGGGGACA	111	FR874847	Salas-Leiton et al., 2012
	R	CCCCATCTAAAGTGACCCATGCGGTA			
ubq	F	AGCTGGCCCAGAAATATAACTGCGACA	93	AB291588	Infante et al. 2008
	R	ACTTCTTCTTGCGGCAGTTGACAGCAC			

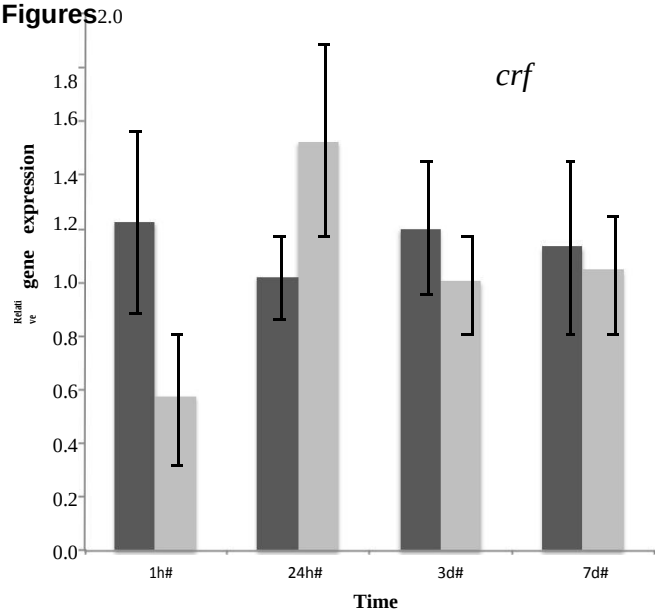
gr, glucocorticoid receptor; *hsp*, heat shock protein; *crh*, corticotrophin release hormone; *crhbp*, corticotrophin release hormone binding proteins; *pomc*, proopiomelanocortin; *ubq*, ubiquitin.

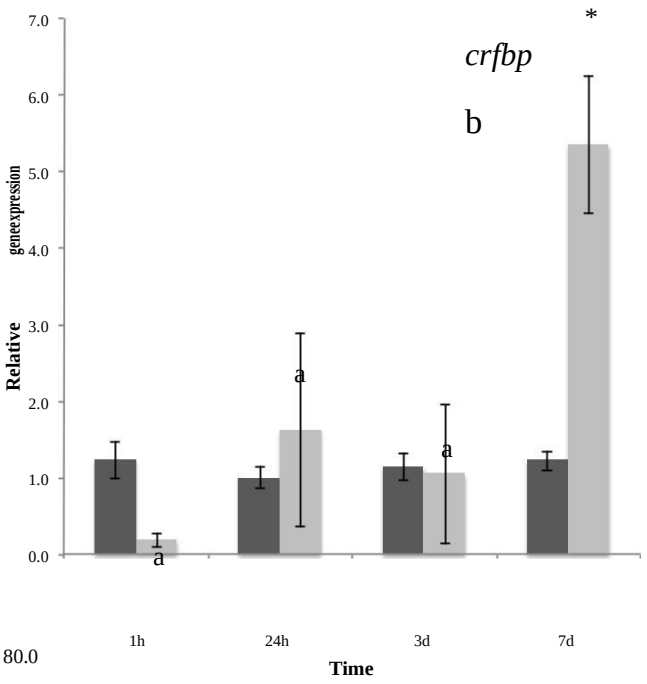
Tables

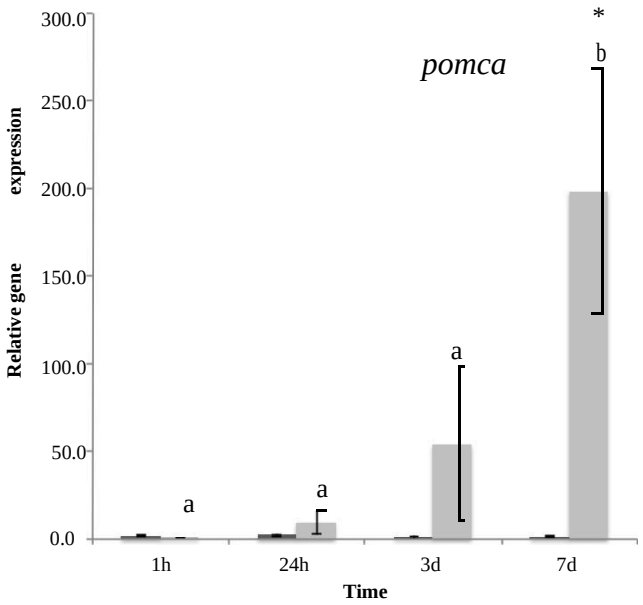
Table 2.- Calculated *hsp90*/*gr* ratios in Senegalese sole subjected to a thermal stress challenge.

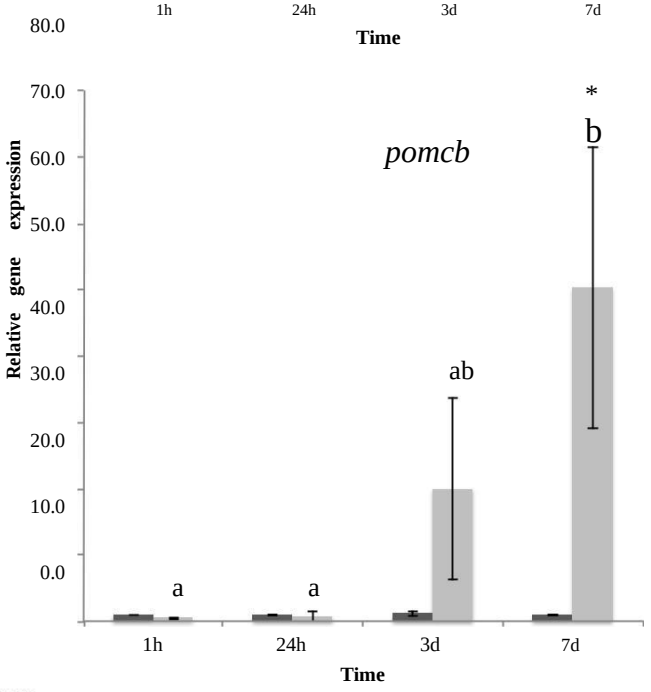
		18 °C	**24 °C**
Liver	*1 h*	0.81	0.83
	24 h	0.98	2.44
	3 d	0.97	1.06
	7 d	1.13	1.35
Muscle	*1 h*	1.13	2.01
	24 h	0.98	0.3
	3 d	1.04	0.43
	7 d	0.99	2.18
Intestine	*1 h*	0.99	2.91
	24 h	0.98	0.70
	3 d	0.99	0.94
	7 d	0.99	0.42
Gill	*1 h*	0.95	0.54
	24 h	0.93	12.86
	3 d	1.04	1.10
	7 d	0.96	0.71
Brain	*1 h*	0.97	27.99
	24 h	1.02	1.40
	3 d	1.00	0.78
	7 d	0.93	6.26

Figures2.0



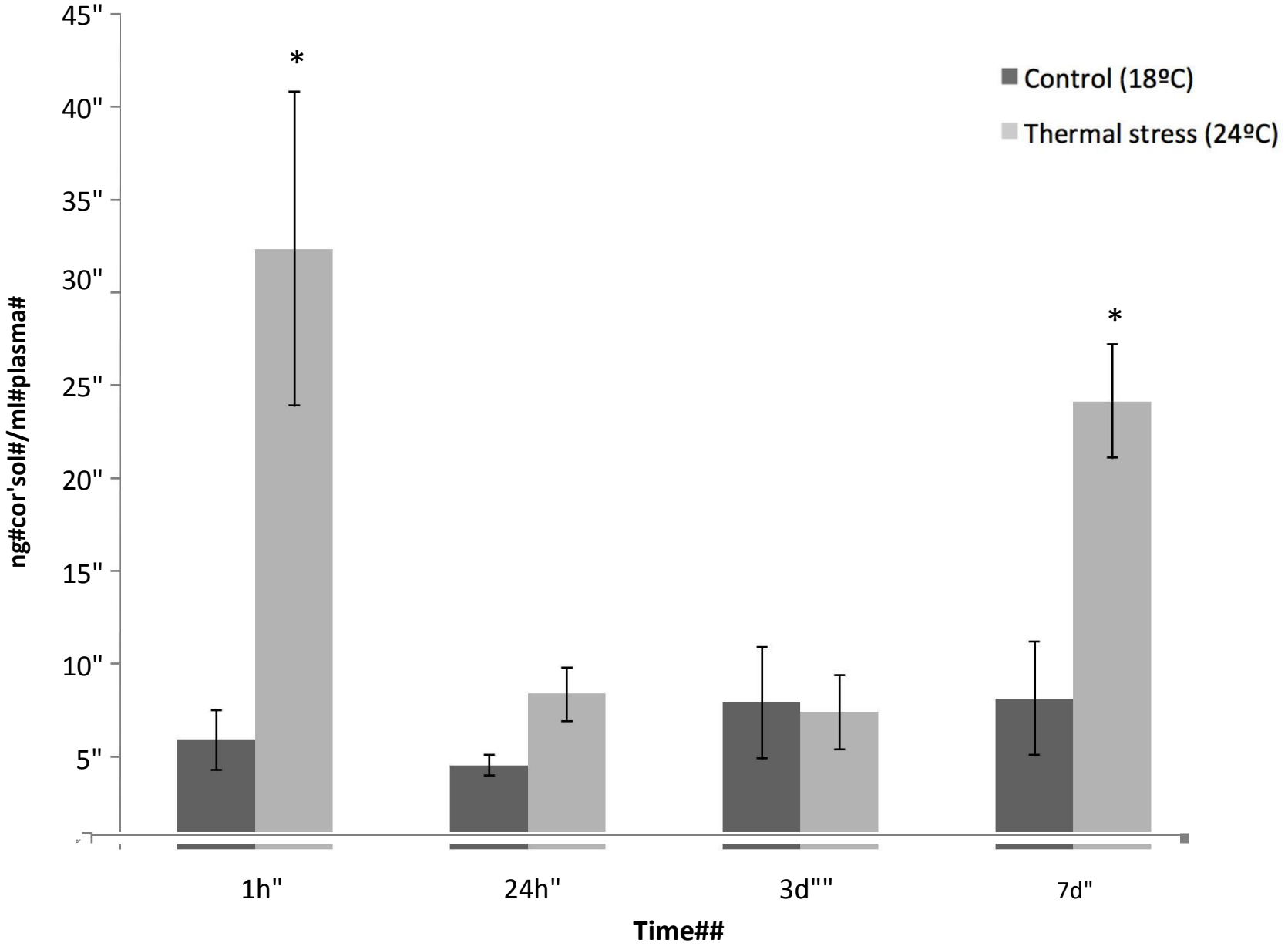




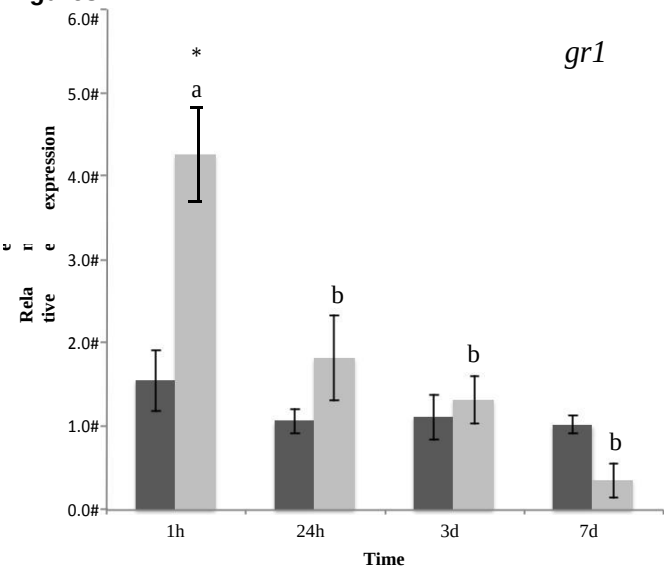


Control (18ºC)
Thermal stress (24ºC)

Figures


45"
40"
35"
30"
25"
20"
15"
10"
5"
ng#cor'sol#/ml#plasma#
*
*
Control (18ºC)
Thermal stress (24ºC)
1h"
24h"
3d""
7d"
Time##

Figures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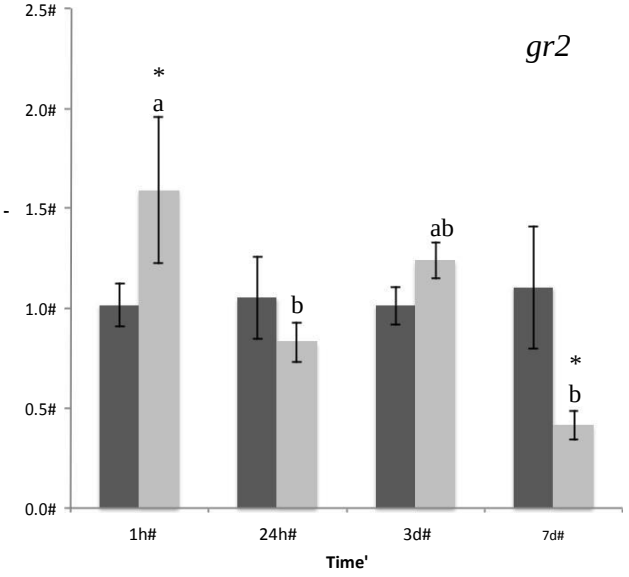
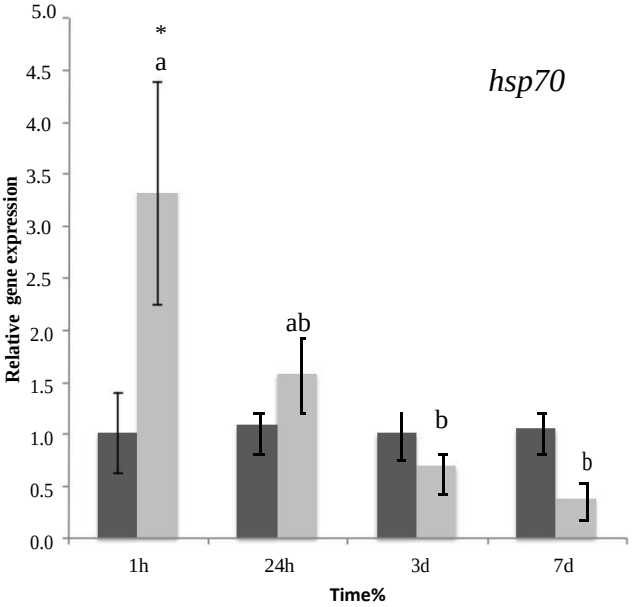
Relative expression
6.0#
5.0#
4.0#
3.0#
2.0#
1.0#
0.0#
*
a
b
b
b
1h
24h
3d
7d
Time


gr2
2.5#
2.0#
1.5#
1.0#
0.5#
0.0#
*
a
b
ab
*
b
1h#
24h#
3d#
7d#
Time'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Relative gene expression
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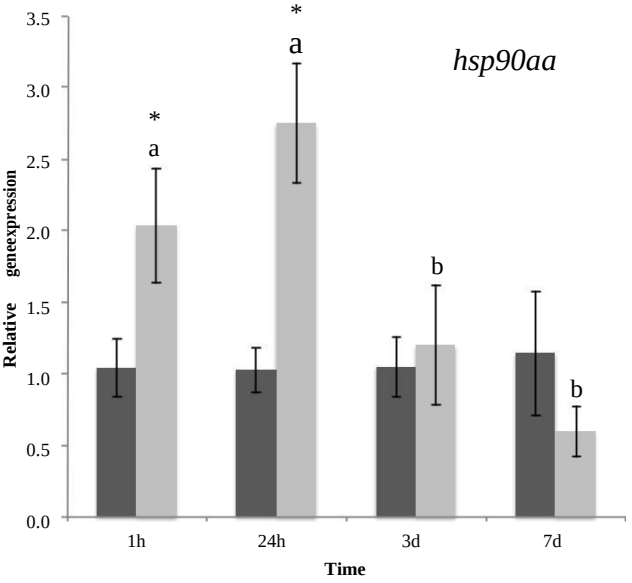
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
a
ab
b
b
1h
24h
3d
7d
Time%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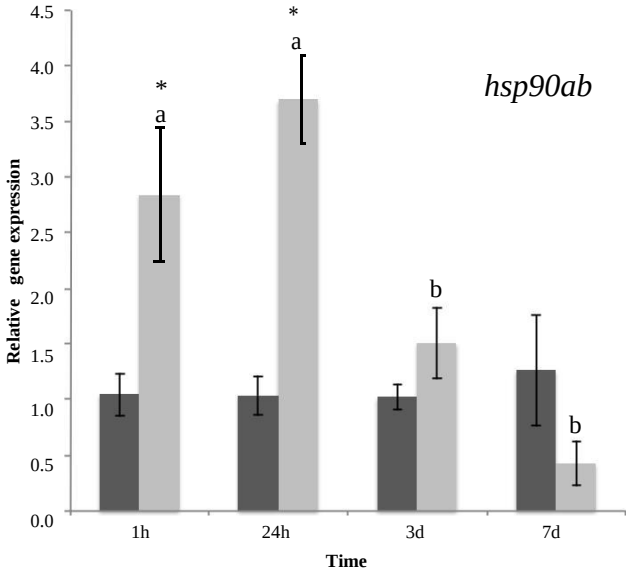
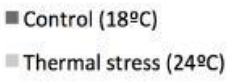
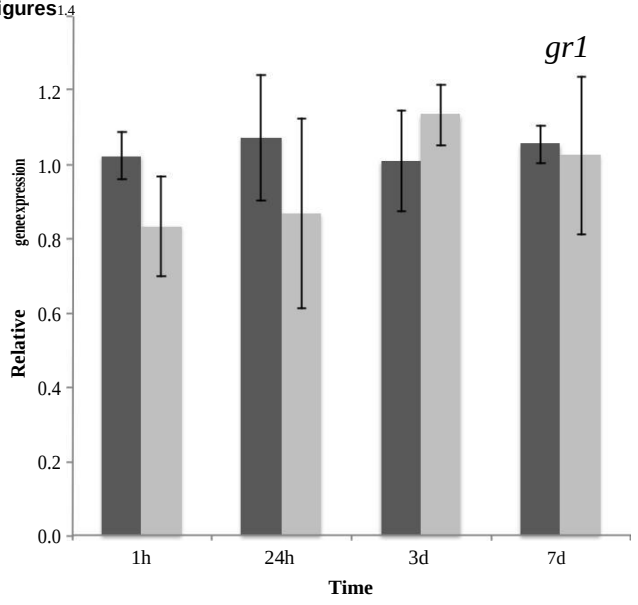
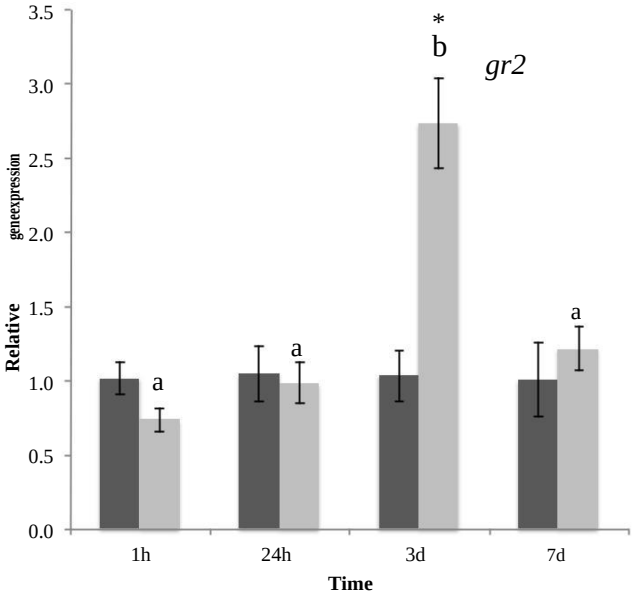
Relative geneexpression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
a
*
a
b
b
1h
24h
3d
7d
Time

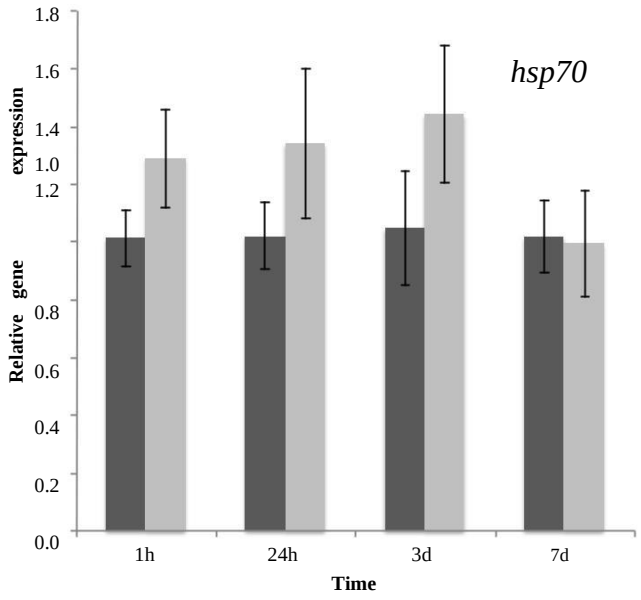

hsp90ab
Relative gene expression
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
a
*
a
b
b
1h
24h
3d
7d
Time

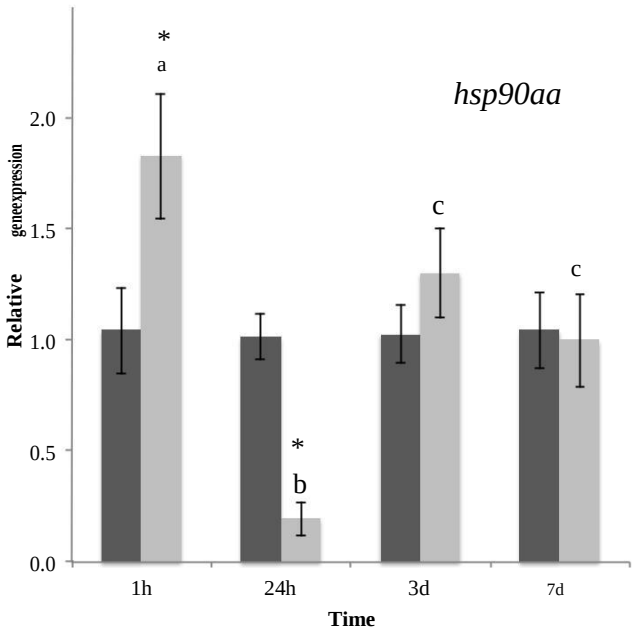

Control (18ºC)
Thermal stress (24ºC)

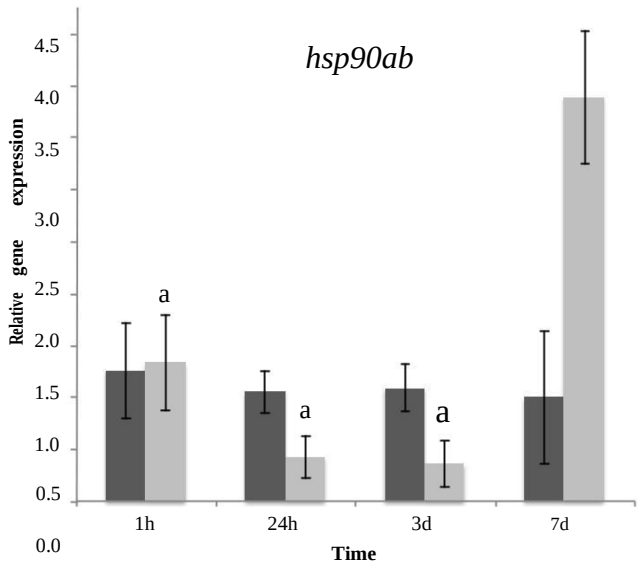
Figures

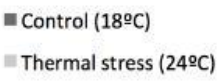

gr1
Relative geneexpression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1h
24h
3d
7d
Time


gr2
Relative geneexpression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
b
a
a
a
1h
24h
3d
7d
Time

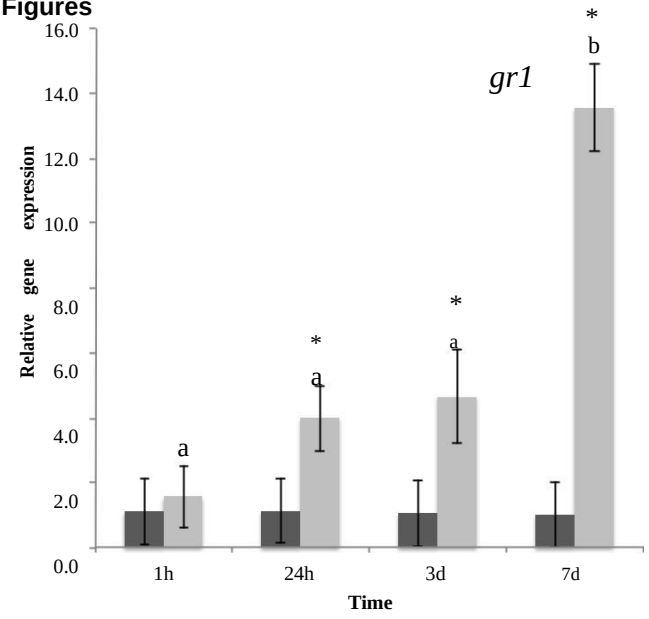

hsp70
Relative gene expression
1.8
1.6
1.4
1.0
1.2
0.8
0.6
0.4
0.2
0.0
1h
24h
3d
7d
Time

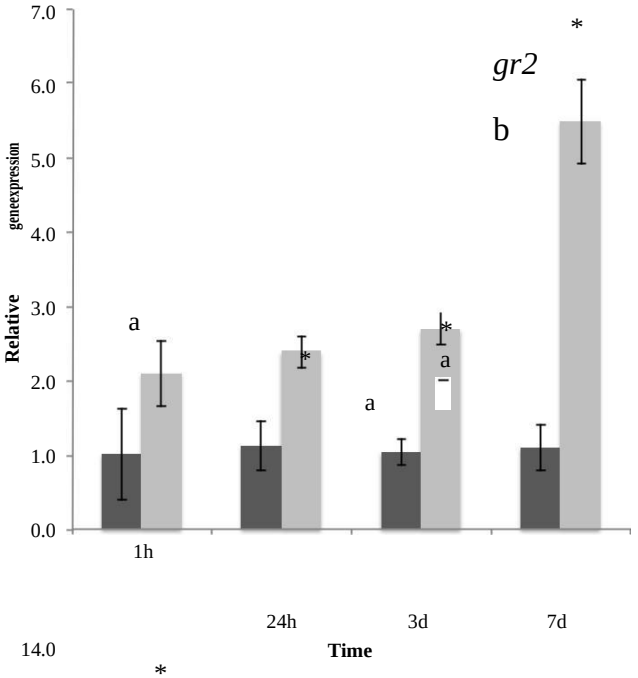

hsp90aa
Relative geneexpression
2.0
1.5
1.0
0.5
0.0
*
a
c
*
b
c
1h
24h
3d
7d
Time


hsp90ab
Relative gene expression
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
a
1h
24h
3d
7d
Time


Control (18ºC)
Thermal stress (24ºC)

Figures


gr1
Relative gene expression
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
a
a
a
b
*
*
*
1h
24h
3d
7d
Time


gr2
Relative geneexpression
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6.0
5.0
4.0
3.0
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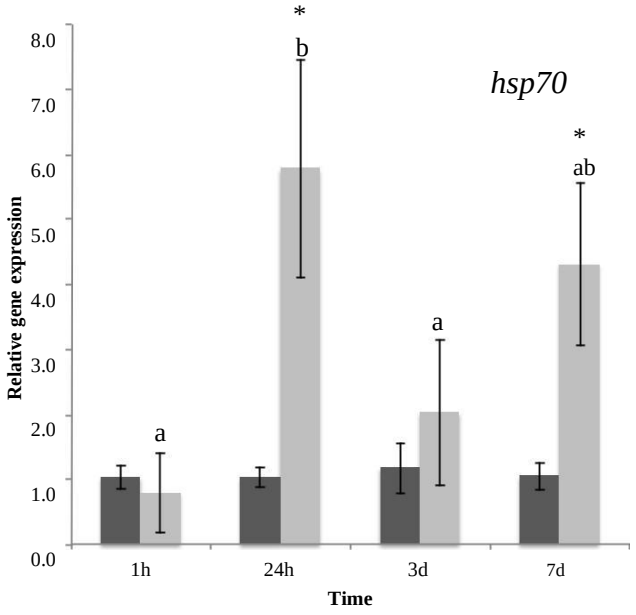
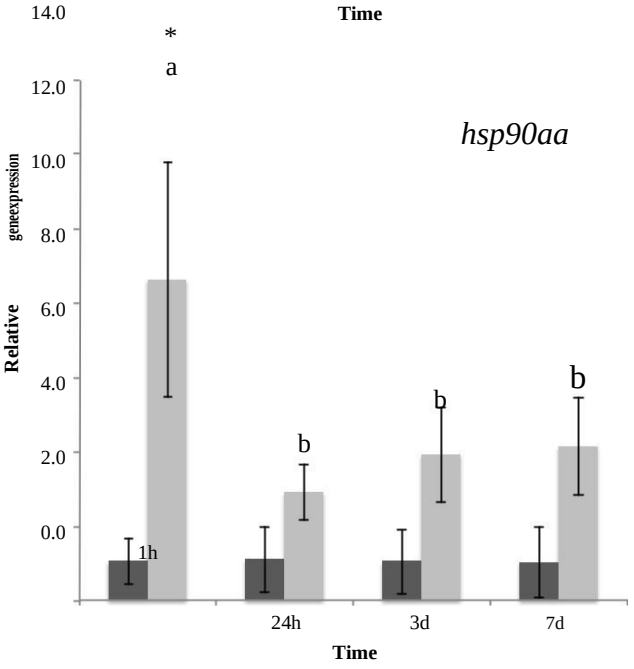
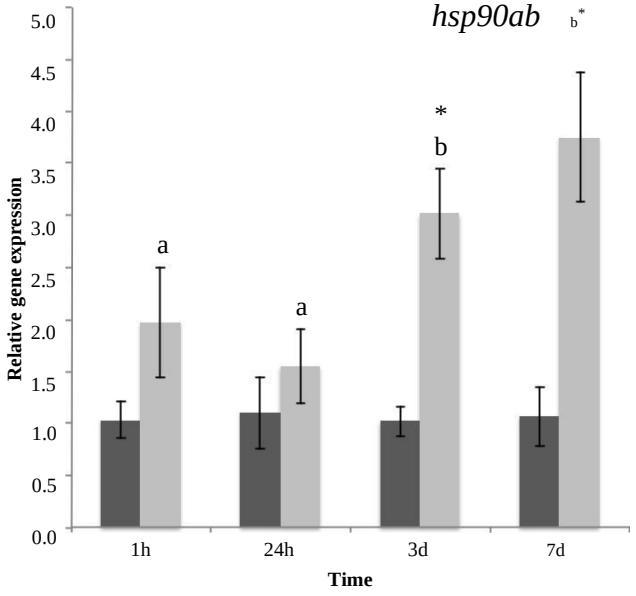
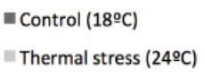
1.0
0.0
a
a
a
b
*
*
*
1h
24h
3d
7d
Time

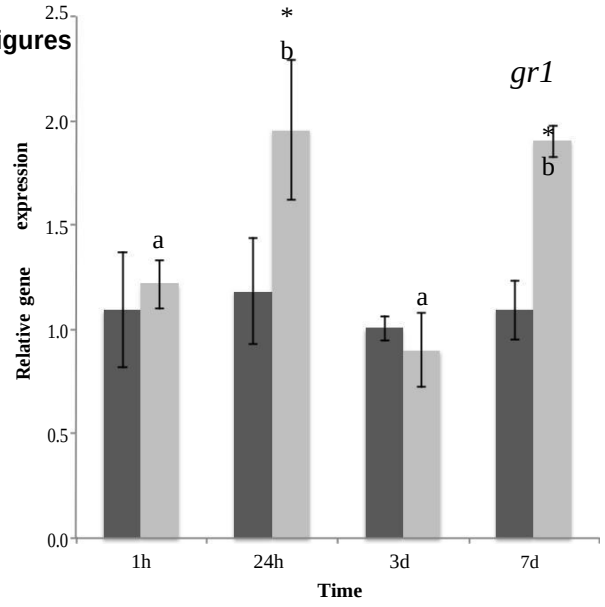

hsp70
Relative gene expression
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
a
a
b
ab
*
*
1h
24h
3d
7d
Time

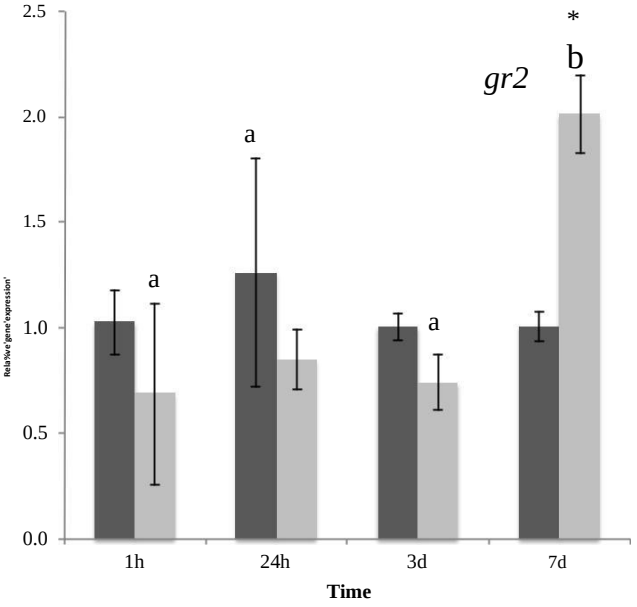

hsp90aa
Relative geneexpression
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
a
b
b
b
*
1h
24h
3d
7d
Time

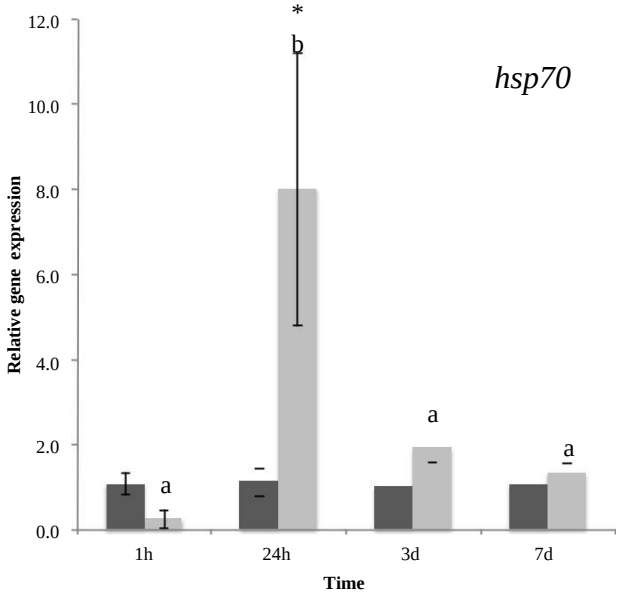

hsp90ab
Relative gene expression
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
*
1h
24h
3d
7d
Time

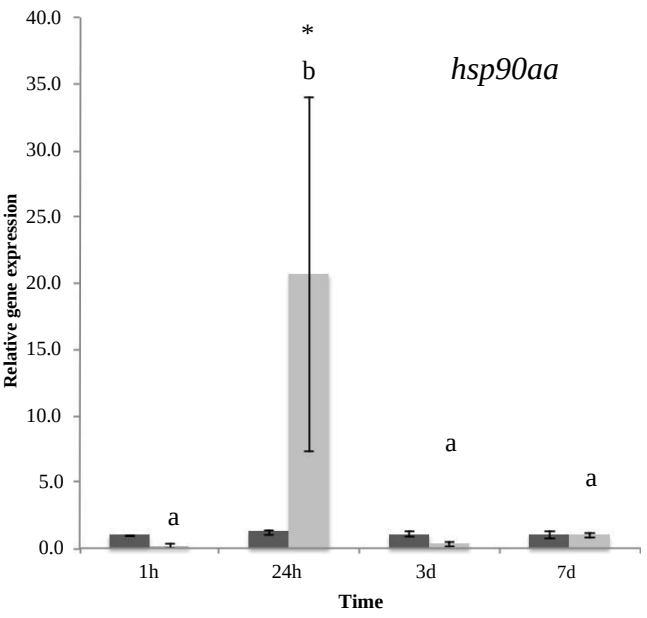

Control (18ºC)
Thermal stress (24ºC)

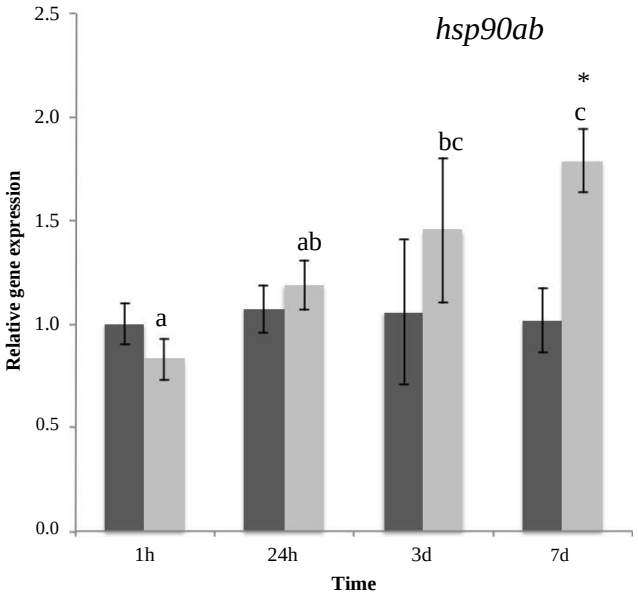
Figures

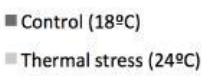

gr1
Relative gene expression
Time
1h
24h
3d
7d
a
b
a
b
*
*


gr2
Time
1h
24h
3d
7d
a
a
a
b
*

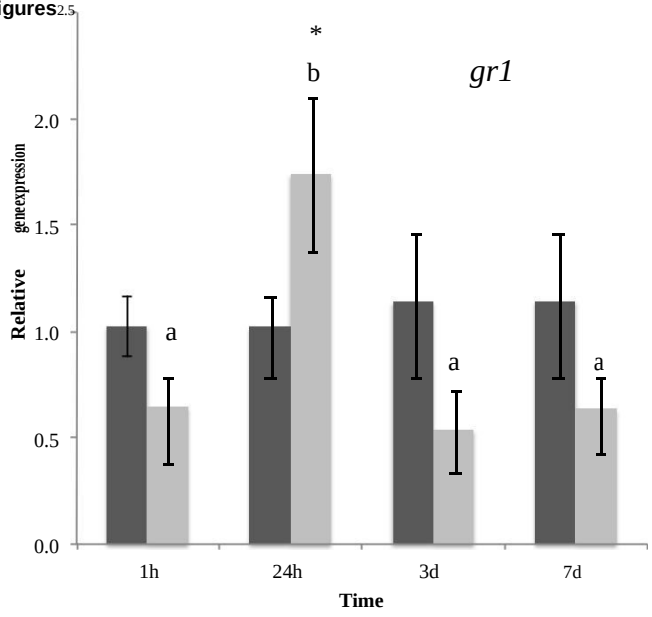

hsp70
Relative gene expression
Time
1h
24h
3d
7d
a
b
a
a
*

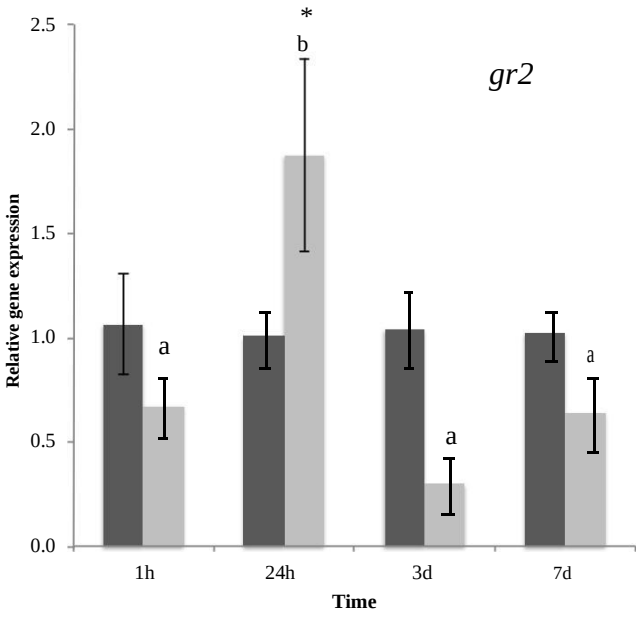

hsp90aa
Relative gene expression
Time
1h
24h
3d
7d
a
b
a
a
*


hsp90ab
Relative gene expression
Time
1h
24h
3d
7d
a
ab
bc
c
*


Control (18ºC)
Thermal stress (24ºC)

Figures


gr1
Relative geneexpression
2.5
2.0
1.5
1.0
0.5
0.0
*
b
a
a
a
1h
24h
3d
7d
Time


gr2
Relative gene expression
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2.0
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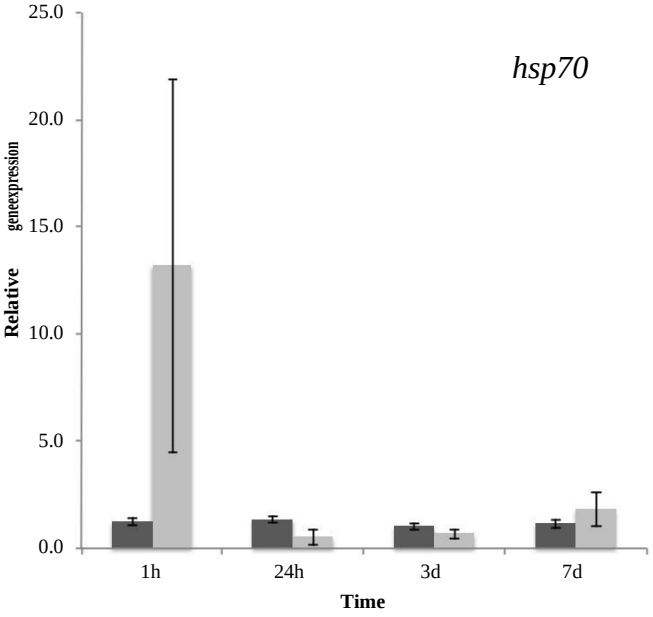
1.0
0.5
0.0
*
b
a
a
a
1h
24h
3d
7d
Time


hsp70
Relative geneexpression
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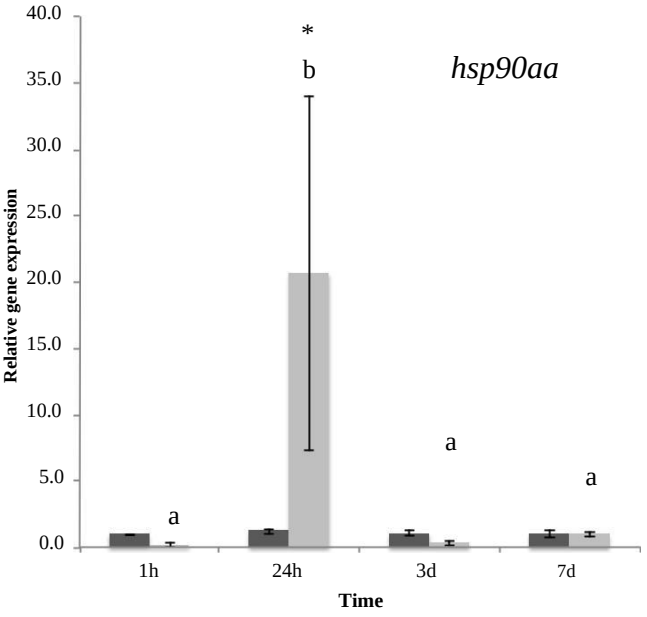
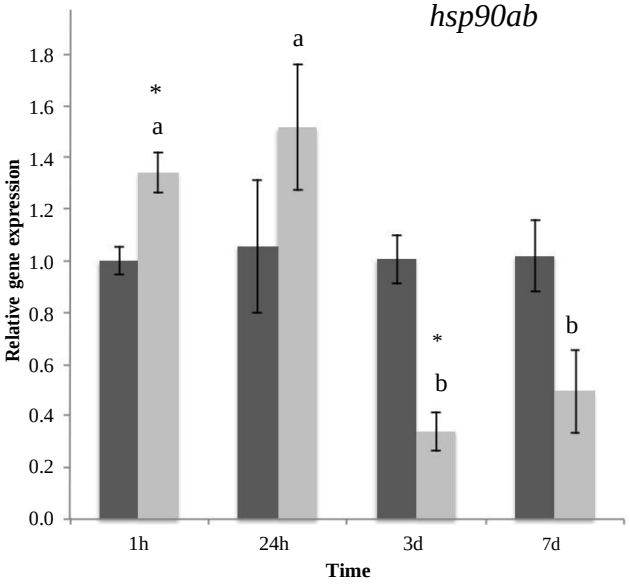
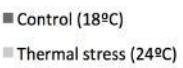
15.0
10.0
5.0
0.0
1h
24h
3d
7d
Time


hsp90aa
Relative gene expression
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
*
b
a
a
a
1h
24h
3d
7d
Time


hsp90ab
Relative gene expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
a
a
*
b
b
1h
24h
3d
7d
Time


Control (18ºC)
Thermal stress (24ºC)