Author's version (accepted manuscript):

Chronic stress impairs the local immune response during cutaneous repair in gilthead sea bream (*Sparus aurata*, L.)

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Abbreviations:

COLIVα1, collagen type IV alpha 1 Chain; CRTAC2, cartilage acidic protein 2; CSF-1R, colony stimulating factor 1 receptor; GR, glucocorticoid receptor; HPA, hypothalamic-pituitary-adrenal; HSI, hepatosomatic index; K, condition factor; MMC, melanomacrophage center; MMP9, matrix metallopeptidase 9; MPO, myeloperoxidase; PCNA, proliferating cell nuclear antigen; POMC,

proopiomelanocortin; StAR, steroidogenic acute regulatory protein; TGFβ1, transforming growth factor beta 1.

Abstract

Scale removal in fish triggers a damage-repair program to re-establish the lost epidermis and scale and an associated local immune response. In mammals, chronic stress is known to delay wound healing and to modulate the cutaneous stress axis, but this is unstudied in teleost fish the most successful extant vertebrates. The present study was designed to test the hypothesis that chronic stress impairs cutaneous repair in teleost fish as a consequence of suppression of the immune response. The hypothesis was tested by removing the scales and damaging the skin on one side of the body of fish previously exposed for 4 weeks to a chronic crowding stress and then evaluating cutaneous repair for 1 week. Scale removal caused the loss of the epidermis although at 3 days it was re-established. At this stage the basement membrane was significantly thicker ($p = 0.038$) and the hypodermis was significantly thinner ($p = 0.016$) in the regenerating skin of stressed fish relative to the control fish. At 3 days, stressed fish also had a significantly lower plasma osmolality ($p = 0.015$) than control fish indicative of reduced barrier function. Chronic stress caused a significant down-regulation of the glucocorticoid receptor (*gr*) in skin before damage (time 0, $p = 0.005$) and of *star* at 3 and 7 days ($p < 0.05$) after regeneration relative to control fish. In regenerating skin key transcripts of cutaneous repair, *pcna*, *colivα1* and *mmp9*, and the inflammatory response, *tgfβ1*, *csf-1r*, *mpo* and *crtac2*, were down-regulated ($p < 0.05$) by chronic stress. Irrespective of chronic stress and in contrast to intact skin many hyper pigmented masses, putative melanomacrophages, infiltrated the epidermis of regenerating skin. This study reveals that chronic stress suppresses the local immune response to scale removal and impairs the expression of key transcripts of wound healing. Elements of the stress axis were identified and modulated by chronic stress during cutaneous repair in gilthead seabream skin.

Keywords

Barrier function, cutaneous wound healing, immune response, melanomacrophage centers, peripheral stress axis

1. Introduction

The skin, together with its appendages, serves as a barrier and has an essential role in maintaining whole organism homeostasis and for this reason damage is rapidly repaired. In vertebrates the skin is composed of an outer epidermis, the dermis composed of connective tissue and is separated from underlying structures such as the muscle by a subcutaneous layer of adipose tissue (hypodermis) (Bacha & Bacha, 2000; Kuehnel, 2003). In general vertebrate skin has a relatively well conserved organization, although habitat specific specializations have emerged and fish skin is protected by scales and composed of living non-keratinized epidermal cells covered with a mucous layer (Elliott, 2011; Hawkes, 1974; Spearman, 1973). Despite the obvious differences that exist between piscine and mammalian skin, this tissue is proposed to arise through common developmental pathways and has a similar role in innate immunity (Rakers et al., 2010; Wu et al., 2004).

Recent studies of human skin reveal that in addition to being an endocrine responsive tissue it also produces hormones, neurotransmitters and neuropeptides (Slominski, 2005; Slominski & Wortsman, 2000; Slominski et al., 2007). In particular, the key elements of the hypothalamic-pituitary-adrenal (HPA) axis are present in skin indicating that in addition to the classical central stress axis a second cutaneous stress axis also exists. The cutaneous stress axis in human skin maintains local homeostasis and has a regulatory role in skin immunity and the pigmentary system, although its involvement in cutaneous wound healing is poorly studied (Slominski et al., 2007). The emergence of a cutaneous stress axis in vertebrates is largely unstudied, particularly in the most successful group of extant vertebrates, the teleost fish. Although the results of a recent microarray study of the cutaneous response to an ectoparasite infestation in Atlantic salmon revealed the up-regulation of genes involved in steroid metabolism (Krasnov et al., 2012) and hinted at the existence of a cutaneous stress axis.

The skin is the largest neuroendocrine-immune tissue and is the main barrier between an organism and its external environment and an integrated response of the endocrine, immune and central nervous system is essential for maintenance of internal homeostasis (Brazzini et al., 2003;

Slominski et al., 2012). For this reason damage to this barrier in mammals leads to a rapid multi-phase repair process which relies on the innate immune response (Barrientos et al., 2008). An emerging and widely studied concept in mammals is the cross-talk that occurs between the stress axis and the immune system (Dhabhar, 2008). The release of glucocorticoids as a result of prolonged stress down-regulates pro-inflammatory cytokines (Schleimer et al., 1990) and this delays repair of the barrier in mammals (Glaser et al., 1999; Godbout & Glaser, 2006; Vileikyte, 2007; Walburn et al., 2009). In common with other vertebrates, acute stress in fish is reported to act as an immunoenhancer (Fast et al., 2008; Tort, 2011; Weyts et al., 1999), although chronic stress (prolonged stress) suppresses the immune response (Fast et al., 2008; Tort, 2011; Weyts et al., 1999).

Scale loss caused by aggressive interactions between fish, abrasion and other environmental factors causes loss of the coating mucous layer, the epidermis and the superficial dermis attached to the scale and causes a cutaneous wound that reduces the efficiency of the skins barrier function. In teleost fish skin repair is much quicker than in mammals and damaged skin is rapidly covered by an epithelial cell layer and mucous. A second and much slower event during cutaneous repair in teleost fish is the development of the new scale with all the characteristics of the ontogenic scale and the associated reorganization of the connective tissue in the dermis (Bereiter-Hahn, 1986; Quilhac & Sire, 1999; Rakers et al., 2010; Vieira et al., 2011). The regulation of cutaneous wound repair involves the up-regulation of chemokines that attract macrophage to the site of inflammation and the release of growth factors by these cells causes cell cycle activation and up-regulation of extracellular matrix proteins and associated tissue proliferation and remodeling (Ibarz et al., 2013; Vieira et al., 2011). The repair of skin in fish differs from that in mammals as it does not result in scarring (Gurtner et al., 2008; Poss et al., 2003; Robson et al., 2001) making the fish an interesting model for comparative studies. The effect of prolonged exposure to stress on repair of skin after scale removal in teleost fish has never been explored.

The present study was designed therefore to test the hypothesis that chronic stress impairs teleost fish cutaneous repair as a consequence of the suppression of the immune response. We

tested the hypothesis by damaging the skin on one side of the body by removing scales from fish pre-exposed to chronic stress for 4 weeks and then observed cutaneous regeneration during a further week of stress. Undamaged (control) and damaged skin was collected and analysed from the same fish. Histology and histomorphometrics were used to assess changes in skin organization and to establish how the barrier changed. Cutaneous repair over time was studied by histology allied to qPCR to, i) evaluate changes in gene transcripts associated with wound healing and the cutaneous immune response and ii) to measure the infiltration of the damaged skin by dark pigmented masses.

2. Material and methods

Manipulation of animals was performed in compliance with international and national ethics guidelines for animal care and experimentation, under a "Group-I" license from the Portuguese Government Central Veterinary service to CCMAR and conducted by a certified investigator (DMP).

2.1. Experimental fish and long-term exposure to crowding stress

Gilthead seabream (*Sparus aurata*) were supplied by the Instituto Português do Mar e da Atmosfera (IPMA, Olhão) and transferred to Ramalhete research station (CCMAR, University of Algarve, Faro, Portugal), where the experiment took place between December of 2014 and January of 2015. Sixty-four fish (87.09 ± 5.54, mean weight ± standard deviation) were randomly distributed into four polyethylene tanks of 100 L and allowed to acclimate to the experimental circuit for 12 days at an initial stocking density of 16 $kg.m^{-3}$ under natural photoperiod. Tanks were supplied with a constant flow-through of aerated seawater at a temperature of 15 ± 1 ºC, salinity (33-36 ‰) and oxygen saturation (>80 %). Fish behavior was monitored daily and they were fed twice daily with a commercial diet (Aquasoja, Portugal) at 1% of body weight (kg food / kg of fish in tank).

Two groups of fish, the control (fish kept at 16 $kg.m^{-3}$) and stress (crowded fish kept at 45 $kg.m^{-3}$) were established in randomly chosen replicate tanks (n = 16 / tank, 2 tanks per group) in the same open circuit (eg. water was not in recirculation). Chronic stress was induced to fish of stress group for 4 weeks before the cutaneous repair challenge by increasing the stocking density of fish from 16 to 45 $kg.m^{-3}$ using a plastic net. In this way fish were confined to a reduced water volume (35 L), but the total tank water volume remained the same as the control to ensure water conditions were equivalent between the control and treatment group (Fig. 1A). Fish from the control group were maintained throughout the experiment at the initial stocking density of 16 $kg.m^{-3}$. After 4 weeks in these conditions, fish (n = 4 / tank; weight, length, condition factor [K] and hepatosomatic index [HSI] (in Table 1) were sampled after exposure for 4 weeks to chronic stress prior to the initiation of cutaneous wound healing (0 time).

2.2. Cutaneous wound healing challenge and sampling procedures

In order to evaluate the effect of chronic stress on cutaneous wound healing, the skin of fish from the control and chronic stress groups was damaged by removing approximately 50 % of the scales from the left side of the body (superior to lateral line, from head do caudal fin) with the blunt side of a knife. Fish were allowed to recover from anesthesia in separated buckets containing aerated seawater before being returned to the original tanks of the experimental circuit. Cutaneous wound healing was monitored by serial sampling of fish from the replicate tanks at 12 hours (0.5 day), 3 and 7 days after scale removal (Fig. 1B; n = 4 / tank; weight, length, K and HSI in Table 1). To minimize stress during sampling the water level in tanks was reduced by 50 % and 4 fish quickly netted and placed in a 10 L bucket containing anaesthetic (phenoxyethanol 0.01 %, Sigma-Aldrich, USA). The water volume in tanks was adjusted after sampling to maintain the control and crowding stress density conditions during cutaneous wound healing. The feeding regime (twice daily at 1 % of body weight (kg food / kg fish in tank, Aquasoja, Portugal) was maintained throughout the experiment, although fish were fasted for 24 hours before sampling. One chronically stressed fish died at 3 days after scale removal for unidentified reasons.

For sampling, fish were sacrificed with an overdose of phenoxyethanol (0.1 %, Sigma-Aldrich, USA), blotted dry and blood collected from the caudal vein using a heparinized syringe. Blood was centrifuged at 10,000 rpm for 4 minutes at 4 ºC, and the separated plasma immediately frozen in liquid nitrogen and stored at -80 ºC. Skin samples of about 1.5 x 2 cm^2 were removed from the left side of the fish under the dorsal fin (damaged side) and also from the equivalent position on the right side of the fish (undamaged side) and placed in ice-cold 4 % paraformaldehyde (PFA), pH 7.4. Tissues were fixed overnight at 4 ºC under constant agitation, washed with three changes of phosphate buffered saline, and finally with sterile DEPC water, and stored in 70 % ethanol at 4 ºC until processing. Skin samples from both the damaged and undamaged side of the fish were also collected for molecular analysis and were snap frozen in liquid nitrogen and stored at -80 ºC until analysis.

2.3. Plasma cortisol and osmolality measurements

Plasma cortisol ($ng.mL^{-1}$) was measured in duplicate using a validated radioimmunoassay (RIA, Rotllant et al., 2005) and plasma osmolality ($mmol.kg^{-1}$) was determined using a vapor pressure osmometer (Vapro Wescor 5520, Utah, USA).

2.4. Skin histology and morphometric analysis

Skin samples with ontogenic scales or skin 7 days after scale regeneration were decalcified for 24 hours in 0.5M EDTA pH 8.0 in the dark. Decalcified tissue was then washed several times with sterile DEPC water and skin samples (n = 6/group/time point) from both damaged and undamaged sides of the same fish were dehydrated through a graded series of ethanol (70 % to 100 %) and embedded in low melting point paraffin wax using an automated tissue processor (Leica TP1020, Leica®). Serial longitudinal sections of 5 µm were cut using a rotary microtome (Leica RM 2135) and mounted on glass slides coated with 3-amino-propyltriethoxysilane (APES; Sigma-Aldrich, USA). One section per individual was stained with hematoxylin and eosin to assess the general histology and 3 slides containing 3 serial sections/slide with a gap of 5 sections (25 µm) between consecutive slides were stained in 0.1 % Picro-Sirius Red (Junqueira et al.,

1979). Stained sections were observed using a microscope (Leica DM2000) equipped with a digital camera (Leica DFC480) and 1 section per slide was photographed at different magnifications for measurement the thickness of basement membrane, basal cell layer, epidermis and hypodermis. Infiltration of the epidermis and dermis by dark pigmentation and the number of goblet cells were also evaluated. Histomorphometric analysis of the photographed skin was performed using Fiji v1.47p software (Schindelin et al., 2012).

Two contiguous and non-overlapping images of skin sections (total length of 2 mm and total area of 2.82 mm^2) stained with Picro-Sirius Red were analyzed (n = 6/group/time point, 100x magnification). Images were used to measure the number of mucous cells and the area infiltrated with dark pigmentation in the dermis of the regenerating and undamaged skin from control or chronically stressed fish. Assessment of the infiltration of dark pigmentation was used as a proxy for relative melanin content and performed using the Otsu method for colour threshold setting (Papadopulos et al., 2007; Zhang & Hu, 2008), which highlighted as red all dark pigment cells. The presence of dark pigment agglomerations in the epidermis was evaluated by semi-quantitative analysis using the following scale (Klopfleisch, 2013): 0 - no pigment in the epidermis, 1 – low agglomeration and 2 – moderate to high agglomeration.

For the basement membrane, basal cell layer and epidermis thicknesses, measurements were taken from the mid-region of the section and 2 equidistant flanking regions in each histological section. Skin sections were photographed at 400x magnification and analyzed using Fiji v1.47p software (Schindelin et al., 2012). The thickness of the hypodermis was measured in images of two contiguous fields of each section (100x magnification).

2.5. Molecular analysis

The impact of chronic stress on the expression of specific transcripts related to cutaneous wound healing, immune response and the cutaneous HPA axis was evaluated using qPCR. Collagen type IV alpha 1 Chain (*colivα1)* was amongst the candidate genes selected for analysis and since this gene has not previously been described in gilthead seabream, database mining and

phylogenetic analysis was used to confirm that the sequence retrieved corresponded to seabream *colivα1*.

2.5.1 Database mining and seabream colivα1 sequence retrieval

The gilthead seabream *colivα1* transcript was identified by searching an in house reference transcriptome assembly generated from diverse seabream tissue (Louro et al., 2016) using the basic local alignment search tool (BLAST) algorithm. The sequence of zebrafish (*Danio rerio*) *colivα1* (XP_694040) was used to retrieve homologues from other teleosts with sequenced genomes: salmon, *Salmo salar*; *Takifugu rubripes*; Nile tilapia, *Oreochromis niloticus*; medaka, *Oryzias latipes*; cavefish, *Astyanax mexicanus* and from a primitive freshwater ray-finned fish, spotted gar (*Lepisosteus oculatus*) and the lobe-finned fish, the coelacanth (*Latimeria chalumnae*) and the marine lamprey (*Petromyzon marinus*) genomes, all available from ENSEMBL (http://www.ensembl.org, accessed in December 2015) and NCBI (http://www.ncbi.nlm.nih.gov/, accessed in December 2015). The genome of the cartilaginous elephant shark (*Callorhinchus milii*, http://esharkgenome.imcb.a-star.edu.sg) was also investigated.

The tetrapod genomes including human (*Homo sapiens*), mouse (*Mus musculus*), cow (*Bos taurus*), chicken (*Gallus gallus*) and the amphibian (*Xenopus tropicalis*) available from NCBI (http://www.ncbi.nlm.nih.gov/, accessed in December 2015) were also explored for ColIVα1 genes using a similar strategy to that outlined above. *colivα1* in the genomes of the early chordates, the cephalochordate amphioxus (*Branchiostoma floridae*) and the tunicate Ciona (*Ciona intestinalis*) and in the protostome genomes of the fruit fly (*Drosophila melanogaster*) and *Caenorhabditis elegans* was also analyzed (sequences retrieved from http://www.ncbi.nlm.nih.gov/, accessed in December 2015).

2.5.2. Phylogenetic analysis

Phylogenetic analysis was performed using the deduced amino acid sequences of *colivα1* (accession numbers in Supplementary Table S1). The sequences were aligned using CLUSTALW (1.83) (available from http://www.genome.jp/tools/clustalw/, Thompson et al., 1997) and the alignment was assessed using ProtTest (2.4) and the best model for analysis of protein evolution

selected using the Akaike Information Criterion (AIC) statistical model (Abascal et al., 2005). A phylogenetic tree was constructed using the maximum likelihood (ML) method and the accuracy of the phylogenetic clades assessed using 100 bootstrap analysis (Felsenstein, 1985). ML analysis was constructed in the PhyML program (3.0) (Guindon et al., 2010) available from http://atgc.lirmm.fr/phyml/ using the JTT+I+G substitution model (Jones et al., 1992) with a fixed proportion of invariant sites (0.24), 4 gamma-distributed rate categories and gamma shape parameter (0.912). The ML tree was displayed and edited using FigTree software v.1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). The phylogenetic tree of *colivα1* was rooted with the sequences of amphioxus, the tunicate Ciona, *D. melanogaster* and *C. elegans*.

2.5.3. Analysis of gene expression by quantitative real-time PCR (qPCR)

Total RNA from skin samples (n = 8/group/time point, corresponding to 4 samples per replicate tank) was extracted using a Maxwell 16 System (Promega, USA) following the manufacturer's instructions. The concentration and quality of the extracted RNA was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). To eliminate contaminating genomic DNA total RNA (2 – 9 µg) was treated with DNase using a DNA-free kit (Ambion, UK). cDNA synthesis was carried out in a 20 µL reaction volume containing 500 ng of DNase-treated RNA, 200 ng of random hexamers (Jena Biosciences, Germany), 100 U of RevertAid reverse transcriptase (Fermentas, Thermo Fisher Scientific, USA), 8 U of RiboLockRNase Inhibitor (Fermentas) and 0.5 mM dNTPs. The reaction mix was incubated for 10 min at 20 ºC followed by 50 min at 42 ºC and the enzyme inactivated by heating for 5 min at 72 ºC.

Quantitative Real-Time PCR (qPCR) was used to analyze the mRNA expression of proopiomelanocortin (*pomc α1* and *pomc α2*), glucocorticoid receptor (*gr*), steroidogenic acute regulatory protein (*star*), proliferating cell nuclear antigen (*pcna*), *colivα1*, matrix metallopeptidase 9 (*mmp9*), transforming growth factor beta 1 (*tgfβ1*), myeloperoxidase (*mpo*), colony stimulating factor 1 receptor (*csf-1r*), cartilage acidic protein 2 (*crtac2*) in skin. Specific primers for each transcript were designed using Primer Premier 5.0 software (Premier Biosoft

Int., CA, USA). Primer sequence, amplicon size, amplicon melting temperature and the accession numbers of the genes are listed in Table 2.

Transcripts were quantified by qPCR using the relative standard curve method in duplicate 15 µL reactions containing 10 ng of cDNA (25 ng for *pomc α1* and *pomc α2* mRNA expression analysis) and 300 nM of specific primers (Table 2) and EvaGreen (Sso Fast EvaGreensupermix, Bio-Rad Laboratories, USA). The thermocycle was carried out in an iCycler (iQ Real Time Detection System thermocycler) and data was analysed using the iCycler iQ Real Time Detection System Software v2.4.10.0 (BioRad, USA). qPCR cycling conditions were 30 sec at 95 ºC, 40 cycles of 5 sec at 95 ºC and 10 sec at 60 ºC followed by a final melt curve between 60 and 95 ºC, which gave single product/dissociation curves in all reactions.

Standard curves relating amplification cycle to initial template quantity (in copy number, calculated as previously reported (Vieira et al., 2012) were generated using serial dilutions of specific qPCR products for each gene (obtained using the same species, tissues and primers for qPCR analysis). All amplicons were sequenced to confirm qPCR specificity. Control reactions included a no-template control and a cDNA synthesis control to detect genomic contamination (reverse transcriptase omitted from the reaction). Candidate reference genes tested in qPCR included beta actin (*β-actin*) and ribosomal protein S18 (*rps18*). *Rps18* did not vary significantly between any of the samples and was adopted as the reference gene. Results of qPCR were calculated by dividing the detected copy number of the target gene by the reference gene in each cDNA sample and were expressed as Log2 Fold Change and compared to the expression levels of the control group at 0 hours.

2.6. Statistics

Two-way ANOVA was applied to assess if significant differences existed between control and stress groups across time for plasma cortisol and osmolality. Significant differences between control and stress groups at each sampling time point were evaluated using a student t-test for independent samples. One-way ANOVA was used to evaluate if significant differences

existed within a group at different time points and Tukey's post hoc test was used to establish if differences occurred during cutaneous wound healing.

Three-way ANOVA was performed to assess general significant differences in both histomorphometric and qPCR analysis, considering as factors, stress (Control/Stress), skin damage (Damaged/Undamaged) and time after scale removal (0, 0.5, 3 and 7 days). The following strategy was used to identify if significant differences existed between the main factors: i) a student's t-test for independent samples was performed to identify significant differences between the control and chronic stress groups at time 0; ii) a student's t-test for independent samples was performed to compare each group within the same time period (control undamaged vs stress undamaged; control damaged vs stress damaged; control damaged vs control undamaged; stress damaged vs stress undamaged); iii) one-way ANOVA was used to identify if significant differences existed within a treatment group at different time points of cutaneous repair and Tukey's post hoc test was used to identify those particular differences.

All statistical analysis was performed using SPSS 22.0 software (SPSS Inc., 2013) and significance taken at $p < 0.05$. Replicate tanks for each treatment group were pooled since a student's t-test for independent samples failed to reveal significant tank effects. Log10 transformation of data was used whenever necessary to achieve either normal distribution or equal variance assumptions. Data is presented as mean ± standard error of the mean (SEM) unless otherwise stated.

3. Results

3.1. Biometric parameters

The general physiological status of fish after 4 weeks of chronic stress (0 hours) and during cutaneous wound healing (12 hours, 3 and 7 days after scale removal) was assessed by measuring body weight, total length, K and HSI (Table 1). Two-way ANOVA showed that total length, K and HSI were not affected by time after scale removal or stress, or by the interaction between these effects ($p > 0.05$). At 0 hours, no significant differences were detected between

control and the stress group, for any of the biometric parameters analyzed. No significant differences were detected for K and HSI between the control and chronically stressed fish during cutaneous repair.

3.2. The physiological response to chronic stress and to cutaneous wound healing

Two-way ANOVA revealed that plasma cortisol levels were significantly affected by time ($p < 0.05$) and by the interaction between time and stress ($p < 0.05$; Fig. 2). In general, the plasma cortisol levels were highly variable in all groups analyzed with the exception of 0 hours and 7 days. At 0 hours, the unstressed (control) fish had significantly ($p < 0.01$) higher levels of cortisol (9.1 ± 2.05 ng.mL^{-1}) than the chronically stressed fish (4.0 ± 0.54 ng.mL^{-1}). But 7 days after scale removal, the chronically stressed fish exhibited a significantly ($p = 0.001$) greater increase in cortisol levels (77.7 ± 15.70 ng.mL^{-1}) compared to unstressed (control) fish (10.1 ± 3.93 ng.mL^{-1}). The cortisol levels of chronically stressed fish undergoing cutaneous repair were significantly increased at 12 hours, 3 and 7 days after scale removal (One-way ANOVA, $p = 0.003$, $p = 0.04$, $p < 0.001$, respectively, Fig. 2).

Plasma osmolality was significantly affected by time after scale removal ($p < 0.001$; Two-way ANOVA; Fig. 2). At 0 hours, no significant differences ($p > 0.05$) were detected between unstressed and chronically stressed fish, but 3 days after scale removal, chronically stressed fish displayed significantly ($p = 0.015$) lower osmolality (337.3 ± 1.60 mmol.kg^{-1}) than unstressed (control) fish (342.6 ± 0.84 mmol.kg^{-1}). One-way ANOVA revealed that there was a significant increase in plasma osmolality across time for both the unstressed and stressed fish ($p = 0.002$ and $p = 0.009$, respectively). Seven days after scale removal, plasma osmolality was significantly increased relative to 0 and 12 hours ($p = 0.001$ and $p = 0.02$, respectively) in the unstressed fish. The chronically stressed fish also had significantly increased osmolality 7 days after scale removal relative to 12 hours and 3 days ($p = 0.03$ and $p = 0.01$, respectively).

3.3. Histological modifications in skin as a consequence of chronic stress and during cutaneous healing

Transverse histological sections of skin from all experimental time points (0 hours, 12 hours, 3 and 7 days after scale removal; Fig. 3) were used to detect and monitor the modifications occurring in skin morphology after scale removal. The effects of chronic stress before (0 hours) and during cutaneous wound healing was compared in undamaged and damaged skin from unstressed (control) and chronically stressed fish. Seabream skin is typically composed of a layered multicellular epidermis overlying a compact dermis that contacts with the underlying adipose tissue of the hypodermis. Scales are enclosed in a scale pocket and anchored in the stratum spongiosum and approximately 50 % of each scale projects into the epidermis.

The main modifications observed after removal of the scales (12 hours) was the disruption of the epidermis, dermis and the scale pocket, which left the latter two elements exposed to the external environment (Fig. 3). Within 3 days of damage the epidermis was re-established and the new scale pocket was formed, although calcified scales were only visible in the skin samples taken after 7 days of cutaneous repair. Comparison of damaged and undamaged skin from unstressed (control) and chronically stressed fish revealed that irrespective of stress the regenerating skin contained many small highly pigmented masses in the epidermis (Fig. 3).

3.3.1. Highly pigmented infiltration in the epidermis and dermis

Three-way ANOVA revealed that pigmentation (used as a proxy for melanin) of the epidermis assessed by histology was significantly modified during the experiment (time: $p < 0.001$; cutaneous repair: $p < 0.001$; interaction between time and cutaneous repair: $p < 0.001$, Table 3). Hyperpigmentation in both epidermis and dermis was significantly reduced in chronically stressed fish relative to the unstressed (control) fish at 0 hours ($p = 0.025$ and $p = 0.009$, Table 3 and Fig. 4, respectively). There was a significant increase in hyperpigmented agglomerations in the repaired epidermis relative to undamaged skin of unstressed (control) fish at 3 and 7 days ($p = 0.013$ and $p < 0.001$, respectively). At 7 days, the repairing epidermis of chronically stressed fish was significantly ($p < 0.001$) more pigmented than the undamaged epidermis from the same fish. One-way ANOVA revealed pigmentation was significantly ($p < 0.001$) increased in the repairing epidermis of both unstressed (control) and stressed fish at 3 and

7 days compared to 12 hours ($p < 0.001$, Table 3). The pigmentation of the epidermis of undamaged skin in unstressed (control) fish significantly decreased at day 7 of the experiment relative to time 0 ($p = 0.035$, Table 3). A significant decrease ($p = 0.04$) in the pigmentation of the dermis occurred in damaged skin of chronically stressed fish at day 7 relative to 12 hours after scale removal (Supplementary Table S2).

3.3.2. Mucous cells

A further difference observed between undamaged and damaged skin was the number of mucous cells (Fig. 5). Three-way ANOVA revealed that time had a significant effect ($p = 0.001$) on mucous cell number and that this was also affected by a significant interaction ($p = 0.003$) between stress and cutaneous repair. Twelve hours after scale removal since most of the epidermis was removed from the damaged skin no mucous cells were observed, and the undamaged skin from the same fish had significantly more mucous cells in both the unstressed (control) and chronically stressed fish ($p = 0.024$ and $p = 0.027$, respectively). Three days after the skin was damaged, the undamaged skin of unstressed (control) fish still had significantly ($p < 0.05$) more mucous cells than the damaged skin. At 3 and 7 days undamaged skin of unstressed (control) fish had significantly ($p < 0.05$) more mucous cells than undamaged skin of chronically stressed fish.

3.3.3. Epidermis and basal cell layer thickness

Three-way ANOVA revealed that time significantly affected the thickness of epidermis and basal cell layer ($p < 0.001$; Fig. 6). The thickness of the basal cell layer was also significantly modified during cutaneous repair ($p < 0.001$), and the interaction between time and repair impacted on total epidermis thickness ($p < 0.001$). No significant differences between the skin of unstressed (control) and chronically stressed fish was evident in the thicknesses of epidermis and basal cell layer at 0 hours and at any experimental time point subsequently after scale removal (for both damaged and undamaged sides, $p > 0.05$). Unsurprisingly 12 hours after the skin was damaged the epidermis and the basal cell layer of the undamaged skin were significantly ($p < 0.001$) thicker than the damaged skin in both the unstressed (control) and chronically stressed

fish. At 3 days, basal cell layer is significantly thicker at damaged skin compared to undamaged skin in chronically stressed fish. Seven days after scale removal, the thickness of the basal layer was significantly ($p < 0.01$) greater in damaged skin compared to undamaged skin in both unstressed (control) and chronically stressed fish. The epidermal thickness of damaged skin from chronically stressed fish was also significantly ($p < 0.001$) thicker at 7 days than the undamaged skin from the same fish.

One-way ANOVA revealed that the thickness of the epidermis and basal layer of the damaged skin significantly increased ($p < 0.001$) during cutaneous repair in both unstressed (control) and chronically stressed fish and by 3 days the epidermis and basal layer thickness were significantly increased ($p < 0.001$) relative to 12 hours (Supplementary Table S3). The thickness of the basal layer significantly ($p = 0.03$ and $p < 0.001$, respectively) decreased in undamaged skin of both unstressed (control) and chronically stressed fish from time 0 to 7 days. The thickness of the epidermis in undamaged skin of unstressed (control) fish was significantly ($p < 0.05$) thinner at 3 days compared to time 0.

3.3.4. Basement membrane thickness

Three-way ANOVA revealed that the thickness of the basement membrane was affected by time ($p < 0.001$), stress ($p = 0.005$) and cutaneous repair ($p < 0.001$), and that there was an interaction between time and cutaneous repair ($p < 0.001$) and time and stress ($p = 0.013$; Fig. 6). The basement membrane thickness was not significantly different between damaged and undamaged skin irrespective of the group (unstressed or chronically stressed). The exception was at 3 days when the basement membrane of chronically stressed fish was significantly thicker ($p = 0.038$ and $p = 0.006$, respectively) than the control fish in both damaged and undamaged skin. At 12 hours post damage the basement membrane was significantly ($p < 0.001$) thicker in undamaged skin compared to damaged skin from the same fish in both unstressed (control) and chronically stressed fish. But 3 days after damage the basement membrane of the damaged skin was significantly thicker ($p = 0.008$ and $p = 0.021$, respectively) than the undamaged skin in both

control and chronically stressed fish. The thickness of the basement membrane in the damaged skin was time dependent and in both unstressed (control) and chronically stressed fish its thickness was significantly ($p < 0.001$, one-way ANOVA, Supplementary Table S3) decreased at 12 hours, compared to all other time points ($p < 0.001$). Time also affected the thickness of the basement membrane in undamaged skin of unstressed (control) fish and it was significantly thinner ($p = 0.046$ and $p = 0.009$, respectively) at 3 days relative to 12 hours and 7 days.

3.3.5. Hypodermis thickness

The thickness of the hypodermis was affected by the interaction between stress and cutaneous repair during the experiment ($p = 0.021$, three-way ANOVA; Fig. 6). The hypodermis was not significantly modified between damaged or undamaged skin irrespective of the group (control or chronically stressed fish). An exception occurred 3 days after damage when the hypodermis was significantly ($p = 0.037$) thicker in the repairing skin relative to the undamaged skin from the unstressed fish. Also at 3 days the thickness of the hypodermis in damaged skin was significantly thinner ($p = 0.016$) in the chronically stressed fish relative to the unstressed fish.

3.4. Gene expression

3.4.1. Identification of colivα1 and phylogeny

The identity of the incomplete seabream *colivα1* transcript (1008 bp), selected as a transcript for monitoring cutaneous wound healing, was confirmed by phylogenetic analysis. The phylogenetic tree revealed that the seabream sequence clustered with other teleost fish *colivα1* genes (Supplementary Fig. S1).

3.4.2. Stress axis transcripts

Time affected the transcript abundance of *gr* and *star* in skin during the experiments ($p < 0.001$, $p = 0.045$, respectively, three-way ANOVA; Fig. 7). Transcript abundance in skin was also significantly affected by stress in the case of *gr* ($p = 0.018$) and by cutaneous repair in the case of *star* ($p < 0.001$).

A significant ($p = 0.005$) down-regulation of *gr* transcripts occurred in the skin of chronically stressed fish relative to control fish at time 0. No other significant differences in the transcript abundance of *gr* were found between groups until 7 days when both damaged and undamaged skin from the same unstressed fish and the damaged skin of the chronically stressed fish were significantly ($p = 0.02$) up-regulated relative to the undamaged skin of the unstressed (control) fish (0 hours). A significant ($p < 0.01$) up-regulation of *gr* occurred in both damaged and undamaged skin from the control fish and the damaged skin of the chronically stressed fish at 7 days relative to 12 hours (Supplementary Table S4).

No significant differences were found in the relative abundance of *star* between unstressed (control) and stressed fish at time 0. *Star* transcripts in the damaged skin of chronically stressed fish were significantly down-regulated at 3 ($p = 0.003$) and 7 ($p = 0.021$) days relative to undamaged skin from unstressed fish at time zero. Seven days after cutaneous damage, *star* was significantly ($p < 0.01$) down-regulated in the damaged skin of chronically stressed fish relative to the damaged skin of unstressed fish and significantly up-regulated in undamaged skin relative to damaged skin in the unstressed ($p < 0.05$) and chronically stressed fish ($p < 0.001$). A significant ($p = 0.016$, one-way ANOVA, Supplementary Table S4) down-regulation of *star* transcripts occurred in damaged skin of chronically stressed fish 3 days after scale removal relative to the undamaged skin of chronically stressed fish at time 0.

Pomc α1 and *pomc α2* transcripts were detected in seabream skin but were below the detection threshold for reliable quantification by qPCR and so were excluded from the analysis (data not shown).

3.4.3. Transcripts associated with wound healing

The relative abundance of *pcna*, *coliva1* and *mmp9* transcripts in undamaged and damaged skin from unstressed (control) and chronically stressed fish during the experiment was affected by time ($p < 0.01$, three-way ANOVA; Fig. 8). Both *pcna* and *coliva1* transcript abundance in undamaged and damaged skin during the experiment was also affected by stress (p

< 0.05, three-way ANOVA), and damage also significantly affected *colivα1* and *mmp9* expression ($p < 0.01$ and $p < 0.001$, respectively). Chronic stress interacted with time and affected the relative abundance of *mmp9* ($p < 0.05$), while the interaction of time with cutaneous repair significantly ($p < 0.001$) affected *pcna* transcript abundance.

At time 0, the relative abundance of *pcna* was significantly ($p < 0.05$) down-regulated in stressed fish relative to undamaged skin of unstressed (control) fish. PCNA transcripts were also significantly ($p = 0.003$) down-regulated at 12 hours after scale removal in skin of chronically stressed fish in relation to undamaged skin of unstressed (control) fish. No more significant differences were found between groups at any time point studied during the experiment. The relative abundance of *pcna* was also modified between damaged and undamaged skin irrespective of group and in damaged skin from unstressed fish a significant ($p < 0.01$ and $p < 0.001$, respectively) up-regulation of *pcna* transcripts occurred relative to the undamaged skin, 3 and 7 days after scale removal. In chronically stressed fish, *pcna* transcripts were significantly ($p < 0.01$) down-regulated at 12 hours and significantly ($p = 0.001$) up-regulated at 3 days in damaged skin relative to undamaged skin in the same fish. The relative abundance of *pcna* was significantly ($p < 0.01$) up-regulated at 3 and 7 days relative to 12 hours in the damaged skin of both unstressed and chronically stressed fish (Supplementary Table S5).

The *colivα1* in the undamaged skin of chronically stressed fish was significantly ($p < 0.05$) down-regulated in relation to control fish at time 0. The relative abundance of *colivα1* was not significantly modified between damaged or undamaged skin irrespective of the group (unstressed or chronically stressed fish) during the experiment. An exception occurred at 3 days when *colivα1* transcripts were significantly ($p < 0.05$) down-regulated in damaged skin of stressed fish relative to undamaged skin of the same fish. A significant ($p < 0.05$) up-regulation of *colivα1* occurred in the undamaged skin of chronically stressed fish at 3 days in relation to time 0 (Supplementary Table S5).

Mmp9 was significantly ($p < 0.05$) down-regulated in the undamaged skin of chronically stressed fish relative to undamaged skin of unstressed (control) fish at time 0. Transcripts for *mmp9* were significantly ($p < 0.05$) down-regulated in the undamaged skin of both stressed and unstressed (control) fish at 12 hours relative to the undamaged skin of control fish at time 0. A significant ($p < 0.01$) down-regulation of *mmp9* occurred at 12 hours in undamaged skin of chronically stressed fish relative to damaged skin. Seven days after scale removal the undamaged skin of both unstressed (control) and stressed fish had a significant ($p < 0.05$) down-regulation of *mmp9* relative to damaged skin. Time significantly ($p < 0.05$, Supplementary Table S5) modified the relative abundance of *mmp9* and in damaged skin of both stressed and unstressed (control) fish it was significantly ($p < 0.05$) up-regulated at 3 and 7 days relative to time 0.

3.4.4. Transcripts associated with immune response

The relative abundance of *tgfβ1*, *csf-1r* and *crtac2* transcripts in undamaged and damaged skin from control and chronically stressed fish during the experiment was affected by time, stress and cutaneous repair ($p < 0.01$). *Mpo* transcripts were also affected by time and cutaneous repair ($p < 0.001$) and by the interaction of time and cutaneous repair ($p < 0.01$) and the same was true for *csf-1r* ($p = 0.02$; Fig. 9).

Mpo transcripts were significantly ($p < 0.001$ and $p < 0.05$, respectively) down-regulated in the damaged skin of both unstressed (control) and chronically stressed fish at 12 hours relative to undamaged skin of control fish at time 0. Damaged skin of unstressed fish exhibited a significant ($p < 0.05$) down-regulation of *mpo* transcripts relative to undamaged skin at 12 hours and 3 days. The damaged skin of stressed fish also had a significant ($p < 0.05$) down-regulation of *mpo* relative to undamaged skin at 3 days. Time after scale removal had no impact on the relative abundance of *mpo* irrespective of the experimental group with the exception of the damaged skin of control fish, in which it was significantly ($p < 0.01$) down-regulated at 12 hours relative to the other time points (Supplementary Table S6).

Csf-1r was significantly ($p < 0.01$) down-regulated in undamaged skin of chronically stressed fish at time 0. Significant ($p < 0.001$) down-regulated of *csf-1r* occurred in damaged skin of both control and chronically stressed fish at 12 hours and significant ($p < 0.05$) up-regulation occurred in undamaged skin of control fish at 7 days, relative to undamaged skin of control fish at time 0. A significant ($p < 0.05$) up-regulation of *csf-1r* was found in the undamaged skin of control fish relative to undamaged skin of chronically stressed fish at 7 days. At 12 hours *csf-1r*, in the damaged skin of both control and chronically stressed fish was significantly ($p < 0.05$) down-regulated relative to the undamaged skin from the same fish. A significant up-regulation ($p < 0.05$) of *csf-1r* occurred in the undamaged skin relative to damaged skin of stressed fish at 3 days. A significant ($p < 0.05$) up-regulation was also observed in the damaged skin of both control and chronically stressed fish at 3 and 7 days relative to 12 hours after scale removal and a significant ($p < 0.05$) up-regulation was observed in the undamaged skin of both control and stressed fish at 7 days relative to time 0 (Supplementary Table S6).

The relative abundance of *crtac2* was significantly ($p < 0.001$) down-regulated in the skin of chronically stressed fish throughout the experiment in relation to undamaged skin of unstressed fish at time 0. The exception was 7 days when no significant differences in *crtac2* transcript abundance occurred between the undamaged skin of chronically stressed fish and undamaged skin of control fish. A significant ($p < 0.05$) down-regulation of *crtac2* transcripts occurred in damaged skin of control fish at 12 hours relative to time 0 and a significant ($p < 0.05$ and $p < 0.01$, respectively) up-regulation of *crtac2* occurred in both damaged and undamaged skin of control fish at day 7 relative to time 0. The skin of chronically stressed fish exhibited a significant ($p < 0.001$) down-regulation of *crtac2* in relation to the skin of control fish irrespective of skin damage. The relative abundance of *crtac2* was significantly ($p < 0.05$) up-regulated at 3 and 7 days relative to 12 hours irrespective of the group and skin damage (Supplementary Table S6).

Tgfβ1 was significantly ($p < 0.01$ and $p < 0.05$, respectively) down-regulated in both undamaged skin (time 0) and damaged skin of chronically stressed fish at 12 hours relative to undamaged skin of control fish (time 0). The relative abundance of *tgfβ1* was significantly ($p <$

0.05) up-regulated in the skin of chronically stressed fish at 3 days relative to 0 and 12 hours irrespective of skin treatment (Supplementary Table S6).

4. Discussion

The present study demonstrates for the first time that chronic stress impairs cutaneous repair in gilthead seabream. The impairment in repair is likely due to the immunosuppression elicited by chronic stress and also modulation of other transcripts associated with cutaneous repair. This study also reveals for the first time that elements of a cutaneous stress axis exists in gilthead seabream and that modulation of *gr* transcripts in the skin seems to be linked to the down-regulation of immune transcripts such as *tgfβ1*, *csf-1r* and *mpo* but also to the down-regulation of markers linked to cell proliferation, such as *pcna*. The appearance of many hyperpigmented masses in the epidermis during cutaneous wound healing irrespective of the stress status may indicate that they have a role in skin repair in fish. However, the function in cutaneous repair and exact identity of these highly pigmented masses was not established. A recently identified gene in fish that stimulates epithelial cell outgrowth, *crtac2* (Anjos et al., 2013), may be a promising skin biomarker of chronic stress, since its expression was down-regulated in both undamaged and damaged skin of chronically stressed fish. Furthermore, although chronic stress had a deleterious effect on the inflammatory response during cutaneous repair, this did not interfere with the reestablishment of the barrier as no major histological modifications were identified in repaired skin of the chronically stressed and unstressed fish. In previous studies on mammals an impaired inflammatory response was compensated by reduced cell death and increased phagocytic capacity by stimulating phagocytic behaviour by cells such as platelets and fibroblasts (Martin et al., 2003; Stojadinovic et al., 2007).

4.1. Chronic stress and wound healing impact on plasma parameters

The results of the present study revealed that plasma cortisol in the chronically stressed seabream was similar to that previously reported in gilthead seabream exposed to chronic stress of crowding (Tort et al., 2011). Although plasma cortisol levels of control fish were significantly higher than those of chronically stressed fish at 0 hours, the levels of cortisol were similar to the resting levels (10 $ng.mL^{-1}$) observed in other teleost fish, including other sparids (Tort et al., 2011). High individual variability was observed in plasma levels of cortisol in control fish at 12 hours and 3 days after scale removal as previously reported (Martins et al., 2006; Schreck et al., 2001). Although sampling has been shown to act as an acute stress (Castillo et al., 2008; Rotllant et al., 2001), this may not explain the increase in cortisol observed in our study. An increase in cortisol levels has previously been reported in other fish that were subjected to scale removal from 10-50% of the body surface (Gadomski et al., 1994; Olsen et al., 2012; Zydlewski et al., 2010).

In the present study the combined effect of chronic stress and removal of scales from the left flank presumably caused the observed decrease in plasma osmolality in the chronically stressed fish at 3 days. The loss of scales has been associated with osmotic dysfunction in other teleosts (Black & Tredwell, 1967; Olsen et al., 2012; Smith, 1993). It is well-established that skin as an osmoregulatory function in teleosts (Marshall & Grosell, 2006) and disruption of the barrier caused by the loss of scales has been associated with osmotic dysfunction in teleosts (Black & Tredwell, 1967; Olsen et al., 2012; Smith, 1993). Mucous cells are known to play an important role in the osmoregulatory activity of skin (Shephard, 1994) but since mucous cell number was similar between experimental groups this is unlikely to explain the difference in plasma osmolality (Fig. 5).

4.2. Cutaneous barrier is reestablished in chronically stressed fish without major histological modifications

Cutaneous repair following scale removal involved morphological modifications similar to what was reported in gilthead sea bream (Costa et al., 2017; Vieira et al., 2011), sea bass (Guerreiro et al., 2013) and other teleosts (Ogawa et al., 2010; Ohira et al., 2007; Quilhac & Sire,

1999). No major histological and histomorphometric modifications were seen between control and chronically stressed fish in both regenerated and undamaged skin. The exception was the basement membrane of damaged and undamaged skin of chronically stressed fish which was thicker than the basement membrane of damaged and undamaged skin of control fish at 3 days. The present results contrast with those of human bronchial biopsies treated with corticosteroids in which the thickness of the basement membrane was reduced (Hoshino et al., 1998). In addition, a thinner hypodermis layer was observed in damaged skin of chronically stressed fish relative to control fish at 3 days after scale removal. This may be linked to the suppression of rate-limiting enzymes of fatty acids synthesis pathway by glucocorticoids (Stojadinovic et al., 2007). Although no significant difference was detected in the thickness of the re-established epidermis between control and chronically stressed fish in the present study, reduced re-epithelialization has previously been associated with impaired cutaneous wound healing in chronically stressed mice (Padgett et al., 1998; Romana-Souza et al., 2010) and in human skin treated with glucocorticoids (Lee et al., 2005).

The changes in mucous cell number in undamaged skin of both unstressed and stressed gilthead sea bream was similar to that previously reported in carp maintained in water contaminated with organic fertilizer or acidified (Iger et al., 1988; Iger & Bonga, 1994) and in sea bass exposed to elevated levels of ammonia (Kalogianni et al., 2011). Chronic stress caused a change in mucous cell recruitment in undamaged skin relative to undamaged control skin 3 and 7 days after scale removal from the other flank. Furthermore, the variability in mucous cell number in damaged skin relative to undamaged skin was higher in chronically stressed fish relative to the unstressed fish. Further studies will be required to establish the underlying mechanisms responsible for this change.

Collection of damaged and undamaged skin from the same fish allowed the direct comparison between regenerating and control skin in order to investigate if any histomorphometric modification was only local or a systemic effect of having 50% of disrupted skin in one flank. Our results suggest that cutaneous repair of 50% of one damaged flank may induce to systemic rearrangements on skin in order to compensate for the transient loss of the

cutaneous barrier, as histomorphometric modifications in epidermis and basement membrane thicknesses were observed across cutaneous repair in the undamaged skin of control fish. However, these results are not supported by those of Costa et al. (2017) in which epidermis and basement membrane thicknesses remained constant throughout the repair process in gilthead sea bream. Nonetheless, they reported systemic modifications in the number of blood vessels during cutaneous repair.

4.3. Cutaneous wound healing induces the appearance of highly pigmented agglomerations in the epidermis

One interesting finding in the present study was the appearance of many highly pigmented masses of low to moderate size in the epidermis of the repaired skin in both control and chronically stressed fish. These pigmented masses resembled melanomacrophage centers (MMC), which are aggregates of pigmented macrophages normally seen in the stroma of haemopoietic tissue of spleen, head kidney and liver, which constitute the extracutaneous pigmentary system (Agius & Roberts, 2003). The occurrence of hyperpigmentation at wound sites has previously been described in zebrafish (*Danio rerio*, Lévesque et al., 2013), rohu (*Labeo rohita*, Rai et al., 2012), rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*, Schmidt et al., 2013) and has been linked to the role of MMCs in immunity (Blazer et al., 1997).

4.4. Gilthead seabream expresses elements of the stress axis in skin, which are modulated during cutaneous wound healing

The existence of a cutaneous stress axis in fish has not previously been explored. Expression of transcripts normally associated with the central HPA axis such as, *star*, *pomc α1* and *pomc α2*, in gilthead seabream skin in previous and the present study (Cardoso et al., 2011; Stocco, 2000) suggests the presence of a cutaneous stress axis in fish. This suggestion is supported by a recent microarray study in which genes involved in steroid metabolism, particularly those linked with cortisol production, were up-regulated in Atlantic salmon with damaged skin due to a sea lice infestation (Krasnov et al., 2012). Furthermore, *pomc* expression, associated with the

pigmentary system, was also reported in the skin of barfin flounder (*Verasper moseri*, Takahashi et al., 2005), channel catfish (*Ictalurus punctatus*, Karsi et al., 2004) and African lungfish (*Protopterus annectens*, Masini et al., 1999), although levels of expression were very low.

The present results not only demonstrate the existence of elements of stress axis in fish skin, but also indicate that chronic stress can modulate the expression of cutaneous *star*, the rate-limiting enzyme in the synthesis of cortisol (Stocco, 2000) and also *gr* transcripts that mediate the effects of cortisol (Bury et al., 2003; Cruz et al., 2013), and that this is associated with impairment of cutaneous repair. Although in the present study we did not analyze other steroidogenic enzymes the results obtained for *star* in the present study are in agreement with those of the microarray study of Atlantic salmon chronically stressed by sea lice infestations (Krasnov et al., 2012). Previous studies on skin in chronically stressed mice (Nabors & Berliner, 1969; Tiganescu et al., 2014) and human skin models (Vukelic et al., 2011) revealed that cutaneous cortisol synthesis/biotransformation and local activation of steroidogenic enzymes are required to accelerate wound healing.

The absence of major differences in the morphology of damaged skin from control and chronically stressed gilthead seabream during wound healing in the present study is puzzling since expression of steroidogenic enzymes in skin are known to modulate the effects of stress on mammalian skin morphology (Morgan et al., 2014; Tiganescu et al., 2013). The apparently different response to damage of fish and mammalian skin may be linked to differences in the extent of damage, methods of detection and the multiple molecular factors that interplay during wound healing (Lee et al., 2005; Pérez, 2011). The down-regulation of *gr* transcripts in undamaged skin of chronically stressed fish has also been observed in the skin of chronically stressed mice (Pang et al., 2014) and is linked to systemic negative feedback (Acerete et al., 2007; Berkovitz et al., 1988; Cruz et al., 2013; Shrimpton & Randall, 1994) to minimize the effects of systemic cortisol on target tissue (Frentzel, 2008).

4.5. Chronic stress impairs the expression of key genes of cutaneous wound healing

Pcna, an indicator of epidermal proliferation (Braiman-Wiksman et al., 2007), was down-regulated in the skin of chronically stressed fish 12 hours after scale removal, indicating that

chronic stress may delay cutaneous repair. Down-regulation of *pcna* and *crtac2*, during cutaneous repair in the chronically stressed fish may indicate that the negative impacts of stress occur via reduced epidermal migration and proliferation, although further studies are required to test this hypothesis.

Expression of *colivα1*, one of the major components of the basement membrane (Abreu-Velez & Howard, 2012) and *mmp9*, which plays a role in the degradation of extracellular matrix (Van den Steen et al., 2002) and also in inflammation (Chadzinska et al., 2008) were not significantly modified during wound healing in the chronically stressed seabream. Nonetheless, the expression of *colivα1* and *mmp9* transcripts was affected by prolonged exposure to stress as they were down-regulated in the undamaged skin of chronically stressed fish relative to unstressed fish at time 0. These results are in line with those of Stojadinovic et al. (2007) and Oikarinen et al. (1987) that reported decreased expression of collagen type IV, a process that may be mediated by GR signaling, in human epidermis and in human fibrosarcoma cells, respectively after treatment with dexamethasone.

4.6. Chronic stress suppresses the immune response during cutaneous wound healing

The inhibitory effects of glucocorticoids on cutaneous wound healing in mammals are linked to the suppression of inflammatory cytokines and growth factors (Dhabhar & McEwen, 1999) and to the inhibition of leukocyte recruitment to the wound area (Wahl, 1989). Down-regulation of *tgfβ1* transcripts, a cytokine with pleiotropic effects during wound healing (O'Kane & Ferguson, 1997), down-regulation of *csf-1r* transcripts, a macrophage marker in gilthead seabream (Roca et al., 2006) and down-regulation of *mpo* transcripts, a marker of leukocyte activation in gilthead seabream (Rodríguez et al., 2003) in the damaged skin of chronically stressed fish demonstrates the deleterious effects of chronic stress on the immune response during wound healing. Similar observations have been made in murine wound healing models (Frank et al., 1996; Hans-Dietmar et al., 2000) and in primary human keratinocyte cultures (Stojadinovic et al., 2007) in which dexamethasone suppressed the expression of TGFβ1 and key genes involved in leukocyte recruitment. The inhibition of macrophage recruitment suggested by the down-

regulation of *csf-1r* transcripts in the present study may explain the down-regulation of *tgfβ1* and *mpo*, since activated macrophages are an important source of these factors during wound healing (Rappolee et al., 1988; Roberts & Sporn, 1988).

5. Conclusion

The results of this study report for the first time in a teleost fish that chronic stress changes the molecular mechanisms of cutaneous repair after scale removal. Specifically, chronic stress suppresses the local inflammatory response and was associated with down-regulation of key elements of the repair process. This study also suggests that chronic stress modulates the expression of elements of the stress axis identified in skin and that this may impaircutaneous repair. Surprisingly, no major histological modifications were observed in damaged skin of chronically stressed fish relative to damaged skin in control fish and reestablishment of the cutaneous barrier was similar. The identification of many dark pigmented masses as potential MMCs in the regenerated epidermis irrespective of stress is of great interest and future research should be directed at understanding their role in cutaneous repair. This study providesinsight into he molecular interplay of the peripheral neuroendocrine-immune responses during cutaneous repair in fish exposed to chronic stress, and as such has relevance for aquaculture.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

The authors would like to thank Dr. Pedro Pousão (IPMA, Olhão) for kindly providing the gilthead seabream used in this study and also Mr. João Reis (Ramalhete Station) for animal care during the experiment. This work was supported by the European Regional Development Fund through COMPETE and the Portuguese Foundation for Science and Technology (FCT)

(CCMAR/Multi/04326/2013), (PTDC/MAR/122296/2010). JCRC is funded by an auxiliary research contract under the project UID/Multi/04326/2013 and LA by a post-doctoral fellowship (SFRH/BPD/79105/2011) from FCT, the Ministry of Science and Higher Education.

Authors' contributions

DMP conceived and planned the project. APM performed the practical work including histology, plasma analysis and molecular biology. APM, LA and JCRC performed the bioinformatics analyses. DMP and APM analyzed and interpreted the data. DMP and APM drafted the manuscript and LA and JCRC revised it critically for important intellectual content. All authors have given their final approval of the version to be published.

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Table 1: Body weight (g), total length (cm), HSI (%), calculated as 100 x (liver mass/body mass) and condition factor (K), calculated as 100 x (body weight/total length^3), of fish from control and stress groups, at 0 hours, 12 hours (0.5), 3 and 7 days after scale removal (n = 8/group).

Biometric parameters	Weight		Length		K		HSI	
Time	Control	Stress	Control	Stress	Control	Stress	Control	Stress
0	89.4 ± 7.2^{a}	89.5 ± 5.3^{a}	17.6 ± 0.9	17.1 ± 0.8^{ab}	1.7 ± 0.2	1.8 ± 0.2	3.3 ± 0.3	3.4 ± 0.3
0.5	89.4 ± 5.9^{ab}	89.8 ± 5.4^{a}	17.4 ± 0.8	16.9 ± 0.7^{a}	1.7 ± 0.2	1.9 ± 0.2	3.4 ± 0.5	3.3 ± 0.5
3	95.4 ± 9.3^{ab}	98.3 ± 5.3^{b}	17.5 ± 0.5	18.0 ± 0.8^{b}	1.7 ± 0.2	1.7 ± 0.2	3.5 ± 0.3	3.5 ± 0.4
7	99.0 ± 4.6^{b}	94.6 ± 6.8^{ab}	18.1 ± 0.4	17.3 ± 0.6**^{ab}	1.7 ± 0.1	1.8 ± 0.2	3.6 ± 0.4	3.5 ± 0.5

Different letters indicate significant differences between time points. Asterisks denote significant difference between control and stress groups within same time point: ** p = 0.01. These significant differences in growth parameters can be attributed to bias in the fish size distribution during sampling. The results are shown as mean ± SD; Two-way ANOVA; p < 0.05.

Table 2: List of primers used for gene expression analysis by quantitative RT-PCR. Gene name, accession number, primer sequence, amplicon length (bp), annealing temperature (Ta, ºC), qPCR efficiency (%) and R^2 are indicated for each primer pair (F = forward and R = reverse primer).

Gene	Accession No.	Sequence (5´ to 3´)	Amplicon (bp)	Ta (ºC)	Efficiency (%)	R^2
*colivα1**	isotig36976	F: GGGCTGAACTTCCGTAGAC R: ATTCCGCAGTGTCCTGATG	128	60	100	0.999
crtac2	(Redruello et al., 2010)	F: TCTTCACCTATTCGCACAGTCATCG R: GCATCGGCTCTCCTCAACACC	131	58	98	0.987
csf-1r	AM050293	F: ACGTCTGGTCCTATGGCATC R: AGTCTGGTTGGGACATCTGG	129	62	96	0.996
gr	DQ486890	F: CCATCACCTCTGCCGCATCTG R: TCTGGAGGAACTGCTGCTGAACC	195	64	100	0.995
mmp9	AM905938	F: ATTCAGAAGGTGGAGGGAAGCG R: CATTGGGGACACCACCGAAGA	151	60	96	0.997
*mpo***	AM957359 FP335321 FM148574	F: TTGGTCCAGACATCCTCG R: ATGGGCAAAGCGGTAG	110	62	95	0.996
pcna	KF857335	F: GAGCAGCTGGGTATTCCAGA R: CTGTGGCGGAGAACTTGACT	148	60	97	0.995
star	EF640987	F: ACATCGGGAAGGTGTTCAAG R: TCTCTGCAGACACCTCATGG	177	62	99	0.998
tgfβ1	AF424703	F: TCAGAACTGGCTCAAAGGGAAC R: ACGGTGGTTGCTTCGTCATA	167	60	90	0.998
rps18	AM490061	F: AGGGTGTTGGCAGACGTTAC R: CTTCTGCCTGTTGAGGAACC	164	60	99	0.993
β-actin	X89920	F: CCCTGCCCCACGCCATCC R: TCTCGGCTGTGGTGGTGAAGG	94	60	97	0.998

* Obtained from database assembly for gilthead seabream (Louro et al., 2016).

** Deduced from the seabream ESTs AM957359, FP335321 and FM148574.

Table 3: Histomorphometric assessment of hyperpigmented agglomerations in epidermis. The presence of dark pigment masses in the epidermis was evaluated by semi-quantitative analysis using the following scale: 0 - no pigment in the epidermis), 1 – low agglomeration, 2 – moderate to high agglomeration. Cardinal (#) indicates significant difference between undamaged skin of control fish and undamaged skin of chronically stressed fish. Asterisks indicate significant differences between damaged and undamaged skin: * $p < 0.05$, *** $p < 0.001$. Different letters were used to identify significant differences between time points. The results are shown as mean ± SEM; Three-way ANOVA; $p < 0.05$; n = 6 individuals per group (3 slides per individual).

Time	Control Undamaged	Control Damaged	Stress Undamaged	Stress Damaged
0	0.8 ± 0.25^{a}	-	$0.1 \pm 0.12^{\#}$	-
0.5	0.6 ± 0.22^{ab}	0^{b}	0.3 ± 0.21	0^{a}
3	0.5 ± 0.22^{ab}	$1.4 \pm 0.22^{*ac}$	0.5 ± 0.27	1.3 ± 0.29^{b}
7	0^{b}	$1.6 \pm 0.16^{***c}$	0.1 ± 0.05	$1.5 \pm 0.17^{***b}$

Figures and Figure captions

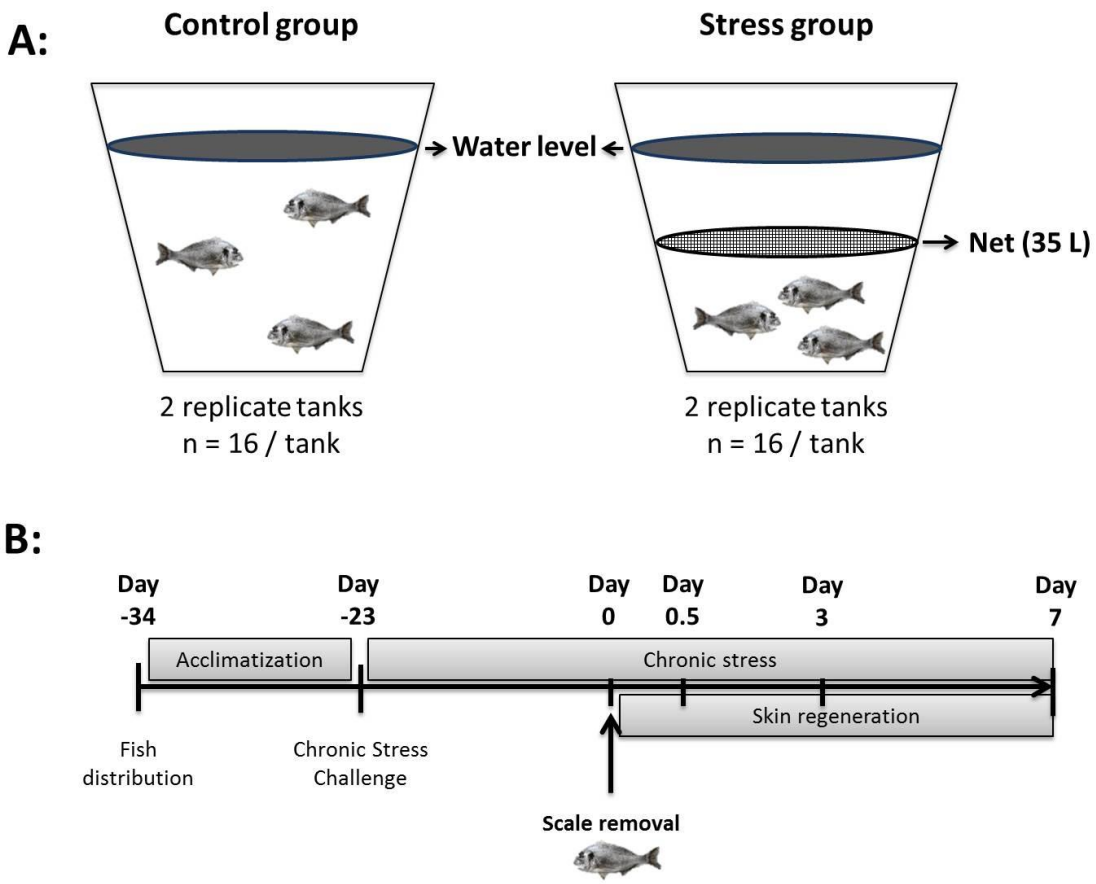


Fig. 1: Experimental set-up. A: Scheme of the methodology used to expose fish to chronic stress. Fish (n = 16/tank, 2 replicate tanks per group) were subjected to prolonged exposure to stress through crowding for 4 weeks. The level of the water was similar between control and stress groups, but stressed fish were confined to a smaller volume of water by a net placed to limit the available tank volume for fish to 35 L. Fish from the control group were maintained at a density of 16 $kg.m^{-3}$ and fish from stress group were subjected to a stressful density of 46 $kg.m^{-3}$. B: Scheme of the skin damage challenge. Fish (n = 4/tank) from control and stress groups were sampled 23 days after the onset of the chronic stress (time 0). The remaining fish were subjected to scale removal from the left side of the body and this was taken as the start point of the skin repair. Fish (n = 4/tank/sampling) were then sampled at 0.5 (12 hours), 3 and 7 days after scale removal. Fish from the chronically stressed group were continuously exposed to the crowding stress during cutaneous repair.

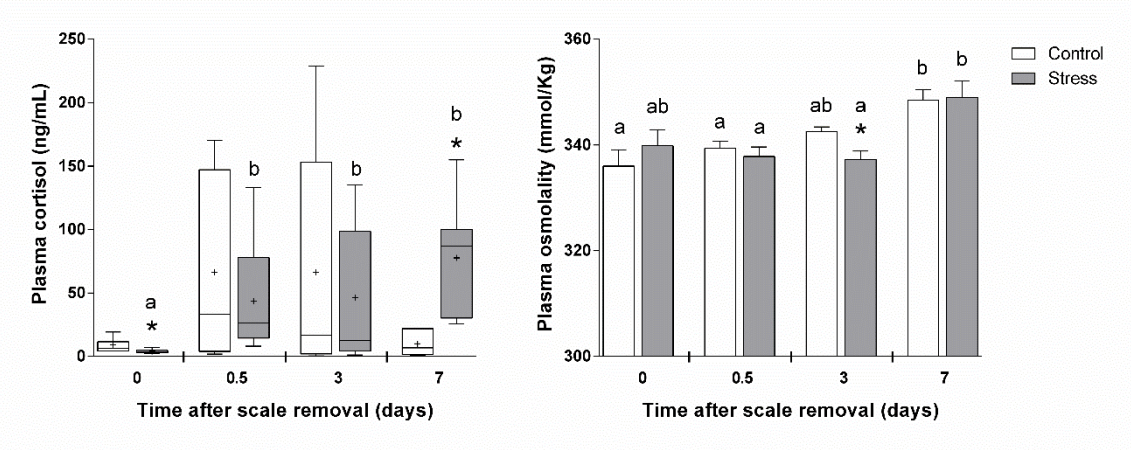


Fig. 2: Changes in plasma levels of cortisol and osmolality. Plasma osmolality of control and stress groups at the different sampling times during the skin damage challenge (0, 0.5, 3 and 7 days after scale removal) are represented as mean ± SEM and plasma cortisol as mean ± SEM in a Tukey box and whiskers plot. '+' represents the mean and asterisk (*) indicates significant difference between control and stress groups at each time point. Different numbers denote significant differences between different time points, within same group. Two-way ANOVA; $p < 0.05$; $n = 8$ per group.

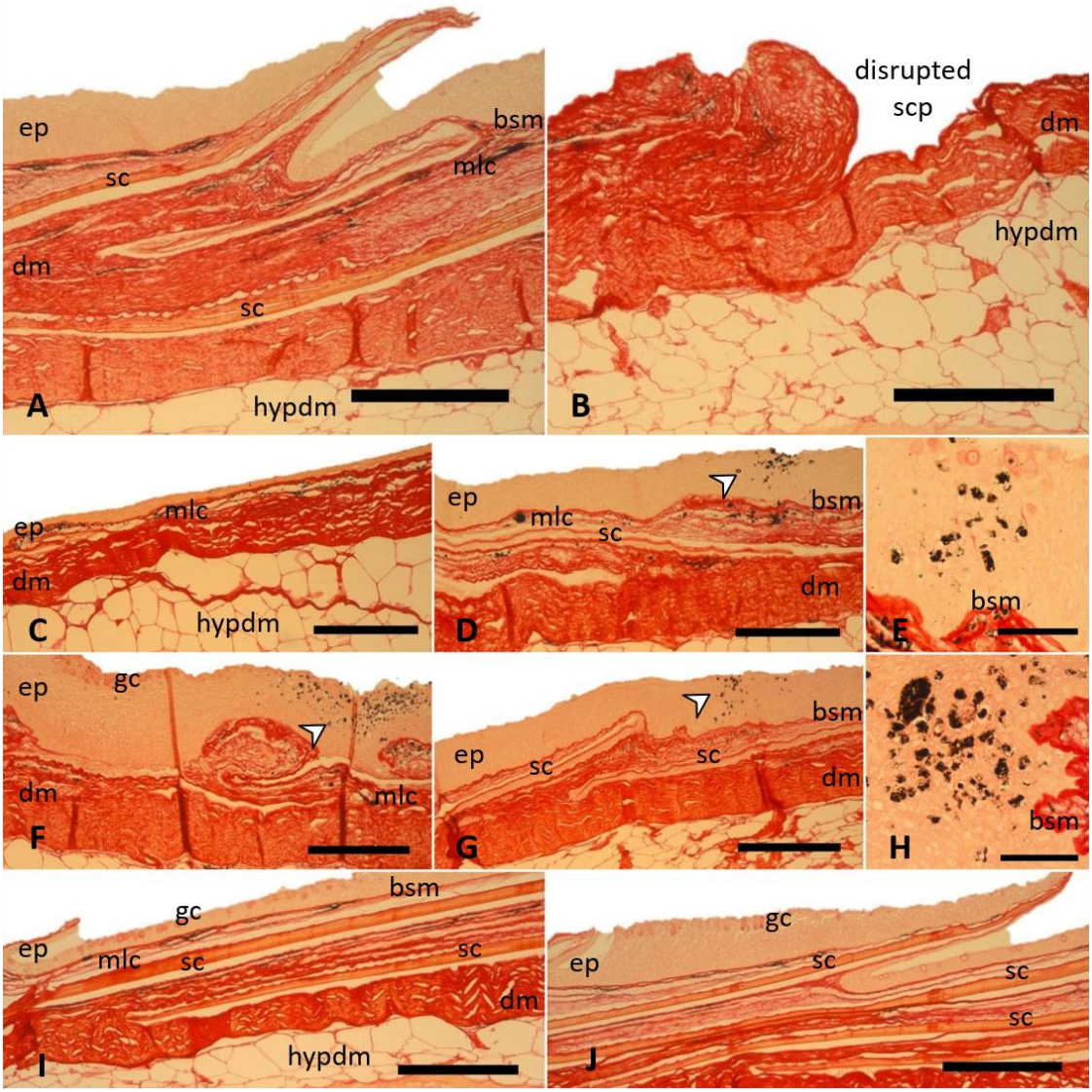


Fig. 3: Transverse histological sections (5 µm) of skin from all experimental time points. The typical organization of seabream skin, stained with Picro-Sirus Red, was evident at 0 hours (A) and it was composed of a thick epidermis (ep) overlying a compact dermis (dm) with the underlying adipose tissue of the hypodermis (hypdm). The scale (sc) was enclosed in a scale pocket (scp) with the posterior region of the scale projected into the epidermis and in the image it is orientated to the right. Scale removal caused the loss of the epidermis and the disruption of the scale pocket (B), leaving the underlying dermis exposed to the external environment at 12 hours. Within 3 days, the epidermis was re-established and the scale pocket was formed in both control and chronically stressed fish skin (C and F, respectively), although a thin newly formed scale was seen within the scale pocket in both control and chronically stressed fish skin only at 7

days (D and G, respectively). Comparison of the damaged skin (C, D, F and G) and undamaged skin at 3 and 7 days (I and J, respectively) revealed that the damaged skin contained many small highly pigmented masses in the epidermis in both unstressed and chronically stressed fish (white arrowhead). These highly pigmented masses could be seen as a small/low agglomeration (E) or as a large/high agglomeration of melanin containing cells (H). Goblet cells – gb; melanocytes – mlc; basement membrane – bsm. Scale bars: A,B,C,D,F,G,I, J - 100 μm; E, H - 50 μm.

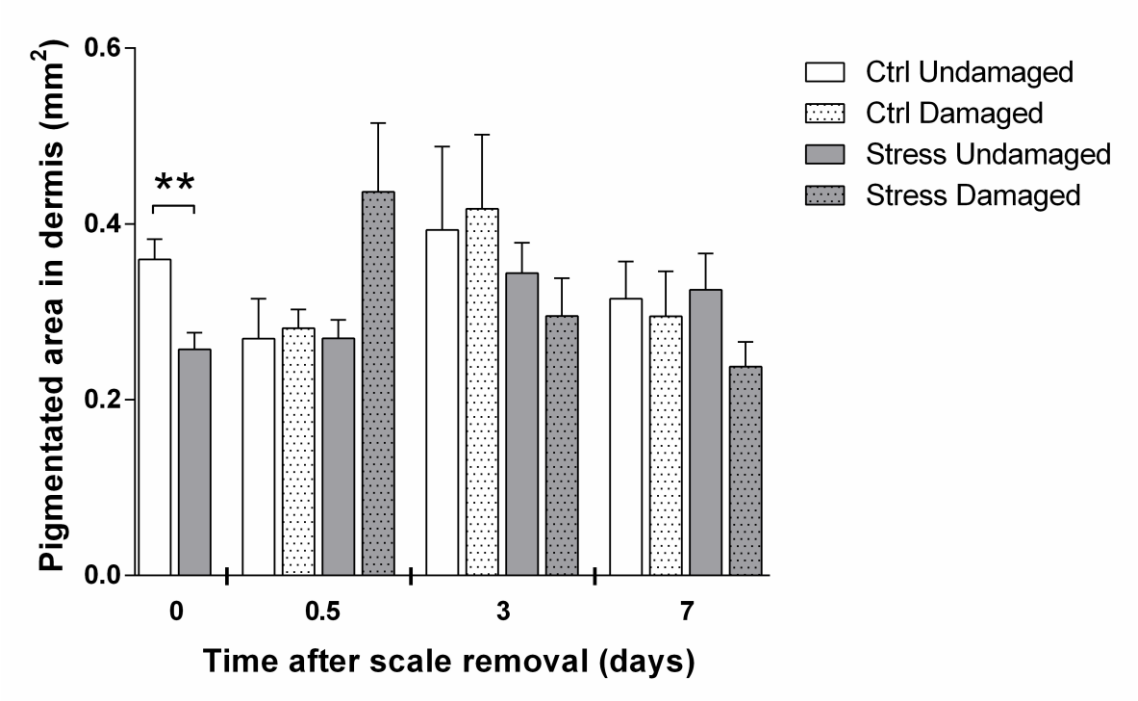


Fig. 4: Histomorphometric analysis of the dark pigmentation infiltration in dermis. Area of dermis occupied by dark pigmentation was measured in two contiguous and non-overlapping images corresponding to a total length of 2 mm (total area of 2.82 mm^2) at 100x magnification. Control undamaged skin, control damaged skin, stress undamaged skin and stress damaged skin and the different sampling times during cutaneous repair (0, 0.5, 3 and 7 days after scale removal) are represented. Asterisks indicate significant differences: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The results are shown as mean ± SEM; Three-way ANOVA; $p < 0.05$; n = 6 individuals per group (3 slides per individual).

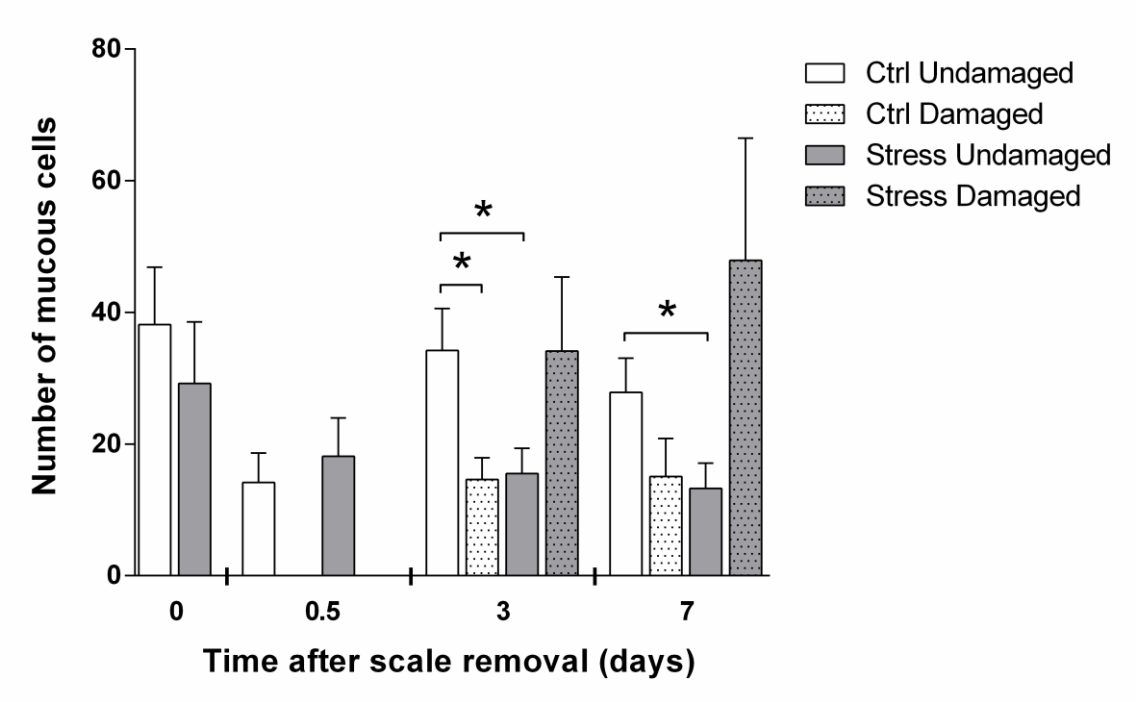


Fig. 5: Histomorphometric analysis of the number of mucous cells in the epidermis. The number of mucous cells was measured at 100x magnification of two contiguous and non-overlapping images with a total length of 2 mm (total area of 2.82 mm^2). Control undamaged skin, control damaged skin, stress undamaged skin and stress damaged skin and the different sampling times during cutaneous repair (0, 0.5, 3 and 7 days after scale removal) are represented. Asterisk (* $p < 0.05$) indicates significant differences between the control and chronically stressed fish at each time point. The results are shown as mean ± SEM; Three-way ANOVA; $p < 0.05$; $n = 6$ individuals per group (3 slides per individual).

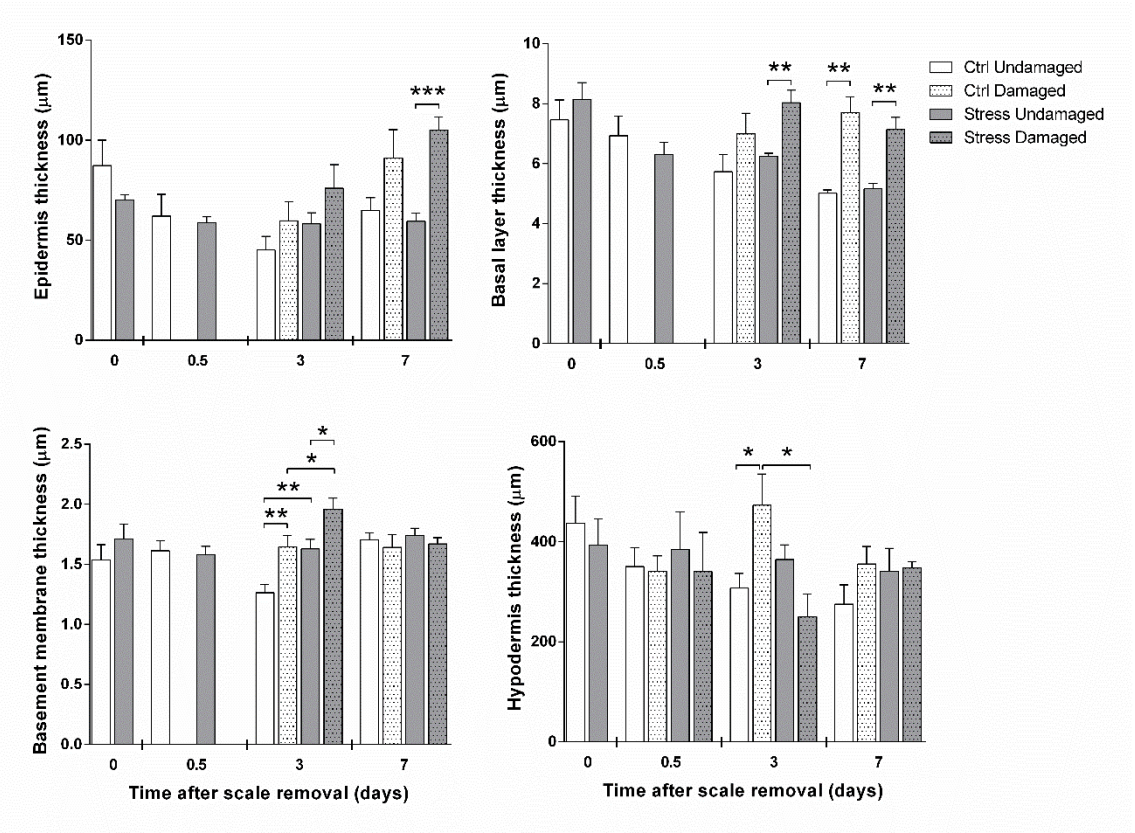


Fig. 6: Histomorphometric analysis of the thickness of the various skin layers. Analysis of epidermis, basal layer and basement membrane thickness was performed in 3 different zones of each section (n = 3/fish) at 400x magnification. The measurement of the hypodermis thickness was performed using imaged at 100x magnification of two different fields of each section. Control undamaged skin, control damaged skin, stress undamaged skin and stress damaged skin and the different sampling times during cutaneous repair (0, 0.5, 3 and 7 days after scale removal) are represented. Asterisks indicate significant differences: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The results are shown as mean ± SEM; Three-way ANOVA; $p < 0.05$ n = 6 individuals per group (3 slides per individual).

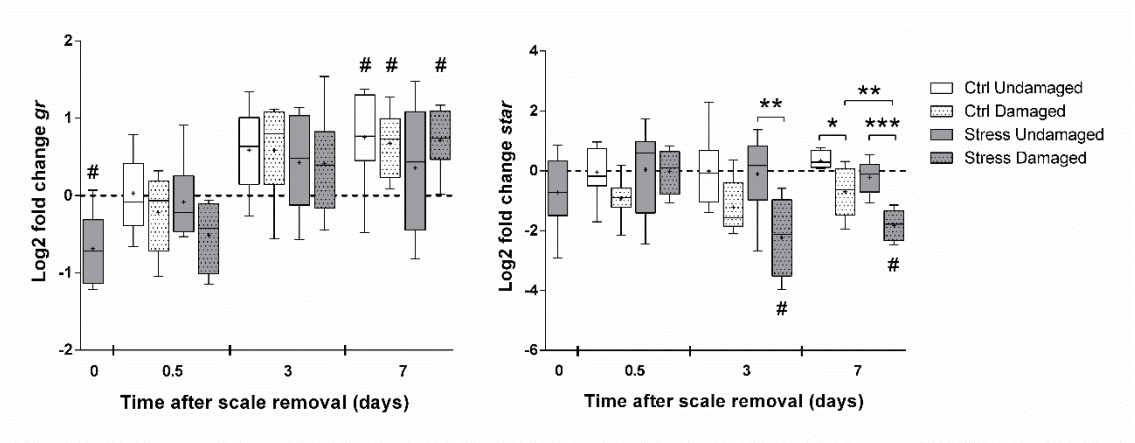
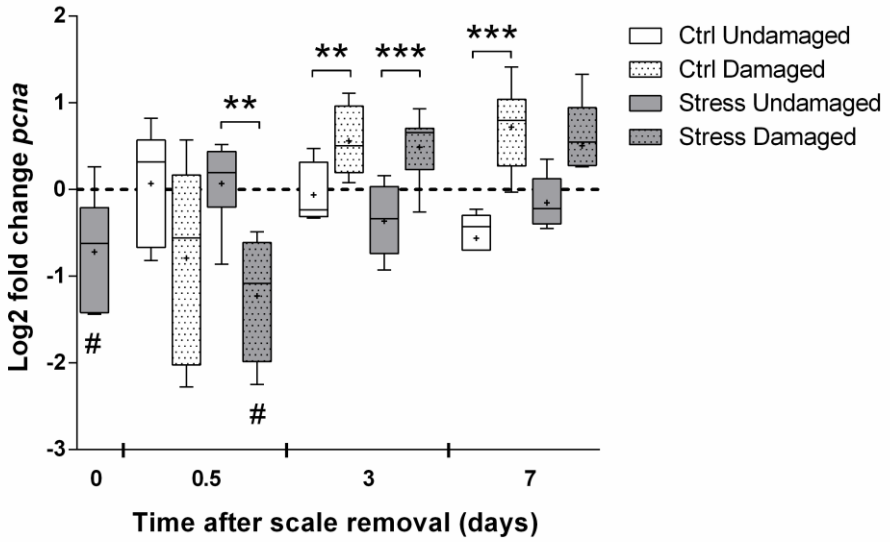
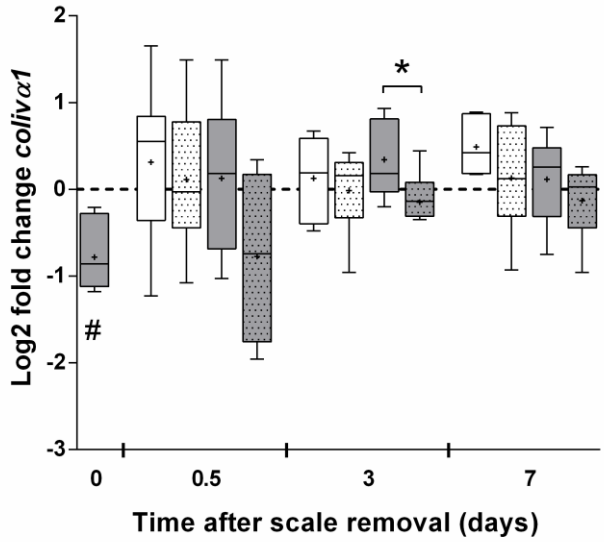


Fig. 7: Overall response of stress related transcripts in the skin to chronic stress and wound healing. Transcript abundance of *star* and *gr* at 0, 0.5, 3 and 7 days after scale removal were determined by qPCR and results were normalized using *rps18* transcript abundance. The normalized results are expressed as Log2 Fold Change and were calculated relative to the undamaged skin of control fish at time 0. Control undamaged skin, control damaged skin, stress undamaged skin and stress damaged skin are plotted in Tukey box and whiskers graphs and '+' represents the mean. Significant up- or down-regulation relative to the undamaged skin of control fish at time 0 (control group) is denoted by cardinal (#). Significant differences between groups at the same time point are indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Three-Way ANOVA; n = 8 per group.


Ctrl Undamaged
Ctrl Damaged
Stress Undamaged
Stress Damaged
Log2 fold change pcna
Time after scale removal (days)
0
0.5
3
7
**
**
***
***
#
#


Log2 fold change colivα1
Time after scale removal (days)
0
0.5
3
7
*
#

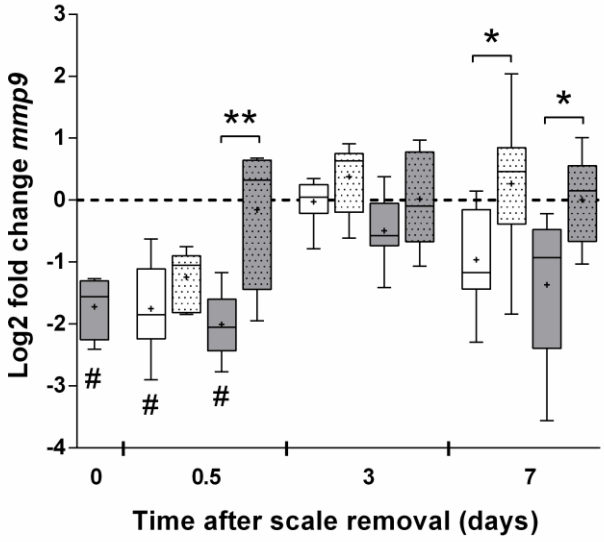

Log2 fold change mmp9
Time after scale removal (days)
0
0.5
3
7
**
*
*
#
#
#

Fig. 8: Assessment of transcripts associated with cutaneous wound healing. Transcript abundance of *colivα1*, *pcna* and *mmp9* at 0, 0.5, 3 and 7 days after scale removal were determined by qPCR and the results were normalized using *rps18* transcript abundance. The normalized results are expressed as Log2 Fold Change and are calculated relative to the undamaged skin of control fish at time 0. Control undamaged skin, control damaged skin, stress undamaged skin and stress damaged skin are plotted in Tukey box and whiskers graphs and '+' represents the mean. Significant up- or down-regulation relative to the undamaged skin of control fish at time 0 (control group) is denoted by cardinal (#). Significant differences between groups of the same time are indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Three-Way ANOVA; n = 8 per group.

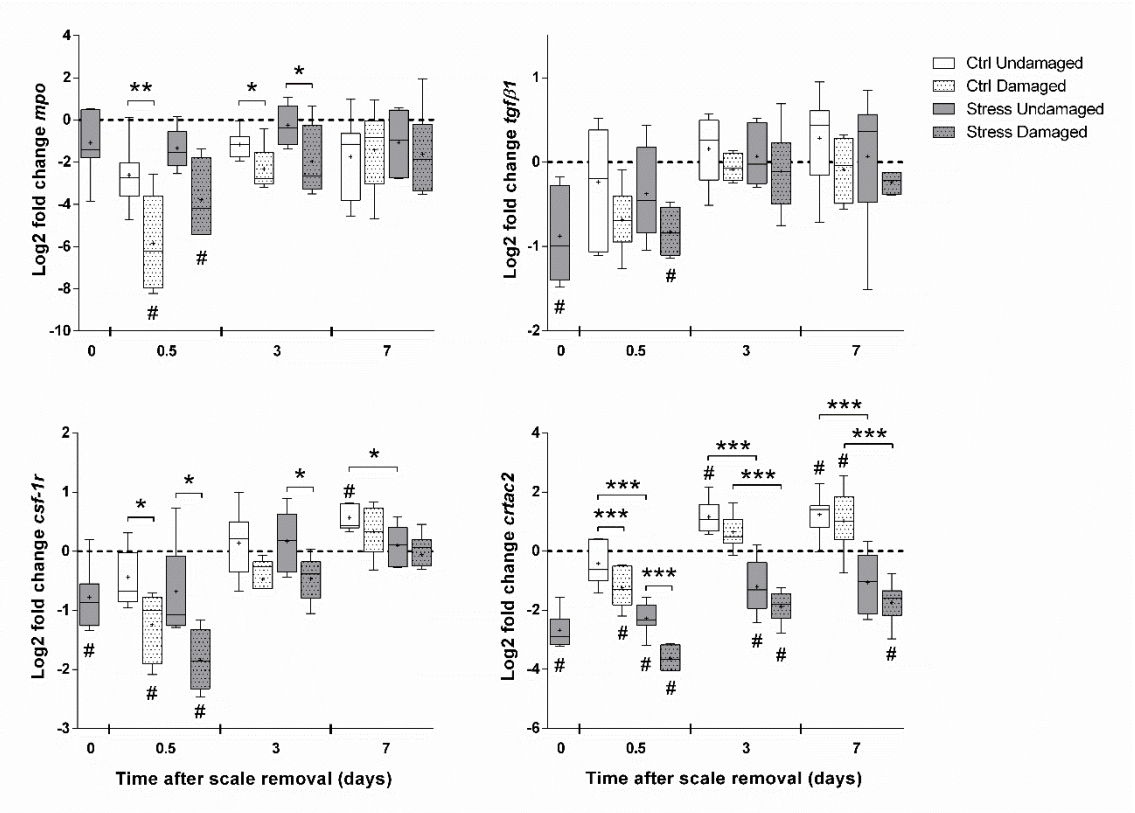


Fig. 9: Assessment of transcripts indicative of the overall immune response during cutaneous wound healing. Transcript abundance of *mpo*, *csf-1r*, *crtac2* and *tgfβ1* at 0, 0.5, 3 and 7 days after scale removal were determined by qPCR and the results were normalized using *rps18* transcript abundance. The normalized results are expressed as Log2 Fold Change and are calculated relative to the undamaged skin of control fish at time 0. Control undamaged skin, control damaged skin,

stress undamaged skin and stress damaged skin are plotted in Tukey box and whiskers graphs and '+' represents the mean. Significant up- or down-regulation relative to the undamaged skin of control fish at time 0 (control group) is denoted by cardinal (#). Significant differences between groups at the same time point are indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Three-Way ANOVA; $n = 8$ per group.

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116 ## Supplementary Tables

117 **Supplementary Table S1:** Accession numbers of the sequences used for phylogenic analysis.

Specie	Collagen IVα1 ID
Homo sapiens (Human)	NP_001836
Mus musculus (Mouse)	NP_034061
Bos taurus (Cow)	NP_001159983
Gallus gallus (Chicken)	NP_001155871
Xenopus tropicalis (Xenopus)	XP_002933064
Latimeria chalumnae (Coelacanth)	H3B0R5-1
Callorhynchus milii (Elephant shark)	SINCAMP00000024535
Lepisosteus oculatus (Spotted gar)	W5MRD0-1
Takifugu rubripes (Fugu)	XP_003961761
Oreochromis niloticus (Nile tilapia)	XP_005457918
Sparus aurata (Seabream)	isotig36976
Oryzias latipes	BAI66290

(Medaka)	
Salmo salar	XP_014029658
(Atlantic Salmon)	
Danio rerio	XP_694040
(Zebrafish)	
Astyanax mexicanus	XP_007228052
(Cavefish)	
Branchiostoma floridae	XP_002593481
(Amphioxus)	
Ciona intestinalis	XP_002120982
(Ciona)	
Caenorhabditis elegans	NP_001022662
(Worm)	
Drosophila melanogaster	NP_477190
(Fruit fly)	

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Supplementary Table S2: The effect of time after scale removal on hyperpigmentation of dermis and on the number of mucous cells. Analysis of significant differences within each group (Control undamaged, control damaged, stress undamaged and stress damaged) at different time points of the experiment (0, 0.5, 3 and 7 days after scale removal), by One-Way ANOVA. Different letters were used to identify significant differences between time points. Tukey's post hoc test; $p < 0.05$; $n = 6$ individuals per group (3 slides per individual).

Parameter	Hyperpigmentation of Dermis				Mucous Cell Number			
Time	0	0.5	3	7	0	0.5	3	7
Control Undamaged								
Control Damaged					a	b	a	a
Stress Undamaged								
Stress Damaged	ab	a	ab	b	ab	a	ab	b

Supplementary Table S3: The effect of time after scale removal on the thickness of epidermis, dermis and hypodermis. Analysis of significant differences within each group (Control undamaged, control damaged, stress undamaged and stress damaged) at different time points of the experiment (0, 0.5, 3 and 7 days after scale removal), by One-Way ANOVA. Different letters were used to identify significant differences between time points: Tukey's post hoc test; $p < 0.05$; n = 6 individuals per group (3 slides per individual).

Parameter	Epidermis Thickness				Basal Layer Thickness				Basement Membrane Thickness			
Time	0	0.5	3	7	0	0.5	3	7	0	0.5	3	7
Control Undamaged	a	ab	b	ab	a	ab	ab	b	ab	a	b	a
Control Damaged	a	b	a	a	a	b	a	a	a	b	a	a
Stress Undamaged					a	b	b	b				
Stress Damaged	a	b	a	c	a	b	a	a	a	b	a	a

Supplementary Table S4: The effect of time after scale removal on the relative abundance of *gr* and *star* transcripts. Analysis of significant differences within each group (Control undamaged, control damaged, stress undamaged and stress damaged) at different time points of the experiment (0, 0.5, 3 and 7 days after scale removal), by One-Way ANOVA. Different letters were used to identify significant differences between time points. Tukey's post hoc test; $p < 0.05$; $n = 8$ per group.

Transcript	*gr*				*star*			
Time	0	0.5	3	7	0	0.5	3	7
Control Undamaged	a	a	ab	b				
Control Damaged	ab	a	bc	c				
Stress Undamaged	a	ab	b	b				
Stress Damaged	a	a	b	b	a	ab	b	ab

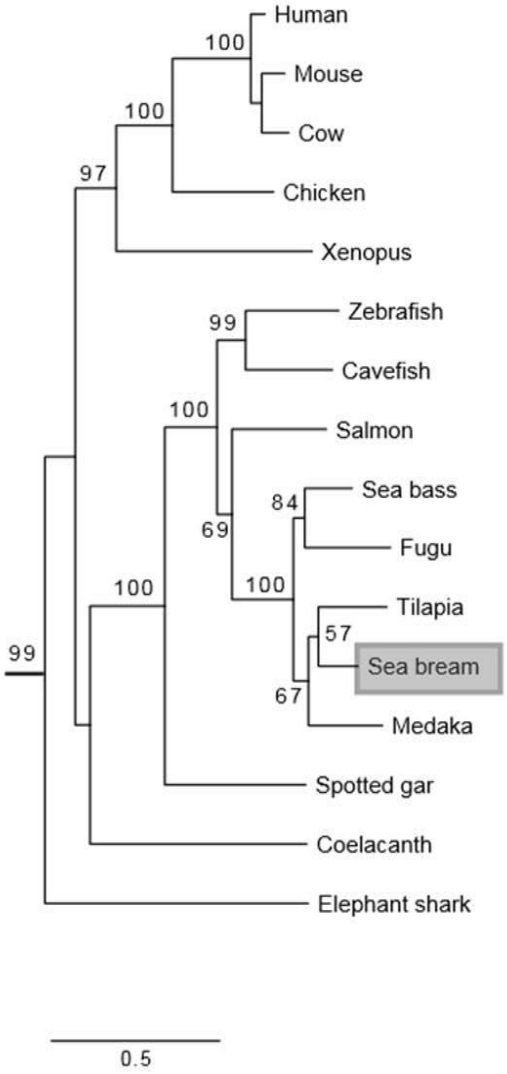
Supplementary Table S5: The effect of time after scale removal on the relative abundance of *pcna*, *colivα1* and *mmp9* transcripts. Analysis of significant differences within each group (Control undamaged, control damaged, stress undamaged and stress damaged) at different time points of the experiment (0, 0.5, 3 and 7 days after scale removal), by One-Way ANOVA. Different letters were used to identify significant differences between time points. Tukey's post hoc test; $p < 0.05$; $n = 8$ per group.

Transcript	*pcna*				*colivα1*				*mmp9*			
Time	0	0.5	3	7	0	0.5	3	7	0	0.5	3	7
Control Undamaged									a	b	a	ab
Control Damaged	ab	a	b	b					ab	a	b	b
Stress Undamaged	a	b	ab	ab	a	ab	b	ab	a	a	b	ab
Stress Damaged	a	a	b	b					a	ab	b	b

Supplementary Table S6: The effect of time after scale removal on the relative abundance of *mpo*, *csf-1r*, *crtac2* and *tgfβ1* transcripts. Analysis of significant differences within each group (Control undamaged, control damaged, stress undamaged and stress damaged) at different time points of the experiment (0, 0.5, 3 and 7 days after scale removal), by One-Way ANOVA. Different letters were used to identify significant differences between time points. Tukey's post hoc test; $p < 0.05$; $n = 8$ per group.

Transcript	*mpo*				*csf-1r*				*crtac2*				*tgfβ1*			
Time	0	0.5	3	7	0	0.5	3	7	0	0.5	3	7	0	0.5	3	7
Control Undamaged					a	a	ab	b	a	a	b	b				
Control Damaged	a	b	a	a	ac	b	a	c	a	b	ac	c				
Stress Undamaged					a	ac	b	bc	a	a	b	b	a	ab	b	b
Stress Damaged					ab	b	a	a	ac	a	bc	b	a	a	b	ab

Supplementary Figure



Supplementary Fig. S1: Phylogenetic analysis of the seabream *coliva1* with the metazoan homologues. Tree was built using the ML method and reliability of internal branching was assessed with 100 bootstrap replicates. Only branch support values higher than 50% are represented. Species names and accession numbers are available in Supplementary Table S1.