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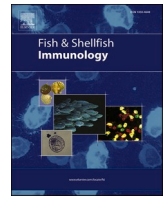
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Full length article



## Thermal imprinting during embryogenesis modifies skin repair in juvenile European sea bass (*Dicentrarchus labrax*)

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### ABSTRACT

Fish skin is a multifunctional tissue that develops during embryogenesis, a developmental stage highly susceptible to epigenetic marks. In this study, the impact of egg incubation temperature on the regeneration of a cutaneous wound caused by scale removal in juvenile European sea bass was evaluated. Sea bass eggs were incubated at 11, 13.5 and 16 °C until hatching and then were reared at a common temperature until 9 months when the skin was damaged and sampled at 0, 1 and 3 days after scale removal and compared to the intact skin from the other flank. Skin damage elicited an immediate significant ( $p < 0.001$ ) up-regulation of *pcna* in fish from eggs incubated at higher temperatures. In fish from eggs incubated at 11 °C there was a significant ( $p < 0.001$ ) up-regulation of *krt2* compared to fish from higher thermal backgrounds 1 day after skin damage. Damaged epidermis was regenerated after 3 days in all fish irrespective of the thermal background, but in fish from eggs incubated at 11 °C the epidermis was significantly ( $p < 0.01$ ) thinner compared to other groups, had less goblet cells and less melanomacrophages. The thickness of the dermis increased during regeneration of wounded skin irrespective of the thermal background and by 3 days was significantly ( $p < 0.01$ ) thicker than the dermis from the intact flank. The expression of genes for ECM remodelling (*mmp9*, *colXa*, *col1a1*, *sparc*, and *angptl2b*) and innate immunity (*lyg1*, *lalba*, *sod1*, *csf-1r* and *ppary*) changed during regeneration but were not affected by egg thermal regime. Overall, the results indicate that thermal imprinting of eggs modifies the damage-repair response in juvenile sea bass skin.

### 1. Introduction

Fish skin is a multifunctional tissue and its direct contact with the aquatic environment make it an important osmoregulatory organ and mucosal barrier of innate immunity. Other conserved functions include the role of skin in communication, sensory perception, locomotion, and thermal regulation [1,2]. The morphology of fish skin (epidermis, dermis and subcutaneous adipose tissue) is similar to skin in other vertebrates, despite their evolutionary distance and the unique specializations for their aquatic habitat [3]. The scales are specialized calcified appendages in fish that are anchored in the dermis and serve as

an ion reservoir and sustain and protect the upper layers of the skin [4]. Scales are continuously lost by natural shedding [5] or by physical damage to the skin surface [6] and in aquaculture scale loss is frequent due to the characteristics of holding facilities, manipulation and aggressive interactions between fish [7]. Loss of scales reduces the protective epithelium/mucous layers and exposes the fish internal milieu to microorganisms and can severely affect homeostasis and health [8–11]. Extensive scale loss is a fish welfare issue as it can lead to disease and even mortality if the mucosal barrier is not re-established quickly [12,13]. Rapid reestablishment of the mucosal barrier occurs through a well-orchestrated repair process composed of

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reepithelialisation, inflammation, granulation, tissue remodelling and finally scale regeneration [9,11,14,15].

One fascinating aspect of fish skin is that it integrates all 3 levels of fish immunity within the same organ; it is a physical barrier and possess elements of both innate (e.g. mucus and enzymes) and adaptive (e.g. skin-associated lymphoid tissue [SALT]) immunity [2,16,17]. It is generally accepted that the fish immune system is modulated by environmental temperature [18–20]. Similarly, reestablishment of barrier function through repair of cutaneous wounds in fish also depends on temperature [21,22]. At lower temperatures cutaneous wounds heal slowly due to the delay in reepithelialisation and the inflammatory response [23,24]. Interestingly, regenerated scales were reported to be stronger and to have improved mechanical properties in common carp maintained at higher temperatures than normal [25]. Despite evidence that exposure to suboptimal temperatures during early life stages suppresses the immunity of juvenile and adult fish [26–29], if temperature during embryogenesis can influence cutaneous wound healing in older fish has not been established. However, this is of relevance from an aquaculture management perspective and in the context of current sea water temperature warming and unpredictable marine heatwaves due to climate change [30,31]. Furthermore, fish skin is formed before gastrulation and from nonneuronal ectoderm, evolving an envelope layer (bilayered primary epidermis) that produces a primary collagenous stroma at 24 h postfertilization (hpf) and it is functional before hatch (e.g. performs osmoregulation before the appearance of gills) [32–34]. This process occurs during the critical embryonic stage when fish are more susceptible to changes in ambient water temperature since epigenetic modifications are imprinted during embryonic development [35–37], with consequences for the physiology of juvenile and adults, both in the wild and in aquaculture [38,39].

The economically important European sea bass (*Dicentrarchus labrax*,

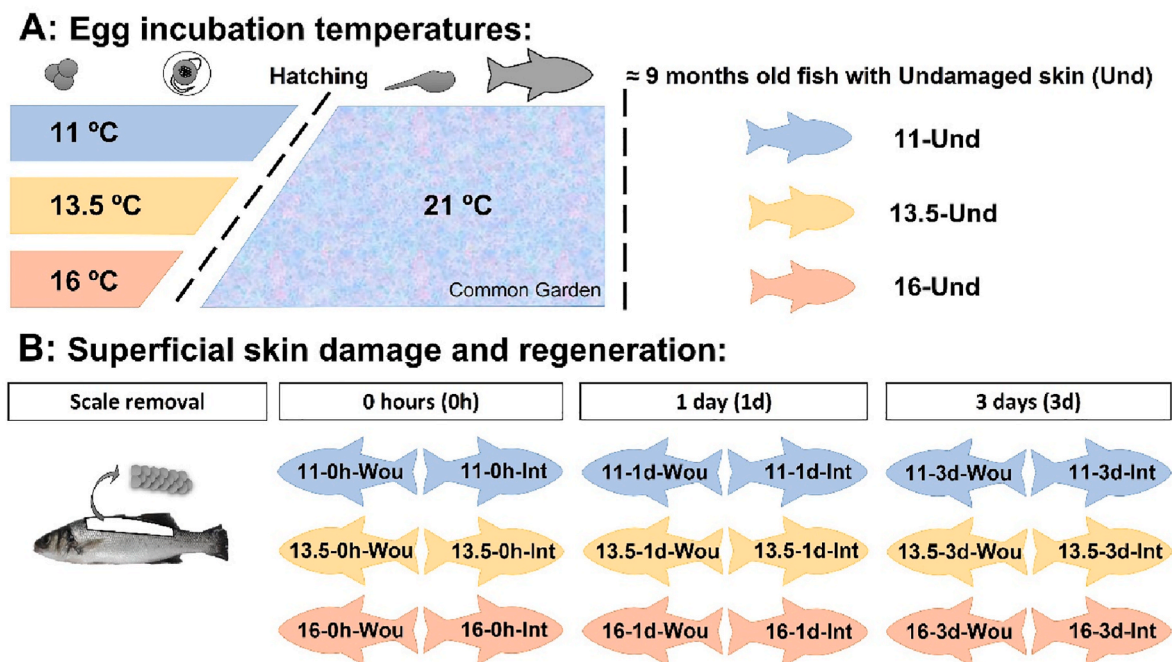
Linnaeus, 1758) and other species that live in near-shore areas, including estuaries and lagoons, are likely to experience the greatest increase in sea surface temperatures caused by climate change [31,40,41]. This is likely to affect early development of eggs and larval stages through temperature induced epigenetic marks [35–37,42]. Thus, the objective of the present study was to determine the effect of early life temperature on skin repair in juvenile European sea bass. This was accomplished by manipulating egg incubation temperatures and analysing the regeneration of a superficial cutaneous wound caused by scale removal in juveniles using histology and histomorphometry. The changes in expression of gene transcripts associated with the mucosal immune barrier and the reepithelization/remodelling response, was assessed using reverse transcription quantitative polymerase chain reaction (RT-qPCR).

## 2. Materials and methods

Rearing of European sea bass (*Dicentrarchus labrax*), as well as the skin regeneration experiment were performed at the research station of Ifremer, Palavas-Les-Flots, Montpellier, France. Experiments were authorized by the institutional ethics committee, approval APA-FIS#10745 and all procedures involving animals were carried out in accordance with the ethical standards of the institution and followed the recommendations of Directive 2010/63/EU.

### 2.1. Thermal regimes during early development

European sea bass eggs from a West Mediterranean population were obtained by combining eggs from 10 females with frozen sperm of 13 males by *in vitro* fertilization using a full factorial mating design, in October 2016. Eggs were distributed into seawater tanks at 11 °C,



**Fig. 1.** Experimental setup. A: Schematic representation of temperatures used during European sea bass development. Three different temperatures within the standard range for European sea bass egg incubation were used: 11 °C, 13.5 °C (most common egg incubation temperature used for European sea bass) and 16 °C. Eggs were maintained at these temperatures until hatching, which occurred at different times for each temperature. After hatching, larvae that came from different thermal backgrounds as eggs were included in the same tank to give a common garden design and reared under the same controlled (optimal) temperature conditions for about 9 months until the beginning of the next phase of the experiment. Nine month old fish ( $n = 8$ /thermal background) were sampled for biometric measurements, blood and skin (undamaged skin = Und fish). B: Superficial skin damage and regeneration. The remaining fish were subjected to a cutaneous wound by removing 50% of the scales from the left flank (white area) and then immediately sampled for wounded skin samples (0h-Wou; left flank,  $n = 8$ /thermal background) and intact skin samples (0h-Int; right flank,  $n = 8$ /thermal background) using the same fish. Fish were then sampled at days 1 (1d-Wou and 1d-Int groups) and 3 (3d-Wou and 3d-Int groups) to monitor the skin regeneration in fish from different thermal backgrounds ( $n = 8$ /thermal background/time point).

13.5 °C or 16 °C, which corresponds to the optimal temperature range (11–16 °C) and the median and most common egg incubation temperature (13.5 °C) used for European sea bass [43] and incubated until hatching (Fig. 1A). Larvae from the three thermal backgrounds (3 tanks per thermal background) were reared at 15 °C until December 29th, when the temperature was slowly increased and reached 25 °C by January 3rd of 2017. On February 15th, a random subpopulation of 600 fish per tank was transferred into a larger rearing tank at 21 °C (3 tanks per thermal background). Between the 6th and 13th of April, a random subpopulation of 225 fish per thermal background (equally extracted from all rearing tanks) was tagged and mixed with fish from other thermal backgrounds (common garden design) in triplicate tanks (for a total of 675 tagged fish per tank). All tanks were reared at 21 °C until fish were ~9 months old. The experiment produced a large stock of juvenile fish (total of 2025 tagged fish) that were also used for other experiments [44,45].

To assess if thermal programming during egg incubation caused modifications in the general physiology of juvenile sea bass, eight ~9 month old fish from each of the three thermal backgrounds were euthanized with an overdose of 225 mg L<sup>-1</sup> benzocaine (E1501, Sigma, USA), measured (nearest mm) and weighed (nearest g) and the condition factor determined (Table 1), and a sample of blood and intact skin was collected (Undamaged fish, Fig. 1A). Blood was collected from the caudal vein in a heparinized syringe and the plasma separated by centrifugation at 10,000 rpm for 4 min at 4 °C and stored at -20 °C until analysis. One skin sample of about 1.5 cm<sup>2</sup> was removed from the left flank of the fish under the dorsal fin and immediately stored in RNAlater® (Sigma-Aldrich) at -20 °C until molecular analysis.

## 2.2. Superficial skin damage and sampling

The remaining ~9 month old fish from the common garden experiment were randomly distributed between 2 tanks, so that an equal number of fish from each thermal background were placed in duplicate experimental tanks (n = 16/thermal background/tank) and acclimated for 3 weeks prior to the start of the experiment to evaluate the impact of early thermal imprinting on the regeneration of fish skin. After acclimation, the fish were anesthetized with 37.5 mg L<sup>-1</sup> benzocaine and a superficial skin wound was inflicted on the left dorsal region above the lateral line, by removing the scales along the length of the dorsal fin with the blunt side of a knife (Fig. 1B). Damage did not provoke bleeding. Fish (n = 4/thermal background/tank) were sampled immediately after the superficial skin damage (0h), 24 h (1d) and 72 h later (3d). For sampling, fish were sacrificed with an overdose of benzocaine, blotted dry, and blood collected as outlined above, and two skin samples of about 1.5 cm<sup>2</sup> were removed from the left flank of the fish under the dorsal fin (wounded skin [Wou]) and from an equivalent position from the right flank (intact skin [Int], Fig. 1B). Skin samples of wounded and intact skin were collected and immediately stored in RNAlater® (Sigma-Aldrich) at -20 °C until molecular analysis and further samples were fixed overnight in 4% paraformaldehyde (PFA) pH 7.4 at 4 °C under constant agitation. After tissue fixation samples were washed in three changes of phosphate buffered saline, followed by sterile DEPC water,

and stored in 70% ethanol at 4 °C until processing.

## 2.3. Plasma cortisol and glucose

Plasma cortisol (ng.mL<sup>-1</sup>) was measured using a validated radioimmunoassay [46]. Glucose (mM) was measured with a glucose oxidase-peroxidase colorimetric commercial kit (Spinreact 1001190, Spain), following the manufacturer's instructions. Analysis of glucose was performed in 96-well plates and colour development was evaluated at 505 nm using a Microplate Biotek Synergy 4 reader (BioTek Instruments, Inc., USA).

## 2.4. Histology and histomorphometric analysis of intact and damaged skin

Following fixation, intact skin samples were decalcified in 0.5 M EDTA (pH 8.0) in the dark for 24 h and washed several times with sterile DEPC water. Both intact (decalcified) and wounded skin were dehydrated through a graded ethanol series (70–100%) and embedded in low melting point paraffin wax using a tissue processor (Leica TP1020, Leica). Longitudinal sections parallel to the scales (5 µm) were cut using a rotary microtome (Leica RM 2135) and mounted onto glass slides coated with 3-amino-propyltriethoxysilane (APES; Sigma-Aldrich). To assess the general histology of the skin, one section per individual (n = 8/thermal background/timepoint) was stained with haematoxylin and eosin. For histomorphometric analysis (n = 8/thermal background/timepoint), 3 slides containing 3 serial sections/slide with a gap of 25 µm between consecutive slides were stained with Alcian blue-Periodic Acid Schiff (AB-PAS) for two purposes: a) to stain goblet cells, and distinguish neutral (magenta) and acidic (blue) mucins; and b) to stain the basement membrane (magenta) [47]. Other components of the tissue were also stained according to their affinity with the dyes: epithelium (purple blue), acid hyaluronic (light blue) and collagen fibres (pale pink). Stained sections were observed using a light microscope (Leica DM2000) equipped with a digital camera (Leica DFC480). One section per slide (3 slides/intact and 3 slides/wounded skin per individual) was photographed at 100x magnification for measurement of the dermis and hypodermis thickness, and at 200x magnification to count the number of goblet cells and for measurement of the epidermis, basal cell layer and basement membrane thickness.

The thickness of the epidermis, basal cell layer, basement membrane, dermis (stratum compactum) and hypodermis were measured in three equidistant positions across each histological section (~1 cm length; 9 measurements for the intact or wounded skin from each fish). Goblet cells were counted on the epithelium surface (500 µm in length) of the 3 equidistant regions selected. Measurements were performed with Fiji v1.52p software [48].

## 2.5. Analysis of gene expression

Intact and wounded skin samples (n = 8/temperature background/timepoint) were disrupted with β-mercaptoethanol and iron beads in a Qiagen Retsch MM300 TissueLyser (Retsch, Germany) at a frequency of

**Table 1**  
Biometric parameters of juvenile sea bass from different thermal backgrounds.

Thermal background	Weight (g)			Length (cm)			K		
	11 °C	13.5 °C	16 °C	11 °C	13.5 °C	16 °C	11 °C	13.5 °C	16 °C
Undamaged	37.6 ± 10.2	36.7 ± 7.6	39.6 ± 13.6	14.1 ± 1.2	13.8 ± 0.9	14.1 ± 1.2	1.3 ± 0.1	1.4 ± 0.1	1.4 ± 0.2
0h	35.3 ± 12.3	32.9 ± 10.7	32.3 ± 8.6 ab	13.2 ± 1.5	12.9 ± 1.2	13.0 ± 1.2 ab	1.5 ± 0.1	1.5 ± 0.1	1.4 ± 0.1
1d	34.8 ± 9.7	32.1 ± 6.5	27.4 ± 6.6a	12.9 ± 1.2	12.9 ± 1.0	12.0 ± 1.0a	1.6 ± 0.2	1.5 ± 0.1	1.5 ± 0.2
3d	36.9 ± 13.9	31.8 ± 7.0	39.5 ± 8.4b	13.2 ± 1.7	12.8 ± 0.7	13.9 ± 1.0b	1.5 ± 0.1	1.5 ± 0.1	1.4 ± 0.0

Body weight (g), standard length (cm) and condition factor (K), calculated as 100 x (body weight/length<sup>3</sup>), of fish exposed to different temperatures during egg incubation. Data are shown as the mean ± SD. Different letters indicate significant differences between thermal groups. One-way ANOVA (Undamaged groups) and Two-way ANOVA (Regeneration groups); p < 0.05.



30 Hz (four cycles of 30 s each) and total RNA was extracted using an E.Z.N.A® Total RNA Kit I (R6834, Omega). Contaminating genomic DNA was removed from RNA extracts with an E.Z.N.A® RNase-Free DNase Set I kit (E1091, Omega) according to the manufacturer's instructions. The concentration and quality of the extracted RNA was determined with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). cDNA synthesis (with 500 ng of DNase treated RNA) was performed using random hexamers (200 ng, Jena Biosciences, Germany), and RevertAid reverse transcriptase (100 U, Fermentas, Thermo Fisher Scientific, USA) as previously described [49].

Quantitative Real-Time PCR (qPCR) was used for analysis of mRNA expression of immune transcripts and tissue reepithelialisation/remodelling markers. The immune response to superficial skin damage was evaluated using genes linked to innate immunity (colony stimulating factor 1 receptor [*csf-1r*], G-type lysozyme 1 [*lyg1*] and lactalbumin [*lalba*] and antioxidant defence markers (catalase [*cat*], copper/zinc superoxide dismutase [*sod1*]) and peroxisome proliferator-activated receptor gamma [*ppary*]). Skin and scale regeneration after superficial damage was evaluated by assessing the expression of keratin 2 (*krt2*), proliferating cell nuclear antigen (*pcna*), collagen type I alpha 1a (*col1a1*), collagen type X (*colxa*), osteonectin (*sparc*), myosin light chain 2a (*mlc2a*) and 2b (*mlc2b*), matrix metalloproteinase 9 (*mmp9*), angiopoietin-like 2b (*angptl2b*) and phosphoenolpyruvate carboxykinase 1 (*pck1*). The primer sequence, accession numbers, annealing temperature and qPCR efficiency are listed in Table 2.

Transcript levels were quantified following the relative standard

curve method [50] in duplicate 10 µL reactions containing 10 ng of cDNA, 300 nM of specific primers (Table 2) and EvaGreen (Sso Fast EvaGreensupermix, Bio-Rad Laboratories, USA). Thermocycling was performed in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, USA) and conditions were: 30 s at 95 °C, 40 cycles of 10 s at 95 °C and 10 s at 59–62 °C (depending on the primer pair, Table 1) followed by a final melt curve between 60 and 95 °C. Single product/dissociation curves were obtained in all reactions. Amplicons obtained from primer pairs were sequenced to confirm the specificity of the reaction and control reactions included a no-template control and a cDNA synthesis control (reverse transcriptase omitted).

Bio-Rad CFX Maestro 1.0 software (Bio-Rad Laboratories, USA) was used for data analysis and the CFX Maestro Reference Gene Selection Tool [51] was used to compare gene stability using the GeNorm algorithm [52]. The CFX Maestro software tool identified heat shock protein 70 (*hsp70*), elongation factor one alpha (*ef1a*) and 18S ribosomal RNA subunit (*18s*) (in this order) as the most stable and ideal reference genes and therefore the geometric mean of the three reference genes was calculated and used for normalization. The qPCR results were then calculated by dividing the copy number of the target gene by the geometric mean of the reference genes and was expressed as Log2 fold change, calculated relative to the undamaged skin of the 13.5 °C group (13.5-Und), which is the most common egg incubation temperature used for European sea bass [43].

**Table 2**

List of the primers used for gene expression analysis by quantitative Real-Time PCR. The gene symbols, accession numbers, primer sequences, annealing temperatures (Ta, °C), qPCR efficiencies (%) and R2 are indicated for each primer pair (F = forward and R = reverse primer).

Gene	Accession No.	Sequence (5' to 3')	Ta (°C)	Efficiency (%)	R <sup>2</sup>
<i>18s</i>	[53]	F: TGACGGGAAGGGCACCACCAG R: AATCGCTCCACCAACTAAGAACGG	60	100	0.998
<i>angptl2b</i>	[8]	F: TGCTGCACGAGATCATCAGGAA R: GTACTGTGCTCGAGATCTTT	60	108	0.998
<i>cat</i>	DLAgn_00171080 <sup>a</sup>	F: TTTGCTGATGGCTACCCG R: TGGCATAATCTGGTTGGTG	62	101	1
<i>col1a1</i>	DLAgn_00196080 <sup>a</sup>	F: AGACCTGCGTATCCCAACTC R: GCCACCGTTCATAGCCCTCTCC	59	102	1
<i>colxa1</i>	DLAgn_00066250 <sup>a</sup>	F: TGGGAATGAGTGAGGTTATGG R: GGATGCTGTAGGCAAAATAGT	60	107	0.999
<i>csf-1r</i>	KM225787.1	F: ACGTCTGGTCCATATGGCATC R: AGTCTGGTTGGGACATCTGG	62	101	0.997
<i>ef1a</i>	[54]	F: GACACAGAGACTTCATCAAG R: GTCCGTTCTAGAGATACCA	60	97	0.998
<i>hsp70</i>	Dla_2G14_00124 <sup>a</sup>	F: GCTCCACTCGTATCCCCAAG R: ACATCCAGAAGCAGCAGGTC	60	102	0.999
<i>krt2</i>	DLAgn_00060200 <sup>a</sup>	F: TCTGCTGTCAGTGTGGTAT R: TAACTGGATGGAAGTGGGAT	60	103	0.999
<i>lyg1</i>	[55]	F: AGGGAAGAGCCACGGGTCAG R: ATGCTTCACTTGTCTTTGGC	60	100	0.998
<i>lalba</i>	[55]	F: TGAAGGACGCTCTAATGACGG R: CGCCATATACCCCTCTTCTTCT	62	105	0.999
<i>mlc2a</i>	ENSDLAT00005025294.1 <sup>b</sup>	F: TGACCACCAACAACCCACA R: TCGTCCTTGCTGATGATGCC	62	100	0.998
<i>mlc2b</i>	ENSDLAT00005016731.1 <sup>b</sup>	F: AGCAAAGATGACCTGAGGGACG R: GGAAGACGGTGAAGTTGATGGG	60	102	0.999
<i>mmp9</i>	ENSDLAT00005045003.1 <sup>b</sup>	F: TGACGCTTCGCCAGAGC R: GCCAAAAGACCATCCTTACCA	60	104	1
<i>sparc</i>	[54]	F: AAGAAGGGCAAAGTGTGTGAGG R: TGGCAAAGAAGTGGCAAGAGG	60	101	1
<i>pck1</i>	DLAgn_00097810 <sup>a</sup>	F: GCTTTTAGCTGGCAACACGG R: TGTAGCCGAAGAAGGGACGC	62	100	0.995
<i>pcna</i>	JQ755266.1	F: GAGCAGCTGGTATTCCAGA R: CTGTGGCGGAGAAGTACT	60	97	0.999
<i>ppary</i>	[56]	F: CATCCGCTCTCCTTGTCTCC R: TGTCTGCTCCGTCCTGTAG	60	103	1
<i>sod1</i>	DLAgn_00043240 <sup>a</sup>	F: CTAAGACGGGCAATGCTGG R: GGTCTTAAGTGTGTGGGAA	62	99	0.999

<sup>a</sup> Accessed in the European sea bass genome available on UCSC Genome Browser database (<http://seabass.mpipz.mpg.de/index.html>).

<sup>b</sup> Accessed in the genome browser Ensembl (<http://www.ensembl.org/index.html>).

## 2.6. Statistical analysis

Collection of low plasma volumes in some samples and the presence of lipemic samples caused an imbalance in the number of samples analysed in the experimental groups of undamaged animals. Statistical analysis was performed using IBM® SPSS® Statistics 28.0 for Windows (IBM Corp., NY, USA) and graphs were constructed with GraphPad Prism 6.01 for Windows (GraphPad Software, CA, USA). One-way ANOVA was used to evaluate the effect of egg incubation temperatures on biometric parameters, biochemistry and gene expression of undamaged groups, while the lack of homoscedasticity in the glucose samples, prompted the use of the Kruskal-Wallis H Test. Two-way ANOVA was used to evaluate the effect of egg incubation temperatures on plasma parameters at different times after skin damage. Three-way mixed ANOVA was used to evaluate the effect on histomorphometry and gene expression of egg incubation temperatures, skin condition (wounded versus intact skin) and time after skin damage. A log10-transformation was used whenever necessary to normalise the data and when this procedure was used it is indicated in the figure legends. Simple main effects analysis was conducted with a Bonferroni control for Type I errors. For all the analysis performed statistical significance was set at  $p < 0.05$  and results are expressed as the mean  $\pm$  SEM, unless otherwise stated.

## 3. Results

Characterization of undamaged fish (Supp. Fig. 1 and Supp. Fig. 2), biometric parameters and a description of the intact and wounded skin morphology after superficial damage are provided as Supplementary Results.

### 3.1. Physiological response to superficial skin damage

Two-way ANOVA revealed that plasma levels of cortisol and glucose were significantly affected by time of wounding ( $p < 0.001$ ) and by thermal background ( $p < 0.05$ ). Cortisol was also significantly ( $p = 0.03$ ) modified by the combination of these two factors (Fig. 2 and Supp. Table S1). One day after skin damage the 16-1d group of fish had significantly higher ( $p < 0.001$ ) cortisol levels than fish from other thermal backgrounds and significantly higher ( $p < 0.01$ ) glucose levels compared to the 11-1d group of fish (Fig. 2). Three days after cutaneous damage plasma cortisol significantly decreased in all groups when compared to levels at 0h, but the 16-3d group of fish maintained significantly higher ( $p < 0.001$ ) cortisol levels compared to fish from other thermal backgrounds. At day 3 after skin damage, glucose levels were significantly ( $p < 0.01$ ) decreased compared to 0h in all groups of

fish, the exception was the fish reared at 11 °C, for which glucose levels were not significantly ( $p > 0.05$ ) modified during the experiment (Supp. Table S1).

### 3.2. Histological modifications in intact and wounded skin after superficial damage

Sea bass skin from the right flank of fish incubated as eggs at 13.5 °C was collected immediately after the skin of the left flank was damaged (13.5-0h-Int) and served as a control for the wounded (0h) and regenerated skin at 1 and 3 days after scale removal. Samples before skin damage (undamaged groups) were not included in this analysis since the intact 0h skin samples served as the control for undamaged skin.

The intact skin of fish from eggs hatched at different water temperatures was morphologically similar (normal organization is seen in Fig. 3A) apart from the thickness of the basement membrane when the skin on the other flank was damaged (1d, see sections below). The re-establishment of the epidermis in damaged skin followed the same general program irrespective of the egg incubation temperature, although some significant differences were identified in the regenerative process between groups (Figs. 3 and 4, see next sections).

#### 3.2.1. Number of goblet cells

Two types of goblet cells were detected in skin samples and classified according to their reaction to AB-PAS staining: AB-PAS positive, which stained blue/purple, and AB-PAS negative, cells that were transparent (no trace of cytoplasm nor evident nucleus). We assigned the AB-PAS negative cells to newly differentiated goblet cells during the regeneration of fish skin [57,58].

Three-way mixed ANOVA revealed that the number of AB-PAS positive and negative goblet cells was significantly affected by time ( $p = 0.024$  and  $p = 0.006$ , respectively) and skin condition (intact versus wounded,  $p < 0.001$ ). The number of AB-PAS negative cells was also significantly modified by the combination of time and skin condition ( $p = 0.01$ ) and by the interaction between thermal background, time and skin condition ( $p = 0.046$ ). AB-PAS positive and negative goblet cell number was not significantly different between the intact skin of fish from different thermal backgrounds (Fig. 5). Both goblet cell types were lost during skin damage (0h) and after 1 day of regeneration a small number of AB-PAS positive goblet cells were observed in the wounded skin (Supp. Table S2). After three days of regeneration AB-PAS positive goblet cell number had increased but remained significantly lower ( $p < 0.001$ ) than in the intact skin irrespective of the thermal background. AB-PAS negative cells reappeared in the epidermis on the 3rd day of regeneration but were also significantly less abundant ( $p < 0.001$ ) compared to the intact skin in all groups, except in fish from eggs

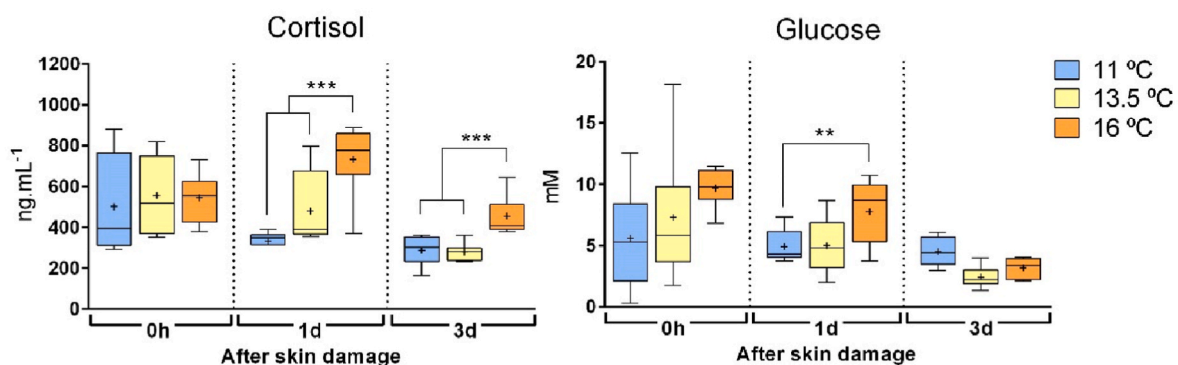
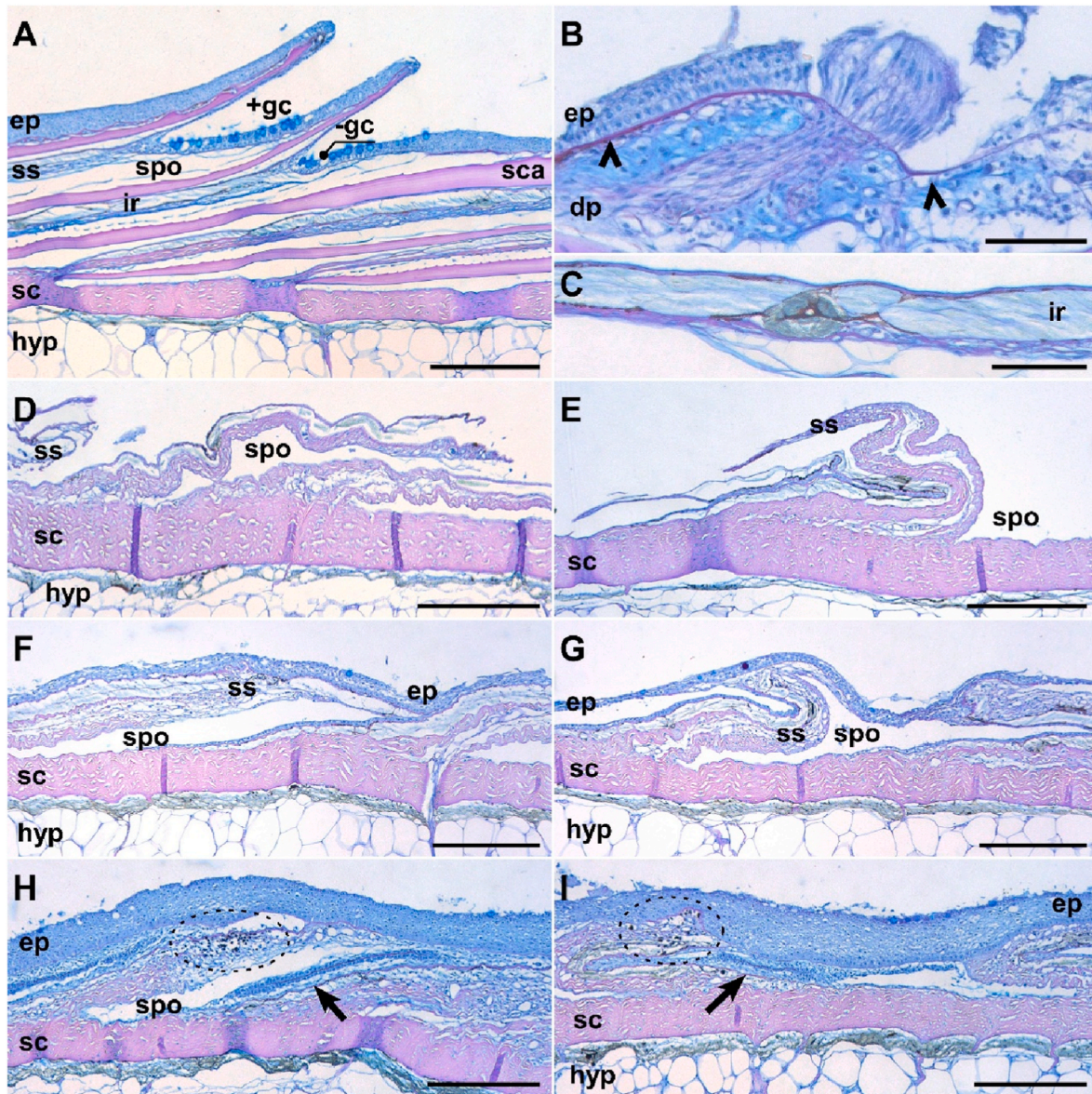


Fig. 2. Plasma levels of cortisol and glucose in European sea bass with different thermal backgrounds. Results are plotted in a Tukey box and whiskers plot, in which '+' represents the mean. Both plots show the plasma levels of cortisol and glucose for three thermal backgrounds (11 °C, 13.5 °C and 16 °C egg incubation temperatures), immediately after (0h) and at 1 and 3 days (1d and 3d, respectively) after skin damage by scale removal. Asterisks (\*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ) indicate significant differences between fish from different thermal backgrounds at each time point. Plasma cortisol levels were Log10 transformed for analysis and statistical significance (two-way ANOVA) was set at  $p < 0.05$ ;  $n = 8$  per thermal background/time point.





**Fig. 3.** Histological sections (5  $\mu\text{m}$ ) of European sea bass skin perpendicular to the epidermis stained with AB-PAS. The normal organization of the intact skin in the European sea bass is shown (A), with the epidermis (ep, purple blue) covering the scales (sca, pink) and showing AB-PAS positive goblet cells (+gc) stained blue and AB-PAS negative goblet cells (-gc) unstained. Scales are inserted in scale pockets (spo) enclosed between two strata of the dermis, stratum spongiosum (ss, pink to light blue) and stratum compactum (sc, pink). The subcutaneous tissue, hypodermis (hyp) is rich in adipocytes, blood vessels and nerves. Occasionally, taste buds are observed in the epidermis (B), separated from dermal papilla (dp) by a well-defined basement membrane (arrowhead, magenta). A layer (ir) consisting of iridophores (silver/gray) and dark melanophores (C) is observed near the stratum spongiosum and between the dermis and hypodermis. As soon as the scales are removed (0h, D and E) the epidermis, AB-PAS positive and negative goblet cells are also lost. The scale pocket is left open and exposes the dermis to the aquatic environment. The direction and pressure of water flow on the loose stratum spongiosum influences how the scale pocket will close and the regenerating epidermis will cover the dermis: with a flattened (D, F and H) or folded (E, G and I) stratum spongiosum. At day one (F and G) the scale pocket is closed, and a thin regenerating epidermis is observed. At 3 days (H and I) the thickness of the regenerating epidermis increases substantially. Hyperpigmented masses (melanomacrophages) appear in the stratum spongiosum (enclosed by dashed line) and in the epidermis. Arrows indicate a two-cell layer enclosing a very thin regenerating scale (pink line). These cells, the scleroblasts, are hyper-basophilic, almost cuboidal, and have a well-developed cytoplasm and a rounded central nucleus. Scale bars: A, D – I – 200  $\mu\text{m}$ ; B and C – 50  $\mu\text{m}$ .

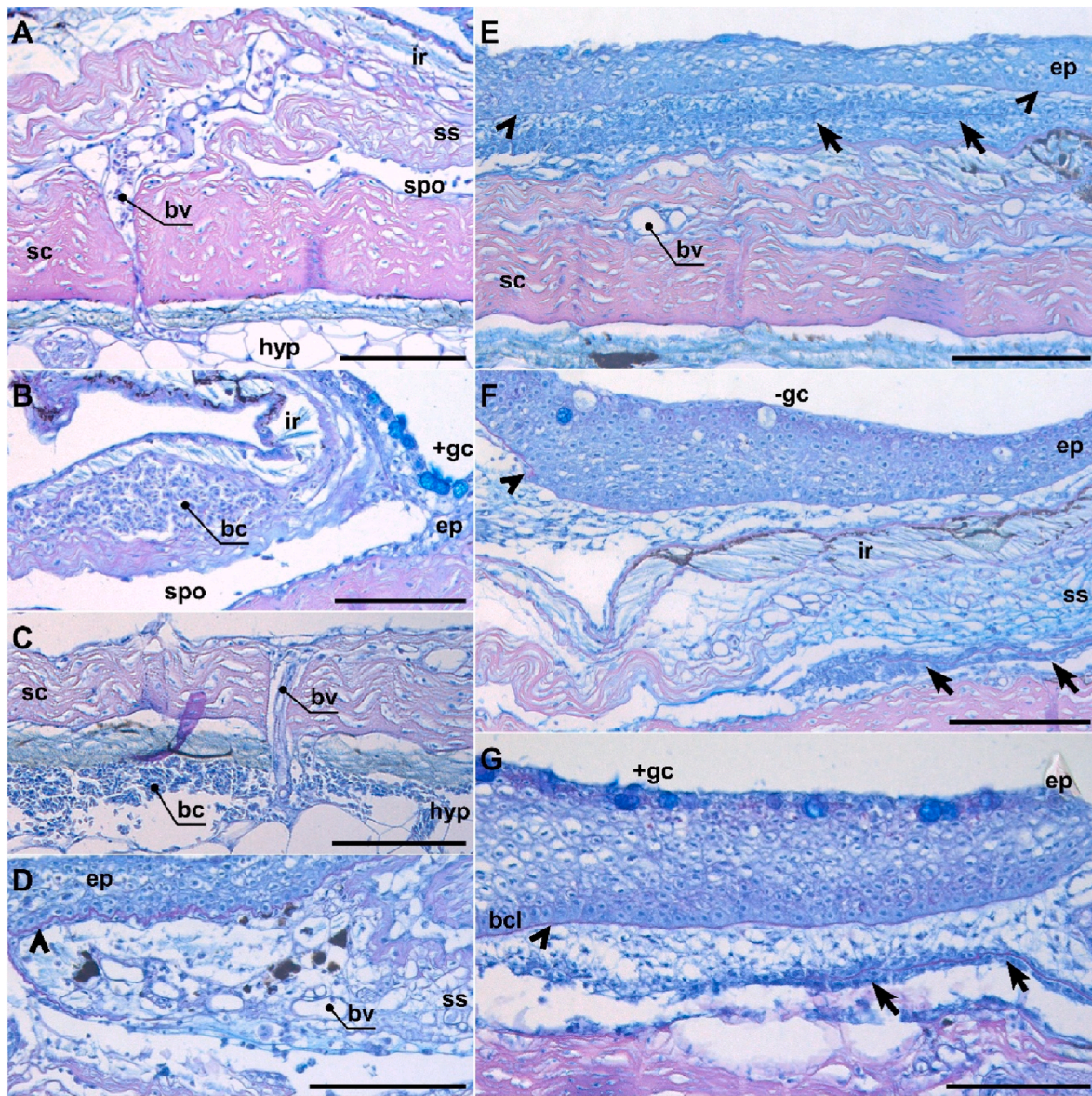
hatched at 13.5  $^{\circ}\text{C}$ , which displayed a similar number of AB-PAS negative cells between wounded and intact skin. At 3 days, the number of both AB-PAS positive and negative cells was significantly more abundant ( $p < 0.001$ ) in the wounded skin of fish from eggs hatched at 13.5  $^{\circ}\text{C}$  compared to the wounded skin of fish from eggs hatched at 11  $^{\circ}\text{C}$ .

### 3.2.2. Epidermis and basal cell layer thickness

Three-way mixed ANOVA demonstrated that the thickness of the

epidermis and basal cell layer was significantly affected by time, skin condition and by the interaction between these two factors ( $p < 0.001$ ) (Fig. 5). The thickness of the epidermis was significantly modified by the thermal background ( $p = 0.016$ ) and by the interaction between the factor's thermal background, time, and skin condition ( $p = 0.004$ ). Basal cell layer thickness was also significantly affected by the combination of time and thermal background ( $p = 0.033$ ). After 1 day regeneration of wounded skin the epidermis and basal cell layer were significantly thinner than the intact skin irrespective of the thermal background. In





**Fig. 4.** Detailed histological sections (5  $\mu\text{m}$ ) of events during skin regeneration after scale removal in the European sea bass (section perpendicular to the epidermis stained with AB-PAS). Cutaneous wound healing involved the infiltration of many blood vessels (bv) and blood cells (bc) in the dermis (pink), specifically the stratum spongiosum (ss, A and B) and in the hypodermis (hyp, C). It is possible to see that epidermal cells (ep, purple blue) infiltrate the scale pocket (spo, B). The melanomacrophages that appeared in the epidermis or stratum spongiosum (D) are mainly dark and variable in size (from cell size to larger mass). Detailed images of the regenerated epidermis 3 days after scale removal is shown (E–G), and it is separated from the dermis by a basement membrane (arrowhead, magenta). AB-PAS positive (+gc, blue) and negative (-gc, unstained) goblet cells appear 3 days after skin damage and maintain the same characteristics as those of the intact skin. The epidermis and basal cell layer (bcl) of fish from eggs hatched at 11  $^{\circ}\text{C}$  (E) is significantly thinner than the epidermis of fish from eggs hatched at 13.5  $^{\circ}\text{C}$  (F) and 16  $^{\circ}\text{C}$  (G). The regenerating scale (pink line) is enclosed by a layer of scleroblasts (arrows). Ir – iridescent layer, sc – stratum compactum. Scale bars: 100  $\mu\text{m}$ .

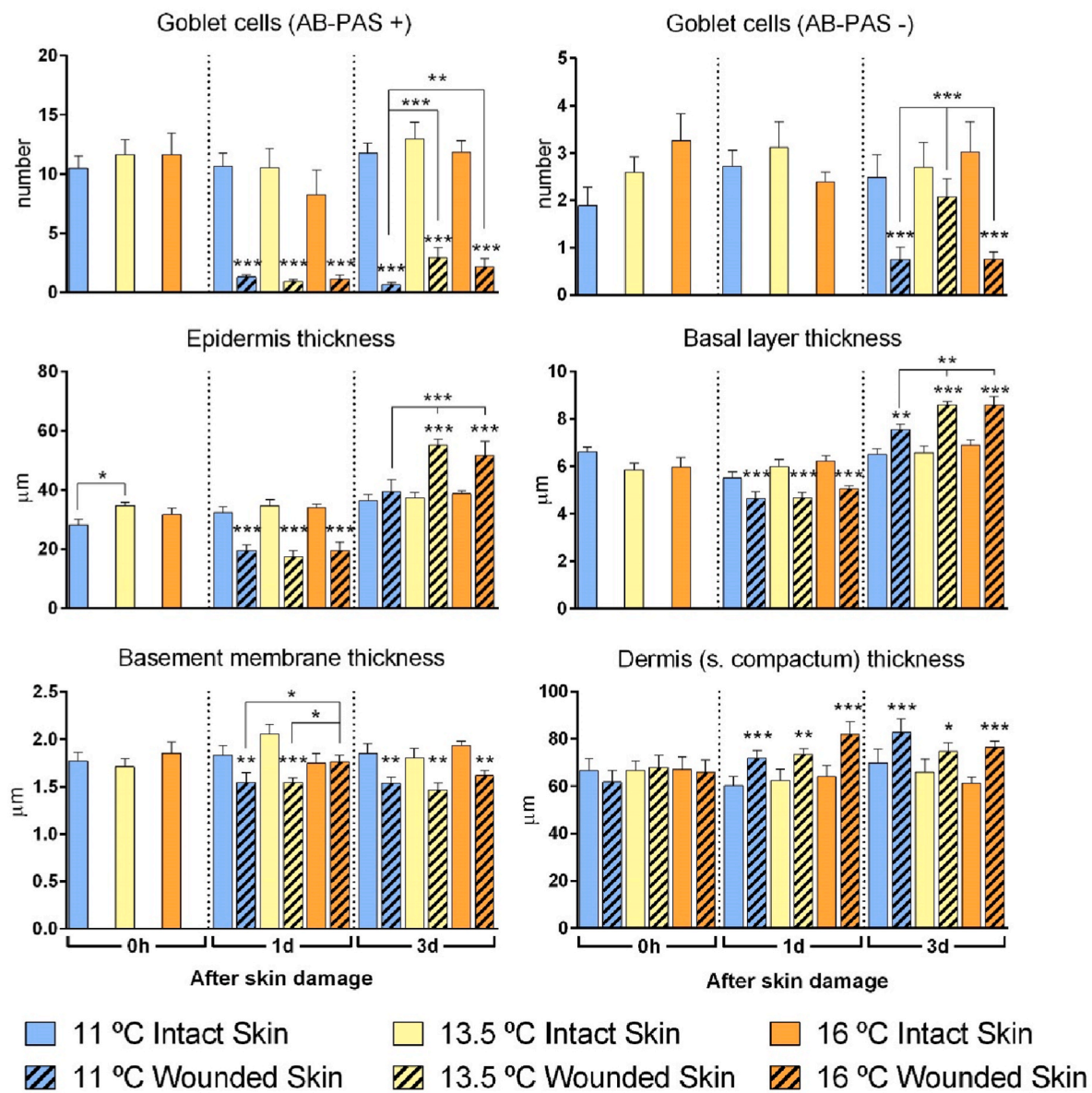
fish from the thermal background groups, with the exception of 11-3d-Wou fish, 3 days after skin regeneration both epidermis and basal cell layer was significantly thicker ( $p < 0.001$ ) than the intact skin. The increase in the thickness of the epidermis and basal cell layer after 3 days regeneration was significantly higher ( $p < 0.01$ ) in fish from eggs hatched at 13.5 and 16  $^{\circ}\text{C}$  compared to 11  $^{\circ}\text{C}$ .

The epidermis thickness of intact skin was significantly modified by the thermal background at 0h and was significantly thicker ( $p = 0.037$ ) in 13.5-0h-Int fish compared to 11-0h-Int fish (Fig. 5). Damage to the skin of the left flank affected the skin on the intact right flank. Comparison of intact skin at 0h (start of experiment) and 3 days revealed that the epidermal thickness was significantly increased ( $p = 0.004$  and  $p = 0.02$ , respectively) in the intact skin of fish from eggs hatched at 11  $^{\circ}\text{C}$  and 16  $^{\circ}\text{C}$  (Supp. Table S2).

### 3.2.3. Basement membrane thickness

The thickness of the basement membrane was significantly affected by time and skin condition ( $p < 0.001$ ), by the interaction between time and skin condition ( $p < 0.001$ ) and by the interaction between time, skin condition and thermal background ( $p = 0.041$ ; three-way mixed ANOVA). The basement membrane was lost together with epidermis when the scales were removed but was restored 1 day after damage in all groups of fish irrespective of thermal background, but 16-1d-Wou fish displayed a significantly thicker ( $p = 0.035$ ) basement membrane compared to the wounded skin of other fish (Fig. 5). The thickness of the basement membrane was not re-established in wounded skin and was significantly thinner after 3 days of regeneration ( $p < 0.01$ ) than in intact skin, irrespective of the thermal background. Damage to one flank caused a significant change in the basement membrane of the intact skin





**Fig. 5.** Histomorphometric analysis of the main cells (number) and layers (thickness in  $\mu\text{m}$ ) of European sea bass skin. Results for each thermal background group (11 °C, 13.5 °C and 16 °C), for both wounded and intact skin and at each time point after superficial skin damage (0h, 1d and 3d;  $n = 8/\text{thermal background/skin condition/timepoint}$ ) were plotted as the mean +s.e.m. Significant differences between wounded and intact skin are indicated: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; Three-way mixed ANOVA.

on the other flank, which became significantly thicker ( $p = 0.037$ ) in 13.5-1d-Int fish compared to 13.5-0h-Wou (control) (Supp. Table S2).

### 3.2.4. Dermis and hypodermis thickness

Three-way mixed ANOVA revealed that dermis thickness was significantly affected by skin condition ( $p < 0.001$ ) and by the interaction between skin condition and time ( $p < 0.001$ , Fig. 5 and Supp. Table S2). No significant differences were found in the dermis thickness between fish from different thermal backgrounds at any time point, in both intact and wounded skin. Removal of the scales with the epidermis caused a significant modification in the underlying dermis and at 1 and 3 days of regeneration the dermis was significantly thicker ( $p < 0.01$ ) in wounded skin compared to the intact skin irrespective of the thermal background of the fish.

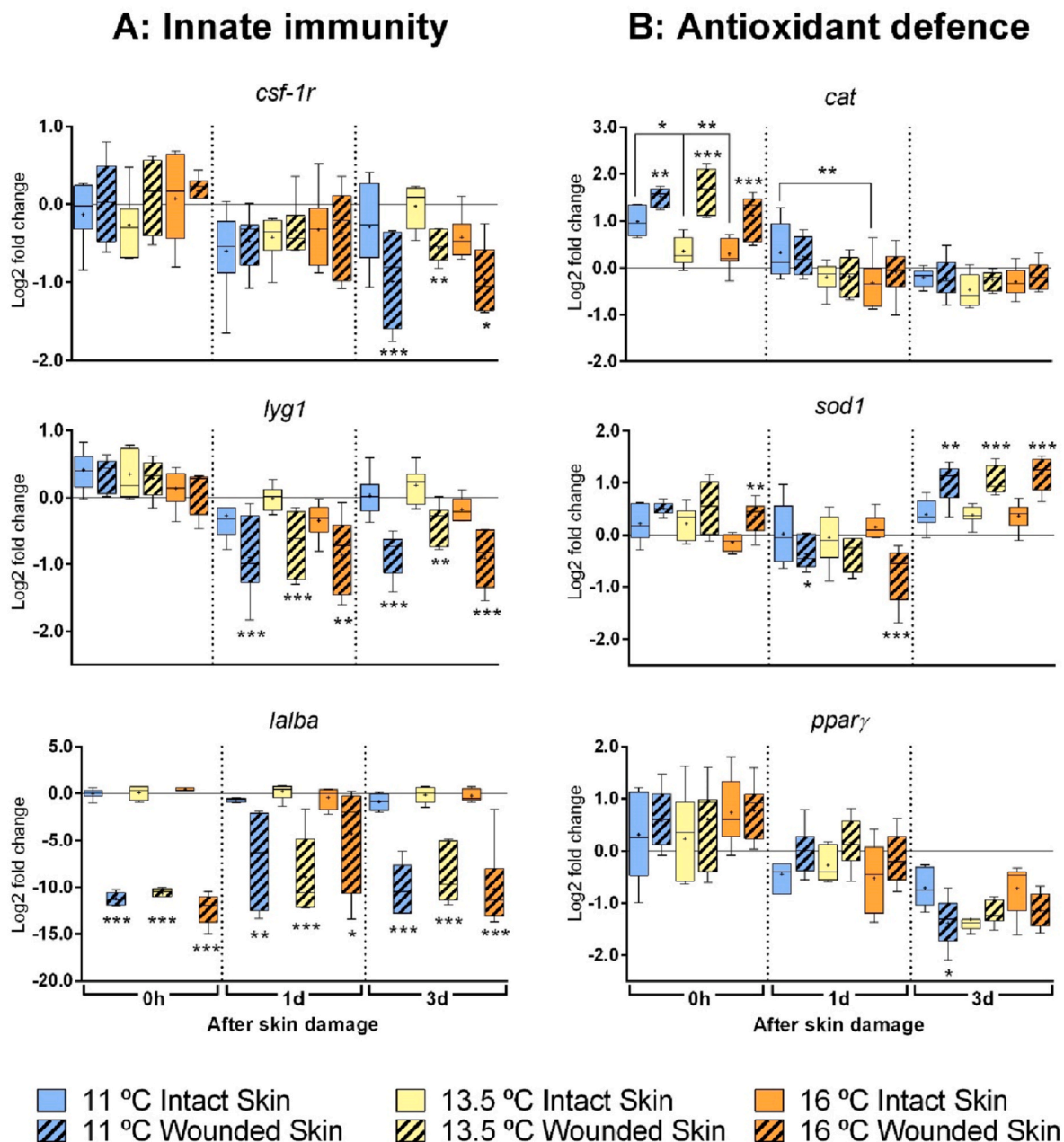
No significant differences were detected in the hypodermis between fish from eggs hatched under different thermal backgrounds at any time point, in both intact and wounded skin (Supp. Fig. 3 and Supp. Table S2).

### 3.3. Gene expression

It is difficult to collect skin samples without including some muscle. For this reason to assess the relative contribution of muscle to the skin total RNA extracts the expression of markers of muscle hyperplasia (*mcl2a*) and hypertrophy (*mcl2b*) [72], was determined. Three-way mixed ANOVA revealed that the expression of *mcl2a* ( $p < 0.01$ ) and *mcl2b* ( $p < 0.001$ ) was significantly influenced by time and skin condition (Supp. Fig. S4 and Supp. Table S3 in Supplementary Results).

#### 3.3.1. Transcripts associated with immune response

Three-way mixed ANOVA revealed that transcripts associated with innate immunity, *csf-1r*, *lyg1* and *lalba*, were significantly ( $p < 0.001$ ) affected by time and significantly ( $p < 0.001$ ) affected by the interaction between time and skin condition (Fig. 6). Expression of *lyg1* and *lalba* was also significantly ( $p < 0.001$ ) affected by skin condition. Skin damage (0h) caused significant ( $p < 0.001$ ) down-regulation of *lalba* in



**Fig. 6.** Expression analysis by qPCR of transcripts during cutaneous wound healing and associated with: A) innate immunity (*csf-1r*, *lyg1* and *lalba*) and B) antioxidant defence (*cat*, *sod1* and *ppar $\gamma$* ). Results were normalised using the geometric mean of *hsp70*, *ef1a* and *18s* and then expressed as Log<sub>2</sub> fold change, calculated relative to undamaged skin from the 13.5 °C group (considered the control temperature for European sea bass egg incubation). Results for each thermal background (11 °C, 13.5 °C and 16 °C), for both wounded and intact skin and at each time point (n = 8/thermal background/skin condition/timepoint) were plotted in Tukey box and whiskers graphs and '+' represents the mean. Significant differences between wounded and intact skin are indicated: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; Three-way mixed ANOVA.

the wounded skin compared to intact skin irrespective of thermal background and it remained down-regulated for the duration of the experiment. *Lyg1* expression was significantly ( $p < 0.01$ ) down-regulated in wounded skin 1 and 3 days after damage compared to intact skin in all thermal groups. Expression of *csf-1r* was significantly ( $p < 0.05$ ) down-regulated 3 days after damage in wounded skin compared to intact skin.

Transcripts associated with antioxidant defence (*cat*, *sod1* and *ppar $\gamma$* ) were significantly ( $p < 0.001$ ) affected by time and by the interaction of time with skin condition ( $p < 0.001$ , Fig. 6, three-way mixed ANOVA). Expression of *cat* was also significantly affected by thermal background ( $p = 0.001$ ) and skin condition ( $p < 0.001$ ) and by the interaction of

thermal background with skin condition ( $p = 0.042$ ). Expression of *sod1* was also significantly ( $p = 0.007$ ) affected by skin condition. Skin damage (0h) caused a significant up-regulation of *cat* ( $p < 0.01$ ) in wounded skin, irrespective of the thermal background, and up-regulation of *sod1* ( $p < 0.01$ ) in wounded skin relative to intact skin of fish from eggs hatched at 16 °C. In fish from eggs hatched at 11 °C the expression of *cat* was significantly up-regulated ( $p < 0.05$ ) in the intact skin compared to the intact skin of fish from eggs hatched at 13.5 °C and 16 °C. The expression of *cat* significantly decreased ( $p < 0.05$ ) 1 day after skin damage in both intact and wounded skin irrespective of the thermal background (Supp. Table S3), but expression of *cat* remained significantly increased ( $p = 0.007$ ) in the intact skin of fish from eggs hatched

at 11 °C compared to intact skin of fish from eggs hatched at 16 °C. In wounded skin regenerating for 1-day levels of *sod1* were significantly decreased ( $p < 0.05$  compared to intact skin, but *sod1* expression was significantly increased ( $p < 0.01$ ) after 3 days of regeneration compared to intact skin and to wounded skin at 0h ( $p < 0.05$ ), irrespective of the

thermal background (Fig. 6 and Supp. Table S3).

The expression of *ppar $\gamma$*  significantly ( $p < 0.05$ ) decreased in both wounded and intact skin throughout the experiment irrespective of thermal background (Fig. 6 and Supp. Table S3). At day 3, the expression of *ppar $\gamma$*  was significantly ( $p < 0.05$ ) down-regulated in 11-3d-Wou fish

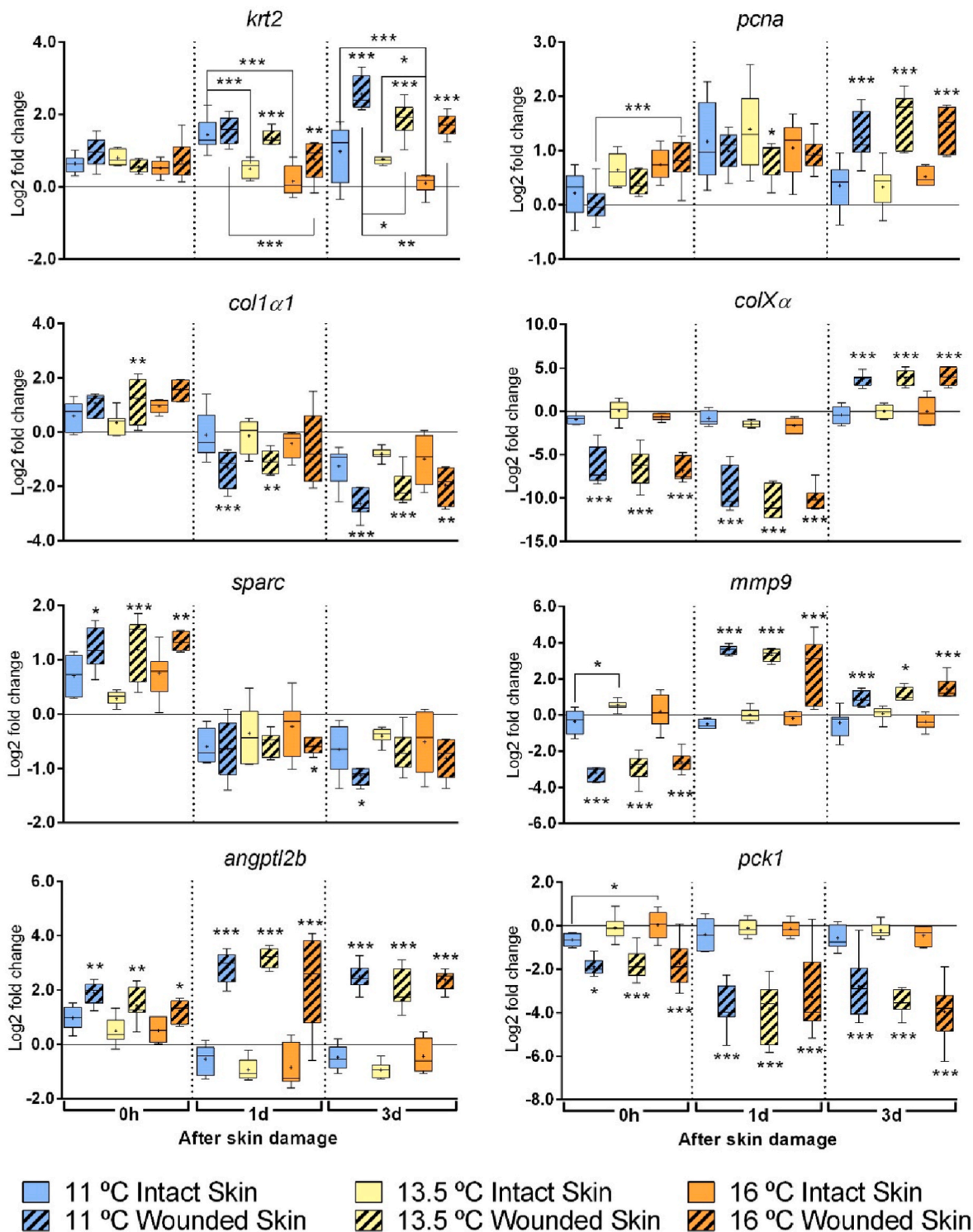


Fig. 7. Relative abundance of transcripts associated with reepithelialization and tissue remodelling during cutaneous wound healing (*krt2*, *pcna*, *col1a1*, *colx $\alpha$* , *sparc*, *mmp9*, *angptl2b* and *pck1*). Results were normalised using the geometric mean of *hsp70*, *ef1a* and *18s* and then expressed as Log<sub>2</sub> fold change, calculated relative to undamaged skin from 13.5 °C group (considered the control temperature for European sea bass egg incubation). Results for each thermal background (11 °C, 13.5 °C and 16 °C), for both wounded and intact skin and at each time point (n = 8/thermal background/skin condition/timepoint) were plotted in Tukey box and whiskers graphs and ‘+’ represents the mean. Significant differences between wounded and intact skin are indicated: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; Three-way mixed ANOVA.



relative to 11-3d-Int fish (Fig. 6).

### 3.3.2. Transcripts associated with skin regeneration

Three-way mixed ANOVA demonstrated that the expression of all transcripts associated with skin regeneration was significantly ( $p < 0.001$ ) modified by time, skin condition and by the interaction between these two factors (Fig. 7 and Supp. Table S3). The exceptions were *angptl2b* and *mmp9* that were not significantly ( $p > 0.05$ ) affected by time, and *sparc* that was not significantly ( $p > 0.05$ ) affected by skin condition. Expression of *krt2* and *pcna* in skin was significantly ( $p < 0.001$  and  $p = 0.041$ , respectively) affected by thermal background. Expression of *krt2* was also significantly ( $p = 0.004$ ) modified by the combination of time and thermal background.

After skin damage (0h), the expression of *colXa*, *mmp9* and *pck1* in wounded skin was significantly down-regulated ( $p < 0.001$ ) and the expression of *angptl2b* and *sparc* was significantly up-regulated ( $p < 0.05$ ) compared to intact skin irrespective of the thermal background (Fig. 7). *Col1a1* in 13.5-0h-Wou fish was significantly up-regulated ( $p = 0.004$ ) compared to 13.5-0h-Int fish. *Pcna* expression was significantly up-regulated ( $p < 0.001$ ) in the wounded skin of fish from eggs hatched at 16 °C compared to 11 °C. Damage to the skin on one flank modified gene expression of skin from the intact flank. At 0h the expression of *mmp9* in 13.5-0h-Int fish was significantly up-regulated ( $p = 0.013$ ) compared to 11-0h-Int fish and *pck1* was significantly up-regulated ( $p = 0.045$ ) in the 16-0h-Int compared to 11-0h-Int fish.

One day after skin damage, expression of *col1a1* significantly ( $p < 0.05$ ) decreased and the expression of *mmp9* significantly ( $p < 0.001$ ) increased in wounded skin relative to intact skin (Fig. 7). The transcript *krt2* was significantly ( $p < 0.01$ ) up-regulated in wounded skin relative to intact skin and was significantly ( $p < 0.001$ ) higher in 11-1d-Wou than in 16-1d-Wou. The intact skin of fish from eggs hatched at 11 °C also had a significantly ( $p < 0.001$ ) increased expression of *krt2* compared to other fish. Expression of *pcna* transcripts in the intact skin was modified after skin damage and was significantly ( $p < 0.001$ ) increased at day 1 compared to 0h in fish from eggs hatched at 11 °C and 13.5 °C (Fig. 7 and Supp. Table S3). Expression of *pcna* in the wounded skin significantly ( $p < 0.05$ ) increased after skin damage and was significantly ( $p < 0.001$ ) up-regulated relative to intact skin 3 days after scale removal, regardless of the thermal background.

Three days after skin damage, expression of *colXa* significantly ( $p < 0.001$ ) increased in wounded skin and was significantly ( $p < 0.001$ ) up-regulated relative to intact skin, irrespective of the thermal background (Fig. 7 and Supp. Table S3). Expression of *mmp9* significantly ( $p < 0.001$ ) decreased in wounded skin at day 0 compared to intact skin but was significantly ( $p < 0.001$ ) up-regulated compared to intact skin at day 1 and 3, irrespective of thermal background. Expression of *krt2* at 3 days was significantly up-regulated ( $p < 0.001$ ) in wounded skin relative to intact skin, and significantly ( $p < 0.01$ ) higher in fish hatched at 11 °C compared to other fish. Intact skin from fish hatched at 11 °C had a significantly increased ( $p < 0.05$ ) expression of *krt2* compared to fish from eggs incubated at 16 °C. Irrespective of the thermal background, expression of *angptl2b* and *pck1* was significantly ( $p < 0.001$ ) up- and down-regulated, respectively, in wounded skin compared to intact skin throughout the experiment.

## 4. Discussion

This is the first study to demonstrate that thermal imprinting at the egg stage modifies the repair response of superficial skin wounds in juvenile fish. Higher temperatures during egg incubation (16 °C) favoured the up-regulation of *pcna*, a common marker of cell proliferation/division [59,60], as soon as the scales were removed (0h), and produced a thicker regenerated epidermis compared to fish from eggs hatched at 11 °C. Lower temperatures (11 °C) promoted the up-regulation of *krt2*, a marker for epithelial cell migration [61], 1 and 3 days after scale removal, and was associated with thinner regenerated epidermis and

less goblet cells compared to fish from other thermal backgrounds. These results suggest that higher temperatures during embryogenesis may stimulate the regeneration of the epidermis by proliferation of epithelial cells, while re-epithelialization in fish incubated as eggs at lower temperatures may be activated later and propelled by cell migration. This is a very interesting observation that needs further investigation.

As has been previously reported in sea bream [8–10], the response to skin damage was not restricted to the damaged flank but affected the cutaneous barrier as a whole since both wounded and non-damaged (intact) flanks responded. When skin was damaged, fish incubated as eggs at lower temperatures (11 °C) had a significantly enhanced expression of *cat*, suggesting a stronger antioxidant response compared to fish incubated as eggs at higher temperatures (16 °C). An interesting finding of our study was the association between the expression of antioxidant enzymes and the appearance of melanomacrophages after skin damage. Qualitative assessment of melanomacrophages (data not shown) indicated that fish with an increased expression of *cat* at 0h and 1 day appeared to have more melanomacrophages in wounded skin, and this was most notable in fish that were incubated as eggs at lower temperatures (11 °C). The impact of thermal imprinting on melanomacrophages has previously been reported in seabream [26], in which acute stress reduced the number of small melanomacrophages centers (MMCs) in the head kidney of adult fish incubated as eggs and larvae at higher temperatures. Additionally, the appearance of melanomacrophages in the epidermis of wounded skin appeared to be associated with a thickening of the epidermis and at 3 days after damage, a shift in the presence of melanomacrophages occurred and fish which exhibited a thicker epidermis (13.5-3d-Wou and 16-3d-Wou), also had more melanomacrophages than fish incubated as eggs at 11 °C. The occurrence of dark pigmented cells resembling small MMCs is now well-documented in the wounded skin of fish [62–66], but our observations reinforce the involvement of melanomacrophages in the regeneration of damaged fish skin.

The temperature of egg incubation and therefore embryogenesis did not modify the characteristics of the cutaneous barrier in juvenile fish before skin damage as judged by the molecular indices evaluated. The exception was a significantly thicker epidermis in the intact flank of fish from eggs hatched at 13.5 °C compared to 11 °C observed at 0h. This contrasts with the situation in Atlantic salmon [67], since higher rearing temperatures decreased the epidermal thickness and modified the skin transcriptome, but measurements were performed on post-smolts and four weeks after thermal conditions.

Superficial skin damage also provoked a significant increase in circulating cortisol levels of all fish irrespective of their thermal background, as expected based on the response of other fish [10,12,68,69]. However, the magnitude and resilience of the stress response in fish incubated at different temperatures as eggs differed. The highest egg incubation temperature (16 °C) elicited a higher and more prolonged cortisol response compared to fish incubated as eggs at 11 °C and 13.5 °C. Interestingly, the response of plasma glucose was similar to cortisol in relation to the egg thermal background and was higher in fish from eggs incubated at 16 °C compared to 11 °C one day after skin damage. The change in cortisol and glucose is a common “fight-or-flight” response to acute stressors in sea bass and other fish [70,71] and did not seem to be a consequence of thermal imprinting. However, long lasting effects on the responsiveness of the hypothalamic-pituitary-interrenal (HPI) axis have previously been reported in thermally imprinted sea bream [26].

Overall, our results indicate that skin and HPI-axis development is responsive to egg incubation temperatures in agreement with previous studies [32,72–74]. Although not established in this study, phenotypic plasticity of skin and the HPI-axis was likely induced by epigenetic marks driven by early life temperatures [37,42,75,76]. Such epigenetic marks include DNA methylation, histone modification and chromatin organization, and have the ability to activate or silence specific gene expression without changing the DNA sequence, but by controlling the



DNA availability to the transcription machinery [35,36]. Although it should be noted that non-coding RNAs, particularly microRNAs (miRNAs), also have a wide range of transcriptional and post-transcription activities and regulate the abundance and translation of mRNAs [35, 36]. It will be important in the future to establish which of these epigenetic mechanisms are behind the phenotypic plasticity observed in our study.

#### 4.1. Modulation of skin re-epithelialization by embryonic temperatures

Two key transcripts, *pcna* and *krt2*, proxies for re-epithelialization after skin injury [59–61,77], were significantly modulated by egg incubation temperatures. In fish from eggs incubated at 16 °C, the expression of *pcna* in the skin was significantly increased as soon as the scales were removed, in the wounded skin (16-0h-Wou) compared to 11-0h-Wou fish. In contrast, expression of *krt2* increased 1 day after scale removal but in fish incubated as eggs at 11 °C (11-1d-Wou and 11-1d-Int) relative to 16-1d-Wou and 16-1d-Int fish. Despite the differences in the response of the skin in fish exposed to different temperatures as eggs, the epidermis was regenerated by day 3 after damage in all fish irrespective of the thermal background. However, the regenerated epidermis (including the basal cell layer) of 11-3d-Wou fish was significantly thinner compared to the skin in other groups. Bullock et al. [21] observed that skin regeneration at lower temperatures in adult plaice (*Pleuronectes platessa*) also resulted in a thinner epidermis compared to those regenerated at higher temperatures, suggesting the thinning of the regenerated epidermis was most likely caused by migration of pre-existing cells from the wound bed. Ai-Jun et al. [78] found a decrease in keratins during epithelial regeneration in adult turbot maintained at higher temperatures than usual (>9 °C). The observations in plaice and turbot are corroborated by our own results, since 11-3d-Wou fish had increased expression of *krt2*, a marker for cell migration [61], relative to the skin of the other groups (13.5-3d-Wou and 16-3d-Wou). Furthermore, 11-3d-Wou fish had significant down-regulation of *ppary* compared to 11-3d-Int. Down-regulation of *ppary* has been linked in mammals to a thinner epidermis due to inhibition of keratinocyte proliferation [79] and stimulation of keratinocyte terminal differentiation and keratin production [80], suggesting an impairment of the cutaneous barrier after injury [81]. Additionally, goblet cells (both AB-PAS positive and negative) were significantly decreased in 11-3d-Wou fish compared to other fish incubated as eggs at higher temperatures (13.5 °C and 16 °C). Since the epidermis and basal cell layer thickness was also decreased in 11-3d-Wou fish compared to the skin in other fish, we speculate that these cells may contribute to proliferation during the regeneration of the wounded epidermis and that thermal imprinting modified their response and the overall cutaneous barrier repair in the juvenile sea bass that were incubated as eggs at 11 °C.

It might be argued that the results reported for some of the transcripts in sea bass skin in the present study are a reflection of differing amounts of muscle in tissue samples and the associated hyperplasia/hypertrophy of muscle induced by higher rearing temperatures [82–84]. However, care was taken during sampling to minimise contamination by muscle and the ratio between the expression of the muscle specific gene transcripts, *mcl2a* and *mcl2b*, in the skin cDNA was not significantly induced by higher developmental temperatures as observed in the literature, so this supports the notion that a skin specific response was captured.

#### 4.2. Expression of lysozymes, components of the innate immune response, following skin damage

Overall, the innate immune response of sea bass to superficial skin damage was similar in fish from different thermal backgrounds. Although G-type lysozyme (Lyg1) and lactalbumin (Lalba), both members of the lysozyme family [55,85,86], are highly abundant in fish skin

[55], and mucous [2,87], their role in fish skin is not understood. In rats, it was reported that LALBA improves cutaneous wound healing in burned skin by enhancing fibroblast migration and collagen I synthesis [88], but this has not been evaluated in fish. Both *lyg1* and *lalba* were significantly down-regulated in the wounded skin of fish irrespective of the egg incubation temperatures. Expression of *lalba* was decreased as soon as the scales were removed (0h), while the expression of *lyg1* only decreased after 1 day. This different modulation of lysozyme family members suggests that they might diverge not only in their biological function, but also in their tissue origin. Considering that the embryonic origin of the mammary gland (known to produce LALBA) is the epidermis [89], we speculate based on the results in the sea bream that the epidermis is the main source of *lalba*, since the apparently immediate down-regulation of *lalba* in skin from which the scales were removed may be a reflection of the loss of the epidermis, that produces the transcript rather than down-regulation of expression. The origin of innate humoral factors in fish skin will be something to explore in the future.

#### 4.3. Antioxidant responses following skin damage in thermally imprinted fish

The impact of temperatures during the egg stage was most evident in the expression of antioxidant markers compared to transcripts associated with innate immunity. When the skin of fish irrespective of thermal background was damaged, expression of *cat* was increased relative to the intact skin. The expression of *sod1* was also increased in 16-0h-Wou fish relative to 16-0h-Int fish. Damaging the skin of Gilthead seabream in previous studies elicited a similar antioxidant response [11,90] to that seen in the present study. However, we are the first to demonstrate that thermal imprinting in the egg stage modifies the antioxidant response in damaged skin undergoing repair. Curiously, thermal imprinting also modified the antioxidant response of the intact skin of fish with skin damage on the other flank. This was evident in fish incubated as eggs at lower temperatures (11-0h-Int and 11-1d-Int), where the skin had significant increased *cat* expression compared to fish incubated as eggs at higher temperatures (16-0h-Int and 16-1d-Int, respectively). This may indicate that the antioxidant response to oxidative damage after injury, specifically H<sub>2</sub>O<sub>2</sub> detoxification, was improved in sea bass with higher mRNA levels of *cat* as has previously been observed in model fish species [91].

Increased resistance to oxidative stress due to improvements in antioxidant capacity has been observed in fish with a higher melanin content in the skin [92,93]. Qualitative evaluation of the presence of melanomacrophages (data not shown) revealed that fish from eggs incubated at 11 °C had more melanomacrophages in the dermis (*s. spongiosum*) of wounded skin than fish from other thermal backgrounds, one day after scale removal. But at 3 days, melanomacrophages surfaced in the regenerated epidermis, and this event overlapped with the up-regulation of *sod1* in the wounded skin compared to the intact skin of all fish irrespective of thermal regime. Although 13.5-3d-Wou and 16-3d-Wou fish appeared to display more melanomacrophages than 11-3d-Wou fish. The expression of *csf-1r*, a marker of fish macrophages [94], the melanin-containing cells of melanomacrophages [95], may support this observation, since expression of *csf-1r* was more down-regulated in 11-3d-Wou relative to 11-3d-Int than in other fish.

#### 4.4. Dermis was also modulated during skin regeneration

One day after scales were removed, the stratum spongiosum of the dermis in the damaged skin became intensely infiltrated by blood vessels and blood cells, in common with observations in Atlantic salmon [96] and the Antarctic fish, *Nototenia coriiceps* [77]. There was also an increase in the thickness of the stratum compactum of the dermis during the regeneration of wounded skin, and a thicker dermis than the intact skin was observed in all fish irrespective of the thermal background.

Similar observations have been made in rats [97,98]. The expression of transcripts associated with ECM remodelling (*mmp9*, *colXa* and *col1a1*) were also modified between the wounded and intact skin of fish irrespective of the thermal background. Curiously, expression of *col1a1*, a major component of fish skin [99], was significantly down-regulated in wounded skin compared to intact skin in fish incubated as eggs at 11 °C and 13.5 °C, but not in 16-1d-Wou. This is at odds with the observations in Gilthead sea bream that higher embryonic temperatures increase *col1a1* expression and if the explanation for the difference is linked to species specific characteristics or the experimental regime remains to be established [100].

Expression of *colXa* was significantly down-regulated in all the seabass as soon as the scales were removed from the skin. But after 3 days repair a shift occurred and the expression of *colXa* in wounded skin became significantly up-regulated relative to intact skin, irrespective of thermal background, and this overlapped with the appearance of a very thin regenerating scale, as previously reported in the gilthead seabream [9]. Expression of *colXa* by scleroblasts and by adjacent basal epidermal cells have been linked to the regeneration of a new scale [101] and to the production of dermal bone ECM [102,103]. Although no significant differences were found between the thickness of the ontogenetic scales of the intact skin and the thickness of regenerating scales of the wounded skin (data not shown), at 3 days 11-3d-Wou fish had significantly less *sparc* mRNA levels, a well-established marker of osteoblasts [104], compared to 11-3d-Int fish. It would be interesting in the future to investigate the impact of thermal imprinting on the regenerating scales since our previous studies [100,105] demonstrated thermal imprinting modified bone development and its responsiveness to a cold challenge.

## 5. Conclusion

This study revealed for the first time that egg incubation temperatures, even though within the accepted range for embryo development in European sea bass, caused thermal imprinting with significant modifications in skin regeneration after superficial damage 9 months after the thermal treatment. The most significant finding of our study was that embryonic temperatures modulated mechanisms underpinning skin re-epithelialization after damage in juveniles. The significant differences in expression of *krt2* and *pcna* in fish from eggs incubated at 11 °C and 16 °C, suggests fundamental changes occurred in cell migration and cell proliferation during barrier repair. The cutaneous barrier was fully recovered 3 days after damage irrespective of the thermal regime but juvenile sea bass from eggs incubated at 16 °C had thicker skin compared to those incubated at 11 °C corroborating the qPCR results suggesting higher cell proliferation in the former group. Although sea bass skin regenerated following the same general program previously reported in the literature, it was clear that thermal imprinting caused a change in the response of fish from eggs incubated at the lowest and highest temperatures of the accepted range, with specific modifications shown by both histology and gene expression. Future work will be required to establish the underlying epigenetic mechanisms behind the plasticity of this response.

## Credit author statement

**Ana Patrícia Mateus:** Methodology, Formal analysis, Validation, Investigation, Visualization, Writing – original draft; Writing – review & editing; **Rita A. Costa:** Methodology, Formal analysis, Visualization, Writing – original draft; **Bastien Sadoul:** Methodology, Writing – review & editing; **Marie-Laure Bégout:** Writing – review & editing, Project administration; **Xavier Cousin:** Conceptualization, Methodology, Writing – review & editing; **Adelino VM Canario:** Resources, Writing – review & editing, Supervision, Project administration; **Deborah M. Power:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

## Declarations of competing interest

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

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2023.108647>.

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