Short- and long-term effects on growth and expression patterns in response to incubation temperatures in Senegalese sole

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Abbreviations: CDH, cumulative degree-hours; dnmt, DNA methyltransferases; dph, days post-hatch; EST, expressed sequence tag; H3, histone 3; HPT, Hypothalamic-pituitary-thyroid; IGFs, insulin growth factors; RA, retinoic acid.

ABSTRACT

In this study, the short- and long-term effects of embryo incubation temperatures (16, 18 and 20ºC) on development and growth of the flatfish Senegalese sole (*Solea senegalensis*) was determined by investigating the expression patterns of the epigenetic regulators DNA methyltransferases (dnmt) and histone 3 (H3) and genes belonging to the retinoic acid (RA), insulin-like growth factors (IGFs) and hypothalamic-pituitary-thyroid (HPT) axes. Results indicated that egg incubation temperature affected embryo development, but not survival, and incubation at 16ºC significantly delayed development. Coincident with these effects, levels of muscle-specific *dnmt3aa* transcripts and histone H3 protein levels were significantly different between the 16 and 20ºC groups at hatch. The larvae from eggs incubated at 20ºC relative to the 16ºC group had significantly higher transcript levels of four genes belonging to the HPT axis (*trhr1a*, *tshr*, *thrb* and *dio2*), four genes of the RA axis *(aldh1a2*, *cyp26a1*, *rara2*, *rarg*), *igfbp1* and the glycolytic enzyme *gapdh2*. Taken together the data suggest that higher egg incubation temperatures enhance energy production, which accelerates cell proliferation and larval development and that hatching is a key moment for the regulation of epigenetic mechanisms. Long-term effects of egg and larval incubation temperatures were revealed by a higher mRNA abundance of the thyroid-related genes *tgb* and *tpo* and the RA degrading enzyme *cyp26a1* in pre- and metamorphic larvae when they were incubated at 20ºC as embryos and may be related to the earlier initiation of metamorphosis in the pelagic larval stages. Evaluation of growth in pelagic larvae and juveniles after weaning (one trial from 42 to 119 and another from 164 to 247 days post-hatch using a longitudinal approach) revealed that juveniles from embryos incubated at 20ºC had a higher growth rate. All these data demonstrate that the thermal regime during embryogenesis modulated mechanisms that regulate larval plasticity and caused imprinting evident in juvenile sole as persistent changes in key endocrinological pathways and growth performance.

Keywords: *Solea senegalensis*; epigenetics; thermal plasticity; Dnmt; histone; growth

1. Introduction

Environmental temperature is a major factor that governs fish development and growth imposing severe changes in metabolism, physiology, behavior and morphology (Pittman et al., 2013). This notable response to environmental temperature is linked to the absence of thermal homeostasis (they are poikilotherms) and the specific evolutionary consequences for development are relatively poorly explored. Normally, early life stages develop faster at higher temperatures due to the modifications induced in molecular and metabolic responses (Campos et al., 2013c; Camus and Koutsikopoulos, 1984; Das et al., 2006; Martell et al., 2006; Politis et al., 2017b; Radonic et al., 2005; Thépot and Jerry, 2015). However, when the environmental temperature is outside of the thermal tolerance range, survival rates decrease and the incidence of malformations substantially increases (Das et al., 2006; Little et al., 2013). In addition to the rapid cellular and metabolic responses to temperature, thermal regimes also modulate embryo and larval phenotype and have long-term effects (also known as developmental or transgenerational plasticity). This epigenetic programing of early life stages has been reported to strongly influence metabolic rates and acclimation capacity, sex determination and muscle development in juvenile fish (Pittman et al., 2013; Schnurr et al., 2014; Schulte et al., 2011). This may be accomplished by DNA methylation, histone modifications or chromatin remodeling (Kim and Kaang, 2017).

DNA methylation is controlled by the DNA methyltransferases Dnmt1 and 3 that are involved respectively in maintenance and de novo DNA methylation and regulate chromatin state transitions (Goll and Bestor, 2005). Moreover, chromatin structure and remodeling is highly dependent on post-translational modifications of histone proteins (mainly methylation and acetylation) that drive stable changes in gene expression patterns and result in different animal phenotypes (Kim et al., 2009; Kim and Kaang, 2017). The histone family has been highly conserved during evolution and there is a high level of redundancy in the genome (Cheung et al., 2000; Maehara et al., 2015; Okada et al., 2005; Ren and van Nocker, 2016). Histone H3 is an important target for epigenetic modifications that affect chromatin structure and remodeling

processes. H3 isoforms include the replication dependent or canonical H3, the replication independent or replacement histones (H3.3) that are uncoupled from DNA replication and expressed throughout the cell cycle and the tissue- and centromere-specific forms (Akiyama et al., 2011; Ren and van Nocker, 2016).

One well studied reprogramming effect of temperature during early development of fish occurs in muscle and leads to modified fibre composition and growth patterns in adults (Johnston, 2006). A long-lasting influence of rearing temperature on muscle structure and somatic growth related the modifications induced in hypertrophy and hyperplasia of muscle fibres has been reported for several fish species (Alami-Durante et al., 2007; Albokhadaim et al., 2007; Johnston et al., 2009; Johnston et al., 2003; Johnston et al., 2000a; López-Albors et al., 2008; Macqueen et al., 2008; Steinbacher et al., 2011). However, temperature during early development can also influence other traits such as appetite and feeding behavior in juvenile fish (Albokhadaim et al., 2007) and sex differentiation, which causes skewed population ratios of sex in some species (Chen et al., 2014; Luckenbach et al., 2009; Navarro-Martín et al., 2011; Piferrer and Guiguen, 2008; Wen et al., 2014). Recently, the role of thermal imprinting during early development was shown in sea bream and was associated with a modified bone response to a cold challenge in juveniles, which was associated with modifications in the response of the thyroid, IGF-GH and cortisol axes (Mateus et al., 2017a). Additionally, in the sea bream and sea bass adult stress responsiveness was also significantly modified when eggs and larval fish were reared under different thermal regimes (Fokos et al., 2017; Mateus et al., 2017b). All these data indicate that the temperature regime during fish development modifies their developmental trajectory. To understand how early temperature regimes in hatchery stages affect juveniles and aquaculture productivity more studies are urgently needed.

Senegalese sole (*Solea senegalensis*) is an eurytherm flatfish that has optimal survival and growth rates over a wide thermal range (from 13 to 28°C) in the wild (Vinagre et al., 2006). It is an increasingly popular aquaculture species in the Mediterranean due to its high commercial value

and its prolonged reproductive season (in spring and autumn) since spawning can occur over a broad thermal range, 13 and 23 ºC, although fecundity is highest between 15-21 ºC (Anguís and Cañavate, 2005). This extremely wide thermal range for spawning means that embryonic and larvae development may occur under highly divergent environmental conditions with consequences for physiological traits. Campos et al. (2013c) demonstrated that larval growth, muscle phenotype and the expression pattern of myogenesis-related genes were different in post larval sole of larvae reared at 15 to 21ºC until hatch. Moreover, Campos et al. (2013b) demonstrated that metamorphic larvae reared at 15ºC had increased methylation of the *myog* promoter and its expression was lower in the skeletal muscle when compared to larvae grown at higher temperatures (18 and 21ºC). However, lower temperatures during the pelagic stage resulted in reduced larval survival at settlement but not at 100 dph (Campos et al., 2013a).

In addition to the effect of epigenetics on growth, Blanco-Vives et al. (2011) demonstrated that daily thermocycles oscillating between 19-22ºC from hatch to 97 dph had a strong impact on the timing of gonad differentiation and sex ratios in sole populations. Moreover, incubation temperature was a key regulator of bone development and the incidence of skeletal deformities in sole juveniles (Dionísio et al., 2012). All these data demonstrate that in early developmental stages temperature can program key production traits in early developmental stages of sole and this makes the species an interesting model for understanding epigenetic mechanism behind thermally induced phenotypic plasticity and how this may impact on aquaculture performance. In this context, the present study aimed to: i) quantify the expression of DNA methyltransferases and histone H3 during embryogenesis and establish their regulation by temperature; ii) quantify the short- and long-term responses of genes modulating growth and metamorphosis, eg. HPT, GH-IGF and RA axes, in response to incubation temperatures; and iii) evaluate the long-term effects of incubation temperatures on somatic growth and metamorphosis. The knowledge generated in this study will provide new data of interest for the aquaculture industry since it will reveal how manipulation of temperature during embryonic development may benefit somatic growth in juveniles and adults.

2. Materials and Methods

2.1 Fish trials

2.1.1 Embryo incubation trial and larval rearing

All procedures were authorized by the Bioethics and Animal Welfare Committee of IFAPA and given the registration number 06–11–15-337 by the National authorities for regulation of animal care and experimentation.

Fertilized eggs for Senegalese sole were obtained from CUPIMAR (San Fernando, Cadiz, Spain). Broodstock was fed daily with polychaeta, mussels and squid (~1% biomass). Eggs were collected early in the morning (9:00 a.m.) and transferred to a 1,000 mL measuring cylinder to separate buoyant (viable) from non-buoyant (non-viable) eggs. The number of viable eggs in each fraction was estimated using a volumetric method (1,100 eggs mL^{-1}). Water temperature and salinity in the broodstock tank (20 animals; ratio 2M:1F) during spawning were 18ºC and 32 ppt, respectively. Fertilized embryos were collected and randomly distributed between nine cylindro-conical tanks (500 L) at a density of 140 eggs L^{-1} in an open seawater circuit supplied with gently aerated seawater. The temperatures selected to evaluate the effects of thermal reprogramming were based on the thermal range, 16-20ºC, tolerated for sole reproduction and used in the hatchery stage by the Mediterranean aquaculture industry. When embryos were at the beginning of gastrula (50% epiboly), the water temperature was shift progressively from 18ºC to the target incubation temperatures, 16ºC, 18ºC and 20ºC. The temperature change occurred over 1h by mixing water at 20ºC (well water) and 13ºC (cooled using a water cooler carrier) and treatments were carried in triplicate tanks. During the experiment the temperature was continuously recorded with temperature data loggers (HOBO PENDANTs Onset Computer Corporation, Massachusetts) located in each of the experimental tanks. The average temperatures for each treatment group were 15.5 ± 0.9, 18.0 ± 0.3 and 20.3 ± 0.2 (Suppl. file 1). The embryos exposed to the 18ºC and 16ºC treatments were maintained at these water temperatures for 42h and 52h, respectively to ensure the change in thermal regime occurred at the same developmental stage in all temperature

treatment groups. Thereafter, the water temperature in all experimental tanks was increased in approximately 1 h to the temperature normally used by industry (~20ºC) (Fig. 1).

To monitor embryo development, tanks were sampled every hour or every two hours after temperature treatments were initiated. The developmental stage of the embryos (10-20 embryos per sampling point) was recorded using a Nikon SMZ800 dissecting microscope connected to a Leica DC320 camera. At each sampling time point a pool of embryos (n = 30) was collected from each incubator and snap frozen in liquid nitrogen. The embryonic stages were classified according to Kimmel et al. (1995). To facilitate the monitoring of embryonic development during the experiment, a developmental index, similar to the metamorphic index developed by Fernández-Díaz et al. (2001), was assigned to each sample (Suppl. file 2). As development of the sole embryos and larva is not fully synchronous, the score assigned to each sample collected from the triplicate tanks of each experimental group was corrected by the percentage of embryos in each developmental stage. At hatch, all the larvae were transferred to nine 400L tanks at an initial density of 50 to 60 larvae L^{-1} and cultured at 20ºC until 20 days post-hatch (dph) as previously described (Fernández-Díaz et al., 2001). At hatching and during larval rearing, the survival rates of each experimental group was estimated and larvae were sampled at 3, 7, 11 and 20 dph to measure dry weight as described in Fernández-Díaz et al. (2001). To follow metamorphosis, two pools of larvae (n= 20-30 larvae) between 11 to 20 dph were taken from each of the triplicate thermal regime tanks, euthanized in MS-222 (200 ppm) and one pool used to determine the metamorphic index (Fernández-Díaz et al., 2001) and the other was rinsed and placed in RNA-later for subsequent gene expression analysis. Before expression analysis, all larval samples were classified according to their metamorphic stage, S0, S1, S2, S3 and S4 (Fernández-Díaz et al., 2001).

To quantify the expression of histone 3 (H3) during development under different thermal regimes, 2,000 embryos in gastrula (50% epiboly stage) were incubated in triplicate 1L beakers at 16ºC and 20ºC. Incubation conditions and management procedures were those described in Firmino et

al. (2017). To compare equivalent larval development stages for the two thermal regimes, the sampling time was corrected to take into account differences in water temperature using cumulative degree-hours (CDH) as follows: Gastrula 12 CDH; pharyngula 66 CDH; hatching 140 and 210 CDH. Embryo and larvae were sampled and stored in RNAlater as indicated above for gene expression analysis.

2.1.2 Juvenile trials for growth evaluation

After completion of metamorphosis, sole post-larvae (20 dph) were pooled by thermal regime and transferred to new five-m^2 circular tanks in an open seawater circuit with continuous aeration at an initial density of 3.000 individuals m^{-2}. They were cultivated following the standard production procedures established in CUPIMAR aquaculture facilities according to Cañavate and Fernandez-Diaz (1999). Weaning was completed at 40 dph. Total tank water renewal occurred approximately every two hours to ensure tank self-cleaning. Animals were provided with dry feed (Gemma Micro Skretting, Spain) and all tanks received the same ration, which corresponded to 3-4% of the total tank biomass. The water temperature (20ºC) and salinity (20 ppt) were optimal and maintained stable throughout the experiment. Growth was monitored from 42 to 119 dph and the weight increase was determined by weighing at least 20 juveniles from each tank at 42, 74, 91 and 119 dph. The individual wet weight of larvae was measured and later photographed to determine total length using ImageJ v1.47 software as previously described (Firmino et al., 2017). At the end of the growth trial, 200 specimens from the 16ºC and 18ºC thermal regime and 100 specimens from the 20ºC thermal regime were euthanized, dissected and the sex recorded.

To further validate the long-term effects of incubation temperature on growth, sole juveniles at 119 dph (~120 specimens from each triplicate tank/thermal regime) were moved from CUPIMAR aquaculture facilities to El Toruño (El Puerto de Santa María, Cádiz, Spain). Animals from the 16ºC and 18ºC thermal regimes were acclimated to separate 1 m^2 circular tanks in an open seawater circuit for 20 days and then 220 sole of a similar size from each thermal regime were tagged using a nano intraperitoneal (IP) transponder (Trovan®). Only 110 specimens from the

20ºC thermal regime group were available due to a technical problem in one tank. The different thermal treatment groups were maintained in self-cleaning rectangular tanks (1.0 m × 0.5 m, surface = 0.2 m^2) in an open seawater circuit using the same conditions outlined above. Daily mortality and tag loss was recorded over 15 days. Water temperature and salinity were 20.1 ± 0.5 ºC and 37 ppt, respectively. Fish were treated weekly with a hydrogen peroxide (100 ppm) bath to facilitate IP wound healing and prevent disease. All the fish recovered and the tag retention rates were higher than 80%. At 164 dph, 177, 171 and 87 tagged individuals from the 16ºC, 18ºC and 20ºC treatments, respectively were pooled together and redistributed between three self-cleaning rectangular tanks as indicated above and the growth trial initiated. The starting average size was 0.885 ± 0.139g, 0.903 ± 0.144g, 0.915 ± 0.143g for the 20ºC, 18ºC and 16ºC experimental groups, respectively. The length and weight of individual fish was measured at 25, 40, 60 and 83 days after starting the trial (Fig. 1). Fish identity (read using the nano transponder), weight and length were automatically recorded using the FISH Reader Weight (Zeuss, Trovan, Spain). The average accumulative mortality during the growth trial was 13% and the mortality of each of the three experimental groups were randomly and equally represented in each of the three replicated experimental tanks.

2.2 RNA isolation and RT-qPCR analysis

For gene expression analysis, only embryos and larvae samples from the thermal regimes at 16 and 20ºC were selected. Pools (~40 mg wet weight) of embryos (n=20) and hatched larvae (n=15) and S0 and S4 metamorphic sole larvae (n = 5) were collected from each experimental tank (n=3 per thermal treatment) . Samples were homogenized using a Fast-prep FG120 instrument (Bio101) and Lysing Matrix D (Q-Bio- Gene) for 40s at speed setting 6. Total RNA was isolated using the RNeasy Mini Kit (Qiagen). All RNA isolation procedures were carried out in accordance with the manufacturer's protocol. To avoid DNA contamination total RNA was treated twice with DNase I using an RNase-Free DNase kit (Qiagen). RNA quality was checked by agarose gel electrophoresis and quantified using a Nanodrop ND-8000 (Thermo Scientific).

For DNA methyltransferases (*dnmt*) analysis total RNA (1 µg) was reverse-transcribed using an iScript™ cDNA Synthesis kit (Bio-Rad) according to the manufacturer's protocol. Real-time analysis was carried out on a CFX96™ Real-Time System (Bio-Rad). Real-time reactions were performed in duplicate in a 10 µl reaction volume containing cDNA generated from 10 ng of RNA template, 300 nM each of specific forward and reverse primers, and 5 µl of SYBR Premix Ex Taq (Takara, Clontech) as previously described (Firmino et al. (2017). *dnmt* expression data was normalized using the geometric mean of 18S rDNA and *actb1* both of which had a stable expression across the experimental samples. A qPCR-openarray based on 56 × 48 Taqman probes was used to determine gene expression of 56 transcripts related to: i) retinoic acid (RA) metabolism (17), ii) hypothalamus-pituitary-thyroid axis (HPT; 13), iii) the GH-IGF (20) axis and iv) other endocrine regulatory functions (3). The cDNA synthesis, qPCR reactions and data analysis were done as previously described (Boglino et al., 2017). Data were normalized using the geometric mean of *eef1a* and *ub52* (Infante et al., 2008). Relative mRNA expression was determined using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001)

2.3 Western Blot analysis for H3

2.3.1 Sample preparation

Pools of embryos and larvae (n=3 per developmental stage) reared at 16 and 20 ºC were defrosted on ice, weighed and then 5 volumes of extraction buffer (0.8M Urea, 5mM Tris, 10 mM NaCl, pH 7.6) added. Eggs were homogenized using a plastic pestle and a 1.5 ml microcentrifuge tube. Homogenates were left on ice for 20 minutes with occasional mixing and then centrifuged (10,000 rpm, 20 min at 4ºC) and the supernatant collected. Protein in the supernatant was quantified using a colorimetric assay (#500-0006, BioRad, USA) and a standard curve prepared using bovine serum albumin (Quick Start BSA Standard Set, #500-0207, BioRad, USA) and read using a micro plate reader (Benchmark, BioRad, USA) set at the appropriate wavelength (595 nm for protein).

2.3.2 SDS-PAGE and Western blotting

Protein extracts were analyzed by SDS-PAGE (14% polyacrylamide gels) using the Laemmli method (1970) followed by Western blotting. In brief, after electrophoresis of samples the proteins were transferred to nitrocellulose membranes (Bio-Rad, Germany) using 300mAmps constant current. Membranes were rinsed in Tris (0.1M, pH 7.6) and then blocked by placing them in a solution of protein (3% defatted milk power and 2% sheep serum) overnight at 4ºC. Immunodetection of histone H3 was carried out using a polyclonal rabbit anti-human Histone H3 (Sigma-Aldrich, Madrid, Spain, H0164) antisera. In brief, membranes were incubated for 2h at room temperature with rabbit anti-human Histone H3 antisera (1/30,000) followed by 1h with goat anti-rabbit immunoglobulin G conjugated to peroxidase anti-peroxidase (1/80,000, Sigma-Aldrich, A0545). Detection of immune complexes was performed using the ECL™ Prime system (GE Healthcare, UK) and the images captured in the ImageQuant LAS 500 imaging analysis system (GE Healthcare, Sweden). The histone 3-like levels were determined by densitometry using the ImageJ ij148 software. Exclusion of the negative control from immunoblotting reaction (negative control) gave no signal.

The likely cross-reactivity of the anti-human histone H3 antisera with sole histone H3 was assessed by comparing the conservation of the amino acid sequence used for antisera production (aa 125-136). The sequences encoding histone H3 in *S. senegalensis* were retrieved from SoleaDB (Benzekri et al., 2014) and the draft genome and compared to human histone H3 retrieved from HistoneDB2.0 (Draizen et al., 2016). The sole and human histone H3 sequences were aligned using the software suite DNAstar and amino acid sequence the similarity determined. The molecular weight of sole histone H3 was also deduced to confirm that the immunoreactive proteins detected by Western blotting had approximately the same molecular weight as that predicted for sole histone H3 proteins.

2.4 Statistical analysis

All data were checked for normal distribution with the Kolmogorov–Smirnov test as well as for homogeneity of variance with a Levene′s test and when necessary a log transformation was applied. Hatching rates were analyzed using a Kruskal-Wallis test. To identify significant differences in the weight and length of larva and non-tagged juveniles, the average tank weight at each sampling point (n=3) was used in the one-way ANOVA. To compare growth in tagged juveniles, a mixed ANOVA with repeated measures was carried out using thermal treatment and tank as fixed factors. A Greenhouse-Geisse correction was used to evaluate time interaction effects. To identify significant differences in gene expression associated to temperature and embryonic stages, a two-way ANOVA was performed using stage and thermal treatment as fixed factors followed by a Student t-test when significant differences were found. For qPCR-array analysis, all data were log transformed and processed using one- (at hatch) or two-way (metamorphic stages) multivariate analysis of variance (MANOVA) followed by an FDR correction as reported in Boglino et al. (2017). For Western blot analysis of histone H3, a one-way ANOVA was used to identify significant differences in abundance between the same developmental stage in eggs and larvae maintained at 16ºC or 20ºC. Statistical analyses were performed using SPSS v21 software (IBM Corp., Armonk, NY, USA) and Statistix 9 (Analytical Software, Tallahassee, FL, USA).

3. Results

3.1 Short-term effects of incubation temperature until hatching

3.1.1 Effects on embryo development

The development of embryos incubated at 16ºC from gastrula was delayed relative to embryos at 18 and 20ºC (Fig. 2). Embryos incubated at 20ºC from gastrula hatched at 24h after the start of the temperature trial. Embryos incubated at 18ºC and at 16ºC from gastrula hatched at 36h and 48h, respectively. The main developmental delay occurred during the pharyngula stage (Fig. 2).

Hatching rates were not affected by thermal regime and were 76.3 ± 4.0, 60.3 ± 14.7 and 53.6 ± 16.1% for embryos incubated at 20ºC, 18ºC and 16ºC, respectively.

3.1.2 Effects on the expression methyltransferases (dnmt) and histone H3

To assess the effect of incubation temperature on the expression of four *dnmt* genes (*dnmt1*, *dnmt3aa*, *dnmt3ab*, and *dnmt3bb.1*) during embryo development, samples at a similar developmental stage (gastrula, segmentation and hatch) at 16 and 20ºC were selected and transcript levels quantified (Fig. 3). Two-way ANOVA showed that *dnmt3aa* and *dnmt3ab* had a significantly (P<0.05) increased expression from gastrula to hatch whereas *dnmt3bb.1* had a significantly (P<0.05) reduced expression. The high variation for *dnmt1* in gastrula and for *dnmt3ab* at hatch could be due to a small asynchrony between biological replicates. A significant interaction between developmental stage and temperature was found for *dnmt3aa* indicating that hatching larvae from the 16ºC group had significantly higher *dnmt3aa* mRNA levels (P<0.05) than the 20ºC group.

To evaluate if incubation temperature also affected the expression of histones, Western blot was used to quantify the levels of H3 in the 16 and 20ºC groups. An intense band at ~16 kDa was detected by western blotting. The approximate molecular weight of the H3 immunoreactive protein detected by western blotting was in agreement with the deduced molecular weight of Senegalese sole H3 transcripts and genes extracted from the SoleaDB (15.3 and 15.4 kDa for H3 and H3.3, respectively) (Benzekri et al., 2014). Sequence analysis of canonical H3 (identified in two different genome scaffolds) and replacement type H3.3 (distributed in four scaffolds) with respect to the human H3 paralogs revealed they shared amino acid (aa) sequence similarity higher than 95%. Furthermore, sole H3, sole H3.3 and human H3 shared 100% aa sequence conservation of the epitope recognized by the human H3 antisera used in the study (Suppl. file 3). The centromeric cenH3 form, retrieved from the sole genome, which shared only 46.7 and 47.4% similarity with sole H3 and H3.3, respectively, was unlikely to bind the antisera. Analysis of sole H3 expression during embryonic development (gastrula, pharyngula and after hatching at 140 and

210 CDH) revealed significant differences in H3 abundance between embryos incubated at 16 and 20ºC at 140 CDH (just after hatching) (Fig. 4).

Table 1. Differentially expression genes of HPT, GH-IGF and RA axes and glucolytic pathway in hatched larvae after incubation at 16ºC and 20ºC. Data were expressed as the mean fold change (mean±SD, n=3) from the calibrator group (16ºC).

Pathway	Gene	16ºC	20ºC
Thyroid axis	*trhr1a*	1.02±0.14	1.60±0.01
	tshr	1.00±0.04	1.88±0.13
	thrb	1.01±0.09	1.74±0.05
	dio2	1.02±0.14	2.27±0.18
Retinoic acid axis	*aldh1a2*	1.01±0.08	1.84±0.13
	cyp26a1	1.00±0.05	1.63±0.16
	rara2	1.00±0.02	1.27±0.01
	rxra	1.00±0.04	0.79±0.03
	rarg	1.00±0.07	1.28±0.03
IGFs	*igfbp1*	1.00±0.05	1.26±0.05
Energy	*gapdh2*	1.00±0.06	1.63±0.13

3.1.3 Effects on the expression of genes related to HPT, retinoic acid and GH-IGF axes temperature at hatch

A total of 11 out of the 56 genes quantified using a qPCR chip were significantly modified at hatch (Table 1). Four transcripts of the HPT axis (*trhr1a*, *tshr*, *thrb* and *dio2*), five transcripts associated with RA signalling pathway (*aldh1a2*, *cyp26a1*, *rara2*, *rxra*, *rarg*) and two with

339 growth and metabolism, *igfbp1* and *gapdh2*, respectively (Table 1) were differentially expressed
340 at hatch in larvae incubated at 16ºC or 20ºC after FDR correction. The transcript abundance of
341 the differentially expressed genes (except *rxra*) was higher in the 20ºC group.

342 *3.2 Long-term effects of embryo incubation temperature*

343 *3.2.1 Long-term effects on larval growth and metamorphosis progress*

344 After hatching, larval rearing was carried out at 20ºC for the 16, 18 and 20ºC groups. At 96 h
345 post-hatch, the total larval length was significantly (P<0.05) shorter (3.12 ± 0.18 mm) in larvae
346 from the 16 ºC treatment relative to 18ºC (3.28 ± 0.13) and 20ºC (3.32 ± 0.17). Significant
347 differences in larval size were still evident at 7 dph when all larvae had been transferred to 20ºC
348 (Fig. 5A) and the dry weight of larvae from the 16ºC group (0.072 ± 0.012 mg larvae^{-1}) was
349 significantly (P< 0.05) lower than the 18ºC (0.095±0.005 mg larvae^{-1}) and 20ºC groups (0.09 ±
350 0.007 mg larvae^{-1}). However, larval dry weight was no longer significantly different at the
351 beginning (10 dph) or end of metamorphosis (20 dph). When larval length was determined, larvae
352 from the 16ºC treatment were significantly (P<0.05) shorter than the 20ºC group throughout the
353 trial. At the end of metamorphosis, the larvae from the 20ºC treatment group were significantly
354 (P<0.05) longer than the 16 and 18 ºC groups (Fig. 5B). All larvae from the temperature treatment
355 groups successfully completed metamorphosis without visual malformations (Fig. 5C), although
356 in the 16ºC group a delay in the start of metamorphosis occurred. Survival rates at metamorphosis
357 were similar across treatments and ranged between 65.9 and 66.4%.

358 *3.2.2 Long-term effects of incubation temperature on the expression of genes related to*
359 *the HPT, retinoic acid and GH-IGF axes during metamorphosis*

360 Expression analysis of 56 transcripts related to endocrine pathways before start of metamorphosis
361 (S0: 11 and 12 dph for larvae from 16 and 20ºC, respectively) and in post-metamorphic larvae
362 (S4: 17 and 19 dph, respectively) revealed that *thrb*, *slc5a5*, *cyp26a1*, *igf1*, *igfbp2* and *igfbp5*
363 increased significantly at the end of metamorphosis. Two genes involved in TH synthesis, *tgb* and

tpo, and the RA degrading enzyme *cyp26a1*, were significantly (P<0.05) more abundant in larvae from the 20 ºC group relative to the 16ºC group (Fig. 6).

3.2.3 Long-term effects on juvenile growth

To evaluate the effects of egg incubation temperature on juvenile growth, post-larvae (maintained at 20ºC) were weaned at 40 dph. Weaning survival rates ranged between 33.6 and 37.5% for the three treatment groups. No differences in wet weight or standard length existed between the 16, 18 and 20ºC groups at 42, 74 and 91 dph (Fig. 7A), although by 119 dph the juveniles from the 16ºC (3.29 ± 0.52 cm) group were significantly (P<0.05) smaller than the 18ºC (3.65±0.59 cm) and 20ºC (3.63±0.61 cm) groups (Fig. 7A). The sex ratio determined at 119 dph revealed that the percentage of males was 67.1±3.3%, 73.0±1.4%, and 71.4±13.4% for 16ºC, 18ºC and 20ºC, treatments, respectively.

To establish the effect of egg incubation temperature on growth potential the growth rate of the IP tagged mixed population of size matched sole from the three temperature treatment groups was determined from 164 to 247 dph (Fig. 7B). Accumulated mortality throughout the experiment ranged from 15.6 and 18.9%, and occurred randomly after sampling. A rapid dispersion in the size range of sole during grow-out was observed (Fig. 7B). ANOVA with repeated measures showed a significant time×treatment interaction $F(3.70, 675.0) = 3.47$, P=0.01 and a significant reduction in weight gain of soles from the 16ºC treatment relative to the other two groups was confirmed (Fig.7B).

4. Discussion

The short and long-term effects of embryo incubation temperature on growth, development and expression of gene transcripts associated with endocrine pathways and epigenetic regulating mechanisms in sole were investigated. The incubation trial showed that higher temperatures

accelerated the progress of embryo development (as determined by the time they entered in blastula, pharyngula, segmentation and hatch stages). This association between environmental temperature and hatching time has previously been reported in other fish species including sole (Campos et al., 2013c; Camus and Koutsikopoulos, 1984; Martell et al., 2005; 2006; Radonic et al., 2005; Thépot and Jerry, 2015). The delay of ~10-15h between the hatching times reported by Campos et al. (2013c) and in our study using a similar thermal range may be due to the developmental stage at which the experiments commenced (3 h in blastula Campos et al. (2013c) and 8h in mid-gastrula in our study). In both studies, no significant differences in hatching rates were found (range 44.2-52.9% (Campos et al., 2013c) and 53.6-76.3%, this study) indicating that the thermal range assayed was relevant for implementation of programming strategies in commercial hatcheries.

DNA methylation is a major epigenetic mechanism that modifies gene expression patterns under different temperatures and controls fish larval plasticity (Campos et al., 2014; Campos et al., 2013b; Navarro-Martín et al., 2011). DNA methyltransferases encoded by *dnmt1* and *dnmt3* paralog genes control DNA methylation and have different spatio-temporal expression patterns in embryos and lecitotrophic larvae and are proposed to regulate the formation of organs such as the eye, muscle, brain, kidney, digestive organs, and/or hematopoietic cells (Campos et al., 2013b; Firmino et al., 2017; Seritrakul and Gross, 2014; Takayama et al., 2014). A previous study in sole demonstrated that the expression of the *dnmt1* and *dnmt3ba* is modulated by temperature in metamorphic larvae and change the methylation patterns of the *myog* gene promoter (Campos et al., 2013b). Our results demonstrate that the muscle-specific isoform *dnmt3aa* was specifically up-regulated at hatch in embryos incubated at low temperature confirming previous observations in lecitotrophic larvae (Firmino et al., 2017) and suggesting that the thermal regime can modify methylation patterns. Changes in DNA methylation may modify myogenesis and several other developmental processes in embryos and larvae and lead to overt changes in the characteristics of juvenile and adult fish (Burgerhout et al., 2017; Macqueen et al., 2008; Mateus et al., 2017a; Mateus et al., 2017b). Moreover, the results of our study on sole suggest that the environmental

conditions at hatch when larvae are released from the egg chorion is a key developmental moment for DNMT regulation and we propose can be exploited by hatcheries to improve aquaculture production.

Chromatin replication and remodeling are essential to propagate epigenetic modifications that have persistent long-term (and intergenerational) effects on gene expression (Kim et al., 2009; Kim and Kaang, 2017). Histone H3 is a key component of the nucleosome and highly influenced by post-translational modifications. A genome and EST scan in sole identified two canonical H3 genes, five H3.3 genes and the centromeric CeH3. These genes were highly conserved with respect to their counterparts in mammals (Cheung et al., 2000; Maehara et al., 2015; Okada et al., 2005; Ren and van Nocker, 2016) including the epitope recognized by the commercial H3 antisera used in this study (Rivera-Casas et al., 2017). Western blot identified a highly abundant protein with expected size of sole H3 (15.3-15.4 kD) as well as a smaller secondary band, probably an enzyme cleavage product (Howe and Gamble, 2015). The organogenesis and associated processes during embryogenesis require an active cell proliferation, differentiation, migration and chromatin remodeling with increased levels of H3 for DNA replication and nucleosomes formation (Bogenberger and Laybourn, 2008; Mazurais et al., 2011). Interestingly, H3 was also differentially expressed in response to temperature at hatch. Although the approach taken did not identify post-translational modifications, the data obtained indicated higher levels of H3 in embryos maintained at 20ºC, which may be indicative of accelerated cell proliferation. This also ties in with the results of previous studies that reported thermal imprinting during embryonic development increased muscle precursor cell proliferation and differentiation and resulted in enhanced growth in adults (Johnston et al., 2000b; Matschak and Stickland, 1995; Steinbacher et al., 2011). Further research will be necessary to identify if the changes in H3 identified are also associated with histone modifications or a switch in the H3 isotype to enhance and better modulate the epigenetic changes during embryogenesis in sole.

Expression analysis of endocrine pathways revealed clear short-and long-term responses associated with modified thermal regimes during embryogenesis. At hatch, coordinated activation of genes related to HPT (4 genes) and RA axes (4) as well as the *igfbp1* and the glycolytic-related *gapdh2* occurred in larvae incubated at 20ºC. These HPT and RA axes play a key role in fish embryogenesis controlling cellular proliferation, differentiation and apoptosis and also crosstalk to modulate signaling pathways (Boglino et al., 2017; Bohnsack and Kahana, 2013; Power et al., 2001). The activation of thyrotropin and thyroid hormone receptors and deiodinases indicate an early response to thermal regime by of the thyroid axis. The early activation of genes related to the thyroid, IGF-GH axes and heat shock proteins at temperature higher than the normal incubation temperature was also identified in *Anguilla anguilla* and linked to accelerated organogenesis (Politis et al., 2017a; Politis et al., 2017b). Moreover, the increased mRNA levels of the glycolytic enzyme *gapdh2* in sole at 20ºC is suggestive of a switch to aerobic glycolysis to meet the energy demands of rapid cell proliferation and formation of skeletal muscle precursors (Tixier et al., 2013). All the qPCR data indicate that higher temperatures during embryogenesis accelerate not only development but also shift gene expression patterns linked to energy production, organogenesis and cell differentiation.

In addition to the thermally induced gene expression patterns at hatch, we found persistent effects on genes related to the thyroid (*tgb* and *tpo*) and RA degradation (*cyp26a1*) in metamorphic stages. Both the thyroid and RA axes play a key role in flatfish metamorphosis and control asymmetric pigmentation and eye migration as well as thyroid follicle development, skeletogenesis and mineralization (Fernandez et al., 2017; Shao et al., 2017). We hypothesize that the increased expression of *tgb* and *tpo* both of which are involved in central TH biosynthesis is associated with an enhanced activity or number of thyroid follicles that in turn promote the production of THs. The earlier initiation of the TH driven metamorphosis, observed in sole larvae incubated at 20ºC relative to 16ºC in this study, supports this hypothesis. Moreover, the HPT-axis also plays a key role in larval thermal adaption and is sensitive to temperature in early stages of European eel development (Politis et al., 2017b). The observation that the embryo and larval

incubation temperature influences the response of the bone to thermal challenge in juvenile sea bream and is associated with the differential expression of TH receptors alpha and beta (Mateus et al., 2017a) identifies the HPT as a key regulatory axis involved in embryonic thermal imprinting response. In contrast, thermal imprinting in sole embryos had no effect on IGF transcript abundance in metamorphic larvae indicating that they have a major role in somatic growth during larval ontogeny but are unlikely to be important in thermal imprinting (Campos et al., 2013c; Politis et al., 2017a).

Incubation temperatures had no effect on the sex ratio but had a profound effect on somatic growth both in the pelagic and benthic stages. In the present study the proportion of males ranged from 67-71%, which is a typical range for the sex ratio routinely found in sole populations produced in commercial aquaculture (Morais et al., 2014; Viñas et al., 2012). In contrast, the growth rate of the pelagic and benthic sole stages up to 247 dph was reduced when embryos were incubated at 16ºC. These results extend previous observations that sole larvae incubated at low temperatures (15ºC) have fewer and smaller muscle fibres than those incubated at higher temperatures (Campos et al., 2013c). Intriguingly, thermal imprinting of sole in our study caused a significant difference in length but not in weight of pelagic larvae. The fast growth rates and the energy demands to successfully fulfill metamorphosis indicate energy is allocated to increase lipid reserves (Hachero-Cruzado et al., 2014; Roman-Padilla et al., 2017) and this may explain the small differences in weight and significant differences in total length. Moreover, the two growth trials in juveniles after weaning in the present study revealed an association with embryo incubation temperature. All these data demonstrate that somatic growth of sole larvae and juveniles is highly influenced by embryo incubation temperature and reveals the importance of the hatchery phase for subsequent performance. Further research will be necessary to confirm if transgenerational plasticity occurs in sole through imprinting of the primordial germ cells (Chen et al., 2014; Pittman et al., 2013).

In conclusion, this study demonstrates that egg incubation temperatures not only modified embryo development but also the initiation of metamorphosis and the growth performance of pelagic larvae and juveniles. Significant changes in the expression of DNA methyltransferases and histone H3 observed at hatch indicates that the contact of the embryo with the surrounding water is a key moment for the modulation of larval plasticity. Moreover, the short and long-term responses of genes related to the HPT, RA and IGF axes indicate there is a coordinated response to temperature, which presumably modulates embryogenesis and is linked to persistent effects mainly on central TH precursors and RA signaling. The present results reveal for the first time in sole that egg incubation temperature has a long-term impact on fish performance and is of high relevance for aquaculture. The results open the door for innovative and low technology strategies to modulate fish performance and complement the classical genetic breeding procedures and will contribute to smart and sustainable production of sole.

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Authors' contributions

MM: planned, supervised and provided funds; CC, JF, LA and SS carried out the experiments and analysis; MM and DMP analyzed, critically reviewed and interpreted the results. MM drafted the manuscript and DMP critically revised the manuscript contents; All authors read, revised and approved the final manuscript.

Declarations of interest

None

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Captions

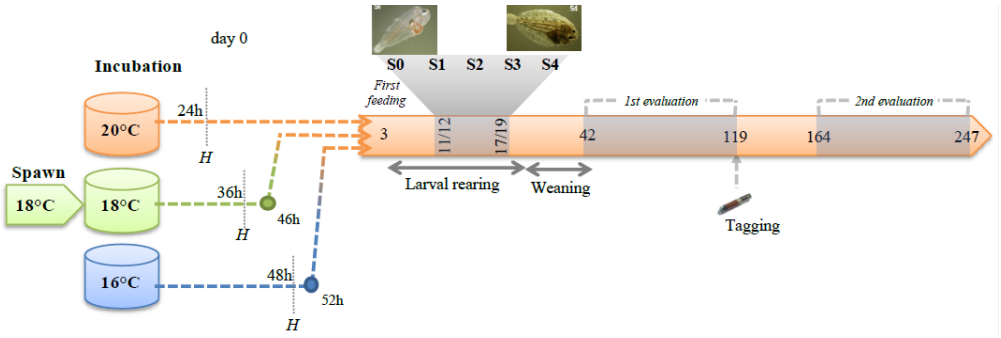


Figure 1. Experimental design to evaluate the short- and long-term effects of embryo incubation temperatures on larval and juvenile sole performance. Embryos were collected in gastrula embryonic stage (50% epiboly) and incubated in triplicate tanks at 16ºC, 18ºC and 20ºC. After hatching, the water temperature of all treatment groups was raised to 20ºC (at 42h and 52h for 18ºC and 16ºC, respectively) and then water temperature was maintained constant at 20ºC thereafter. To evaluate the short-term effects of the treatments, samples were collected in gastrula, segmentation and at hatch. To evaluate long-term effects, larval growth in pelagic stages and during metamorphosis (between 11/12 and 17/19 depending on the incubation temperature) were sampled. Moreover, juvenile growth after weaning between 42 and 119 dph and between 164-247 dph was monitored with intraperitoneally tags.

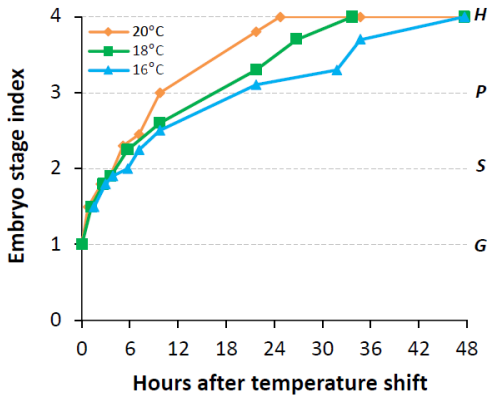


Figure 2. Development of embryos at different incubation temperatures (16ºC, blue; 18ºC; green; 20ºC, orange). Embryo stage index was calculated as indicated in the M&M. The main embryonic

developmental stages are indicated on the right: G; gastrula; S, segmentation; P, pharyngula; H, hatch).

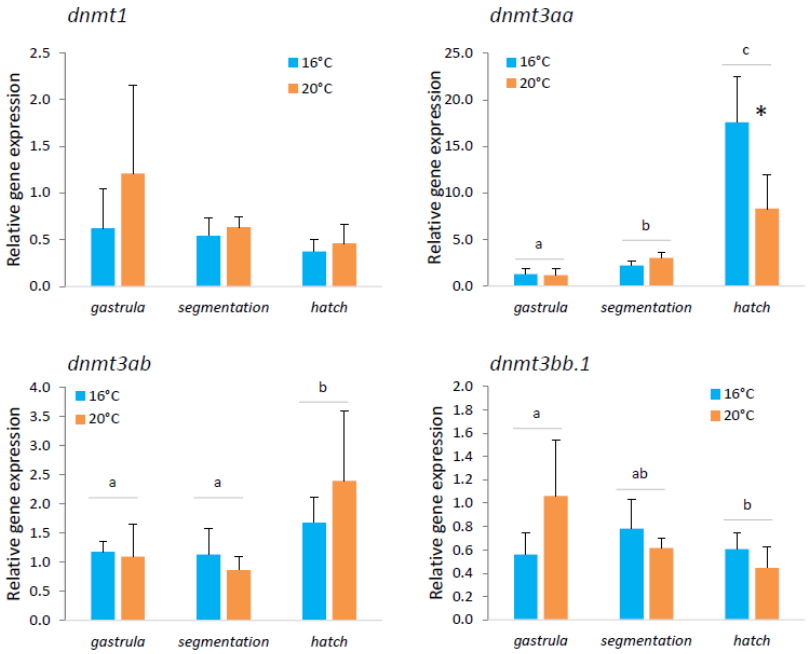


Figure 3. Expression levels of four DNA methyltransferases (*dnmt1*, *dnmt3aa*, *dnmt3ab* and *dnmt3bb.1*) in embryos incubated at 16ºC (blue) and 20ºC (orange). Expression levels were quantified in gastrula, segmentation and at hatch. Data were expressed as the mean fold change (mean±SD, n=3) relative to the calibrator group (gastrula). Different letters denote significant differences among development stages (P<0.05) and the asterisk indicates significant differences between temperatures at a specific developmental stage.

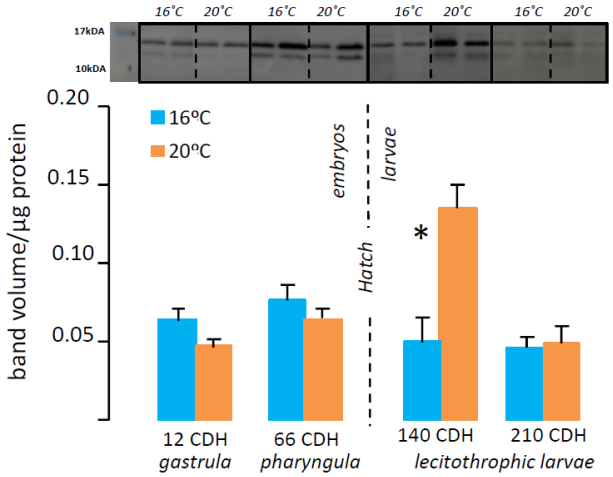


Figure 4. Western blot of H3. Cumulative-degree hour was used to normalize larval developmental stages (gastrula, pharyngula and hatch) between different temperature groups. Densitometry data per stage are expressed as the mean fold change (mean±SD, n = 3). Asterisks denote significant differences between the detected H3 protein levels for the different temperature treatments for the same CDH (P<0.05).

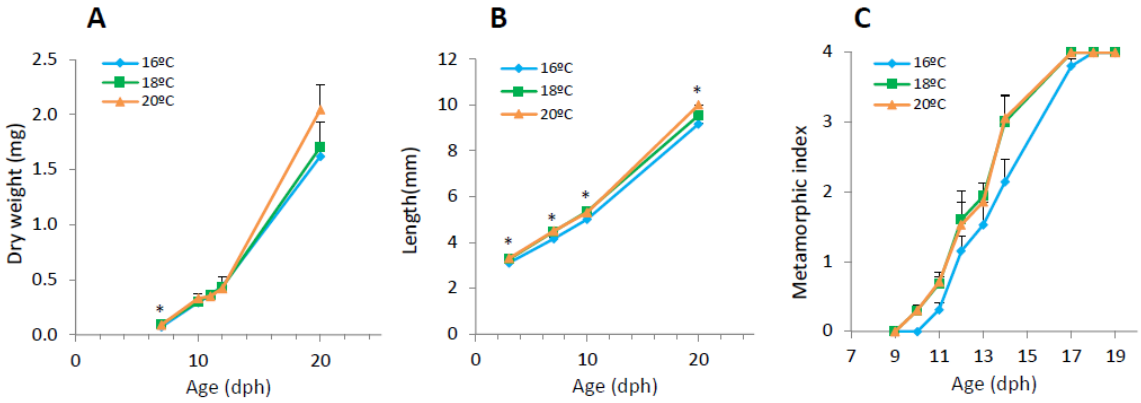


Figure 5. Dry weight (A), total length (B) and metamorphic progress (C) of larvae from embryos incubated at 16ºC (blue), 18ºC (green) and 20ºC (orange) until hatch. Data are expressed as the mean weight±SD (n=3).

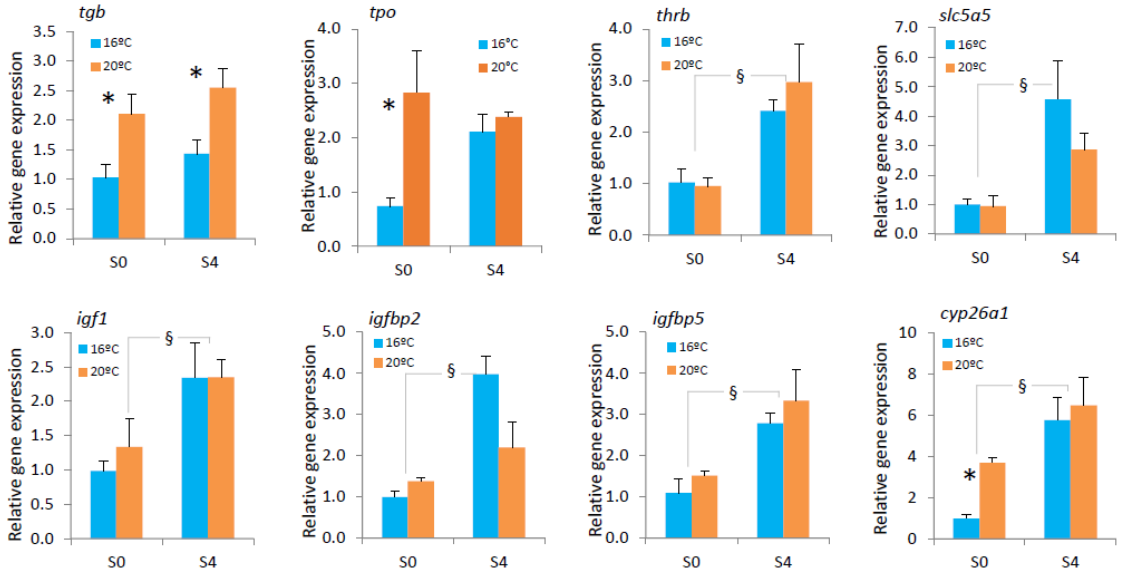
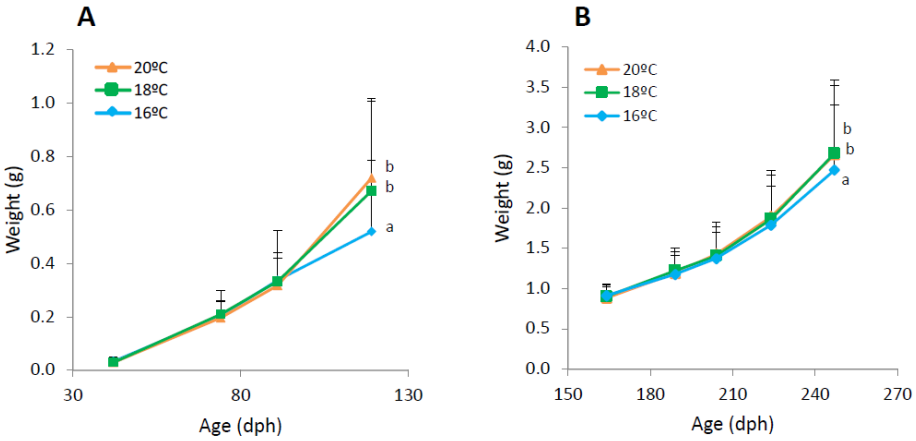


Figure 6. Expression levels of *tgb*, *tpo*, *thrb*, *slc5a5*, *igf1*, *igfbp2*, *igfbp5* in larvae incubated at 16ºC (blue) and 20ºC (orange) before metamorphosis (S0) and at the completion of

777 metamorphosis (S4). Data were expressed as the mean fold change (mean±SD, n=3) from the
778 calibrator group (S0). Asterisks denote significant differences between temperatures at a specific
779 developmental stage and § significant developmental differences.



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781 ***Figure 7.*** Weight of juveniles from embryos incubated at 16ºC (blue), 18ºC (green) and 20ºC
782 (orange). A) Weight at 42, 74, 91 and 119 dph. (n=3); B) Weight of tagged juvenile soles from
783 164 to 247 dph. Data are expressed as the average tank weight±SD (n=3). Letters denotes
784 significant differences among thermal treatments (P<0.05) at a specific sampling point

785 ***Suppl file 1.*** Staging scheme used to monitor the development of embryos. The embryonic stages
786 were classified according to Kimmel et al. (1995).

Stage	Score	Developmental status
Blastula	0.5	All embryos <1k-cell
	0.9	All embryos high-dome
	1.0	30% of embryos in epiboly
Gastrula	1.25	All embryos at 50% epiboly
	1.5	All embryos at 75% epiboly
	1.9	All embryos at 90% epiboly
	2	All embryos at bud
Segmentation	2.25	All embryos at 5-somites
	2.5	All embryos at 14-somites
	2.9	All embryos at 20-somites
	3	All embryos at 26-somites
Pharyngula	3.5	All embryos at Prim15
	3.9	All embryos at Prim25
	4	Hatch

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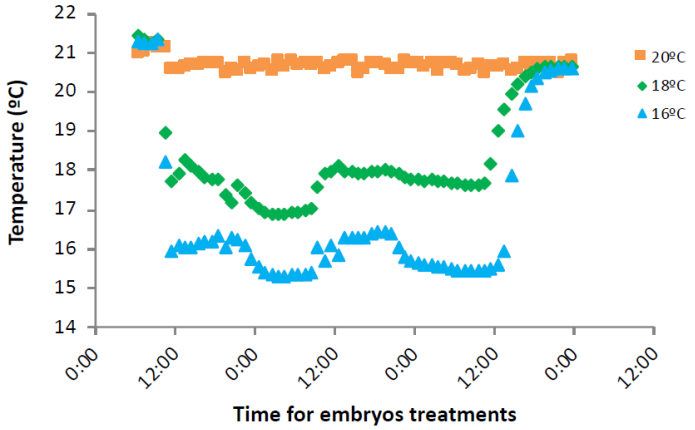
788

Suppl file 2. Temperatures registered during the incubation trial. 16ºC blue; 18ºC green; 20ºC orange.

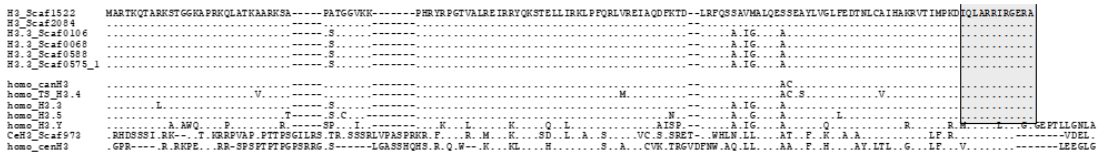


Suppl file 3. H3 sequences in *S. senegalensis*.

A) Type, accession numbers (Merlo et al., 2017[1] and SoleaDB[¥]) for transcripts and genome scaffolds and number of introns for each H3 gene

Type	Transcript	Scaffolds	Introns
Canonical H3	solea_v4.1_unigene 9814[¥] AC275286[1]	Scaf2084	--
	solea_v4.1_unigene192898[¥] AC275288[1]	Scaf1522	--
H3.3	solea_v4.1_unigene616227[¥]	Scaf0068	2
	solea_v4.1_unigene70190[¥]	Scaf0575	2
	solea_v4.1_unigene31341[¥]	Scaf0588	2
	solea_v4.1_unigene 670926[¥]	Scaf0106	2
CeH3	solea_v4.1_unigene32153[¥]	Scaf0973	3

B) Sequence alignments of H3 from Senegalese sole and humans (homo). Data from humans were retrieved from HistoneDB2.0. Dots represent identity. The conserved region recognized by antibody H3 (Sigma Ref H0164) (Rivera-Casas et al., 2017) is shaded



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