Deliverable Report

Deliverable No:	D25.2	**Delivery Month:**	42
Deliverable Title	Mucus defenses of greater amberjack analyzed and immune potential characterized		
WP No:	25	**WP Lead beneficiary:**	P5: UNIABDN
WP Title:	Fish health - greater amberjack		
Task No:	25.1 & 25.3	**Task Lead beneficiary:**	P5: UNIABDN
Task Title:	Identification of immune markers		
Other beneficiaries:	P2 (FCPCT)		
Status:	**Completed**	**Expected month:**	39
…….			

Lead Scientist preparing the Deliverable: Secombes C.J. (UNIABDN)

Other Scientists participating: Montero D. (FCPCT), Milne D.J. (UNIABDN), Acosta F. (FCPCT), Fernández-Montero, A. (FCPCT)

Objective: Identify and describe the immune potential of greater amberjack (*Seriola dumerili*) mucus defenses.

Description: Variation of standardized immune markers has been studied in relation to different culture conditions. Immune potential of mucus defenses has been studied from the systemic point of view (including lysozyme and bactericidal activities, and gene transcript level) and from the histological point of view. The variations of routine aquaculture practices such as stocking density and standardized protocols for weighing the fish (manipulation) together with water temperature have been also evaluated. A histological study of a healthy greater amberjack juvenile was conducted to obtain reference values and slides to be used as a control in other deliverables such as the parasitic infection and the effects of immune stimulants incorporated into functional feeds on this parameter (D25.3).

Effect of routine aquaculture-related procedures

In a first step, a trial was conducted in order to determine how aquaculture-associated stressful conditions are affecting selected parameters. The processes selected were manipulation (handling fish for less than 5 minutes as handling procedures standardized for weighing the fish) and stocking density (kg/m^3), both are related with aquaculture practices and necessary to manage stocks of greater amberjack.

Four variables were considered in this trial 1, taking into account previous standardized protocols for this species in the facilities that University of Las Palmas de Gran Canaria has in the FCPCT:

-High density (HD): Initial density of 8 kg/m^3
-Low density (LD): Initial density of 4 kg/m^3
-High manipulation (HM) level: Weight and length sampling was conducted weekly
-Low manipulation (LM) level: Weight and length sampling was conducted monthly

Four treatments combining these parameters were obtained, placed in twelve 1 m^3 tanks:

-Low density & low manipulation level (LDLM)
-High density & low manipulation level (HDLM)
-Low density & high manipulation level (LDHM)
-High density & high manipulation level (HDHM)

Initial fish whole body weight was 450 ± 75 (mean ± SD) and fish were subjected to the different treatments during 60 days. At the end of the experimental period, a stress challenge test was conducted, consisting of a "shallow water condition" for 1 h in a flow-through regime with a 100% renovation of water each 15 min to avoid effects of low oxygen or high ammonia concentration in the water. Samples of skin mucus where obtained at 1 h, 24 h and 7 days post stress. In addition, samples of skin and gills were also obtained for histological studies, including goblet cell counts.

After 60 days, the treatment HDHM reduced growth performance when compared to LDLM, denoting that greater amberjack is highly sensitive to aquaculture procedures. High stocking density, as described for other species, induced a reduction of growth (see **Figure 1**).

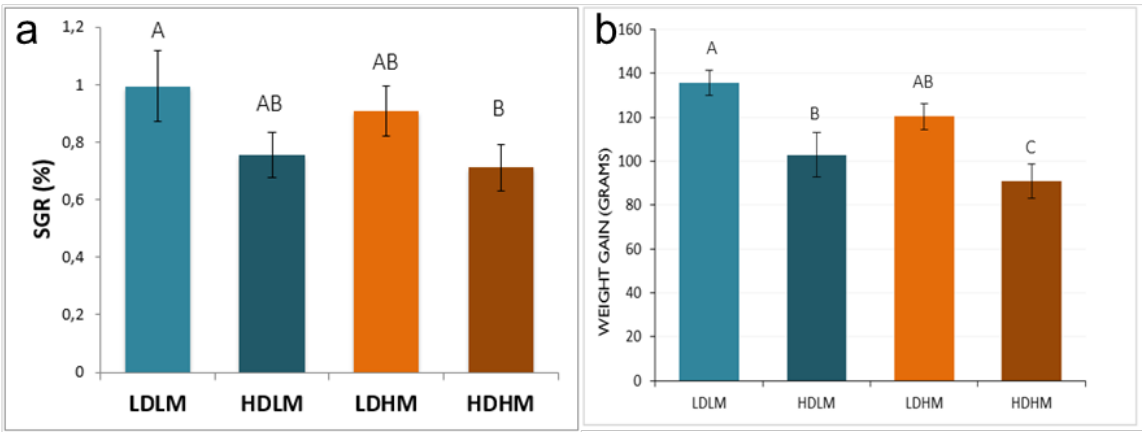

Figure 1. Specific growth rate (a) and weight gain (b) for each treatment; LDLM: low density low manipulation; HDLM: High density low manipulation; LDHM: Low density high manipulation; HDHM: High density high manipulation. Different letters denote significant differences among treatments (P<0.05).

High stocking density induced a decrease in mucus lysozyme activity (see **Figure 2**). A similar effect was obtained for bactericidal activity of the mucus (see **Figure 2**) but no effects were found in peroxidase activity.

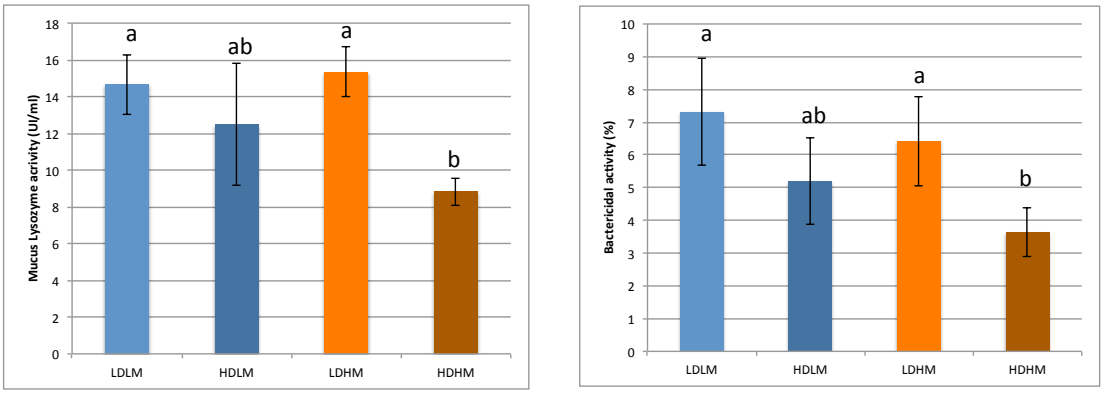

Figure 2. Mucus lysozyme activity (a) and bactericidal activity (b) for each treatment; LDLM: low density low manipulation; HDLM: High density low manipulation; LDHM: Low density high manipulation; HDHM: High density high manipulation. Different letters denote significant differences among treatments ($P<0.05$).

When shallow water stress was applied to each treatment, fish from LDHM treatment had significantly lower ($P<0.05$) plasma cortisol when compared with fish from HDLM treatment after 1 h. Plasma cortisol was higher at 1 h after stress for fish from all the treatments when compared to 24 h and 7 days. At 24 h post stress, fish from HDHM treatment showed significantly ($P<0.05$) higher plasma cortisol. No differences were found in plasma cortisol concentration among fish from different treatments at 7 days after stress (see **Figure 3.a**). No significant differences were found in skin mucus cortisol among fish from different treatments, although fish from LDHM treatment showed the lowest ($P<0.05$) mucus cortisol concentration after 1 h of stress (see **Figure 3.b**). Skin mucus cortisol concentration was shown to be highly correlated to plasma cortisol concentration: $y=73.161x-20.613$; ($P<0.01$), with $R^2 = 0.731$.

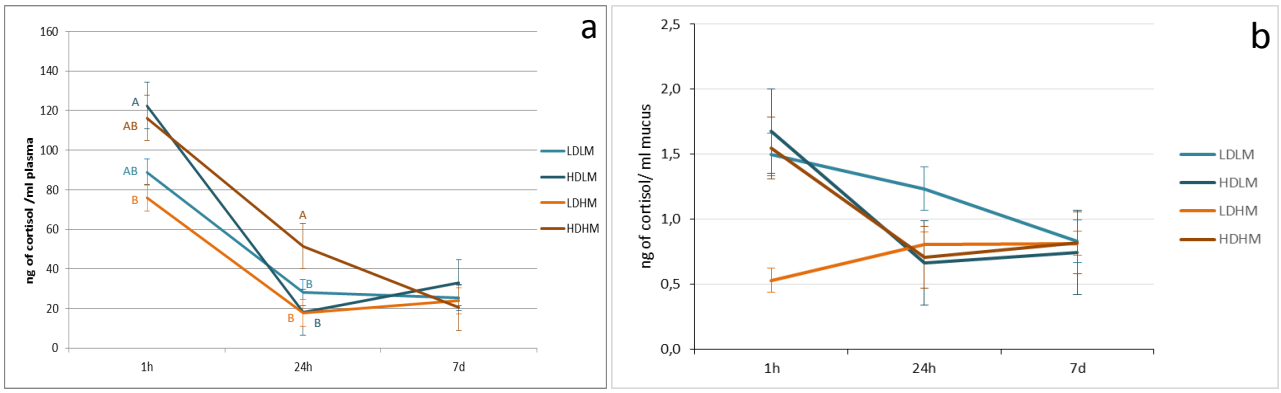

Figure 3. Cortisol levels measured in serum (a) and skin mucus (b) for each treatment; LDLM: low density low manipulation; HDLM: High density low manipulation; LDHM: Low density high manipulation; HDHM: High density high manipulation. Different letters indicate significant differences among treatments ($P<0.05$).

Histological studies showed no effect of either stocking density or manipulation on the number of goblet cells in skin. Although no differences were recorded in the number of goblet cells measured in gills among treatments, fish subjected to LDLM treatment showed a tendency to have more goblet cells after shallow water stress (see **Figure 4**).

	Proximal area		Distal area	
Treatments	1H	7D	1H	7D
LDLM	785,42	579,76	2455,47	2285,56
HDHM	614,57	475,84	2381,23	1915,34

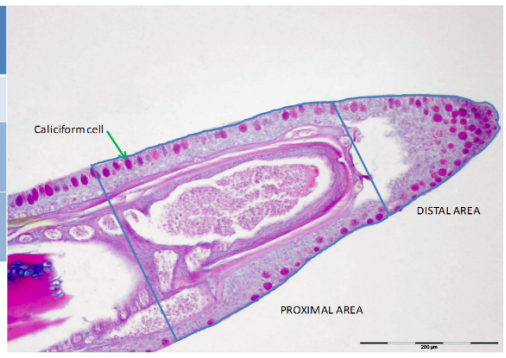

Figure 4. Quantification of goblet cells in gills x nm^2 in distal area and proximal area.

Once the culture conditions were determined for this species, an experiment to study the effect of temperature was conducted. Greater amberjack juveniles were held at three different temperatures in RAS, and some immunological and animal welfare parameters were obtained after 120 days.

Four hundred and fifty greater amberjack juveniles of 19.5 ± 4.1 g body weight and 9.8 ± 0.7 cm body length were distributed in 18 cylindroconical 500 L tanks (25 individuals per tank). Each set of three tanks were controlled by one RAS, with two RAS for each experimental temperature (17, 22 and 26ºC). Fish were fed to apparent satiety for 120 days, with growth data collected each month. At the end of the experimental period, basal cortisol level and immunological parameters where measured in serum and mucus. After that, the system was changed to open circulation for a short period of time, leaving the possibility for spontaneous ectoparasite infections to occur, and data of prevalence and parasite level were recorded.

Fish held at 26º C showed a higher ($P<0.05$) growth performance when compared with fish held at 22ºC. On the other hand fish held at 17ºC showed the lowest ($P<0.05$) final body weight (see **Figure 5**).

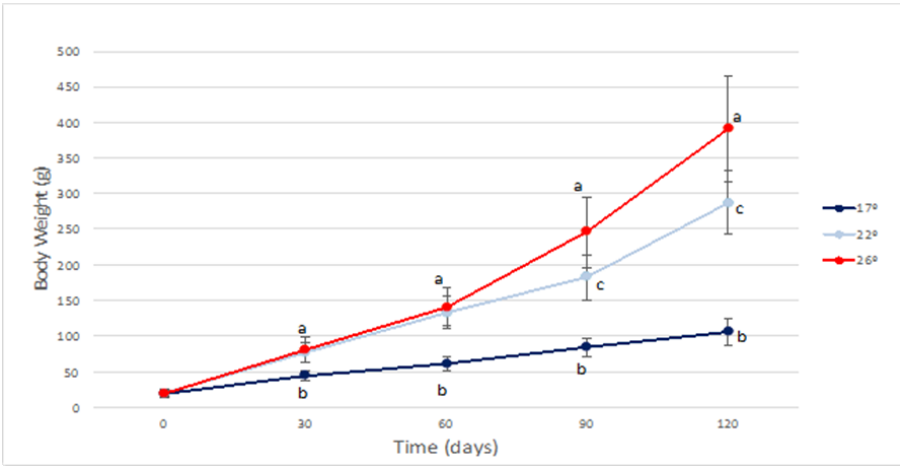

Figure 5. Growth performance among three temperatures over the 120 day trial. Different letters indicate significant differences among treatments ($P<0.05$).

Serum lysozyme and peroxidase activity, as well as basal cortisol level showed no significant differences ($P<0.05$) among different temperatures (**see Figure 6**), denoting the capability of this species to acclimate to those selected temperatures that are within the natural range for this species.

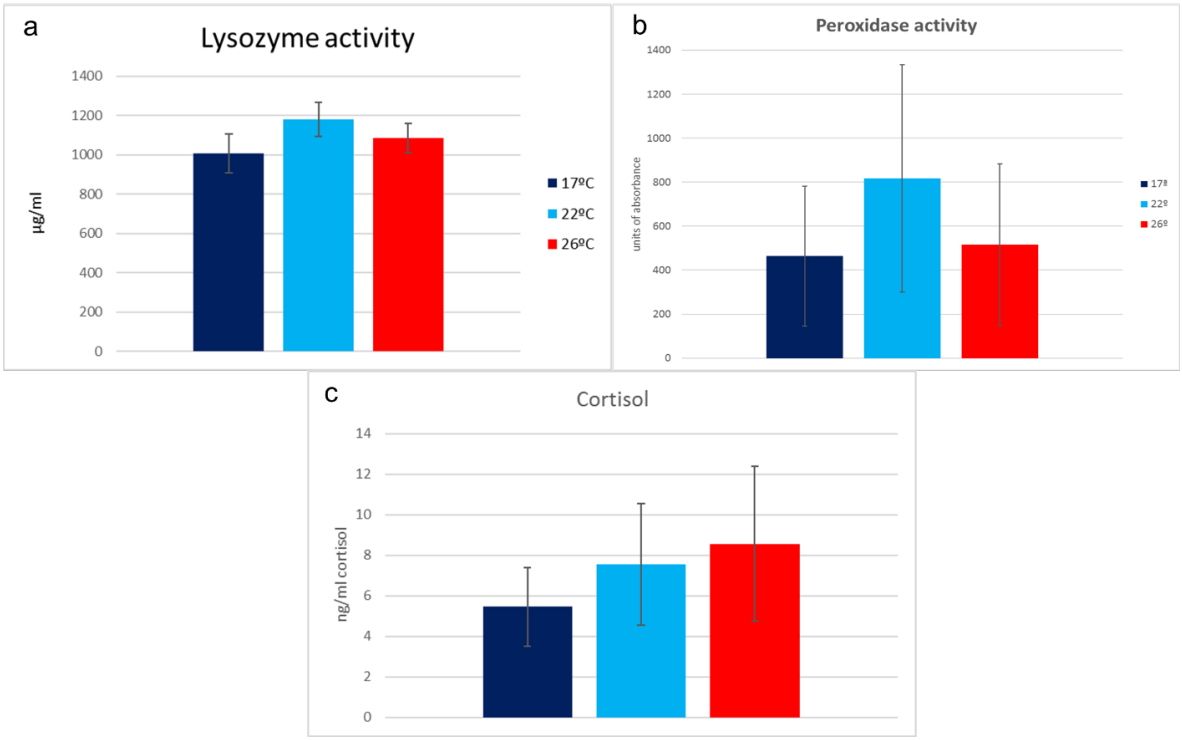

Figure 6. Serum Lysozyme activity (a), serum peroxidase activity (b) and basal cortisol level (c) among three temperatures during the 120 days of trial.

Mucus lysozyme activity was lower in fish held at 17ºC (see **Figure 7**), whereas no effect was obtained in peroxidase activity. Regarding bactericidal activity (see **Figure 8**), although no significant differences were found among fish held at different temperatures, maybe due to the high standard deviation observed, a clear tendency to increased activity was seen when temperature increased.

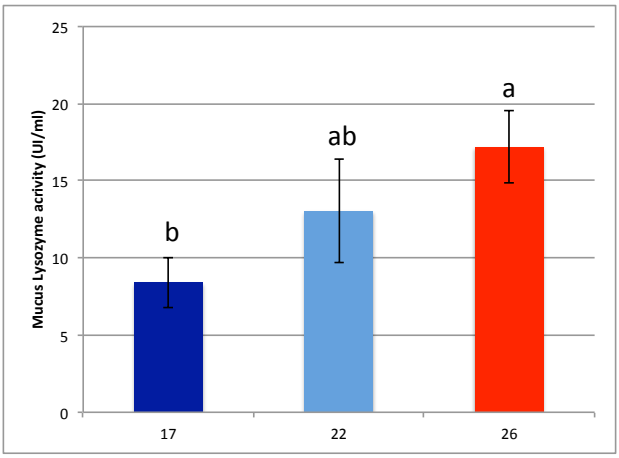

Figure 7. Skin mucus lysozyme activity from fish held at three different three temperatures during the 120 days of trial.

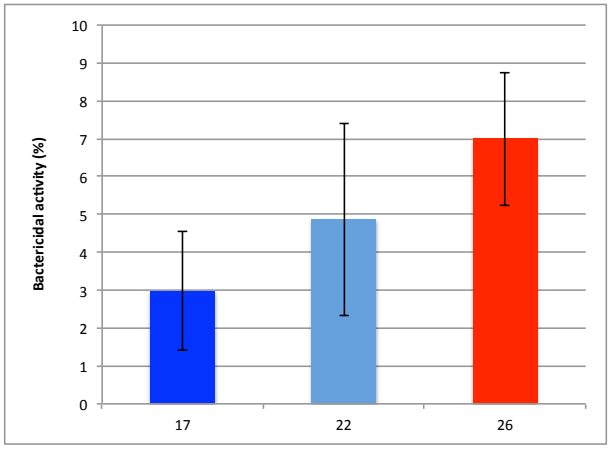

Figure 8. Skin mucus bactericidal activity from fish held at three different three temperatures during the 120 days of trial.

Immune genes potentially involved in mucosal defences were also analysed for their transcript expression level in mucosal tissues (gut and gills) in comparison to classical systemic immune tissues (head kidney and spleen). In the case of immunoglobulins, IgM levels were significantly higher in gut vs gills, but both were lower than in the systemic immune tissues (see **Figure 9**). In the case of IgT it was also higher in gut but although lower than in HK or spleen, these differences were not significant (see **Figure 10**).

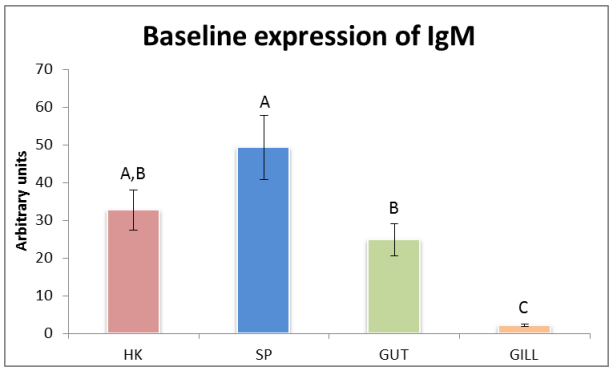

Figure 9. Transcript level of IgM in amberjack head kidney (HK), spleen (SP), gut and gills. Different letters indicate significant differences among tissues (P<0.05). N=10.

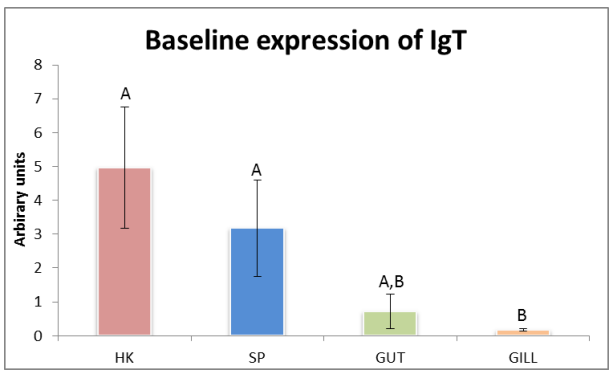

Figure 10. Transcript level of IgT in amberjack head kidney (HK), spleen (SP), gut and gills. Different letters indicate significant differences among tissues (P<0.05). N=10.

The cytokines IL-17 and IL-22 are known to have a role in eliciting antimicrobial defences at mucosal sites, and hence their expression was also analysed. IL-17A/F expression was relatively high in the gut and HK (see **Figure 11**), whilst in contrast IL-17D expression showed the opposite trend with higher expression in gills and spleen (see **Figure 12**). Clearly these different IL-17 isoforms show differential expression between tissues.

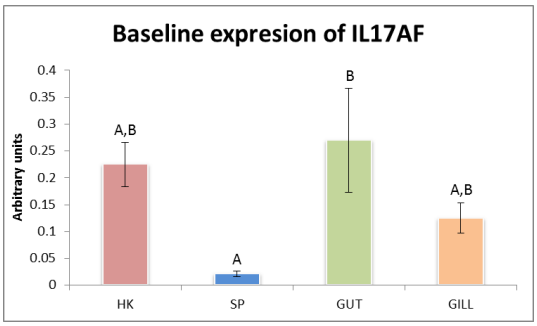

Figure 11. Transcript level of IL-17A/F in amberjack head kidney (HK), spleen (SP), gut and gills. Different letters indicate significant differences among tissues (P<0.05). N=10.

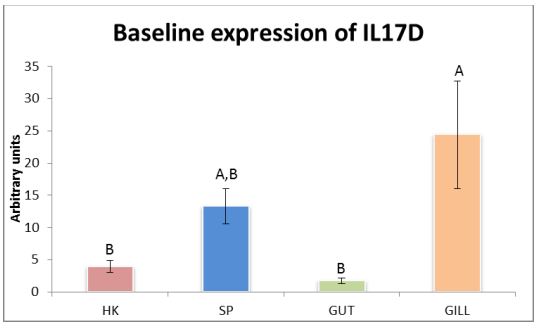

Figure 12. Transcript level of IL-17D in amberjack head kidney (HK), spleen (SP), gut and gills. Different letters indicate significant differences among tissues (P<0.05). N=10.

IL-22 and iNOS were also studied as important immune molecules associated with mucosal defences. IL-22 expression was relatively high in gut and gills, as well as in HK (See **Figure 13**). Interestingly, constitutive expression of iNOS was highest in gills relative to the other tissues, with nitric oxide potentially representing an important antimicrobial agent at this site (see **Figure 14**).

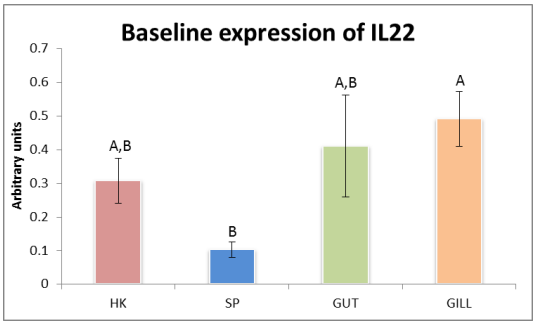

Figure 13. Transcript level of IL-22 in amberjack head kidney (HK), spleen (SP), gut and gills. Different letters indicate significant differences among tissues (P<0.05). N=10.

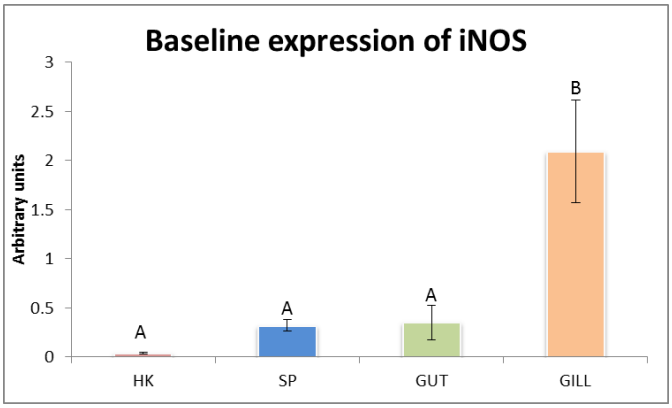

Figure 14. Transcript level of iNOS in amberjack head kidney (HK), spleen (SP), gut and gills. Different letters indicate significant differences among tissues (P<0.05). N=10.

Histological description of greater amberjack immune potential

In this section, a morphological description of the skin barrier is included to provide information of the different aspects of a healthy greater amberjack juvenile to be used in other deliverables, where effects of parasite infection will be evaluated (deliverable 25.3). In **Figure 15**, a standard histological section is shown, to identify the different cells and layers.

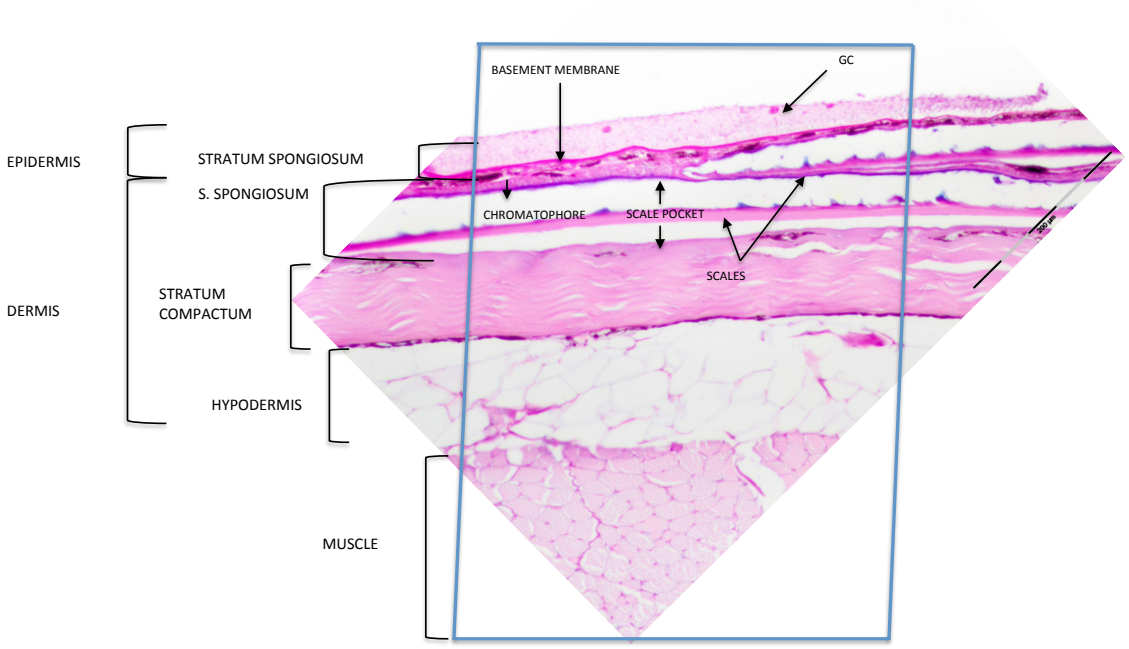

Figure 15. Histological section of the greater amberjack skin includes the epidermis, dermis, scales, hypodermis and muscle. The epidermis includes goblet cells (GC) and secretory cells. The basement membrane (BM) separates the epidermis from the dermis. The dermis is composed by the *stratum spongiosum* or loose connective tissue and the *stratum compactum*. The hypodermis separates the dermis from the muscle layer.

A specific study on epidermis and upper dermis from a healthy greater amberjack juvenile is presented in **Figure 16**. Some epidermal secretory cells can be observed, including goblets cells (GC) and unidentified secretory cells (S). Some infiltrated leucocytes are also identified close to the basement membrane and in the loose connective tissue of the dermis

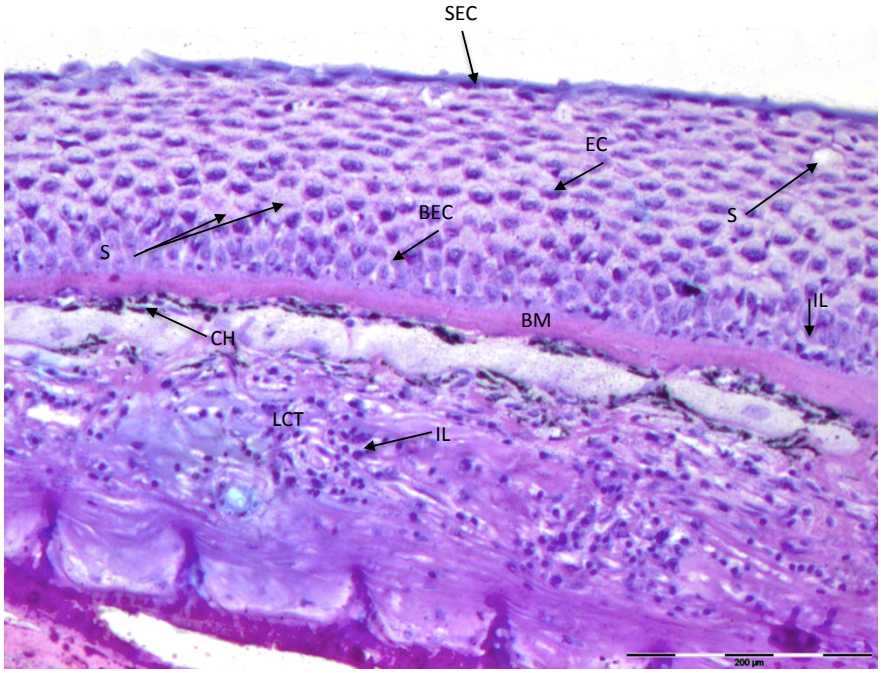

Figure 16. Histological section of the skin of greater amberjack including the epidermis and upper dermis. Basement membrane (BM) separates the epidermis from the upper part of the dermis (*stratum spongiosum;* loose connective tissue, LCT)). Epithelial cells of the epidermis include the basal layer epithelial cells (BEC), which have rounded to elongated nuclei arranged perpendicular to the basal membrane (DM), mid-layer epithelial cells (EC) with rounded nuclei and surface-epithelial cells (SEC) with elongated nuclei arranged parallel to the basal membrane (BM). Epidermal secretory cells include goblet cells (GC), and unidentified secretory cells (S). Several infiltrated leucocytes are also identified close to the BM and in the LCT of the dermis. Pigment cells (chromatophores, CH) are present in the upper dermis.

Finally a transverse section of the epidermis of a healthy greater amberjack is presented in **Figure 17,** in which goblet cell distribution is described, to verify the normal distribution of these cells.

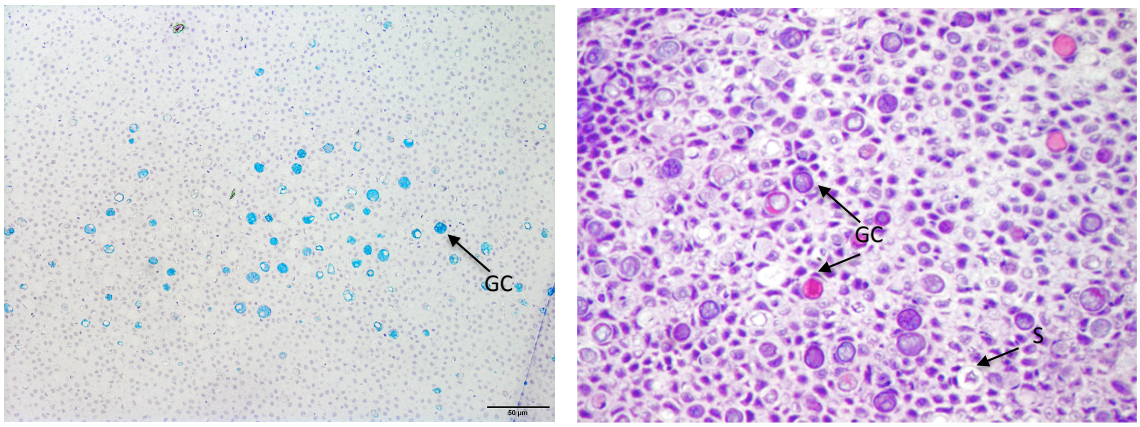

Figure 17. Transverse section of greater amberjack epidermis. (A) Alcian Blue (pH=2.5) staining. See goblet cells (GC) secreting acid mucins. (B) Alcian-Blue-PAS-Giemsa staining. Note the PAS - and PAS+ GC.

Conclusions

This DL demonstrates the immune potential of skin mucus of amberjack, and shows that relative to other species the mucosal surfaces include a full repertoire of antimicrobial defences. We also demonstrate these defences can vary with certain environmental conditions, and that they are especially sensitive to aquaculture-associated stressful conditions

Deviations

None.

Co-funded by the Seventh Framework Programme of the European Union