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Skin infection of greater amberjack (*Seriola dumerili*) by monogenean ectoparasite *neobenedenia girellae*: A morphological and histopathological descriptive study

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ABSTRACT

Neobenedenia girellae is considered an epizootic infection for intensively cultured fish species. Particularly, for greater amberjack (*Seriola dumerili*) *N. girellae* causes high mortality rates and supposes a bottleneck during its on-growing period. Thus, the objective of this work was to describe the skin morphological alterations caused by a *N. girellae* infection on greater amberjack. Greater amberjack juveniles were sampled pre and post experimental infection with *N. girellae* obtaining cranial and dorsal skin samples. Samples were processed for morphological and ultrastructural studies and revealed clear differences in the structure of both regions, confirming the cranial region as the most susceptible region to be parasitized due to an absence of scales and lower goblet cells density. *N. girellae* adhesion disrupted the structure of epidermal epithelial cells by overpressure. *Stratum spongiosum* surface-epithelial cells located near the parasite presented a clear cell degradation process, associated in some cases with cellular detachment. *N. girellae* infection induced epidermal hydropic degeneration and, in some cases, focal spongiosis. Tissue ulcerative lesions caused by the parasite's attachment structures were characterized by a specific mobilization of leucocytes to the fixation areas. Thus, *N.girellae* induces important alterations in greater amberjack epidermis independently of the skin region that explain the appearance of secondary infections and associated mortalities.

1. Introduction

Fish skin is considered the first physical barrier against external pathogens and stressors (Whitear and Mittal, 1986). This tissue is well-organized in a pre-epithelial barrier mainly composed by skin mucus, an epithelial barrier which differs in structure depending on the fish region studied, and a scattered skin associated lymphoid tissue (SALT) (Salinas et al., 2011). It is well documented how an altered biochemical and immunological composition of the fish skin mucus may result in an increased susceptibility to pathogen infection or to winter syndrome (Fast et al., 2002a; Contessi et al., 2006), in a wide range of fish species, such as Coho salmon (*Oncorhynchus kisutch*), Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*) or gilthead seabream (*Sparus aurata*). Besides, the fish skin epithelial layer and its SALT characteristics and status modulate fish susceptibility to pathogen infections in relation to prevent pathogen physical attachment (Ourth and Chung, 2004; Griffin and Mitchell, 2007). However, fish skin presents a limited morphological response to injury (Esteban, 2012). Skin cellular injury derived from pathogen attachment causes cell membrane damage, an imbalance of epithelial cells and limits mitochondrial ATP production (Esteban, 2012). Moreover, some pathogens as *Moritella viscosa* or some hematophagous parasites as sea lice (*Lepeophtheirus salmonis*) inhibit the epidermal regeneration capacity of keratocytes (Karlsen et al., 2012). Therefore, skin integrity and functionality are key features to maintain fish health (Caruso et al., 2010; Esteban, 2012; Fernández-Montero et al., 2019), especially in species with high susceptibility to ectoparasites.

The effects of ectoparasites on fish skin are specific of each host and parasite. For example, a sea lice infection on Atlantic salmon skin produces an ulcerative process caused by the second antennae and an inflammatory status of the dermis characterized by epidermal thickness variations, cell detachment, presence of necrotic areas and leucocytes mobilizations (Jones et al., 1990). Whereas *Gyrodactylus derjavini* infection is characterized by hydropic degenerated epidermal cells,

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cellular reorganization, increased goblet cells density and epidermis narrowing (Appleby et al., 1997; Buchmann and Bresciani, 1997). Other monogenean infection, as *Neobenedenia melleni* has been related with surface epithelium denudation and interstitial edema in red hybrid tilapia (*Oreochromis* spp.) (Robinson et al., 1992).

Neobenedenia girellae Hargis, 1955, is a marine warm water monogenean ectoparasite of special importance for marine cultured species, which affects significantly to greater amberjack (*Seriola dumerili* Risso,1810), representing the main bottleneck for its production (Shirakashi et al., 2013). *Neobenedenia sp*. has been related with high mortalities ranging between 100 and 70 % (Ogawa et al., 1995; Shinn et al., 2015). At early infection stages, *N. girellae* shows preference on its fixation for the fins and the cranial skin region, whereas for long-term infections its moves preferentially towards ventral and dorso-lateral skin regions (Hirayama et al., 2009; Hirazawa et al., 2011). It has been previously described as *N. girellae* infection induces variations on greater amberjack skin epidermis thickness accompanied of an increased density of goblet cells (Hirayama et al., 2009; Hirazawa et al., 2010, 2016), in relation to an unbalanced osmoregulatory and respiratory functioning of the skin (Hirayama et al., 2009; Hirazawa et al., 2016). Moreover, *N. girellae* infection in this fish species usually is associated with skin secondary bacterial infections, derived from an altered fish behaviour characterized by scratching its skin with the tanks and nets and thus, causing important skin wounds (Ogawa et al., 1995; Hirayama et al., 2009). In addition, morphological differences in skin structure in different areas have already been observed for other species, like Atlantic salmon or rainbow trout (Hawkes, 1974; Ashley, 1975). Furthermore, there is still a lack of information about greater amberjack skin morphology, and despite of its relation with ectoparasite fixation preferences for other species, information about the skin morphology could help to understand the undelaying mechanism of site preference of *N. girellae* in greater amberjack.

Thus, the objective was to study the associated skin morphological alterations induced by an experimental parasitization with *N. girellae* and to better understand the underlying mechanism for *N. girellae* infection site.

2. Material and methods

The present study was conducted at the Scientific and Technologic Park of the University of Las Palmas de Gran Canaria (Las Palmas, Canary Islands, Spain).

In order to ensure that animal welfare standards are maintained, anaesthetic was used within the sampling procedures. All animal experiments described in this manuscript fully comply with the recommendations in the Guide for Care and Use of Laboratory Animals of the European Union Council (2010/63/EU).

2.1. Experimental fish and experimental conditions

The experimental greater amberjack juveniles used for the present study come from a batch reared in a recirculated system to ensure no previous infection with ectoparasites, being selected in this particularly life-stage due to its higher susceptibility to *N. girellae* compared with adult stages. Greater amberjack juveniles of initial weight 343.0 ± 53.0 g were divided randomly in two experimental groups of twenty-five individuals. The first group was euthanized with an anaesthetic overdose (clove oil, 5 mL/L; Guinama S.L; Spain, Ref. Mg83168) and sampled as control non-parasitized fish. The other twenty-five fish were distributed in 5 cylindroconical 500 L tanks (5 fish/tank), feeding to apparent satiety during 30 days with a commercial diet (Europe 22, Skretting). Temperature (22 °C ± 0.5) and dissolved oxygen (7.4 ± 0.9 mg L^{-1}) were monitored daily.

2.2. Neobenedenia girellae experimental infection

Neobenedenia girellae infection was performed as previously described in Fernández-Montero et al. (2019) with some modifications. Briefly, eggs where collected from a formerly greater amberjack parasitized tank by entanglement of the eggs in a 5 mm pore net during 24 h. Nets with the eggs were disposed in the experimental tanks for 30 days to let them hatch and allow the oncomiracidia fix to the fish. After those 30 days of parasitization challenge, all the experimental fish were checked to be all parasitized and in a similar parasitization level using the score-methodology described in Fernández-Montero et al. (2019).

2.3. Sampling procedures

First sampling was conducted pre-parasitization of the first experimental group. Skin samples were obtained from two different regions, cranial and dorso-lateral (Fig. 1). Skin regions were selected according to previous results obtained by Hirayama et al. (2009) based on the differences observed in parasite fixation and prevalence.

Final sampling was conducted after thirty days, when all the animals showed visual signs of parasitization, as behavioural scratches, pallid skin mucosal colour and haemorrhagic skin and fins processes. Sampling was conducted in the same way as in non-parasitized group.

2.4. Morphological studies

Sections of skin (Fig. 1) from 25 fish per group were removed and fixed in buffered 4% formalin. Samples were dehydrated in a series of ethanol dilutions and embedded progressively in Technovit (Electron Microscopy Sciences, PA, USA) following the method of Pittman et al. (2013). Briefly, sections were stained with Alcian Blue-PAS-GIEMSA (Martoja and Martoja-Pierson, 1970). Stained sections were observed under an Olympus cx41rf optic microscope (Tokyo, Japan) and evaluated. Micrographs were obtained with an adapted camera Olympus xc50 (Tokyo, Japan). Microscopy measurements were conducted with Image Pro Plus software (Media Cybernetics, Silver Spring, USA) (n = 30 slides /region/treatment) and presented as an average with standard deviations.

2.5. Ultrastructural studies

Three skin samples for Scanning Electron Microscopy (SEM) from three fish of each group where dehydrated through a graded series of ethanol, followed by a critic point dried (Hitachi HCP-2, Chiyoda, Tokyo, Japan) washing the samples in CO_2. The dried samples were mounted on aluminium stubs and metallization was conducted by sputter coated with argon-gold (Gibbons, 1986). SEM samples were examined and photographed with a JEOL JSM-6335 F field emission scanning electron microscope (JEOL USA, Inc, USA).

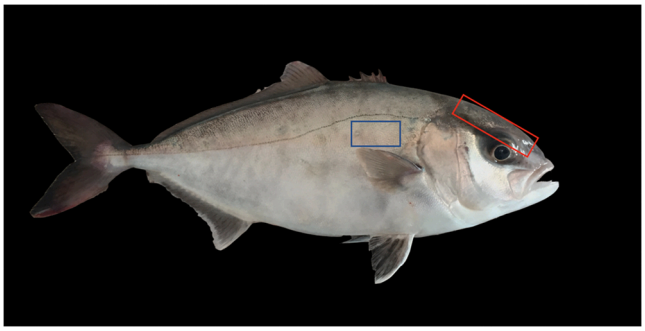

Fig. 1. Greater amberjack selected skin sections for histological analyses. Red colored corresponds with cranial region; blue-colored corresponds with dorso-lateral region.

Three skin samples of the two studied regions from three fish of each experimental group were dissected (n = 9 samples/ treatment), cut in small pieces and immediately fixed in 2.5 % glutaraldehyde in 0.15 M HEPES buffer (pH = 7.4), post fixed in 2% osmium tetroxide and 2% uranyl acetate, dehydrated in graded ethanol series, and embedded individually in an Embed 812 (Electron Microscopy Sciences (EMS), PA, USA) resin block. Semithin (1 μm) serial transverse sections were contrasted with toluidine blue and examined under light microscopy (Hoffman et al., 1983). Ultrathin (50 nm) sections were contrasted with lead citrate and examined with a JEOL JEM-1011 Transmission Electron Microscope (TEM; JEOL USA, Inc, USA) equipped with a digital camera MegaView III soft imaging system CCD Camera (EMSIS GmbH, Germany).

3. Results

3.1. Morphological description of greater amberjack dorso-lateral and cranial region

The two different skin regions selected for the study showed some morphological variations in the non-parasitized fish. Skin dorso-lateral region presents a thinner epidermis (177 ± 37 μm; n = 10) with a higher presence of goblet cells (Fig. 2. A) when compared to cranial region (279 ± 48 μm; n = 10) (Fig. 2. B). Despite this difference in width, the disposition of the epidermal cell layers followed a similar morphological pattern in both regions. Briefly, epithelial cells of *stratum basale* presented a cuboid appearance, with an oval shaped nucleus disposed centrally or slightly displaced and perpendicularly to the basal membrane. Epithelial cells of *stratum spinosum* presented ovoid to polygonal shapes with a variable nuclei size, which was disposed centrally and in transversal or parallel disposition in relation to the basal membrane. The surface layer of the epidermis, *stratum superficiale*, was constituted by 2–3 irregular rows of flattened cells containing irregular, elongated and in some cases pyknotic nuclei disposed parallelly to the basal membrane (Fig. 2. A, B). In addition, cranial region presents an apparent better alignment of the basal epithelial cells along the basal membrane (Fig. 2. B).

3.2. Effects of parasite attachment on epidermis

The epidermis morphological alterations derived from *N. girellae* fixation, did not differ between cranial and dorso-lateral regions. In general terms, a slight edema and sloughing of epidermal cells were observed around the site of attachment. No signs of hyperplasia or inflammation were observed. Similarly, and regardless of the region where *N. girellae* was fixed, it produced an epithelial overpressure by its haptor attachment structures, which induced a disruption of the epidermal layer structure by over-flattening *stratum superficiale* located epidermal cells (Fig. 3. A) in comparison with non-parasitized fish (Fig. 3. B). This alteration resulted in a disruption of the normal cellular linear organization of surface epithelial cells, however it was not accompanied of cellular hypertrophy.

The parasite surrounding adhesion region was characterized by cell detachment of the first epithelial layer (Fig. 4. A). TEM study revealed also a partial digestion of the *stratum superficiale* cells near the parasite adhesion area, where cells presented a less electron-dense cytoplasm appearance and signs of necrophanerosis, as disruption of microridges and intercellular junctions structures (Fig. 4. C, D), which lead to cellular detachment and to the appearance of intercellular spaces between them (Fig. 4. E, F) compared to non-parasitized areas of the *stratum superficiale*. Cellular organelles showed signs of degradation and nuclei were under a pyknotic or karyorrhexic process (Fig. 4. F).

N. girellae hooks and, specially, the anchors produced a deep perforation of epidermis (Fig. 5. A, B), inducing a reduction of its thickness (158 ± 49 μm and 220 ± 67 μm for dorsal and cranial region, respectively, n = 10). These punctures had different width (around 50 μm) and depth (around 130 μm) depending on the size of the parasite attachment structures (Fig. 5. B). The mechanical perforation altered the structure of the epithelial cells surrounding the lacerations in both, the *stratum superficiale* and *stratum spinosum*, which led to architectural changes to an ovoid and/or flattened nucleus epidermal cell shape compared to a conserved round nucleus in non-parasitized fish (Fig. 5. B).

The incidence of *N. girellae* caused an increase in the number of scattered goblets cells in greater amberjack epidermis compared with non-parasitized fish. Goblet cells were mainly disposed in one single layer in the *stratum spinosum* and also in the *stratum superficiale* (Fig. 6. B). Goblet cells mucin contents included neutral (PAS positive) and acidic (Alcian blue positive) mucus, not showing a preference and being

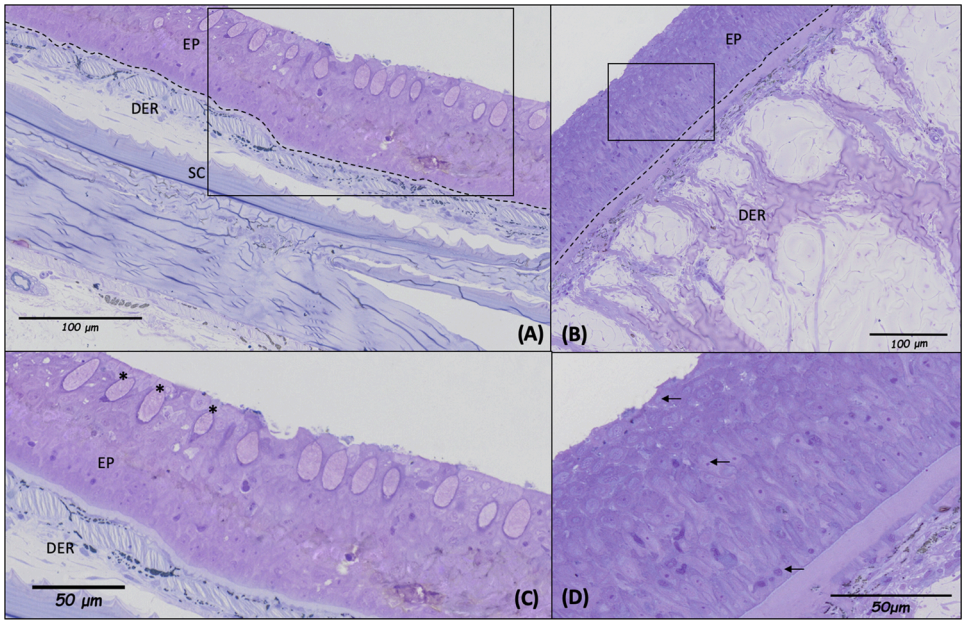

Fig. 2. Dorso-lateral (A) and cranial (B) semithin sections of greater amberjack (*Seriola dumerili)* skin stained with Alcian blue-PAS-GIEMSA and toluidine blue (pH: 2.5). Observe the evident differences in the thickness of the epidermis and dermis, the irregular *stratum basale* alignment pattern of dorso-lateral region (–) and the lack of imbricated scales (SC) in cranial region dermis (n = 10). Detail of dorsolateral (C) and cranial (D) epidermis regions and layers disposition. Observe the variations on the epithelial cells shape and nuclei disposition (→). *Stratum basale* epithelial cells presented a central/basal nuclear position, perpendicular to the basal membrane. *Stratum spinosum presented* round-shaped epithelial cells with a centric and small nucleus transversal/ parallel to the basal membrane. *Stratum superficiale* presented flat-shaped cells with irregular a centric/parallel to the basal membrane nucleus. Observe the higher density of goblet cells (*) on greater amberjack dorso-lateral region compared to cranial region (C vs D). Alcian blue-PAS-GIEMSA and toluidine blue, pH: 2.5. EP: epidermis; DER: dermis; SC: scales; SB: *stratum basale*; SSP: *stratum spinosum*; SS: *stratum superficiale*.

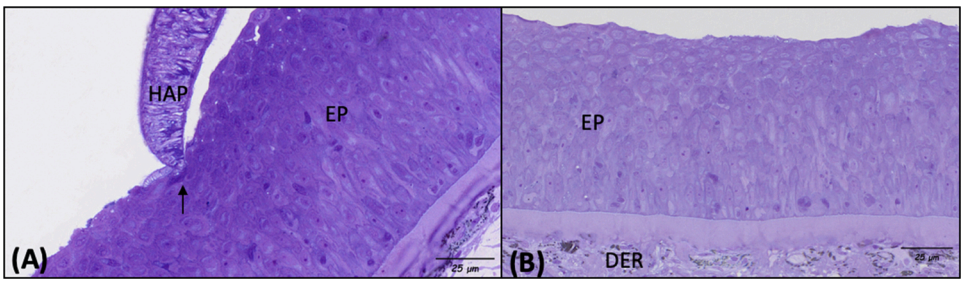

Fig. 3. (A) Semithin section of *Neobenedenia girellae* haptor fixed to greater amberjack (*Seriola dumerili)* skin. Observe the induced cellular disruption and disorganization of epidermal *stratum superficiale* (→) in relation to a non-parasitized greater amberjack (B). Alcian blue-PAS-GIEMSA, pH = 2.5. HAP: haptor; EP: epidermis; DER: dermis.

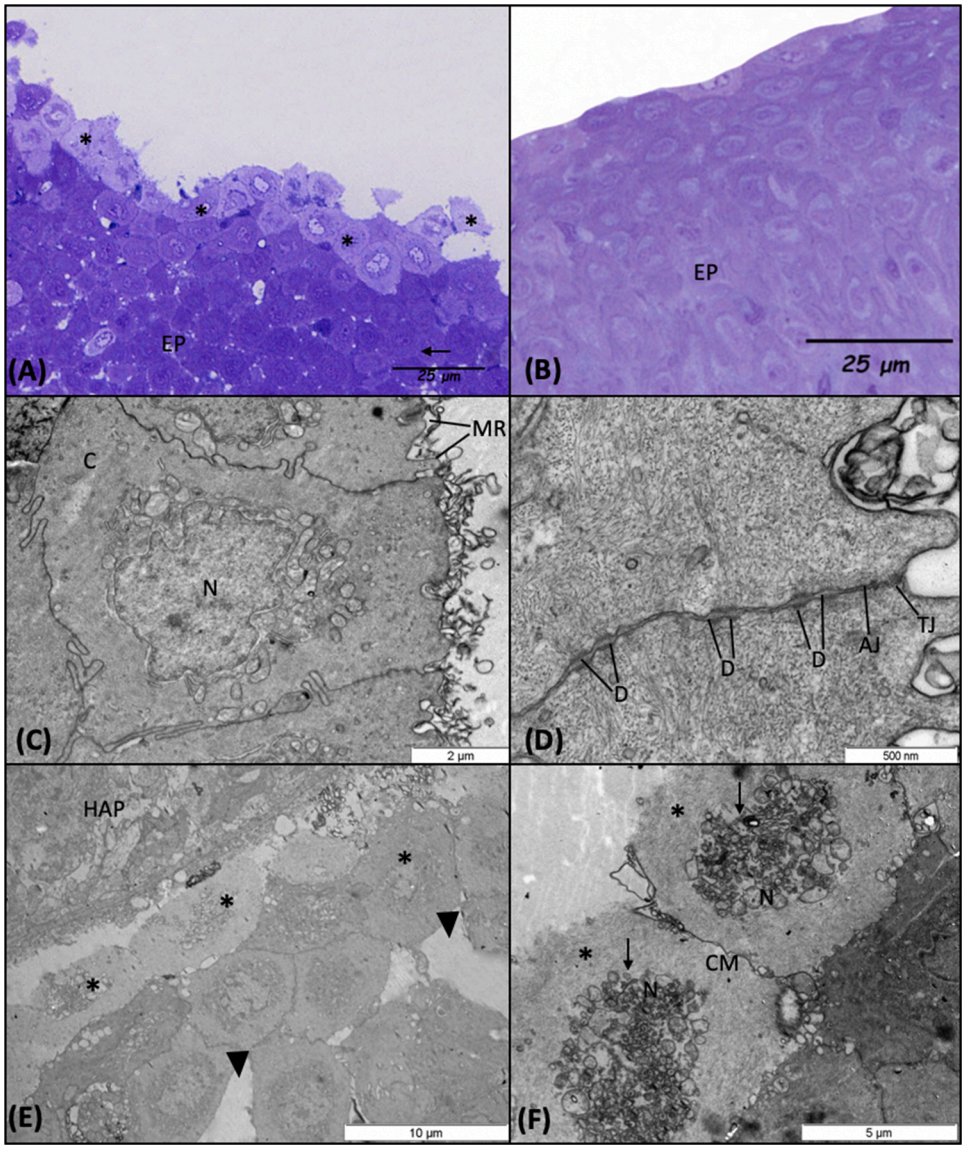

Fig. 4. Detailed micrographs of greater amberjack (*Seriola dumerili*) parasitized and non-parasitized skin. (A) surface epithelial cells of a greater amberjack parasitized skin. Observe the partially digested surface epithelial cells (*) compared to non-parasitized fish skin (B). Alcian blue-PAS-GIEMSA, pH = 2.5. (C) *Stratum superficiale* epithelial cells of non-parasitized skin areas, observe the maintenance of the cellular structure and micro-ridges. (D) Detail of the cell structure of non-parasitized areas with tight junctions, adherens junctions and desmosomes still maintaining their structure and cells cohesion. (E) TEM micrograph of surface parasitized greater amberjack (*Seriola dumerili)* epithelial cells in contact with *Neobenedenia girellae* haptor, where focal acantholytic processes represented as intercellular spaces (Δ) could be observed with degraded cells (*). (F) Detailed TEM micrograph of parasitized greater amberjack degraded cells (*) due to the parasite attachment. Observe the necrophanerosis signs as digested apical membrane, pyknotic or karyorrhexic nucleus (→) and the absence of intercellular junctions. EP: epidermis; IS: intercellular spaces; N: nucleus; C: cytoplasm; AJ: adherens junctions; TJ: tight junctions; D: desmosomes; MR: micro-ridges; CM: cellular membrane.

variable as well as cell size among individuals. Mucins were packed inside the goblet cells in mucosomes of similar electron-density and different size, which fill almost the whole cytoplasm of the goblet cells and the nuclei was located basally (Fig. 6. C).

Oedematous areas were observed around the perforation sites, where hydropic degenerated epithelial cells could be observed mainly in the *stratum spinosum* and the *stratum basale* of infected fish. In those areas, affected epithelial cells presented intracytoplasmatic hydropic vacuoles, which caused nuclei displacement (Fig. 7. A, B). These morphological alterations entailed a cellular hypertrophy pattern (diameter: 50 µm), which altered the typical *stratum basale* cell cubic appearance observed in non-pathologically-altered areas. In this cell layer, non parasitized skin regions presented a well conserved oval nuclei disposed in the center of the cell (diameter: 21.3 ± 2.2, n = 10) and an organized

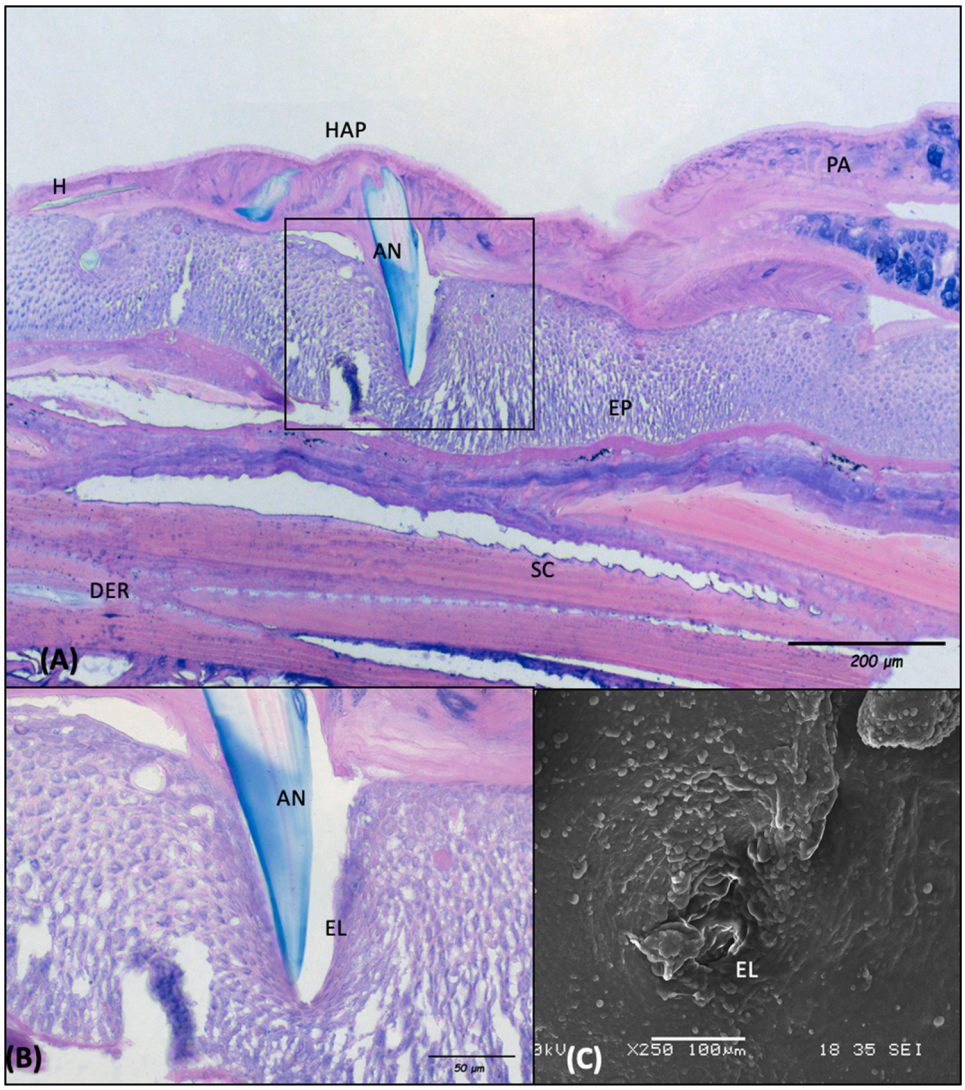

Fig. 5. (A, B) Transversal section of *Neobenedenia girellae* attached to greater amberjack (*Seriola dumerili*) skin. Observe how anchors reach ¾ of the epidermis, induce lacerations and pack epidermal cells while hooks are fixed more superficially. Alcian blue-PAS-GIEMSA, pH = 2.5. (C) Detailed SEM micrograph of the laceration produced by *N. girellae* attachment. PA: parasite; EP: epidermis; H: hooks (hamuli); HAP: haptor; AN: anchor (accessory sclerites); DER: dermis; SC: scales; EL: epidermal lacerations.

continuous cellular layer, with some digitations in their cell membranes that increase cellular cohesion perpendicular to the plane of the basal surface (Fig. 2. D; Fig. 8. D). In consequence, epidermis of infected fish presented widening of intercellular spaces and spongiosis (Fig. 7. C), unlike with the non-parasitized fish (Fig. 7. D).

Mobilizations of lymphocytic-like cells were observed mainly focused in the *stratum basale*, near the parasite adhesion region. In some cases, these mobilizations reached higher stratums of epidermis as *stratum spinosum* surrounding the anchors and hooks of the attached parasite (Fig. 8. A, B). Intraepithelial lymphocytes (IELs) could be observed near the spongiotic foci (Fig. 8. C, D).

3.3. Dermis

No mechanical alterations were observed in the dermis of greater amberjack after *N. girellae* fixation, since haptoral attachment structures did not reach the dermis. However, the cranial region presented a wider dermis (753 ± 118 µm) than the dorsal region (430 ± 34 µm) (Fig. 2). Two different layers mainly constitute the dermis: The *stratum compactum* and the *stratum spongiosum*. The *stratum spongiosum* is located just below the epidermis and is mainly composed by vascular connective tissue and imbricated scales, however in this fish species scales were observed only in dorso-lateral region. The *stratum compactum* is located below the *stratum spongiosum* and limits with the hypodermis, and it is composed by long fibres of collagen disposed in curly waves with chromatophores in apical disposition of this layer.

No hydropic degeneration was found in the dermis of infected fish, but IELs focuses were observed in the *stratum spongiosum* of infected fish in relation mainly to parasite adhesion areas. The incidence of IELs focuses was higher in the dermis than in the epidermis, and in some cases was also not associated to a focal point (Fig. 9).

4. Discussion

Fish skin morphology varies depending on the fish species and skin region studied (Hawkes, 1974; Ashley, 1975). In this sense, due to the proximity to cranial bones, cranial region skin has a thicker epidermis than dorso-lateral region, being also a region particularly susceptible to the appearance of blows and wounds. Previous studies based on the study of the fixation mechanism of *N. girellae* suggest that the most susceptible regions are the fins (Hirayama et al., 2009) and the cranial region at early infection stages (Hirazawa et al., 2011), whereas for long-term infections its moves preferentially towards ventral and dorso-lateral skin regions (Hirayama et al., 2009; Hirazawa et al., 2011), similarly to Atlantic salmon and sea lice infection (Genna et al., 2005). The existence of morphological and physical differences between the cranial and the dorso-lateral region in this fish species may explain partially the different incidence ratios depending on the fish skin region

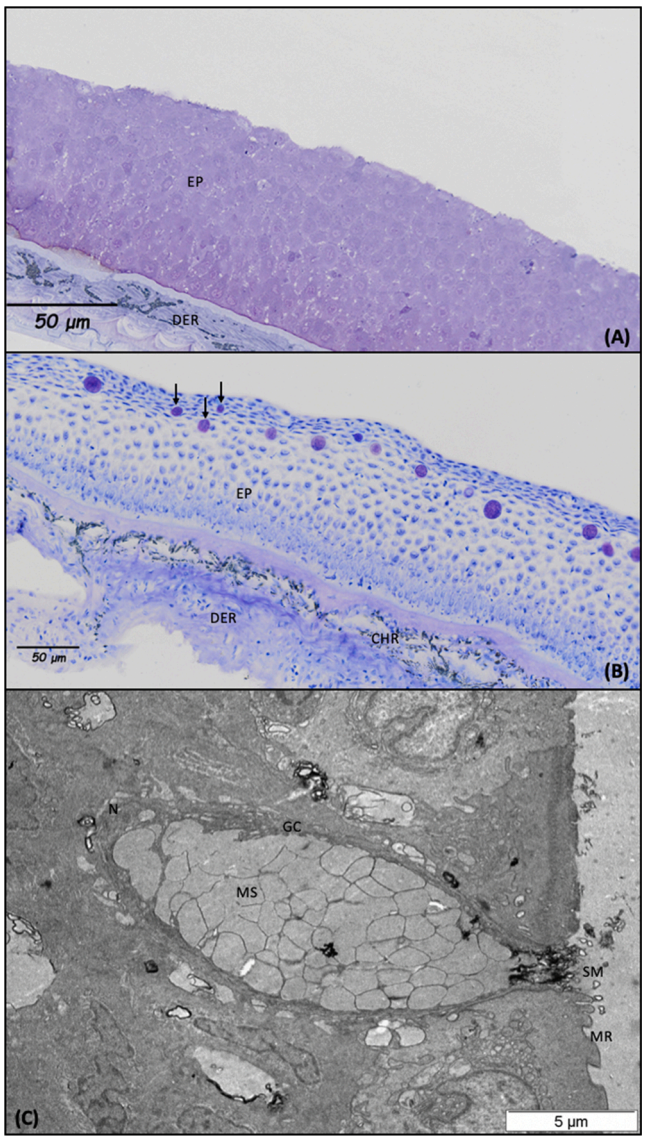

Fig. 6. Semithin sections micrographs of skin dorso-lateral region detailing the differences between non-parasitized fish (A) and infected (B) greater amberjack (*Seriola dumerili*) epidermal goblet cells density (→). Alcian blue-PAS-GIEMSA, pH = 2.5. Observe the higher density of goblet cells in parasitized fish skin. (C) TEM micrograph of a greater amberjack skin goblet cell. EP: epidermis; DER: dermis; GC: goblet cell; CHR: chromatophores; MS: mucosomes; MR: micro-ridges; SM: secreted mucus: N: nucleus.

studied and the grade and phase of infection as described in other fish species. For example, the dorso-lateral skin morphology of salmonids is characterized by a thin epidermis layer disrupted by the insertion of the scales (Fast et al., 2002a). Despite greater amberjack scales size and morphology differ from Atlantic salmon scales (Jonsdottir et al., 1992; Mazzola et al., 2000), their presence in dorso-lateral region supposes to *N. girellae* oncomiracidia an extra physical barrier to overcome in order to fix themselves to the fish skin compared to fins and cranial region at early infection stages (Hirazawa et al., 2011). Our results, in terms of a higher density of scattered goblet cells in the epidermis of the dorso-lateral region compared to cranial region, are in agreement with previous studies in other fish species, such as Atlantic salmon (Pittman et al., 2013). Moreover, the monogenean *Gyrodactylus derjavini* fixation is strongly correlated with a lower density of goblet cells per skin region, as described by Buchmann and Bresciani (1997), who highlighted the preference of this region in early infection stages. In addition, Yokoyama et al. (2019) observed a reduction of *N. girellae* infection in greater amberjack associated to an increase of skin mucus production and lysozyme activity. Additionally, greater amberjack cranial region skin has been pointed to have low acquired protection against *N. girellae* infection (Hirazawa et al., 2011). Altogether, our findings on the morphological differences between cranial and dorsolateral region could complement this mentioned studies and give extra information to site/region-specific preferences for attachment of *N. girellae* in greater amberjack.

Besides the morphological differences of cranial and dorso-lateral regions, *N. girellae* fixation induced similar morphological alterations in both regions. An overpressure over epidermis caused by parasite haptor fixation has already been reported for other species such as Atlantic salmon during sea lice infection (Jones et al., 1990). This process is related with a disruption of cell organization and cellular hyperplasia as a reaction to a chronic irritation, which finally uses to be related with a cellular necrotic process (Jones et al., 1990; Jones, 2001). The fixation of *G. dejarvini* to rainbow trout has been associated to an up-regulation of proinflammatory cytokines as *il1-β*, which may contribute to cause hyperplasia of the surrounding epithelial cells (Buchmann and Brescianni, 1997). However, for greater amberjack and in contrast with other fish species, such as salmonids, no severe hyperplasia associated to *N. girellae* infection was observed on the epidermis. On the other hand, the observed epidermal over-flattening and crushing of *stratum superficiale* cells could derive in a medium to long-term basis in cellular necrosis and increased cell detachment (Jones et al., 1990) for this fish species.

The occurrence of epithelial cellular apoptosis foci in the first layer of *stratum superficiale* is a normal pattern during regeneration process of fish skin (Hawkes, 1974). Nevertheless, the appearance of this process associated to more than one layer especially near the adhesion regions of the haptor and adhesive glands, and on larger surfaces compared with no parasitized fish denotes a pathological origin. Indeed, an epidermis damage induced by sonication in goldfish (*Carassius auratus*) by Frenkel et al. (1999) presented a similar cellular detachment and necrotic pattern in the first layers of epidermal cells as the one described in the present study. This process has already been observed for ectoparasite infections, as sea lice infection in Atlantic salmon (Jones et al., 1990; Jonsdottir et al., 1992), and *N. girellae* in Cobia (*Rachycentrom canadum)* (Ogawa et al., 2006) and barramundi (Trujillo-González et al., 2015). As it mentioned above, the process of fixation and overpressure caused near the frontal filament and second antennae is related with cellular detachment and with ulcerative processes during sea lice infections, which imply a complete lose and erosion of skin layers via destabilization of epithelial cells intercellular junctions (Boxshall, 1977; Roubal, 1986; Jensen et al., 2015), similarly as founded in the present study, but without reaching such a severe level. In fact, ectoparasites produce specific proteases during the adhesion process, which act as immune disruptors and virulence factors (Esteban, 2012). In sea lice infections, proteases detected during the attachment to Atlantic salmon are mainly composed by serine-proteases (Ross et al., 2000), which specifically digest cellular membranes and intercellular junctions. Hirazawa et al. (2006) reported also the existence of some *N. girellae* specific serine-proteases with presumed similar functions to those described for sea lice. *N. girellae* digestion patterns of epithelial cells observed in the present study agree with the ones described for sea lice, losing the structure of intercellular junctions and micro-ridges at initial stages and disappearing during the subsequent cell degradation process (Nolan et al., 1999). Sea lice feeding activity in Atlantic salmon, Coho salmon and rainbow trout has been associated with a reduction of the epidermis thickness (Fast et al., 2002a, 2002b; Holm et al., 2015), as observed for greater amberjack infected with *N. girellae* in the present and previous studies (Hirayama et al., 2009; Hirazawa et al., 2016). This effect is highly dependent on the body site studied (Trujillo-González et al., 2015), and particularly for greater amberjack, the most affected skin areas are the ventral and the dorsal region (Hirazawa et al., 2011), as observed in the present study. Thus, summarizing the process of

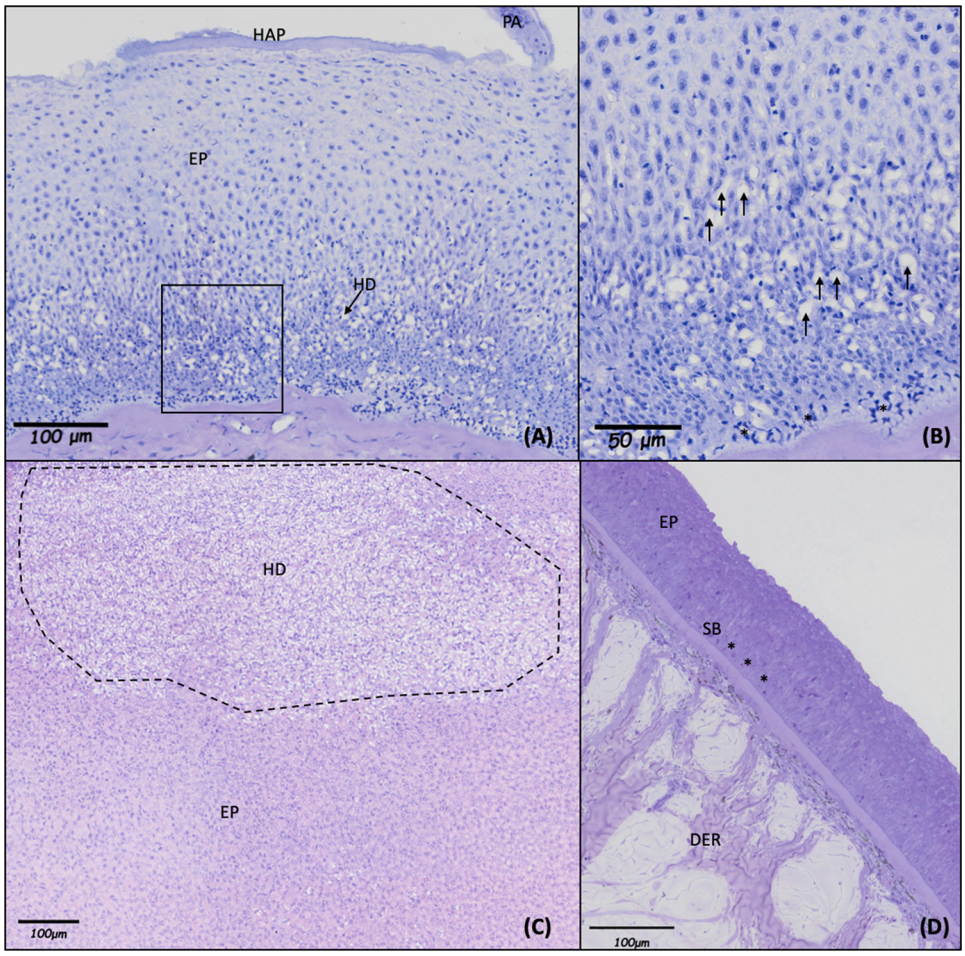

Fig. 7. (A) Transversal section of parasitized cranial skin of greater amberjack, *Seriola dumerili*. Observe, the parasite-induced water imbalance on *stratum spinosum* and *basale* cells with hydropic degeneration (→). Intraepithelial lymphocytes could be also observed (*) in a higher magnification of the selected region (·) of Figure A (B). Alcian blue-PAS-GIEMSA, pH = 2.5. (C) Longitudinal section of greater amberjack cranial skin. Observe the hydropic degeneration morphological pattern covering extensive areas of the tissue (spongiosis) (–) (Alcian blue-PAS-GIEMSA, pH = 2.5) in relation to a transversal section of no parasitized greater amberjack cranial skin (D) where this symptomatology is absent. Observe cellular cohesion and conserved structure of *stratum basale* (*) with oval nuclei disposed in the center of the cell. Alcian blue-PAS-GIEMSA, pH = 2.5. PA: Parasite; HAP: haptor; EP: epidermis; HD: hydropic degeneration.

N. girellae fixation to greater amberjack skin starts by a fixation of the haptor to the epidermis, and the parasite anterior adhesion glands contribute to adhere the oral cavity to skin and produce proteases in order to facilitate *N. girellae* feeding from epithelial cells. In fact, *N. girellae* after anchoring is able to pivot around the anchor for feeding (Whittington, 2011). Besides the morphological alterations described above, *N. girellae* attachment structures haptor penetrate at least the epidermal *stratum superficiale* and/or the *stratum spinosum*, generating mechanical damage in form of epidermal lacerations. Previous studies with blood feeding ectoparasites like polyopisthocotylea or sea lice have evaluated the mechanical damage induced on host skin (Jones et al., 1990; Jonsdottir et al., 1992) and gill (Montero et al., 2004; Mansell et al., 2005). Particularly for sea lice chalimus, which gets fixed to its host with the second antennae, associated ulcerations disrupting the basement membrane according to their blood-feeding have been observed (Jones et al., 1990). Contrary for greater amberjack and since the basal membrane structure was perfectly conserved in all the sections studied, this mechanical damage was limited to first skin layer and directly conditioned by the attachment structures size. Besides, greater amberjack skin damage caused by *N. girellae* is also associated to an induced scratching behaviour, which facilitates the appearance of secondary infections (Roubal and Bullock, 1988; Svendsen and Bøgwald, 1997; Sutherland et al., 2011; Stien et al., 2013).

Infected greater amberjack also presented a skin hydropic degeneration process. Hydropic degeneration caused by an ectoparasite infection has been also described for other cultured fish infected with *Amyloodinium ocellatum* and developmental stages (Paperna, 1980). In the present study, the hydropic degeneration was mostly focused near the parasite adhesion region, however, in some of the evaluated fish the hydropic degeneration was massive, making unfunctional the epithelium and classified as a typical pattern of spongiosis (Paperna, 1980; Speare et al., 1991). Other ectoparasites have demonstrated to produce this kind of lesions, as *Ichthyobodo necator* in salmonids or *Benedenia epinepheli* in *Ephinephelus aeneus* (Robertson, 1985; Eissa et al., 2016)

Meanwhile all these mechanical effects are surrounding the fixation of the parasite, host response presented different strategies. *N. girellae* infection in the present study induced an increase in the density of goblet cells present in the epidermis of infected fish, being an increase on mucus production one of the most evident effects in the host response to avoid parasite fixation and to trigger an specific immune response against the parasite (Paperna, 1991; Fast, 2014; Fernández-Montero et al., 2019; Hirazawa et al., 2016; Yokoyama et al., 2019). In contrast, Buchmann and Bresciani (1997) denoted the reduction of quantity of goblet cells during long-term parasitization of rainbow trout with *G. derjavini*, probably due to host response saturation. For this reason, more studies need to be addressed to understand mucus production dynamics and its regulation along infection periods.

On the other hand, host immune response entails an adaptive and an innate immune response. *N. girellae* infection produced an acute response to the parasite infection characterized by appearance of IELs focuses in the dermis. In a long-term response, IELs focuses could become massive, filing the dermis. Besides that, no associated inflammatory process was observed in dermis, as in dermatitis. A dermatitis histopathological status has been observed in other species with pathogens causing skin diseases (Lunder et al., 1995; Rizgalla et al., 2016). Besides, ectoparasite infections suppose a source of acute and chronic stress, which entails a cortisol response from the host related with a limited capacity of host to carry out a successful wound healing process,

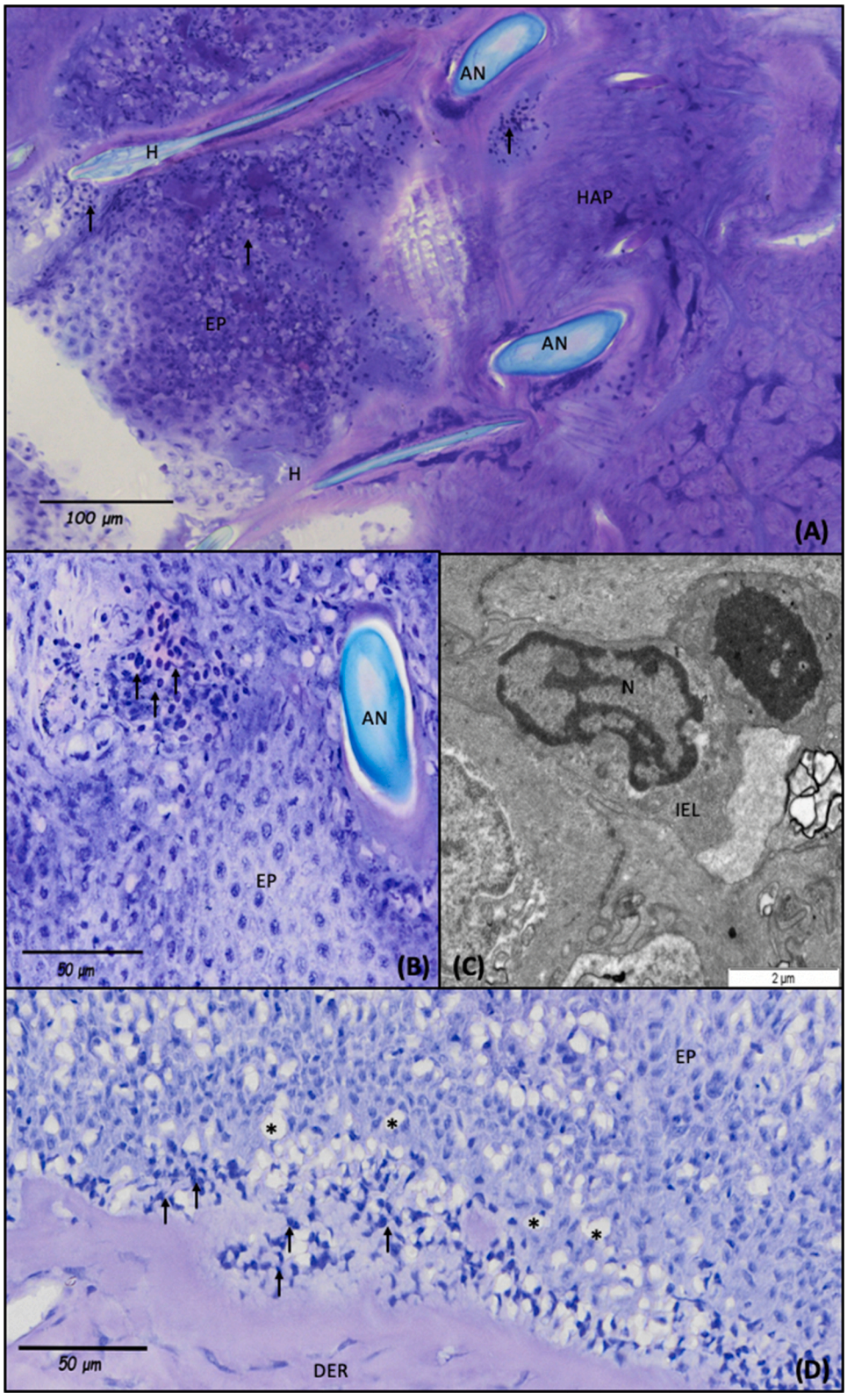

Fig. 8. (A, B) Longitudinal section of *Neobenedenia girellae* haptor fixed to greater amberjack, *Seriola dumerili* skin. Observe the lymphocyte infiltrations (→) near the parasite anchors and hooks. Alcian-blue-PAS-GIEMSA, pH = 2.5. (C) TEM micrograph of intraepithelial lymphocyte (IEL) in the epidermis of a parasitized fish. (D) Detail of an epidermis area presenting IEL infiltrations (→) near the basal membrane associated to hydropic degeneration processes (*), near the parasite adhesion regions and mainly in *stratum basale and spinosum*. Alcian-blue-PAS-GIEMSA, pH = 2.5. EP: epidermis; HAP: haptor; AN: anchors (accessory sclerites); H: hooks (hamuli); IEL: intraepithelial lymphocyte; N: nucleus; DER: dermis.

but also with immunosuppression and the appearance of secondary infections by opportunistic pathogens (Krasnov et al., 2012).

In summary, a higher incidence of *N. girellae* fixation in greater amberjack cranial region could be related with the morphological characteristics of this region as less quantity of goblet cells and lack of imbricated scales. The attachment of *N. girellae to greater amberjack skin* induced a mechanical damage characterized by focal epithelial vacuolization, epithelial cell digestion, cellular detachment and epidermis disorganization. Besides, *N. girellae* infection induces an increased density of epidermal goblet cells and a migration of IELs to the fixation area. In a long-term basis and due to the observed skin lesions, an infection of greater amberjack with *N. girellae* may favour the appearance of

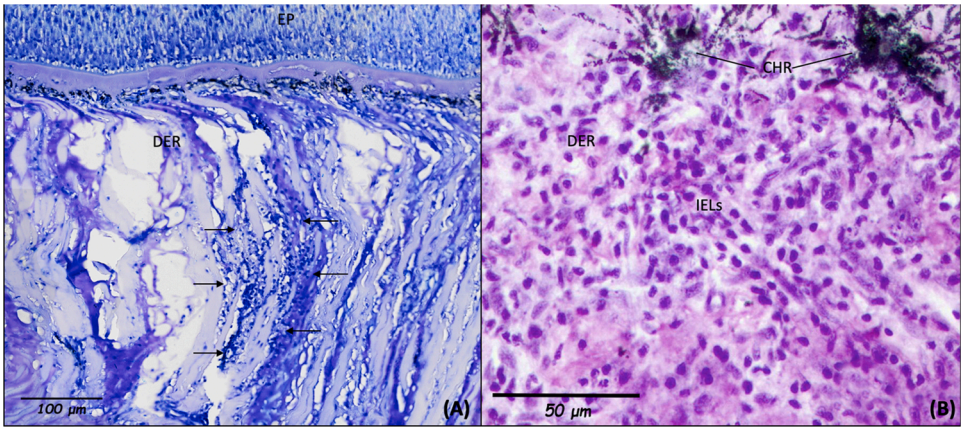

Fig. 9. (A) Detail of greater amberjack (*seriola dumerili*) cranial region transversal section. Observe the focal dermic extravasation of intraepithelial lymphocytes (IELs) (→) associated a focal point near blood vessels. Alcian blue-PAS-GIEMSA, pH = 2.5. (B) Detail of IELs focuses in the dermis *stratum spongiosum*. Hematoxilin-eosin, pH = 4.3. EP: epidermis; DER: dermis; IEL: Intraephithelial lymphocytes; CHR: chromathophores.

secondary bacterial infections caused by opportunistic pathogens, limiting the production performance of this species in sea cages.

Authors' contributions

AF carried the infection challenge, make the samplings, conduct the histological analyses and evaluations and wrote the manuscript. DM conceived of the study and participated in its design and coordination and reviewed and helped to draft the manuscript. MSI helped with the acquisition of the grant and participated in the design of the study. FA participated in the manuscript revision. MJC conceived of the study and participated in its design and coordination, helped with the SEM processing and participated in the histopathological evaluations. ST conducted the ultrastructural evaluations, helped with the histopathological evaluations and helped to draft the manuscript. All the authors read and approved the final manuscript.

Author agreement/ disclosure statement/ role of funding sources

The authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We confirm that all data are fully available without restriction. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property. We further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript. We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from alvarofmontero@gmail.com. We also confirm that the funding sources listed in the present manuscript had no role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Declaration of Competing Interest

The authors report no declarations of interest.

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