

Mortality and cellular response in the skin and gills of plaice (*Pleuronectes platessa* L) to parasite and bacteria infection

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Abstract

During the culture of wild-caught plaice, *Pleuronectes platessa* L. gross skin lesions and patches of dark brown pigmentation were observed on the ocular side of the fish. Mortality increased approximately nine months after capture, and after 10 months, 21 of the 27 fish had died. The presence of *Gyrodactylus unicopula* (Glukova 1955) was demonstrated by histology and light microscopy studies of skin samples from affected fish. The parasite, probably assisted by secondary bacterial infection, caused extensive disruption of the skin and underlying tissue. No parasites or bacteria were seen in gill sections. However, epithelial hyperplasia was observed in the gills of affected fish, and suspected to be a result of the parasite infection. Histologically, malpigmentation was confirmed to be caused by melanocytes in the dermis and, to some extent, underlying tissues. These findings of high mortality and extensive ulceration of the skin suggest a direct cause-and-effect link to the specific parasite. However, the possibility of an interaction, or an additive effect of bacteria cannot be ruled out. This is the first report of mortality and skin responses of *G. unicopula* infection in plaice.

Introduction

Plaice, *Pleuronectes platessa* L, is a popular fish among consumers in many countries. However, reduced catches (FAO, 2007) and increased prices could make this a possible candidate for aquaculture.

In wild fish stocks, parasites from a wide variety of groups are normally present in low numbers living in balance with their host. However, in aquaculture because of higher density of fish (Barton and Iwama, 1991) stressful conditions may increase susceptibility to diseases (Pickering and Pottinger, 1989) and epizootics may occur if conditions are suitable (Johnson et al., 1996). The gills and skin of fish are highly susceptible to environmental influences and

disruptions of these organs may have serious consequences for fish viability (Wendelaar Bonga and Lock, 1992; Noga, 2000). Different responses may occur in these organs including the occurrences of melanin-containing cells in inflammatory lesions (Roberts, 1975). The effects of parasites on wild and farmed stocks are receiving increased attention due to economic losses and the impact on fish welfare (Scholz, 1999). The present histological study was conducted to investigate the aetiology of grossly visible skin hyperpigmentation and lesions in skin and increased mortality in a broodstock of plaice.

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Materials and methods

Diseased fish

In October 2007, 27 plaice, *P. platessa*, were trawled at a depth of approximately 20 m from a sandy bottom near the research station in Bodø (671448N, 142418E). The mean weight of the fish was approximately 1.5 kg, and the fish appeared healthy with no gross signs of disease. The fish were captured for studies of reproduction and stripped of eggs and sperm during the subsequent spawning season from February to May. The fish were held for 11 months in 2 m x 2 m gel-coated fibreglass tanks. Ambient deep sea water was taken from an inlet at a depth of 50 m, aerated, and filtrated through a Bernoulli filter to remove particles above 300µm. The temperature was $7.4 \pm 0.9^\circ\text{C}$, and salinity approximately 34 psu. Dissolved oxygen as measured in the outlet water was $89 \pm 4.1\%$. Fish were hand fed ad lib a commercial dry pellet feed for marine fish (Biomar, Myre, Norway).

During the 11 months, 21 fish died, 15 of these during the last two months. The remaining six fish were grossly investigated. All fish had skin lesions and abnormal pigmentation, and were showing signs of disease. Four of these were selected for further histological investigations as described below.

Control fish

To have healthy fish with normal and unaffected skin and gills as a reference, two plaice, weight 1.3 kg and 1,4 kg, were caught by a fish pot from the same location approximately 1 year after the experimental fish.

Sampling, fixation, and histology

All sampling of skin and gills of diseased fish

was conducted during the month before termination of the project, i.e. 10 months after capture, whereas sampling from control fish was conducted immediately after capture. For sampling, fish were netted into 40 L of seawater containing 75 mg/L of Fiquel (tricaine methanesulfonate, Argent). After 4-6 min, the fish became anesthetized and the diseased fish were photographed using a digital camera (Olympus C-750 Ultra Zoom, 4.0 mega pixels). All fish were euthanized by decapitation and gills and skin samples collected as described below.

Skin and gills samples from four diseased and from two healthy (control) fish were fixed in 10% neutral buffered formalin, dehydrated in ethanol, cleared in xylene and embedded in paraffin. 3 µm sections were stained with haematoxylin and eosin (HE). Sections were examined with an Olympus BX 61 microscope, and photographed using a digital camera (Olympus Camedia C - 3040).

For TEM, skin samples of one affected fish were fixed in 2.5% glutaraldehyde + 2.5% paraformaldehyde in 0.05M cacodylate buffer, adjusted to 330 mOsM and stored at 6°C (Ottesen and Olafsen, 1997). Samples were washed in phosphate buffer prior to post-fixation in 1% aqueous OsO₄ for 2 hrs at 4°C. The tissue was then stained in 2% aqueous uranyl acetate for 1.5 hrs at 20°C, dehydrated in a series of graded ethanols, and subsequently embedded in Epon/Araldite. Semi- and ultrathin sections were cut on an ultramicrotome (RMC MT-7, RMC, Tucson, Arizona, USA). Ultra-thin sections were examined and photographed with a Jeol JEM1010 transmission electron microscope operated at 80kV.

Results

Generally, healthy plaice have an even and normally pigmented skin. A micrograph of histology section of normal skin is shown in Figure 1. Melanin-containing cells were observed only in the dermis, not the epidermis. All six diseased fish had at the time of sampling grossly visible and demarcated areas of dark pigmentation on the ocular side. The hyperpigmented area ranged from areas with grossly visible ulcers penetrating into the dermis (Figure 2) interspersed with a varying number of black pigment patches, to areas without gross observable skin lesions. In some cases there were patches of pale areas outside the hyperpigmented areas.

In histological sections of skin of the ocular side of all four affected fish with extensive hyperpigmentation and ulceration, parasites were observed. Based on morphology of the parasite (Figure 3 and 4) and the characteristic opisthaptor (Figure 3) the parasite was identified as *Gyrodactylus unicopula*. The parasites occurred

most frequently in the epidermis (Figure 3), but were also seen attached to the basement membrane, and in the dermis (Figure 4).

The epidermis was characterized by foci of hyperplasia of epithelial cells and of mucous cells, and skin erosions in which the epidermis was sloughed off. Frequently, the skin was ulcerated with a disintegrated, or partly absent, basal membrane. The dermis (Figure 4), and underlying muscle (Figure 5) were affected in some cases, resulting in the degeneration of muscle fibres and haemorrhagic tissue. Extensive pigmentation was seen under and close to the basal membrane, and sporadically elsewhere in the dermis and the muscle layers. In dermis this was confirmed by electron microscopy to be free melanosomes and melanocytes (Figure 4 and 6). Pigments, or pigment cells were not observed in the epidermis. Rod-shaped bacteria were observed in the lesions, both in the epidermis and in the dermis (Figure 6).



Figure 1. Histological section of normal skin on the ocular side of *Pleuronectes platessa*. Epidermis (E), scale pocket (SP), basal membrane and dermis (D) are shown. HE. Bar 200µm.

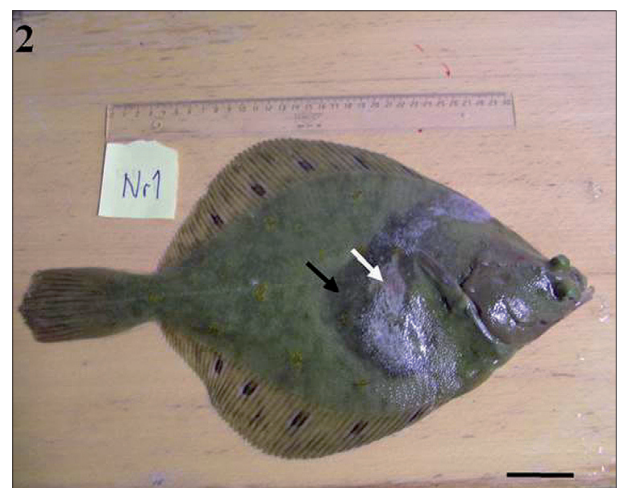


Figure 2. Extensive hyperpigmentation (black arrow) in the skin, and skin where the epidermis are sloughed or eroded (white arrow) in *Pleuronectes platessa*. Bar 5cm.

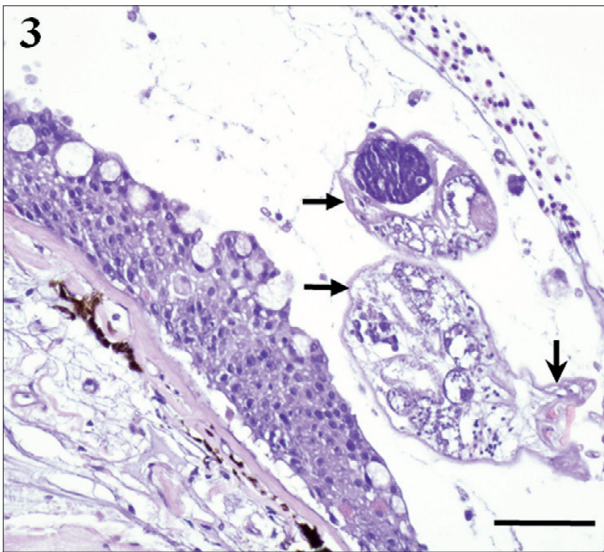


Figure 3. *Gyrodactylus unicopula* (filled arrowheads) on the epidermis of *Pleuronectes platessa*, the opisthohaptor (stealth arrowhead) is shown. HE. Bar 50µm.

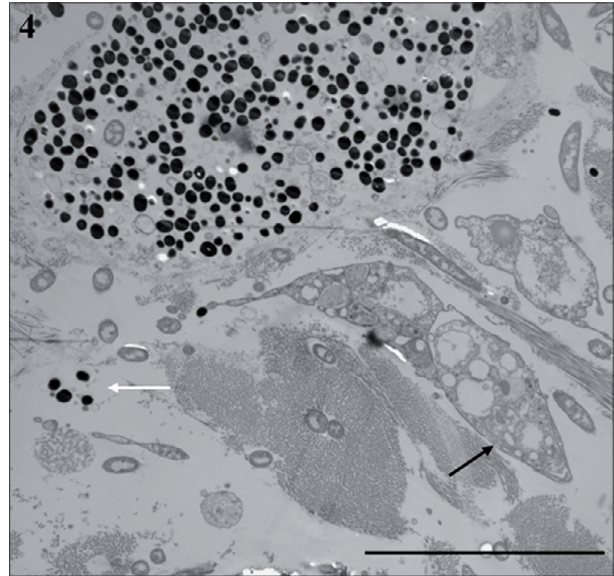


Figure 4. The *Gyrodactylus unicopula* (black arrow) in dermis of *Pleuronectes platessa*. Massive disruption of the dermis and melanocytes are seen. Free melanosomes are indicated (white arrow). TEM. Bar 10µm.

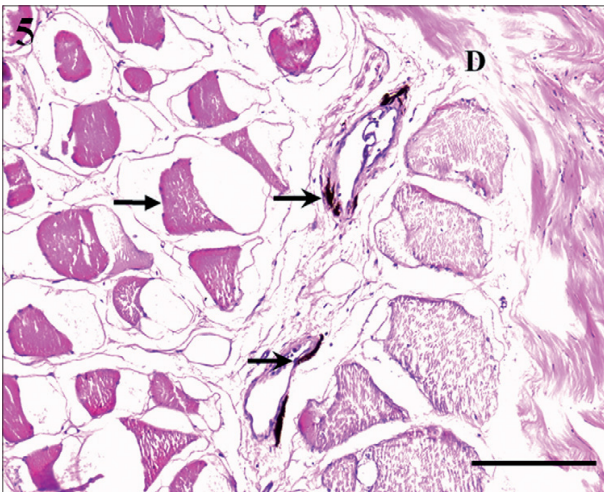


Figure 5. Section of skin *Pleuronectes platessa* where the basal membrane was partly disrupted and the dermis thickened. Degenerated muscle fibers are indicated (filled arrow) and perivascular pigmentation (stealth arrowheads). HE. Bar 50µm.

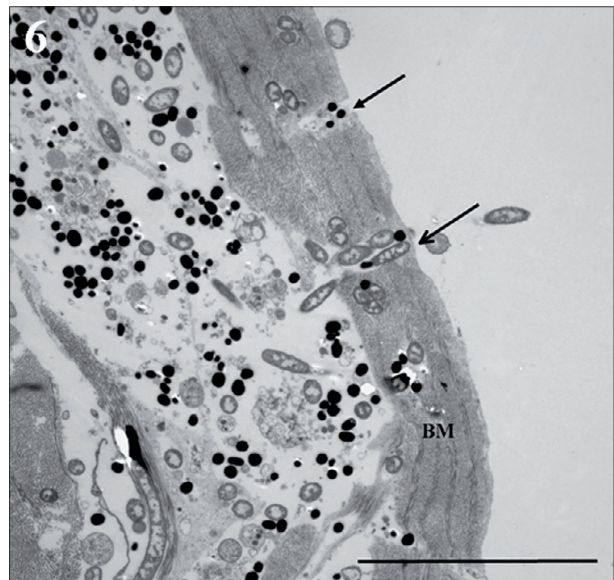


Figure 6. Transmission electron micrographs of the basal membrane (BM) and dermis of *Pleuronectes platessa* skin. The epidermis is sloughed, the intact basal membrane are penetrated by bacteria (open arrowhead). The structure of dermis is disrupted and a high density of bacteria can be seen. Free melanosomes can be seen both within the basal membrane (filled arrowhead), and in the dermis. Bar 10µm.

Bacteria or parasites were not seen in any of the gill sections of affected fish. However, hyperplasia of mucous cells and an increase in leucocytes, with high numbers of eosinophilic granule cells were seen (Figure 7). Normal structure of gills

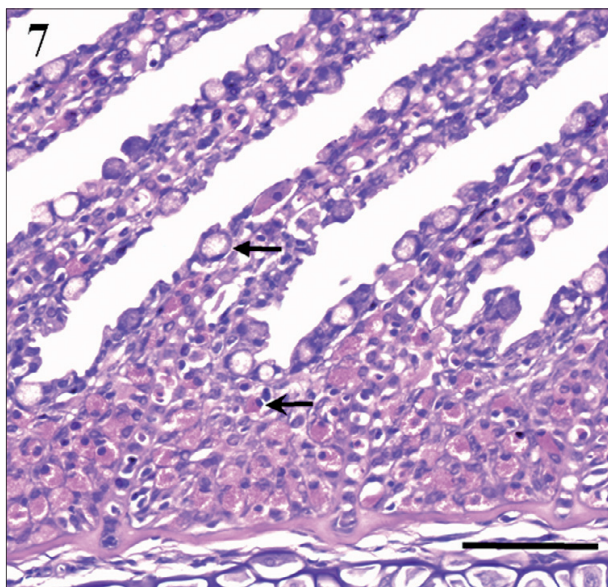


Figure 7. Increased number of eosinophilic granulocytes (stealth arrowhead) and mucous cells (filled arrowhead) in the secondary lamellae of gills of affected *Pleuronectes platessa*. HE. Bar 20µm.

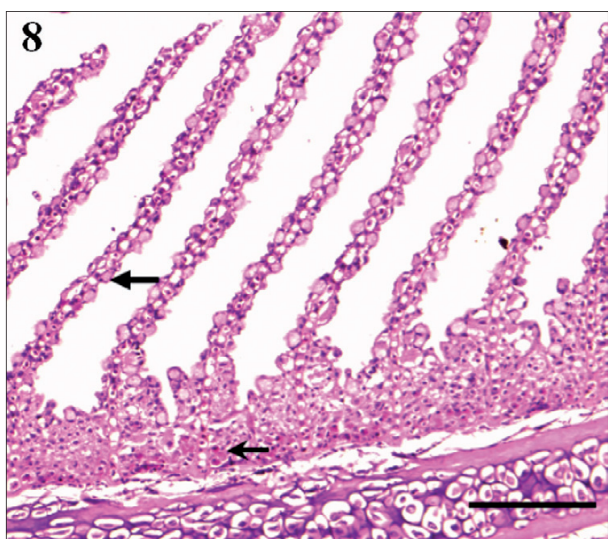


Figure 8. Histological section of normal gills of healthy *Pleuronectes platessa*. Primary and secondary lamella with eosinophilic granulocytes (stealth arrowhead) and mucous cells (filled arrowhead) are shown. HE. Bar 50µm.

of wild caught and healthy plaice are shown in Figure 8.

Discussion

In the present study, plaice showed severe signs of disease and increased mortality after 9 months in captivity. At capture, there was no gross sign of infection; however, the number of ectoparasites is normally very low in wild fish (Berland, 1993). Microscopic investigation of skin samples of diseased fish revealed extensive damages and abnormal distribution of melanin. Parasites, and sporadically rod shaped bacteria, were seen in skin lesions. The parasite was determined to be the marine ectoparasite *G. unicipula*, belonging to the monogeneans which have a direct single host life cycle that can be completed within a closed system. Monogeneans are known to be pathogenic to various flatfish under culture conditions (Thoney and Hargis, 1991), and have been found in the gill filaments of young plaice (Mackenzie K, 1970). The parasite was not observed on the skin of the plaice and mortality was not reported. The same author also stated that *G. unicipula* is the only *Gyrodactylus* that exploits plaice. A close relative to *G. unicipula*, *Neoheterobothrium affine* (Linton, 1898) causes an inflammatory response in the gills of summer flounder, and *Entobdella* species have been reported to cause problems in brood stocks of Dover sole (McVicar and Mackenzie, 1977), supporting our evidence of the pathogenic nature of this group of parasites.

There was a clear relationship between the prevalence of externally visible hyperpigmented lesions and observed structural damage in skin tissue. Parasite infections may cause aggregation of melanin in skin of fish (Lopez et al., 2002). In some dermatopathies, such as ulcerative dermal

necrosis and furunculosis (Roberts, 2001), melanocytes grow into the healing epithelialized scar tissue and eventually differentiate into melanophores. The present morphological results suggest that these pigment cells are probably melanocytes or melanophores. These cells and free melanosomes were observed in the dermis beneath the basal membrane in the affected area of the skin. The role of the extensive distribution of melanin pigments and pigment cells in the affected tissue is not known, but could be a part of the healing response.

Some of the parasites appeared to be anchored to the basal membrane and severe damage from attachment and feeding activities were seen in all layers of the affected skin. The damages observed at the site of infection in the present study are comparable to earlier reports of the direct effects of sea lice attachment and feeding on the body surface of salmon (Johnson et al., 1996). There were no observations of parasites or bacteria in the gills. A possible relationship between the infection of parasites and cellular responses in gills, in particular the high numbers of eosinophilic granular cells seen in gills and skin should be investigated in future studies.

Fish are surrounded by and share the milieu with micro organisms, many of which are pathogens (Law, 2001). Deleterious effects caused by bacteria were also seen in affected tissues. Toxins released from parasites and physical damage from tissue grazing and feeding activity will damage the skin and allow invasion by secondary lesion- and ulcer-causing pathogens (Burkholder et al., 2001), as observed by the presence of rod-shaped bacteria in the lesions. Moreover, parasites themselves may

be pathogen carriers (Cusack and Cone, 1986). Nevertheless, even if assisted by the parasites in intruding the host, the bacteria themselves were obviously able to penetrate tissues as shown in the present investigation.

Our findings suggest a direct cause-and-effect link between mortality and the specific parasite, *G. unicopula*, found within the severe damage of the skin and underlying tissues. However, secondary infections of bacteria clearly had severe effects and may have contributed to high mortality observed in the present study. This is the first report on mortality and skin responses related to infection of *G. unicopula* in *P. platessa*.

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