Aspects of the Atlantic salmon immune response during infection with the salmon louse, Lepeophtheirus salmonis 1837

Dr. Keith Walton,
Senior faculty, Institute of Aquaculture,
University of Stirling, Stirling, Scotland.
Email: kei.walt@gmail.com

Abstract

Atlantic salmon (*Salmo salar*) were experimentally infected with *Lepeophtheirus salmonis* copepodids and aspects of the host's immune response investigated. Copepodid secretory/excretory product (SEP) produced during early settlement was analysed using fast-protein liquid chromatography (FPLC), sodium dodecyl sulphate (SDS)-electrophoresis and zymography. Following establishment and the appearance of the chalimus stages, the expression of the chemokine interleukin-8 (IL-8) in the heart, spleen, head kidney, fins, liver and pyloric cæca was investigated using real-time (quantitative) PCR (qPCR). Furthermore, the secretions of *L. salmonis* chalimus were analysed for the presence of the prostanoid PGE_2 using commercially available enzyme-linked immunoassay (EIA) kits.

Analysis of copepodid secretory/excretory product suggested that any immunosuppressive component is not proteinaceous in nature. Whilst there was a definite increase in protein concentration of SEP relative to control SEP, further analysis using subtractive chromatographic analysis did not reveal any unique fraction present in either SEP or CSEP that was absent in the other. Interleukin-8 expression levels in tissues changed following *L. salmonis* infection, with heart and spleen showing significant increases in IL-8 gene expression, whilst the head kidney, fins, liver and pyloric cæca showed no significant increase. The increase in splenic IL-8 expression may be linked to its role as one of the major secondary lymphoid organs. However, this is the first record of increase in IL-8 expression in cardiac tissue. The secretions of *L. salmonis* chalimus were found to contain quantifiable levels of PGE_2, albeit in highly variable quantities. This concurs with already published findings for adult *L. salmonis* (see Fast, *et al.* 2004). It is proposed that the chalimus states us the PGE_2 to modulate the hosts' immune response at the site of attachment and feeding.

Key word: Aquaculture, immunosuppressive, Endoplasmic reticulum, Parasitic antigens

Introduction

Eicosanoids comprising mainly of prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs), are lipid based mediators with a short half-life that act in an autocrine and paracrine manner (Smith, 1989, Daugschies and Joachim, 2000). They are synthesised *de novo* from polyunsaturated fatty acids (PUFAs; see Figure 23) and share a chain length of 20 carbon atoms (C-20) as a common feature (Cayman Chemical Company, 2005). The prefix *eicosa-* or icosa- (from the Greek for 20) denotes the C-20 (Beare-Rogers *et al.*, 2001). According to their biochemical properties and biosynthetic pathways eicosanoids are classified into epoxids, hydroxyeicosatetraenic acids (HETEs), hydroxyoctadecadienic acids (HODEs), hydroxyperoxyeicosatetraenic acids (HPETEs), lipoxins, prostaglandins (PGs), thromboxanes (TXs) and hepoxilins (Smith, 1989, Kühn and Borngräber, 1998). PGs have a cyclopentane structure whereas TXs are characterised by an oxane ring; both are collectively named prostanoids (Slater and McDonald-Gibson, 1987).

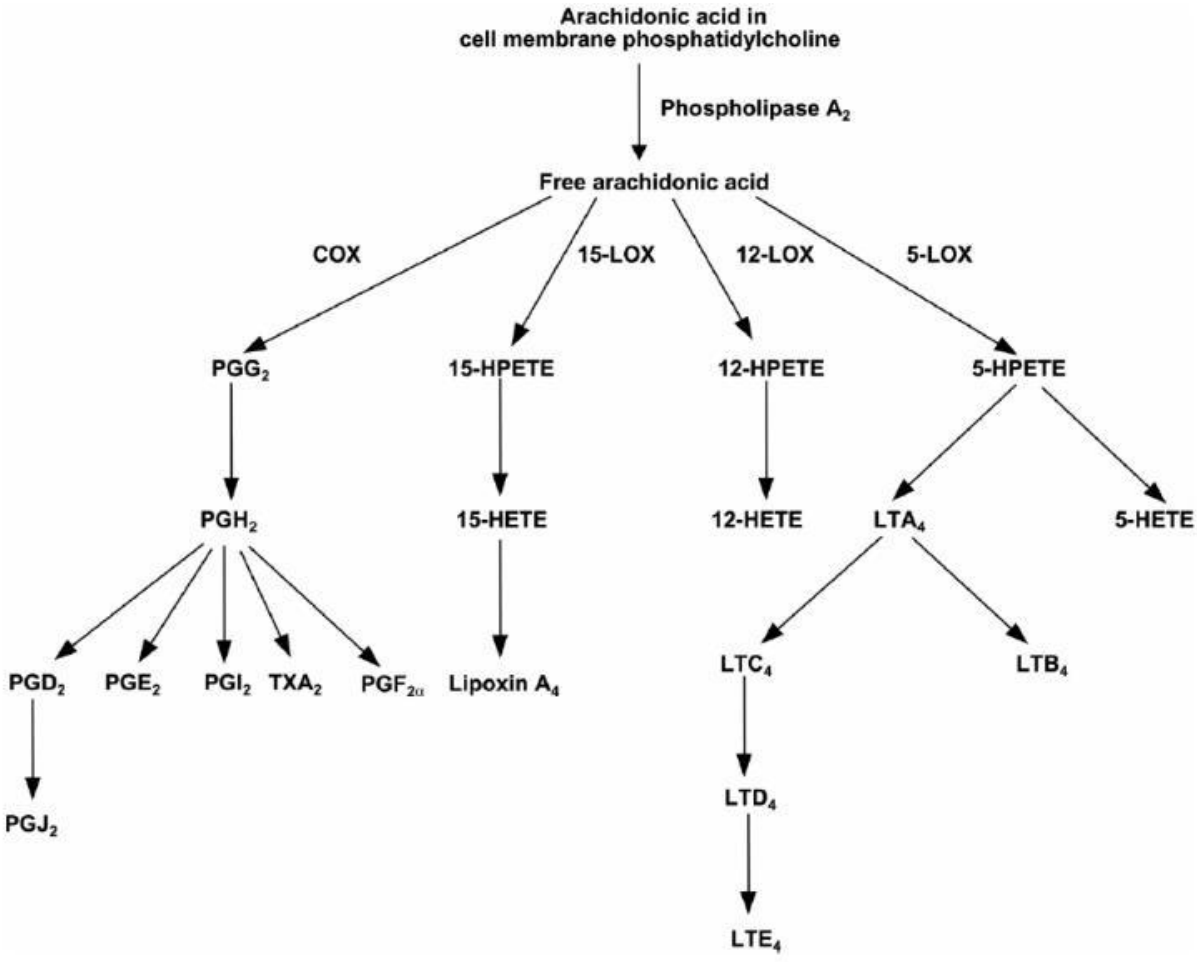

Figure : Simplified scheme of pathways of eicosanoid synthesis (Calder, 2005)

In vertebrates cyclooxygenase (COX) is the key enzyme for prostanoid synthesis (see Figure 23), catalysing the conversion of arachidonic acid first to the intermediate PGG_2, which is subsequently peroxidised to PGH_2 (Belley and Chadee, 1995). This is then enzymatically converted to the various bioactive prostanoids (Smith, 1989).

In mammals there are 2 isoforms of COX: COX-I, which is expressed constitutively (synthesised in the absence of any particular stimulus) and is slightly upregulated by hormones, and COX-II, which is highly inducible (Belley and Chadee, 1995, Kühn and Borngräber, 1998). COX-I is a glycoprotein complex of two identical haem-containing sub units of 70 kDa and is a monotopic membrane protein found primarily in the endoplasmic reticulum of mammalian cells. In contrast, COX-II is believed to be a glycoprotein doublet containing several *N*-linked glycosylation sites with a molecular mass of up to 74 kDa (Belley and Chadee, 1995). *N*-linked glycosylation is required for maximum activity of both isoforms (Belley and Chadee, 1995). Belley and Chadee (1995) report that arthropod-derived COX acts primarily in a constitutive manner (COX-I), however, the discovery of COX-II in mammals has led to a renewed interest in the possibility that to whether parasites may produce a similar enzyme that can be used in pathogenesis and/or immunoregulation (Belley and Chadee, 1995).

Weinheimer and Spraggins (1969) were the first to discover eicosanoids in an invertebrate animal, the octocoral *Plexaura homomella*. Since that time eicosanoid synthesis has been demonstrated to occur in other invertebrate species (Daugschies and Joachim, 2000), playing important roles in ion transport, neurobiology and reproduction (Smith, 1989, Stanley-Samuelson, 1991, De Petrocellis and Di Marzo, 1994, Stanley and Miller, 1998, Ogg and Stanley-Samuelson, 1992). Whilst mammalian eicosanoids are synthesised *de novo*, it is unknown whether this is true for invertebrates. De Petrocellis and Di Marzo (1994) reported that non-parasitic invertebrates are able to store eicosanoids as lipoxin derivates in certain tissues.

Prostaglandins are very important in the biology of invertebrate animals, regulating events within tissues and cells (Stanley-Samuelson, 1994b). The presence and significance of eicosanoids in invertebrates has been presented from various perspectives in several reviews (Brady, 1983; 1985; Stanley-Samuelson and Loher, 1986; Stanley-Samuelson, 1987; 1993; 1994a; 1994b, Sauer *et al.*, 1993; Lamacka and Sajbidor, 1995; Stanley-Samuelson and Pedibhotla, 1996; De Petrocellis and Di Marzo, 1994). Most of the work focusing on COX-II expression in parasites has concentrated on those parasites of public importance such as *Schistosoma mansoni* (Salafsky and Fusco, 1985; Fusco *et al.*, 1993; Salafsky *et al.*, 1984;

Salafsky and Fusco, 1987b;a), *Entamoeba histolytica* (Belley and Chadee, 1995), *Trichobilharzia ocellata* (Nevhutalu *et al.*, 1993) and *Taenia taeniaeformis* (Leid and McConnell, 1983). Recently, however, more resources have gone into studying haematophagous arthropods such as ticks and the effects that PGs can have on their feeding.

The saliva of many tick species has been shown to contain a complex cocktail of pharmacologically active compounds such as immunosuppressants, analgesics, anticoagulants and anti-platelet aggregatory compounds that facilitate feeding (Bowman *et al.*, 1996). Normally a host's haemostatic processes would stop leakage from a blood vessel damaged through haematophagous parasite feeding. This would involve circulating platelets adhering to the damaged vessel wall, being activated and then aggregating to form a plug in the gap and to provide a scaffold for the coagulation process and fibrin clot. Most of the haematophagous arthropods studied to date, however, inhibit platelet aggregation by secreting the enzyme apyrase (Bowman *et al.*, 1996). Those arthropods that do not secrete apyrase instead secreted other anti-platelet aggregatory compounds such as PGI_2 and PGD_2 (Bowman *et al.*, 1996).

The PGs have been detected in the saliva of the cattle tick, *Boophilus microplus,* and the dog tick *Amblyomma americanum* by bioassay and chromatography techniques (Stanley-Samuelson, 1994a) and in the saliva of *L. salmonis* (Fast *et al.*, 2004). There are reports of PGs in other tick tissues; however, the physiological roles of these compounds remains to be elucidated (Stanley-Samuelson, 1994a). Kemp and Bourne (1980) report that histamine causes ticks to detach from their host but other mediators such as bradykinin, PGE_2 and 5-HT (serotonin) have no behavioural effect. The authors conclude that PGs alter the behaviour of the tick to increase the likelihood of finding a suitable host.

The prostaglandins PGE_2 and PGI_2, and to a lesser extent PGD_2, are potent vasodilators that cause dilation of the vascular smooth muscle thereby increasing blood flow (Bowman *et al.*, 1996). In general PGs are able to induce vasodilation without increasing plasma leakage and the associated pain by preventing mast cell degranulation. Furthermore, potent vasoconstriction peptides that are released by the vascular endothelium in response to mechanical injury, shear, stretch, turbulent flow or inflammatory mediators at the site of insult are countered by PGs and in particular PGE_2. The saliva of the tick *Ixodes dammini* (*Ixodes scapularis*) contains substantial quantities of 6-keto-PGF1", the stable degradation product of PGI_2 (Ribeiro *et al.*, 1988). Fezza *et al*. (2003) report that PGE_2 within the salivary gland of ixodid ticks can act in

either an autocrine or paracrine manner through its interactions with PGE_2 receptors. This induces exocytosis (secretion) of bioactive proteins.

Despite prolonged and continuous attachment of many haematophagous arthropods natural hosts mount immune responses that are ineffective (Bowman *et al.*, 1996). Experimental evidence from Wikel (1996) has suggested that rather than evading the host's immune system, haematophagous arthropods suppress it via components of their saliva. The salivary homogenates of several tick species have been shown to impair T-cell function, possibly due to a reduction in the production of cytokines that are vital for ontogeny of the immune response, including recruitment, activation and proliferation of immune cells and also the inflammatory response (Bowman *et al.*, 1996). Ribeiro *et al.* (1985) report the PGE_2 content of *I. dammini* saliva has an inhibitory effect on IL-2 production by T-cells. Further experiments by Inokuma *et al*. (1994) indicate that that PGE_2 content of *Boophilus microplus* saliva was responsible for the inhibition of T-cell proliferation. A conflicting report from Urioste *et al*. (1994), however, reported a similar T-cell suppression from *I. dammini* saliva which lacked PGE_2. This led the authors to conclude that salivary PGE_2 plays a minor role in the immunosuppressive activity of tick saliva. Bowman (1996) proposes that factors other than PGE_2 are potentially immunosuppressive but PGs may exert a limited immunosuppressive effect.

The PGs are intricately involved with pain and inflammation (Bowman *et al.*, 1996). It would be logical to assume that during feeding haematophagous arthropods cause pain to the host thereby increasing the likelihood that grooming will dislodge the parasite. Examination of the process of pain and inflammatory events, however, suggests that ticks not only render the pro-inflammatory properties of PGs ineffective but saliva-derived PGs exhibit anti-inflammatory actions at the feeding site (Bowman *et al.*, 1996).

Lepeophtheirus salmonis infections of susceptible hosts, e.g. Atlantic salmon, are notable in that no significant inflammatory response is elicited (Johnson and Albright, 1992a). However, a well-developed inflammatory response is associated with resistance to infection in coho salmon *(Oncorhynchus kisutch*) (Johnson and Albright, 1992a). Fast *et al*. (2004) and Johnson and Fast (2004) both propose that immunomodulation of the host in the absence of high cortisol levels is responsible for the lack of response in Atlantic salmon. The identification of trypsin and PGE_2 in adult *L. salmonis* secretions by Fast *et al*. (2004, 2003) and Firth *et al*. (2000) have given validity to this hypothesis.

The aim of this study was to determine if secretions of *L. salmonis* chalimus stages contain the prostanoid PGE_2. This chapter describes the commercial enzyme-linked immunoassay (EIA) technique used to measure PGE_2 levels in *Lepeophtheirus salmonis*.

Finding and Discussion

Prostaglandin E_2 (PGE_2) is an eicosanoid that is known to play a variety of roles in the feeding and avoidance of host immune responses in arthropod parasites. The present study has demonstrated that *L. salmonis* chalimus stages do indeed produce quantifiable levels of PGE_2.

Salmonis at concentrations in the same range as those in the saliva of several other arthropod parasites. The authors also recorded the absence of any other type of PG, which correlates with the results for most the saliva of most tick species (Aljamali *et al.*, 2002). During the present study, no type of PG other than PGE_2 was detected, which agrees with the findings of Aljamali *et al*. (2002) and Fast *et al.* (2004). However, it must be noted that EIA kits used in this study have a high specificity for PGE_2. Therefore, the likelihood of detecting non- PGE_2 prostaglandins was minimal.

This study also found a high level of variation in PGE_2 production by *L. salmonis* even within groups that had been off the host for the same amount of time. This concurs with the findings of Fast *et al*. (2004), who reported similar variation in adult lice. Aljamali *et al*. (2002) reported a fivefold variability in levels of prostaglandin in the saliva of *Amblyomma americanum* from the same population following dopamine stimulation. The authors also noted that prostaglandin production can also vary depending on the length of time since the most recent feeding. Based on the results of this study it is proposed that the variation seen in the levels of PGE_2 production of *L. salmonis* chalimus stages are the result of minor ontogenetic differences between the chalimus on the samples. Furthermore, as chalimus stages feed a minimal amount compared to the later developmental stages, this suggests that length of time since feeding can be discounted as a possible explanation.

Fast *et al*. (2005) demonstrated that PGE_2 at physiologically meaningful levels, and in the absence of a stress response, was able to inhibit expression of IL-1!, COX-II and MH class I and II genes and Pinge-Filho *et al*. (1999) reported PGE_2 down-regulates the pro-inflammatory cytokines IL-1! and TNF". Furthermore, Fast *et al*. (2006) concluded that, as blood constitutes a (minor) component of the sea louse's diet (Brandal *et al.*, 1976; Bricknell *et al.*, 2003; Haji Hamid *et al.*, 1998) PGE_2 could be used by *L. salmonis* to increase blood to the feeding site but

also to prevent leukocyte recruitment and presentation of parasitic antigens to T lymphocytes. This concurs with the findings of Papadogiannakis *et al*. (1984), Papadogiannakis and Johnsen (1987) and To and Schrieber (1990) who all propose that PGE_2 may also adversely affect site-specific leukocyte recruitment and function. Fast *et al*. (2004) propose that the role of PGE_2 as a systemic modulator is unlikely, citing its high instability, which leads to it losing its activity following one passage through the circulatory system in mammalian models. The findings of Fast *et al.* (2004) and the findings of the present study may explain Jónsdóttir *et al*.'s 1992 and Johnson and Albright's 1992b findings that there is minimal tissue response in Atlantic salmon to *L. salmonis* beneath the site of active feeding and attachment yet an inflammatory response in tissues surrounding the lesion.

Down-regulation of host inflammatory cytokines has been observed in several other host-parasite relationships such as *Rhipicephalus sanguineus* (Ferreira and Silva, 2001), ixodid ticks (Fuchsberger *et al.*, 1995), *Rhipicephalus appendiculatus* (Gwakiska *et al.*, 2001), *Ixodes ricinus* (Kopecky *et al.*, 1999) and *Ixodes scapularis* (Schoeler *et al.*, 1999; 2000). Ferreira and Silva (1998) report that the saliva from *Rhipicephalus sanguineus* ticks also impairs T cell proliferation and IFN-#-induced macrophage microbial activity. Prostaglandin E_2 can cause polarisation towards a Th_2 lymphocyte response by downregulating pro-inflammatory cytokines (Betz and Fox, 1991), which has also been observed in other arthropod parasites hosts (Ramachandra and Wikel, 1992; Schoeler and Wikel, 2001) and can delay the clearance of secondary bacterial infections (Dalton and O'Neill, 2002).

It cannot be discounted that the production of host mucus may also be increased due to the presence of PGE_2 (Fast *et al.*, 2004). This is of particular importance in *L. salmonis* infections as it forms part of their diet. Nettesheim and Bader (1996) and Tani *et al*. (2002) report that PGE_2 encourages mucin secretion from rat tracheal and gastric epithelial cells. Furthermore, Nolan *et al*. (1999) observed increased mucus production by Atlantic salmon skin epithelia following infection with low numbers of *L. salmonis* adults. However, the potential role of PGE_2 in this observation has yet to be elucidated.

In artificial infections in the laboratory, infection with high numbers of *L. salmonis* commonly results in host mortality at the moult from chalimus to pre-adult stage without the development of lesions (Bjorn and Finstad, 1997; Grimnes and Jakobsen, 1996; Ross *et al.*, 2000). Additionally, Fast *et al.* (2002) and Mustafa *et al.* (2000b) both report reduced macrophage function and increased susceptibility to secondary infection in infected fish without

the presence of a cortisol response. PGE_2 or other *L. salmonis*-derived compounds may be responsible at least in part for these observations (Fast *et al.*, 2004). For example the sudden and high level of host mortalities reported at the moult from chalimus to the pre-adult stage has parallels with a toxic shock response. Whilst the role of PGE_2 in toxic shock is unknown, prostanoids release has been demonstrated to occur early in the course of shock. The inhibition of such a release has been shown to significantly increase survival in mammalian toxic shock models (Ball *et al.*, 1986; Lefer, 1983).

Conclusion

In conclusion there is a good body of evidence to support the hypothesis that *L. salmonis* immunomodulates its hosts by producing substances such as prostaglandin E_2. Fast *et al*. (2004) reported adult *L. salmonis* are capable of producing PGE_2, which they propose modulates the host's immune system. The present study has demonstrated that the chalimus stages of *L. salmonis* are also capable of producing PGE_2 and whilst its exact purpose has not been investigated, many of the observations reported during the early stages of *L. salmonis* can be attributed to PGE_2.

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