Hepatocellular fibrillar inclusions in European flounder (*Platichthys flesus*): endocrine disruption in the marine environment?

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Abstract

Hepatocellular fibrillar inclusions (HFI) are an unusual pathology of unknown aetiology affecting the flatfish species European flounder (*Platichthys flesus*) and have previously been observed in UK estuaries impacted by endocrine disrupting chemicals. Using a multidisciplinary approach, this study explores the hypothesis that induction of vitellogenin as a result of exposure to endocrine disrupting chemicals is associated with the development of HFI. Thus HFI could serve as a potential histological biomarker of endocrine disruption in male *P. flesus.* Liver samples (n= 200) were collected from *P. flesus* at selected UK estuaries during autumn 2010, processed for histopathology and examined for incidences of HFI. The prevalence of HFI in UK estuaries was ranked in the following order: Mersey (79.3 %) > Tyne (78.6 %) > Thames (63.6 %) > Medway (44.7 %) > Humber > (34.5 %) > Alde (3.7 %). The severity of HFI was evaluated using a qualitative scoring index and revealed that severity appeared to be more pronounced in those estuaries with higher prevalence of HFI (r= 0.98). The data also demonstrated that male fish exhibited a higher prevalence of HFI compared to females (p= <0.001). No association between HFI and *P. flesus* age was observed (p= 0.648). Electron microscopy examination confirmed that HFI were the result of extensive disorganisation and proliferation of the rough endoplasmic reticulum (RER). The *P. flesus* from the Mersey and Tyne provided robust biological material for further study and were collectively categorised into biological groups of HFI severity stages. Real-time Polymerase Chain Reaction (qPCR) demonstrated no significant differences between biological groups and transcription of vitellogenin (VtG) (p= 0.3098) and choriogenin (CHR) (p= 0.5317). Plasma VtG concentrations greater than 1 µg ml^{-1} were only observed in 10 fish during the entire study and 6 of these originated from the Tyne and Mersey (range 1.7-1944.0 µg ml^{-1}). The low level of gene transcription accompanied by near background concentrations of plasma VtG made it difficult to identify relationships between gene transcription, HFI formation and protein translation. Despite this, immunohistochemistry (IHC) successfully identified VtG with an immediate association with HFI. The IHC labelling was clear and distinct exhibiting no unspecific binding of primary antibody. This study was unable to confirm previous reports of microtubules within HFI using IHC

incorporating a α/β- tubulin polyclonal primary antibody for zebrafish. Whilst this study was unable to provide conclusive evidence concerning the implication of endocrine disruption on HFI development, the detection of VtG within HFI of male fish does highlight endocrine disruption as a potential avenue for future laboratory studies concerning the pathogenesis of HFI. However, until laboratory experiments are able to establish links, it is important to remember that other mechanisms may still be implicated.

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Abbreviations and Definitions

ATUB	α-tubulin
BEQUALM	Biological Effects Quality Assurance in Monitoring
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
Cefas	Centre for Environment, Fisheries and Aquaculture Science
CHR	Choriogenin
CNS	Central nervous system
CSEMP	Clean Seas Environmental Monitoring Programme
DES	Diethylstilbestrol
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
EDC	Endocrine Disrupting Chemicals
EDMAR	Endocrine Disruption in the Marine Environment
EF1	Elongation factor 1
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
FACT	F-actin
FCA	Foci of cellular alteration
FFPE	Formalin fixed paraffin embedded
FSH	Follicle stimulating hormone
GES	Good Environmental Status
GnRH	Gonadotrophin releasing hormone
GtHl/ll	Gonadotrophin l/ll
GVBD	Germinal vesicle breakdown
HE	Haematoxylin and eosin
HIER	Heat-induced epitope retrieval
HPG axis	Hypothalamus-pituitary-gonadal axis
HFI	Hepatocellular Fibrillar Inclusion
ICES	International Council for the Exploration of the Seas
IHC	Immunohistochemistry

IMS	Industrial methylated sprit
JAMP	Joint Assessment Monitoring Programme
LH	Luteinising hormone
LMD	Laser Microdissection
MIS	Maturation-inducing steroids
MPF	Maturation -promoting factor
mRNA	Messenger ribonucleic acid
MSFD	Marine Strategy Framework Directive
NBF	Neutral Buffered Formalin
nPR	Nuclear progesterone receptor
OCT	Optimal Cutting Temperature
OSPAR	Oslo-Paris Commission
PAH	Polycyclic Aromatic Hydrocarbons
PAS	Periodic acid Schiff's
PCB	Polychlorinated biphenyls
PKC	Protein kinase C
qPCR	Real-time Polymerase Chain Reaction
RER	Rough endoplasmic reticulum
SGIMC	Study Group on the Integrated Monitoring of Contaminants
STW	Sewage treatment works
TBS	Tris buffered saline
TEM	Transmission Electron Microscopy
UBQ	Ubiquitin
VtG	Vitellogenin
WGBEC	Working Group for the Biological Effects of Contaminants
Zr	*Zona radiata*

1.0 Introduction

Marine pollution has long been the subject of concern for conservationists and environmental managers alike. As such there has been a growing need to measure both spatial and temporal trends of anthropogenic chemicals to help in the mitigation of their environmental impact. Pollution of the aquatic environment originates from numerous anthropogenic sources including mining; fuel combustion; agricultural use of pesticides and herbicides; chemical and materials manufacturing; industrial and domestic waste; and environmental chemical spills (Kime, 1998). Until relatively recently, there has been little enforcement in environmental monitoring of marine contaminants and the biological effects they induce in organisms. The European Union (EU) Marine Strategy Framework Directive (MSFD) is an important piece of legislation whereby all member states must achieve Good Environmental Status (GES) by the year 2020. This is characterised by a collection of qualitative "descriptors" which state that anthropogenic activities must not adversely affect the marine environment in relation, but not limited to, biodiversity, food webs, fisheries and pollution. This legislation now underpins the marine monitoring effort undertaken in the UK and EU member states.

Historically, environmental monitoring programmes have primarily focused on measuring chemical concentrations in water, sediments and biota; however in recent years there has been growing concern regarding the biological effects of contaminants on marine organism health. Descriptor 8 of the MSFD stipulates that concentrations of contaminants in the marine environment must not be at levels that give rise to pollution effects (MSFD, 2008). To this end, the International Council for Exploration of the Seas (ICES) Study Group on the Integrated Monitoring of Contaminants (SGIMC) has developed a comprehensive integrated monitoring programme utilising fish and invertebrate biomarkers to assess the biological effects of contaminants within inshore and coastal waters (Davies and Vethaak, 2012). This programme has been adopted by the UK and taken forward under the Clean Seas Environmental Monitoring Programme (CSEMP). By

integrating chemical endpoints with whole organism, tissue and sub-cellular biomarkers, the programme provides a comprehensive picture of the health status of the marine environment. In addition to investigating causal links, the identification of specific chemical and biological interactions concerning existing and emerging chemical compounds is particularly desirable. Programmes such as the CSEMP help environmental managers and government policy makers to make improvements to existing measures designed to mitigate the effects of anthropogenic chemicals on aquatic ecosystems. Unfortunately these ideals are seldom achieved due to the complexities encountered when the growing numbers of chemicals present in the aquatic environment are considered. In light of this, marine monitoring programmes are adopting a weight of evidence approach to investigate causal links between classes of contaminants and biomarkers of biological effects, whilst attempting to identify specific chemical and biological interactions. This task is undoubtedly challenging, although previous studies have been successful in the identification of specific relationships between anthropogenic chemicals and their effects, particularly in the field of hepatocarcinogenicity and endocrine disruption (Waite *et al*., 1991; Sumpter and Jobling, 1995; Matthiessen *et al*., 1995; Harries *et al*., 1999; Allen *et al*., 1999b; Raut and Angus, 2010; Chow *et al*., 2013).

1.1 Endocrine regulation of gametogenesis in fish

A large number of physiological functions are dependent on specific interactions between messaging of the central nervous system (CNS) and endocrine system. The CNS utilises rapid electrical messaging for the initiation of immediate biological responses, whereas hormone messaging is generally slow and facilitates comparatively longer lasting changes. Hormones are chemical messengers secreted by endocrine glands into circulating blood plasma, which only act on specific target sites despite their distribution throughout an organism. This specificity exists due to the presence of specific receptors (membrane and nuclear) that preferentially bind corresponding hormones. Once a hormone has bound to a receptor site, it gives rise to intracellular messaging or direct binding of this hormone-receptor complex to regulatory elements for a particular gene; both of

which bring about changes in cell function. Some hormones, such gonadotrophin-releasing hormone (GnRH) do not require transport in the bloodstream and instead target tissues within the immediate proximity of release.

The endocrine regulation of sexual development and maturation in teleost fish is dictated by numerous exogenous environmental factors such as, temperature, food availability, physiological and nutritional state, light and social behaviour. This ensures optimal conditions for successful reproduction and offspring survival. This multifactorial influence stimulates the CNS and subsequently the endocrine system via the hypothalamus and pituitary resulting in the release of gonadal hormones that facilitate sexual development. This interaction is widely referred to as the hypothalamus-pituitary-gonadal (HPG) axis and is tightly regulated by feedback mechanisms (figure 1).

Following stimulation of the hypothalamus, secretion of several neurotransmitters and neuropeptides influence the behaviour of GnRH neurons located in the pre-optic area and medial basal hypothalamus. GnRH is released from nerve terminals and binds to receptors on the plasma membrane of nearby gonadotrophin cells (gonadotropes) situated within the anterior pituitary. Once this hormone-receptor complex has been formed, a series of intracellular signalling pathways are triggered that regulate the synthesis and secretion of a group of hormones called gonadotrophins. Gonadotrophins are hormones responsible for stimulating the synthesis of sex steroids (androgens, oestrogens and progestins) that, depending on the sex, target specific tissues so that gametogenesis, reproduction, sexual phenotype and behavioural characteristics can be successfully regulated. Neurotransmitters such as dopamine and serotonin may also have an influence on the secretion of GnRH and gonadotrophins through direct stimulation of the pituitary (Van der Kraak, 1997). The reproductive cycle is controlled by two gonadotrophins: follicle stimulating hormone (FSH) and luteinising hormone (LH) (Nagahama, 1994; Janz, 2000; Thomas, 2008). Whilst the physiological functions of these gonadotrophins have not been fully described in all fish species, they have been studied extensively in salmonids and their function is dependent on the sex of

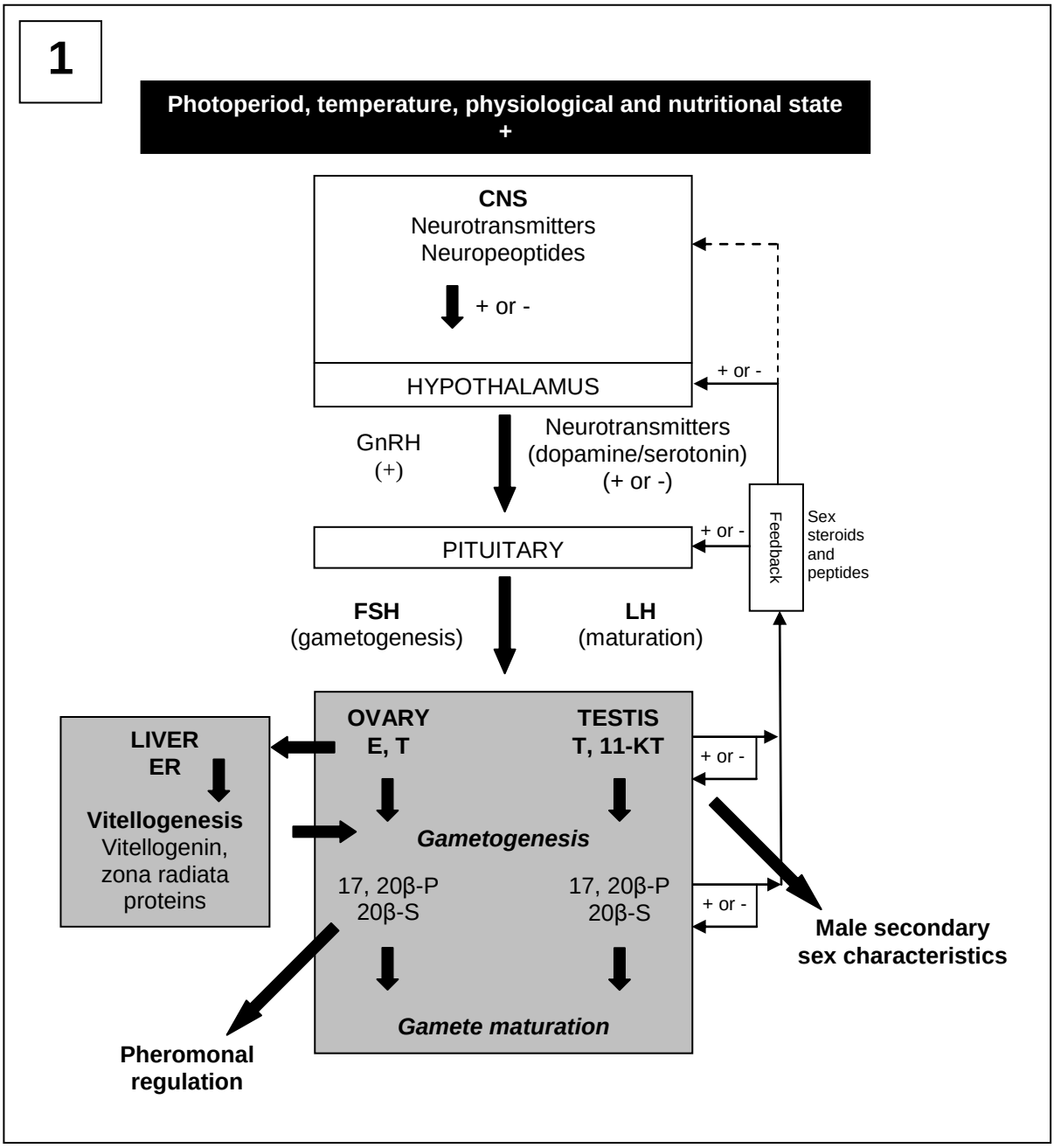


Figure 1: Schematic diagram of hypothalamus-pituitary-gonadal (HPG) axis adapted from Thomas (2008).

the fish. FSH plays an important role during early gonadal development, vitellogenesis and spermatogenesis. Conversely, LH generally regulates the final stages of the reproductive cycle such as oocyte maturation, ovulation and spermiation. FSH stimulates spermatogonial proliferation during early gonadal development; however it is not believed to have direct control over early development of females (oogonial proliferation and primary oocyte growth) as identified in males. FSH and LH both play an important role in the latter stages of gamete growth and maturation during oogenesis and spermatogenesis.

Oogenesis is the process associated with oogonial recruitment, oocyte development and ovulation. Following recruitment of oogonia, a single layer of granulosa cells develops around the oocyte. A layer of connective tissue surrounding the granulosa cells produces an additional layer called the theca. The granulosa and thecal cells are responsible for sex steroid production following hormonal stimulation (Nagahama, 1994; Janz, 2000). During the early stages of oogenesis, stimulation from the CNS results in the release of GnRH from nerve terminals within the hypothalamus which brings about the subsequent release of FSH and LH by gonadotropes of the anterior pituitary. In females, FSH is primarily responsible for the initiation of vitellogenesis and zonagenesis, whereas LH is involved in the final steps of oocyte maturation and ovulation (Thomas 2008). Following release from gonadotropes, the concentration of FSH increases within circulating blood plasma and binds to receptors situated on the thecal cell layer. This hormone-receptor complex results in the synthesis of testosterone that subsequently diffuses into the granulosa cell layer where it is transformed via aromatisation into 17 β-estradiol (Janz, 2000). The 17 β-estradiol regulates proteins that make up the vitelline envelope (*zona radiata*) of developing oocytes. It is secreted by granulosa cells into the blood plasma, travels to the liver and binds to oestrogen receptors within hepatocytes. This results in the synthesis of the yolk protein precursor vitellogenin (VtG) (vitellogenesis) and eggshell protein *zona radiata* (Zr) (zonagenesis) by hepatocytes through the activation and transcription of their respective genes (Arukwe, 2001; Thomas 2008). VtG and Zr are secreted by hepatocytes into the blood plasma and travel to the ovary where they diffuse

across the theca and granulosa. VtG binds to specific receptors on the oocyte surface where it is absorbed and undergoes cleavage into the major yolk proteins lipovitellin and phosvitin. These proteins are subsequently utilised for oocyte growth and development. Zr forms a non-cellular protective coat between the granulosa and developing oocyte. Once the concentration of 17 β-estradiol reaches a specified threshold, a negative feedback loop is initiated that inhibits the otherwise continued production of GnRH by gonadotropes (Thomas 2008). As the oocyte continues to grow, there is a decrease in FSH concentration and a concomitant increase in LH that trigger the production of maturation-inducing steroids (MIS) within the ovarian wall. Oocyte maturation occurs in two stages (1) the MIS independent stage and (2) the MIS dependant stage. The MIS independent stage prepares the oocytes for interaction with the MIS, which is reflected by an up regulation of the MIS receptor and connexin (the main constituent of gap junctions), at the oocytes plasma membrane. However, some species can demonstrate a decrease in connexcins that may be species specific (Cerdá *et al*., 1993). The MIS dependant stage begins with the migration of the nucleus (germinal vesicle) to the animal pole region of the oocyte where germinal vesicle breakdown (GVBD) takes place (Nagahama, 1994). GVBD is initiated following the binding of MIS to specific progestin receptors situated at the plasma membrane. In addition, this complex results in a decrease in cellular concentrations of cyclic adenosine monophosphate (cAMP) allowing meiosis to continue through the production of Maturation Promoting Factor (MPF). The ooplasm undergoes further changes including lipid coalescence, hydration and the formation of an oil droplet to increase the relative buoyancy of pelagic eggs (species specific). The completion of oocyte maturation is followed by ovulation (Janz, 2000). MIS also binds to a nuclear progesterone receptor (nPR) in the ovarian follicle wall which causes activation of protein kinase C (PKC). PKC stimulates the synthesis of arachidonic acid which is enzymatically transformed into prostaglandin. Prostaglandin is subsequently used to regulate the contraction and relaxation of smooth muscle situated in the follicle wall that eventually results in oocytes becoming released (Thomas, 2008).

In comparison to oogenesis, the regulation of spermatogenesis is relatively unstudied. Spermatogenesis broadly consists of three distinct stages (1) proliferation of spermatogonia through mitosis of stem cells (2) meiosis of spermatocytes and (3) transformation of spermatids into spermatozoa. Two cell types have supporting roles during spermatogenesis. Spermatogonia are provided with physical and chemical support during spermatogenesis by Sertoli cells situated within immediate proximity to the spermatogonia. Leydig cells are situated within the adjacent connective tissue where they carry out the synthesis of sex steroid hormones for spermatogenesis. Similarly to females, these sex steroids also serve as a regulatory feedback mechanism to the hypothalamus and pituitary. FSH is generally present at elevated concentrations right up until spawning occurs, whilst LH is typically low throughout the period of growth and becomes elevated when spawning commences (Nagahama, 1994). Following secretion by gonadotropes, FSH regulates the proliferation of spermatogonia and the production of nutrients and growth factors by the Sertoli cells. These nutrients and growth factors are also under the influence of testosterone and 11-ketotestosterone (androgens) and trace amounts of 17 β-estradiol, which are produced by the Leydig cells following interaction with circulating LH. Similarly to females, LH controls the maturation process (development of spermatozoa) through the triggering of maturation-inducing steroids (MIS) production.

The HPG-axis is tightly regulated through the production of several sex steroid hormones and the feedback mechanisms that they evoke (figure 1). The integrity of these regulatory mechanisms and the biological responses they elicit are at considerable risk in the presence of exogenous chemicals possessing similar hormonal properties to, or preventing the function of endogenous hormones. Unfortunately, many laboratory and field studies have successfully identified numerous chemicals possessing so called endocrine disrupting effects.

1.2 Endocrine disruption

Endocrine disruption was brought to the attention of the public and scientific populous in 1996 with the publication of "*Our Stolen Future"* (Colborn *et al*., 1996).

This widely acclaimed scientific paper-novel crossover reviews the scientific literature and describes how anthropogenic chemicals can possess similar properties to those of hormones, thus having serious effects on the endocrine system (endocrine disruption). Endocrine disrupting chemicals (EDCs) are chemical compounds (both natural and synthetic) that interfere with the production and regulation of natural hormones and their subsequent effects. They are thought to have five main mechanisms by which they bring about adverse biological effects. EDCs may:

1. Have a similar structure to hormones and are able to bind to receptors that are intended to be the target for natural circulating hormones.
2. Block receptors thus preventing natural hormones from binding and fulfilling their role and function.
3. Bring about an increase in the number of receptors thus amplifying natural hormonal effects.
4. Directly/indirectly interact with natural circulating hormones resulting in a change of hormonal messaging.
5. Increase/decrease hormone production and their regulation by affecting feedback mechanisms resulting in a hormonal imbalance.

Since the late 1920s there has been a plethora of synthetic chemicals manufactured for medicinal, agricultural and industrial purposes. Laboratory and field studies have demonstrated that many of these chemicals have caused biological abnormalities in humans, mammals, birds and fish (Colborn *et al*., 1996). In human medicine, the synthetic oestrogen diethylstilbestrol (DES) was routinely administered to “at risk” pregnant woman to prevent miscarriages, frequently leading to the formation of vaginal clear-cell cancer and severe defects to the reproductive tract of the offspring during adulthood (Herbst *et al*., 1971). Elsewhere, artificially induced increased levels of oestrogen (both natural and synthetic) in laboratory rats during pregnancy were found to cause significant physical reproductive defects (Greene *et al*., 1938). The production of industrial chemical compounds and their subsequent by products are frequently released

into the aquatic environment where they accumulate in exposed organisms. Compounds such as methoxychlor, polychlorinated bophenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT), primarily manufactured for use in pesticides, plastics, electrical transformers and other industrial applications, have been found to possess significant oestrogenic activity (Arukwe, 2001). The more persistent of these compounds bio-accumulate in successive levels of biological food chains where they have negative effects on reproduction (Colborn *et al*., 1996).

There has been increasing concern in recent years regarding anthropogenic inputs from domestic, agricultural and industrial effluents, into the aquatic environment and their potential endocrine disrupting effects; particularly those chemical compounds possessing oestrogenic properties (Kime, 1998; Arukwe and Goksoyr, 1998; Scott *et al*., 2006a; Raut and Angus, 2010). This has resulted in a significant body of research investigating the effects of EDCs on aquatic organisms through laboratory and field studies. Many of these studies investigate the ability of EDCs to interfere with production and regulation of sex steroid hormones through oestrogenic and/or androgenic effects.

1.3 Endocrine disruption in the freshwater environment

Much of the work on aquatic endocrine disruption has focussed on the induction of vitellogenesis in male fish that does not ordinarily occur owing to the absence of endogenous 17 β-estradiol. Despite this, the presence of oestrogen receptors in liver of male fish can lead to the expression of the VtG gene in the presence of a natural or synthetic oestrogen (Sumpter and Jobling, 1995). The result is the hepatocellular production and secretion of VtG into the blood plasma. The absence of oocytes to sequester the protein effectively can lead to an increase in circulating concentrations of VtG. The measurement of VtG concentrations in the blood plasma of male fish therefore serves as a real-time biomarker of acute oestrogenic activity in the aquatic environment (Purdom *et al*., 1994; Sumpter and Jobling, 1995; Folmar *et al*., 1996; Kime *et al*., 1999). Vitellogenesis has been used effectively to investigate oestrogenic effects following prior observations of

individual fish with both male and female sex characteristics downstream of a sewage treatment works (STW). A subsequent study reported that caged male rainbow trout (*Onchorynchus mykiss*) positioned in STW effluent at several freshwater field locations exhibited a 500 to 100,000-fold increase in VtG blood plasma concentration that was indicative of exposure to environmental oestrogens (Purdom *et al*., 1994). Numerous studies have since adopted this approach to investigate oestrogenic activity in laboratory and field studies (Folmar *et al*., 1996; Harries *et al*., 1999; Folmar *et al*., 2001; Kleinkauf *et al*., 2004; Liney *et al*., 2005; Scott *et al*., 2006b; Scott *et al*., 2007). Furthermore, considerable efforts have been made to investigate potential endocrine disrupting chemicals from other sources including heavy metals, pesticides, PCBs and polycyclic aromatic hydrocarbons (PAHs). Many of these have been found to have, amongst others, oestrogenic properties (Wester *et al*., 1985; Wester and Canton, 1986; Kime, 1998; Vega-Lopez *et al*., 2006; Rodas-Ortiz *et al*., 2008; Pait and Nelson, 2009; Yum *et al*., 2010). Endocrine disruption can manifest itself in a more dramatic manner. The development of ovotestis (intersex) in male fish has previously been demonstrated in fish sampled from sites impacted by compounds possessing oestrogenic activity. Roach (*Rutilus rutilus*) sampled from rivers contaminated with treated sewage effluent exhibited the presence of ovotestis of varying severity (Jobling *et al*., 2002). Harris *et al.* (2011) further reported that a high prevalence of ovotestis in *R. rutilus* could have serious implications at the population level. Naturally, the population level effects of EDCs are concerning and worthy of further investigation (Mills and Chichester, 2005; Ford *et al*., 2007).

1.4 Endocrine disruption in the marine environment

Marine fish species have similarly been implicated in aquatic endocrine disruption studies with considerable efforts undertaken to investigate effects and monitor corresponding trends (Allen *et al*., 1999a; Allen *et al*., 1999b; Kleinkauf *et al*., 2004; Kirby *et al*., 2006; Scott *et al*., 2006b; Scott *et al*., 2007; Velasco-Santamaria *et al*., 2010). In the UK, the Endocrine Disruption in the Marine Environment (EDMAR) programme extensively investigated the effects of endocrine disruption on the reproductive health of marine fish and invertebrates from several estuaries. During

this study, VtG concentrations in the blood plasma of male European flounder (*Platichthys flesus*) were particularly elevated at the Clyde, Tyne, Tees and Mersey estuaries (Allen *et al*., 1999a; Allen *et al*., 1999b). Subsequent temporal trend analysis revealed that whilst a relative decline in VtG levels was evident in male *P. flesus* sampled from the worst affected estuaries, VtG concentrations still remained high (Kirby *et al*., 2004).

Elevated VtG concentrations in marine fish have primarily been reported in estuarine species presumably due to higher anthropogenic impacts in these locations, although there is evidence that offshore species can be affected (Scott *et al*., 2006b; Scott *et al*., 2007). Similarly to freshwater fish species, ovotestis has been observed in several euryhaline and marine fish species including viviparous blenny (*Zoarces viviparous*), three spined stickleback (*Gasterosteus aculeatus*), thick lipped grey mullet (*Chelon labrosus*), Mediterranean swordfish (*Xiphias gladius*) and European flounder (*Platichthys flesus*) (Gercken and Sordyl, 2002; Vethaak *et al*., 2002; De Metrio *et al*., 2003; Lyons *et al*., 2004; Pettersson *et al*., 2007; Puy-Azurmendi *et al*., 2013). In the UK, ovotestis in *P. flesus* has been reported from locations demonstrating high VtG concentrations in male blood plasma (Simpson *et al*., 2000; Stentiford *et al*., 2003; Kirby *et al*., 2004). Reports of ovotestis in offshore species are relatively rare with affected fish primarily consisting of estuarine and coastal fish species (Stentiford and Feist, 2005).

1.5 Molecular tools in environmental monitoring

Biomarker techniques and tissue level changes of endocrine disruption that can detect recent effects are invaluable for investigating GES under descriptor 8 of the MSFD. Fishes are particularly sensitive to oestrogenic EDCs during the early phases of sexual differentiation (Devlin and Nagahama, 2002). Ovotestis is potentially a chronic legacy effect providing an indication of past exposure, whereas elevated plasma VtG concentrations in male fish provide insight into immediate effects. Vitellogenesis and ovotestis in male fishes are established as reliable biochemical and tissue level biomarkers of endocrine disruption, demonstrated by numerous field and laboratory studies (Folmar *et al*., 1996; Tyler

et al., 1998; Harries *et al*., 1999; Oberdörster and Cheek, 2001; De Metrio *et al*., 2003; Liney *et al*., 2005; Scott *et al*., 2006a). With recent advances in molecular based techniques and approaches, there is increasing interest in their application to investigate chemicals and their pollution effects (Rotchell and Ostrander, 2003; Marchand *et al*., 2006; Williams *et al*., 2007; Scholz and Mayer, 2008; Santos *et al*., 2010; Nogueira *et al*., 2010; Evrard *et al*., 2010a; Evrard *et al*., 2010b; Bozinovic and Oleksiak, 2011; Zhao and Hu, 2012; Robertson and McCormick, 2012). The Real-time polymerase chain reaction (qPCR) has gained particular popularity to investigate differential gene expression in aquatic molecular toxicology studies (Marchand *et al*., 2006; Zhang *et al*., 2008; Tyler *et al*., 2009; Nogueira *et al*., 2010; Evrard *et al*., 2010a; Zhao and Hu, 2012; Gagne *et al*., 2013; Truter *et al*., 2014). The application of qPCR offers numerous potential benefits including high specificity and throughput of biological samples at comparatively low cost; in toxicological studies, the ability to investigate gene expression pertaining to PAH, PCB, metal and oestrongenic exposure in a single procedural step; relative ease in gene expression quantification compared to the challenges encountered when using other techniques i.e. gene microarrays and Northern blotting; the advantage of using small quantities of starting material for subsequent analysis; and the potential to conduct non-invasive collection of biological samples for analysis (Larkin *et al*., 2003; George *et al*., 2004; Kubista *et al*., 2006). The use of relatively small samples for qPCR is particularly attractive for those investigating vitellogenesis in small fish models (Scholz and Mayer, 2008) where availability of blood volume may be limited. Unsurprisingly, qPCR has been implemented in laboratory studies investigating the induction of hepatic VtG and choriogenin mRNA expression as a reliable and sensitive biomarker for investigating oestrogenic effects (Lee *et al*., 2002; Scholz *et al*., 2004; Yu *et al*., 2006; Tian *et al*., 2009; Moffatt *et al*., 2010; Lange *et al*., 2012; Ferreira *et al*., 2013). The future application of qPCR in biological effects field studies has potential to provide additional evidence when investigating oestrogenic effects in the aquatic environment.

1.6 The Hepatobiliary System

The liver is the major organ responsible for the absorption, metabolism and storage of nutrients from the digestive tract before release into the blood for use by other organs and tissues. It is the primary barrier between the digestive system and the blood therefore playing an important role in the detoxification of toxins, carcinogens, drugs and their metabolites, through the formation of bile and subsequent excretion via the intestine (Hinton and Couch, 1998). The liver is also implicated in extensive protein synthesis, crucially VtG and choriogenin during oogenesis in fish.

The liver contains an extensive network of blood vessels and bile canals that vary in size depending on their location within the intrahepatic tree of the vascular and biliary system. The primary functional unit of the liver is the hepatocyte which comprises the largest majority of the overall organ volume. Hepatocytes are polygonal cells radially arranged into tubular chords (trabeculae) appearing as flattened plates of one or two cells thickness (figure 2). Separated by an endothelial lining, a labyrinth of irregularly dilated capillaries (sinusoids) is located between the hepatocellular plates. The endothelial lining is separated from hepatocytes by the perisinusoidal space, known as the space of Disse. Blood fluids readily pass across the endothelial lining into this space where immediate contact is made with numerous protruding microvilli of the hepatocellular plasma membrane. This allows metabolites and macromolecules to pass effectively between the sinusoid lumen and the hepatocyte. Situated immediately between hepatocytes of adjacent trabeculae is the bile canaliculus. Possessing no endothelial lining, this small tubular space (1-2 µm) follows along the length of trabeculae separated only from hepatocytes by the plasma membrane. Canaliculi are the beginning of the biliary hierarchal tree and, via transitional preductules, exit into larger bile ductules (cholangioles) lined with cuboidal biliary epithelial cells. The cholangiolar network connects with larger intrahepatic bile ducts that exit the liver into the common bile duct of the extrahepatic biliary system. For substances to pass from blood plasma to the biliary system, they must first cross the sinusoidal membrane into the space of Disse where they are absorbed into hepatocytes via

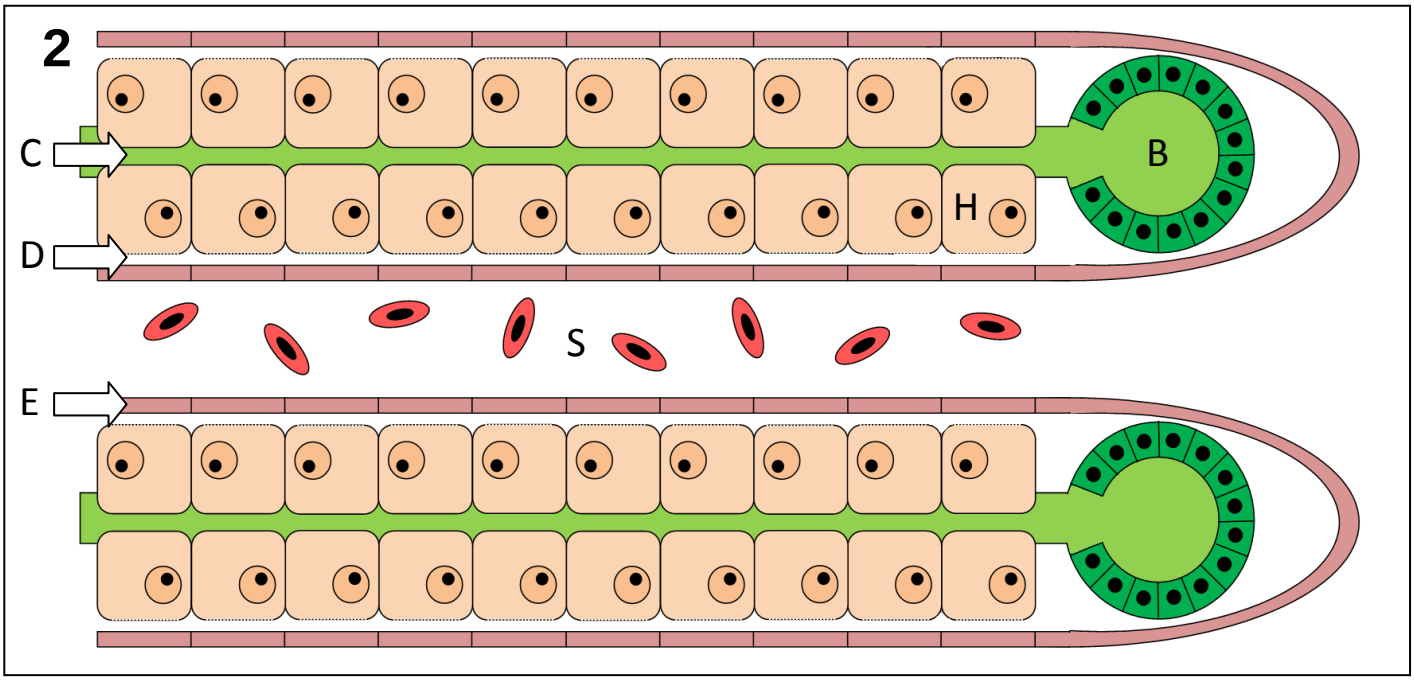


Figure 2: Schematic diagram of canaliculus functional unit of liver. Hepatocytes (H), Bile ductule (B), sinusoid (S), canaliculus (C), space of Dissé (D), sinusoid endothelium (E).

endocytosis. A period of intracellular transport and metabolism follows prior to elimination of potential harmful metabolites across the apical hepatocellular plasma membrane into the canaliculus.

The liver plays a central role in detoxification as a consequence of its central location within the circulatory system, receiving inputs from both the gills and intestine (Hinton *et al*., 2008). Relatively hydrophilic xenobiotics favour the gills as root of entry whereby those relatively lipophilic xenobiotics primarily enter the circulatory system through the intestine via food. Hepatocytes differ between fish regarding nutrient storage with some preferentially storing large quantities of glycogen compared to lipid observed in other species. The amount and type of storage product also varies depending on sex, gametogenesis, nutrition, physiological condition and temperature resulting in differential sensitivity to potentially toxic lipophilic xenobiotics (Hinton *et al*., 2001). Chemicals can directly impact upon liver structure and function causing acute hepatocellular toxic injury and necrosis following intracellular metabolism of a parent compound into highly reactive molecules (electrophiles). These electrophiles readily combine with molecules such as proteins, DNA and RNA, impairing routine biological functions. Xenobiotics may also impart their effects indirectly by interfering with the interaction and signalling between cells. The continued assault of the liver by these mechanisms can result in the formation of chronic toxic liver injury e.g. carcinogenesis (Hinton and Couch, 1998; Hinton *et al*., 2008). For these reasons, the liver is a key target organ for investigating the toxic effects of contaminants in the aquatic environment.

1.7 Liver histopathology

Histopathology is the study of abnormal changes in relation to cells and tissues at the microscopic level. In addition to providing insight into infectious diseases, histopathology is used to investigate tissue changes in toxicological studies. Histopathology has previously been used to investigate the cause-effect relationship between environmental contaminants and the presence of toxicopathic liver lesions in numerous flatfish species (Köhler, 1990; Myers *et al*., 1990; Myers

et al., 1998; Stentiford *et al*., 2003; Stehr *et al*., 2004; Lang *et al*., 2006). Flatfish are a particularly sensitive biomarker species resulting from their sedimentary habitat and a diet primarily consisting of the organisms that live within. As such they are continuously exposed to potentially harmful sediment-associated environmental contaminants. Toxicopathic liver lesions are largely grouped into several categories including non specific and inflammatory lesions; pre-neoplastic foci of cellular alteration (FCA); benign and malignant neoplasms; and non-neoplastic toxicopathic lesions (Feist *et al*., 2004).

Hepatocellular fibrillar inclusions (HFI) are a visually striking non-neoplastic toxicopathic lesion of unknown aetiology. Characterised by the presence of “brush” or “needle-like” structures within the cytoplasm of affected hepatocytes, their aetiology has been the subject of previous speculation (Köhler, 1989; Köhler, 1990; Vethaak and Wester, 1996; Stentiford *et al*., 2003; Lyons *et al*., 2004; Pal *et al*., 2011; Carrola *et al*., 2013). Marine monitoring undertaken by Cefas shows this pathology is prevalent in *P. flesus* from several UK estuaries (table 1) but rarely seen in flatfish sampled offshore (Cefas, unpublished data). Incidences of HFI at the Clyde, Tyne, Tees and Mersey, those estuaries previously demonstrating high VtG concentrations in blood plasma and ovotestis (Allen *et al*., 1999a; Allen *et al*., 1999b; Stentiford *et al*., 2003; Kirby *et al*., 2004), have been recorded as high as 84.0 %, 53.3 %, 60.0 % and 80 % prevalence respectively. Moreover, they are particularly prevalent at these locations compared to designated reference sites sampled during the same period (Stentiford *et al*., 2003; Lyons *et al*., 2004). HFI have also been seen at relatively high prevalence (≈35 %) in the Douro River estuary, Portugal (Carrola, 2013). Köhler (1989, 1990) reported that ultrastructural analyses of HFI in *P. flesus* sampled from Elbe estuary, Germany revealed proliferating cytoskeletal microtubules and/or rough endoplasmic reticulum (RER) associated with hepatocellular regeneration (Köhler, 1989). Immunohistochemistry later confirmed that HFI were comprised of microtubules (Köhler, unpublished data). HFI have been observed in laboratory exposures of Common carp (*Cyprinus carpio*) to dietary heavy oil with no observations noted in corresponding controls (Pal et al., 2011). The aetiology of HFI remains unconfirmed. The role of the RER

in the formation of HFI should be considered further in the context of endocrine disruption. The RER is an interconnecting network of membrane bound tubules and flattened sacks (cisternae) situated throughout the cytoplasm of eukaryotic cells. Proportions of

Laboratory Reference	Year	Month	Season	Location	*n*=	Prevalence (%)	Sex ratio (M/F)
RA00045	2000	May	Spring	Tyne	30	10	1/3
RA00048	2000	May	Spring	Tees	3	0	0/3
RA00051	2000	May	Spring	Mersey	30	26.7	4/2
RA00054	2000	May	Spring	Alde	30	3.3	1/0
RA00074	2000	September	Autumn	Tyne	30	43.3	9/4
RA00079	2000	September	Autumn	Tees	15	60	6/1
RA00082	2000	October	Autumn	Mersey	30	80	11/13
RA00085	2000	October	Autumn	Alde	30	0	0/0
RA01129	2001	NR	Autumn	Tyne	30	53.3	12/3
RA01135	2001	NR	Autumn	Tees	30	43.3	NA
RA02028	2002	March	Spring	Clyde	30	56.7	8/9
RA02105	2002	September	Autumn	Clyde	50	84	24/22
RA02110	2002	NR	Autumn	Forth	44	56.8	11/13
RA02120	2002	NR	Autumn	Tyne	50	30	11/3
RA02125	2002	September	Autumn	Mersey	50	62	18/11
RA02130	2002	October	Autumn	Southampton	44	36.4	13/1
RA02135	2002	NR	Autumn	Thames	49	16.3	3/5
RA02140	2002	November	Autumn	Alde	50	16	5/3
RA02154	2002	October	Autumn	St Andrews Bay	48	25	6/6
RA06033	2006	May	Spring	Tyne	40	17.5	5/2
RA06035	2006	April	Spring	Alde	50	0	0/0
RA06041	2006	April	Spring	Mersey	50	36	11/7
RA06042	2006	April	Spring	Morecambe Bay	50	2	1/0
RA07027	2007	April	Spring	Tyne	41	0	0/0
RA09159	2009	September	Autumn	Tyne	50	6	3/0
RA09152	2009	October	Autumn	Alde	50	0	0/0
RA09077	2009	November	Autumn	Mersey	50	34	15/2

Table 1: Historical hepatocellular fibrillar inclusion (HFI) prevalence data collected between 2000 and 2009 during Cefas estuarine monitoring programmes.

cisternae possessing ribosomes studded along the outer membrane, which translate mRNA code for protein synthesis, can vary considerably depending on

the metabolic state of the cell. Cell types that are particularly active regarding protein synthesis, such as those found in the liver and pancreas, often exhibit a relatively high proportion of RER. If we consider that HFI are particularly prevalent in UK estuaries reportedly exhibiting significant oestrogenic activity (Allen *et al*., 1999b), the high prevalence may indicate disorganisation of RER in relation to abnormal levels of vitellogenesis. However, the implication of cytoskeleton elements (Köhler, 1989) is also worthy of further investigation. The cytoskeleton is a multifunctional organelle comprised of three major components (microtubules, microfilaments and intermediate filaments) each with distinct cellular roles. Microtubules consist of the protein tubulin and exist as vast networks throughout the cell. They play an important role in numerous cellular functions such as mitosis, but also hepatocellular processes including plasma protein secretion and intracellular translocation of organelles and secretory vesicles from the Golgi apparatus to the cell surface (Agius, 1996). Elevated levels of tubulin have previously been described in pathological conditions of rats whereby hepatocytes exhibit increased plasma protein secretion (Maurice *et al*., 1980).

We speculate that HFI are implicated in the abnormal translocation and secretion of proteins (such as VtG) and may serve as a phenotypic pre-cursor to elevated VtG concentrations in blood plasma. As such, it may offer a histological method for the detection of recent exposure to oestrogenic chemicals. This study utilises histology, immunohistochemistry (IHC), transmission electron microscopy (TEM) and the real time polymerase chain reaction (qPCR) technique to investigate a previously observed pathology of unknown aetiology in the liver of the *P. flesus* as part of the CSEMP. The results are discussed within the context of endocrine disruption in the marine environment.

2.0 Aims & Objectives

This project will explore the hypothesis that induction of vitellogenin and choriogenin resulting from exposure to endocrine disrupting chemicals, is associated with the development of HFI (figure 3). *P. flesus* will be sampled from several UK estuaries as part of Cefas's Clean Seas Environmental Monitoring Programme (CSEMP). Liver and gonad will be sampled and processed for formalin fixed paraffin embedded (FFPE) histology and analysed for liver histopathology lesions following the guidelines of the BEQUALM environmental monitoring programme (Feist *et al*., 2004). Additional liver samples will also be snap-frozen for differential gene expression analysis. The relationship between the different measured endpoints will be analysed statistically and results discussed in the context of the use of HFI in the liver of *P. flesus* as a potential histological biomarker of exposure to endocrine disruption in the marine environment.

2.1 Objective 1: Collate all historical data on HFI from UK waters

Historical data held at Cefas will be analysed and used to produce a summary of the known locations and prevalence of HFI in UK waters. This will provide an overview of all HFI data held at Cefas and identify UK "hot-spots" for this pathology

RESULTS: *Histology*

2.2 Objective 2: Quantify the prevalence and severity of HFI from several UK estuaries to identify worst affected estuaries in 2010.

The prevalence of HFI in *P. flesus* liver samples collected during this study will be determined via histological analysis. A simple semi-quantitative scoring index will also be used to provide data concerning the severity of HFI at specified sites, thus complimenting previously collected data on prevalence.

MATERIALS AND METHODS: *Histology*

RESULTS: *Histology*

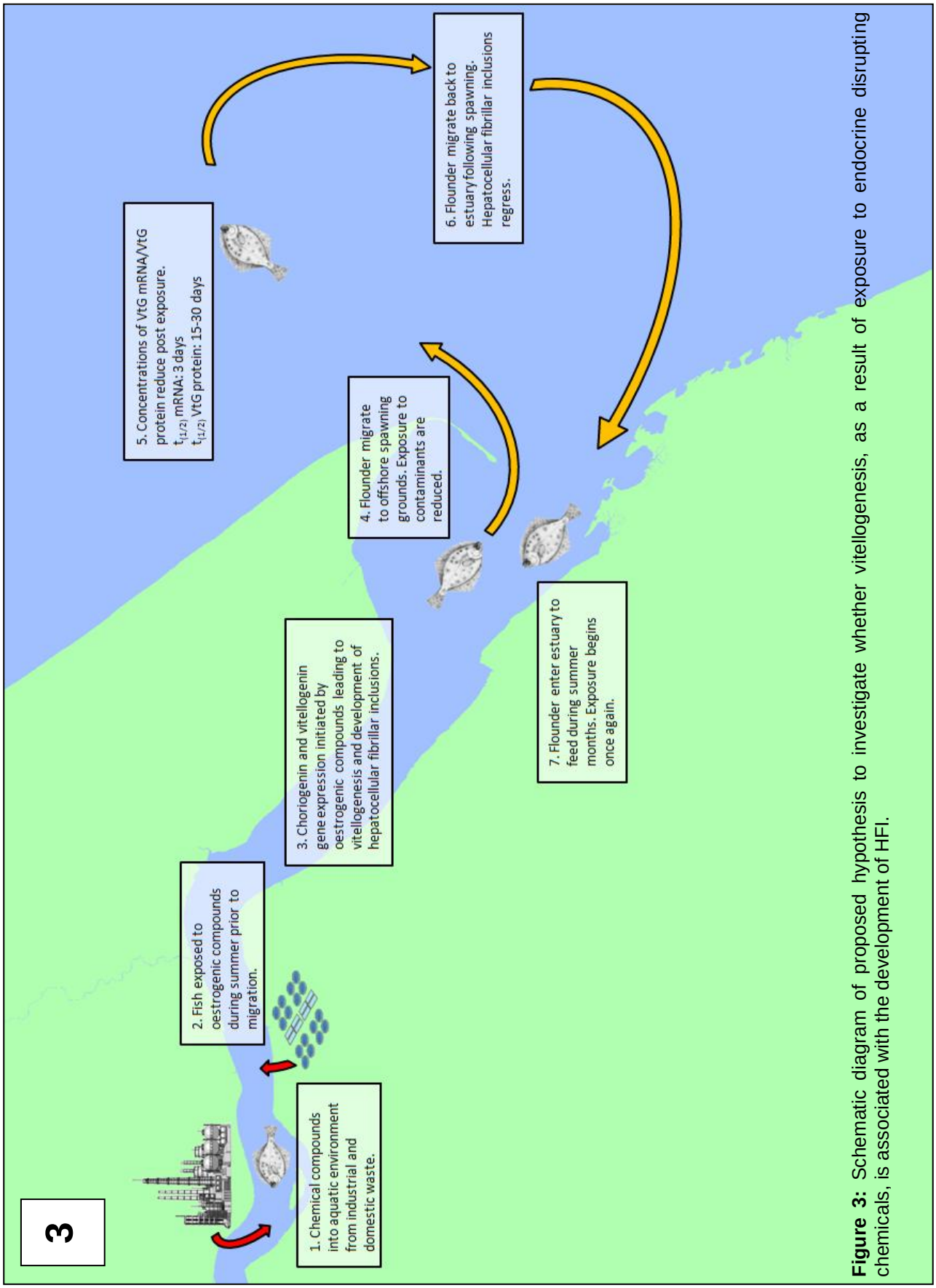


Figure 3: Schematic diagram of proposed hypothesis to investigate whether vitellogenesis, as a result of exposure to endocrine disrupting chemicals, is associated with the development of HFI.

The observation of elevated HFI prevalence at estuaries impacted by oestrogenic compounds (Allen *et al*., 1999a; Allen *et al*., 1999b) and reports that extensive RER might be implicated in their formation (Köhler, 1989); suggests that xenobiotic induced VtG synthesis could be an important consideration in HFI development. Furthermore, the role of microtubules in HFI formation (Köhler, 1989) and their observation in pathological conditions concerning increased secretion of protein into blood plasma (Maurice *et al*., 1980), is worthy of further investigation. Selected corresponding formalin fixed paraffin embedded (FFPE) material will be processed for IHC for the detection of VtG and microtubule α/β- tubulin within hepatocytes affected with HFI.

MATERIALS AND METHODS: *Immunohistochemistry*

RESULTS: *Immunohistochemistry*

2.4 Objective 4: Describe the ultrastructure of HFI using transmission Electron Microscopy (TEM)

Selected representative samples will be processed for TEM to investigate previous reports that HFI may be comprised of RER and/or microtubules. Corresponding samples will be collected from all *P. flesus* obtained during the 2010 sampling programme and stored in glutaraldehyde prior to processing.

MATERIALS AND METHODS: *Transmission Electron Microscopy (TEM)*

RESULTS: *Transmission Electron Microscopy (TEM)*

2.5 Objective 5: Investigate differential VtG and choriogenin *gene expression between different HFI severity stages*

Differential analysis of VtG and choriogenin gene transcription will be quantified using real time PCR (qPCR) on snap frozen liver samples to provide evidence of potential links to endocrine disruption. Liver samples will be from corresponding male *P. flesus* obtained during the 2010 sampling programme. Correlations between gene expression and phenotype will be investigated i.e. prevalence and severity of fibrillar inclusions.

MATERIALS AND METHODS: *RNA extraction, Reverse transciption, Real-time Polymerase Chain Reaction (qPCR)*

RESULTS: *Real-time Polymerase Chain Reaction (qPCR)*

2.6 Objective 6: Investigate oestrogenic activity in UK estuaries through the quantification of VtG blood plasma concentration in male P. flesus

Correlations between vitellogenin concentrations and HFI will be investigated to provide evidence of potential links to endocrine disruption. Plasma samples will be from corresponding male *P. flesus* obtained during the 2010 sampling programme. Correlations between VtG concentrations and phenotype will be investigated i.e. prevalence and severity of fibrillar inclusions.

MATERIALS AND METHODS: *VtG Analysis*

RESULTS: *VtG Analysis*

3.0 Materials and Methods

3.1 Field Sampling

P. flesus (n=50) were sampled each from the Alde, Humber, Medway, Thames, Tyne and Mersey estuaries (figure 4 and table 2) during autumn 2010 as part of an annual monitoring programme to investigate the biological effects of contaminants. Fishing at each estuary was conducted using a 2 m beam trawl and 10 mm mesh cod end for standardised durations of 20 minutes. *P. flesus* were transferred to aerated flow through seawater tanks prior to sampling. Following euthanasia, blood was sampled using a heparinised syringe from the caudal vein and immediately centrifuged at 10000 rpm for 5 mins. Plasma was transferred to a cryovial and snap frozen in liquid nitrogen prior to storage at -80 °C. Viscera were removed and a standardised 3-4 mm liver cross section (in addition to gonad, kidney and spleen tissues) was obtained for formalin fixed paraffin embedded (FFPE) histology. For tissue preservation, organs were placed into 10 % Neutral Buffered Formalin (NBF) (Pioneer Research Chemicals Ltd., UK) for 48 h prior to transferring into 70 % Industrial Methylated Sprit (IMS) (Pioneer Research Chemicals Ltd., UK) and subsequent histological processing. Otoliths were sampled from each fish for age determination (Easey and Millner, 2008)[1]. For transmission electron microscopy (TEM) and real-time PCR (qPCR), corresponding liver tissues were dissected and placed into glutaraldehyde (Agar Scientific, UK) and snap frozen in cryovials using liquid nitrogen respectively. All frozen samples were stored in a dry shipper prior to transportation back to the laboratory where there were transferred to a -80 °C freezer.

3.2 Histology

Tissues were processed for FFPE histology in a Leica Peloris vacuum infiltration processor using standard histological protocols (appendix 1) and embedded in paraffin wax. Sections of 3-4 µm were obtained using a rotary microtome and subsequently stained with haematoxylin and eosin (HE) for histological examination

[1] Preparation of otoliths for age determination was conducted by Mr. Glenn Saunders (Cefas, Lowestoft). Otolith sections were analysed for age determination by Mr. Mark Etherton (Cefas, Lowestoft).

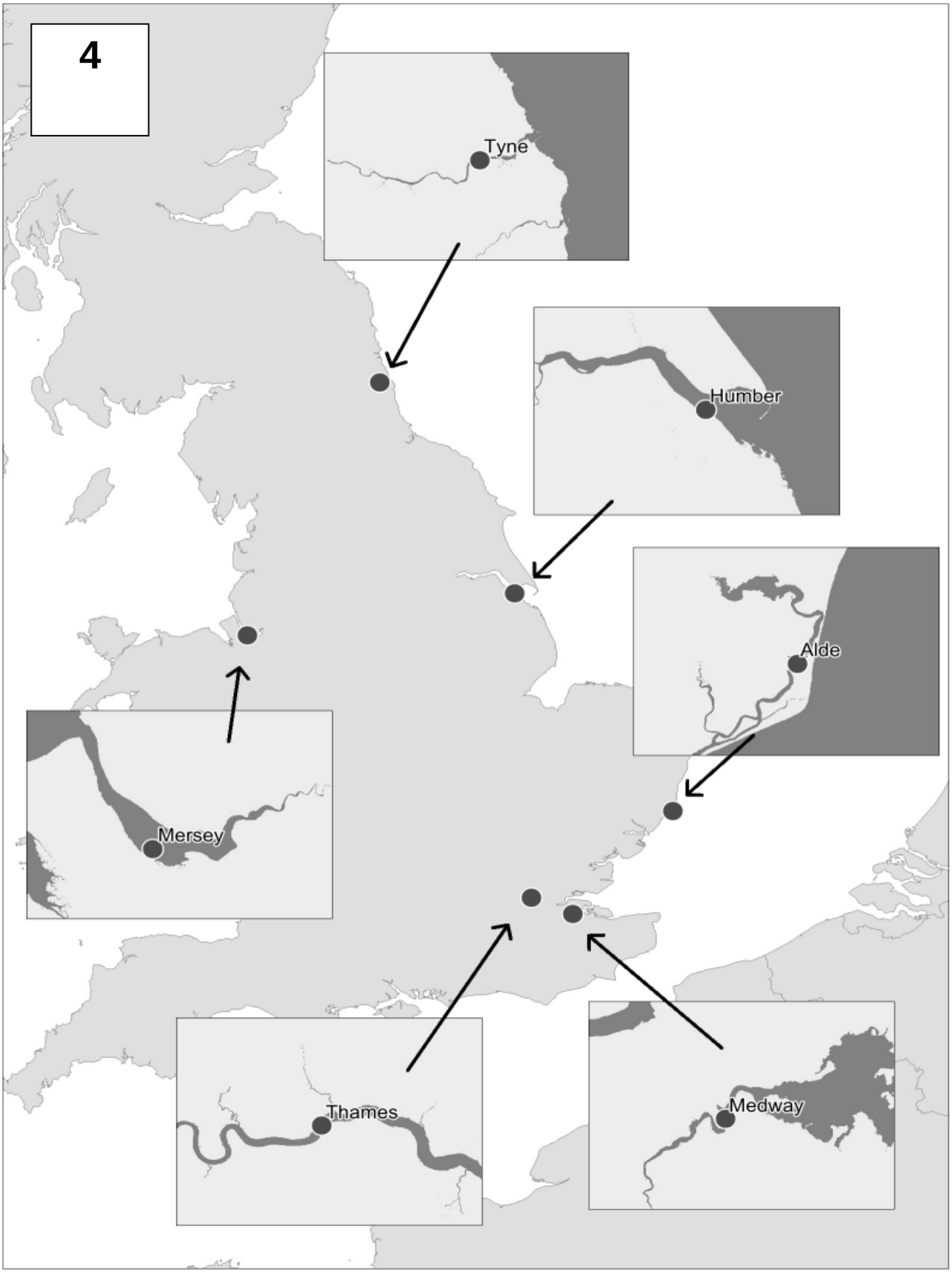


Figure 4: Geographical locations of Mersey, Tyne, Thames, Medway, Humber and Alde estuaries used in 2010 sampling programme.

(appendix 2). Where applicable, tissue sections were also stained with the periodic acid Schiff's (PAS) technique. Slides were examined for lesions indicative of contaminant exposure according to quality assured criteria (Feist et al, 2004) using a Nikon Eclipse Ni-U microscope and Nikon NIS Elements BR image analysis software (Nikon, UK). HFI prevalence and severity were recorded using a semi-quantitative index (Table 3) and used to identify samples for immunohistochemistry, TEM, and qPCR.

Location	Latitude	Longitude
Medway	51°23.3194'N	0°31.2808'E
Thames	51°30.2592'N	0°04.7829'E
Mersey	53°18.3649'N	2°53.0278'W
Humber	53°35.3959'N	0°04.2323'W
Tyne	54°59.2499'N	1°29.7802'W
Alde	52° 6'48.83"N	1°34'28.91"E

Table 2: Latitude and longitude coordinates of estuarine sampling locations.

Stage	Type	Description
0	Absent	No hepatocytes affected.
1	Present	Few scattered cells containing HFI within parenchyma. Only present in limited/few fields of view. HFI mostly observed within small/condensed hepatocytes.
2	Elevated	Approximately 25-50 % of hepatocytes contain HFI. HFI are present in relatively few numbers (10-25 % of hepatocytes) of those fields of view affected.
3	Intermediate	Approximately 50 % of hepatocytes contain HFI appearing both small/condensed and enlarged with many fibrils.
4	Abundant	Large percentage of hepatocytes affected with HFI (≥80 %). Hepatocytes appear enlarged with many condensed fibrils.
5	Degenerative/ unknown	Hepatocytes appear to contain degenerative material. Uncertain if related to HFI.

Table 3: Histological scoring criteria developed for grading HFI severity in *P. flesus*.

3.3 Immunohistochemistry (IHC)

Following histological analysis, further tissue sections (3-5 µm) were obtained for immunohistochemical detection of VtG and microtubule α/β- tubulin. Sections were selected from representative livers (including females) for confirmation of IHC positive labelling. Further sections were also obtained from all corresponding male flounder liver samples processed for qPCR from the Tyne and Mersey estuaries. Briefly, sections were dewaxed and rehydrated prior to heat-induced epitope retrieval (HIER) being undertaken. This was achieved by transferring sections into a suitable vessel containing 50mM Sodium citrate buffer placed within a pressure cooker. Once maximum pressure was achieved using a hotplate, sections remained for 10 minutes prior to removal, gentle cooling and washing in distilled water. Endogenous biotin activity was blocked by application of 0.05 % avidin and 0.05 % biotin in Tris buffered saline (TBS) with intermediate and final washing steps in TBS. Sections were subsequently treated using a modified protocol of the Leica Immunohistochemistry Peroxidase Detection System (RE7110, Leica UK) incorporating a (1) polyclonal anti-rabbit flounder VtG primary antibody (1:5000) and (2) a commercially available α/β- tubulin polyclonal primary antibody for zebrafish (#2148, Cell Signaling Technology, USA) (1:50). A negative control was used by substituting primary antibody for Tris buffered saline (TBS).

3.4 Transmission Electron Microscopy (TEM)[2]

Selected corresponding samples of interest were processed for TEM. Semi-thin sections were obtained using an ultramicrotome, stained with toludine blue and analysed for areas of interest using light microscopy. Targeted areas were further sectioned for the production of ultra-thin sections and stained with osmium tetroxide. Sections were analysed using a Joel JEM-1400 electron microscope (Joel, UK).

3.5 RNA Extraction

Following histological and immunohistochemical examination, gene expression analysis was undertaken on representative samples to investigate the relationship

[2] Laboratory preparation of samples for TEM analysis was conducted by Mr. Stuart Ross and Miss Kelly Bateman (Cefas, Weymouth).

between HFI severity and gene expression. Male flounder from the Tyne and Mersey estuaries were selected for this purpose given that a range of severity stages were observed at these sites. Total RNA was extracted using the GenElute Mammalian Total RNA Miniprep Kit (Sigma, Poole, UK). Briefly, frozen liver samples (up to 40 mg) were homogenised in 500 µL of lysis solution prior to extraction and purification according to the manufacturer's protocol. RNA concentration was quantified using a Nanodrop spectrophotometer (Applied Biosystems, UK).

3.6 Reverse Transcription

For each reaction, oligo dTs (0.25 µL) (Invitrogen, Life Technologies, UK), random primers (0.6 µL) (Promega, UK) and nuclease free water (1 µL) (Fisher Scientific, UK) was added to 1 µg of total RNA. The reaction mixture was denatured at 70 °C for 5 minutes followed by cooling on ice for further 5 minutes. First strand buffer (4 µL), dNTPs (1 µL) and Superscript-II reverse transcriptase (1 µL) (Invitrogen, Life Technologies, UK) was added to each reaction mix. Reaction mixes were kept on ice prior to reverse transcription of incubation at 25 °C, 50 °C and 70 °C for 5 minutes, 60 minutes and 15 minutes respectively followed by final incubation step of 4 °C. Resulting cDNA template was diluted to 400uL with nuclease free water (1:20) and stored at -20 °C prior to use.

3.7 Real-time Polymerase Chain Reaction (qPCR)

Quantification of VtG and choriogenin (CHR) (table 4) transcription was undertaken by qPCR. Each reaction was undertaken in duplicate using an Applied Biosystems Step-One Plus qPCR system, with relative transcription levels normalised to levels of ubiquitin (UBQ), elongation factor 1 (EF1), F-actin (FACT) and α-tubulin (ATUB) housekeeping genes (table 4). Each reaction mix was comprised of the following: 10 µL of Promega GoTaq® qPCR Master Mix (Promega, UK), 0.4 µL (0.2 µM) of each forward and reverse primer (Table 4), 4 µL of cDNA template (1:20 dilution) and 5.6 µL of nuclease free water for a total reaction volume of 20 µL. The qPCR conditions comprised of thermal activation of Taq DNA polymerase at 94 °C for 2 min, followed by 40 cycles of denaturation at 94°C for 15 seconds, annealing and

elongation at 60°C for 1 minute. This was followed by melt curve analysis at 95-60 °C with fluorescence recorded every 0.3 °C. Reaction data were exported to the LinRegPCR analysis software for determination of baseline fluorescence, PCR efficiency and Cq value (Ruijter *et al*., 2009). Subsequent data were exported to Relative Expression Software Tool (REST) 2009 (Corbett Research Pty Ltd, Germany) for relative gene expression analysis (Pfaffl *et al*., 2002). Comparison of *P. flesus* expression data was conducted between biological groups comprised of HFI severity stage. Severity stages of 3 and 4 were combined resulting from low numbers of fish exhibiting stage 4.

	Acronyms	Acc. Number	Forward Primer (5'-3')	Reverse Primer (5'-3')	Reference
Target genes					
Vitellogenin	VtG	AJ416327	CTGACCTTCGTGGATATTGAG	ATCTGAGCCTCGGCATTG	Williams 2003
Choriogenin	CHR	DV570128	CCTCCCAGAAGTCCAGTGAA	GTGGCAGGGCATTGAGTTAC	Falciani 2008
Housekeeping genes					
Alpha tubulin	ATUB	AJ291985	CACAGCCTCACTTCGTTTTG	AGATGACAGGGGCATAGGTG	Williams 2003
F-actin capping protein β subunit	FACT	DV567493	GAGGTGCAGGAGAAGTCCAG	TGTCCTTGGTCTTCCCAAAG	Falciani 2008
Elongation factor alpha	EF1	AJ291984	TGTCCCATCTGCTAAGGCTG	CTTGAGGCGTTCTGTCTCCT	Williams 2003
Ubiquitin	UBQ	AJ298327	GGACATGTTTCACTGGCAA	GGATGTCAAGGCAGATGCT	This publication

Table 4: Primer sequences used for qPCR.

3.8 VtG analysis[3]

VtG analysis was conducted as part of a parallel study using an enzyme-linked immunosorbent assays (ELISA) (Kirby *et al*., 2004). VtG concentrations were determined to investigate the extent of vitellogenesis in male *P. flesus* sampled from all estuaries. Data were used to investigate potential links between gene expression, HFI formation and plasma VtG were subsequently investigated.

[3] ELISA for VtG analysis was conducted by Dr. Marion Sebire (Cefas, Weymouth).

3.9 Data analysis

Chi-square test, Mann-Whitney rank sum test and linear regression analysis were undertaken in SigmaPlot version 11.0, from Systat Software, Inc. (San Jose California USA). Kruskal-Wallis rank sum test was undertaken in R v.2.7.0 (R Development Core Team, 2008)[4].

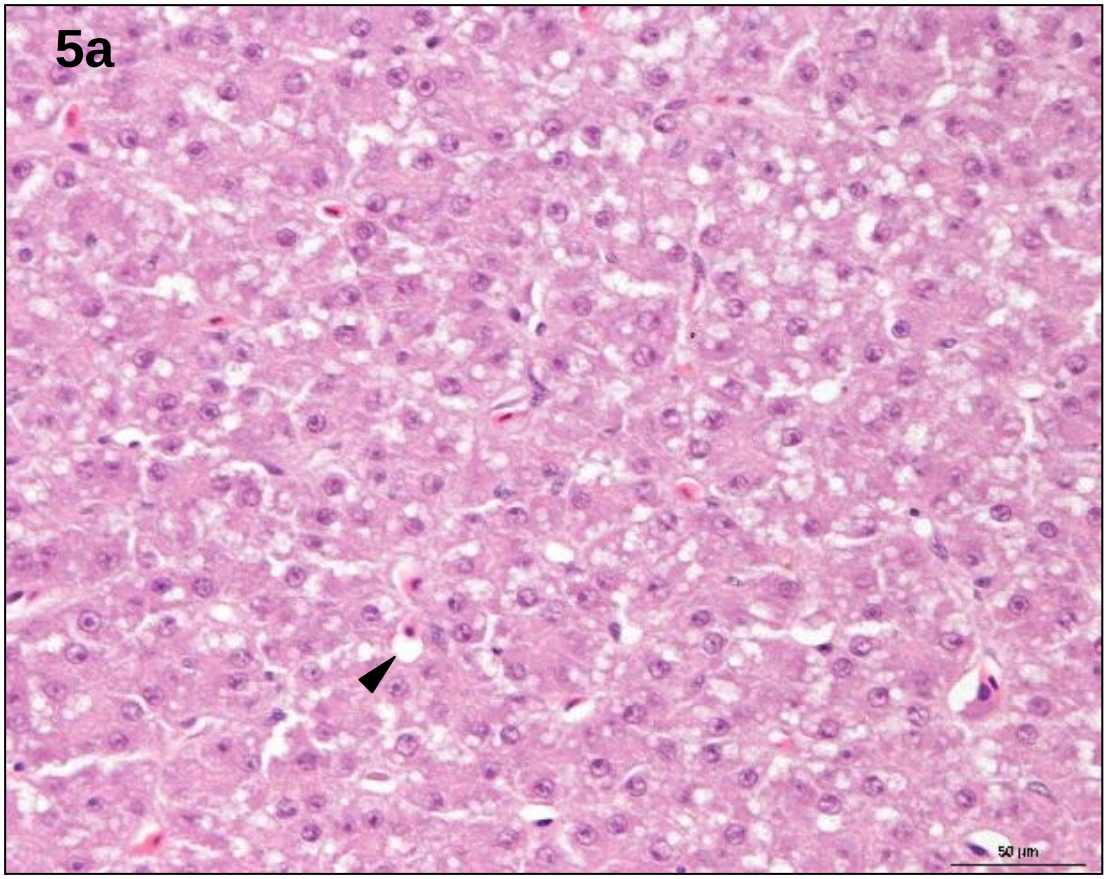
4.0 Results

4.1 Histology

Histological analysis using light microscopy revealed the presence of HFI with representative captured images demonstrating key features of the condition (figures 5-12). Affected hepatocytes contained fine “brush” or “needle-like” filaments arranged into array assemblies. HFI appeared crystalline and were associated with cellular atrophy. Affected hepatocytes possessed a cytoplasm that was angular in appearance when observed at low magnifications. Closer analysis revealed basophilic filaments in standard haematoxylin and eosin (HE) sections (figure 5) and were observed at varying degrees of cytoplasmic coverage between and within individual samples. Some hepatocytes possessed relatively few filaments whereas severely affected cells exhibited many compact filaments occupying the majority of the cytoplasm volume with an amorphous appearance (figure 7). The orientation of HFI varied between cells with longitudinal sections exhibiting HFI directed across hepatocytes towards the sinusoid (figure 6) and transverse sections revealing clusters of individual basophilic specks or spots (figure 7). Transverse sections of HFI appeared to harbour discrete vacuoles in one particular female demonstrating particularly elevated HFI severity (figure 8). Upon further inspection, these vacuoles were believed to correspond to intra-fibrillar spaces observed in longitudinal sections from the same fish (figure 9). However, this female had an atypical appearance, even so far as HFI are concerned, as characterised by a particularly pronounced distension of hepatocytes and accompanying polygonal appearance. HFI vary considerably in appearance therefore it is possible this observation was an extreme case of

[4] Kruskal-Wallis rank sum test was programmed in R by Mr. Jon Barry (Cefas, Lowestoft).

hepatocellular pleomorphism resulting from their formation. On occasion HFI were observed displaced towards the periphery of hepatocytes possessing increased lipid content (such as those livers exhibiting micro and macrovesicular steatosis). Affected cells under these circumstances were notably difficult to identify. HFI were observed in relatively few cells scattered throughout the liver as well as discrete patches that appeared inconsistent with respect to their distribution. The most severely affected fish revealed HFI throughout the majority of their liver with several histological sections appearing to show near 100 % coverage. A further observation within affected hepatocytes was the presence of an eosinophilic proteinacious material situated both free within the cytoplasm and amongst individual filaments (figure 10 and 11). This material was present at varying quantities and was not observed in all examples. The eosinophilic substance was also observed in hepatocytes that did not appear to exhibit HFI upon histological inspection and appeared similar to the condition “phospholipoidosis” (Feist *et al*., 2004).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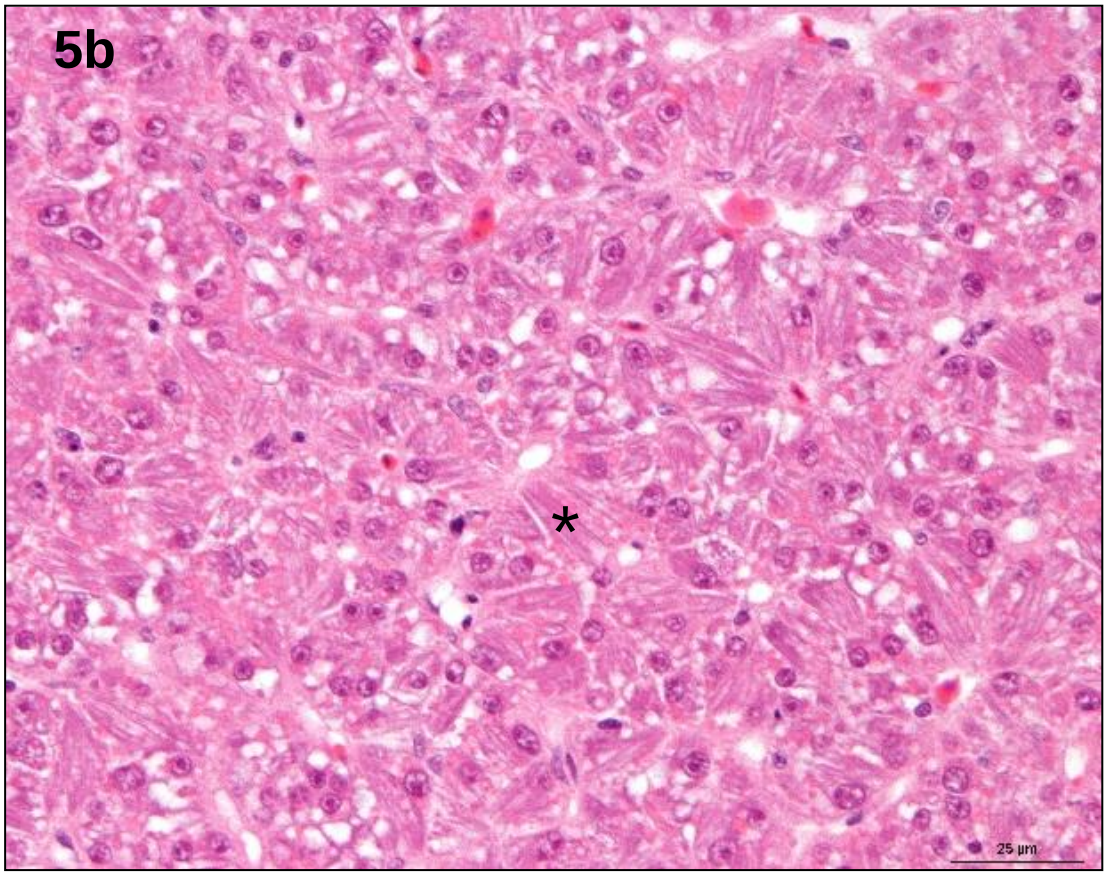
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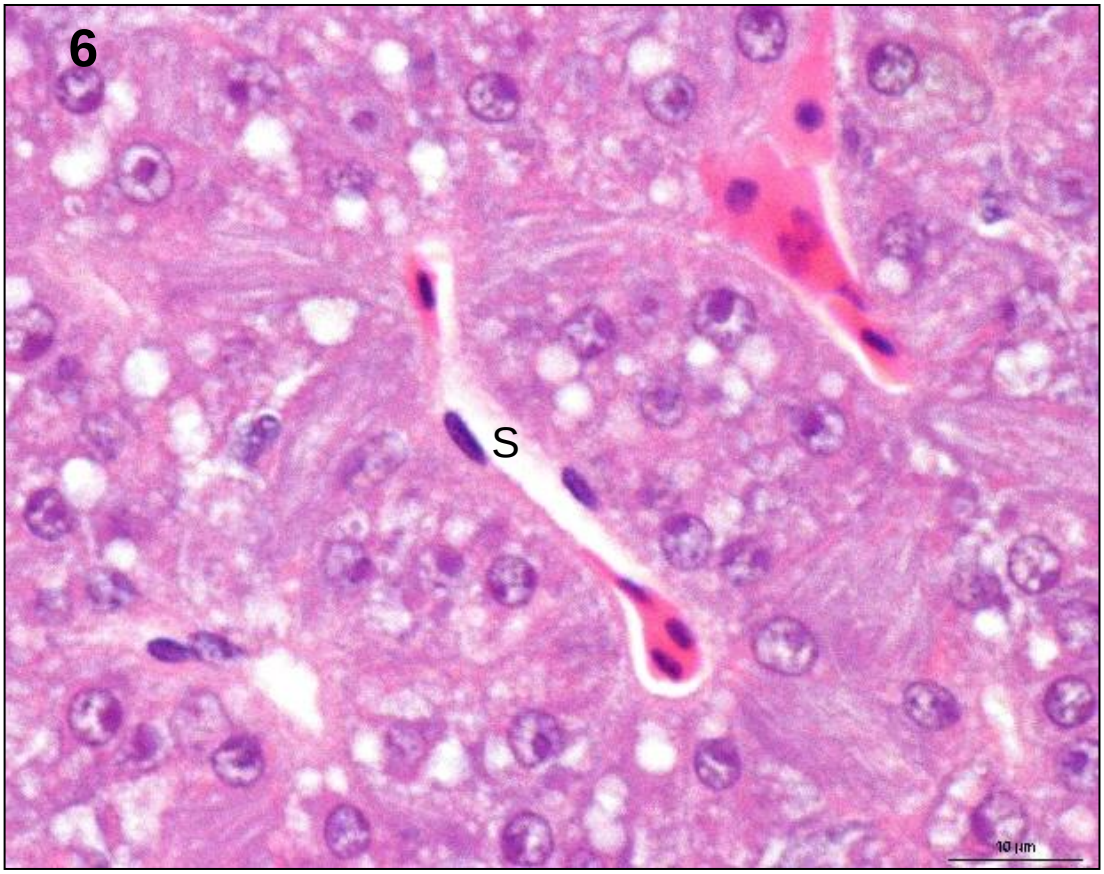
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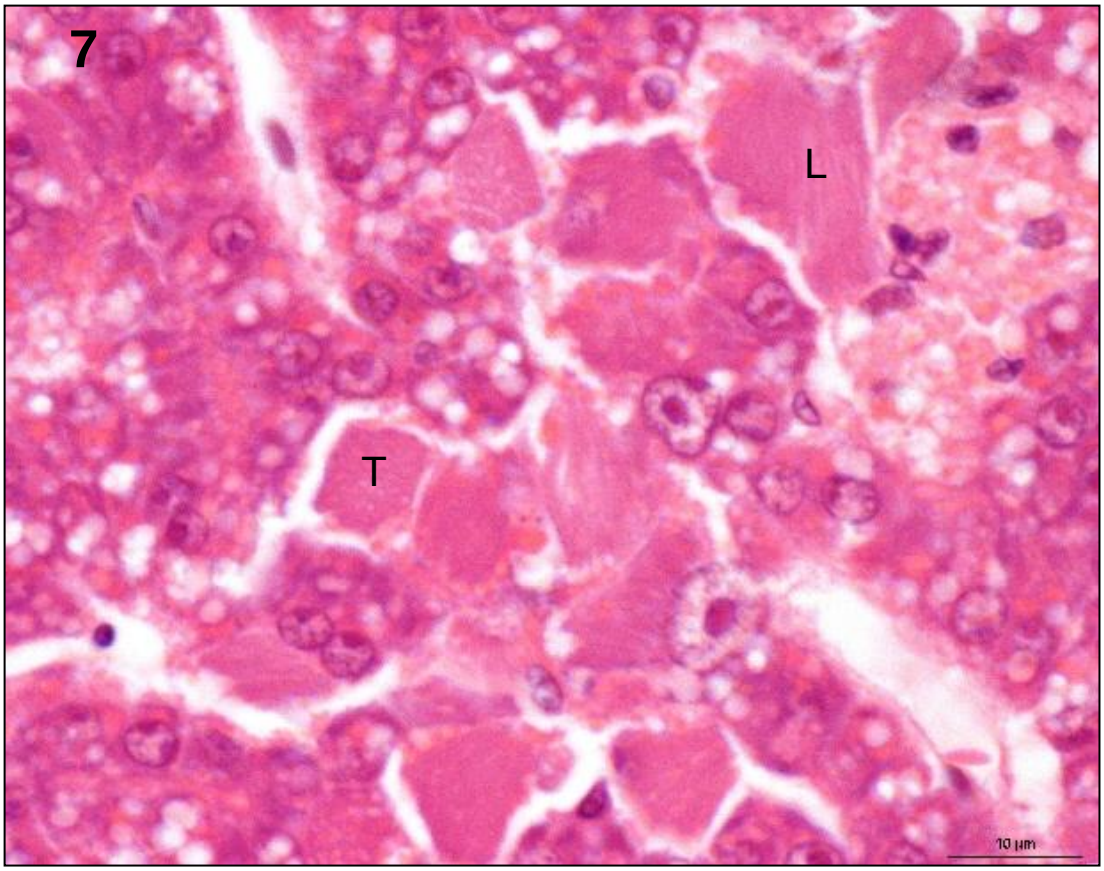
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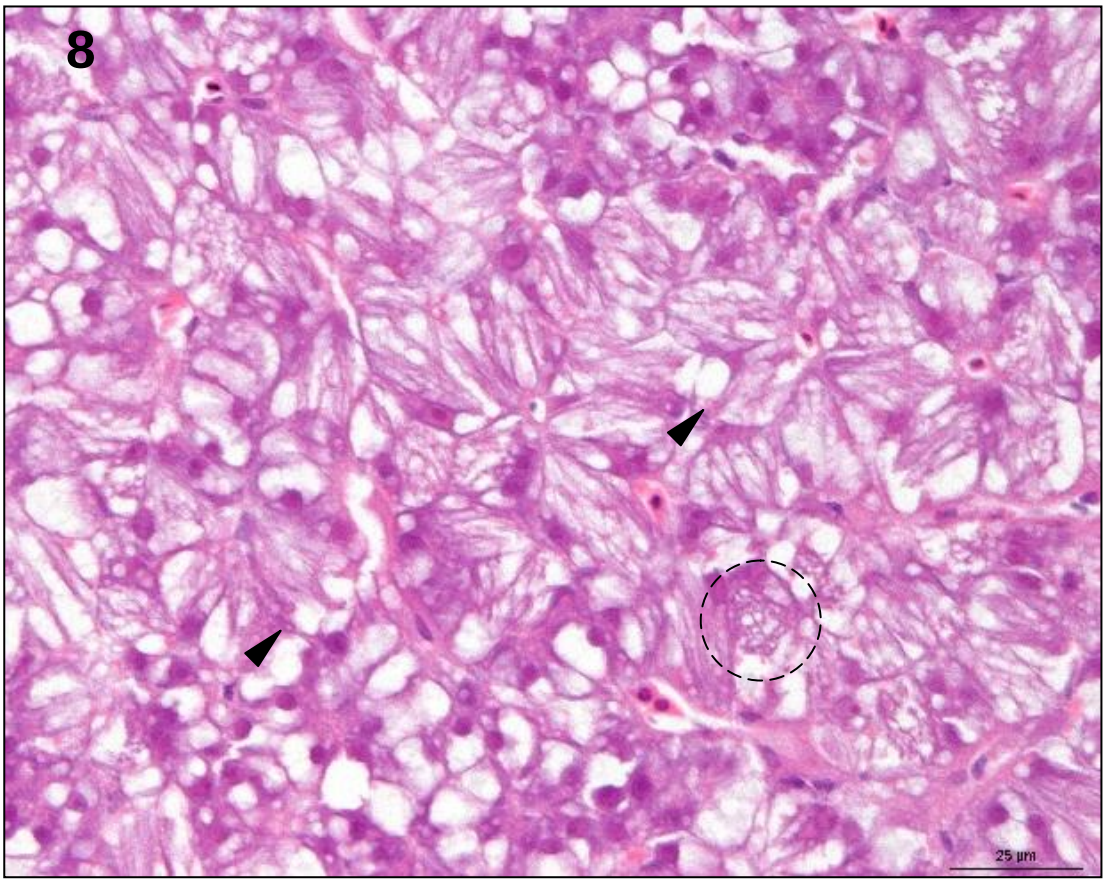
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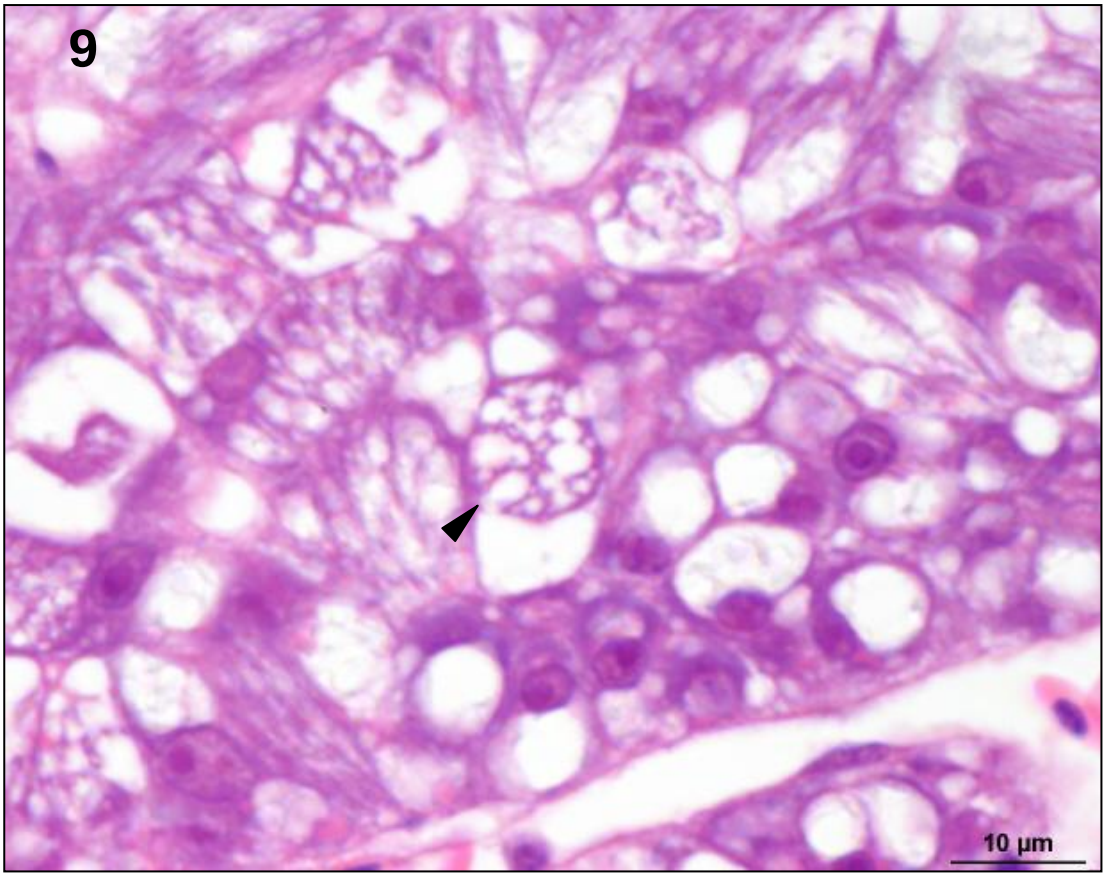
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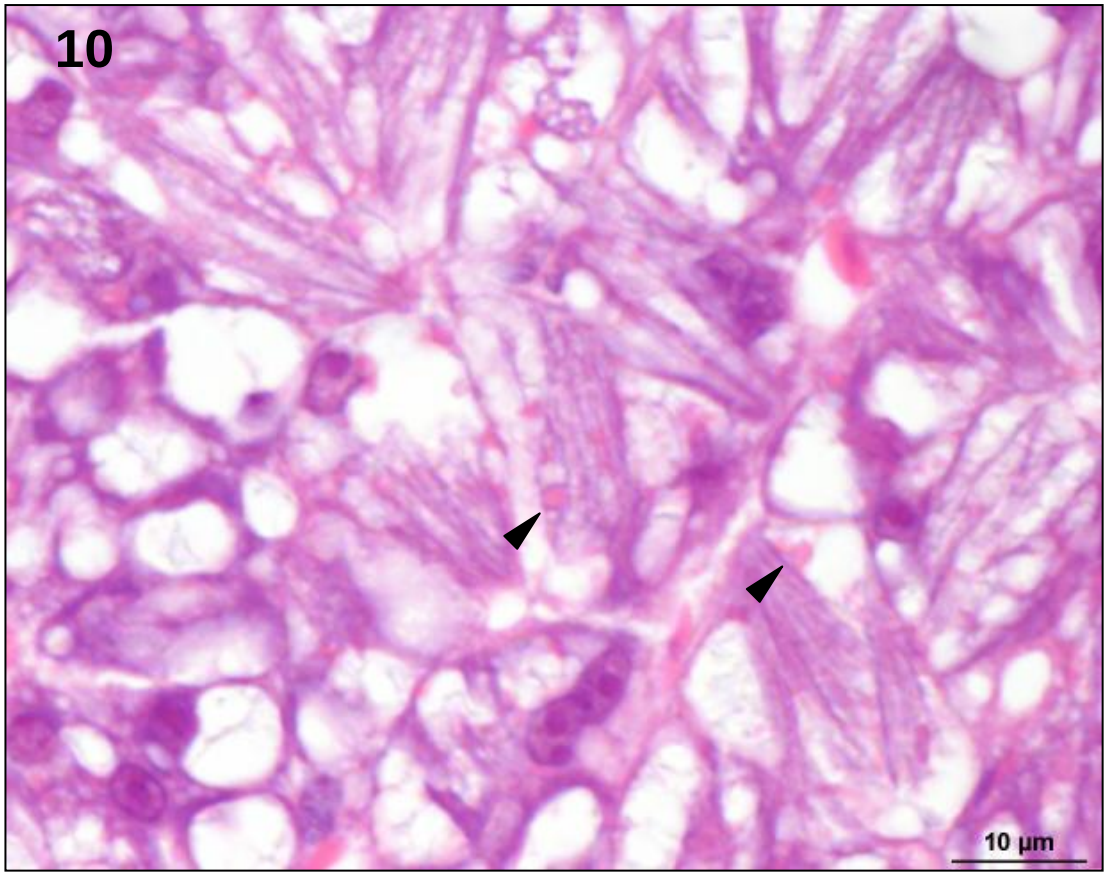
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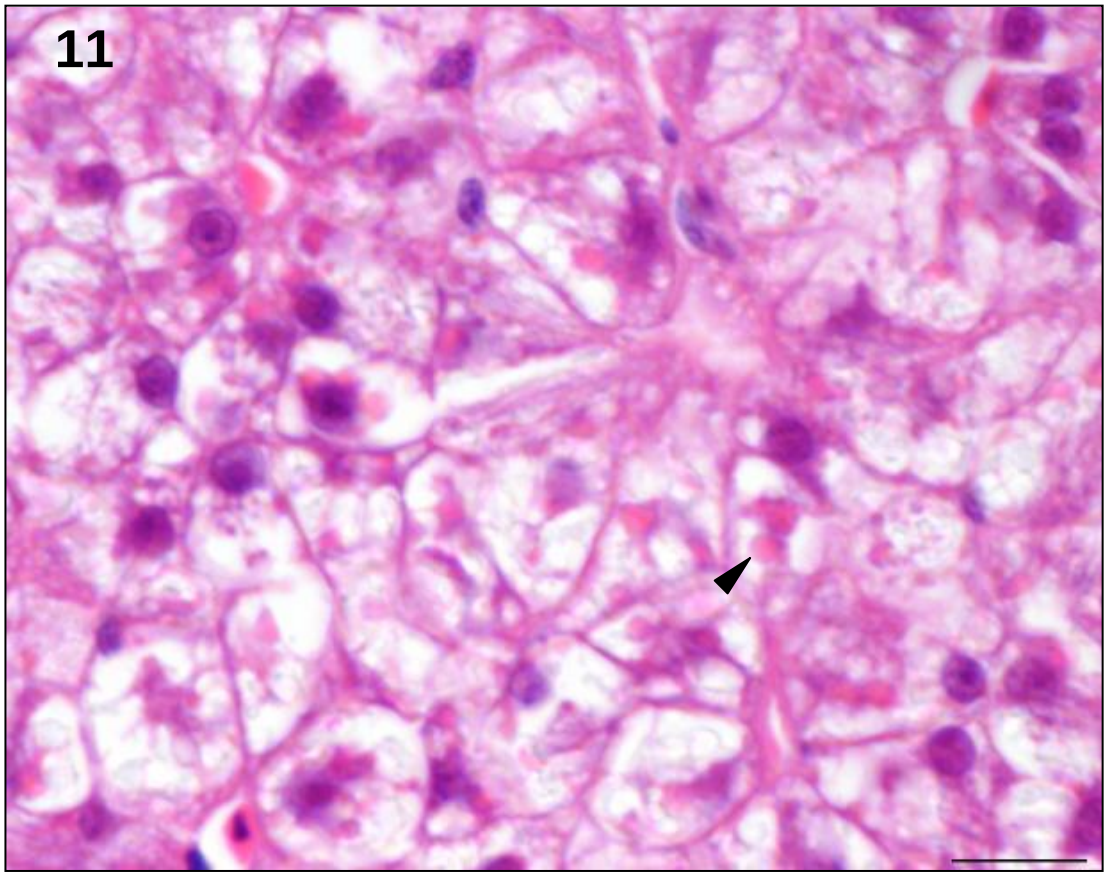
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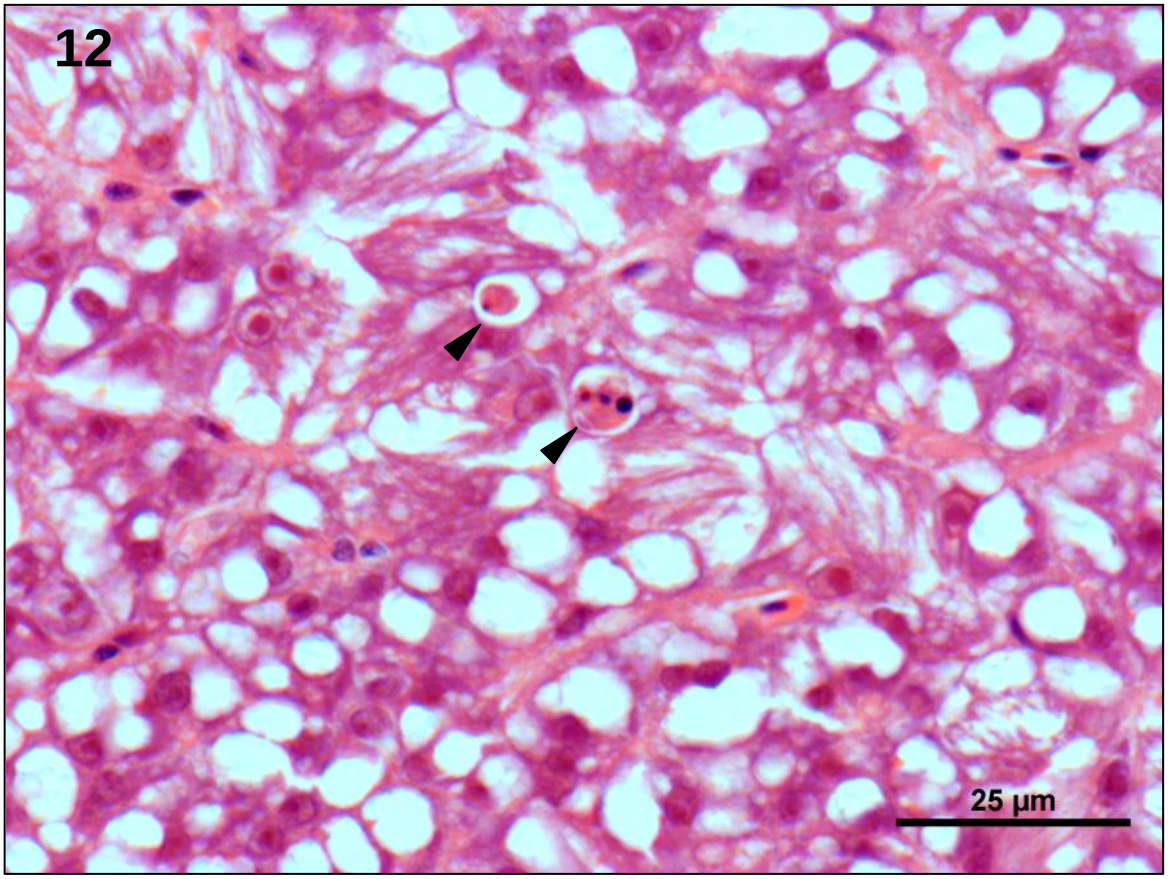


Figure 5a: Normal histological section of male *P. flesus* liver from the River Alde with no abnormalities detected (NAD). Each sinusoid (arrowhead) was surrounded by a single layer of normal hepatocytes. HE. Scale bar, 50 µm. **Figure 5b:** HFI in female *P. flesus* from the River Mersey. HFIs here are characterised by significant numbers of pronounced longitudinal basophilic brush-like structures within hepatocytes (*). HE. Scale bar, 25 µm. **Figure 6:** Longitudinal cross section of sinusoid in male *P. flesus* from River Alde. Note the presence of HFI arrays directed towards sinusoid (S) within surrounding hepatocytes. HE. Scale bar, 10 µm. **Figure 7:** Dense HFI arrays exhibiting longitudinal (L) and transverse (T) orientations in male *P. flesus* from River Mersey (age= 2 years). HE. Scale bar, 10 µm. **Figure 8:** Liver section from female *P. flesus* from River Tyne exhibiting hepatocyte vacuolation and many distinct angular HFIs throughout. Eosinophilic material within cytoplasm (arrowhead) could often be seen. Note the presence of transverse cross sections of putative HFI bundles (circle) in relatively empty hepatocytes. HE. Scale bar, 25 µm. **Figure 9:** High powered view of *P. flesus* in figure 8 shows transverse section of putative HFI. Inclusions contained distinct round vacuolation between fibrillar arrays and often compartmentalised within hepatocytes (arrowhead). HE. Scale bar, 10 µm. **Figure 10:** *P. flesus* shown in figure 8 and 9 demonstrating presence of eosinophilic material (arrowhead) located between fibrillar arrays. HE. Scale bar, 10 µm. **Figure 11:** Male *P. flesus* from River Mersey demonstrating pronounced eosinophilic material (arrowhead) located within hepatocytes. The quantity of eosinophilic material appeared more prevalent in cells containing fewer HFIs. HE. Scale bar, 10 µm. **Figure 12:** Elevated levels of apoptosis (arrowhead) characterised by shrinkage of cytoplasm, pyknosis and karryorhexis of nucleus, were sometimes observed in individuals exhibiting increased severity of HFIs. HE. Scale bar, 25 µm.

The HFI were identified at all sampling sites of varying prevalence and severity (table 5, figure 13a and 13b) and as such were not observed in all hepatocytes of affected fish. With respect to prevalence, the Mersey and the Alde were the worst and least affected sites. The results show that male and female were differentially affected (figures 13a and 13b) with male *P. flesus* exhibiting 30 % HFI prevalence compared to 11.7 % observed in females (Chi-square test, $p<0.001$). The following figures in parenthesis indicate the percentage prevalence affected for all fish, male fish and female respectively: Mersey (77.0, 79.3, and 57.1 %), Tyne (60.0, 78.6, and 36.4 %), Thames (46.0, 63.6, and 32.1 %), Medway (36.0, 44.7, and 8.3 %), Humber (26.0, 34.5 and 14.3 %), and Alde (6.0, 3.7, and 8.7 %). A simple qualitative index was developed to assist in the assessment of HFI severity. Increased HFI prevalence appeared related to increased severity, with those sampling locations demonstrating a relatively high prevalence also possessing larger numbers of *P. flesus* with increased HFI frequency (figure 13a and 13b). Linear regression analysis revealed a strong positive correlation between prevalence and mean HFI severity stage ($r= 0.98$). Liver tissues from the Mersey and Tyne estuaries possessed numerous severity stages of the condition therefore male fish from these estuaries were selected for further immunohistochemical, ultrastructural and molecular analysis.

4.2 Age determination

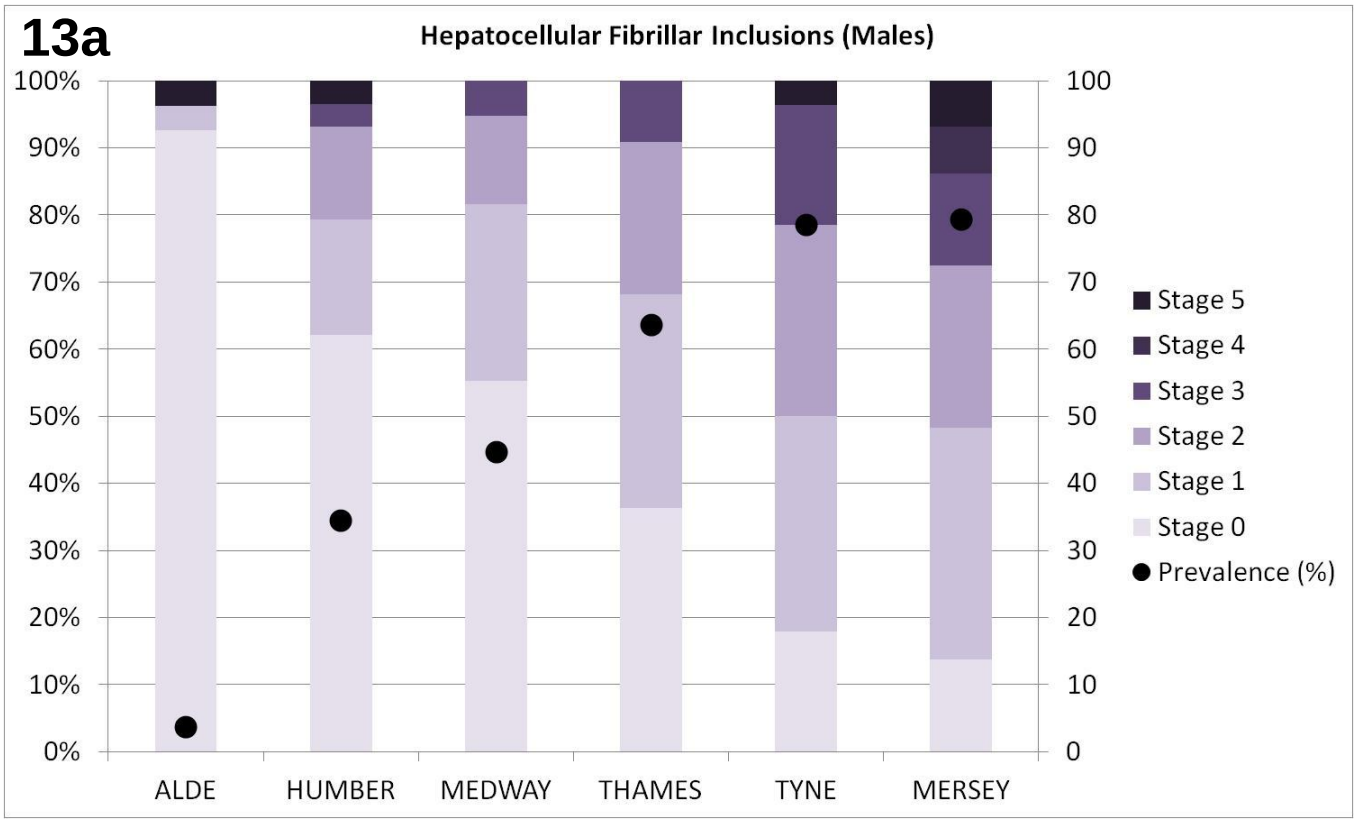
Length and weight ranges exhibited considerable overlap between individual ages (table 6). The most common ages of *P. flesus* sampled from all estuaries was 2-4 yrs (figure 14aa). Further analysis of age at individual estuaries demonstrated that the Humber and Tyne estuaries had a comparatively lower number of 2 yr old fish, although 3 and 4 yr old fish remained frequent. Analysis of age determination and HFI from all estuaries revealed that the prevalence of *P. flesus* with HFI was approximately 40 % for ages 1-6 yrs (figure 14b). This prevalence was approximately 70% for ages 7 and 8 yrs. Furthermore, the mean age for fish with and without HFI was 3.076 and 3.098 respectively indicating no association between age and HFI prevalence (Mann-Whitney Rank Sum Test, $p=0.648$).

Male	Alde	Humber	Medway	Thames	Tyne	Mersey
%	3.7	34.5	44.7	63.6	78.6	79.3
Stage 0	25	18	21	8	5	4
Stage 1	1	5	10	7	9	10
Stage 2	0	4	5	5	8	7
Stage 3	0	1	2	2	5	4
Stage 4	0	0	0	0	0	2
Stage 5	1	1	0	0	1	2
Female	**Alde**	**Humber**	**Medway**	**Thames**	**Tyne**	**Mersey**
%	8.7	14.3	8.3	32.1	36.4	57.1
Stage 0	21	18	11	19	14	9
Stage 1	1	2	1	4	2	2
Stage 2	0	1	0	1	4	4
Stage 3	1	0	0	4	1	2
Stage 4	0	0	0	0	1	4
Stage 5	0	0	0	0	0	0

Table 5: HFI prevalence (%) and frequency of male and female *P. flesus* exhibiting each severity stage.

Age (yrs)	Length range (mm)	Weight range (g)
1	193-225	79-116
2	201-329	83-393
3	220-365	106-518
4	237-379	148-664
5	260-335	173-453
6	290-371	272-499
7	316-346	307-411
8	316-386	289-616

Table 6: Length and weight ranges demonstrated significant overlap for *P. flesus* across all age groups sampled from all estuaries during this study.



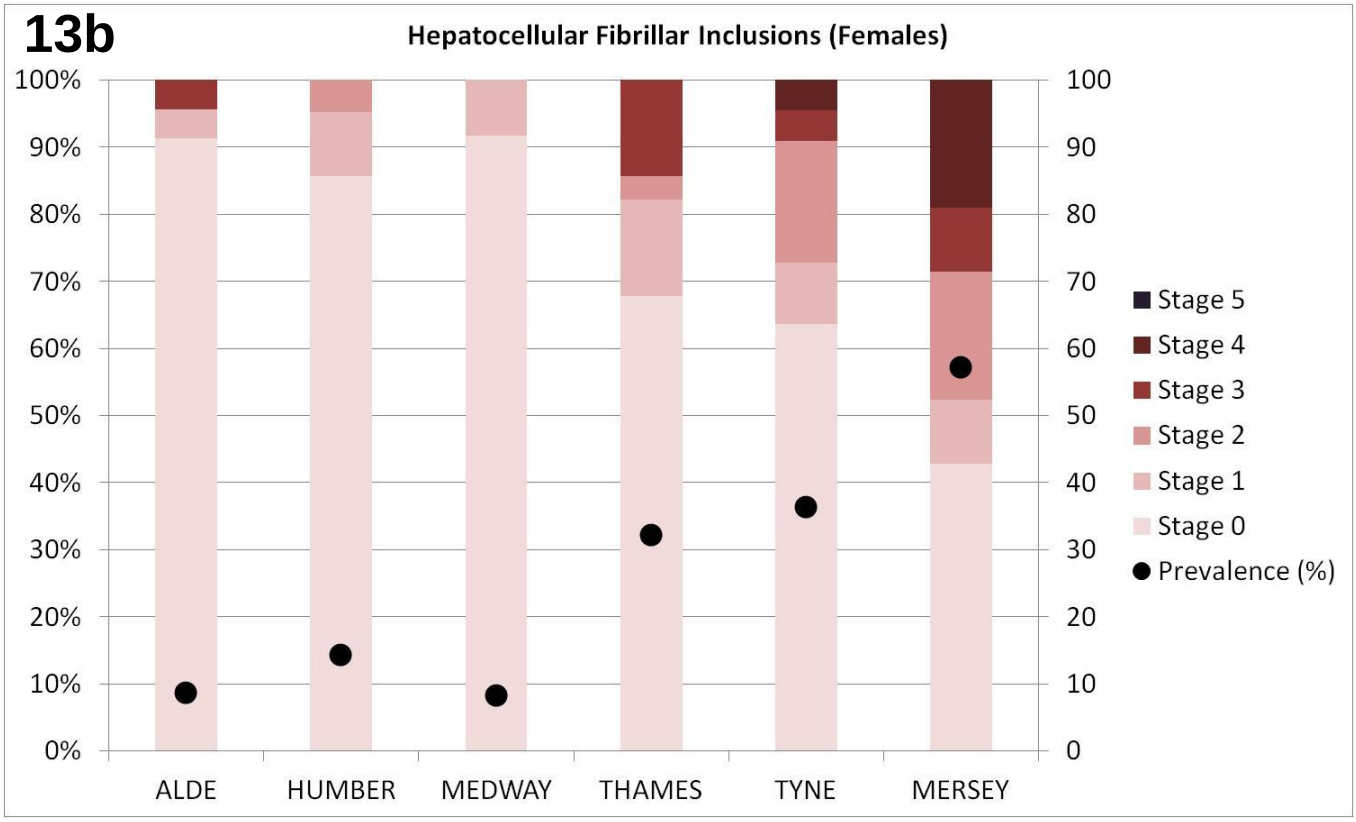
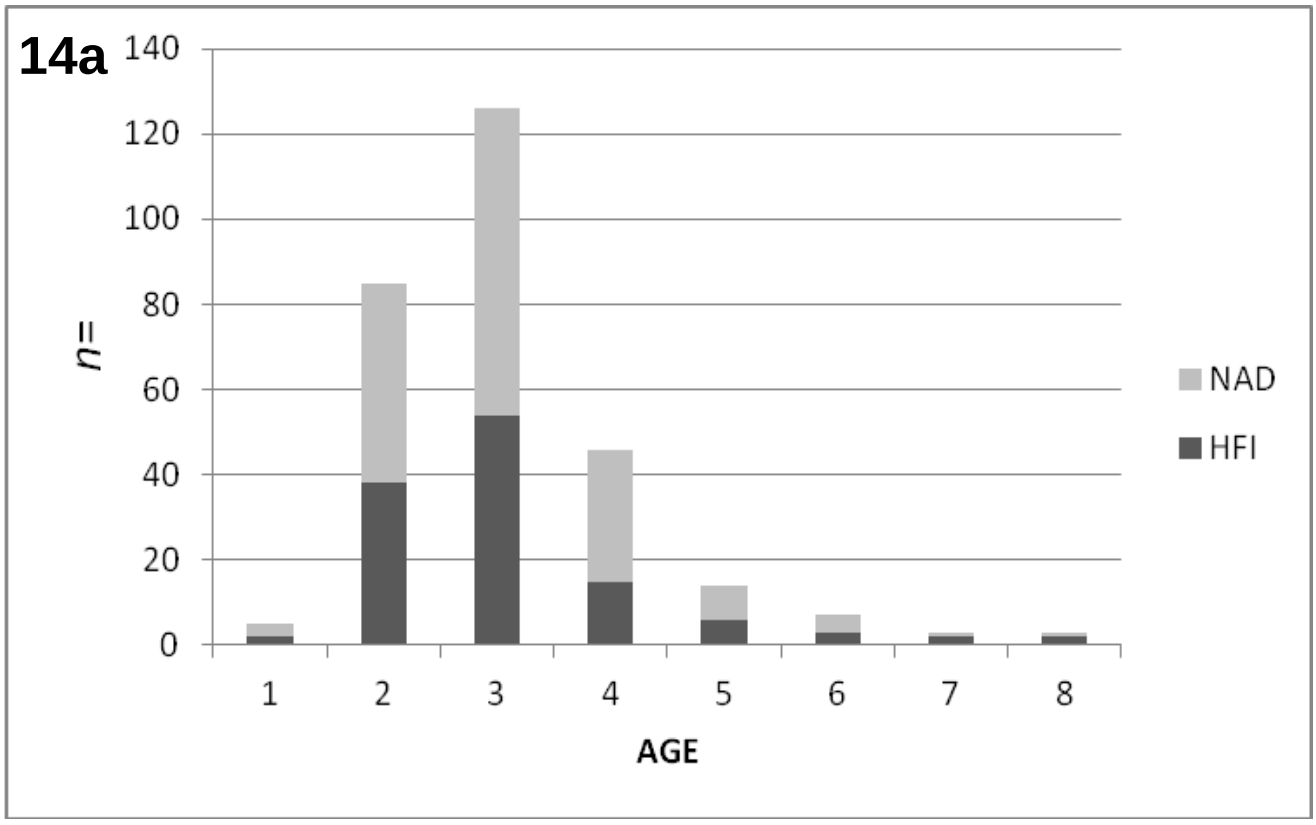


Figure 13a: 100% stacked column chart demonstrating prevalence and proportion of male *P. flesus* exhibiting each HFI severity stage described in table 3. HFI stage 5 not included in calculation of prevalence. **Figure 13b:** 100% stacked column chart demonstrating prevalence and proportion of female *P. flesus* exhibiting each HFI severity stage described in table 3. HFI stage 5 not included in calculation of prevalence.



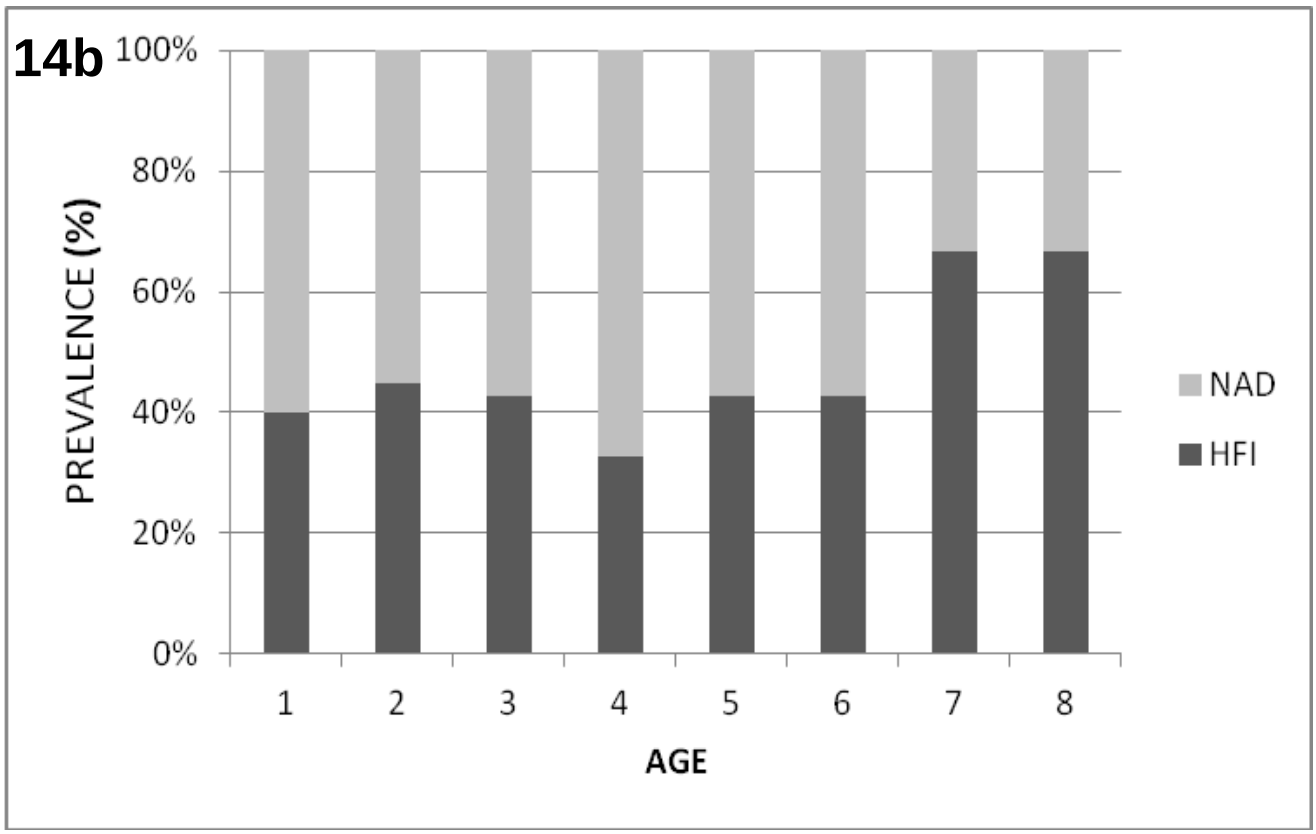


Figure 14a: Age distribution histogram demonstrating proportion of *P. flesus* exhibiting HFI and no abnormalities detected (NAD). **Figure 14b:** 100% stacked column chart demonstrating proportion of *P. flesus* of all ages from all sampling locations exhibiting HFI and no abnormalities detected (NAD).

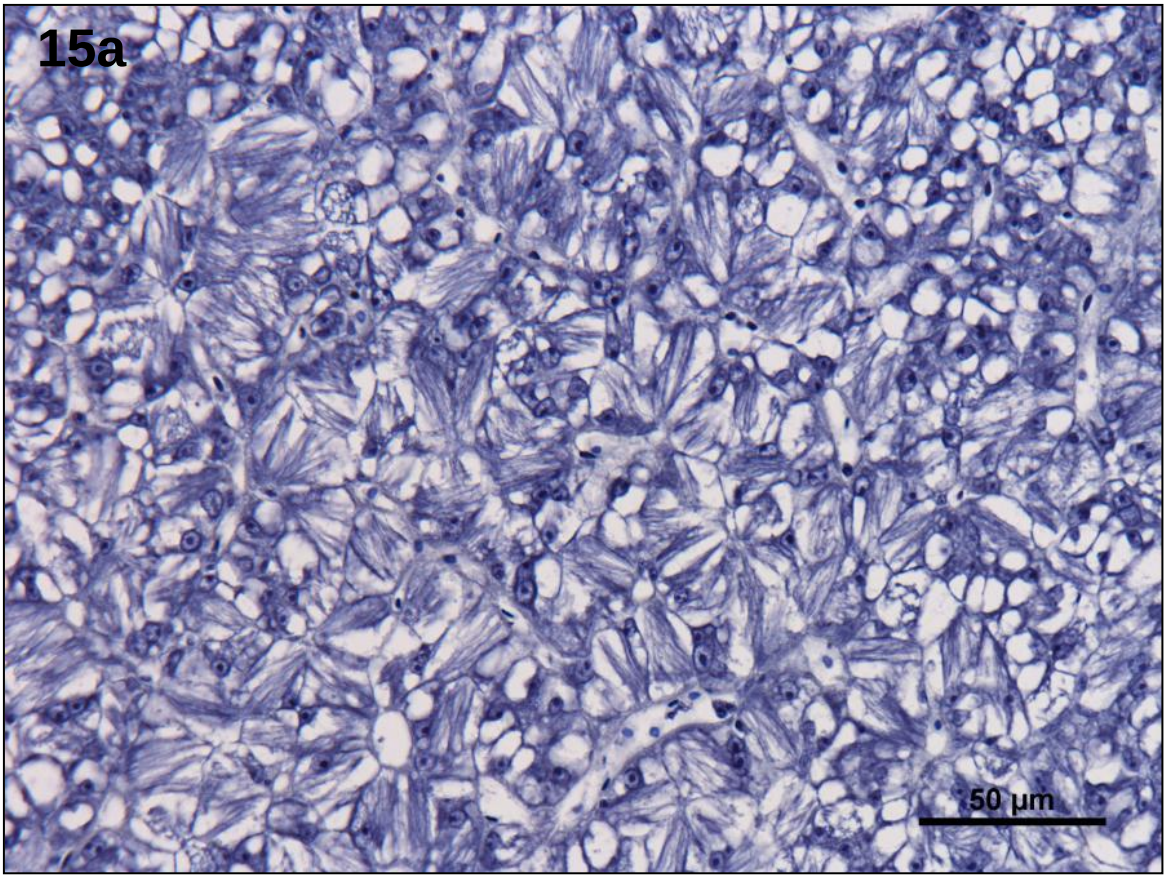
4.3 Immunohistochemistry (IHC)

The IHC was undertaken on representative samples selected from the Mersey and Tyne estuaries using a polyclonal anti-rabbit *P. flesus* VtG primary antibody. Negative controls (substitution of primary antibody for TBS) demonstrated no background staining of diaminobenzidine chromogen (figure 15a). IHC revealed the presence of VtG within hepatocyes and readily associated VtG with HFI (figure 15b). Labelling was clear and distinct with IHC demonstrating good definition of individual filaments of HFI, with minimal background and non-specific staining. The previously identified eosinophilic material in HE sections (figures 10 and 11) appeared to correspond to a substance demonstrating positive labelling for VtG (figure 16). Positive labelling was also observed at the periphery of the majority of sinusoids in all sections stained for VtG in IHC sections. This could result from residual VtG in the sinusoids, although it was also observed in sections where HFI were absent. This observation may be artefact resulting from the retention of primary antibody as is commonly observed at the periphery of IHC sections following insufficient washing. A comparison of HFI severity was conducted between histological HE and IHC sections to determine if all HFI identified demonstrated positive VtG labelling (figures 17-20). VtG labelling of HFI of all severity stages was generally positive, although many liver sections from the Mersey exhibited decreased labelling as characterised by a dirty opaque appearance (figure 20b). This was despite evidence of HFI in HE histological sections (figure 20a). In these specimens, IHC labelling of HFI appeared relatively weak and less defined compared to HFI from other locations.

The hypothesis that HFI is the result of an abnormal proliferation of cytoskeleton microtubules was tested. Microtubules are comprised of α/β- tubulin protein dimers therefore IHC was undertaken using a commercially available α/β- tubulin polyclonal antibody claiming cross reactivity for zebrafish. IHC demonstrated negative labelling for α/β- tubulin.

4.4 Transmission Electron Microscopy (TEM)

Normal hepatocytes possessed round nuclei with well defined nucleoli and typically exhibited no cellular atrophy. Mitochondria, Golgi complex and lysosomes were clearly visible (figure 21). Nuclei were surrounded by stacks of RER cisternae with ribosomes studded along the outer membrane (figures 22). Hepatocytes with HFI demonstrated significant atrophy characterised by disruption of ultrastructure, distended cytoplasm and polygonal appearance. HFI were numerous and appeared as flattened arrays orientated across the longest hepatocellular axis (figure 23-25). Close inspection revealed ribosomes (28-32 nm) studded along their full length (figure 26) confirming that HFI were RER cisternae exhibiting significant disorganisation. HFI were mostly interspersed with mitochondria and occasional non-membrane bound large lipid like inclusions (figure 23) appearing to correspond to eosinophilic material previously observed in histological HE sections (figure 10 and 11). Similar inclusions were also seen in normal hepatocytes, although many hepatocytes containing HFI were observed elsewhere within the same liver. The identification of other organelles in HFI affected hepatocytes was often troublesome due to displacement caused by the cellular volume that distended RER occupied. No signs of the observed transverse sections of putative HFI (figures 8 and 9) were observed during TEM analysis. HFI in female livers were frequently electron dense in appearance (figure 27 and 28) and were caused by increased numbers of free ribosomes interspersed between cisternae (figure 29). Electron dense hepatocytes always contained a shrunken pyknotic pleomorphic nuclei (2-3 µm) with poorly defined nucleoli and irregular border (figure 28). Hepatocytes containing HFI that were not electron dense contained a nucleus that was similar in size (5-6 µm) to normal hepatocytes. HFI often exhibited a degree of fragmentation appearing to result in vacuous spaces (figure 23) of varying size. These spaces gave hepatocytes a “moth-eaten” appearance. This was not believed to be the exclusive result of ineffective fixation as demonstrated by the presence of mitochondria with well defined cristae. However, swollen mitochondria exhibiting loss of cristae structure were occasionally observed in other hepatocytes containing HFI. A number of hepatocytes exhibited clear demarcation of intracellular areas with and without vacuous spaces (figure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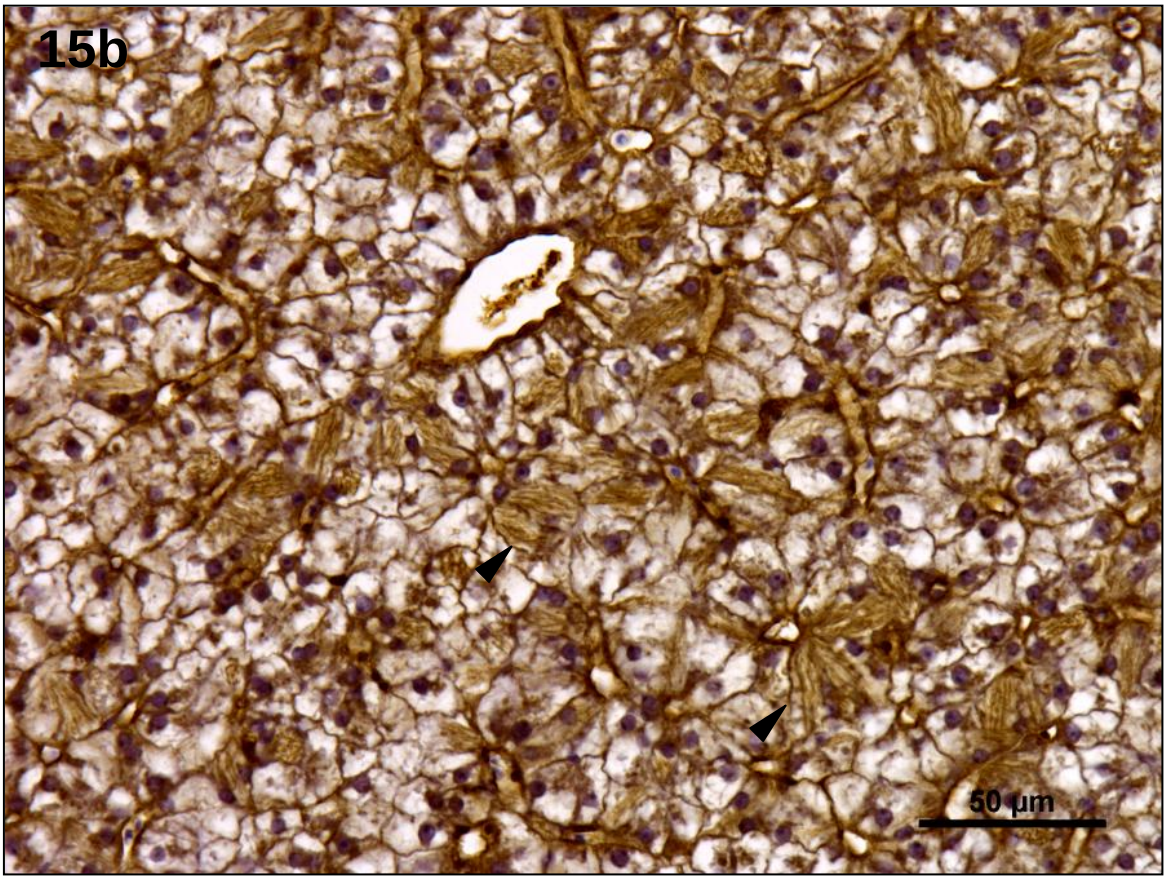
15b
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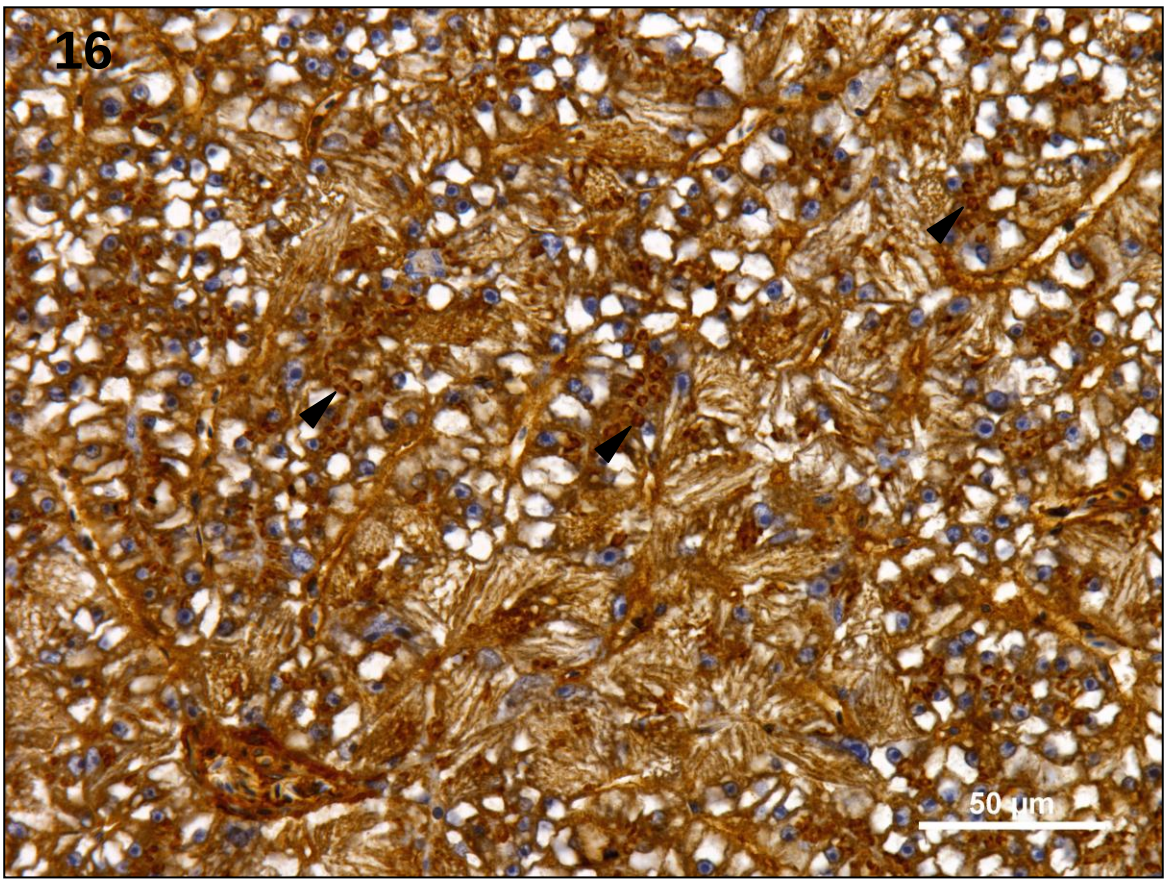
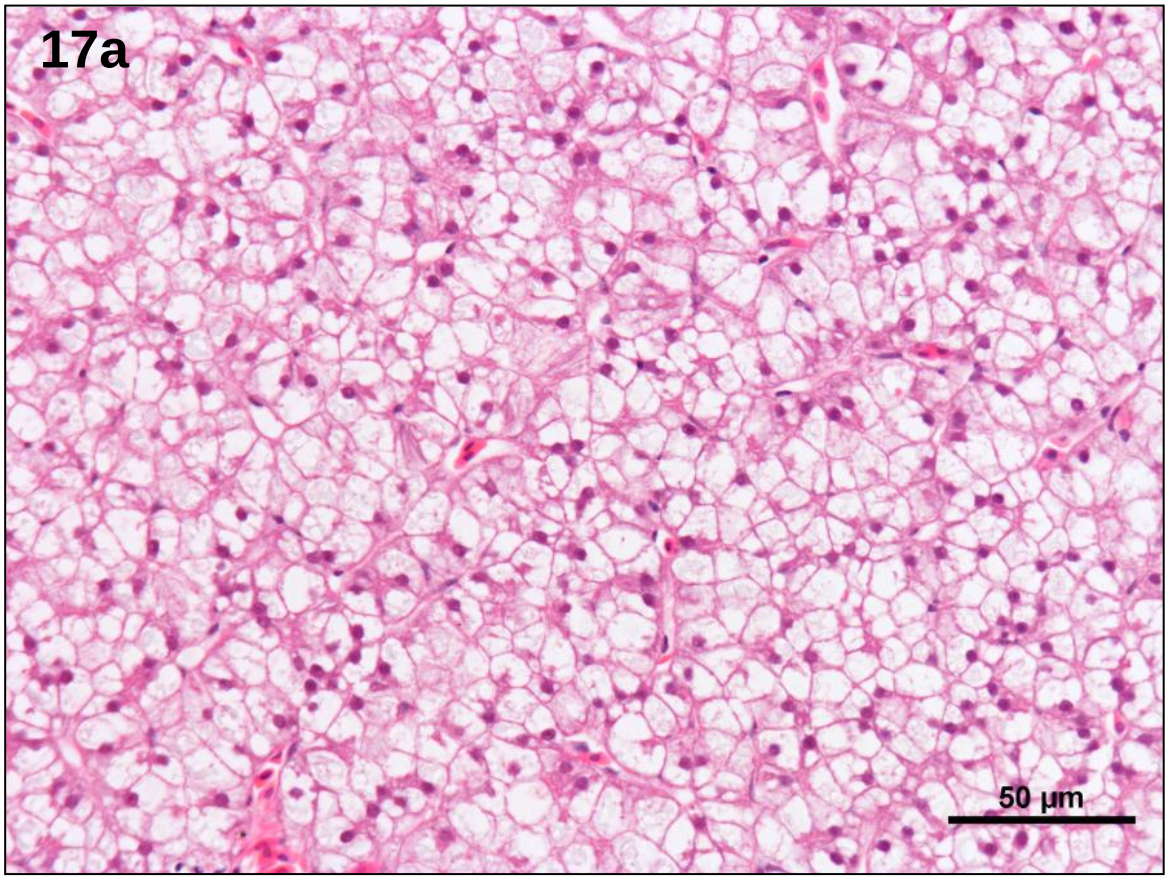


Figure 15a: Negative control (no antibody) for IHC labelling of VtG. Scale bar, 50 µm. **Figure 15b:** IHC labelling of VtG in male *P. flesus* using polyclonal anti-rabbit *P. flesus* VtG antibody (1:5000). Positively labelled VtG demonstrated immediate association with HFIs. Individual HFI fibrils can clearly be seen within hepatocytes (arrowhead). Scale bar, 50 µm. **Figure 16:** IHC labelling of VtG using polyclonal anti-rabbit *P. flesus* VtG antibody (1:5000). Positively labelled VtG demonstrated immediate association with HFIs. IHC appeared to reveal positive labelling of previously identified eosinophilic material present in hepatocytes (arrowhead). Scale bar, 50 µm.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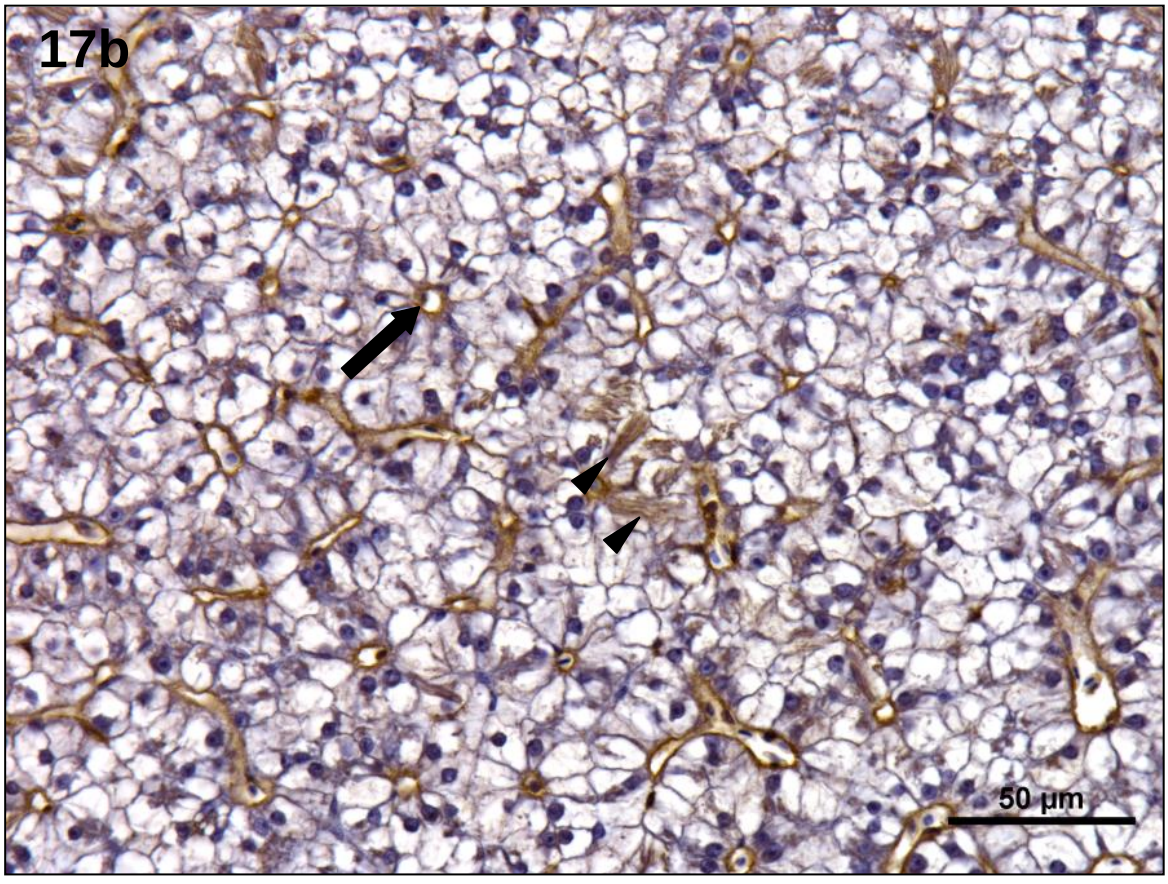
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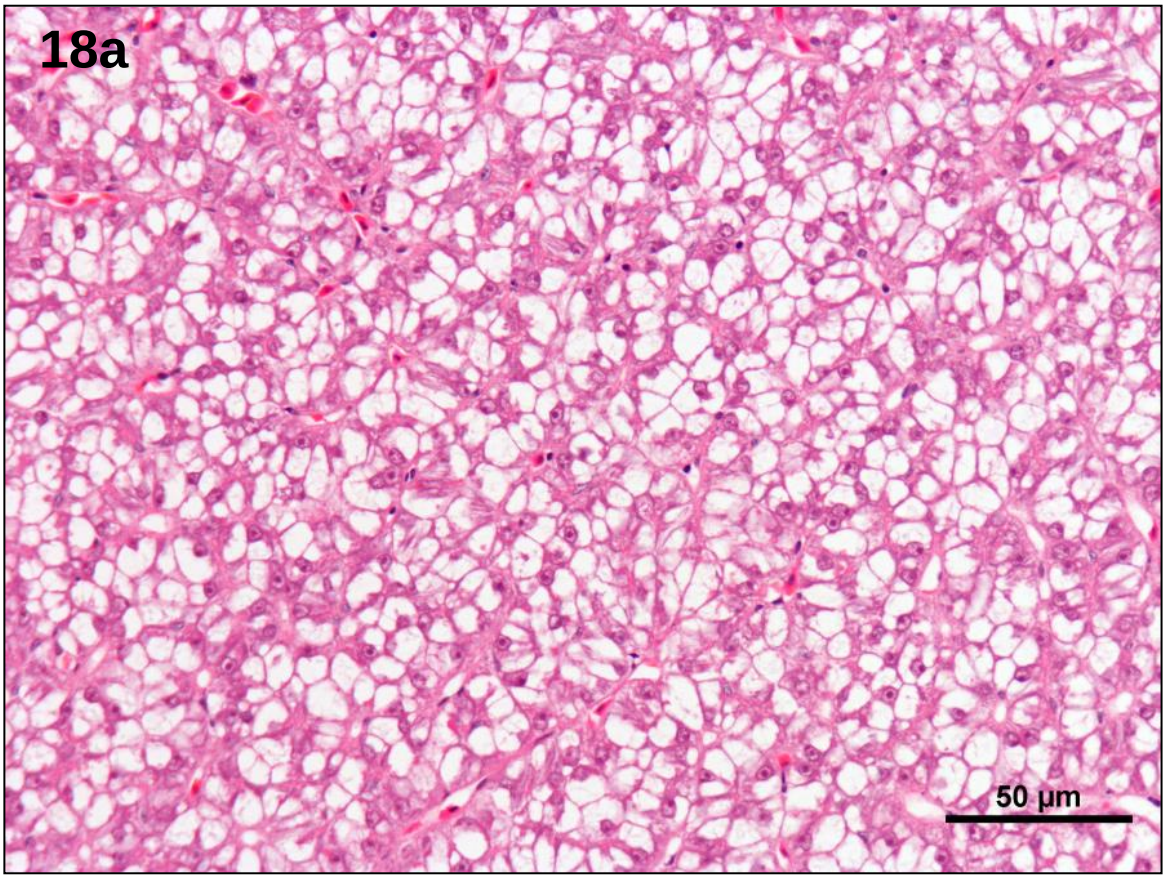
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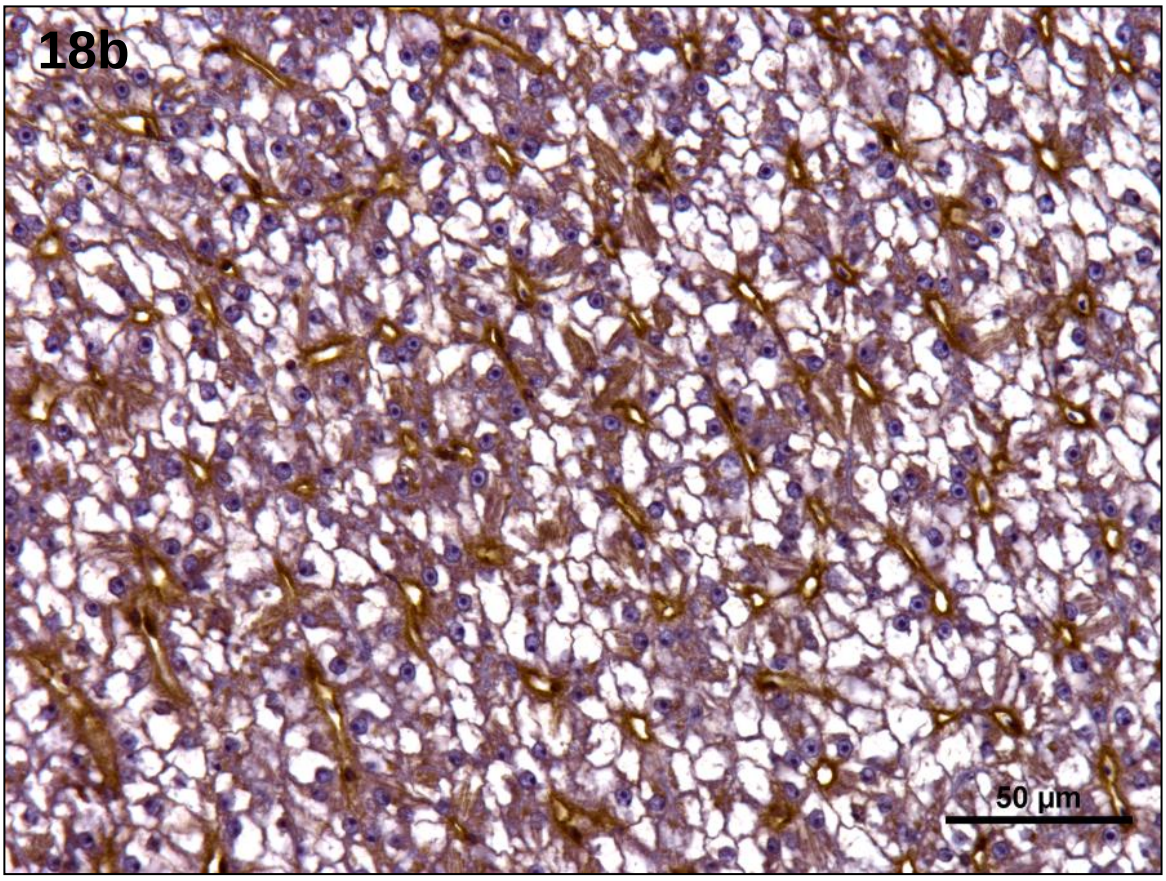
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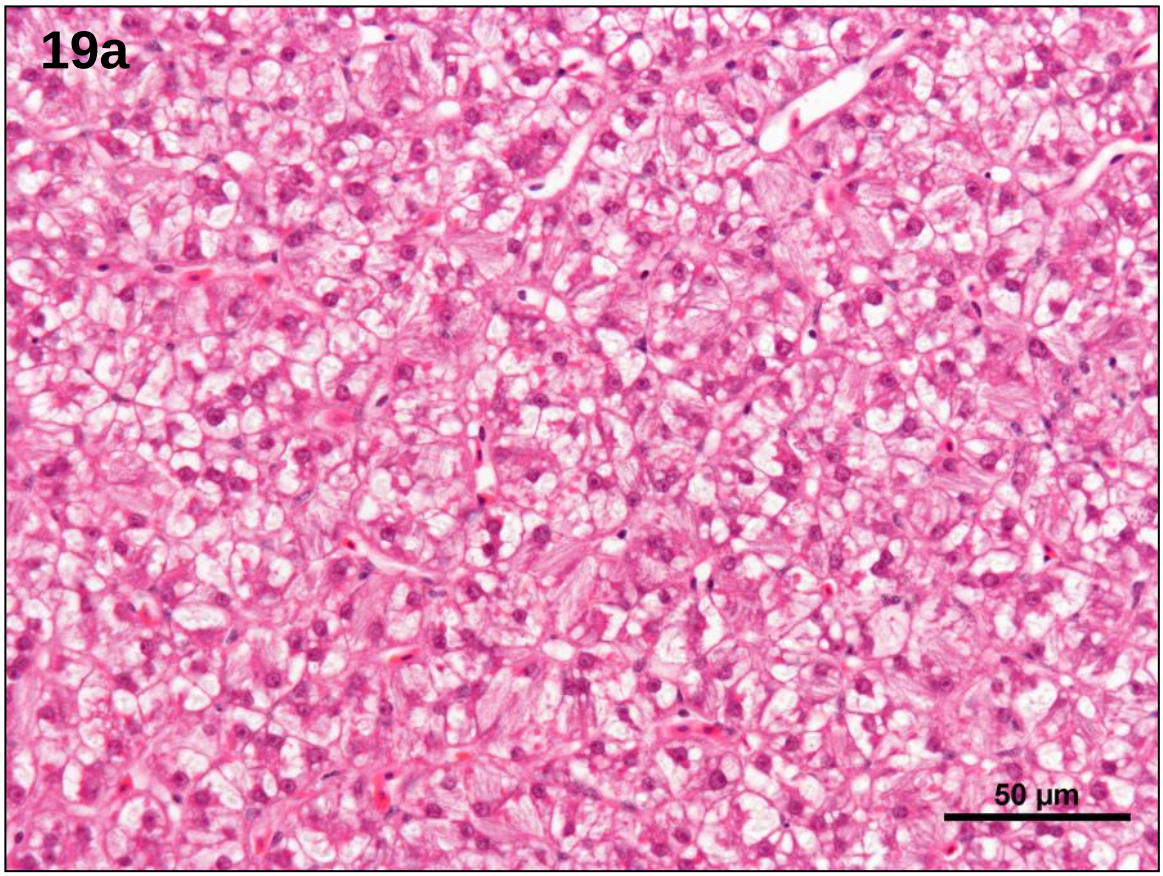
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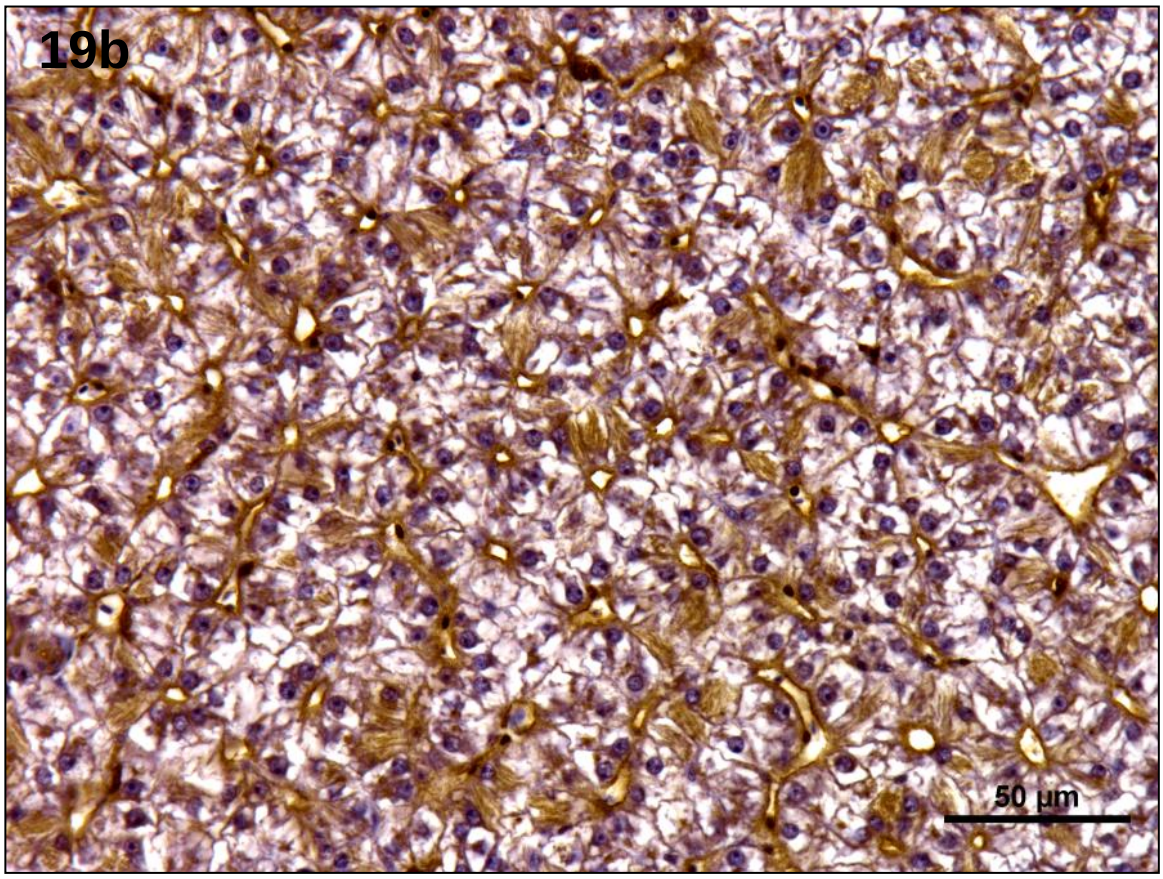
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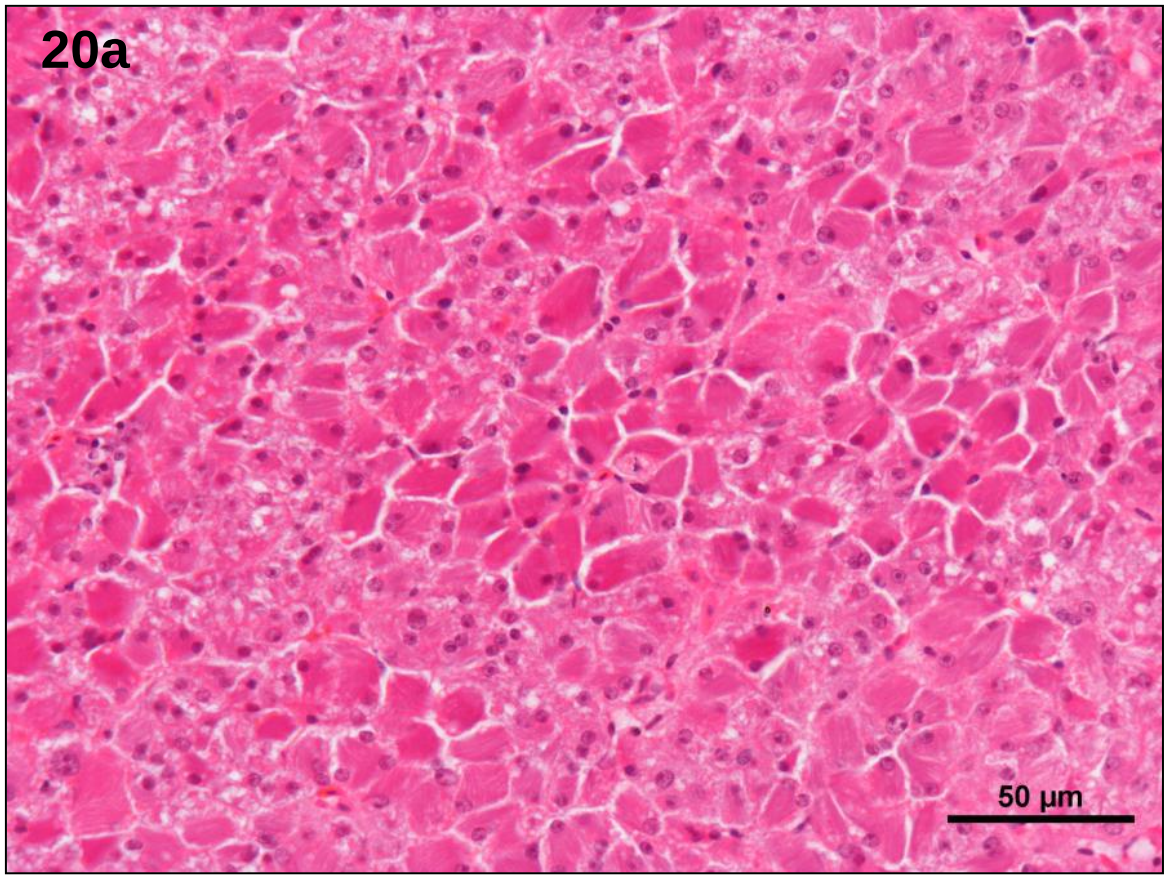
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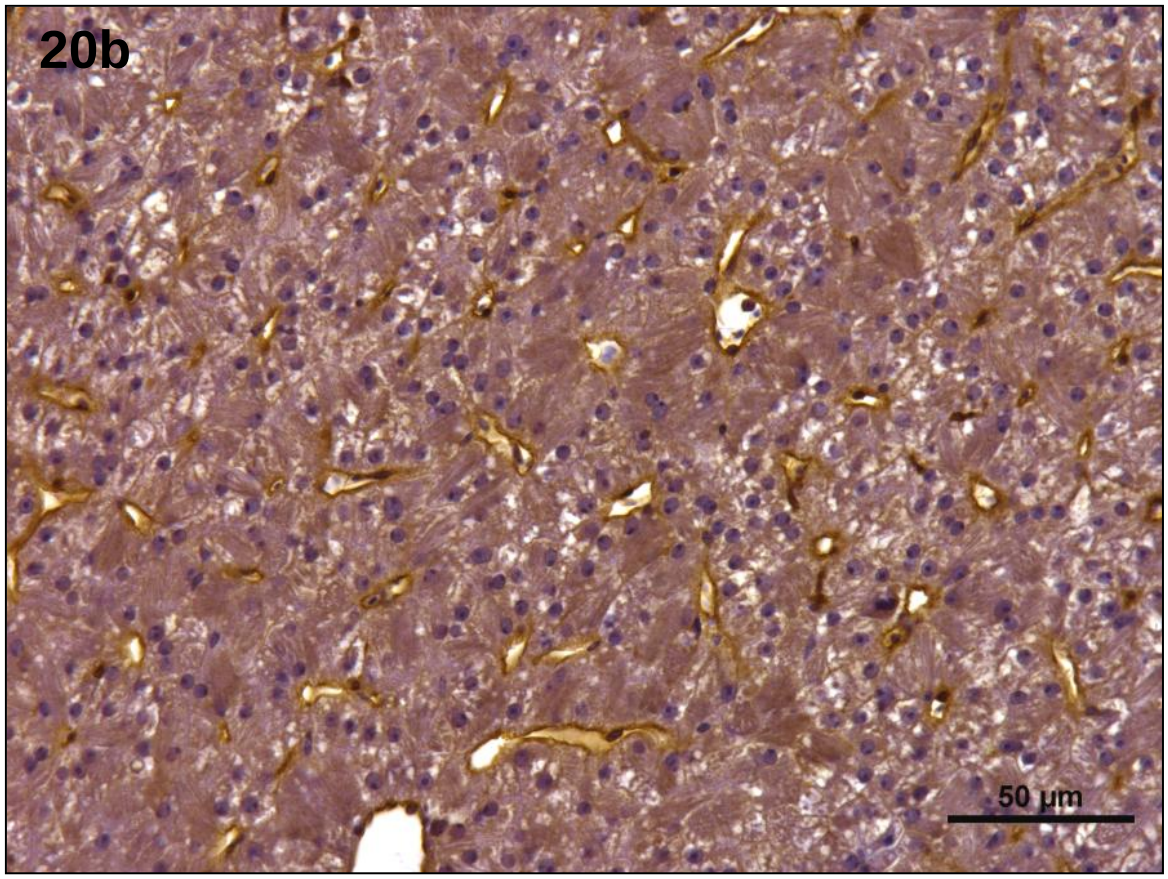
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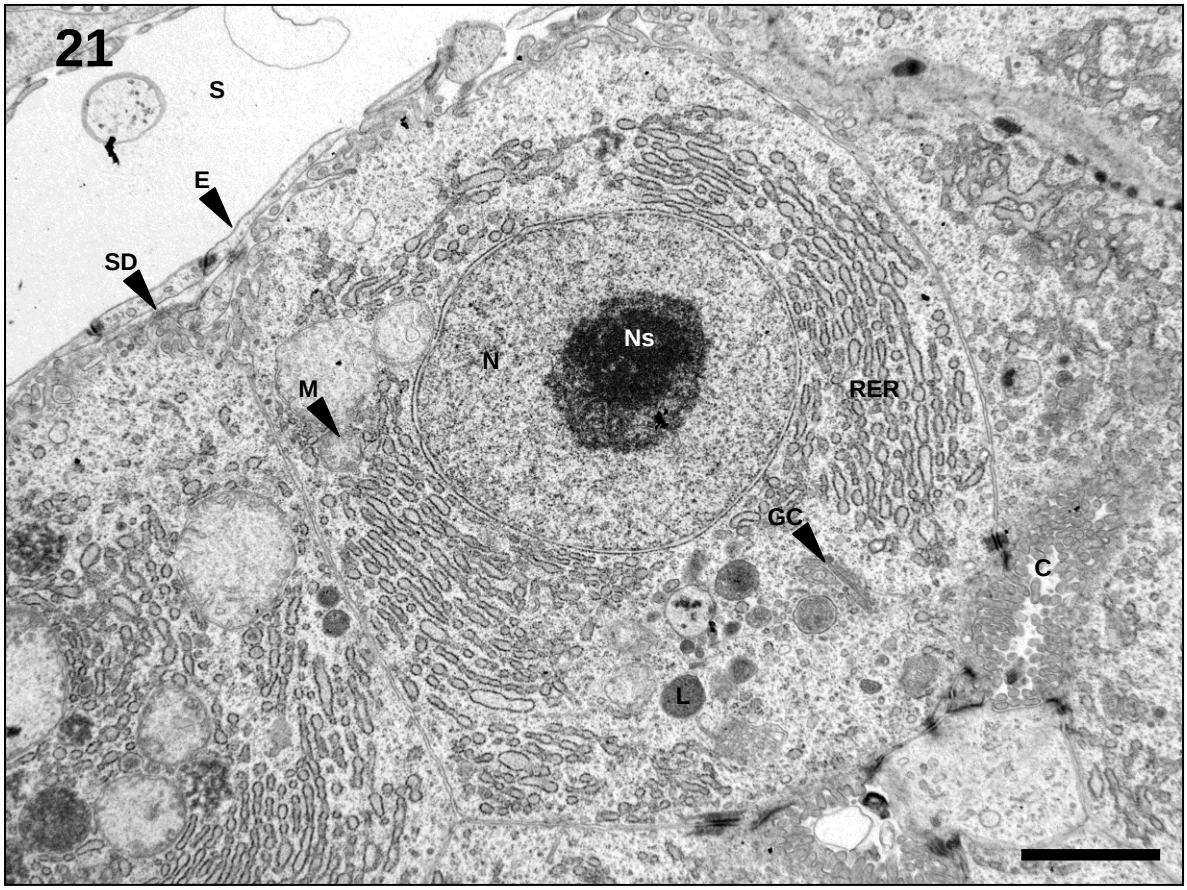
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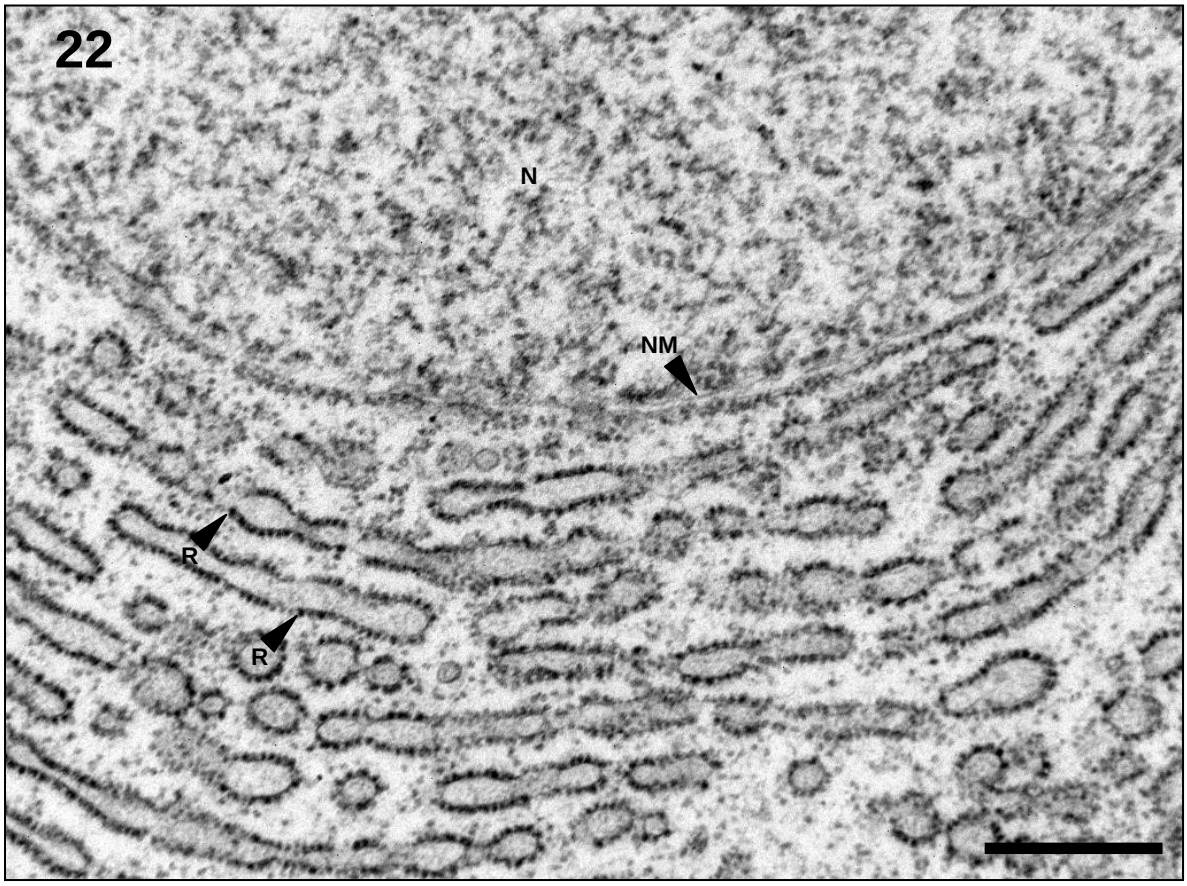
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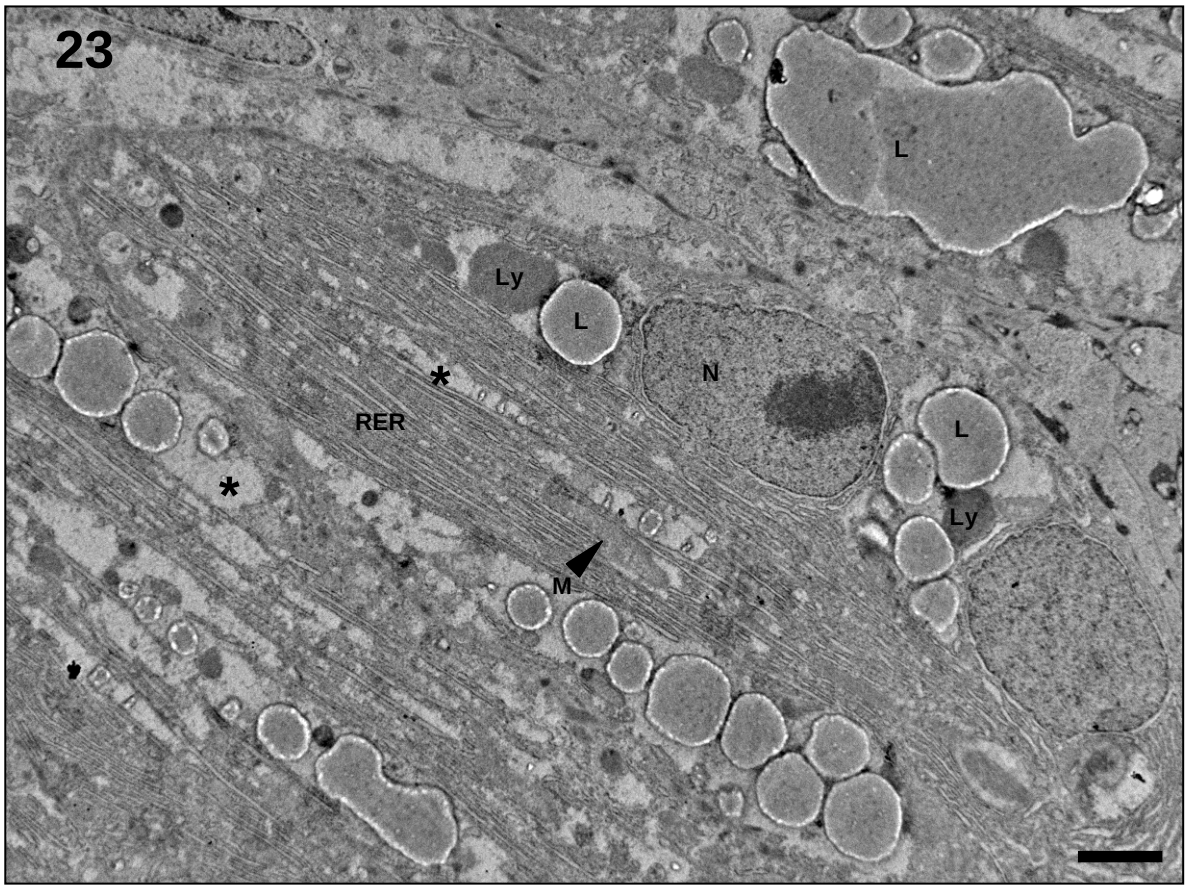
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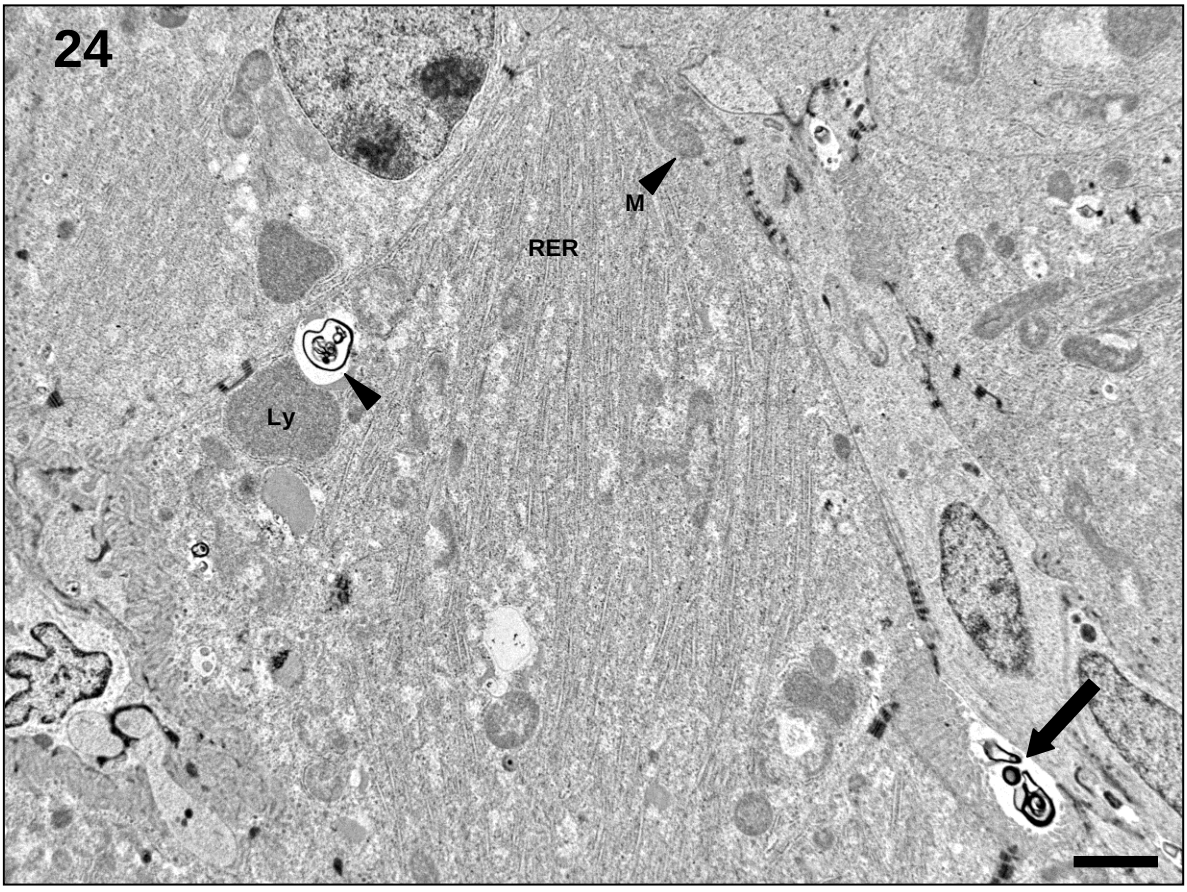
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Ly

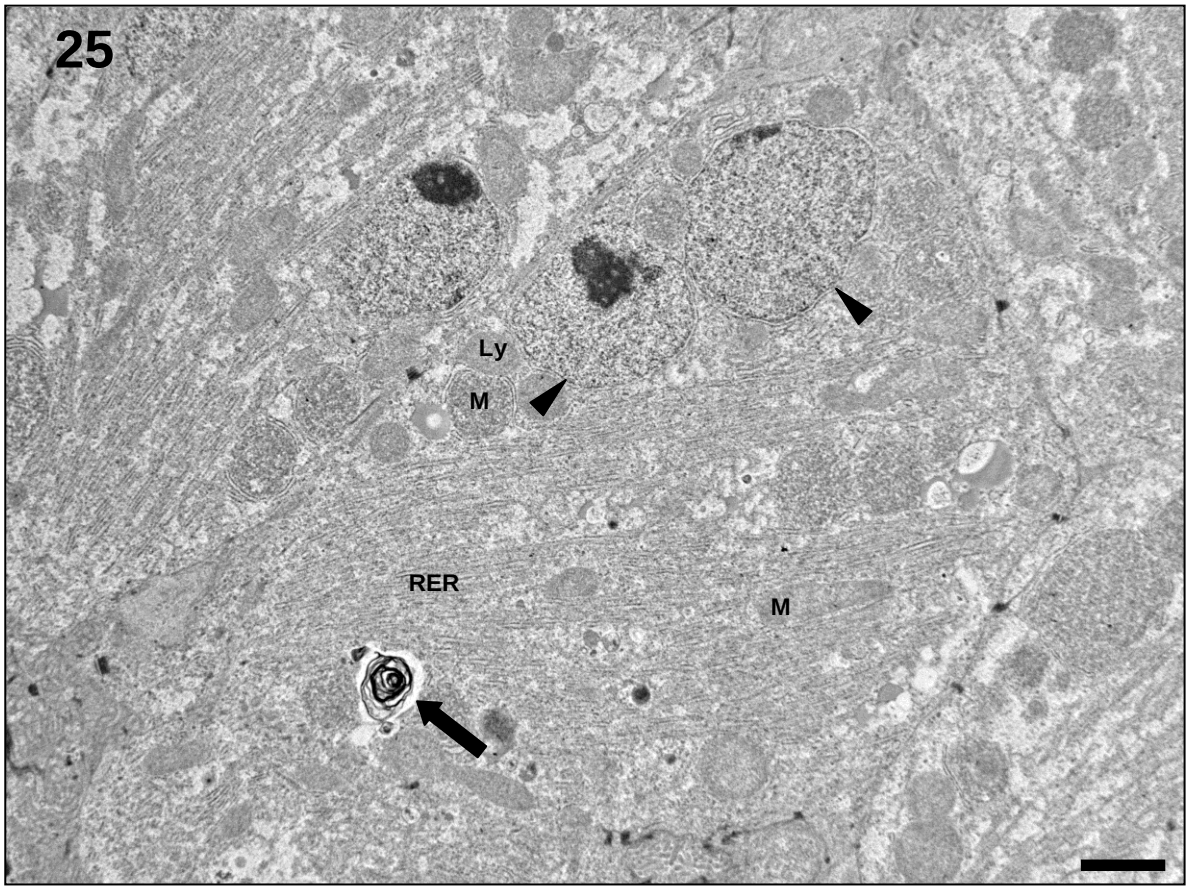


Figure 17a: HE histological section of male *P. flesus*. HFI severity stage 1. Scale bar, 50 µm. **Figure 17b:** IHC histological section of figure 17a. HFI severity stage 1. Note presence of HFIs (arrowhead) and false positive labelling of VtG (arrow) associated with endothelium of sinusoids. Scale bar, 50 µm. **Figure 18a:** HE histological section of male *P. flesus*. HFI severity stage 2. Scale bar, 50 µm. **Figure 18b:** IHC histological section of figure 18a. HFI severity stage 2. Scale bar, 50 µm. **Figure 19a:** HE histological section of male *P. flesus*. HFI severity stage 3. Scale bar, 50 µm. **Figure 19b:** IHC histological section of figure 19a. HFI severity stage 3. Scale bar, 50 µm. **Figure 20a:** HE histological section of male *P. flesus*. HFI severity stage 4. Scale bar, 50 µm. **Figure 20b:** IHC histological section of figure 20a. HFI severity stage 4. Note the presence of atypical staining demonstrated by relatively opaque appearance of HFI. Scale bar, 50 µm. **Figure 21:** Transmission electron microscopy (TEM) of normal *P. flesus* hepatocyte. The nucleus (N) is surrounded by stacks of rough endoplasmic reticulum (RER). Nucleolus (Ns), the Golgi complex (GC), sinusoid (S), mitochondrian (M), space of Dissé (SD), lysosome (Ly), sinusoid endothelium (E), caniculus (C). Scale bar, 2 µm. **Figure 22:** Normal hepatocytes displaying ribosomes (R) studded along the outer membrane of rough endoplasmic reticulum. Nucleus (N), nuclear membrane (NM). Scale bar, 500 nm. **Figure 23:** Hepatocyte from same fish as figure 8-10 and 16. HFI are confirmed here as rough endoplasmic reticulum (RER) orientated across full axis interspersed with mitochondria (M), lysosomes (Ly), and lipid like substance (L). Note the immediate association of lysosomes (Ly) with lipid. Vacuous spaces between apparent disintegrating RER were frequently observed (*). Scale bar, 2 µm. **Figure 24:** Hepatocyte displaying HFI. Note coalescence of lysosome (Ly) with autophagasome (arrow) containing membranous material. Occasionally, similar material appeared to have been ejected into the bile canaliculi (arrow). Rough endoplasmic reticulum (RER), mitochondrian (M). Scale bar, 2 µm. **Figure 25:** Bi-nucleate hepatocyte exhibiting HFI. Single lysosome (Ly) appears to undergo coalescence with swollen degenerate mitochondrian (M). Note the presence of autophagosome containing membranous material (arrow). Nuclei (N). Scale bar, 2 µm.

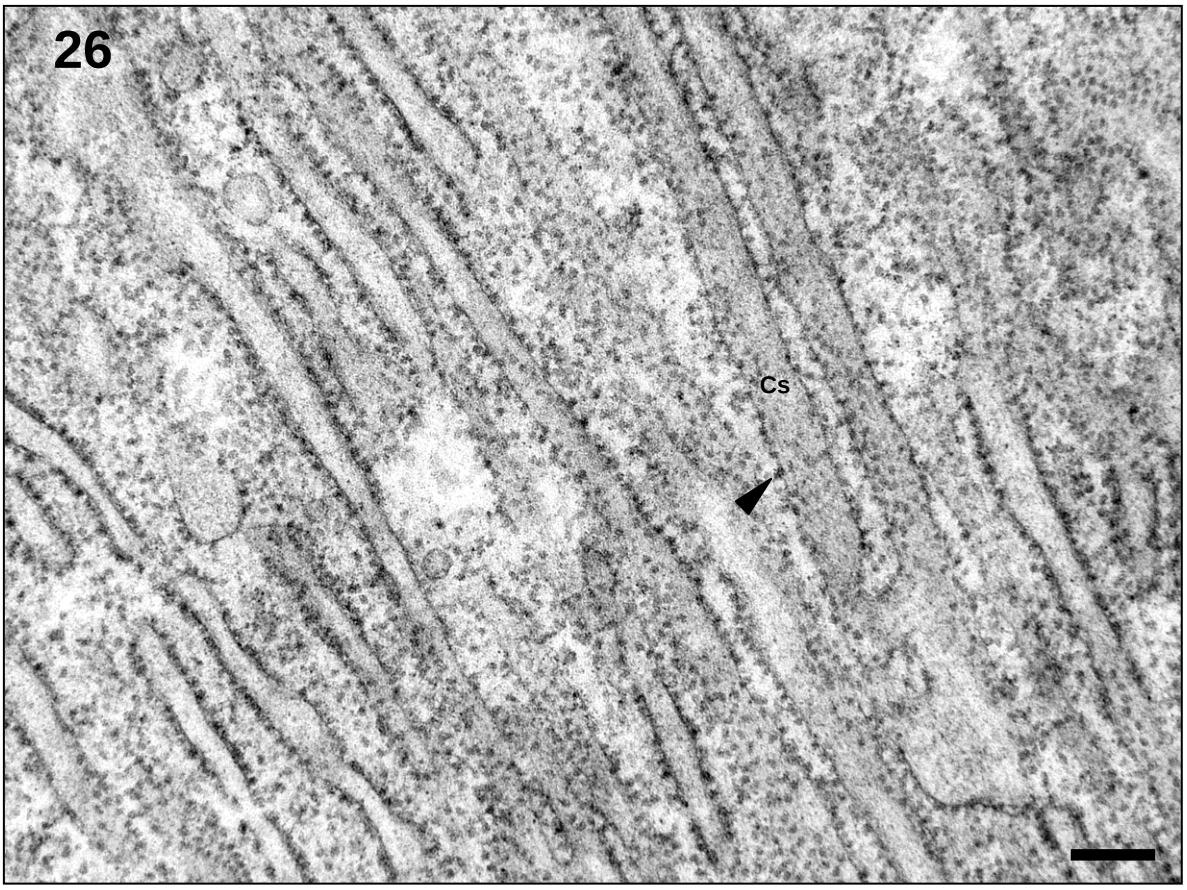
30). These spaces were less common in electron dense hepatocytes. The observation of autophagosomes containing a degenerate membranous substance is worthy of note and indicated that the process of autophagy was active in hepatocytes containing HFI. Membranous material was frequently seen within the bile canaliculi. Autophagosomes were often seen undergoing coalescence with or immediately associated with lysosomes (figures 24 and 28). Similarly, lysosomes occasionally demonstrated coalescence and immediate association with lipid (figure 23 and 30). One hepatocyte exhibited a structure that was similar in appearance to so called 'macrotubules' (Köhler, 1989) within the lumen of few cisternae (figure 31 and 32). These electron dense structures were situated in the centre of the cisternae lumen and appeared to orientate along the entire length of individual cisternae. No other examples were observed in this study.

4.5 Real-time Polymerase Chain Reaction (qPCR)

The reaction efficiency for qPCR of VtG, CHR, UBQ, EF1, FACT and ATUB was 1.826, 1.822, 1.963, 1.850, 1.940 and 1.790 respectively. Analysis of gene transcripts was carried out for VtG and CHR in liver tissue of male *P. flesus* sampled from the Mersey and Tyne estuaries. Linear regression analysis between VtG and CHR transcripts showed a strong positive linear correlation (r= 0.981) indicating a reliable qPCR (figure 33). Similar relative quantities of VtG and CHR gene transcripts were observed across biological groups (figure 34a and 34b). Kruskal-Wallis rank sum test revealed no association between relative quantities of VtG and CHR gene transcripts, and biological groups (p= 0.3098 and p=0.5317 respectively). The difference between the lowest and highest levels of VtG gene transcription was an 8060 fold increase.

4.6 VtG analysis of plasma

The ELISA successfully determined VtG concentrations in 171 plasma samples of male *P. flesus* (table 7). VtG concentrations were low for a large proportion of *P. flesus* sampled from all estuaries. Overall, ten fish exhibited VtG concentrations >1 µg/ml (range 1.7-1944.0 µg ml^{-1}) with the remainder of fish demonstrating concentrations similar to baseline levels observed during previous monitoring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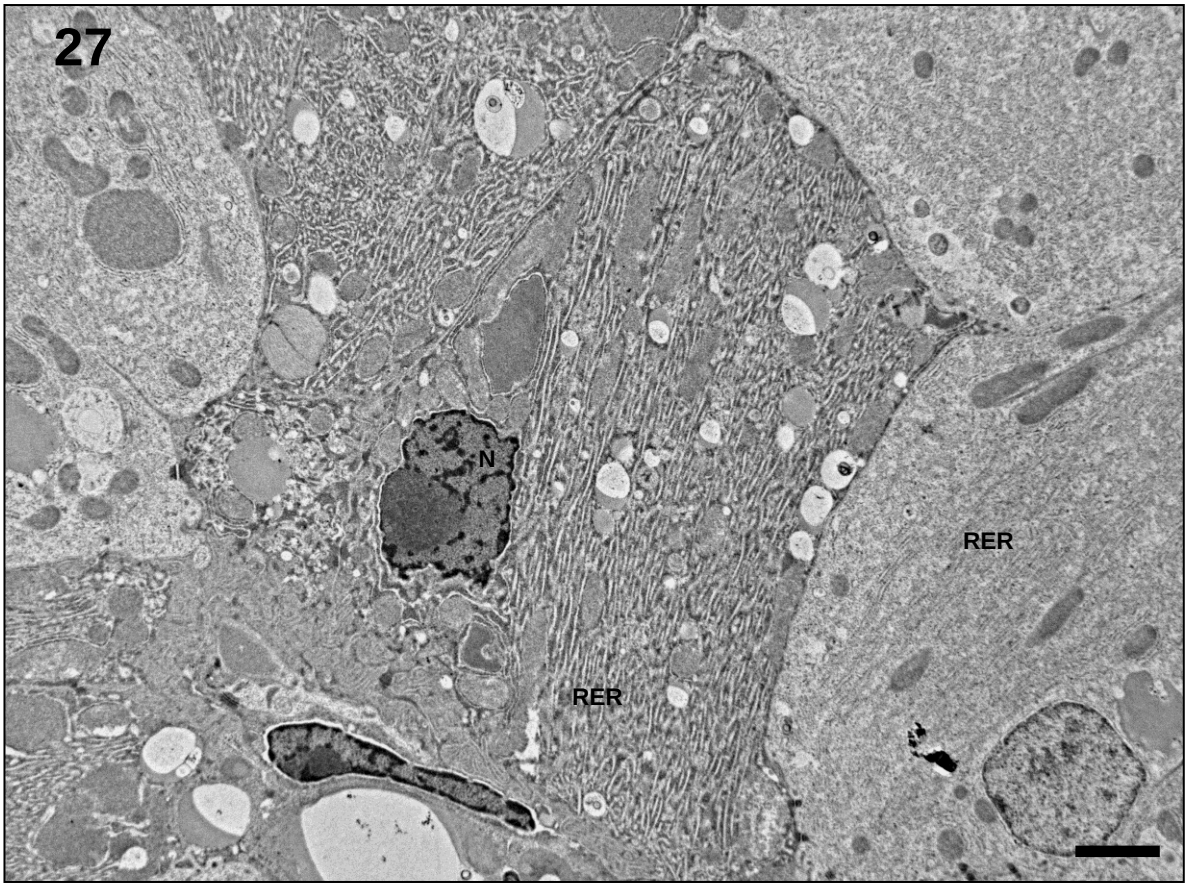
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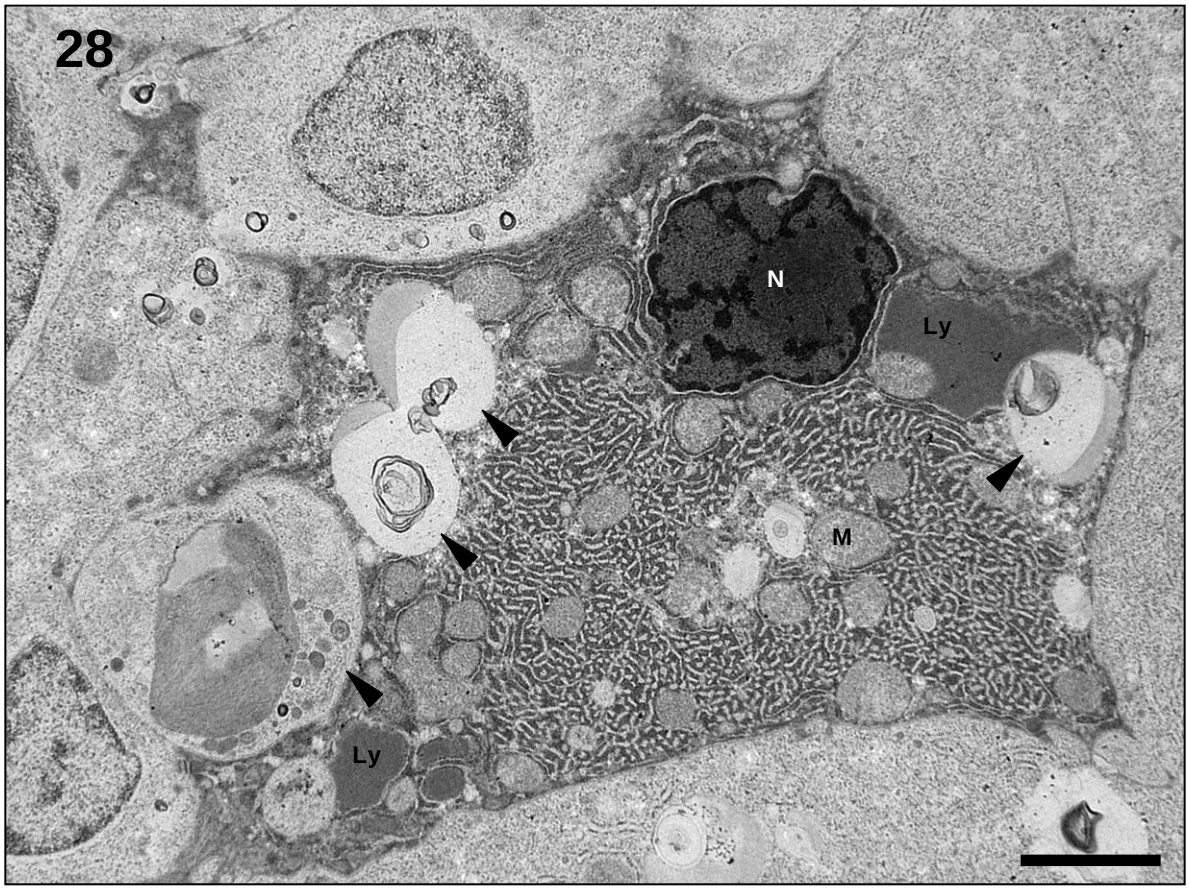
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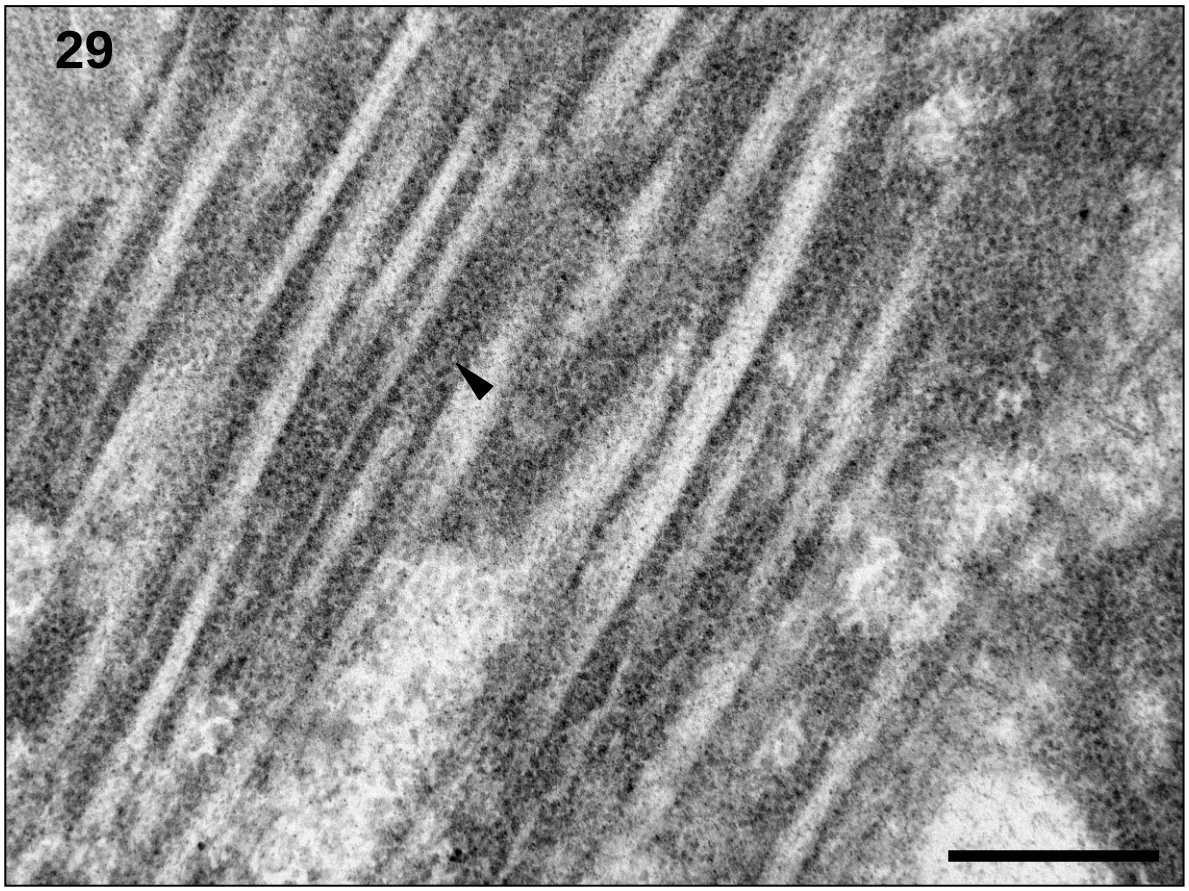
M
Ly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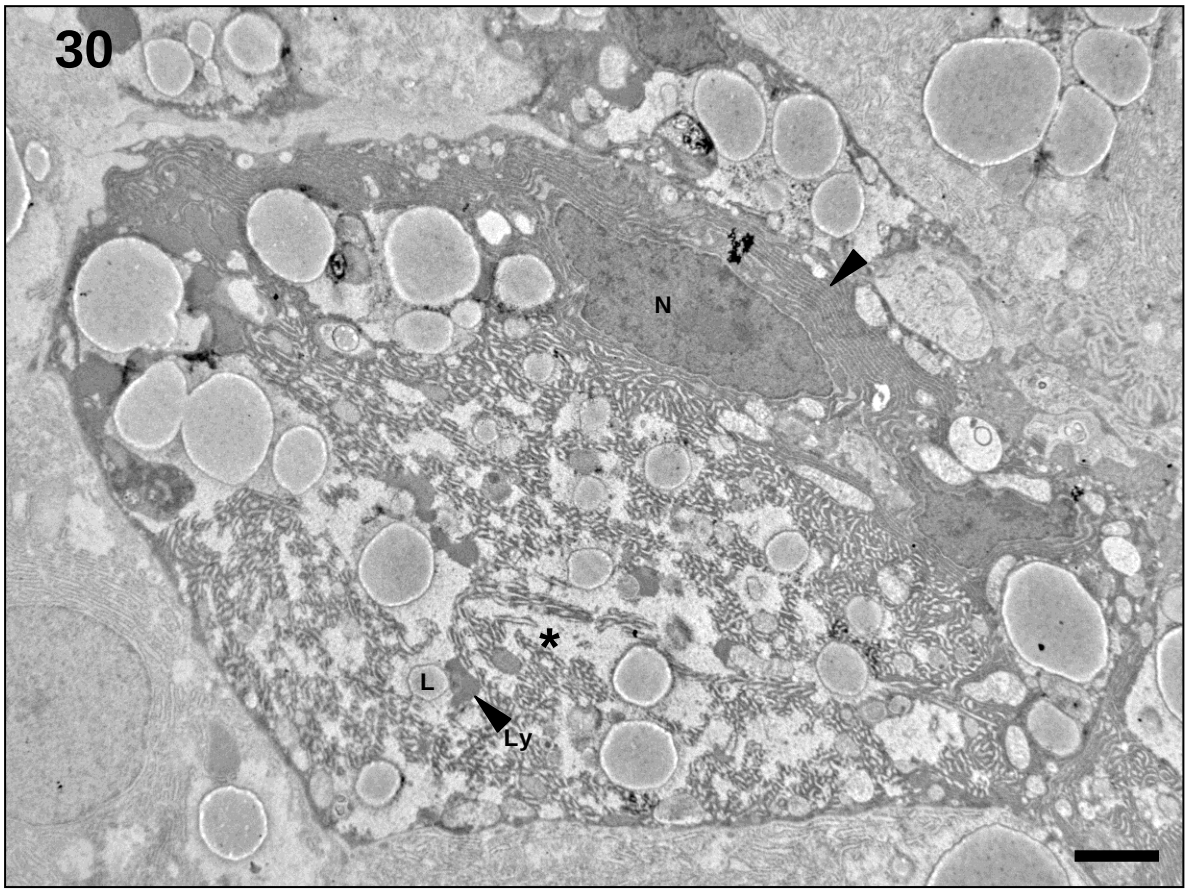


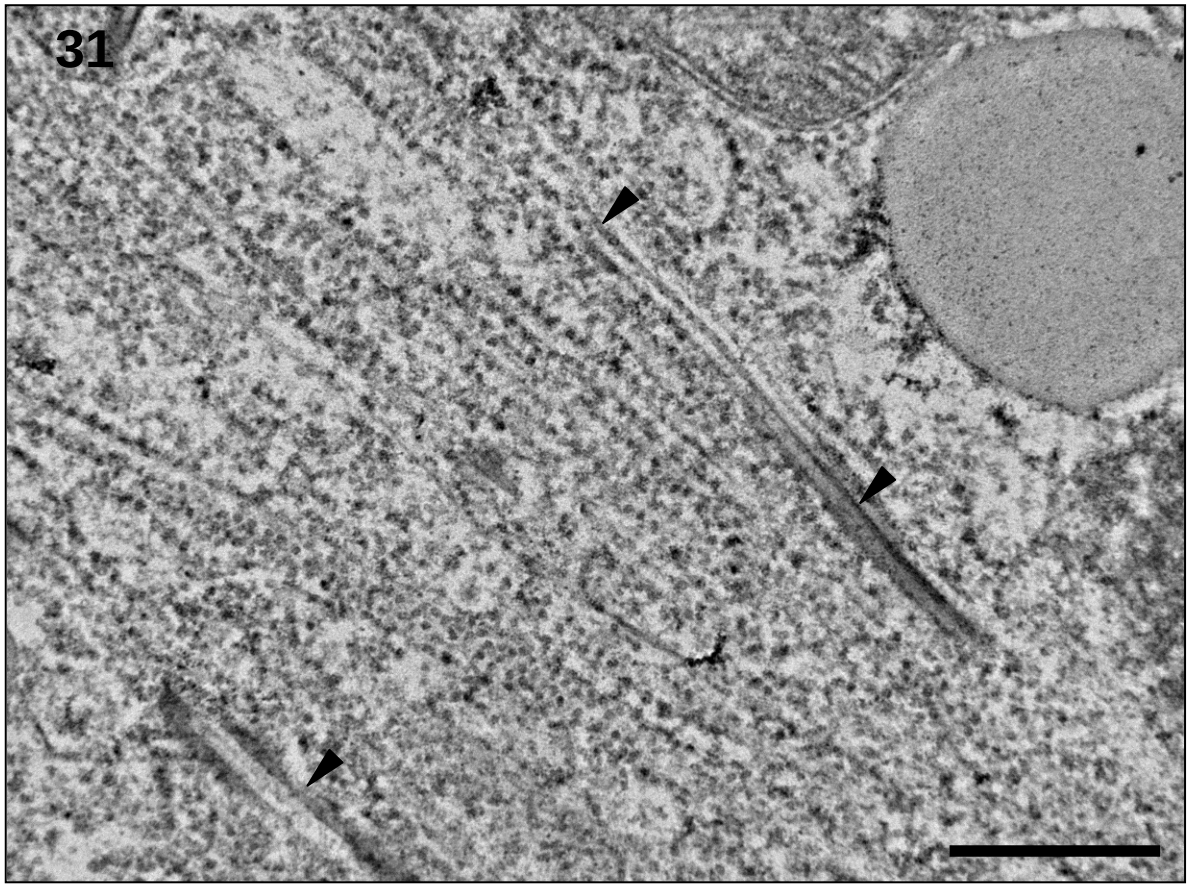
Figure 26: Detail of HFI revealed ribosomes (arrow) studded along full length of RER cisternae (Cs). Scale bar, 500 nm. **Figure 27:** Electron dense hepatocyte exhibiting HFI; affected nucleus with condensed chromatin (pyknosis) and irregular border. Note the adjacent hepatocytes (*) with decreased electron density. Scale bar, 2 µm. **Figure 28:** Hepatocyte exhibiting putative HFI in transverse cross section. Numerous autophagosomes (arrowhead) can be seen containing membranous material and immediate association with lysosomes (Ly). Mitochondria (M) appear swollen between RER cisternae. Pyknotic nucleus (N). Scale bar, 2 µm. **Figure 29:** High power view of electron dense areas reveal an increase in the frequency of free ribosomes (arrowhead) situated between RER cisternae. Scale bar, 500 nm. **Figure 30:** Hepatocytes occasionally demonstrated regions of both intact (arrowhead) and disintegrating RER (*) with “moth-eaten” appearance. Note the immediate association of lysosomes (Ly) with lipid (L). Scale bar, 2 µm.

programmes (Kirby *et al*., 2004). Four outliers were observed from the Mersey and Tyne (table 8) for which relative quantity of gene transcripts and HFI stage were determined. Overall, mean plasma VtG concentrations were ranked as follows: Tyne > Mersey > Alde ≈ Humber ≈ Medway ≈ Thames.

5.0 Discussion

Histological biomarkers of endocrine disruption in the aquatic environment have primarily been confined to observations of ovotestis in the gonads of male fish (Harries *et al*., 1999; Allen *et al*., 1999a; Jobling *et al*., 2002; Stentiford and Feist, 2005; Tyler and Jobling, 2008; Bizarro *et al*., 2013). This study investigated incidences of HFI as a potential histological biomarker of endocrine disruption in the liver of *P. flesus* using a multidisciplinary approach. Histological examinations revealed that the prevalence of HFI in *P. flesus* remains high in several UK estuaries, particularly the River Mersey and Tyne. Previous studies on diseases of flatfish have highlighted the importance of age as a potentially confounding factor in the understanding of chronic disease development (Vethaak *et al*., 1992; Vethaak and Jol, 1996; Stentiford *et al*., 2009; Stentiford *et al*., 2010).

This study investigated the effect of age on the prevalence of HFI for the first time. Overall, analysis of data did not demonstrate any correlation between age and the formation of HFI. The majority of *P. flesus* sampled during this study were aged between 2 and 4 years old. These ages also contained the largest number of *P. flesus* exhibiting HFI. The data shows that although the 2 to 4 year old age class possessed greater numbers of fish with HFI, all ages contained approximately equal proportions of affected and unaffected fish (figure 14b). Overall, closer inspection of data showed that the prevalence of HFI was approximately 40 % with the exception of 7 and 8 year olds (≈ 70 %). This apparent increase may result from a low sample number with only three fish caught within these older age groups. Carolla *et al.* (2013) showed that the smallest (25.3±5.1 cm) and largest (35.5±3.3 cm) *P. flesus* sampled from the Douro estuary, Portugal, exhibited a significantly lower prevalence of HFI. Length is commonly used as a surrogate for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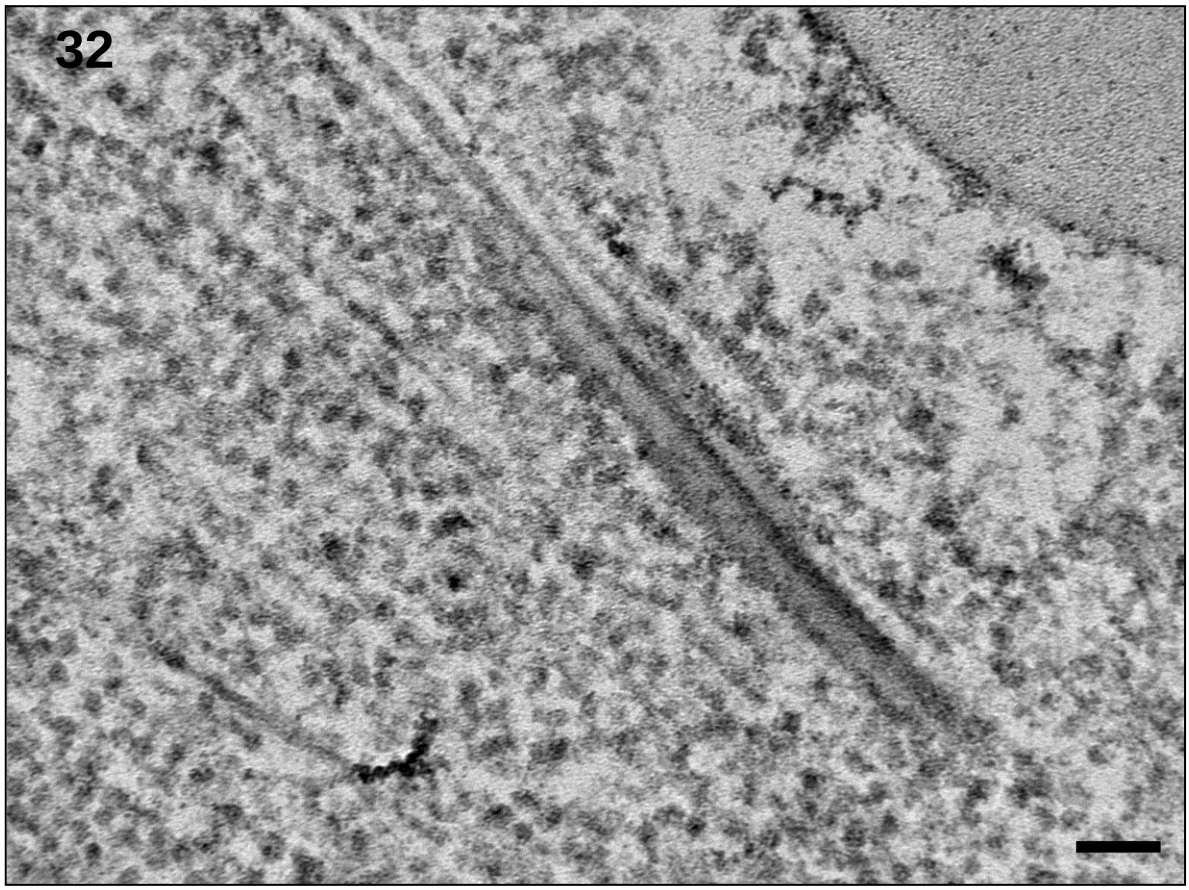
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33

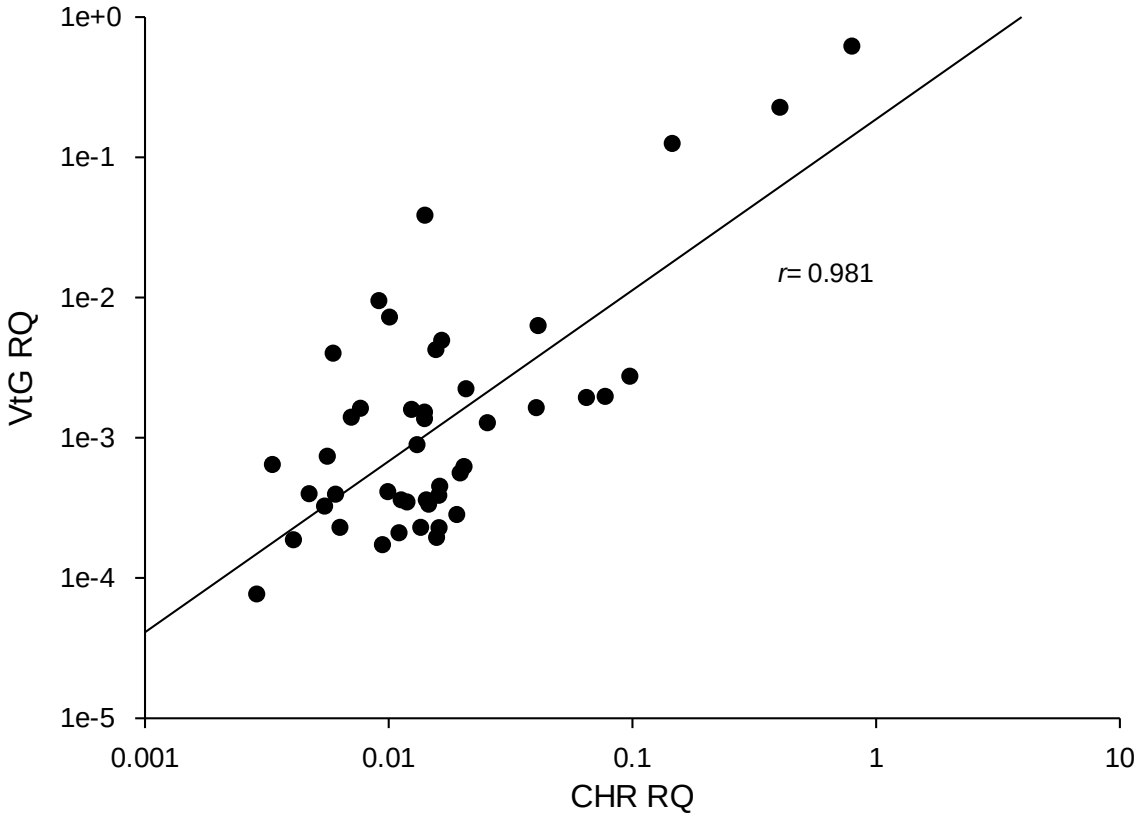
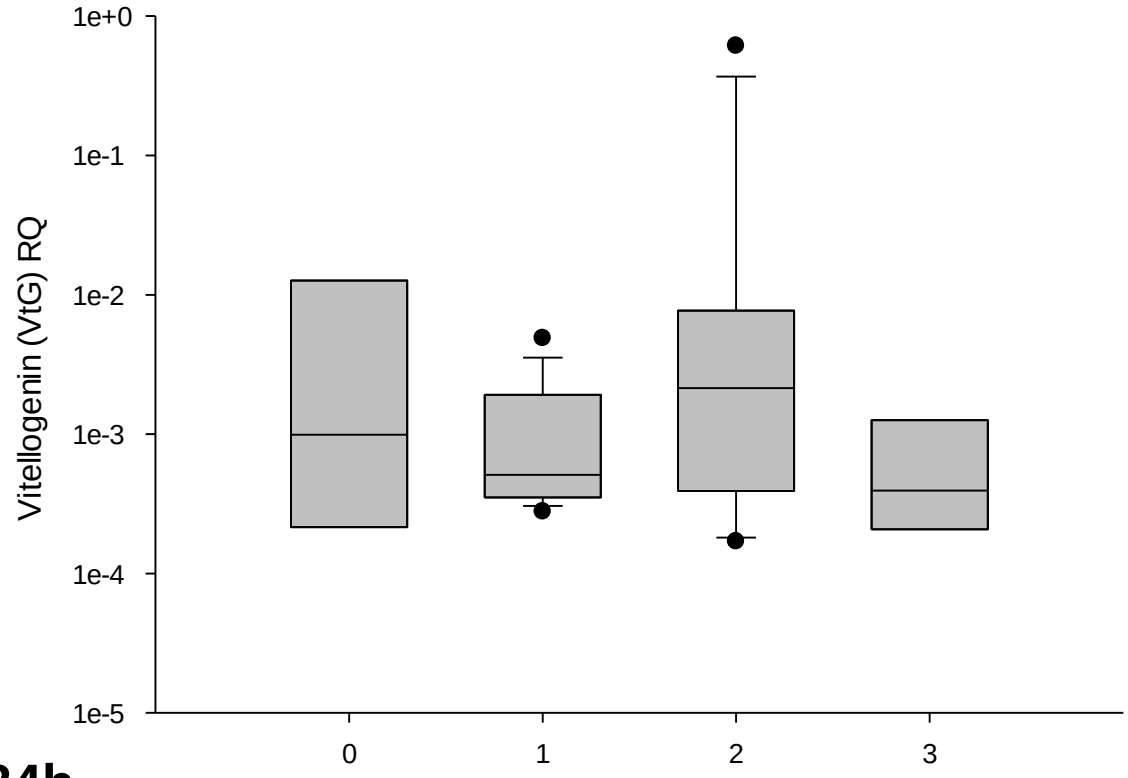
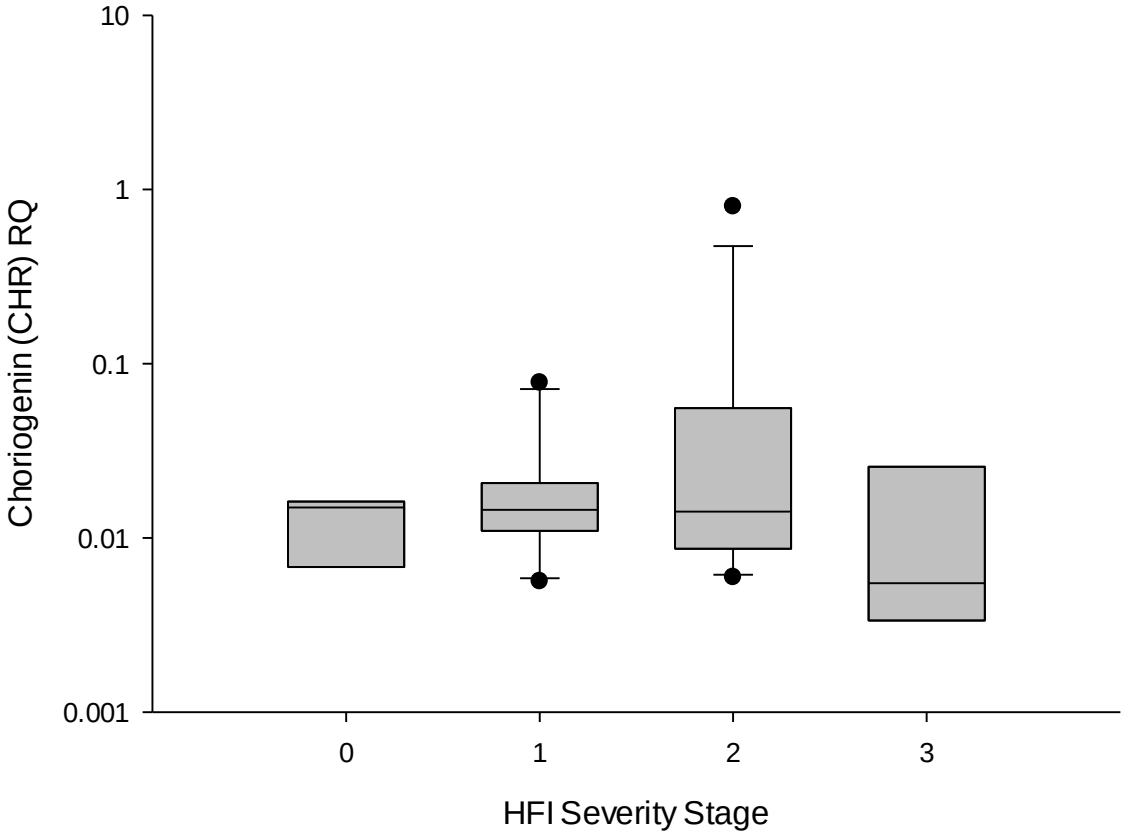


Figure 31: Hepatocyte exhibiting 'macrotubule' structures (arrow) as described by Kohler (1989). Tubules potentially traverse entire length of cisternae lumen, although disappear from plane of section in examples seen during this study. Scale bar, 500 nm. **Figure 32:** Close up of tubular structures situated within cisternae lumen. Scale bar, 100 nm. **Figure 33:** Correlation (r=0.981) between relative quantity of VtG and CHR gene transcripts in male *P. flesus* from the Tyne and Mersey.

34a


1e+0
1e-1
1e-2
1e-3
1e-4
1e-5
Vitellogenin (VtG) RQ
0
1
2
3

34b


10
1
0.1
0.01
0.001
Choriogenin (CHR) RQ
0
1
2
3
HFI Severity Stage

Estuary	n=	Mean plasma concentration ($\mu g\ ml^{-1}$)	Standard Deviation
Alde	27	0.73	1.592
Humber	29	0.28	0.420
Medway	38	0.20	0.002
Thames	22	0.20	0.001
Tyne	28	101.8	398.838
Mersey	29	23.7	124.860

Table 7: Mean plasma concentrations for male *P. flesus* from all estuaries sampled during this study.

VtG Conc. ($\mu g\ ml^{-1}$)	VtG mRNA (fold change*)	CHR mRNA (fold change*)	HFI Stage
Mersey			
8.7	0.124	0.147	2
672.9	0.6	0.8	2
Tyne			
897.9	0.038	0.014	0
1944.0	0.2	0.4	5

Table 8: Comparison of VtG plasma concentrations, VtG/CHR mRNA relative quantity and HFI severity stage between four *P. flesus* exhibiting elevated concentrations of plasma VtG. (*) Relative to female control.

age, although it is reported that length is not necessarily reliable for this purpose and varies considerably between geographical regions (Stentiford *et al*., 2010). The extrapolation of these lengths to our data shows that the smallest and largest Douro fish were potentially between 1 and 6 yrs old. Consequently, it is impossible to estimate and accurately compare the age of Douro *P. flesus* to our own data concerning the relationship between age and HFI prevalence. Based on our observations that HFI prevalence is approximately equal across all ages, we predict that HFI are likely to be an acute response and not a chronic pathological condition such as liver neoplasms reported in flatfish elsewhere (Myers *et al*., 1990; Myers *et al*., 1998; Stentiford *et al*., 2003; Stehr *et al*., 2004; Stentiford *et al*., 2009).

Stentiford *et al.*, (2003) observed seasonal differences of HFI prevalence with a higher prevalence observed during autumn compared to spring, suggesting a metabolic basis for their occurrence. *P. flesus* were also sampled during autumn in this current study; therefore the high prevalence we observed perhaps provides further evidence that season may affect incidences of HFI. A review of historical Cefas data could not determine seasonal influence on the development of HFI. This resulted from limited data availability within year for the majority of data, with the exception of the data reported by Stentiford *et al.*, (2003) i.e. one sampling event in any specified year (table 1). Stentiford et al., (2003) conducted sampling during spring and autumn within a single year thus allowing direct seasonal comparisons to be made within year. Historical Cefas data from the Mersey, Tyne and Alde estuaries demonstrated a decreasing trend in HFI prevalence during autumn of 2000, 2002 and 2009 (table 1). Attempts to conduct year-to-year seasonal comparisons using this data are therefore inappropriate. HFI prevalence was not statistically different between spring-summer and autumn-winter in *P. flesus* sampled from the Douro estuary (Carrola *et al*., 2013). *P. flesus* primarily reside within estuaries and begin annual migrations to open ocean spawning grounds during the autumn once sexual maturity is reached at 2-3 yrs of age (Summers, 1979). Spawning typically occurs between February and May, after which *P. flesus* return inshore to feed during the summer. If contaminants are

involved in the acute development of HFI, it is feasible that seasonal differences are related to migratory movements of *P. flesus*, with fish sampled during autumn-winter having already spent several months within an estuary, where contaminants are considered more concentrated. Conversely, spring-summer sampling events are likely to acquire fish that are returning (or just returned) to an estuary having spent several months at sea where concentrations of contaminants are reduced. Despite this, atypical migration patterns whereby *P. flesus* remain in brackish estuarine environments to spawn, are reported in some populations (Florin and Hoglund, 2008; Morais *et al*., 2011). The study of HFI in these and similar populations such as these may not reveal seasonal differences. An interesting relationship potentially exists concerning the behaviour of *P. flesus* in relation to sexual maturity, migratory patterns and potential exposure to estuarine contaminants. It is also important to consider environmental and potentially species specific biological factors that may influence HFI development. Ultrastructural changes in hepatocytes have been reported in winter flounder (*Pleuronectes americanus*) associated with the annual synthesis of antifreeze protein during winter (March and Reisman, 1995). This protein is circulated in body fluids and helps fish meet the physiological demands of cold temperatures through the prevention of epidermal ice crystal formation. Whilst HFI were not observed, it highlights a relatively unique function in this species. Indeed, HFI in *P. flesus* may correspond to a biological function concerning migrations from brackish estuaries into marine areas. Previous reports of seasonal differences (Stentiford *et al*., 2003) would also support this explanation, although comparisons to other migratory flounder species would be beneficial. On balance, HFI have been observed, albeit rarely, in the wholly marine flatfish *L. limanda* (Cefas, unpublished data). Furthermore, this study demonstrates relatively large differences in prevalence between estuaries during the same sampling period. As such, it is probable that other differential factors have a role in the development of HFI. Only through further study will the potential relationship between HFI and oestrogenic compounds be accurately determined.

The TEM examination confirmed previous reports that HFI are formed from proliferating RER exhibiting extensive disorganisation patterns. Köhler (1989; 1990) described the presence of enlarged microtubules (macrotubules) within the RER cisternae lumina of *P. flesus* sampled from the river Elbe, Germany, contaminated with heavy metals, PCBs, PAHs and pesticides. It was proposed that macrotubules were the product of the incorporation of tubulin subunits from the cytoplasmic pool into RER cisternae and their subsequent polymerisation within. The polymerisation of tubulin was believed to cause the disorganisation of the RER i.e. HFI. Depuration experiments showed that macrotubules depolymerised during liver regeneration and appeared to bring about a gradual transition of affected HFI into increasing quantities of smooth endoplasmic reticulum (SER). The author describes that macrotubules might act as binding and detoxifying sites for substances inducing the microsomal enzyme system located in the smooth endoplasmic reticulum. Despite extensive examination during our study, structures believed to be macrotubules were only observed in a single hepatocyte. Tannic acid treatment has previously been used to enhance staining of peptides and proteins including microtubules (Mizuhira and Futaesaku, 1971); and was successfully used by Köhler (1990) for the demonstration of macrotubules within RER cisternae. If macrotubules are indeed implicated in the formation HFI, it is possible that their apparent absence in our samples was the result of the non-treatment of liver samples with tannic acid (Köhler, 1989; Köhler, 1990).

The endoplasmic reticulum (ER) is highly dynamic and demonstrates fluctuating quantities depending on the metabolic state of the cell (Wolfe, 1993). Consequently, the proportion of free and membrane bound ribosomes varies considerably, with cells responsible for the synthesis and extracellular secretion of proteins exhibiting significant increases in the number of membrane bound ribosomes. Free ribosomes are primarily responsible for the synthesis of proteins destined for use within the cytoplasm. Ribosomes that are bound to the ER are responsible for the synthesis of proteins destined for use by cellular membranes, or enveloped within vesicles that are stored within the cytoplasm or transported to the plasma membrane for export from the cell. Proteins that are synthesised on ER

bound ribsosomes may pass directly through the cisternae membrane into the RER lumen where they are packaged into vesicles, released from the RER and transported to various cytoplasmic locations, via the Golgi complex; such as the lumen of other organelles or the plasma membrane. With this in mind, it is perhaps unclear how tubulin subunits are incorporated into the RER for the formation of macrotubules, although IHC was successfully used to demonstrate tubulin in macrotubules (unpublished data, see Köhler, 2004). In the present study, IHC incorporating a commercially available α/β- tubulin polyclonal primary antibody was used for the detection of microtubules and their association with HFI. Unfortunately, this study failed to identify microtubules. It is possible that despite confirmed antibody species reactivity for zebrafish (*Danio rerio*) the antibody does not exhibit cross reactivity for *P. flesus*, although α/β- tubulin genes are considered to be highly conserved between species (Wade, 2007). The role of tubulin in the formation of HFI in this study remains inconclusive, highlighting the need for further study.

We considered that HFI could be the result of abnormal vitellogenesis for the reasons that (1) HFI appear to be the result of proliferating RER ordinarily implicated in extensive protein synthesis and (2) HFI are prevalent in UK estuaries previously containing male *P. flesus* demonstrating vitellogenesis. IHC incorporating a polyclonal primary antibody for VtG provided valuable insight following the positive detection of VtG and an immediate association with HFI (figures 15b and 16). This indicates that HFI may somehow be involved during abnormal vitellogenin synthesis. In this study, positive and clearly defined VtG labelling of HFI was evident in all male and female *P. flesus* except for those sampled from the Mersey. This was unexpected because Mersey *P. flesus* exhibited the highest HFI prevalence in histological HE sections.. Instead, Mersey *P. flesus* exhibited low intensity positive staining for VtG with an opaque-like appearance (figure 20b). A potential explanation for this is over fixation resulting in cross linking of antigens and subsequent antigen masking. However, tissues from all sampling locations were treated identically by removing from fixative 48 h post sampling. This time period was not believed to have a detrimental effect on IHC

(Werner *et al*., 2000); nonetheless heat-induced epitope retrieval (HIER) was incorporated into the IHC protocol to ensure effective labelling. Wester *et al.* (1985) reported periodic acid Schiff (PAS) positive VtG staining in the liver of *Poecilia reticulata* following long term exposure to β-hexachlorocyclohexane. When the PAS technique was conducted on two histological sections of Mersey *P. Flesus,* demonstrating severe HFI, the technique revealed negative staining (apart from occasional disassociated staining of glycogen) within HFI. This suggested that absence of VtG labelling by IHC was not a fixation artefact. It is reasonable to propose that the presence of HFI may not exclusively represent concurrent protein synthesis. Instead, a transition period may exist prior to degradation of HFI via autophagy following a period of intense protein synthesis.

Positive VtG labelling of a substance appearing as cytoplasmic droplets was also observed within hepatocytes of male and female *P. flesus*. Direct comparisons between histological HE and corresponding IHC sections suggested this substance was VtG in origin (figures 10, 11 and 16). Similar eosinophilic substances have been observed in hepatocytes of fish exposed to oestrogenic compounds (Wester and Canton, 1986; Folmar *et al*., 2001; Zaroogian *et al*., 2001). Folmar *et al*. (2001) described the occurrence of high levels of a hyaline material accumulating in hepatocytes of male summer flounder (*Paralichthys dentatus*) following injection with 17 β-estradiol. Later, IHC confirmed this material to be VtG, although the same quantities of hyaline material observed in HE sections were not seen in IHC. This was believed to be a failure of the heterologous antibody to detect all VtG; or that the unreactive protein may have been choriogenic in origin. The accumulation of VtG in the cytoplasm of hepatocytes immediately following synthesis initially appears unlikely. Proteins, such as VtG, that are destined for use by tissues elsewhere, are ordinarily synthesised on ER bound ribosomes passing through the cisternae membrane and into the RER lumen (Wolfe, 1993). Here they are released from the RER within vesicles,and transported via the Golgi complex to the plasma membrane within secretory vesicles for extracellular export via exocytosis.

Zaroogian *et al.* (2001) proposed that the occurence of VtG within the cytoplasm of male *P. dentatus* hepatocytes was caused by the lack of oocytes to sequester the protein, resulting in an overwhelmed kidney in efforts to eliminate it. The authors predicted that glomerular damage may allow reabsorbtion of VtG into the circulatory system followed by transportation back to the liver and subsequent accumalation in hepatocellular lysosomes where it is structurally altered. This hypothesis is consistent with the findings of Folmar *et al.* (2001) regarding observations of unreactive protein within *P. dentatus* hepatocytes. In this study, we observed a lipid like substance (figure 23) in TEM sections of *P. flesus* hepatocytes, appearing to correspond to both an eosinophilc substance and VtG in HE and IHC sections respectively (figures 10 and 16). Close examination of TEM sections revealed that this material was not membrane bound, indicating it was not absorbed via endocytosis into the lysosomal compartment (Zaroogian *et al*., 2001). It is important to note that our observations and interpretations are based on the very few samples processed for TEM. Initially, representative samples of HFI were processed for TEM to confirm that HFI were comprised of RER. Consequently, we only identified material (both male and female) that was likely to yield good material for this purpose, with the significance of the lipid-like substance in the description of this pathology not being appreciated fully. The lipid-like substance observed in TEM, was only observed in hepatocytes of female *P. flesus*. Samples of male *P. flesus* processed for TEM did not exhibit the lipid-like material, nor was the eosinophilic substance/VtG observed in corresponding histological HE and IHC sections. As a result, this study is unable to effectively interpret the results in the context of the hypothesis of Zaroogian *et al.* (2001). A comprehensive ultrastructural analysis of larger numbers of male fish is required to complete our understanding of confirmed VtG in IHC sections and HFI formation as a whole.

This study utilised qPCR and ELISA for the detection of hepatic VtG/CHR gene transcripts and blood plasma VtG respectively in male *P. flesus*. *P. flesus* were sampled during the autumn, being likely to have resided in estuaries for several months prior to annual migrations of sexually mature adults to offshore spawning grounds. Their estuarine residence at this time suggests that transcription may

continue in the presence of biologically relevant concentrations of an oestrogenic compound in the marine environment. No significant differences were observed in VtG/CHR transcription levels between male biological groups from the Mersey and Tyne (figure 33). It is feasible that activation of VtG/CHR genes and transcription had already occurred prior to the formation of HFI, with the relatively short VtG mRNA half life of 3 days (Craft *et al*., 2004) resulting in the reduction of quantifiable transcripts. However, ELISA demonstrated low concentrations of plasma in the majority of fish and no statistical differences between biological groups. The mean plasma VtG concentrations of 23.7 and 101.8 µg ml^{-1} for the Mersey and Tyne respectively, result from two outliers at each estuary exhibiting relatively high concentrations of VtG (8.7 and 672.9 µg ml^{-1}; 897.9 and 1944.0 µg ml^{-1} respectively). VtG plasma concentrations in remaining male *P. flesus* from all estuaries were similar to baseline levels previously observed in *P. flesus* from the Alde estuary.

This observation is consistent with Kirby *et al.* (2004) who reported decreasing plasma VtG concentrations from the Mersey (19,226.2 < 3.5 µg ml^{-1}) and Tyne (448.3, 113.0 < 0.5 µg ml^{-1}) between 1996 and 2001. This observation accompanied by the comparatively high VtG half life of 13-15 days (Allen *et al*., 1999a; Craft *et al*., 2004) indicates significant improvements regarding the presence of oestrogenic compounds in those estuarine environment. Analysis of the four outliers (table 8) with high plasma VtG concentrations demonstrated no clear pattern and no insight into the potential relationship between gene transcription, vitellogenesis and HFI. Despite, relative lack of VtG measured by ELISA, positive IHC labelling of VtG and an immediate association with HFI was curious. The, IHC labelling was clearly defined with no background staining (figures 15b, 16, 17b, 18b, 19b, 20b) leaving little doubt that labelling was specific. A potential explanation for this observation is that VtG concentrations in plasma were below the minimum detection limit for ELISA (≤ 0.2 µg ml^{-1}) although were still at detectable levels within hepatocytes using IHC. Alternatively, extracellular export of VtG may have not yet begun, resulting in the detectable concentrations observed in this study. However, this does seem unlikely when considering the number of

samples analysed in total. With all things considered, the potential exists that positive VtG labelling of HFI is purely coincidental. Their development and vitellogenesis may be independent of each other, with disruption to the RER and subsequent HFI formation resulting from other toxicopathic mechanisms and not endocrine disruption. This is feasible considering that estuaries significantly impacted by industrial EDCs are also likely to contain other classes of contaminants. This study was unable to identify clear links between HFI and endocrine disruption. However, it has made several interesting observations in the context of potential endocrine disruption e.g. positive labelling of VtG within HFI of male fish; that warrant further investigation. It is hoped that the conclusions of this study can inform the design of future studies investigating this pathology.

6.0 Conclusions

This study has confirmed that HFI remain high in several UK estuaries and result from significant disorganisation and proliferation of the RER. The observation that approximately equal proportions of *P. flesus* of all ages are affected suggests that HFI are an acute pathological condition. Despite the lack of supporting molecular and plasma protein data, this study provided limited insight into the hypothesis that endocrine disruption is potentially implicated in the development of HFI. This is upheld by positive VtG labelling of HFI via IHC and observations that male and female are differentially affected, with male *P. flesus* exhibiting a higher prevalence of HFI compared to females. The lack of vitellogenesis induction in large numbers of male fish during this study hampered this investigation into the relationships between exposure, transcription, pathology, protein translation and VtG plasma concentrations. Further laboratory studies using controlled experimental conditions will improve the understanding concerning the pathogenesis of this condition and its application as a histological marker of endocrine disruption in future marine monitoring programmes.

7.0 APPENDICES

7.1 Annex 1: Histological processing protocol

	REAGENT	DURATION	TEMPERATURE
1.	70 % Industrial Methylated Spirits (IMS)	Until programme starts	Ambient
2.	90 % IMS	45 mins	Ambient
3.	Absolute IMS	45 mins	Ambient
4.	Absolute IMS	45 mins	Ambient
5.	Absolute IMS	45 mins	Ambient
6.	Absolute IMS	45 mins	Ambient
7.	Xylene	45 mins	Ambient
8.	Xylene	45 mins	Ambient
9.	Xylene	45 mins	Ambient
10.	Xylene	45 mins	Ambient
11.	Paraffin wax @ 60°C	45 mins	65°C
12.	Paraffin wax @ 60°C	45 mins	65°C
13.	Paraffin wax @ 60°C	45 mins	65°C
14.	Paraffin wax @ 60°C	45 mins	65°C
	Finish		

7.2 *Annex 2: Haemotoxylin and eosin (HE) staining protocol*

1) Places slides into xylene/xylene substitute to remove wax from slides for 3-5 minutes.
2) Repeat step 1 in fresh xylene/xylene substitute.
3) Place slides into 100% alcohol for 3-5 minutes to remove solvent from tissue (industrial methylated spirit can be used here)
4) Repeat step 3 in fresh 100% alcohol.
5) Place slides into running tap water for 5 minutes (a bowl set to overflow with running tap water is ideal).
6) Place slides into Haematoxylin staining solution for 3-5 minutes.
7) Place slides into running tap water to “blue” for 5-10 minutes.
8) Differentiate in acid-alcohol.
9) Place slides into running tap water for approximately 10 minutes to “blue”.
10) Check sample slide under a microscope for blue nuclei and clear cytoplasm. If over differentiated, repeat steps 6-9.
11) Place slides into eosin staining solution for 3 minutes.
12) Place slides into running tap water and rinse well for up to 30 seconds.
13) Place slides into 70% alcohol and rinse well for up to 1 minute.
14) Place slides into 100% alcohol for 3 minutes.
15) Repeat step 14 twice with fresh 100% alcohol.
16) Place slides into xylene/xylene substitute for 3-5 minutes.
17) Repeat step 16 twice with fresh xylene/xylene substitute.
18) If slides appear cloudy, repeat steps 14-17 with fresh solutions.
19) Mount in a suitable mountant such as DePeX and leave to dry before examination.

7.3 Annex 3: Immunohistochemistry protocol

SODIUM CITRATE BUFFER

Tri-sodium citrate dihydrate (2.94 g)
Distilled water (1000 ml)

Tween 20 (0.5 ml). Mix to dissolve, adjust pH to 6.0 with 1N HCl before adding Tween 20. Store this solution at room temperature for 3 months or at 4°C for longer storage.

10X TRIS BUFFERED SALINE (TBS)

Tris Base (61 g)
Sodium chloride (90 g)
Distilled water (1000 ml).

Mix to dissolve and adjust pH to 7.6 using concentrated HCl. Add 5 ml Tween 20. Dilute 1:10 with distilled water before use and adjust pH if necessary.

DIAMINOBENZEDENE (DAB) WORKING SOLUTION

Add 50µl of DAB chromogen to 1 ml of DAB substrate buffer.

Protocol

1. De-paraffinise sections in xylene/xylene substitute and re-hydrate through graded alcohols.
2. Rinse slides in running tap water for 5 minutes.
3. Perform antigen retrieval by placing slides into plastic coplin jar containing Sodium citrate buffer and place on steamer rack situated inside pressure cooker. Fill pressure cooker with water so it is at the same level as the level of buffer within the coplin jar once it is placed inside.
4. Place cooker on high heat until full pressure is reached.

5. Reduce temperature, whilst maintaining pressure, and leave on hotplate for 10 minutes.
6. Remove from hotplate and allow pressure cooker to vent in fume hood prior to opening.
7. Remove coplin jar from pressure cooker and replace buffer with warm tap water followed by cool tap water. This is to cool the slides gradually.
8. Block endogenous biotin activity incubating sections with 0.05% Avidin in TBS for 15 minutes. Rinse in water for 2x 5 minutes. Proceed by incubating slides with 0.05% Biotin in TBS for 15 minutes. Rinse in water for 2x 5 minutes.
9. Rinse slides in TBS for 2x 5 minutes.
10. Incubate slides with Protein Block serum for 5 minutes.
11. Rinse slides in TBS for 2x 5 minutes.
12. Incubate with optimally diluted primary antibody (anti-flounder VtG) at 37°C for 60 minutes with gentle orbital rotation.
13. Rinse slides in TBS for 2x 5 minutes.
14. Incubate with biotinylated secondary antibody at room temperature for 60 minutes with gentle orbital rotation.
15. Rinse slides in TBS for 2x 5 minutes.
16. Quench endogenous peroxidase activity by incubating slides with peroxidase blocking reagent (0.3% Hydrogen peroxide in TBS) for 10 minutes at room temperature.
17. Rinse slides in TBS for 2x 5 minutes.
18. Incubate with Streptavidin detection complex at 25°C for 30 minutes with gentle orbital rotation.
19. Rinse slides in TBS for 2x 5 minutes.
20. Incubate with diaminobenzedene (DAB) chromogen working solution for 5 minutes to develop staining. This can be monitored using an inverted microscope to determine optimum time and reduce background staining. The reaction is stopped by immersing slides in water.

21. Perform chromogenic enhancement by placing slides into 0.5% Copper sulfate in PBS for 1-5 minutes at 25°C with gentle orbital rotation. Rinse in distilled water. This step is optional.
22. Counterstain with Harris's haematoxylin for 2 minutes.
23. Rinse slides in water for 5 minutes.
24. Dehydrate, clear and mount.

7.4 Annex 4: RNA extraction

RNA extraction was achieved by using the GenElute Mammalian Total RNA Miniprep Kit (Sigma, Poole, UK). Lysis buffer was initially prepared by transferring 10 µL of 2-mercatoethanol into 1 mL of extraction buffer. For each sample, approximately 40 mg of snap frozen liver was placed into lysis tubes containing glass beads and homogenised for 40 seconds at 6.5 cycles using a fast prep. Homogenised contents were carefully transferred into a filtration column and centrifuged for 2 minutes @ 12-16k rpm. Extraction proceeded by adding 500 µL of 70 % ethanol to filtrate and transferring 700 µL of solution to binding column. Binding column was centrifuged for 15 seconds at maximum centrifugation speed. 500 µL of Wash Buffer 1 was transferred to binding column and was centrifuged for a further 15 seconds at maximum centrifugation speed. Following these steps, 500 µL of Wash Buffer 2 was transferred to binding column and centrifuged for 15 seconds at maximum centrifugation speed. A further wash of the binding column with Wash Buffer 2 was carried out and centrifuged at maximum centrifugation speed for 2 minutes. 50 µL of elution buffer was added to binding column to elute extracted RNA. After 1 minute, binding column was placed in centrifuge for 1 minute at maximum centrifugation speed to collect extracted RNA. RNA concentration was quantified using a Nanodrop (Applied Biosystems) spectrophotometer calibrated with elution buffer.

8.0 References

Agius, L. 1996. The hepatocyte cytoskeleton. Biochemical, physiological, and pathological aspects. In *The Cytoskeleton,* Volume 3. Hesketh, J.E., Pryme, I.F. (eds), pp 71 - 106. JAI Press, Greenwich.

Allen, Y., Matthiessen, P., Scott, A. P., Haworth, S., Feist, S. and Thain, J. E. 1999a. The extent of oestrogenic contamination in the UK estuarine and marine environments - further surveys of flounder. *Science of the Total Environment*, 233, 5 - 20.

Allen, Y., Scott, A. P., Matthiessen, P., Haworth, S., Thain, J. E. and Feist, S. 1999b. Survey of estrogenic activity in United Kingdom estuarine and coastal waters and its effects on gonadal development of the flounder Platichthys flesus. *Environmental Toxicology and Chemistry*, 18, 1791 - 1800.

Arukwe, A. 2001. Cellular and molecular responses to endocrine-modulators and the impact on fish reproduction. *Marine Pollution Bulletin*, 42, 643 - 655.

Arukwe, A. and Goksoyr, A. 1998. Xenobiotics, xenoestrogens and reproduction disturbances in fish. *Sarsia*, 83, 225 - 241.

Bizarro, C., Ros, O., Vallejo, A., Prieto, A., Etxebarria, N., Cajaraville, M. P. and Ortiz-Zarragoitia, M. 2013. Intersex condition and molecular markers of endocrine disruption in relation with burdens of emerging pollutants in thicklip grey mullets (*Chelon labrosus*) from Basque estuaries (South-East Bay of Biscay). *Marine Environmental Research*,

Bozinovic, G. and Oleksiak, M. F. 2011. Genomic Approaches with Natural Fish Populations from Polluted Environments. *Environmental Toxicology and Chemistry*, 30, 283 - 289.

Carrola, J., Fontaínhas-Fernandes, A., Pires, M. J. and Rocha, E. 2013. Frequency of hepatocellular fibrillar inclusions in European flounder (*Platichthys flesus*) from the Douro River estuary, Portugal. *Environmental Science and Pollution Research*, 1 - 10.

Cerdá, J. L., Petrino, T. R., & Wallace, R. A. 1993. Functional Heterologous Gap Junctions in *Fundulus* Ovarian Follicles Maintain Meiotic Arrest and Permit Hydration during Oocyte Maturation. *Developmental biology*, 160(1), 228-235.

Chow, W. S., Chan, W. K.-L. and Chan, K. M. 2013. Toxicity assessment and vitellogenin expression in zebrafish (*Danio rerio*) embryos and larvae acutely exposed to bisphenol A, endosulfan, heptachlor, methoxychlor and tetrabromobisphenol A. *Journal of Applied Toxicology*, 33, 670 - 678.

Colborn, T., Dumanoski, D. and Myers, J. P. 1996. Our stolen future: are we threatening our fertility, intelligence, and survival?: a scientific detective story., Abacus, London. 306 p.

Craft, J. A., Brown, M., Dempsey, K., Francey, J., Kirby, M. F., Scott, A. P., Katsiadaki, I., Robinson, C. D., Davies, I. M. and Bradac, P. 2004. Kinetics of vitellogenin protein and mRNA induction and depuration in fish following laboratory and environmental exposure to oestrogens. *Marine Environmental Research*, 58, 419 - 423.

Davies, I. M. and Vethaak, D. 2012. Integrated marine environmental monitoring of chemicals and their effects. *ICES Cooperative Research Report* No. 315. 277 p.

De Metrio, G., Corriero, A., Desantis, S., Zubani, D., Cirillo, F., Deflorio, M., Bridges, C. R., Eicker, J., De La Serna, J. M., Megalofonou, P. and Kime, D. E. 2003. Evidence of a high percentage of intersex in the Mediterranean swordfish (*Xiphias gladius L*.). *Marine Pollution Bulletin*, 46, 358 - 361.

Devlin, R. H. and Nagahama, Y. 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture*, 208, 191 - 364.

Easey, W. and Millner, R. S. 2008. Improved methods for the preparation and staining of thin sections of fish otoliths for age determination. Sci. Ser. Tech Rep., Cefas Lowestoft, 143. 12pp.

Evrard, E., Marchand, J., Theron, M., Pichavant-Rafini, K., Durand, G., Quiniou, L. and Laroche, J. 2010a. Impacts of mixtures of herbicides on molecular and physiological responses of the European flounder Platichthys flesus. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, 152, 321 - 331.

Evrard, E., Devaux, A., Bony, S., Burgeot, T., Riso, R., Budzinski, H., Le Du, M., Quiniou, L. and Laroche, J. 2010b. Responses of the European flounder *Platichthys flesus* to the chemical stress in estuaries: load of contaminants, gene expression, cellular impact and growth rate. *Biomarkers*, 15, 111 - 127.

Feist, S. W., Lang, T., Stentiford, G. D. and Köhler, A. 2004. Biological effects of contaminants: Use of liver pathology of the European flatfish dab (*Limanda limanda L*.) and flounder (*Platichthys flesus L*.) for monitoring. *ICES techniques in marine environmental sciences*, 1 - 43.

Ferreira, F., Monteiro, N., Vieira, M., Reis-Henriques, M., Castro, L. and Santos, M. 2013. A real-time PCR assay for differential expression of vitellogenin I and II genes in the liver of the sentinel fish species *Lipophrys pholis*. *Toxicology Mechanisms and Methods*, 23, 591 - 597.

Florin, A. B. and Hoglund, J. 2008. Population structure of flounder (*Platichthys flesus*) in the Baltic Sea: differences among demersal and pelagic spawners. *Heredity*, 101, 27 - 38.

Folmar, L. C., Denslow, N. D., Rao, V., Chow, M., Crain, D. A., Enblom, J., Marcino, J. and Guillette, L. J. 1996. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. *Environmental Health Perspectives*, 104, 1096 - 1101.

Folmar, L. C., Gardner, G. R., Schreibman, M. P., Magliulo-Cepriano, L., Mills, L. J., Zaroogian, G., Gutjahr-Gobell, R., Haebler, R., Horowitz, D. B. and Denslow, N. D. 2001. Vitellogenin-induced pathology in male summer flounder (*Paralichthys dentatus*). Aquatic *Toxicology*, 51, 431 - 441.

Ford, A. T., Martins, I. and Fernandes, T. F. 2007. Population level effects of intersexuality in the marine environment. *Science of the Total Environment*, 374, 102 - 111.

Gagne, F., Smyth, S., Andre, C., Douville, M., Gelinas, M. and Barclay, K. 2013. Stress-related gene expression changes in rainbow trout hepatocytes exposed to various municipal wastewater treatment influents and effluents. *Environmental Science and Pollution Research*, 20, 1706 - 1718.

George, S., Gubbins, M., MacIntosh, A., Reynolds, W., Sabine, V., Scott, A. and Thain, J. 2004. A comparison of pollutant biomarker responses with transcriptional responses in European flounders (*Platichthys flesus*) subjected to estuarine pollution. *Marine Environmental Research*, 58, 571 - 575.

Gercken, J. and Sordyl, H. 2002. Intersex in feral marine and freshwater fish from northeastern Germany. *Marine Environmental Research*, 54, 651 - 655.

Greene, R. R., Burrill, M. W. and Ivy, A. C. 1938. Experimental intersexuality: The production of feminized male rats by antenatal treatment with estrogens. *Science*, 88, 130 - 131.

Harries, J. E., Janbakhsh, A., Jobling, S., Matthiessen, P., Sumpter, J. P. and Tyler, C. R. 1999. Estrogenic potency of effluent from two sewage treatment works in the United Kingdom. *Environmental Toxicology and Chemistry*, 18, 932 - 937.

Herbst, A. L., Ulfelder, H. and Poskanzer, D. C. 1971. Adenocarcinoma of the vagina: association of maternal stilbestrol therapy with tumor appearance in young women. *New England journal of medicine*, 284, 878 - 881.

Hinton, D. E. and Couch, J. A. 1998. Architectural pattern, tissue and cellular morphology in livers of fishes: relationship to experimentally-induced neoplastic responses. In *Fish Ecotoxicology*. Braunbeck, T., Hinton, D.E., Streit, B. (eds), pp 141-64. Birkhäuser Verlag, Basel, Switzerland.

Hinton, D. E., Segner, H., Au, D. W., Kullman, S. W. and Hardman, R. C. 2008. Liver toxicity. In *The Toxicology of Fishes*. Giulo, R.T., Hinton, D.E. (eds), pp. 327 - 400. CRC Press, Boca Raton.

Hinton, D. E., Segner, H. and Braunbeck, T. 2001. Toxic responses of the liver. In *Target Organ Toxicity in Marine and Freshwater teleosts*, Volume 1. Schlenk, D., Benson W.H. (eds), pp. 225 - 266. CRC Press, Boca Raton.

Janz, D.M. 2000. Endocrine System. In *The Laboratory Fish*. Ostrander, G. K. (ed), 189-217. Academic Press, London.

Jobling, S., Coey, S., Whitmore, J. G., Kime, D. E., Van Look, K. J. W., McAllister, B. G., Beresford, N., Henshaw, A. C., Brighty, G., Tyler, C. R. and Sumpter, J. P. 2002. Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Biology of Reproduction*, 67, 515 - 524.

Kime, D. E., Nash, J. P. and Scott, A. P. 1999. Vitellogenesis as a biomarker of reproductive disruption by xenobiotics. *Aquaculture*, 177, 345 - 352.

Kime, D. E. 1998. Endocrine disruption in fish. Kluwer Academic Publishers, Norwell, Massachusetts. 396 p.

Kirby, M., Smith, A., Barry, J., Katsiadaki, I., Lyons, B. and Scott, A. 2006. Differential sensitivity of flounder (*Platichthys flesus*) in response to oestrogenic chemical exposure: An issue for design and interpretation of monitoring and research programmes. *Marine Environmental Research*, 62, 315 - 325.

Kirby, M. F., Allen, Y. T., Dyer, R. A., Feist, S. W., Katsiadaki, I., Matthiessen, P., Scott, A. P., Smith, A., Stentiford, G. D., Thain, J. E., Thomas, K. V., Tolhurst, L. and Waldock, M. J. 2004. Surveys of plasma vitellogenin and intersex in male flounder (*Platichthys flesus*) as measures of endocrine disruption by estrogenic contamination in United Kingdom estuaries: Temporal trends, 1996 to 2001. *Environmental Toxicology and Chemistry*, 23, 748 - 758.

Kleinkauf, A., Scott, A. P., Stewart, C., Simpson, M. G. and Leah, R. T. 2004. Abnormally elevated VTG concentrations in flounder (*Platichthys flesus*) from the Mersey Estuary (UK) - A continuing problem. *Ecotoxicology and Environmental Safety*, 58, 356 - 364.

Köhler, A. 1989. Regeneration of contaminant-induced liver lesions in flounder - experimental studies towards the identification of cause-effect relationships. *Aquatic Toxicology*, 14, 203 - 232.

Köhler, A. 1990. Identification of contaminant-induced cellular and subcellular lesions in the liver of flounder (*Platichthys flesus L*.) caught at differently polluted estuaries. *Aquatic Toxicology*, 16, 271 - 293.

Kubista, M., Andrade, J. M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B. and Strömbom, L. 2006. The real-time polymerase chain reaction. *Molecular aspects of medicine*, 27, 95 - 125.

Lang, T., Wosniok, W., Barsiene, J., Broeg, K., Kopecka, J. and Parkkonen, J. 2006. Liver histopathology in Baltic flounder (Platichthys flesus) as indicator of biological effects of contaminants. *Marine Pollution Bulletin*, 53, 488 - 496.

Lange, A., Katsu, Y., Miyagawa, S., Ogino, Y., Urushitani, H., Kobayashi, T., Hirai, T., Shears, J. A., Nagae, M., Yamamoto, J., Ohnishi, Y., Oka, T., Tatarazako, N., Ohta, Y., Tyler, C. R. and Iguchi, T. 2012. Comparative responsiveness to natural and synthetic estrogens of fish species commonly used in the laboratory and field monitoring. *Aquatic Toxicology*, 109, 250 - 258.

Larkin, P., Knoebl, I. and Denslow, N. D. 2003. Differential gene expression analysis in fish exposed to endocrine disrupting compounds. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology*, 136, 149 - 161.

Lee, C., Na, J. G., Lee, K. C. and Park, K. 2002. Choriogenin mRNA induction in male medaka, *Oryzias latipes* as a biomarker of endocrine disruption. *Aquatic Toxicology*, 61, 233 - 241.

Liney, K. A., Jobling, S., Sheers, J. A., Simpson, P. and Tyler, C. R. 2005. Assessing the sensitivity of different life stages for sexual disruption in roach (*Rutilus rutilus*) exposed to effluents from wastewater treatment works. *Environmental Health Perspectives*, 113, 1299 - 1307.

Lyons, B. P., Stentiford, G. D., Green, M., Bignell, J., Bateman, K., Feist, S. W., Goodsir, F., Reynolds, W. J. and Thain, J. E. 2004. DNA adduct analysis and histopathological biomarkers in European flounder (*Platichthys flesus*) sampled from UK estuaries. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, 552, 177 - 186.

March, P. E. and Reisman, H. M. 1995. Seasonal-Changes in Hepatocyte Ultrastructure Correlated with the Cyclic Synthesis of Secretory Proteins in the

Winter Flounder (*Pleuronectes-Americanus*). *Cell and Tissue Research*, 281, 153 - 161.

Marchand, J., Tanguy, A., Charrier, G., Quiniou, L., Plee-Gauthier, E. and Laroche, J. 2006. Molecular identification and expression of differentially regulated genes of the european flounder, *Platichthys flesus*, submitted to pesticide exposure. *Marine Biotechnology*, 8, 275 - 294.

Matthiessen, P., Waldock, R., Thain, J. E., Waite, M. E. and Scropehowe, S. 1995. Changes in Periwinkle (*Littorina-Littorea*) Populations Following the Ban on TBT-Based Antifoulings on Small Boats in the United-Kingdom. *Ecotoxicology and Environmental Safety*, 30, 180 - 194.

Maurice, M., Feldmann, G., Bellon, B. and Druet, P. 1980. Increase in polymerized liver tubulin during stimulation of hepatic plasma protein secretion in the rat. *Biochemical and biophysical research communications*, 97, 355 - 363.

Mills, L. J. and Chichester, C. 2005. Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? *Science of the Total Environment*, 343, 1 - 34.

Mizuhira, V. and Futaesaku, Y. 1971. On the new approach of tannic acid and digitonine to the biological fixatives. *Proceedings of the Electron Microscopy Society of America*, 29, 494 - 495.

Moffatt, L. T., May, C. L., Studer, K. E., Reckhow, D. A. and Arcaro, K. F. 2010. Effects of exposure water volume, depuration time, and feeding status on vitellogenin mRNA induction in male medaka (*Oryzias latipes*) exposed to 17 beta-estradiol. *Ecotoxicology and Environmental Safety*, 73, 1835 - 1841.

Morais, P., Dias, E., Babaluk, J. and Antunes, C. 2011. The migration patterns of the European flounder *Platichthys flesus* (Linnaeus, 1758) (Pleuronectidae, Pisces)

at the southern limit of its distribution range: Ecological implications and fishery management. *Journal of Sea Research*, 65, 235 - 246.

Myers, M. S., Johnson, L. L., Hom, T., Collier, T. K., Stein, J. E. and Varanasi, U. 1998. Toxicopathic hepatic lesions in subadult English sole (*Pleuronectes vetulus*) from Puget Sound, Washington, USA: Relationships with other biomarkers of contaminant exposure. *Marine Environmental Research*, 45, 47 - 67.

Myers, M. S., Landahl, J. T., Krahn, M. M., Johnson, L. L. and Mccain, B. B. 1990. Overview of studies on Liver carcinogenesis in English sole from Puget Sound; evidence for a xenobiotic chemical etiology I: Pathology and epizootiology. *Science of the Total Environment*, 94, 33 - 50.

Nagahama, Y. 1994. Endocrine Regulation of Gametogenesis in Fish. *International Journal of Developmental Biology*, 38, 217 - 229.

Nogueira, P., Pacheco, M., Pereira, M., Mendo, S. and Rotchell, J. M. 2010. Anchoring novel molecular biomarker responses to traditional responses in fish exposed to environmental contamination. *Environmental Pollution*, 158, 1783 - 1790.

Oberdörster, E. and Cheek, A. O. 2001. Gender benders at the beach: Endocrine disruption in marine and estuarine organisms. *Environmental Toxicology and Chemistry*, 20, 23 - 36.

Pait, A. S. and Nelson, J. O. 2009. A survey of indicators for reproductive endocrine disruption in Fundulus heteroclitus (killifish) at selected sites in the Chesapeake Bay. *Marine Environmental Research*, 68, 170 - 177.

Pal, S., Kokushi, E., Cheikyula, J. O., Koyama, J. and Uno, S. 2011. Histopathological effects and EROD induction in common carp exposed to dietary heavy oil. *Ecotoxicology and Environmental Safety*, 74, 307 - 314.

Pettersson, M., Hahlbeck, E., Katsiadaki, I., Asplund, L. and Bengtsson, B. E. 2007. Survey of estrogenic and androgenic disruption in Swedish coastal waters by the analysis of bile fluid from perch and biomarkers in the three-spined stickleback. *Marine Pollution Bulletin*, 54, 1868 - 1880.

Pfaffl, M. W., Horgan, G. W. and Dempfle, L. 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research*, 30,

Purdom, C. E., Hardiman, P. A., Bye, V. J., Eno, N. C., Tyler, C. R. and Sumpter, J. P. 1994. Estrogenic effects of effluents from sewage treatment works. *Chemistry and Ecology*, 8, 275 - 285.

Puy-Azurmendi, E., Ortiz-Zarragoitia, M., Villagrasa, M., Kuster, M., Aragón, P., Atienza, J., Puchades, R., Maquieira, A., Domínguez, C., López de Alda, M., Fernandes, D., Porte, C., Bayona, J. M., Barceló, D. and Cajaraville, M. P. 2013. Endocrine disruption in thicklip grey mullet (*Chelon labrosus*) from the Urdaibai Biosphere Reserve (Bay of Biscay, Southwestern Europe). *Science of the Total Environment*, 443, 233 - 244.

R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Raut, S. A. and Angus, R. A. 2010. Triclosan Has Endocrine-Disrupting Effects in Male Western Mosquitofish, *Gambusia Affinis*. *Environmental Toxicology and Chemistry*, 29, 1287 - 1291.

Robertson, L. S. and McCormick, S. D. 2012. The effect of nonylphenol on gene expression in Atlantic salmon smolts. *Aquatic Toxicology*, 122, 36 - 43.

Rodas-Ortiz, J. P., Ceja-Moreno, V., Chan-Cocom, M. and Gold-Bouchot, G. 2008. Vitellogenin Induction and Increased Plasma 17 beta-Estradiol

Concentrations in Male Nile Tilapia, *Oreochromis niloticus*, Exposed to Organochlorine Pollutants and Polycyclic Aromatics Hydrocarbons. *Bulletin of Environmental Contamination and Toxicology*, 81, 543 - 547.

Rotchell, J. M. and Ostrander, G. K. 2003. Molecular markers of endocrine disruption in aquatic organisms. *Journal of Toxicology and Environmental Health - Part B: Critical Reviews*, 6, 453 - 495.

Ruijter, J. M., Ramakers, C., Hoogaars, W. M. H., Karlen, Y., Bakker, O., van den Hoff, M. J. B. and Moorman, A. F. M. 2009. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Research*, 37,

Santos, E. M., Ball, J. S., Williams, T. D., Wu, H., Ortega, F., Van Aerle, R., Katsiadaki, I., Falciani, F., Viant, M. R., Chipman, J. K. and Tyler, C. R. 2010. Identifying Health Impacts of Exposure to Copper Using Transcriptomics and Metabolomics in a Fish Model. *Environmental Science & Technology*, 44, 820 - 826.

Scholz, S., Kordes, C., Hamann, J. and Gutzeit, H. O. 2004. Induction of vitellogenin in vivo and in vitro in the model teleost medaka (*Oryzias latipes*): comparison of gene expression and protein levels. *Marine Environmental Research*, 57, 235 - 244.

Scholz, S. and Mayer, I. 2008. Molecular biomarkers of endocrine disruption in small model fish. *Molecular and Cellular Endocrinology*, 293, 57 - 70.

Scott, A. P., Katsiadaki, I., Kirby, M. F. and Thain, J. 2006a. Relationship between sex steroid and vitellogenin concentrations in flounder (*Platichthys flesus*) sampled from an estuary contaminated with estrogenic endocrine-disrupting compounds. *Environmental Health Perspectives*, 114, 27 - 31.

Scott, A. P., Katsiadaki, I., Witthames, P. R., Hylland, K., Davies, I. M., McIntosh, A. D. and Thain, J. 2006b. Vitellogenin in the blood plasma of male cod (*Gadus morhua*): A sign of oestrogenic endocrine disruption in the open sea? *Marine Environmental Research*, 61, 149 - 170.

Scott, A. P., Sanders, M., Stentiford, G. D., Reese, R. A. and Katsiadaki, I. 2007. Evidence for estrogenic endocrine disruption in an offshore flatfish, the dab (*Limanda limanda L*.). *Marine Environmental Research*, 64, 128 - 148.

Simpson, M. G., Parry, M., Kleinkauf, A., Swarbreck, D., Walker, P. and Leah, R. T. 2000. Pathology of the liver, kidney and gonad of flounder (*Platichthys flesus*) from a UK estuary impacted by endocrine disrupting chemicals. *Marine Environmental Research*, 50, 283 - 287.

Stehr, C. M., Myers, M. S., Johnson, L. L., Spencer, S. and Stein, J. E. 2004. Toxicopathic liver lesions in English sole and chemical contaminant exposure in Vancouver Harbour, Canada. *Marine Environmental Research*, 57, 55 - 74.

Stentiford, G. D., Bignell, J. P., Lyons, B. P. and Feist, S. W. 2009. Site-specific disease profiles in fish and their use in environmental monitoring. *Marine Ecology Progress Series*, 381, 1 - 15.

Stentiford, G. D., Bignell, J. P., Lyons, B. P., Thain, J. E. and Feist, S. W. 2010. Effect of age on liver pathology and other diseases in flatfish: implications for assessment of marine ecological health status. *Marine Ecology Progress Series*, 411, 215 - 230.

Stentiford, G. D. and Feist, S. W. 2005. First reported cases of intersex (ovotestis) in the flatfish species dab *Limanda limanda*: Dogger Bank, North Sea. *Marine Ecology Progress Series*, 301, 307 - 310.

Stentiford, G. D., Longshaw, M., Lyons, B. P., Jones, G., Green, M. and Feist, S. W. 2003. Histopathological biomarkers in estuarine fish species for the

assessment of biological effects of contaminants. *Marine Environmental Research*, 55, 137 - 159.

Summers, R. W. 1979. Life cycle and population ecology of the flounder *Platichthys flesus* (L.) in the Ythan estuary, Scotland. *Journal of Natural History*, 13, 703 - 723.

Sumpter, J. P. and Jobling, S. 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environmental Health Perspectives*, 103, 173 - 178.

Thomas, P. 2008. The endocrine system. In *The Toxicology of Fishes*. Giulo, R.T., Hinton, D.E. (eds), pp. 457 - 488. CRC Press, Boca Raton.

Tian, H., Ru, S., Wang, Z., Cai, W. and Wang, W. 2009. Estrogenic effects of monocrotophos evaluated by vitellogenin mRNA and protein induction in male goldfish (*Carassius auratus*). *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology*, 150, 231 - 236.

Truter, J. C., van Wyk, J. H., Oberholster, P. J. and Botha, A. M. 2014. The impacts of neutralized acid mine drainage contaminated water on the expression of selected endocrine-linked genes in juvenile Mozambique tilapia *Oreochromis mossambicus* exposed in vivo. *Ecotoxicology and Environmental Safety*, 100, 209 - 217.

Tyler, C. R. and Jobling, S. 2008. Roach, sex, and gender-bending chemicals: The feminization of wild fish in English rivers. *BioScience*, 58, 1051 - 1059.

Tyler, C. R., Jobling, S. and Sumpter, J. P. 1998. Endocrine disruption in wildlife: A critical review of the evidence. *Critical Reviews in Toxicology*, 28, 319 - 361.

Tyler, C. R., Filby, A. L., Bickley, L. K., Cumming, R. I., Gibson, R., Labadie, P., Katsu, Y., Liney, K. E., Shears, J. A., Silva-Castro, V., Urushitani, H., Lange, A.,

Winter, M. J., Iguchi, T. and Hill, E. M. 2009. Environmental Health Impacts of Equine Estrogens Derived from Hormone Replacement Therapy. *Environmental Science & Technology*, 43, 3897 - 3904.

Van der Kraak, G., Chang, J.P. and Janz, D.M. 1997. Reproduction. In *The Physiology of Fishes*, 2nd edn. Evans, D.H. (ed), pp. 465-488. CRC, Boca Raton.

Vega-Lopez, A. N., Martinez-Tabche, L., Dominguez-Lopez, M. L., Garcia-Latorre, E., Ramon-Gallegos, E. and Garcia-Gasca, A. 2006. Vitellogenin induction in the endangered goodeid fish *Girardinichthys viviparus*: Vitellogenin characterization and estrogenic effects of polychlorinated biphenyls. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology*, 142, 356 - 364.

Velasco-Santamaria, Y. M., Bjerregaard, P. and Korsgaard, B. 2010. Gonadal alterations in male eelpout (*Zoarces viviparus*) exposed to ethinylestradiol and trenbolone separately or in combination. *Marine Environmental Research*, 69, S67 - S69.

Vethaak, A. D., Bucke, D., Lang, T., Wester, P. W., Jol, J. and Carr, M. 1992. Fish Disease Monitoring Along A Pollution Transect - A Case-Study Using Dab *Limanda-Limanda* in the German Bight. *Marine Ecology Progress Series*, 91, 173 - 192.

Vethaak, A. D. and Jol, J. G. 1996. Diseases of flounder *Platichthys flesus* in Dutch coastal and estuarine waters, with particular reference to environmental stress factors .1. Epizootiology of gross lesions. *Diseases of Aquatic Organisms*, 26, 81 - 97.

Vethaak, A. D., Lahr, J., Kuiper, R. V., Grinwis, G. C. M., Rankouhi, T. R., Giesy, J. P. and Gerritsen, A. 2002. Estrogenic effects in fish in The Netherlands: Some preliminary results. *Toxicology*, 181-182, 147 - 150.

Vethaak, A. D. and Wester, P. W. 1996. Diseases of flounder *Platichthys flesus* in Dutch coastal and estuarine waters, with particular reference to environmental stress factors .2. Liver histopathology. *Diseases of Aquatic Organisms*, 26, 99 - 116.

Wade, R.H. 2007. Microtubules. In *Microtubule Protocols*, Zhou, J. (ed), pp 1-16. Humana Press Inc., Totowa.

Waite, M. E., Waldock, M. J., Thain, J. E., Smith, D. J. and Milton, S. M. 1991. Reductions in TBT Concentrations in UK Estuaries Following Legislation in 1986 and 1987. *Marine Environmental Research*, 32, 89 - 111.

Werner, M., Chott, A., Fabiano, A. and Battifora, H. 2000. Effect of formalin tissue fixation and processing on immunohistochemistry. American Journal of Surgical *Pathology*, 24, 1016 - 1019.

Wester, P. W. and Canton, J. H. 1986. Histopathological study of *Oryzias latipes* (medaka) after long-term ß-hexachlorocyclohexane exposure. *Aquatic Toxicology*, 9, 21 - 45.

Wester, P. W., Canton, J. H. and Bisschop, A. 1985. Histopathological study of *Poecilia reticulata* (guppy) after long-term ß-hexachlorocyclohexane exposure. *Aquatic Toxicology*, 6, 271 - 296.

Williams, T. D., Diab, A. M., George, S. G., Sabine, V. and Chipman, J. K. 2007. Gene expression responses of European flounder (*Platichthys flesus*) to 17-ß estradiol. *Toxicology Letters*, 168, 236 - 248.

Wolfe, S. L. 1993. Molecular and cellular biology. Wadsworth, Belmont, CA. 1145 p.

Yu, R. M. K., Wong, M. M. L., Kong, R. Y. C., Wu, R. S. S. and Cheng, S. H. 2006. Induction of hepatic choriogenin mRNA expression in male marine medaka: A

highly sensitive biomarker for environmental estrogens. *Aquatic Toxicology*, 77, 348 - 358.

Yum, S., Woo, S., Kagami, Y., Park, H. S. and Ryu, J. C. 2010. Changes in gene expression profile of medaka with acute toxicity of Arochlor 1260, a polychlorinated biphenyl mixture. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology*, 151, 51 - 56.

Zaroogian, G., Gardner, G., Borsay Horowitz, D., Gutjahr-Gobell, R., Haebler, R. and Mills, L. 2001. Effect of 17ß-estradiol, o,p'-DDT, octylphenol and p,p'-DDE on gonadal development and liver and kidney pathology in juvenile male summer flounder (*Paralichthys dentatus). Aquatic Toxicology*, 54, 101 - 112.

Zhang, X., Hecker, M., Park, J. W., Tompsett, A. R., Newsted, J., Nakayama, K., Jones, P. D., Au, D., Kong, R., Wu, R. S. and Giesy, J. P. 2008. Real-time PCR array to study effects of chemicals on the Hypothalamic-Pituitary-Gonadal axis of the Japanese medaka. *Aquatic Toxicology*, 88, 173 - 182.

Zhao, Y. and Hu, J. 2012. Development of a molecular biomarker for detecting intersex after exposure of male medaka fish to synthetic estrogen. Environmental *Toxicology and Chemistry*, 31, 1765 - 1773.