The impacts of chemical discharges on the reproductive biology of the bullhead *Cottus gobio* and the dipper *Cinclus cinclus* in the Tamar catchment

Submitted by

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other university

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ABSTRACT

It is now well established that a wide range of natural and anthropogenic chemicals present in the aquatic environment have the potential to disrupt the endocrine system of many organisms. In fish, many of these effects appear to be of a feminising nature, including stimulation of vitellogenin production and induction of intersex. In piscivorous birds these so called endocrine disrupting contaminants have been shown to impair reproduction, influencing reproductive behaviour, sex ratio, eggshell thickness and reproductive success. The effects seen in fish have been associated with high levels of oestrogenic activity in the effluent from waste water treatments works (WwTWs), but few studies have focused on the effects of WwTWs effluents on birds.

In this thesis, the effects of effluents from WwTWs on fish and birds were investigated in the Tamar catchment, SW England. The work spanned making detailed assessment on the oestrogenic and anti-androgenic activity of 3 WwTWs effluents, using a variety of water sampling techniques and applying both recombinant yeast oestrogen screen (YES) and recombinant yeast androgen screen (anti-YAS) bioassays to quantify the different hormonal activities. A survey was undertaken of the hormonal activities at 13 sites to determine concentrations of contaminants in the surface waters throughout the Tamar catchment, using both recombinant yeast screens and targeted analytical chemistry for specific pollutants (LC/MS-TOF and GCMS). An ELISA was developed to quantify vitellogenin (VTG) in the bullhead (our study fish sentinel) as a biomarker of oestrogen exposure, and evidence of endocrine disruption was investigated in wild populations of the bullhead, *Cottus gobio* and the dipper, *Cinclus cinclus*. Macroinvertebrates from upstream and downstream of three WwTW's effluent discharges and from three sampling sites were also sampled as an index of overall water quality in the Tamar catchment, and as an assessment of food availability for the bullheads and dippers.

For the studies on the hormonal activities in three WwTWs in the Tamar catchment, samples were collected by both spot and passive sampling; passive samplers (in replicate) were placed in the effluent discharges for a three week period, and collected on days 7, 14 and 21, spot samples were taken simultaneously. Measurement of total oestrogenic and total anti-androgenic activity was conducted using the YES and anti-YAS, respectively. Spot and passive samples were collected from 13 sites within the Tamar catchment (sampling sites were >2 km downstream of effluent discharges). Additionally, liquid chromatography mass spectrometry time-of-flight (LC/MS-TOF) was used to measure the concentration of oestrone (E_1), 17β-oestrodiol (E_2) and 17α-ethinylestradiol (EE_2) in each sample. Gas chromatography mass spectrometry (GCMS) was used to measure the concentration of individual PBDE and PCB congeners in the spot samples only.

Levels of oestrogenic and anti-androgenic activity observed in the WwTWs effluent were comparable with those measured in effluents in the UK and in other countries. Surface waters of the Tamar, away from the WwTWs effluent discharges, contained very little oestrogenic activity (<1.1 ng E_2 EQs L^{-1}), and anti-androgenic activity was undetectable. Quantification of oestrogenic activity using passive samplers showed an increasing amount of total oestrogenic activity between days 7 and 21 when measured by the both the YES and LC/MS-TOF. Low levels of PBDE congeners 47, 99, 100, 138 and 153 were detected in the spot samples taken from the Tamar catchment, with BDE 47 being the most abundant. In contrast PCBs were undetectable. Neither PBDEs nor PCBs were detected in any of the extracts from the passive samples.

No assay was available to measure VTG (one of the most widely used biomarkers of oestrogen exposure in fish) in the bullhead and so an enzyme linked immunosorbant assay (ELISA) was developed for application to studies on wild bullheads in the Tamar catchment. The bullhead vitellogenin (bh-VTG) ELISA was developed successfully, and proved to be sensitive and robust, with a detection range between 10.5 and 300 ng bh-VTG mL^{-1} (undiluted), comparing favourably with other fish VTG ELISAs. Plasma VTG concentrations measured in male bullheads (collected from the same sites as for the water samples) ranged from below the limit of detection to 990 ng bh-VTG mL^{-1}. Whether these upper levels in the range reflected VTG induction was difficult to conclude. Because of this controlled caged exposures with bullheads and trout were used to assess the relative levels of oestrogenicity in two key WwTWs effluent discharges and to determine the response sensitivity of the bullheads (and trout) to those effluents. These controlled exposures found no responses in plasma VTG in bullheads (ranging between 126 and 934 ng bh-VTG mL^{-1}) suggesting a lack of sensitivity for VTG induction. This was supported by the inability to induce VTG in fish held in the laboratory and treated with steroidal oestrogens. For the effluent exposures on the caged rainbow trout, it was also found that there was no significant induction of VTG, a species normally sensitive to oestrogens. These findings may indicate that the fish were highly stressed due to the river being in spate and the movement of the cages during the controlled exposures. It may also be the case, however, that the use of immature female rainbow trout with a highly variable baseline plasma VTG concentration may prevent any detection of a response.

There were no signs of sexual disruption in any of the gonads analysed from either male or female wild bullheads, demonstrating that any hormonal activity present in the catchment away from the WwTWs effluents was not sufficient to induce adverse effects on reproductive development. An interesting feature noted in the male testes of the bullheads was the presence of spermatid masses, which have been recorded in 10 other Cottidae species, but not previously in the bullhead.

For the studies on dippers, eggs were collected from the nests of breeding dippers to measure for sperm numbers and morphology from sperm trapped in the perivitelline membrane (PVM), and the yolks were analysed for PBDEs, PCBs and organochlorine pesticides (OCPs) by GCMS, for E_1, E_2, and EE_2 by LC/MS-TOF. Eggs of the dipper were collected from nests at the 13 sampling sites, plus an additional three sites and over three years of field study. The number of sperm trapped in the PVM ranged between three and 188, with a mean of 68.78 ± 8.78 SE. Dipper sperm had not previously been characterised, and was found to be similar to other passerine sperm, in that the head was helical, complemented by a mitochondrial helix or keel, which continued in a spiral around the flagellum. Sperm were classed as ‘abnormal’ if they did not adhere to this typical structure. No assessment of motility could be made in relation to the structural abnormalities seen. Contaminants in the dipper eggs were dominated by BDE 99, an unusual result considering the dippers aquatic lifestyle. PCB 153 was the most common PCB, and *p,p’*-DDE was the most abundant OCP; all other pesticides tested were below the limit of detection, as were the levels of all three steroid oestrogens. There was inter- and intra-nest variability between contaminant burdens in all eggs as well as the number of sperm trapped in the PVM, but there was no relationship between sperm number and the level of contaminant loadings in the eggs.

There were no correlations between contaminants and oestrogenic activity measured in the water samples, and plasma VTG concentrations in bullheads or contaminant loadings in eggs, or indeed sperm number. Analysis of macroinvertebrate assemblages

proved that the surface waters of the Tamar catchment were of ‘very good’ quality, even in close proximity to WwTWs effluent discharges. Indeed the oestrogenicity and contaminant loadings in both eggs and surface waters were very low, and this study agrees with a national risk assessment that there appears to be no risk of intersex in fish in the Tamar catchment.

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This thesis is dedicated to my dad.

DISCLAIMER

All work in this thesis, unless stated otherwise, was carried out by the candidate, including coordination, organisation and implementation of all fieldwork (daily for three to four months of each year), all lab work, including the yeast screens, VTG ELISAs, histopathology and PVM analyses, and all data analyses. Where lab work was carried out by a third party, coordination and organisation was carried out by the candidate. It was assumed previously that all lab work and analyses of samples would be carried out by the candidate at the facilities of the sponsor AstraZeneca (AZ), but, unfortunately due to unforeseen circumstances this was not possible. Therefore an outside supplier was sourced and, where required, funded by AZ. Due to this outsourcing, the candidate did not carry out the chemical analyses of the river water and egg yolk samples.

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LIST OF ABBREVIATIONS

°C	Degrees celsius
µg	Microgram
µL	Microlitres
µM	Micromolar
11-KT	11-ketotestosterone
AHH	Aryl hydrocarbon hydroxylase
ANOVA	Analysis of variance
Anti-YAS	Yeast anti-androgen screen
AR	Androgen receptor
ASD	Androstenedione
B/B0	Realative binding
B0	Maxium binding
BAF	Bioaccumulation factor
BCF	Bioconcentration factor
BDE	Brominated diphenyl ether
bh-VTG	Bullhead VTG
BLAST	Basic Local Alignment Search Tool
BMF	Biomagnification factor
BOD	Biological oxygen demand
bp	Base pair
BSA	Bovine serum albumin
C	Carbon
CA	Cortical alveolus
CAS	Chemical abstract service
cDNA	Complementary DNA
COD	Chemical oxygen demand
c-VTG	Carp VTG
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
DHT	5α-dihydrotestosterone
DNA	Deoxyribose nucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
E1	Oestrone
E2	17β-oestradiol
E2 Eqs	17β-oestradiol equivalents
E3	Oestrone
EA	Environment Agency
EDCs	Endocrine disrupting chemicals
EDTA	Ethylenedinitrilotetraacetic acid
EE2	17α-ethinylestradiol
ELISA	Enzyme linked immunosorbant assay
ER	Oestrogen receptor
EROD	Ethyoxyresorufin 0-deethylase
EtOH	Ethanol
EU	European Union
FLUT	Flutamide
FPLC	Fast protein liquid chromatography
FSH	Folicle stimulating hormone
FSSP	Female-specific serum protein
g	Gram
GCMS	Gas chromatography mass spectrometry
GFC	Gel filtration chromatography

GLM	General linear model
GnRH	Gonadotrophin-releasing hormone
GSI	Gonadosomatic index
GtH	Gonadotrophin hormone
HPG	Hypothalamic-pituitary-gonadal axis
HPLC	High performance liquid chromatography
HSI	Hepatosomatic index
IgG	Immunoglobulin G
IMS	Industrial methylated spirit
IUPAC	International Union of Pure and Applied Chemistry
K	Condition factor
kDa	Kilodalton
L	Litre
LC/MS-TOF	Liquid chromatography mass spectrometry with time of flight
LDL	Low density lipoproteins
LH	Leutinising hormone
M	Molar
MeOH	Methanol
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
M-MLV	Moloney Murine Leukaemia Virus
mRNA	Messenger RNA
MT	Methyltestosterone
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National centre for biotechnology information
ng	nanogram
nm	Nanometre
NP	Nonylphenol
NSB	Non-specific binding
OCP	Organochlorine pesticide
OPD	Ortho-phenyline diamine
p,p'-DDE	Dichlorodiphenyldichloroethylene
P450	Cytochrome P450
Pab	Primary antibody
PAH	Polyaromatic hydrocarbon
PBDEs	Polybrominated diphenyl ethers
PBS	Phosphate buffered saline
PCBs	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PGC	Primordial germ cell
pM	Picomolar
PO	Primary oocyte
POP	Persistent organic pollutants
PVM	Perivitelline membrane
RIA	Radioimmunoassay
RNA	Ribose nucleic acid
Rnase	Ribonuclease
RT-qPCR	Real-time quantitative PCR
rt-VTG	Rainbow trout VTG
Sab	Secondary antibody
SC	Spermatocytes
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGA	Spermatogonia A

SGB	Spermatogonia B
SO	Secondary oocyte
ST	Spermatids
STM	Spermatid masses
TBE	Tris-borate EDTA
TBT	Trbutyltin
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TIU	Trypsin inhibitor unit
Tris-HCl	Tris hydroxymethylaminomethane hydrogen chloride
Tween-20	Polyoxyethylene sorbitan mono laurate
UK	United Kingdom
US	United States
UV	Ultra-violet
VO	Vitellogenic oocyte
VTG	Vitellogenin
WwTWs	Waste water treatment works
YAS	Yeast androgen screen
YES	Yeast oestrogen screen

LATIN NAMES OF SPECIES

Fish

Atlantic cod	*Gadus morhua*
Atlantic croker	*Micropogonias undulatus*
Atlantic salmon	*Salmo salar*
Brown trout	*Salmo trutta*
Bullhead	*Cottus gobio*
Carp	*Cyprinus carpio*
Carrion crow	*Corvus corone*
Chinook salmon	*Oncorhynchus tshawytscha*
Chum salmon	*Oncorhynchus keta*
Clownfish	*Amphiprion spp*
Common Japanese conger eel	*Conger mynaster*
Cutthroat trout	*Oncorhynchus clarki*
European eel	*Anguilla anguilla*
European flounder	*Platichthys flesus*
Fathead minnow	*Pimephales promelas*
Gag grouper	*Mycteroperca microlepis*
Gibel carp	*Carassius auratus gibel carpio*
Goldsinny wrasse	*Ctenolabrus rupestris*
Greenback flounder	*Rhombosolea tapirina*
Gudgeon	*Gobio gobio*
Japanese eel	*Anguilla japonica*
Mangrove killifish	*Rivulus marmoratus*
Medaka	*Oryzias latipes*
Mosquitofish	*Gambusia affinis*
Mottled sculpin	*Cottus bairdii*
Moustached warbler	*Acrocephalus melanopogon*
Mummichog	*Funfulus heteroclitus*
Parrot fish	*Scarus spp*
Perch	*Perca fluvitilus*
Rainbow trout	*Oncorhynchus mykiss*
River sculpin	*Cottus hangiogensis*
Roach	*Rutilus rutilus*
Rockfish	*Sebastes schlegeli*
Sea bream	*Sparus aurata*
Siamese fighting fish	*Betta splendens*
Spoonhead sculpin	*Cottus ricei*
Three-spined stickleback	*Gasterosteus aculeatus*
Zebrafish	*Danio rerio*

Birds

Adelie penguin	*Pygoscelis adeliae*
American dipper	*Cinclus mexicanus*
Arctic tern	*Sterna paradisaea*
Bald eagle	*Haliaeetus leucocephalus*
Barn owl	*Tyto alba*
Black kite	*Milvus migrans*
Blue tit	*Cyanistes caeruleus*
Brown pelican	*Pelacanus occidentalis*
Bullfinch	*Pyrrhula pyrrhula*
Buzzard	*Buteo buteo*
Collared flycatcher	*Ficedula albicollis*
Common tern	*Sterna hirundo*
Domestic fowl	*Gallus domesticus*
Double-crested cormorant	*Phalacrocorax auritus*
Eider duck	*Somateria mollissima*
Forsters tern	*Sterna fosteri*
Golden eagle	*Aquila chrysaetos*
Great cormorant	*Phalacrocorax carbo*
Great crested grebe	*Podiceps cristatus*
Great tit	*Parus major*
Grey heron	*Ardea cinerea*
Guillemot	*Uria aalge*
Herring gull	*Larus argentatus*
Imperial eagle	*Aquila heliaca*
Japanese quail	*Coturnix japonica*
Kestrel	*Falco tinnunculus*
Kingfisher	*Alcedo Atthis*
Little egret	*Egretta garzetta*
Little owl	*Athene noctua*
Long-eared owl	*Asio otus*
Marsh harrier	*Circus aeruginosus*
Night heron	*Nycticorax nycticorax*
Osprey	*Pandion haliaetus*
Ostrich	*Struthio camelus*
Peregrine falcon	*Falco peregrinus*
Prothonatory warbler	*Protonaria citrea*
Ring dove	*Streptopelia risoria*
Ring-necked pheasant	*Phasianus colchicus*
Sparrowhawk	*Accipiter nisus*
Starling	*Sturnus vulgaris*
Tawny owl	*Strix aluco*
White-throated dipper	*Cinclus cinclus*
Zebra finch	*Taeniopygia guttata*

Others

African clawed frog	*Xenopus laevis*
American alligator	*Alligator mississippiensis*
American mink	*Mustela vison*
Bank vole	*Myodes glareolus*
Beluga whale	*Delphinapterus leucas*
Bottlenose dolphin	*Tursiops truncates*
Cecropia moth	*Hyalophora cecropia*
Common dog whelk	*Nucella lapillus*
Eastern mudsnail	*Nassarius obsoletus*
Echiuran worm	*Bonellia viridis*
Fox	*Vulpes vulpes*
Green frog	*Rana clamitans*
Harbour porpoise	*Phocoena phocoena*
Harbour seal	*Phoca vitulina*
Humpback whale	*Megaptera novaeangliae*
Impala	*Aepyceros melampus*
Mink frog	*Rana septentironalis*
Northern leopard frog	*Rana pipiens*
Old field mouse	*Peromyscus polionotus*
Otter	*Lutra lutra*
Polar bear	*Ursina maritimus*
Western spotted frog	*Rana petriosa*
Wood mouse	*Apodemus sylvaticus*
Zebra mussel	*Dreissena polymorpha*