LONG-TERM EFFECTS OF OESTROGENIC EFFLUENT EXPOSURE ON WILD FISH POPULATIONS

A thesis submitted for the degree of Doctor of Philosophy

by

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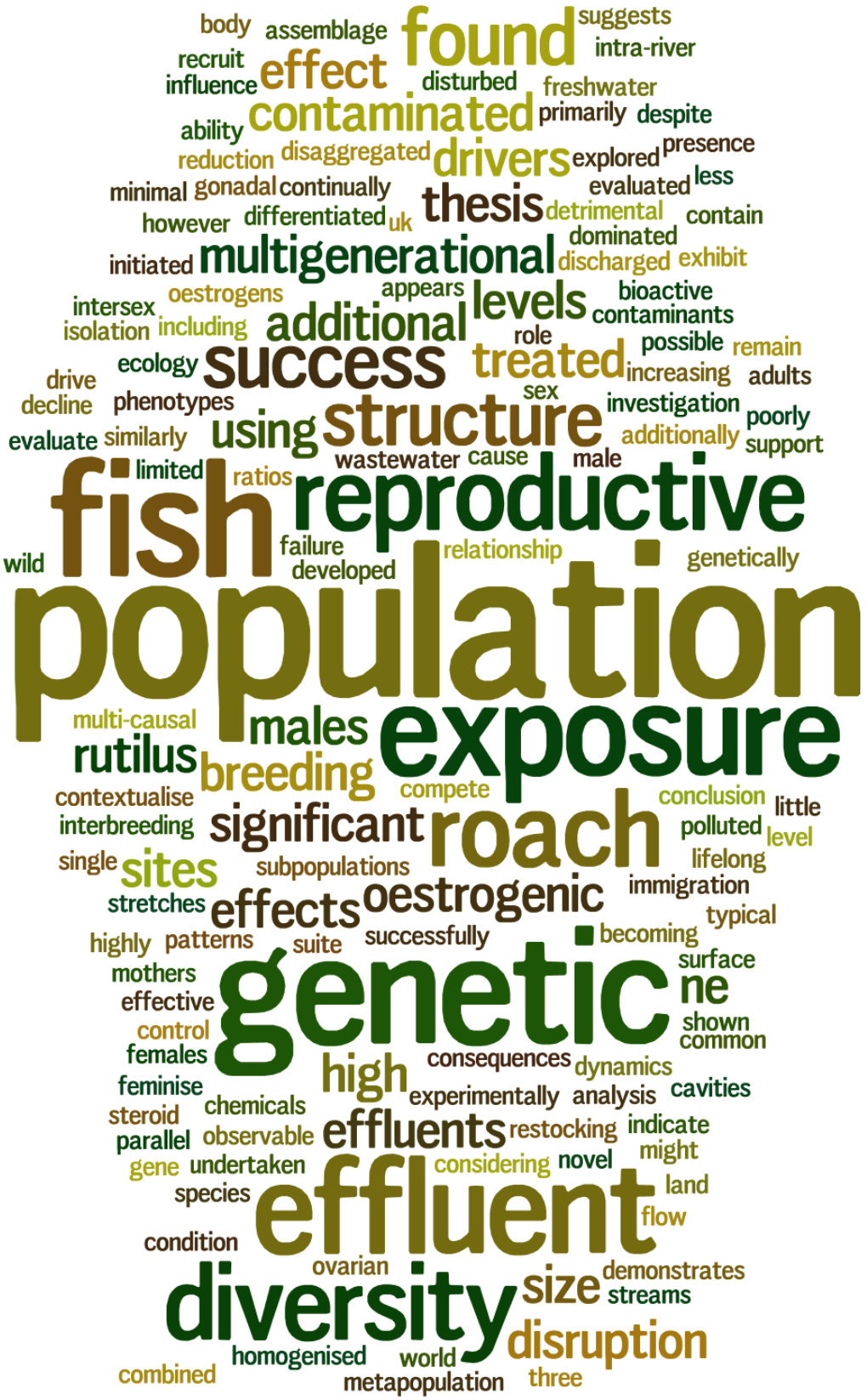
September 2014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DECLARATION

The work submitted in this thesis was conducted between 2010 and 2014 at Brunel University (Uxbridge, Middlesex), University of Exeter (Exeter, Devon) and Essex and Suffolk Water Treatment Works (Langford, Essex). This work was carried out independently and has not been submitted for any other degree.

ABSTRACT

Freshwater streams in the developed world are becoming increasingly dominated by treated wastewater. Continually discharged into most surface waters, these effluents contain a suite of bioactive man-made chemicals, including steroid and non-steroid oestrogens, which have been found to feminise male fish, skew sex ratios, and cause reproductive failure. However, the consequences of reproductive disruption remain poorly explored at the population level. This thesis was initiated to evaluate how oestrogenic contaminants might influence the population ecology of a common cyprinid, the roach (*Rutilus rutilus*). An investigation encompassing population structure, multigenerational exposure and the role of additional drivers of fish population dynamics was undertaken to contextualise the effects of oestrogenic effluents on wild fish populations.

Population genetic analysis of UK roach found they exhibit moderately high levels of genetic diversity and significant intra-river genetic structure. Genetically differentiated local subpopulations indicate little interbreeding and limited gene flow, consistent with a typical metapopulation that has not been homogenised by restocking. Similarly, my thesis demonstrates no significant relationship between effluent exposure and N_e (effective population size) or genetic diversity of roach populations, albeit a 65% reduction in N_e is possible at highly polluted sites. River stretches contaminated with high levels of effluent can support breeding populations, which recruit successfully with minimal immigration from less contaminated sites. Multigenerational effects of effluent exposure on roach were also evaluated experimentally using reproductive success from breeding adults over three generations. Lifelong exposure to 100% treated effluent resulted in feminised phenotypes (ovarian cavities and intersex condition) in males but no observable effect on females. Additionally, despite gonadal disruption in males and effluent exposure of their mothers, I found no detrimental effect on their ability to compete with control fish. Instead, reproductive success was primarily determined by body size. A novel approach considering additional fish population drivers suggests that genetic diversity and species diversity decline in parallel with an increasing presence of disturbed land, when combined with geographical isolation. In conclusion, group assemblage and genetic structure of fish populations appears multi-causal and cannot be disaggregated, such that a single environmental characteristic can be shown to drive patterns of population success.

ACKNOWLEDGEMENTS

This thesis marks the end of my PhD research, yet to get here, many people have been absolutely integral. Firstly, and perhaps most notably, I would like to express my sincerest thanks to both my supervisors Prof. Susan Jobling and Prof. John Sumpter. Their uncompromised support and contrasting views on occasion have been both a continual source of inspiration and enjoyment. Without their passionate supervision and endless positivity I would not have developed the personal and professional qualities that have brought me to this point. Unequivocally, a unique and great pairing.

Critically, this research would not have taken place without the funding of NERC (NE/G019355/1) and DEFRA. For these resources I am incredibly grateful. Similarly, this work was only made possible by working in close allegiance with the Environment Agency. Days spent on a river bank with fisheries teams from across the UK were fantastic fun and every individual went out of their way to be of greatest assistance. You all played a fundamental role (Alex, Adam, Adam, Dan, Dan, Stuart) and it was a superb partnership.

I owe an enormous thank you to Dr Patrick Hamilton at the University of Exeter. Your molecular biology prowess, flow charts and inquiring mind, made this collaborative project a success and a pleasure to work on. Additional gratitude must go to other colleagues at Exeter (Anke, Charles, Greg, Jan) and Richard Williams at CEH who helped extensively throughout.

A great deal of appreciation goes to the staff, post-docs and students at the Institute for the Environment. Many of which came with me on long days in the field, sometimes catching fish…..and sometimes not! I cannot forget to acknowledge Chris, a perfect desk neighbour for the entirety of my Brunel career and a constant source of amusement. Thanks for listening to 4 years worth of moaning and general chitchat Dr Green! Special recognition goes to all the other office post-docs and staff - Alice, Cath, Erin, Luigi, Tamsin- for answering the many questions and concerns of a PhD student and being a vital source of knowledge and much needed assistance at times. You made this a wonderfully brilliant experience and it would’ve been very different without you all.

Finally, but perhaps most significant, are those friends and family that kept me relatively sane and helped me to the finish line (with the help of gin when required). Dad and Gran, you can now stop asking ‘how is the thesis going?’ and a note to my dearest brother, ‘brains’ has finally done it! Special acknowledgement to Rob, who remains the best companion I could ever ask for. Your recognition is a meagre substitution for the endless love and support you selflessly provide. Some sort of work/life balance can hopefully now be reinstated!

With a heavy heart, I dedicate this thesis to my beloved mum. Sorry I didn’t finish it in time for you to witness and ‘thanks a bunch’ for showing such enthusiasm for nature and understanding my love of science. Your memories come as easily as smiling.

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ABBREVIATIONS

CHAPTER 1

AFLP	Amplified Fragment Length Polymorphism
AR	Androgen Receptor
BPA	Bisphenol A
DDT	Dichlorodiphenyltrichloroethane
EDC	Endocrine Disrupting Chemical
ER	Oestrogen Receptor
EST	Expressed Sequence Tags
ESU	Evolutionary Significant Units
EU	European Union
IBD	Isolation By Distance
IPCS	International Program on Chemical Safety
LOEC	Lowest Observed Effect Concentration
mtDNA	Mitochondrial DNA
N_c	Demographic Census Size
N_e	Effective Population Size
NOEC	No Observed Effect Concentration
PAHs	Polycyclic Aromatic Hydrocarbons
PCBs	Polychlorinated Biphenyls
PCDD	Polychlorinated Dibenzodioxin
PCDF	Polychlorinated Dibenzofuran
PCR	Polymerase Chain Reaction
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeat
STR	Short Tandem Repeat
STW	Sewage Treatment Works
TBT	Tributyltin
VTG	Vitellogenin
WHO	World Health Organisation
WWTW	Wastewater Treatment Works
YCS	Year Class Strength

CHAPTER 2

CEH	Centre for Ecology and Hydrology, Wallingford, UK
COD	Chemical Oxygen Demand
EA	Environment Agency

GIS	Geographical Information System
GREAT-ER Rivers	Geography-Referenced Regional Exposure Assessment for European
HWE	Hardy-Weinberg Equilibrium
NFPD	National Fish Population Database
NGR	National Grid Reference
PhATE	Pharmaceutical Assessment and Transport Evaluation
SSR	Simple Sequence Repeat

CHAPTER 3

AMOVA	Analysis of Molecular Variance
AR	Allelic Richness
LFMD	England and Wales Environment Agency Live fish Movement Database
MEGA	Molecular Evolutionary Genetic Analysis
PCA	Principal Component Analysis

CHAPTER 4

ABC	Approximate Bayesian Computation
NFPD	National Fish Populations Database
SA	Sibship Assignment
TPM	Two-Phase Model

CHAPTER 5

EPA	Environmental Protection Agency (U.S) GSI Gonadosomatic Index
HSI	Hepatosomatic Index

CHAPTER 6

HL	Homozygosity by Loci
SL	Standardized Heterozygosity
IR	Internal Relatedness
MEA	Millennium Ecosystem Assessment
NRFA	National River Flow Archive

Chapter 1

General Introduction

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Overview

A myriad of interacting physical, chemical and biological challenges are present across all ecosystems; threatening the fitness, survival, and reproductive success of individuals through mechanisms of habitat degradation, climate change, pollution, disruption of food chains and indirect anthropogenic drivers. Acting at a variety of scales, each has been found to contribute to the decline or subsequent extinction of local populations across a broad spectrum of taxa (Sala et al. 2000, Parmesan and Yohe 2003, Jenkins 2003, Mora et al. 2007). These pervasive factors are indiscriminate throughout both terrestrial and aquatic ecosystems, accelerating environmental modification of niche architecture within the European landscape perhaps more so than anywhere else in the world (Freyhof and Brookes 2011).

With relevance to this thesis, alteration of natural habitats by human activities has proved influential in shaping the distribution and abundance of fish populations globally (Cowx 2001). Statistics suggest at least 37% of Europe's freshwater fishes are now threatened at a continental scale (IUCN 2009). Historically, the more damaging drivers were deemed to be overexploitation and alien introductions; both affecting density- dependence through factors of predation, competition, population density and novel disease vectors. However, more recent focus has shifted towards the significance of habitat fragmentation (Keyghobadi 2007) and water quality influences, which are likely to be fundamentally important in regulating riverine fish populations (Grenouillet et al. 2001, Beardsley and Britton 2012).

Fish are particularly susceptible to water-borne chemicals, to which they are being continually exposed. Adverse outcomes of environmentally relevant concentrations of chemical contaminants have been demonstrated in laboratory studies, nevertheless there remains the question of whether true effects occur in wild fish populations (Mills and Chichester 2005). Current research motivation is therefore aligned with the long-term, population-level implications of chemical contaminants reaching the aquatic environment, which demonstrates the focus of this thesis. The remainder of this chapter will elaborate further on details pertinent to this area of research.

Within the following introduction I will first direct attention towards the scale of the problem, typical sources of chemicals to the environment and elaborate on a well-studied group of contaminants, the Endocrine Disrupting Chemicals (EDCs). Then I present the historical background and motivation for this project, including evidence of known effects of EDCs on wildlife and fish populations, touching on the current study

species adopted in this thesis. The next section moves forward to the utility of modern molecular markers in studying fish populations and the functional importance of integrating variation at the genetic level in ecotoxicological studies. Subsequently, the issue of additional population-level drivers of fish population dynamics are discussed for contextualisation of the problem, including their possible influence on genetic variation of wild populations. Penultimately, I mention the challenges involved in linking individual impacts to the population level, emphasising the difficulties faced in conducting research and the involvement of different approaches. Then finally, I end with the aims and objectives that provide the backbone to this work and an outline of the approach taken to address these within the following experimental chapters.

1.1 Chemicals in the Environment

Major worldwide concern surrounds the range of chemicals being discharged into the biosphere as a result of human activity. Delivery of chemicals into the aquatic environment through the release of treated effluents (point-sources) or surface runoff (via vehicles of air, water and soil) derive from human activities such as agriculture, manufacturing, transportation and energy production. Accordingly, most lotic environments within relative proximity of an urban setting constitute a complex mixture of chemicals, to which resident communities are continually subjected. The sub-lethal effects of such chemical cocktails can be insidious and pervasive. Despite the ubiquitous featuring of chemicals in surface waters, to date their adverse effects on species other than their primary target have only been recognised following a population crash, such as those seen in the *Gyps* vulture genus (a situation where 99.9% of vultures in south Asia were wiped out by a fatal kidney condition having consumed Diclofenac-treated cattle carcasses, subsequently leading to a ban on the use of this chemical; Oaks et al. 2004, Green et al. 2004, Pain et al. 2008).

Total numbers of chemicals in everyday use exceeds 60,000, not including by-products and metabolites (Ternes et al. 1999). Many of these are compounds designed specifically to be stable even when excreted from the body and biologically active at low (ng/l) concentrations (Heberer 2002). A sub-set of all chemical contaminants have been shown to interact with the endocrine system, disrupting reproductive function in many wildlife populations (Jobling and Tyler 2006) and possibly humans. Growing evidence of chemically-induced alterations in sexual development in a wide range of species have been associated with a characteristic group of exogenous compounds, collectively termed EDCs. The presence of complex mixtures of EDCs in the environment — that effectively

act in a similar manner to hormones, disrupting hormonal pathways and the physiological functions they control — pose a major ecological and economic problem on a global scale.

Research initiatives and international recommendations (post-1997) addressing the emergent issue of EDCs demarcated a working definition, as described by the International Program on Chemical Safety (IPCS). This states that *'An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations'* (Damstra et al. 2002). Notably, this incorporates the simultaneous action of chemicals via multiple mechanisms to cause alterations in the endocrine system of an organism, even as a secondary effect. For the purpose of this thesis, therefore, I consider EDCs as exogenous chemicals that evoke mechanistic responses that interact *directly* with the endocrine system, perturbing normal mechanisms of homeostasis.

Recognising the widespread occurrence of EDCs has provoked necessary regulation and research into endocrine-mediated effects throughout a broad spectrum of wildlife (World Health Organisation (WHO) 2012). Taxonomic conservation of hormones and hormone receptors across species helps explain why chemical effects have been demonstrated comprehensively in many wildlife species, examples of which include fish, invertebrates, reptiles, amphibians, birds and mammals (see Pure and Applied Chemistry (2003), volume 75 [11–12] for comprehensive review). Humans, despite being the route of delivery of EDCs to the environment, do not necessarily escape the effect of contamination. Emergent evidence to date unveils the role of chemical burden affecting humans; driving disruptions to reproductive outcomes (infertility, cancers, malformations), thyroid function, brain function, obesity, metabolism, and insulin and glucose homeostasis (WHO 2012).

1.2 Sources and Exposure Routes of EDCs

Populated environments are a primary source of EDCs. Thousands of chemicals are discharged daily into aquatic ecosystems as a by-product of population expansion (through consumption and excretion) and a burgeoning demand for clean water. Disposal of wastewaters, via point-source discharges from sewage/ wastewater treatment works (STW, WWTW) or untreated effluent plumes in the developing world (80%, Gilbert 2012) represent a principal route of delivery for pharmaceuticals and synthetic steroid oestrogens to riverine ecosystems. These chemicals are poorly removed in the sewage

system (Johnson and Sumpter 2001, Kanda and Churchley 2008) amplifying their presence in the aquatic environment. Additionally, the sensitivity of lotic systems to contamination are compounded by the inherent susceptibility of aquatic organisms, the relatively small size of many freshwater systems, restricted distributions of many species, and the inability of species to escape chemical challenge. This results in continual and indiscriminate exposure.

Exposure routes in fish are well defined and include uptake across the gills and skin (Jobling et al. 1998), via ingestion, or even through maternal transfer into developing eggs (Ellis et al. 2005). As expected, the dominant course is via the water, although multiple routes can be affected simultaneously (Sumpter 1998, Tyler and Jobling 2008). This results in substantial impacts of EDCs on aquatic biota, which are amplified by the almost continual exposure to manufactured compounds that are characteristically designed to persist in the environment. Recent evidence from rainbow trout (*Oncorhynchus mykiss*) and adult roach exposed to WWTW effluent have identified the presence of complex mixtures of xenobiotics and their metabolites in plasma samples, crucially showing concentrations increasing in a dose-dependent manner with percentage effluent exposure (Al-Salhi et al. 2012). Many other contaminants accompany these but there is a paucity of data regarding their accumulation and toxicity in fish. The presence of chemicals within the water column (or sediment) may not prove directly toxic to fish but may evoke harmful health repercussions when accumulated or mixed with other chemicals, especially during lifelong exposure. For example, exposure of brown bullhead (*Ameiurus nebulosus*) to a polychlorinated biphenyl (PCB) mixture was found to modulate immune function, which compounded the effects of a later disease challenge, resulting in significantly increased mortality in PCB-exposed fish (Iwanowicz et al. 2009). Furthermore, the majority of these chemicals are not currently regulated or monitored routinely in the European Union (EU) through legislations such as the Water Framework Directive (WFD).

Effective tools and technologies are required to monitor and mitigate environmental pressures on sustainability of wildlife, especially in light of future climate change. Environmental effects, including EDC exposure, can be especially problematic in species with labile sex determination mechanisms. For example, fish are particularly sensitive to EDCs during sexual development and differentiation in early life (Devlin and Nagahama 2002). Therefore, exposure to EDCs at sufficient concentrations, during developmental stages (when endogenous hormonal pathways have not been fully established), can

influence the course of gonadal development and alter sexual differentiation (Piferrer 2001, Devlin and Nagahama 2002). Similar exogenous sex steroids can modulate sex ratios, gonad morphology, quality and quantity of gametes, as well as cause more subtle effects on reproductive behaviour (Jones and Reynolds 1997).

1.3 EDCs in the Aquatic Environment

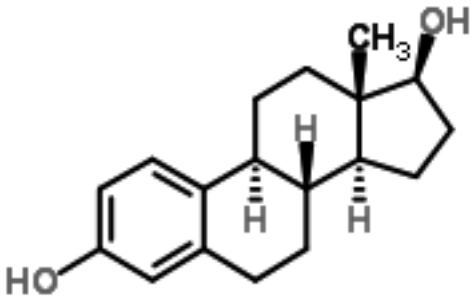
A vast number of EDCs enter the aquatic environment; many of which can perturb the normal physiology of an animal's endocrine system (Colborn et al. 1993). Here I focus on chemicals known to have an oestrogenic potential, due to their significance for the experimental work of this thesis.

Mechanisms of endocrine disruption and the effect of EDCs commonly result from an interaction with relevant nuclear hormone receptors (oestrogen receptors (ERs), or androgen receptors (ARs)). Oestrogenic chemicals diffuse into the cell and then bind to the ER, provoking a conformational change in the receptor and allowing it to modulate gene expression in target cells (Beato 1989). The activation and oestrogenic effect caused by these EDCs is the same as that caused by natural and endogenous steroidal hormone signals in the environment (agonists). However, the binding of chemicals to hormone receptors can also elicit partial agonistic or antagonistic responses (binding to the receptor but not eliciting a response). For example, when considering the ER, a chemical that binds as an antagonist would not elicit the normal response and competitively occupies the receptor site so no other oestrogens can activate it. A partial agonist could be considered mildly oestrogenic as it can elicit some response but can also play a role in blocking the receptor from being fully activated. It must also be noted that ERs have different receptor subtypes in fish (ERα, ERβ), which are agonised or antagonised specifically by different EDCs (Katsu et al. 2007). Some chemicals can also have multiple effects, acting as both agonists and antagonists (Sohoni and Sumpter 1998). Research has primarily identified those EDCs that mimic and compete with endogenous hormones; an overview of the detailed mechanisms of action can be found in Shanle and Xu (2011).

The array of chemicals present in the natural environment can be categorised according to their functionality and the effect that they have on aquatic organisms. Examples of specific chemicals at present that are known to possess oestrogenic properties are discussed briefly below, with an emphasis on compounds with demonstrative effects in aquatic fauna.

1. Natural oestrogens: oestradiol (E2; Figure 1.1), oestrone (E1), oestriol (E3),

2. Synthetic oestrogens: mainly17α-ethinylestradiol (EE2; Figure 1.2)



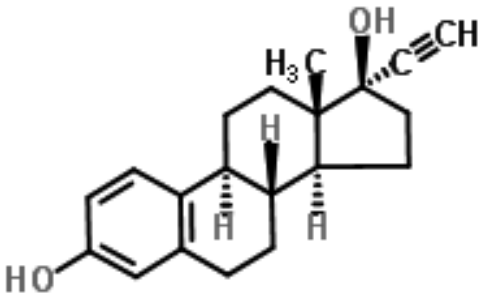


Figure 1.1 17β-oestradiol (E2) **Figure 1.2 Ethinylestradiol (EE2)**

The above all possess a typical steroid structure and have been the most widely studied contributors of oestrogenic effects in WWTW effluents (discussed in the next section).

The following have a chemical structure that in some way resembles all or part of the steroid ring system and are also known to be present in the aquatic environment and some effluents.

3. Phytooestrogens (non-steroidal plant oestogens found in soy products)

4. Xenoestrogens and other chemicals (mimic natural oestrogens)

-Synthetic chemicals such as Bisphenol A (BPA), phthalates, dichlorodiphenyltrichloroethane (DDT), alkylphenols, nonylphenol, PCBs and pesticides

An ever-increasing list of chemicals are being identified as endocrine disruptors, with an expanding number of studies addressing the effects of some xenoestrogens on aquatic fauna. Their potency is considerably lower than the steroid oestrogens but many can compound the loading of EDCs to surface waters, typically delivered by point source effluents. The precise concentrations and known effects of these xenoestrogens are beyond the scope of this introduction and can be found in detail elsewhere (Goodhead and Tyler 2009).

1.4 Delivery of Chemicals to the Aquatic Environment

The regular use of chemicals by every member of the expanding population provokes continual and indiscriminate passage of natural and synthetic compounds into the global environment. Many of these find their way into surface (and ground) waters through

sewage treatment plants, but additional routes from runoff and leaching of agricultural land and industrial facilities contribute widely to the chemical loading (Figure 1.3). Constant delivery of chemicals into the aquatic environment, including EDCs, can be categorised into two mechanistic routes: diffuse or point source.

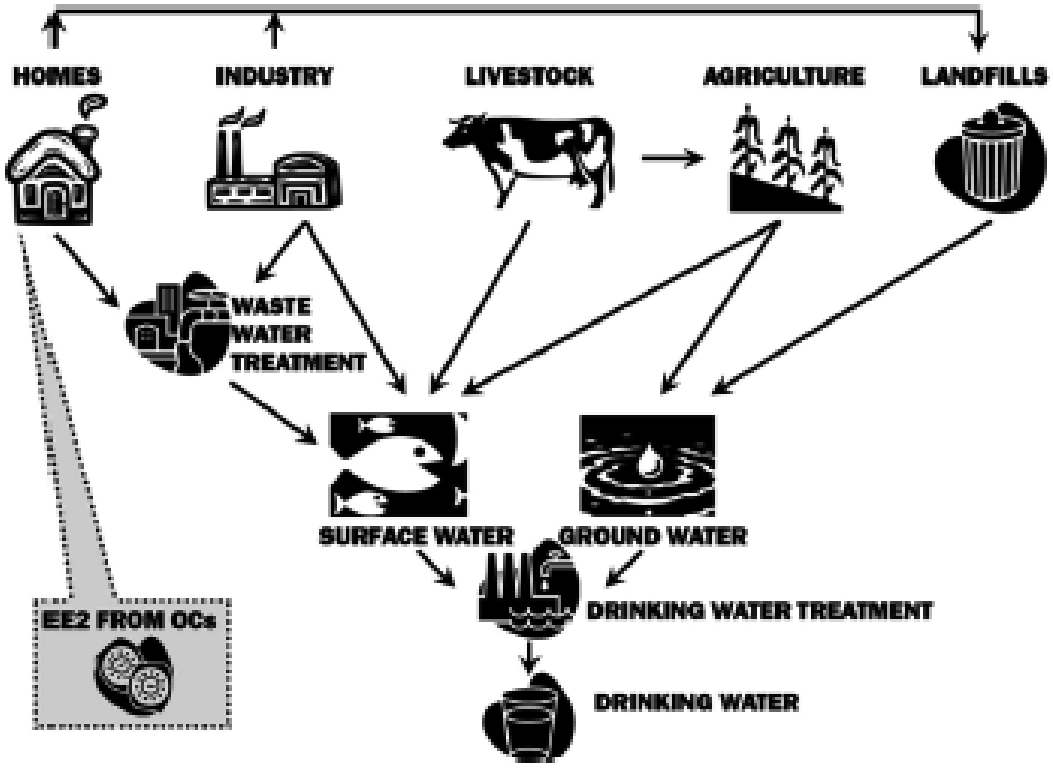


Figure 1.3 Diagram of possible sources of oestrogenic compounds (OC's) to the aquatic environment, taken from Wise et al. (2010)

1.4.1 Diffuse Sources

Diffuse sources of EDCs originate from agricultural runoff (often where animal waste has been applied as fertiliser) and predominantly carry steroidal compounds of E1 and E2. Despite their natural origin, E1 and E2 from farming are present in rivers at concentrations known to mimic or disrupt endocrine function. Early studies suggested chicken manure can contain >1 µmol/g of oestrogen (Shore et al. 1993) and runoff from manure-treated fields following a rain event have contained 1–3 µg/l (Shore and Sheemish 2003). In the UK, passive sampling of E1 and E2 downstream of farms measured oestrogenic activity in the low ng/l range, of 0–10 E2 equivalents (Matthiessen et al. 2006). Therefore despite having the potential to deliver huge quantities of oestrogens to the aquatic environment, final loadings from diffuse sources appear reduced through absorption to particulate matter as well as in-stream dilution.

Mention must also be given to additional EDCs derived from diffuse sources that take the form of herbicides and pesticides applied during crop production. Widely used examples that have oestrogenic properties include atrazine and organochlorine pesticides, such as

DDT. A comprehensive national water quality assessment of streams in the US found pesticides in more than 97% of streams in urban and agricultural areas, including DDT, despite it being banned prior to the study period (Guillom et al. 2007). Their continual detection and presence in the aquatic environment emphasises the significance of surface runoff in the delivery and persistence of potent pesticides, often present at concentrations that may negatively affect aquatic fauna. This becomes obvious when you consider their relative potencies can be 100–1000 times lower than E2, yet detected values can be 10–10000 times higher than natural oestrogens (Wise et al. 2011). However, diffuse pollution is not just limited to surface waters. E2 concentrations ranging from 6 to 66 ng/l have also been measured in mantled karst aquifers in northwest Arkansas. This contamination of groundwater has been associated with poultry litter and cattle manure waste applied originally on the land (Ying 2002), which confirms the ubiquity of chemical contamination and the link between the terrestrial and aquatic environment.

1.4.2 Point Sources

The main source of oestrogens to aquatic ecosystems is considered to be point source effluent discharges from WWTW. Incomplete removal of oestrogens during the wastewater treatment process (Gomes et al. 2003) has been supported by studies performed in different countries, identifying the presence of natural and synthetic oestrogenic substances in influents, effluents and in surface waters downstream of WWTW. Sewage treatment commonly involves two stages, primary and secondary treatment, although additional tertiary treatment processes exist in some areas. Primary treatment is shown to be relatively inefficient in removing oestrogens from wastewater (Johnson et al. 2005), and has been largely superseded by the consistently high oestrogenic removal rates of secondary treatment via biodegradation (83, 99.9 and 78% for E1, E2 and EE2 respectively, Ternes et al. 1999). However, it should be noted that biodegradation products can be more harmful than the parent compound and despite improved removal during secondary treatment; we still see adverse effects of treated effluent on fish (Baynes et al. 2012).

Most effluents reach surface waters through point source discharges, elevating concentrations of natural and synthetic EDCs in receiving waters. The major contributors are WWTW effluents, kraft and pulp mill effluents and industrial discharges. Each effluent has a different chemical composition, containing thousands of EDCs including steroid oestrogens, pharmaceuticals, alkylphenol ethoxylates, phthalates, BPA and heavy metals, to mention just a few (Sumpter 2009). Considerable investment has gone into

upgrading treatment processes to try and remove the majority of these chemicals, but evidence based just on natural and synthetic oestrogens sees them being reported in the low ng/l (see Koh et al. 2008 for review) range in influent, treated effluents and river water. Comprehensive studies by Desbrow et al. (1998) across seven STWs in the UK reported treated effluent concentrations 1.4–76, 2.7–48, 0–7 ng/l for E1 E2 and EE2 respectively. Likewise, 17 STW effluents from plants across Europe found comparable concentrations (E1 0.2–35 ng/l, E2 <0.6–13 ng/l, EE2 <0.8–2.8 ng/l) with the highest concentrations found in a Norwegian plant, which only had primary treatment (Johnson et al. 2005). Measuring chemical concentrations in surface waters is challenging and inherently variable, fluctuating hourly with river flow and effluent discharge rate. All oestrogenic steroids have been detected in surface waters in the low ng/l range, often near the detection limit, in the UK (Williams et al. 2003), Italy (Baronti et al. 2000), the Netherlands (Belfroid et al. 1999), Germany (Kuch and Ballschmitter 2001) and the US (Benotti et al. 2009). Due to the difficulty and variability associated with direct sampling of river water, models have also been developed to predict environmental concentrations (Williams et al. 2009; see Chapter 2 for further discussion). Hannah et al. (2009) report surface water concentrations in the UK of 0.3 ng/l for EE2 (the most potent synthetic oestrogen) which are largely in agreement with measurements taken in effluent in the UK (mean concentrations of EE2 0.7 ng/l in the UK, Williams et al. 2003).

1.5 Historical Background and Justification for this Research

Sensitivity to steroid hormones varies between fish species (Purdom et al. 1994). Effects induced by oestrogenic contaminants can range from biomarker responses (a change in biological response) to gross aberrations in tissue development. Low-level physiological changes detected using biomarkers may not necessarily yield significant health consequences for the organism, yet gross morphological disruption to tissues has the potential to impact the health and reproductive capabilities of the individual. Consequently, under high exposure scenarios oestrogenic exposure can yield substantial physiological and morphological alterations to multiple individuals, increasing the likelihood of population-level effects as a result.

Principle disruptive effects of EDC exposures on wild fish are evident from both freshwater and marine environments. The first study to establish an irrefutable link between effluent / oestrogen exposure and disruption to the endocrine system was that of Purdom et al. (1994). Prior to this, anecdotal reports of grossly hermaphroditic fish in sewage lagoons led to field experiments placing caged trout and carp in effluent plumes

which later showed marked increases in plasma vitellogenin (VTG) concentrations (a blood protein normally synthesised by females during oocyte maturation). The presence of the natural oestrogens [estrone (E1) or 17β-oestradiol (E2)] and a synthetic oestrogen used in birth-control pills [17 α-ethinylestradiol (EE2)] have since been linked with the production of VTG in other species of male fish living downstream of wastewater outfalls(Jobling et al. 1998, Desbrow et al. 1998, Tyler and Routledge 1998). In males, VTG genes normally remain inactive, but can be 'switched on' following the interaction of oestrogenic EDCs with oestrogen receptors, thus stimulating the production of VTG in the liver of male fish. The occurrence of VTG in male fish can remain elevated 21 days after exposure (Panter et al. 2000) and proves damaging to kidney, liver and testicular tissue of males. Rodgers-Gray et al. (2000) have also shown that VTG induction is both dose and time-dependent using graded dilutions of sewage effluent. As a result of these studies illustrating the specificity and sensitivity of the VTG response to oestrogen (Shultz et al. 2001, Tyler et al. 1999), plasma VTG concentrations are now used commonly as a biomarker of oestrogen exposure (Hansen et al. 1998).

Incidences of intersexuality (the simultaneous presence of both testicular and ovarian gonadal tissue) have now been recorded in numerous wildlife and fish species (Barnhoorn et al. 2004, Gross- Sorokin et al. 2006, van Aerle et al. 2001, Vigano et al. 2001, Jobling et al. 1998, 2002, Woodling et al. 2006). Considerable evidence implicates the role of potent oestrogenic compounds in the feminisation of male fish. For example, research on wild roach populations in the UK has demonstrated that feminised male fish are reproductively compromised as a consequence of exposure to oestrogens in WWTW effluents (Jobling et al. 1998, 2002, Liney et al. 2006, Lange et al. 2011, Harris et al. 2011). Specifically, male fish living downstream from treatment works show a range of feminised phenotypes (oocytes in the testis, feminised reproductive ducts); a survey of UK rivers found intersex males at 86% of 51 locations below WWTW discharges and in some rivers all adult male fish sampled were feminised (Jobling et al. 2006). As a consequence of sexual disruption, wild intersex roach produce poor quality gametes with a reduced capacity for fertilisation (determined by *in vitro* fertilisation studies; Jobling et al. 2001, 2002) and have reduced reproductive competitiveness in simulated natural breeding scenarios (Harris et al. 2011). Feminisation and intersexuality of wild freshwater fish has also been documented in other countries across Europe, Japan and America (Kavanagh et al. 2004 and references therein), with a growing number being added from estuarine and marine realms (Caprioli et al. 2007). Importantly, with all of this work, the key causative agents have been identified and controlled laboratory experiments have

demonstrated induction of feminised responses.

Endocrine disrupting effects of contaminants on fish are not limited to those described here. Examples of effects from other EDCs include skewed sex ratios in Swedish eelpouts exposed to pulp mill effluents (Larsson et al. 2000) and reduced sperm counts in guppies exposed to Tributyltin (TBT) through sertoli cell damage (Haubruge 2000). Physiologically, long-term exposure of roach to WWTW effluent can compromise their immune status, induce lesions in the kidney and even cause genetic damage (Filby et al. 2007). Moreover, the sheer volume of chemicals entering the aquatic environment can elicit numerous physiological, behavioural and endocrinological alterations simultaneously as a result of exposure to cocktails of chemicals in everyday use. The possible combination of pollutant mixtures occurring in the environment have been minimally explored, but studies have noted reductions in fecundity of fathead minnows in combined dosing of different oestrogens at concentrations below their effect levels (Brian et al. 2007). The impacts of chemical exposure on behavioural outcomes in fish also warrant further exploration as, for example, it has been shown that they can alter dominance hierarchies and breeding behaviour in reproducing groups of zebrafish, *Danio rerio* (Coe et al. 2008b, 2010). All these potentially important outcomes emphasise the complexity involved in understanding how chemicals can impact fish populations.

The variety and severity of adverse effects of EDCs demonstrated in individuals provokes an interest in their subsequent detrimental impacts on fish populations living in contaminated surface waters (Brown et al. 2003, Coe et al. 2008). Attempts to address this question have explored different avenues of investigation, including lifetime exposures and modelling simulations using data from laboratory exposures and wild fish (Grist et al. 2003, Miller and Ankley 2004, An et al. 2009). With particular reference to synthetic oestrogen exposure, large-scale dosing (6 ng/l EE2) of an experimental lake in Canada led to the collapse of the fathead minnow (*Pimephales promelas)* population in 3 years. Similarly, controlled life-long exposures of zebrafish and fathead minnow in a laboratory setting also resulted in reproductive failure (Nash et al. 2004, Lange et al. 2001). More environmentally relevant concentrations of EE2, below 1 ng/l, caused effects on fecundity and sex ratios in fathead minnows. However, all these studies involved single chemical exposures, which leave uncertainties regarding the long term impact of complex mixtures of EDCs on aquatic fauna. As a consequence, efforts have been directed at exposing fish to WWTW effluent; some of these experiments resulted in complete sex reversal of male roach over a period of 3 years (Lange et al. 2009).

Notwithstanding the results of these exposure studies, evidence is still inconsistent regarding the detection of measurable changes in population sustainability in wild fish (An et al. 2009, Mills and Chichester 2005). Likewise, the complexity of any long-term ramifications of EDC exposure on fish populations, including more subtle changes in behaviour and genetic variation, remain relatively unexplored. With this in mind, there is real cause for concern regarding the unknown impacts of multigenerational exposure to EDCs.

1.6 Chemicals and Aquatic Wildlife Populations

Chemical compounds continue to enter the environment despite uncertainty in the level of risk posed to aquatic fauna (Hutchinson et al. 2006). Given that the existing literature base emphasises the prevalence of sexual disruption in wild fish, together with evidence for sex reversal, compromised immune function and genetic damage in long-term exposure studies, there is an urgent need to understand how fish populations respond to EDCs over multiple generations. Many rivers have been contaminated with effluents since at least the 1960s, therefore these fish populations have experienced a degree of chemical exposure throughout their lifetime. This accentuates the need for additional studies to clarify the repercussions of contaminant-induced physiological, morphological or genetic change at the level of the population. Fundamentally of most concern is the impact of pollutants on the number of individuals (population sustainability).

Severe declines in some coarse fisheries, along with large-scale variations in fishery performance have persisted in many river systems within the UK (Cowx and Frear 2004). Albeit anecdotal, anglers and fisheries scientists agree upon observations of altered population structure and diminished catches in major UK rivers over the last 20 years (Cowx and Welcomme 1998). However, the precise causative factors have never been fully elucidated. Natural variation in stock recruitment accounts for some of this variability but relatively few long-term demographic studies explicitly link human-induced drivers with fish population productivity.

Similarly, few cases have been able to reliably link the sub-lethal effects of chemicals with population decline. Exceptions include a substantial body of literature on heavy metals from mining activities or industrial discharges, which accumulate in the environment, resulting in acute and chronic toxicity triggering population declines. Historically, acid mine wastes in Canada and the US have contributed to declining populations of bull trout (*Salvelinus confluentus*) and other salmonid species (Farag et al.2003). The combination of mining and smelting industries produce high concentrations

of acidic fallout and heavy metal stress in Canadian lakes, which over several decades have forced multiple species extinctions and cessation of ovulation in female fish (Beamish 1976). Likewise, decreased female reproductive condition in wild yellow perch (*Perca flavescens*) has been associated with dietary selenium and copper uptake in contaminated Canadian lakes (Pyle et al. 2005). Toxicological studies support these field observations. For example, controlled exposures using the fathead minnow pair breeding test saw reduced reproductive success in response to cadmium (Sellin and Kolok 2006). Cadmium has also been linked to disruptions in steroidogenesis in humans.

Environmentally persistent, bioaccumulative and potent EDCs such as PCBs have also been cited as the causative agents in the decline of many apex predators. This is believed to be the case with the otter (*Lutra lutra;* Mason and Macdonald 2004), where a large part of their diet is composed of fish, which may act as an important route of exposure. Declines in eel populations have also been attributed to the presence and tissue burden of PCBs (Palstra et al. 2006). Diffuse sources of chemicals, from agricultural runoff, or from the eutrophication of surface waters through nutrient enrichment also provoke indirect impacts (of depleted oxygen levels and turbidity) on aquatic fauna in freshwater and coastal environments globally (discussed further in Chapter 6). Increasing catalogues of chemicals are entering the aquatic environment, many of which are not discussed here, but include: pharmaceuticals, pesticides, fungicides, Polycyclic Aromatic Hydrocarbons (PAHs), dioxins and flame-retardants. The mechanistic actions of these chemicals alone, or in combination, as bioaccumulates of complex mixtures, remain largely unknown, which complicates any assessment of the risk posed to aquatic fauna. Ascertaining effects on fish populations is further confounded by the difficulties in recognition of chronic, sub-lethal effects on naturally fluctuating populations and the complex interactions with additional environmental stochasticity.

Detrimental health effects resulting from exposure to chemical mixtures present in surface waters are likely to be more prolific in future due to the impact of climate change (Johnson et al. 2009, Green et al. 2013). Many European catchments will experience lower river flows and increased environmental challenges will be posed to wildlife that could ultimately impact their sustainability. Given the potential of key chemicals as agents of decline in migratory species, including salmon and spawning eels (Maes et al. 2005), as well as non-migratory species of barbel (*Barbus barbus*) and brown trout (*Salmo trutta*) (Al-Salhi et al. 2012), there is a need for effective tools to monitor the effects of these chemicals on the health and population sustainability of a wider range of

fish species (Tyler et al. 2008).

1.7 Water quality and Fish Population Dynamics

A complex symbiosis between natural lotic environments and human activity pose a pervasive threat that compromises the value of freshwater as a pristine habitat for other successive freshwater organisms (Dudgeon et al. 2006). Furthermore, humans compound the synergistic interactions between existing ecosystem stressors and biota. However, our knowledge of these effects has not kept pace with human-induced alterations to the riverine environment. Concern for river system sustainability is not restricted to countries facing rapid socio-economic change in the near future. Voeroesmarty et al. (2010) recently recognised that the burgeoning population impose acute levels of risk to water security and biodiversity in developed and developing countries, emphasising the threat to global freshwater biodiversity.

Evaluating the influence of chemical pollution on fish populations is complicated by a multitude of biotic and abiotic factors, which can equally provoke fluctuations in abundance. Alterations to normal endocrinology and physiological function in individuals may arise as a consequence of any number of additional drivers; therefore it is important to highlight other possible reasons for stock declines (see Figure 1.4) in an effort to contextualise chemical impacts. To date, significant reductions in wild fish populations in response to EDC exposure are absent from the literature. Likely due to the myriad of additional factors that can influence important life-history parameters of individuals, surreptitiously affecting population sustainability.

Fish populations are inherently stochastic, yet long-term trends denote that >35% of freshwater fish species are considered vulnerable or threatened (IUCN 2009). Unprecedented riverine transformation (through physical alteration, habitat loss and degradation, water extraction, over-exploitation, pollution and the introduction of invasive species) are all responsible for this biodiversity loss (CBD 2005, MEA 2005), which outweighs that seen in either the terrestrial or marine environment (Jenkins 2003). Within the UK, virtually all rivers have a profound legacy regarding, but not restricted to, alterations in flow and water quality that have been accelerating since the 1930s (Petts 1988). It is these impacts that are compounded by the relatively small size and isolated nature of freshwater systems, which accentuate the difficulty to escape environmental change.

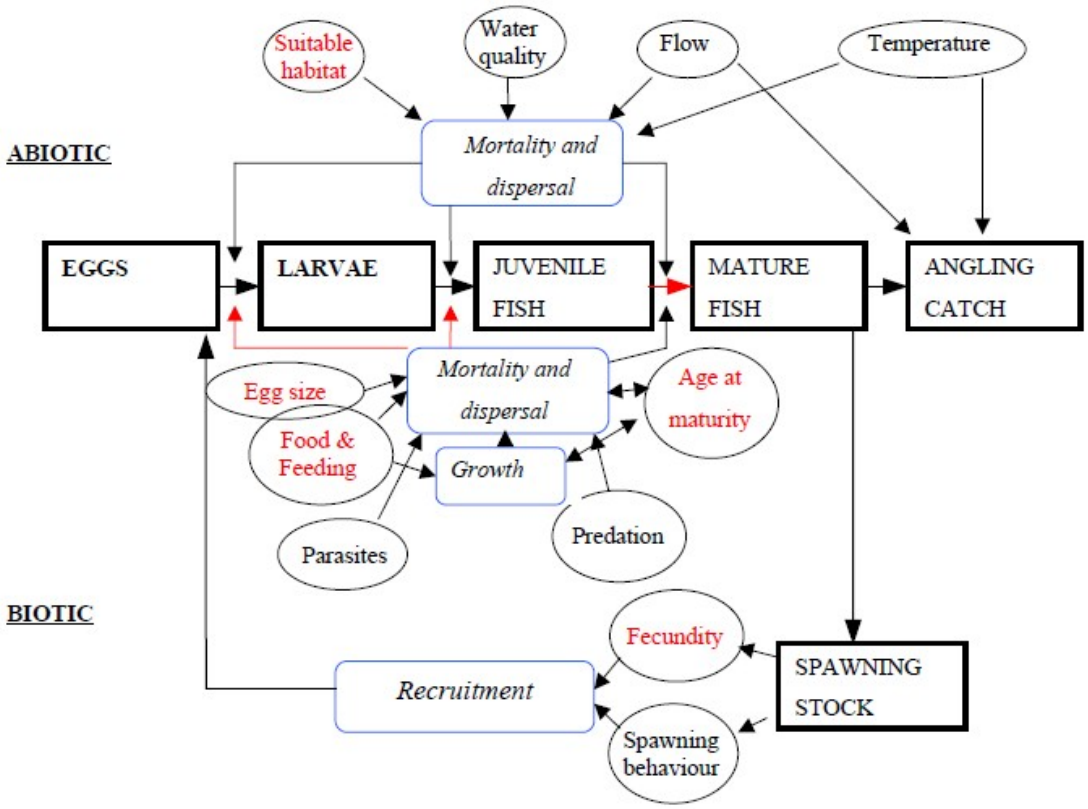


Figure 1.4 Complex flow diagram illustrating the factors that can govern fish abundance in a cyprinid fishery. Current knowledge gaps are highlighted in red. Taken from Musk and Britton (2007)

Disrupting the fabric of the aquatic system drives the global loss in fish biodiversity (Cowx and Portocarrero-Aya 2011). Human-induced modifications of river systems (see Cowx 2002 for review) reduce water quality and accelerate habitat loss; concomitantly intensifying pressure on resident organisms, and giving rise to declines in local fish populations. Large-scale variations in coarse fishery performance have persisted in many river systems within the UK, from the late 1980s onwards (Cowx and Frear 2004). Natural variation in stock recruitment (linked to climatic variation) accounts for some of this variability but relatively few long-term demographic studies discriminate human-induced drivers of population productivity, above other dominant environmental variables. For example, commonalities in environmental factors driving recruitment success (year class strength, YCS) in four cyprinid species in the R. Thames and the Yorkshire Ouse point, in effect, to water temperature governing potential YCS while discharge/flow determines realised YCS (Nunn et al. 2003, Cowx and Frear 2004). These strong year classes contribute significantly to the population and retain a productive legacy; nevertheless, factors governing fish population dynamics are more complex and often site/species specific (Figure 1.4). Water quality impairment is markedly more prolific than air or land pollution throughout England and Wales. Figure 1.5 summarises

the serious incidents (category 1 & 2— deemed major and significant impacts) recorded from 2002 to 2008 and shows the scale and persistence of the issue. Each incident alters the balance of flora and fauna; effects are often unique to the contaminant source and can impinge directly on fish through chronic toxicity, or indirectly through the procurement of unsuitable water quality or unfavourable habitat conditions.

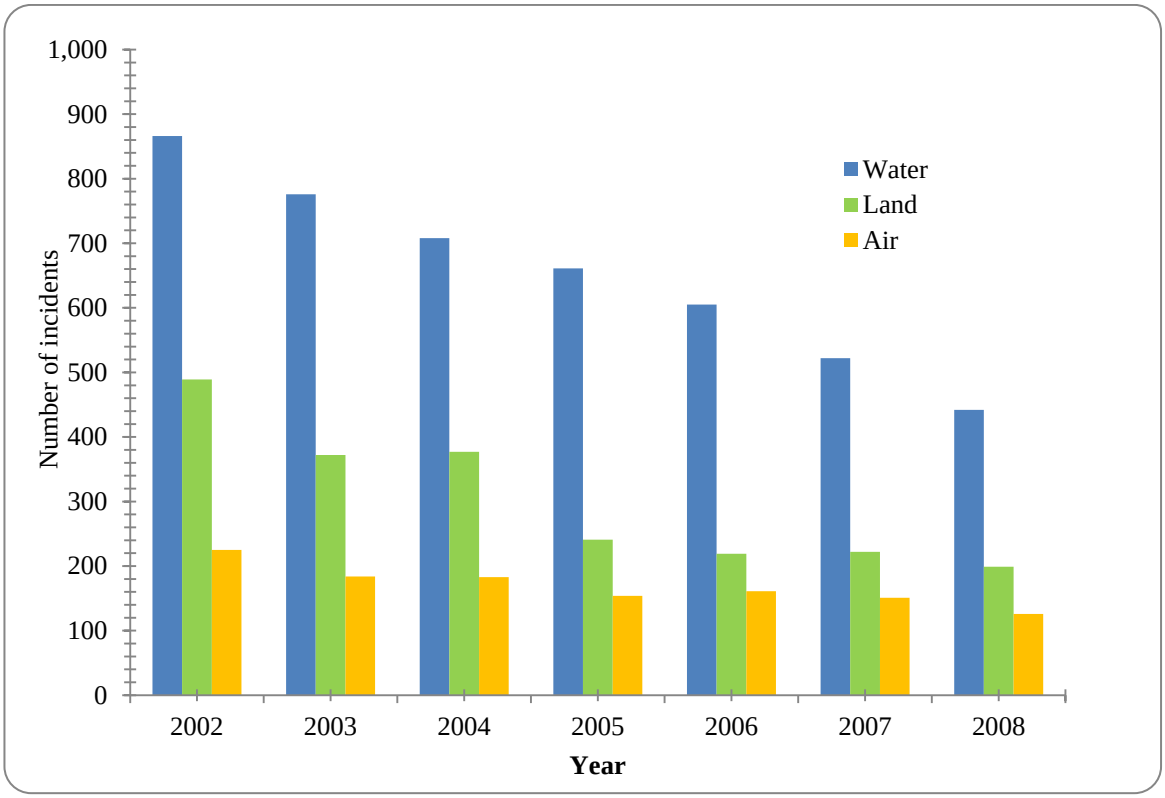


Figure 1.5 Serious pollution incidents affecting water, air and land quality from 2002–2008. Data sourced from the Environment Agency archive (2010)

Demographic impacts of poor water quality can remain subtle, often precluded by sporadic environmental fluctuations. Improvements in water quality are often reflected in stock recovery and the return of sensitive, less tolerant species following 'clean up'. Recovery of migratory salmonids in the River Tyne, UK (Mawle and Milner 2007) and pre-dated species shifts in the River Trent, from roach to chub-dominated communities (Cowx and Broughton 1986) are well-publicised examples of improving water quality impacting on fish populations. Similarly, the River Dearne has a history of heavy pollution, yet periods of fish population improvement were seen in the 1990s when diminishing industrial/mining activities and improved wastewater treatment meant that chemical water quality met coarse fish requirements (Cowx 2004). However, fish stocks still suffer acute exposure to ferrous compounds during high rainfall events so periodic population crashes still hamper fish abundance and successful fisheries developing in this

river. Sustainability and viability of fish populations is therefore an important indicator of river water quality, and remain an important monitoring tool with respect to riverine ecosystem health.

1.8 Fish Population Quantification

Stock assessment and estimation of census size (total number of individuals in a population; N_c) have historically been undertaken in exploited marine species. Nevertheless, it has now become evident that census estimates may provide a misleading impression of genetic integrity of a population (Turner et al. 2002, Hauser et al. 2002, see Palstra and Ruzzante 2008 for a review). Arguably a more robust statistic is effective population size (N_e), which refers to the subset of individuals that successfully contribute to the next generation. N_e can inform on the process of random genetic drift and is a critical parameter affecting the rate at which genetic diversity is lost from a population. For example, threatened species with small population sizes are increasingly at risk of extinction because inbreeding and loss of genetic diversity are deemed unavoidable (Frankham et al. 2002) - vulnerable taxa have a probability of extinction of approximately 10% within 100 years (Frankham 2005). Conceptual application of benchmarks of N_e across many species suggests a threshold value of >50 individuals are required to avoid detrimental loss of genetic variation (Soule 1980) and 500 for long-term considerations of adaptability. The loss of genetic variants can result in decreased population fitness and increased risk of extinction, in addition to adversely affecting the ability of populations to cope with environmental change.

Conservation of populations considers the ratio between N_e and N_c, which differs greatly depending on demography and life history of a species. Given that fish can display diverse reproductive strategies and that N_e is influenced by breeding success and equitability in sex ratio, it is perhaps unsurprising that estimates vary by orders of magnitude. Palstra and Ruzzante (2008) provide a comprehensive, updated review of N_e across a diverse range of taxa and found a median N_e/N_c ratio of 0.14, suggesting that N_e may be orders of magnitude smaller than census sizes. Perhaps unexpectedly, studies in exploited marine species, such as the New Zealand snapper (*Pagrus auratus*) have shown that N_e (176, CI 80–720) was five orders of magnitude smaller than the population estimates derived from fishery data (Hauser et al. 2002). The authors suggest that this may result from the disproportionate contribution of older, more fecund females, which may create bias in reproductive success among individuals and therefore reduce N_e. If such small N_e/N_c ratios are commonplace in fish populations, then many stocks may be

in danger of losing genetic variability, which could result in reduced adaptability, population persistence, and productivity (despite appearing numerous in population surveys). Indeed, Hauser et al. (2002) also saw a decline in genetic diversity in the same population from 1950 to 1998, which was likely due to fewer parental fish contributing during reproductive events.

Therefore, in light of reproductive disruptions caused by chemicals, there is potential for substantial impacts on N_e through changes in sex ratio and skews in reproductive success. Likewise, where only a few individuals are capable of breeding successfully we may also find evidence of a genetic bottleneck, where population numbers may have been reduced dramatically over a short period of time. If this is the case, N_e and genetic diversity may appear reduced in highly polluted environments. Whether long-term impacts of effluent exposure may have compromised breeding dynamics and genetic variation in wild fish species remains to be explored.

1.9 Genetic Variation: Importance, Use and Application for Fish Populations

Accurate quantification of population genetic diversity is of paramount importance in enhancing our understanding of which species are most at risk from anthropogenic pressures. Historically, research has documented extinction rates as a means of ascertaining species rarity and vulnerability. However, a multifaceted approach recognising the importance of retaining genetic diversity — which depicts the allelic and genotypic variation within a population (Frankham et al. 2002)—has now become widely accepted (Bickham et al. 2000).

Genetic variation in a population originates from past mutational events, which generate polymorphic regions in the genome. Polymorphisms are the material basis upon which evolutionary forces then act (Hedrick 2005) to create patterns of genetic variation within and between populations. Genetic structure of populations is created by four key evolutionary processes: mutation, gene flow/ migration, natural selection and random genetic drift (Bagley et al. 2002). Overall, mutation, drift and natural selection act to drive genetic disparity between populations, whereas gene flow/migration reduces differentiation between populations by inducing exchange of individuals/genotypes (Figure 1.6, Frankham 1996). A pre-requisite for an adaptive response to local selection pressure, genetic variation can therefore inform on the evolutionary potential within a species (Hauser et al. 2003).

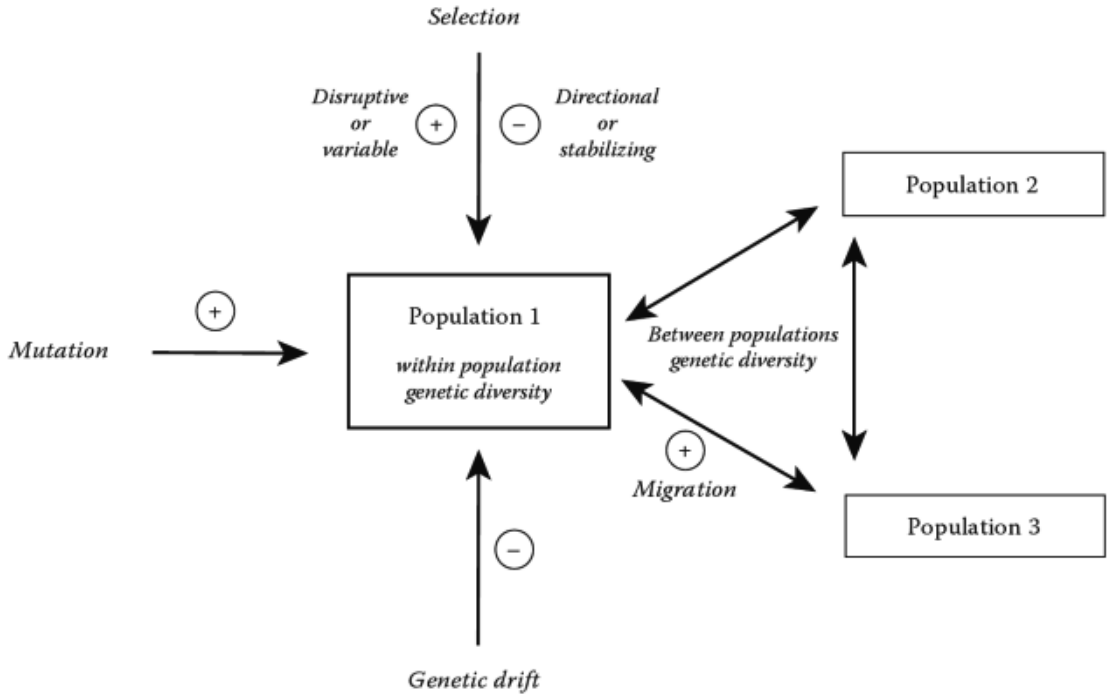


Figure 1.6 Main evolutionary forces acting between and within populations, which bring about changes in within-population genetic diversity (+ or -, increase and decrease respectively). Taken from Amiard-Triquet et al. (2011)

Central considerations to the long-term conservation of fish population viability include preservation of genetic variation through maintaining successful reproductive capability. In many fish species this is complicated by a difficulty to ascertain past fluctuations in population size and breeding dynamics, responsible for generating population structure. For instance, the European eel (*Anguilla anguilla*) has been subject to intense investigation following a long term stock decline across Europe estimated to be around 99% (ICES 2012), however the precise factor responsible for the decline is yet to be defined (Pujolar et al. 2013).

Patterns of genetic diversity between populations are assumed to adhere to a model of isolation by distance (IBD; Wright 1943). Model expectations propose that populations living in close proximity should exhibit a similar genetic composition, as a simple consequence of easy dispersal and a higher rate of gene flow. Therefore, geographically distant populations of the same species are less likely to exchange individuals and genes. However selection pressures exerted by environmental conditions may lead to adaptations. Signatures of selection in natural populations of three-spined stickleback (*Gasterosteus aceleatus*) in the Baltic Sea, have resulted in a convergence in genotype composition (reducing genetic distance) between populations at multiple eutrophic sites in an open environment with no barriers (Lind and Grahn 2011). Impacts in isolated

populations or species with limited dispersal may be greater, emphasising the plasticity required in successful species.

1.10 Genetic Diversity and the Association with Fitness

Fundamental to the long-term sustainability of wild populations, genetic diversity can inform on a cascade of events which shape an organism's phenotype/fitness (Figure 1.7). With this in mind, the potential of EDCs to alter the reproductive contribution of some individuals could cause alterations in genetic variation and patterns of gene flow, with wider implications for population sustainability. Previous studies have investigated whether increased individual fitness arises as a result of increased genetic diversity; a finding that generally appears to hold true in many populations (Reed and Frankham 2003). Conversely, decreasing heterozygosity (indicative of inbreeding) in natural populations of the butterfly, *Melitea cinxia*, significantly increases extinction risk through adverse effects on components of larval survival, adult longevity and egg-hatching rate (Saccheri et al. 1998). Nevertheless, this relationship is not observed ubiquitously. Salmonid research in Finnish populations found genetic diversity and fitness-related traits of offspring showed significant association in two out of three species (Primmer et al. 2003); indicating correlations between genetic diversity and fitness can vary between species.

The link between increased fitness and higher genetic diversity would infer that individuals/populations with more diversity would be at an advantage when resisting effects of toxicant exposure and chemical challenge. This has been demonstrated in multiple strains of the midge, *Chironomus riparius,* where cadmium stress was found to be less likely to affect genetically diverse populations compared to more inbred strains. In the main, increased levels of population diversity generally permit a greater tolerance to chemical exposure due to increased genetic variability. Collectively, the potential benefits of increased genetic variability for reproductive fitness, fitness-related traits (salinity tolerance; Shikano and Taniguchi 2002), enhanced resistance to infectious diseases (Major Histocompatibility Complex (MHC) heterozygosity, Penn 2002) and physical malformations (Shikano et al. 2005) suggest that offspring heterozygosity should be maximised. Knowledge of breeding patterns and mate choice is critical for understanding how chemicals impact populations (Coe et al. 2008), as alterations to parental contributions will subsequently impact the next generation. All heritable differences between individuals are due to differences in their DNA, which can be investigated through the application of advancing molecular methods.

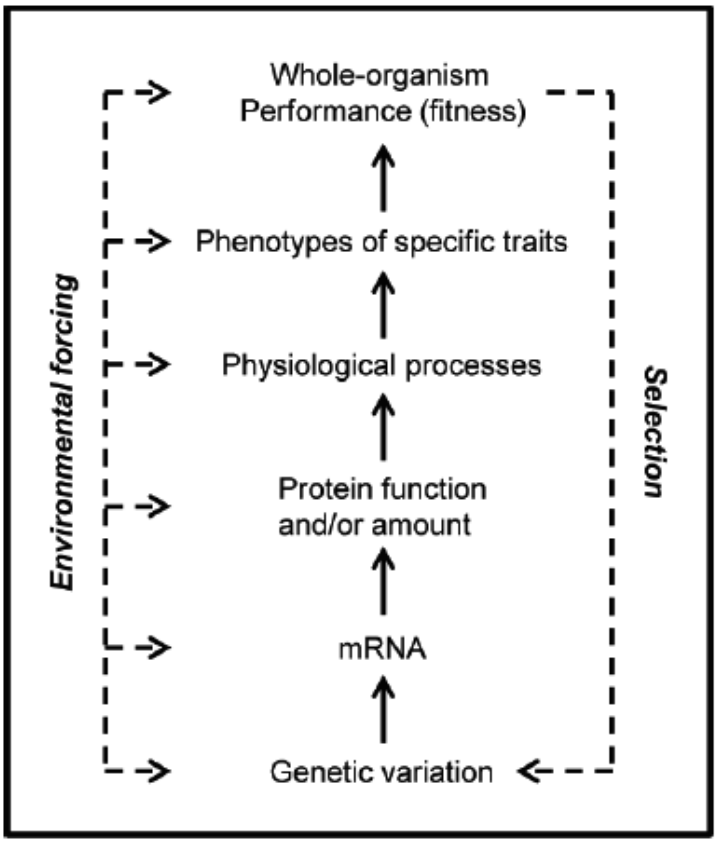


Figure 1.7 Flow chart depicting the intrinsic connection between variation at the genetic level and the cascade of events that then alter whole-organisms fitness

1.11 Molecular Markers in Ecotoxicology and their Application to Fish Populations

Understanding fish population dynamics using genetic applications was, until recently, restricted to model organisms (extensively studied species, usually with full genomic resources). However, significant advancement in the use of molecular applications has fostered the progression of monitoring human impacts on wild organisms and detecting direct repercussions at the population level (Carvajal-Rodriguez et al. 2005). A number of complementary techniques are now available to inform on changes at the genetic level and can be applied to elucidate patterns between and within individual organisms (see Chapter 2).

To advance our understanding of fish populations and investigate contaminant-driven genetic erosion (loss of trait-specific genotypes), the development and application of genotyping approaches have commonly used selective and neutral markers (van Straalen and Timmermans 2002). Examples of these have included, RAPD, RFLP, AFLP, SNPs and DNA microsatellites. For studies of fish population genetics, the most commonly implemented molecular techniques have utilised neutral markers (i.e. those loci not under selection). Over the past two decades, an increasing number of studies have preferentially

chosen the use of DNA microsatellites over genetic markers such as allozymes; recognising their utility in genetic studies of natural populations (Estoup et al. 1998). Features such as hypervariability and extreme abundance in all eukaryotic genomes, makes these short tandem repeats (STRs) or simple sequence repeats (SSRs) display widely varying levels of polymorphism deriving mainly from variability in length (Ellegren 2004). It is this variability in tandemly repeated units (2–5 base pairs in length) between individuals and populations, predominantly due to DNA slippage, that creates distinctly recognisable genotypes. Analysis and genotyping of these microsatellite polymorphisms from small amounts of tissue has become increasingly popular since the late 1980s, with the advent of PCR (polymerase chain reaction); drastically reducing the time and cost involved in genotyping large numbers of samples. The details of this approach are expanded upon in Chapter 2.

Application of microsatellite loci in natural populations yields previously unattainable information on genetic variability within populations. Similarly, by applying theoretical models of microsatellite evolution, geneticists can also discern differences between populations. This foundation has seen their applications extended in the field of fisheries and aquaculture; now used extensively in characterising phylogeny and genetic stocks, along with population and conservation genetics (for review see Chistiakov et al. 2006). For example, salmonid research has consistently employed microsatellite loci to inform on spatial population genetics and philopatry (Dionne et al. 2008) even over small geographic scales (Durrant et al. 2011). Likewise, later studies on European grayling (*Thymallus thymallus*) employed 10 microsatellite loci to examine the genetic structure among 27 UK populations, unravelling the impact of historical stocking and population bottlenecks and how these implicate future management decisions (Dawnay et al. 2011, Swatdipong et al. 2010).

1.12 Use of Microsatellites for Parentage Analysis

Fish species have diverse and complex mating systems that can be understood with the advent of genetic markers. Although initial development can be timely and expensive, microsatellites have proven informative for discriminating parentage and kinship in natural and laboratory populations (Jones and Ardren 2003) and still remain the marker of choice. Unravelling parentage is based on the simple concept of Mendelian inheritance where parents pass on one of two alleles at each locus to the offspring, which therefore carries one allele from each parent. Utilisation of a suite of several microsatellite loci can produce a genotypic profile that is unique to each individual, which is unlikely to be

replicated by another (Chistiakov et al. 2006).

Microsatellite loci offer high resolution for determining maternity and paternity of individuals (Dewoody and Avise 2000). This is done by matching the alleles from multiple microsatellite loci from each offspring with those from the potential parents (Figure 1.8). Extensive application of this technique in fish species is aligned with aquaculture husbandry practices, for the analysis of paternity and inbreeding (Liu and Cordes 2004). Early studies concern farmed salmonids, including Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), where microsatellites can assist in selective breeding of superior progeny through the identification of successful parents (Wright and Bentzen 1994, Norris et al. 2000). Microsatellites markers enable determination of reproductive success, in reference to the different breeding strategies seen in fish. For example, Dewoody and Avise (2000)

summarise the parental contribution of males and females of different species to each nest in multiple sire species (Figure 1.9) assigned using microsatellite determination of parentage. Group-spawning behaviour often complicates assessment of parental contribution, a problem that can be moderated through the use of microsatellite markers; highlighting skewed parental contributions are common in fish (Fessehaye et al. 2006). The degree of multiple paternity in natural populations of a highly social cichlid (*Neolamprologus pulcher*) were unravelled through the use of five highly variable microsatellite markers, finding an extremely high rate of multiple paternity in these cooperative breeders (Dierkes et al. 2008). This study benefited from the ability to sample entire breeding groups, including subordinates, which is impractical especially in wild populations of mobile species where complete parental sampling is not feasible. Reconstruction of kinship groups using statistical methods has gone some way towards addressing this issue and provided further insights. In parentage analysis of wild populations, reconstruction of parent-offspring assignments indirectly using statistical likelihood simulations is now possible, to group individuals into different classes of relationship, often full-siblings, half-siblings and unrelated individuals. From here, the parental genotypes can be reconstructed and used for parentage analysis, although attention must be given to inconsistencies relating to mutations, null alleles and genotyping errors (see Jones et al. 2010 for further discussion).

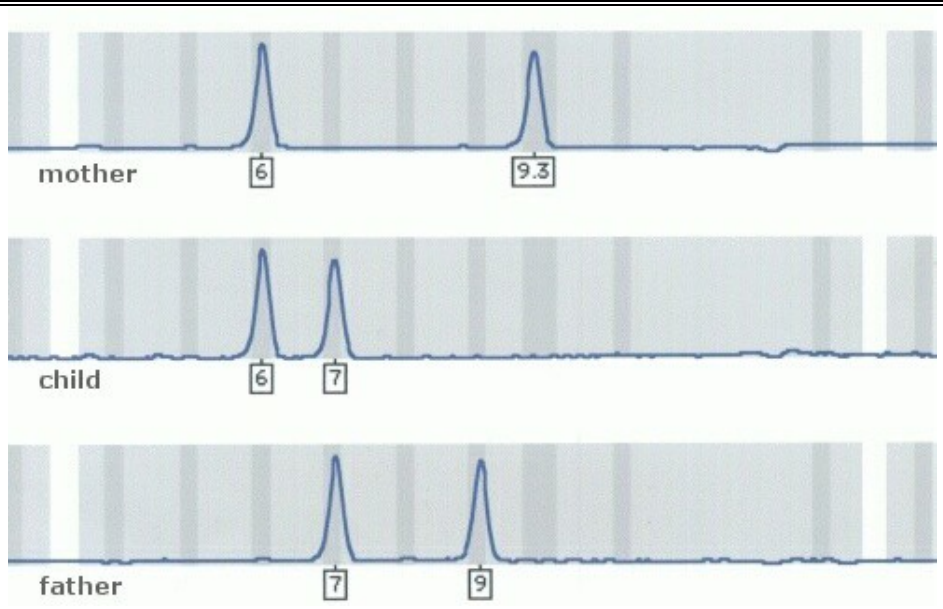


Figure 1.8 Paternity screening using microsatellite markers. The child has inherited markers 6 and 7 from it's mother and father respectively. Taken from Adams (2008)

Similar approaches can be used in ecotoxicology, to examine if chemical exposure can alter the contributions of parental fish to the next generation. Alterations in patterns of parentage between fish colonies in the presence/absence of EDCs will remark on the effects on reproductive success of individuals, from species exhibiting a wide range of breeding strategies. DNA microsatellites were applied to decipher patterns of parentage in zebrafish in the presence of EE2, which subsequently showed altered dominance hierarchies and breeding behaviour of group spawning fish in the laboratory (Coe et al. 2008b, 2009, 2010). If replicated in the wild, this disruption to reproductive hierarchy has potential implications for population genetic diversity. Implications of these alterations at the level of the population, remains unexplored.

1.13 Factors Affecting Genetics of Wild Fish Populations

Historically, the more pervasive factors affecting fish populations were deemed to be overexploitation and introductions of alien species; however, focus has shifted towards drivers such as habitat fragmentation, climate change and pollution in recent years. Derivation of persistent human-induced drivers has most often been deduced from the manipulation of variables experimentally within in a control population. Notwithstanding this, difficulty comes in isolating the precise influence involved, especially in field conditions as factors often act in combination. Emancipated stocking practices play a significant role in the proliferation of alien species within the freshwater and marine environment. Behind habitat fragmentation, the introduction of alien species/disease is

reputedly the biggest threat to global biodiversity (Diamond 1989). Ultimately, such an influx of novel species will be mediated at the population-level through processes such as displacement, predation, hybridisation, and introduction of new diseases/pathogens (McGeoch et al. 2010). Widespread, introductions of rainbow trout (*O. mykiss*) and cutthroat trout (*O. clarki*) in the United States has resulted in loss of diversity in native species because of substantial genetic introgression (Allendorf and Leary 1988, Leary et al. 1993). Analogous research in Poland, Sweden and Italy has documented hybridisation and dominance of introduced or domestic forms of northern whitefish (*Coregonus peled),* American lake trout (*S. namaycush*) and brown trout (*S. trutta*) respectively (Rhymer et al. 1996). Even small introductions of non-natives can impart strong hybrids into the population which out- compete natives and become homogeneous; threatening genetic integrity of well- established and adapted species. Genetic pollution of this fashion can substitute native genotypes with those of the introduced species, thus driving native species closer towards endangerment or extinction.

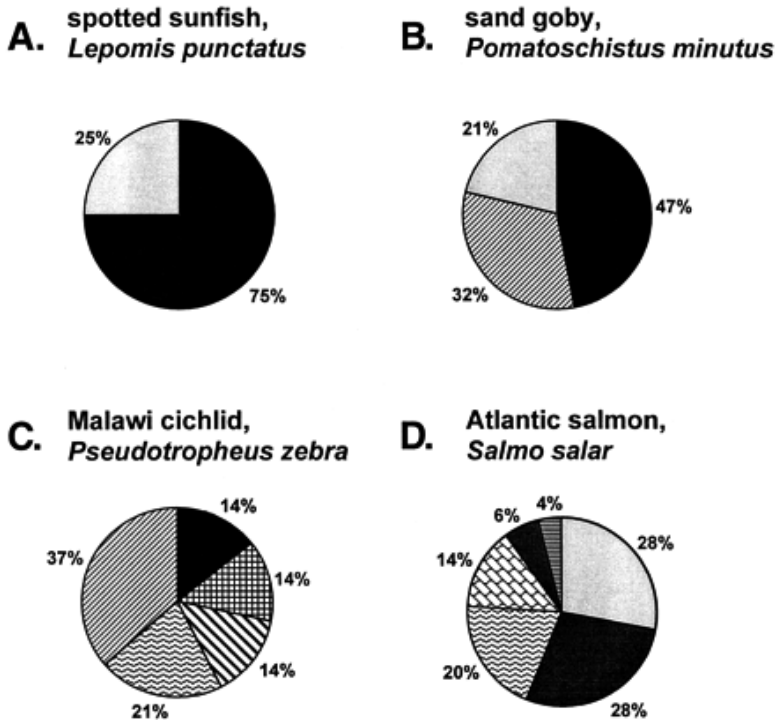


Figure 1.9 Examples of reproductive skew in a representative nest from each of four fish species. Each pie diagram summarises the relative contributions (genetically deduced) of different mothers (A and B) or fathers (C and D) to the pool of embryos within a nest or brood. Taken from Dewoody and Avise (2000)

Habitat fragmentation imparts isolation between members of a population, preventing replenishment and migratory behaviour of individuals, ultimately reducing genetic diversity over time. Motivated by mechanisms of reduced habitat availability and changes in metapopulation structure and quality, we see mounting evidence for erosion of both

neutral and adaptive genetic diversity in transformed habitats (Johansson et al. 2007). A migratory freshwater fish, the white-spotted charr (*Salvelinus leucomaenis*), exhibits significant genetic differentiation between upstream and downstream populations, recently isolated by the construction of artificial dams in Japan (Yamamoto et al. 2004). Reduced genetic diversity in above-dam populations was also attributable to the effect of habitat fragmentation brought about by damming. Maintaining population connectivity minimises the implications of habitat fragmentation, ensuring long-term viability of populations. Any habitat conditions that limit the delivery of new genotypic signatures/alleles will thus compound the effects of genetic inbreeding.

Contrastingly, over-exploitation tends to result in removal of individuals at a rate which is beyond that of normal recruitment. Historically, research addressing genetic changes within and among populations primarily focussed on marine fish/invertebrates and hunted ungulates (Allendorf et al. 2008). Smith et al. (1991) were one of the few to have demonstrated this unambiguously in orange roughy (*Hoplosthethus atlanticus*) prior to the global documentation of the 'collapse' in cod stocks (Pauly et al. 2002). Preferential harvest of heterozygous individuals from the orange roughy population off New Zealand invoked a significant decrease in genetic diversity at three separate fishing grounds; a decline in abundance of the species followed. Additional fish species with wide-ranging extant population sizes and different life histories have also succumbed to intense fishing pressure; common examples include the red drum *Sciaenops ocellatus* (Turner et al. 2002), the New Zealand snapper *Pagrus auratus* (Hauser et al. 2002) and North Sea cod *Gadus morhua* (Hutchinson et al. 2003). Manifestation of this reduction in population size often brings about a subsequent decline in genetic diversity, therefore limiting the inherent ability of a population to adapt to environmental fluctuations.

Broadly speaking, global warming and climate change carry similar connotations both reducing population size. Firstly, temperature shifts may bring about alteration in the availability of habitats, constraining the available habitat size or displacing many individuals toward lower latitudes or finding that suitable niches now exist at higher altitudes (Hughes 2000). Thermo-sensitive processes, such as those concerning reproduction, are sensitive to even slight fluctuations in temperature, rendering entire populations vulnerable to climatic change. Widespread generalist species, namely *Drosophila melanogaster* and *D. sbobscura* appear to be key species, which have shown genetic alterations in response to climate change (Heerwaarden and Hoffman 2006). The polymorphic alcohol dehydrogenase (*Adh*) locus within *D. melanogaster* has been shown

to be under climatic selection and has the capacity to respond relatively quickly to climate change. From this genetic response it should be possible in the future to link shifts in allele frequencies to climate fluctuations, although for now rapid changes in polymorphisms in *D. subobscura* provide some of the first evidence for genetic responses to a changing climate.

Essentially climatic alterations will modify aquatic habitats through a number of interactions with additional environmental stressors. As a result, much of the literature on temperature effects on genetic diversity comes entwined with studies examining multiple environmental stressors (Hill et al. 2006, Oetken et al. 2009, Vogt et al. 2007). Most recently, Oetken et al. (2009) observed the effect of a temperature gradient and TBT exposure on genetically diverse and highly inbred populations of the midge *Chironomus riparius* to elicit the combined response of chemical and temperature stress. Their findings paralleled those of Vogt et al. (2007), suggesting that the interaction between additional stressors including even moderate temperature fluctuations could have severe implications for genetically compromised species.

Culmination of the aforementioned mechanisms is obvious when concerned with population sustainability. Unlike the above factors, the myriad of environmental contaminants to which species are exposed, provokes an array of genetic repercussions at both the local population and species level. Simply speaking, the introduction of a toxic factor to a habitat bestows an advantage to those few individuals with a more resistant genotype (Bol'shakova and Moiseenko 2009). Ultimately this can provoke a gradual loss in genetic diversity over time.

1.14 Links between Genetic Diversity and Pollution

Knowledge of breeding patterns, levels of genetic diversity, population size and structure of wild fish populations are critical for understanding how human disturbance impacts populations. Likewise, fish represent diverse reproductive strategies, including all types of sexuality and breeding systems, paternal care, mate choice, courtship, and breeding behaviour. Chemical exposure has the potential to alter any of these mechanisms and thus impact the next generation, without necessarily modifying demographic indices of biomass and density. Successively, this could alter the genetic makeup of the impacted population and affect their genetic integrity.

Genetic structure and variability of wild fish populations has been poorly addressed with regard to the effects of introduced anthropogenic contaminants. Theoretically, when a population becomes exposed to a novel selective pressure such as a chemical

contaminant, they can respond in a number of ways. Firstly, the population size could decrease dramatically over several generations resulting in population bottlenecks and lowered genetic diversity (Bickham et al. 2000). Similar reductions in genetic diversity of populations can arise through strong selective pressures or directional selection of tolerant genotypes, in response to the new environment. Coors et al. (2009) found evidence in wild populations of *Daphnia magna* of local adaptation to pesticide contamination, which was more pronounced in populations originating from pools surrounded by increasing agricultural land use. Lastly, contaminant driven mutagenicity and genotoxicity of chemicals may drive increases in genetic variation due to high mutation rate and the introduction of new genetic variants in the gene pool (Ellegren et al. 1997).

Consistent demonstrations of hypothesised effects are not always evident in wild populations. A recent study of a moderately pollution-tolerant cyprinid fish, the central stoneroller, (*Campostoma anomalum*) in Ohio, USA compared levels of genetic diversity at impaired sites across a heavily urbanised catchment. Findings suggest no relationship between genetic diversity and habitat quality for central stonerollers, despite earlier work indicating that the allele and genotype frequency shifts they observed in central stoneroller populations were due to selection induced by environmental contaminants (Waits et al. 2008). Similar findings from three-spined stickleback *Gasterosteus aculeatus* (Lind and Grahn 2011) and mummichog (*Fundulus heteroclitus*; McMillan et al. 2006) populations, from pulp-mill effluent and PCB-contaminated environments respectively, showed no difference in genetic variation between polluted and cleaner reference sites. Paradoxically, a more sedentary species, the brown bullhead (*A. nebulosus*), in the Great lakes, showed that genetic diversity estimates from mitochondrial DNA haplotypes were always lower in populations from contaminated sites. The authors suggest that a history of environmental degradation at the contaminated sites may have reduced population size in the past, resulting in reduced present-day genetic diversity (Murdoch and Hebert 1994).

1.15 Challenges in Assessing Chemical Impacts on Populations

The importance of discerning population level impacts of EDCs present in WWTW effluents remains a key area of research (Mills and Chichester 2005), with significant economic implications for the UK and worldwide. Establishment of population level consequences for wild fish populations has commonly exploited one of three methods:

1. Observation of physiological effects and changes in life history patterns that can be directly related to detrimental outcomes at the population level;

2. Direct assessment of the impact of EDCs through the use of fish populations, either in a controlled laboratory setting or those obtained from the wild;
3. Model construction using data on survival, growth and reproduction from laboratory exposures and wild populations to forecast long-term population level effects.

Direct physiological effects of chemical exposure, observed at the level of the individual, have frequently been addressed through the use of model fish species, the fathead minnow (FHM, *P. promelas*), Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) (Ankley and Johnson 2004). Demonstrating population-relevant implications of tissue burdens, biomarker responses and behavioural alterations resulting from exposure, requires strong causal linkages with health outcomes influencing population size.

Laboratory exposures are arguably the most reliable and popular way to assess the health effects of chemicals, as they allow for control for confounding factors and provide levels of replication that are difficult to obtain in the wild. Parrott and Blunt (2005) observed a decrease in fertilisation success of FHM at concentrations of EE2 <1 ng/l and complete feminisation at concentrations >3.5 ng/l. Similarly, environmentally relevant concentrations of 17-β-trenbolone (used for muscle growth in cattle) reduced fecundity of FHM (Ankley et al. 2003). Exposure of adult zebrafish has comparable reproductive outcomes, in reducing the number of eggs produced, altering sperm quality and reducing fertilisation success in breeding colonies exposed to 5 ng/l (Santos et al. 2007) and complete feminisation when exposed to 2 ng/l EE2 (Orn et al. 2003). Effects on successful reproduction are the most extreme demonstrations of disruption, when dictated by EDCs.

Most of these are short-term studies, which have limitations for estimating risk of prolonged, chronic exposure. Long-term exposures are required for increasing relevance at the population level, and have found exposures to ~5 ng/l EE2 during sexual differentiation have resulted in sex reversal of male fish or reproductive failure in several fish species (Nash et al. 2004, Lange et al. 2001, 2009). In both instances, these were in accordance with short-term responses in VTG of males and alteration in sexual differentiation, suggesting that organism-level endpoints can precede effects at higher levels. In summary, laboratory exposures provide valuable information on mechanisms of action and disruption to the reproductive activity/development of fish. However this knowledge cannot, by itself, predict long-term effects on reproduction and population sustainability. For example, full life-cycle exposure of FHM to an anti-cancer drug found

no effects on reproduction at concentrations higher than those typically found in the environment (0.18µg/l) (Williams et al. 2007). This provokes questions regarding the relevance and extrapolation of laboratory experiments to wild fish populations, when conducted at higher concentrations than those found in the environment.

Several studies have utilised computational modelling to investigate the potential long-term implications of alterations to life history traits, such as fecundity and survival. Model construction is typically based on data measuring survival, growth and reproduction, derived from laboratory exposures and through large-scale sampling of wild fish populations. To date, conservation biology has used matrix models to make prognoses of population viability (see work of Caswell) based on variables measured in individuals. The merit of such applications lies in their ability to simulate scenarios that would often be impossible to test experimentally at such a large scale or in certain species. Of particular interest to research on EDC exposure, matrix models can identify a life cycle stage that is experiencing reduced survival probability, and can then model the implications of that reduced survival on population growth. Quantitatively, if the population multiplier (expressed as λ within the model) is < 1, the population has a negative growth rate.

When applied to the question of long term EDC impact, several modeling studies have found similar effects. Grist et al. (2003) used previously published data on FHM (Lange et al. 2001) to construct a matrix model, which found a negative growth rate ensued when the concentration of EE2 exceeded 3.11 ng/l. Likewise, changes in fecundity in the FHM as a result of exposure to 17 β-trenbolone, result in projected decreases in average population size (after 2 years), of 51% following exposure to 0.027 µg/l and 93% following exposure to $\geq$ 0.266 µg/l (Miller and Ankley 2004). Overall, continual exposure to trenbolone, at concentrations equal to or greater than 0.027 mg/l were projected to have average equilibrium population sizes that approached zero. These studies are limited to small model fish species that inhabit freshwater environments. Few address how EDCs could alter population dynamics of larger, long-lived species commonly found in estuarine or brackish environments where WWTW discharges are equally likely. Exposure to EE2 in cunner (*Tautogolabrus adspersus*) caused a reduction in fecundity of 39% and reduction in survival of 15%. Applying these changes in reproductive success and mortality to a matrix model projected a population decrease of 75% after 10 years of exposure (Gutjahr-Gobell et al. 2006).

These studies have all used laboratory exposure data to extrapolate to the population

level, on the assumption that these effects may be mimicked in wild populations. One of the few modelling studies using wild fish data, in particular the proportion of viable embryos from feminised roach (*R. rutilus*) (Jobling et al. 2002) predicted that gonadal feminisation would have a minimal effect on the population growth rate and size of wild roach populations. However the authors did suggest that increased risk was posed to local roach populations when intersex was present in combination with commercial fishing practices (thought to harvest mainly males). All the above modelling studies attempt to link individual effects to potential ecological outcomes on wildlife, through large-scale changes in demographic parameters. To date, subtle shifts in genetic variability and integrity of fish populations have rarely been a considered consequence. Reductions in effective population size, N_e, have been predicted based on female-biased populations (provided sex reversed individuals are fertile, Cotton and Wedekind 2009). However; sex reversal has not, to date, been identified in wild fish.

The principal limitation of modelling approaches is the availability of reliable data to parameterise and validate model outputs. Capturing the complexity of natural population dynamics in a modelling framework is impossible, given the paucity of information on behavioural interactions in fish populations, along with a limited knowledge on the potential role of adaptation and habitat-driven selection. These knowledge gaps are compounded by a relatively poor understanding of density-dependent survival, which can exert a strong regulatory force on the size of fish populations (Lorenzen and Enberg 2002) and thus suppress any effect of reproductive impairment resulting from EDC exposure. Despite these caveats, predictive modelling has demonstrated that chemical exposure could reduce fish populations over many generations; addressing questions that would be impossible to simulate experimentally or answer without the decimation of multiple wild fish populations. Notwithstanding this, population-level effects remain the most important, unanswered, question in the study of EDCs.

1.16 Overall Hypothesis and Aims

My overarching experimental hypothesis for this thesis is that there are significant effects of oestrogenic effluent exposure on wild fish populations.

The aim of this project was therefore focused on determining if population-level consequences of exposure to WWTW effluent are evident in wild fish living in UK rivers, using roach, *R. rutilus*, as the sentinel study species. Limited preexisting research in this area has applied modelling approaches extrapolating from endpoints, such as alterations in physiology and life history parameters, to predict the response of fish populations to long-term effluent exposure. However, these inferences neglect the effect that WWTW effluent may have on patterns of population (and group) genetic structure and variation, and this was one of the key themes addressed within this thesis.

Whilst the array of chemical compounds, including EDCs, entering the aquatic environment is vast, oestrogenic contaminants were a focal constituent throughout this thesis. I aimed to address a sequence of key research objectives and questions outlined below, with a focus towards generating a novel set of genetic information on a popular cyprinid species, which could be used to assess potential impacts of effluents and advise on successful future management and conservation.

1.17 Key Objectives

1. To elucidate the genetic relationships among and between roach populations across the UK, within the wider context of genetic variation across this common cyprinid species. Genetic data for roach are currently limited to a few southern English populations and some from lake environments elsewhere in Europe, with investigations primarily focussed on connectivity and phylogeographic history. Contextualising levels of genetic diversity, variation and differentiation is essential for all other work carried out in this thesis, so is a crucial first step for understanding patterns of gene flow between proximal roach populations and if they conform to typical models of panmixia and isolation by distance.
2. To investigate evidence for small effective population size (N_e) in populations of wild roach inhabiting rivers contaminated with different concentrations of oestrogenic WWTW effluents. Demographic estimates of population size have historically been recorded to inform on temporal fluctuations and sustainability of populations. However, census data can misinform on the viability and size of populations, giving a limited snapshot representation upon which management and conservation decisions are made.

Hence it is important, in the absence of population modelling, to determine and compare the number of breeding individuals (a better long-term indicator) across locations enduring differing levels of exposure.

3. What is the impact of long-term effluent exposure on parentage dynamics of group spawning fish and do males with subtle intersex characteristics breed successfully? Are populations with feminised males sustainable in the long term? In particular, I determine the effects of life-long exposure to an oestrogenic WWTW effluent on reproductive success in breeding roach over two generations, compared with those kept in clean water. Controlled long-term exposures, combined with population- modelling, have suggested that oestrogens could reduce the size of fish populations; however the determination of multi-generational effects of oestrogenic contaminant exposure on reproductive contribution in adult roach and their offspring remains unexplored.

3. To interpret the role of oestrogenic WWTW effluent contamination within the wider context of additional environmental drivers, known to modulate the size of aquatic faunal assemblages. The effect of effluent exposure on roach populations is likely to be one of many factors affecting genetic diversity and population persistence in fish in the wild. Chapters 4 and 5 solely focussed on the role of prolonged oestrogenic contamination in driving population dynamics; however a multitude of other environmental factors can modulate demography. The role of additional land use, habitat and environmental variables in explaining patterns of species diversity and genetic diversity of wild roach populations was therefore the focus of the final experimental chapter (Chapter 6).

Chapter 2

General Materials and Methods

Page 35-66

2.1 Geographical Modelling

To embark on a study focussed on the release of chemicals into the aquatic environment, it is first necessary to assess the level of risk they pose. Chapter 1 outlines the growing concern surrounding the presence of chemical contaminants in aquatic matrices and their adverse effects on living organisms; however it is important to now confirm their presence in UK surface waters and identify areas of greatest concern (and populations most likely to be exposed). To derive the concentration of a chemical in the environment there are commonly two approaches; analytical chemistry and hydrological modelling. This chapter describes how and why a hydrological modelling approach, incorporating the use of geographic mapping, was used to estimate effluent concentrations present in rivers across the UK. From these predictions, it is also possible to derive the steroid oestrogens component present in the river, and denote if concentrations are of sufficient magnitude to produce a potential impact on aquatic organisms, in this case fish.

2.1.1 Introduction to Geographical Information Systems

Geographical Information Systems (GIS), in their most basic sense, offer a tool with which one can create, manipulate, analyse and display information in an integrated fashion. Manipulation of a digital map interface is the most superficial application of this tool which can explore spatial patterns of biological, cultural, demographic, economic, geographic and physical phenomena. Application of geographical information alongside database entries allows execution of statistical analyses and the exploration of predictive models in a visual setting. This ability to examine environmental data at multiple levels of spatial organisation is one of its most advantageous applications, providing holistic simulations of modelled data in a representative 'real world' setting. Popularity of modelling in GIS stems from its simple conceptual representations of geographic features or processes, however the limited complexity compared to the natural landscape warrants careful interpretation.

2.1.2 Modelling with GIS

GIS systems are incredibly useful in hydrology and have been employed to aid representation of unfamiliar geographical features or processes. Often GIS mapping acts in an ancillary capacity, for visualising data modelled in another package. However both applications are becoming more cohesive with the availability of increased computing power and the ability to make calculations using GIS toolboxes. This integration, to form GIS water quality models, has revolutionised hydrological modelling, because it

eliminates the need to make field measurements for every geological aspect.

The approach used here employs a digital data structure of a river network in conjunction with a terrain model, compiled by the Centre for Ecology and Hydrology (CEH Wallingford, UK). Assembling these two matrices allows calculation of rainfall/runoff flow directions, to ascertain the watershed/catchment boundary of any river system of interest (Figure 2.1). Following this, the catchment boundary then acts as a remit within which characteristics can be extracted and quantified (for use in the hydrological model) from the primary input data used in the GIS layers. Additional map layers referencing all the artificial influences (abstractions, discharges, reservoirs) in the area, allow identification of those within the catchment boundary that may additionally alter river flow. Artificial influences / point source discharges (for which daily outflow is known) are then entered into the model to allow estimations of both natural and influenced flow dynamics based on topography.

To date, this hydrological modelling method has been applied extensively by the Environment Agency (EA) and Scottish Environment Protection Agency (SEPA). Utilising this framework provides a consistent source of natural flow statistics, allowing conceptual models to be developed, modified and visualised across any catchment. For example, simple applications of this model offer predictions of the natural flow available to dilute each persons waste, finding a difference of two orders of magnitude between rivers in the West and Northeast of England (Johnson et al. 2007). Likewise, GIS water quality models have proved a vital tool in the prospective simulation of effects of abstraction or discharge changes on riverine conditions, where modification of the natural flow regime could adversely impact stream ecology. In line with ecological guidelines, the mapping component can ultimately provide a visual interpretation of river reaches stressed by artificial influences.

Hydrological models can be highly informative, especially in water stressed areas of the world (Green et al. 2013). However, unmapped catchments require considerable initial investment for development, including large amounts of data on the location, size, human population, and flow for each sewage treatment works. An additional problem comes akin with ungauged catchments, in that there is limited experimental data for model corroboration. Verification of the model used here has taken place on the River Trent and clearly identifies the impact of a large STW discharge on biochemical oxygen demand and orthophosphate signal, in agreement with observed in-situ data (Williams et al. 2009). As a result, similar techniques have been used internationally, in the US (Anderson et al.

2004), Japan (Johnson et al. 2011) and for similar applications deriving effluents and oestrogen river concentrations in Chile (Bertin et al. 2009).

Figure 2.1 Visual depiction of maps consisting of terrain and river network information (A) that are broken down into small catchments related to river stretches. (B) Modelling each river segment separately allows precise quantification of runoff flow and direction over land, along with artificial influences, prior to incorporating interactions with other tributaries

2.1.3 Effluent Exposure Modelling of UK Catchments

All environmental matrices are contaminated to some extent by chemicals; many of which have the potential to impact negatively on the environment. The presence of exogenous chemicals often arises from human usage/consumption, following which, the excreted portion is delivered to sewerage systems and finally to STWs (see Figure 2.2 for an example). Many substances are removed during water treatment (through adsorption and microbial action) and although rates depend on the nature of the chemical (Kuemmerer 2009). Contraceptive hormones are a particular case in point, although a multitude of drug compound residues have been reported in the influent, effluent and receiving water of STWs (Ternes et al.1999). Treated effluents subsequently enter the aquatic environment at multiple entry points more or less continuously; exacerbating accumulation of chemicals in aquatic ecosystems and surreptitiously impacting lotic organisms.

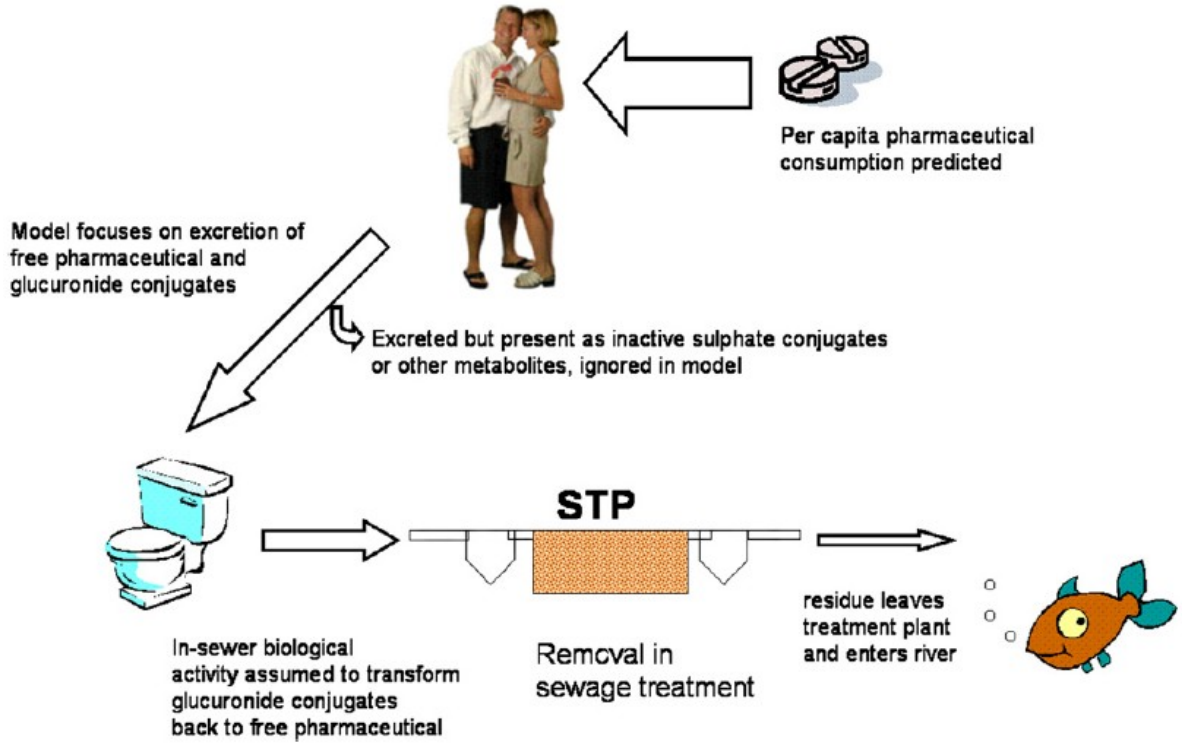


Figure 2.2 Fate and excretion of pharmaceuticals from human consumption, through the body and into the sewerage system; eventually ending up in surface waters. Sourced from Johnson et al. (2007)

2.1.4 Study Region

A core part of this thesis aims to identify population level effects of effluent pollution on native fish populations that inhabit UK rivers. Primary focus was given to the Thames catchment as it is one of the driest, has a history of feminised fish (Jobling et al. 1998, 2002) and has previously been modelled for steroid oestrogen contaminants (Williams et al. 2009). With high levels of urbanisation throughout much of the Thames region, including housing 25% of England's population (Figure 2.3, Environment Agency 2006), it is unsurprising that water resources are facing severe pressure. Such a growing demand will ultimately affect sewage treatment infrastructure and the waters into which effluent is discharged. Several rivers within the region already contain a high proportion of effluent with point source pressures putting 43% of our rivers at risk (Environment Agency 2006). The Thames catchment alone contains 83,000 km of sewers and 344 STWs (Evans 2003), over half of which are without tertiary treatment (Table 2.1 and Box A for full definition of treatment types). Advanced treatment has already been shown to improve micropollutant removal (Lee et al. 2008) and reduce endocrine disruption in wild fish (Baynes et al. 2012, Filby et al. 2010), so the release of poorer quality effluent within the Thames catchment may increase chemical loading in these rivers.

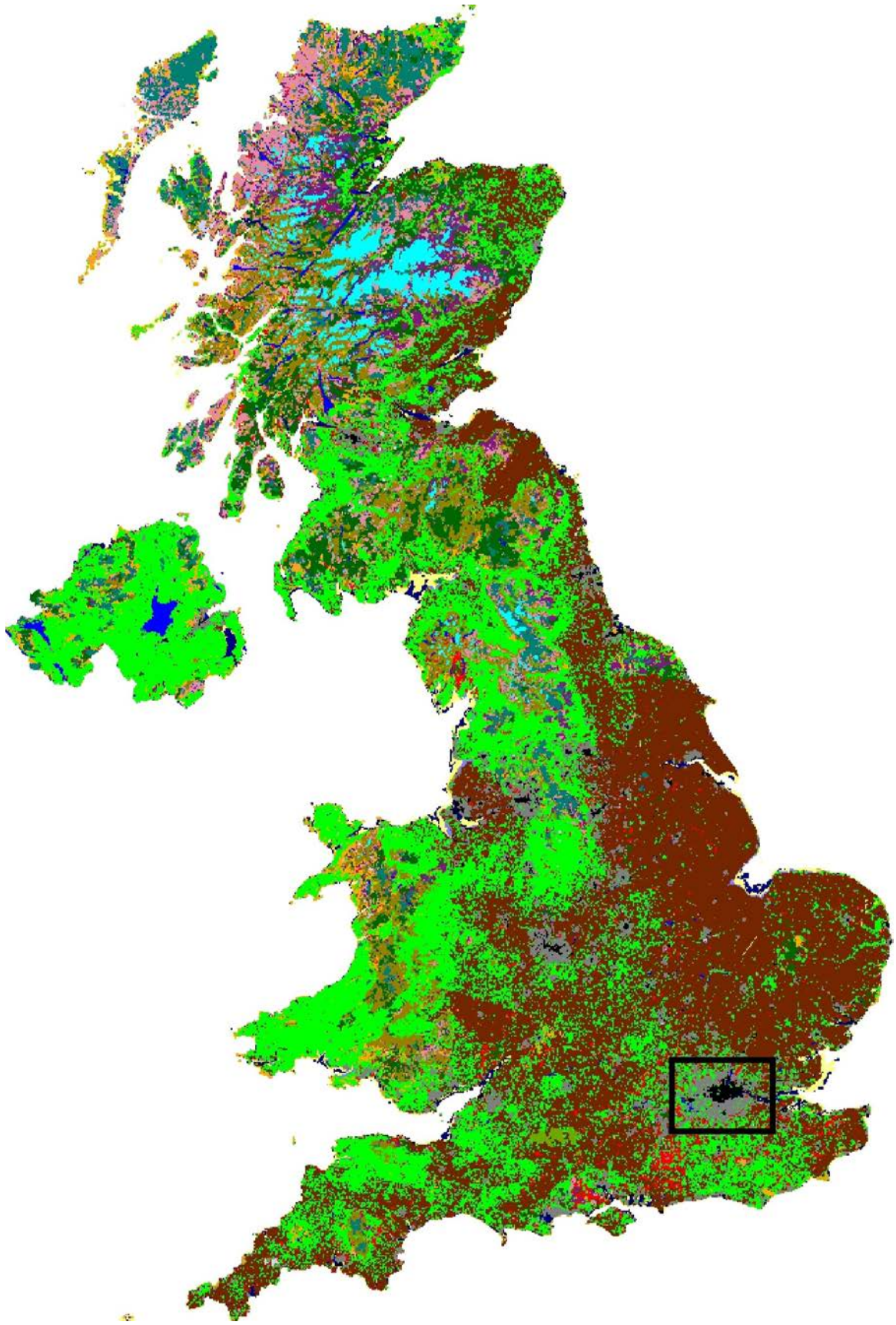


Figure 2.3 Location of the Thames catchment, in reference to the UK (black box). Much of the 12 971 km^2 of catchment is heavily urbanised (grey and black shading) and an average rainfall of 710 mm per annum creates drier conditions than those of Rome. This means increasing volumes of treated sewage are poorly diluted

Box A: Ofwat sewage treatment classification (taken from Williams et al. 2008)

The 2005 scheme classifies STWs into seven different categories.

P (Primary): Includes STWs whose treatment methods are restricted to preliminary and primary treatment (screening, comminution, maceration, grit and detritus removal, pre-aeration and grease removal and storm tanks, plus primary sedimentation, including where assisted by the addition of chemicals such as Clariflow).

SAS (Secondary activated sludge): As primary, plus STWs whose treatment methods include activated sludge (including diffused air aeration, coarse bubble aeration, mechanical aeration, oxygen injection, submerged filters) and other equivalent techniques including deep shaft process, extended aeration (single, double and triple ditches) and biological aerated filters as secondary treatment.

SB (Secondary biological): As primary, plus STWs whose treatment methods include rotating biological contactors and biological filtration (including conventional filtration, high rate filtration, alternating double filtration and double filtration) and root zone treatment (where used as a secondary treatment stage).

TA1 (Tertiary A1): STWs with a secondary activated sludge process whose treatment methods include prolonged settlement in conventional lagoons or raft lagoons, irrigation over grassland, constructed wetlands, root zone treatment (where used as a tertiary stage), drum filters, microstrainers, slow sand filters, tertiary nitrifying filters, wedge wire clarifiers or Clariflow installed in humus tanks, where used as a tertiary treatment stage.

TA2 (Tertiary A2): STWs with a secondary activated sludge process whose treatment methods include rapid-gravity sand filters, moving bed filters, pressure filters, nutrient control using physico-chemical and biological methods, disinfection, hard chemical oxygen demand (COD) and colour removal, where used as a tertiary treatment stage.

TB1 (Tertiary B1): STWs with a secondary stage biological process whose treatment methods include prolonged settlement in conventional lagoons or raft lagoons, irrigation over grassland, constructed wetlands, root zone treatment (where used as a tertiary stage), drum filters, microstrainers, slow sand filters, tertiary nitrifying filters, wedge wire clarifiers or Clariflow installed in humus tanks, where used as a tertiary treatment stage.

TB2 (Tertiary B2): STWs with a secondary biological process whose treatment methods include rapid gravity sand filters, moving bed filters, pressure filters, nutrient control using physico-chemical and biological methods, disinfection, hard COD and colour removal, where used as a tertiary treatment stage.

2.1.5 Choice of Study Sites

Collecting fish samples from the wild was based around sampling sites representing a full span of very low and high effluent concentrations. Previous work has found that the incidence and severity of intersex fish is positively correlated with the proportion of treated effluent from human sources in receiving waters (Jobling et al. 2009). Therefore selecting highly impacted sampling sites may improve the likelihood of finding a significant signature of pollution in wild roach populations. However, to conduct studies into the impact of effluent exposure on roach, it was necessary to predict environmental concentrations of effluent likely to be encountered in all rivers within the chosen study area.

Table 2.1 Composition of treatment types across all modelled STWs in the Thames catchment. Definitions of the treatment type categories can be found in Box A. Despite population density being extremely high around the Thames, we can see that less than half of the STWs have been upgraded to advanced/tertiary treatment processes

	Treatment type of	Number in Thames	% of total STWs	% of total as groups
Primary	P	2	0.58	58
Secondary	SAS	37	10.76	
	SB	160	46.51	
Tertiary	TA1	8	2.33	42
	TA2	30	8.72	
	TB1	76	22.09	
	TB2	31	9.01	
Total		**344**	**100**	**100**

2.1.6 Model Description

Previous risk assessments of aquatic contamination have been conducted using STWs in isolation (EA 2008). More recent developments commonly employ a software system combining GIS for data storage and visualisation, combined with simple mathematical models to predict chemical fate (Feijtel et al. 1997). Evolution of this approach into a more comprehensive exposure assessment necessitates the integration of all discharges and influences along an entire river stretch; accounting for dilution, degradation and addition. Several models have used these principles in order to develop regional risk of down-the-drain chemicals from point source discharges and spatio-temporal variability in river flows: The PhATE (Pharmaceutical Assessment and Transport Evaluation) in US waters (Anderson et al. 2004), GREAT-ER (Geography-referenced Regional Exposure Assessment Tool for European Rivers) in Europe (Feijtel et al. 1997), and Low Flows 2000 Water Quality eXtension model (LF2000 WQX; Wallingford HydroSolutions,

Wallingford, UK) in the UK (Keller and Young 2004, Johnson et al. 2007, Williams et al. 2009).

The basic principles of the model utilised here are built around the existing system for LF2000-WQX. This was an extension of the Low Flows 2000 geographic information systems hydrological model (Young et al. 2003) developed by the Centre for Ecology and Hydrology at Wallingford, UK. LF2000-WQX integrates hydrological and water quality models to generate statistical distributions of down-the-drain chemical concentrations in any river system, accounting for any point source discharge. Realistic predictions have been undertaken using this same model for steroid oestrogens in the UK (Williams et al. 2009). These simulations have been corroborated recently with in- situ sampling, measuring steroid oestrogen concentrations in river water (Williams et al. 2012).

Exact details of the model and its equations are given in Keller and Young (2004). However, only a sub-section of the LF2000-WQX is used here to combine hydrological models of rivers with emission points of STWs. Output data can then be geo-referenced and visualised in ArcGIS v10 (ESRI, Redlands, California). Here I focused on predicted levels of effluent constituting river flow as a means to assess the likelihood of exposure to fish populations. For each river/catchment of interest, every STW is modelled based on the following data obtained from the EA (and water companies); location of the outfall, treatment type (see Box A for explanation); the dry weather flow from the works itself and the domestic population served by that STW (Keller and Young 2004). Natural variability of river flow duration curves (estimated though rainfall, runoff and hydrology of soil types; Keller and Young 2004) are adjusted by the influence of artificial features such as discharges; to gain a reach by reach understanding of monthly influence volumes of additional point source emissions (Williams et al. 2009). Accumulated assessment of emission loading integrates the characteristics of each reach separately, using the flow upstream, any STWs in that reach and any additional tributaries or lateral inflows (Johnson et al. 2007). This same sequence of assessment is applied for each interconnected reach within the river network; cohesively marrying processes of accumulation and dilution of point-source chemicals, to generate river and location specific scenarios of contamination.

The model output of primary interest (percentage of WWTW effluent present in surface waters) depends only on flow rate (dilution), any artificial inputs upstream, and the discharge of effluent from the STW. Flow rate of the river is not entered in the model as a set value for each river reach; instead it is based on extensive flow data archives. Multiple

flow scenarios likely to occur every month (simulating high, moderate and low flow conditions) enter the model to produce a distribution curve of concentrations of effluent in the river reach. For example, the 90th percentile prediction of percentage effluent constituting river flow can be taken as indicative of low flow conditions. Conversely, examining this along with the 10th percentile estimates of effluent concentrations (indicative of high river flow conditions) allows comparisons of best and worst case scenarios for a stretch of river.

2.1.7 Effluent Modelling Output

Once run, the model produced 12 monthly artificially-influenced flow curves and an estimate of the influenced long-term flow duration curve, for each reach. Each reach has an output similar to that seen in Table 2.2, with a separate excel spreadsheet file for every month and also an annual average summary data sheet.

Spreadsheet outputs (of 90^{th} percentile effluent concentration for example) were then applied to existing polyline layers drawn in ArcGIS, which represent all significant rivers within the Thames catchment. Attributing these modelled effluent values to interconnected river reaches using their spatial attributes (national grid references -- NGRs) means they can be manipulated to produce colour-coded maps (Figure 2.4a and b) for all rivers where roach reside. Finally, spatially referenced locations of interest (or sample sites) could then be associated with the upstream and downstream grid references for each reach, inheriting the effluent concentration and flow characteristics modelled for that stretch. Corresponding values for mean, 10^{th} and 90^{th} percentile effluent concentrations were obtained for each site using this same method, assuming continuous flow of effluent from STWs in to all river reaches.

Resulting maps were then utilised to identify 'hotspots' of effluent contamination and therefore act as a focus for obtaining wild roach samples. Similarly, any river stretches that appear relatively unpolluted with effluent may exhibit suitable fish populations from which 'reference' samples could be sourced. Ideally for Chapter 4, paired high and low contaminated sites with viable roach populations would be obtained within relative proximity, using these maps. However this was not always possible. The same process was therefore extended to additional catchments within the UK, when looking to source suitable sites for fish population samples. Application of the same modelling approach was used for the Anglian region, due to its relative proximity to the Thames. Unless otherwise stated, this method was used throughout this thesis to predict levels of effluent expected at sample sites, both the mean effluent concentration and worst case scenario

(the 90th percentile) are used.

Table 2.2 Typical contents of an output file from the LF2000-WQX model. Each individual reach has values for every category seen in this table and once matched with their spatial references (NGRs) they can then be visualised using ArcMap

times	Explanation
RchId	Reach Identifier
USNodeName	Name of top of reach
USNGR	Upstream grid reference of reach
UsNodeType	Upstream river node type (J = junction, D is sewage treatment works discharge, W = water quality monitoring point, U = tributary start point)
DSNodeName	Name of bottom of reach
DSNGR	Downstream grid reference of reach
DsNodeType	Downstream river node type (J = junction, D is sewage treatment works discharge, W = water quality monitoring point, O = Basin outlet)
Q_USMn	Upstream mean flow (m^3/sec)
Q_USSD	Upstream standard deviation flow
Q_US90	Upstream 90th percentile flow
Q_DSMn	Downstream mean flow(m^3/sec)
Q_DSSD	Downstream standard deviation flow
Q_DS90	Downstream 90th percentile flow
Dilut_DSMn	Mean effluent concentration (%)
Dilut_DSSD	Standard deviation of effluent concentration (%)
Dilut_DS90	90th percentile effluent concentration (%)
RchLength	Reach length

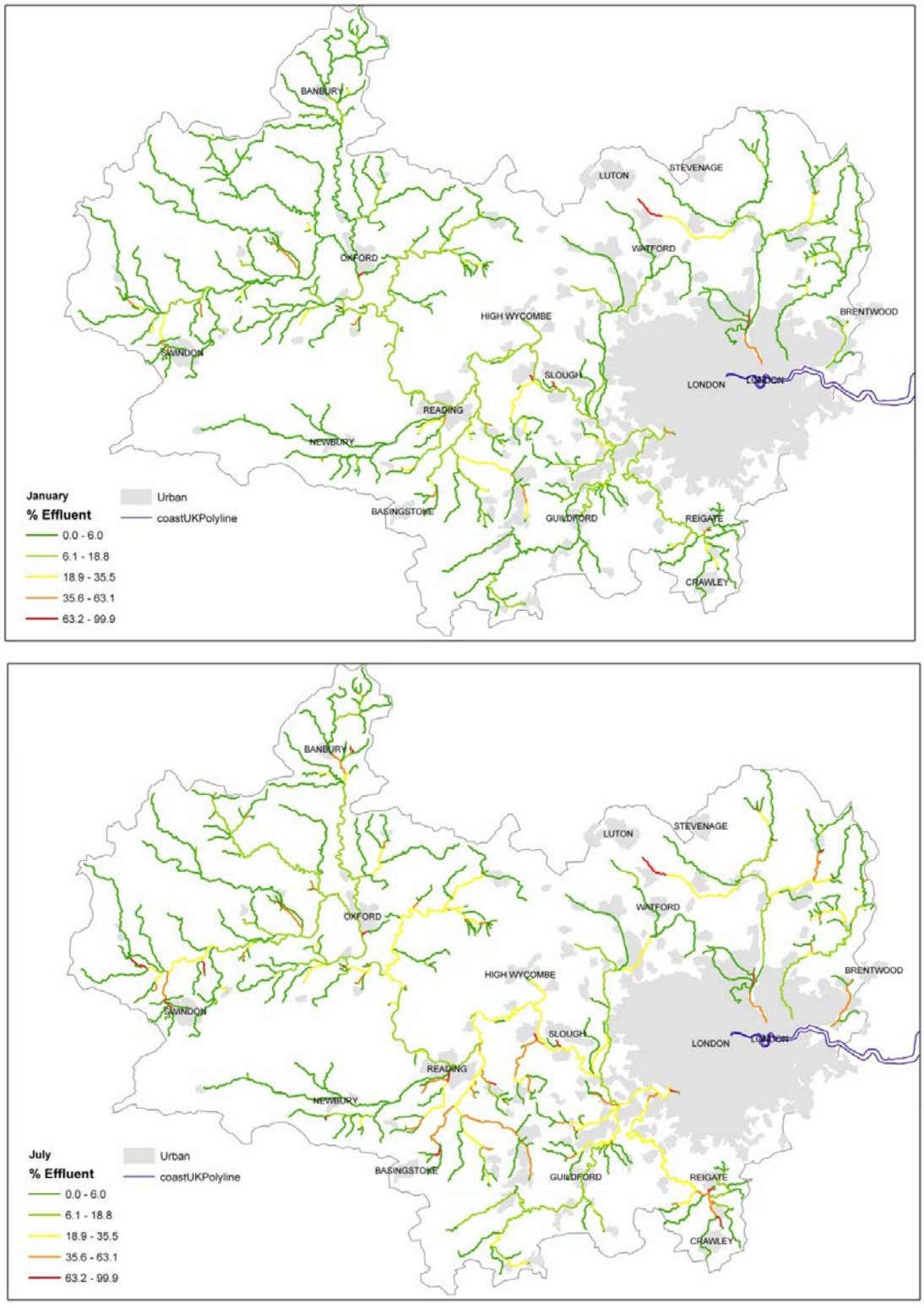


Figure 2.4 Comparative maps of the mean percentage effluent comprising river flow within the Thames river catchment network during (A) January and (B) July. A large proportion of rivers change from green to yellow/orange during the warmer, drier summer months when dilution is less pronounced. Grey areas signify concentrations of urban development and the blue artery is the Thames tideway (saline)

2.1.8 Additional Predictions of Environmental Concentrations of Oestrogens

A complementary risk assessment to map the distribution of steroid oestrogen contamination was used alongside effluent concentrations. Predicting environmental concentrations of steroid oestrogens in effluents was undertaken using the same model and approach provided by Williams et al. (2009). This includes determination of: influent loading of E1, E2, and EE2 from the population served by each STW (Johnson and Williams 2004), oestrogen removal in the STW process, dilution in the receiving water, and biodegradation of steroid estrogens during this course. By combining E1, E2, and EE2 concentrations, each model stretch was given a value in terms of E2 equivalents which later determined its risk category (see classes in Figure 2.5). All river reaches were categorised to: no risk, at risk, or high risk depending on their value derived from the formulae (Figure 2.5). High risk sites have a combined oestrogen concentration above a threshold, where a probable population-level effect would be detected. The risk classes produced using this model have been found to correlate with the incidence and severity of intersex in fish found downstream of the STWs (Jobling et al. 2009). Therefore, undertaking this preliminary modelling work should increase our chance of finding a statistically significant outcome, if effluent and/or oestrogens do indeed influence population genetic parameters of wild roach.

Risk category	Thresholds
No risk	$\frac{[EE2]}{0.1}+\frac{[E2]}{1}+\frac{[E1]}{3}<1$
At risk	$\frac{[EE2]}{0.1}+\frac{[E2]}{1}+\frac{[E1]}{3}>1$ and $\frac{[EE2]}{3}+\frac{[E2]}{10}+\frac{[E1]}{30}<1$
High risk	$\frac{[EE2]}{3}+\frac{[E2]}{10}+\frac{[E1]}{30}>1$

Figure 2.5 Risk categories based on predicted oestrogen concentrations in river stretches. Boundaries between categories are based on the PNEC of total oestrogens. Taken from Williams et al. (2008)

2.1.9 Extension of GIS Work: Incorporating Restocking and Channel Obstructions

For precise characterisation of appropriate sites from which to obtain samples, additional information was incorporated alongside the effluent concentrations projected in ArcGIS. Spatially referenced layers including information on artificial introductions of roach and in-stream obstructions (and passes) to fish movement were added to a base layer of the river network. Information on both these aspects was obtained from relevant individuals (Nigel Hewitt and Darryl Clifton-Dey) within the Environment Agency in early 2010.

Details pertaining to the restocking of roach were extracted from the National Fish Population Database (NFPD), where all monitoring data for England and Wales is stored regarding population surveys, introductions, translocations and estimates of fish population biomass and density. No time period was defined, as the EA centralised database only began in 1999; so all records were extracted from commencement to 2010. All applicable database entries for the Thames region include listings of river name, date, numbers/weight of fish added and NGR for location of release. Prior to 1999, any records would be held on paper by separate regional offices, therefore finding evidence of all historical introductions was unattainable. Nevertheless, little is known to date about the long-term survival and parental contribution of restocked coarse fish in the wild, but their persistence could be low (Aprahamian et al. 2004). The number of generations between any stocking and sampling was taken into account, as well as the location of stocking, to ensure any genetic analysis was not compromised by introduced roach (implications are discussed in more detail in Chapters 3 and 4).

Evidence of barriers to fish movement were sourced from an EA database containing constructions of in-stream obstacles. Initially, these records were created for the purpose of monitoring salmonid population movement within the Thames catchment. Features are defined in categories by type, including; weirs, dams, barrages, mills, locks and waterfalls. Natural and artificial obstructions were listed by river name or water body, alongside NGRs that give their precise location. Approximately 2400 impoundment entries are recorded within the Thames catchment; some of which have fish passes to augment movement of migratory species to spawning grounds in upper reaches. Passes predominantly occur along navigable stretches, including the main stem of the River Thames and the River Kennet/Kennet and Avon canal. Limited data on obstruction height was available to elucidate if the obstructions may be passable to larger roach, so all were deemed impassable. Indeed, many of the in-channel obstructions are known to limit migration to spawning grounds in larger salmonid species (Mills 1991), consequently, smaller roach would likely encounter difficulties traversing these obstacles.

Spreadsheet data for roach introductions and obstructions to fish movement were manipulated in ArcGIS for visualisation in the same manner. All ordnance survey NGRs had to be converted into *X* and *Y* coordinates prior to projection in ArcGIS, using the coordinate converter toolbox. Following translation into the correct spatial reference, datasheets could be added and projected alongside existing basic shapefiles of the Thames catchment river network (see Figure 2.6 and 2.7) in ArcMap.

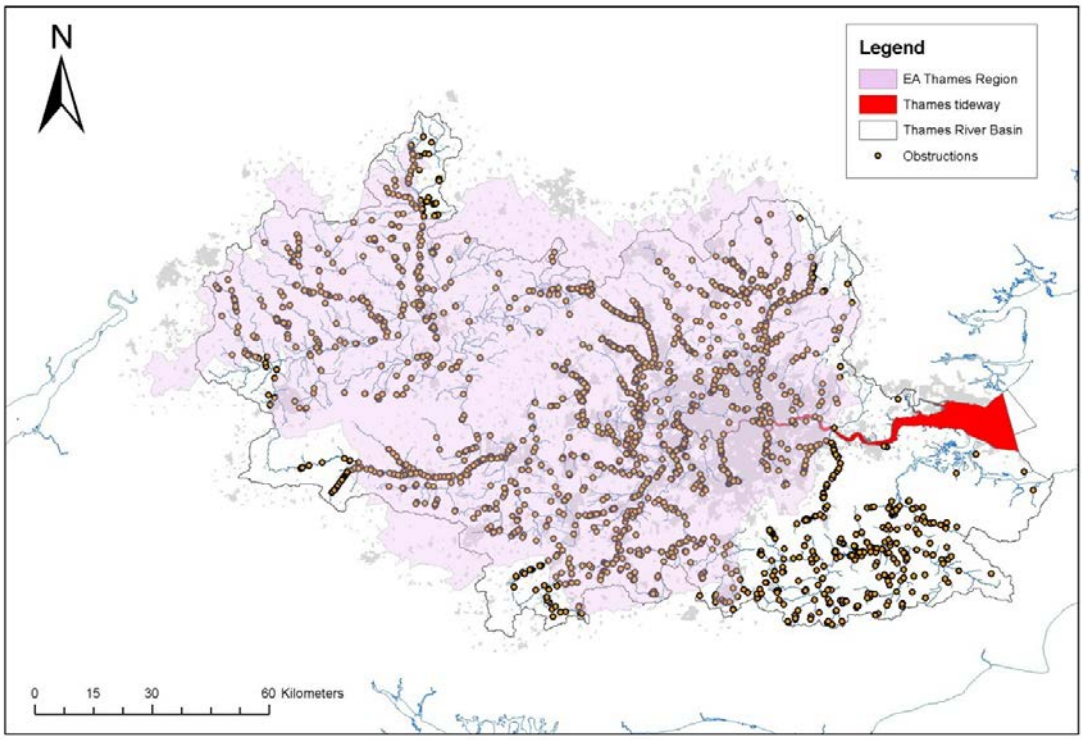


Figure 2.6 Map produced in ArcGIS to highlight all the possible obstructions to fish movement found within the Thames catchment. Very few rivers are unaffected by impoundments, making fish migration difficult in many instances and altering natural river flows. Grey areas depict concentrations of urbanisation. Sample sites and fish populations had to be demarcated by restricting barriers, to limit migration between sites and ensure samples were taken from separate populations confined to effluent-modelled stretches

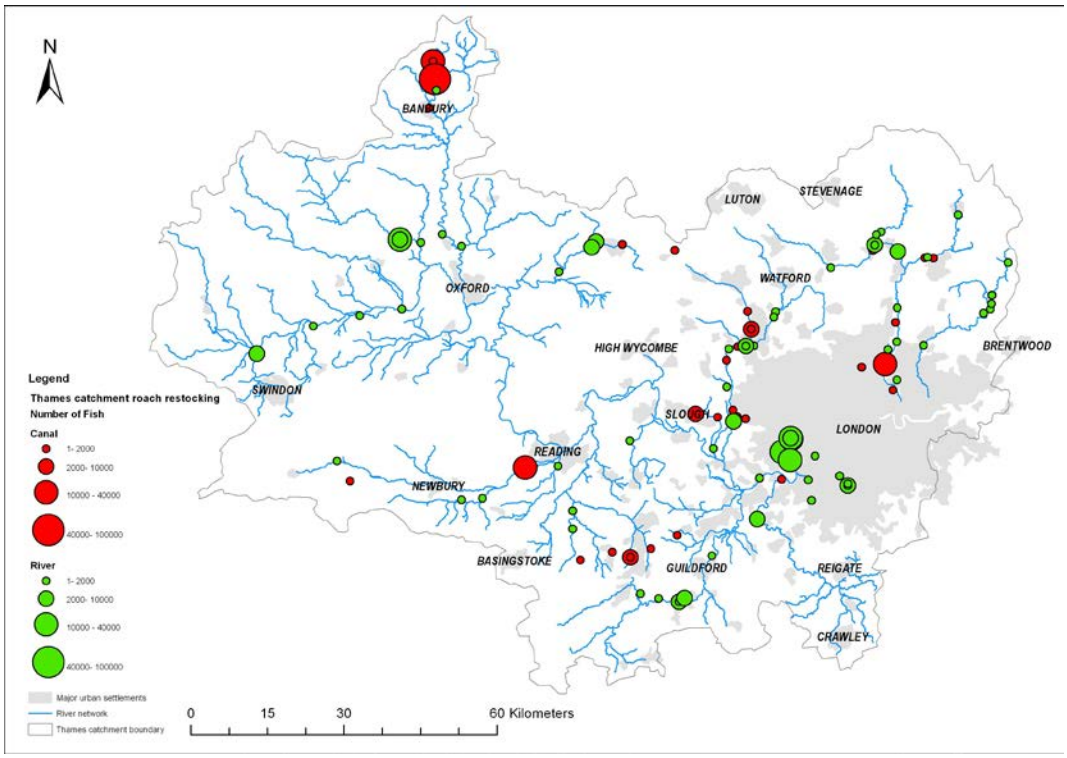


Figure 2.7 Map of the Thames catchment, UK, showing all instances of roach restocking (of age 1+ fish) since 2000. Restocking can occur in rivers and canals along the river network and are represented here in green and red respectively. Proportional circles symbolise the total number of fish added during each restocking occasion. Restocking of still-waters and flood plains has been omitted from this map, as these individuals are likely isolated in disconnected waterbodies and therefore will not compromise any wild fish sampling

2.2 DNA Microsatellite use in Population Genetic Applications

Evolutionary processes act continuously to provoke variation at both the molecular and organism level. Individuals within species display variation at the molecular level, sourced from mutations. This variation arises from base-pair alterations (substitutions, insertions, deletions and chromosomal arrangements) in the DNA molecule that can alter genes and the subsequent expression of different phenotypic characteristics in an organism. Individuals who share similarities at the organism or molecular level can form groups -- intraspecific groups that can differ from one another in their genetic composition. It is this change in frequency of alleles/genes over evolutionary time that is evaluated through the use of genetic markers.

Rapid advancement in microsatellite marker application has revealed previously unattainable information on population structure, unearthing quantifiable variation between individuals and populations of the same species living in relative proximity. Popular applications of microsatellites now include population genetics, conservation biology and the study of evolutionary processes. DNA microsatellites are used throughout this thesis to assess levels of genetic diversity, population structure, estimate effective population size, explore evidence for genetic bottlenecks, and undertake parentage in captive bred populations of roach.

2.2.1 History of Microsatellite Marker Technology

Genes are the factors that determine the phenotypic characteristics of an organism. Identifying genetic variation in natural populations was of scientific interest prior to the published work of Mendel in the 1900s; however it was not until the 1960s that genetic markers became established for unveiling complex population dynamics. Widely used genetic markers are classified as one of two types, protein or molecular (DNA). Techniques include:

- Protein markers- allozymes
- Nuclear DNA markers (biparently inherited) - random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs), Mini/Microsatellites, single nucleotide polymorphisms (SNPs)
- Mitochondrial DNA markers (mtDNA; maternally inherited)- restriction fragment length polymorphisms (RFLPs)

The use of these different techniques is well reviewed within the literature. The review of Liu and Cordes (2004) crucially summarises the principle uses, power, advantages, and disadvantages of the various marker types, along with their applications in a variety of

aquaculture studies.

Most early genetic variation studies were conducted using allozymes in salmonids (Bourke et al. 1997 and references therein), shortly superseded by population genetic studies across different fish species using RAPDs (with the advent of PCR). Collectively, the nuclear DNA methods are grouped under the heading of non-coding, neutral markers, i.e. loci not under selection, and they remain the most widely used category of molecular marker for population-level studies of fish genetics (Ellegren 2004). Bagley et al. (2001) compared RAPDs and AFLPs to analyse patterns of genetic diversity in rainbow trout (*O. mykiss*) finding that AFLPs are preferred due to better reproducibility. More recently, patterns of genetic variation among native fish populations of the Sacramento sucker (*Catostomus occidentalis*) in California were detected using dual approaches of AFLP and microsatellite markers. Fine-scale population structures and estimation of population parameters elucidated by the two marker systems were highly concordant and consistent with biogeography, despite the markers differing in their strengths/weaknesses and reflecting variability in certain parts of the genome.

2.2.2 What are they?

Microsatellites are short DNA sequences of the DNA nucleotides (adenine - A, thiamine - T, guanine - G, cytosine - C) that are perfect or near perfect tandem repeats, dispersed abundantly throughout all eukaryotic genomes. Microsatellites generally have a repeat unit length of 2–5 bases and are in tandem arrays of 5–30 repeats. Most microsatellites (also known as simple sequence repeats, SSRs) are found in non-coding regions of the genome (not in genes), have no known function and are assumed to evolve neutrally. They display a level of polymorphism that is proportional to the underlying mutation rate for each locus (ranging from 10^3 to 10^6 per locus per generation, Ellegren 2000). The (AC) dinucleotide motif is most prevalent throughout all vertebrate genomes, over two times more common than the (AT) motif (Toth et al. 2000). Bhassu et al. (2008), concluded that 30–67% of microsatellites found in fish genomes are dinucleotide, which corresponds well with 47, 52, 64 and 78% for medaka (*Oryzias latipes*), *Fundulus*, zebrafish, and *Xiphophorus* species respectively (Ju et al. 2005). In three-spined stickleback, CA dinucleotides also are the most common type of microsatellites, occurring approximately once every 14 kb (Peichel et al. 2001). For comparison, in the human genome, one microsatellite was found every 6kb and one CA repeat occurred every 30 kb of DNA (Beckmann and Weber 1992).

2.2.3 How do they work?

Microsatellites have characteristic repeated sequences, such as CACACACA, found at the same location (locus) within genomic DNA. The number of repeats can vary widely between individuals, from 3–4 to more than 50. Each diploid individual has two copies of any particular microsatellite segment, which can consist of varying numbers of repeats of the unit in question (See Figure 2.8). In diploid individuals, these two copies are inherited from each parent (codominant). For example, one parent may have a genotype of 15 and 18 repeat units, whereas the other may possess 18 and 21 repeats. Resultantly, their offspring may inherit alleles with lengths of 15 from its mother and 21 from its father. Over time, as individuals interbreed, alleles change in frequency, which can then be recognised at multiple microsatellite loci. Genetic drift, migration, mutation and selection are responsible for changes in allele frequency. Inheritance makes microsatellites particularly useful in parentage analysis, although one single microsatellite cannot be used alone. Several microsatellites are required to 'fingerprint' individuals, as numerous parents could have the same alleles at one microsatellite but are unlikely to share the same pattern of alleles (or repeat-unit length variation) at multiple microsatellite loci.

To detect the differences in alleles, PCR primers are designed that are unique to each specific loci. Primers match the base-pair sequence found on either side of the repeated unit/microsatellite region (Figure 2.8). One primer for each microsatellite is fluorescently labelled and then both primers are added to the target substrate DNA in the PCR, which is denatured and cooled to allow the primers to anneal to their complementary regions. The heating and cooling process is repeated 25–30 times, exponentially increasing the number of copies of the PCR product (see Box B for PCR process). Fluorescently-labelled PCR fragments are then run on a DNA sequencer, to determine their size by recognising the number of repeat units present on each allele. The output of the sequencer (Figure 2.9) shows a unique pattern for multiple labelled microsatellite loci with sizes of each allele.

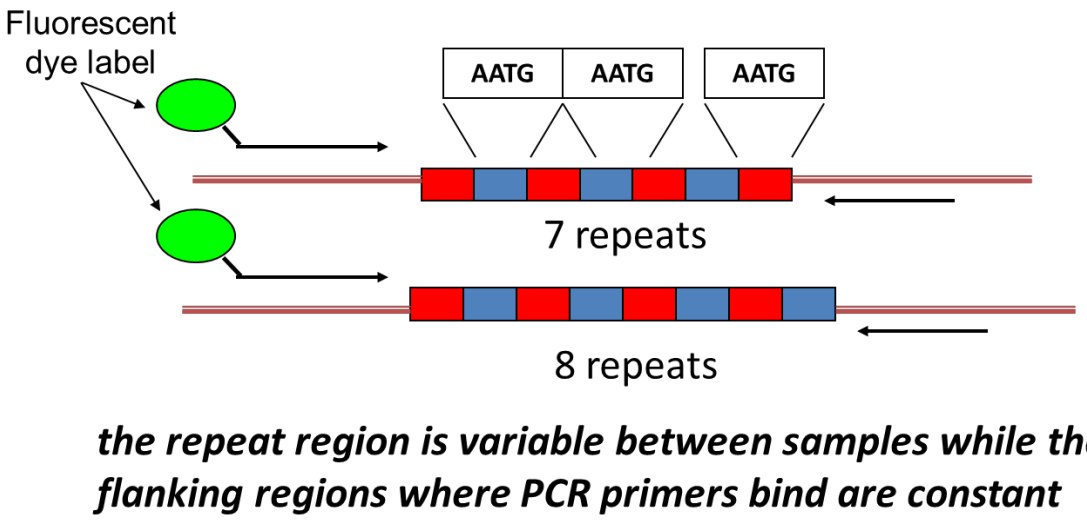


Figure 2.8 Detecting microsatellites from genomic DNA alleles. Two fluorescently-labelled PCR primers (forward and reverse, black arrows) are designed to flank the microsatellite region (blue/red boxes). If there were zero repeats, the PCR product would be the same length as just the two primers. Therefore, by determining the size of each PCR product, you can calculate how many repeat units are present in each microsatellite and establish the size of each allele for an individual. A homozygote for this locus would present alleles of the same length, whereas allele sizes would differ in a heterozygote. Taken from Academic Press (2002)

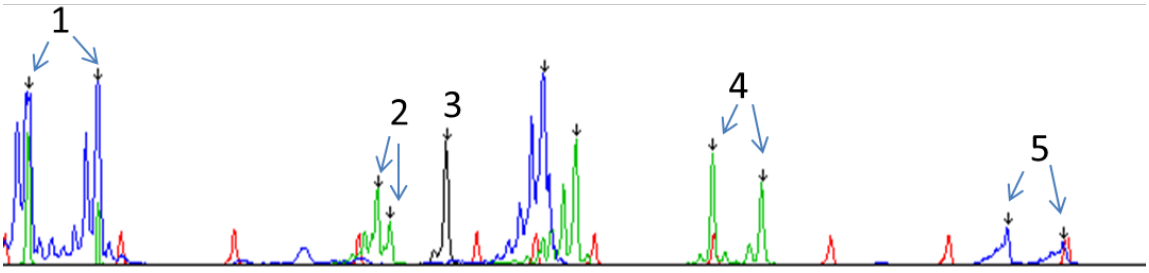


Figure 2.9 Typical electropherogram produced from a labelled DNA sample run on a sequencer. Major peaks of different colours (marked with small arrows) are alleles for microsatellite loci (numbered). A size standard run is run together with the DNA sample. Sequencer software adds numeric values to each axis to reveal the measured fragment length of each PCR product (bp; x- axis) and the intensity (peak height)

Box B: Basic PCR process

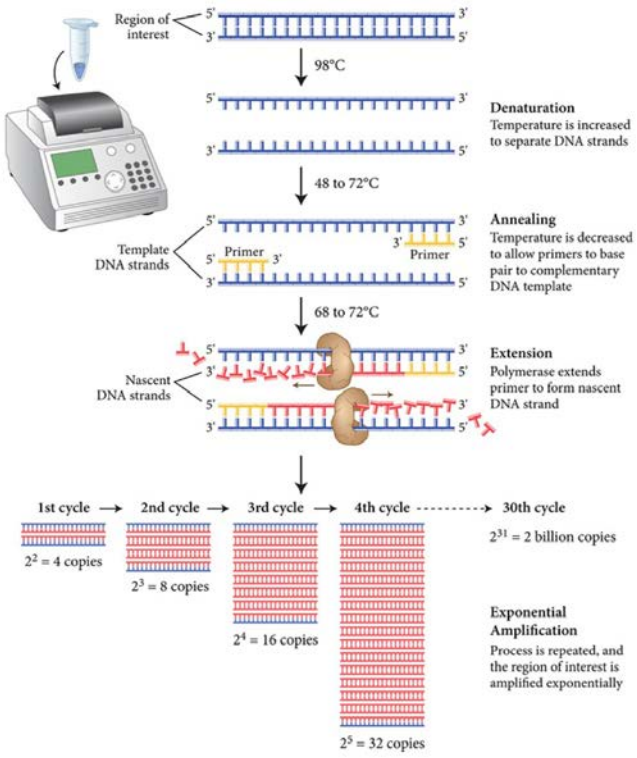


Figure 2.10 Basic PCR steps used to amplify product of interest Picture source: www.neb.com

Arguably one of the most revolutionary techniques to be discovered in molecular biology was the polymerase chain reaction in the early 1980s. Capable of amplifying DNA sequences in any type of organism, PCR combines sensitivity and specificity in 3 basic steps: (1) template denaturation, (2) primer annealing and (3) enzymatic extension (see above figure). A strand of DNA is the template for the reaction and is added alongside a pair of oligonucleotide primers (designed specifically to match the region of interest) and then amplified. Firstly, template DNA is denatured using high temperatures (95^{o}C), to separate single strands upon which the primers (conventionally one forward and another reverse) anneal (~50 °C) with high specificity. Once annealed, extension (~72 ^{o}C) and base-pair matching takes place aided by HotStarTaq Plus DNA Polymerase (Qiagen Type- it Microsatellite PCR Kit) to add nucleotides to the 3' OH-end of the primers. Repetition of these steps several times serves to amplify the intervening regions specified by the primers, exponentially increasing the copy numbers of the PCR product (theoretically, each PCR cycle doubles the amount of amplicon in the reaction). Products from the PCR can then be used for downstream applications.

2.2.4 Advantages of Microsatellite Use

Microsatellites are advantageous molecular markers because they are highly polymorphic. Microsatellite polymorphism is attributable to errors in sequence repeats and replication strand slippage, which ultimately produce differences in length variation in repeat number. In sexually reproducing individuals, no individuals are expected to have an identical genotype for all genes (except identical twins), thus increasing the probability of detecting differences even in closely related individuals/populations (Jarne and Lagoda 1996). The rising popularity and application of microsatellite markers is due to their ability to discriminate between closely related individuals in comparison to allozyme markers (which commonly only exhibit 2/3 alleles). For example, microsatellites have been found to detect fine-scale genetic differentiation among populations of marine fishes, where allozyme markers have failed (Ruzzante et al. 1998, Shaw et al. 1999). This heightened resolution and the potential to provide contemporary estimates of migration and the parentage/relatedness of individuals, makes microsatellites a valuable tool (Queller et al. 1993).

Microsatellites have proved highly beneficial to population genetic analysis due to their abundance and ease of detection by automated systems. Importantly, genotyping can be undertaken using DNA extracted from very small amount of tissue, so fish can be genotyped from samples taken non-destructively such as a small fin clip, or from routinely collected scales. The fact that methods are PCR-based means that highly degraded or historical DNA samples can also be utilised to build a temporal picture of genetic structure of populations and sub-populations across generations/evolutionary context. Temporal stability in population structure has been extensively pursued in studies of salmonid species, where they extract DNA from scales and otoliths from historic reference collections (Hutchinson et al. 1999, Nielsen et al. 1999). This makes it possible to trace genetic variation retrospectively, using archived samples and the PCR technique.

Microsatellites are favoured in the study of population genetics due to their enhanced resolution (linked to their higher mutation rates). For example, SSRs offer increasing utility in recognising recent population expansions, due to the accelerated proliferation of new mutations in rapidly evolving microsatellite loci when compared to SNPs (Guichoux et al. 2011). Contrastingly, RAPDs and AFLPs have reproducibility problems and cannot distinguish heterozygotes due to their binary nature (presence/absence of a band; Table 2.3). Variable microsatellites are codominant, so can resolve homozygote and heterozygote individuals. This characteristic is increasingly important in the wider field of

research concerning the relationship between heterozygosity and fitness, where increased individual genetic diversity can lead to an increase in fitness (Reed and Frankham 2003). Overall, key population parameters can be estimated through microsatellite application, as the genetic constitution of populations is often altered well in advance of extirpation.

Automated methods (sequencing) allow direct sizing of alleles, accompanied by the advent of multiplexing PCR technology to genotype samples. The detection of multiple markers through the pooling of spectrally distinguishable primer sets is now a standard technique in most laboratories. Fluorescently labelled, compatible primers allow different microsatellite alleles to be recognised simultaneously by size and colour. Once the primer multiplex has been designed, tested and produced, the use of those microsatellites becomes rapid and cost effective. The availability of high-throughput capillary sequencers accelerates characterisation of variants across a panel of multiple microsatellite loci (Blohm et al. 2007) that can then be manipulated in statistical programs to estimate bottlenecks, parentage and migration rates (see review in Luikart and England 1999).

Table 2.3 Attributes of markers commonly used in molecular population biology. Adapted from Sunnucks (2000)

	PCR assay	Single locus	Codominant	No. of loci readily available	Connectibility of data among studies	Rapid transfer to taxa	Overall variability [a]
Mitochondrial							
RFLP	No, large	Yes	Yes	Single	Direct	Yes	Low–moderate
Multilocus nuclear							
RAPD	Yes	No	No	Many	Limited	Yes	High
AFLP	Yes	No	No	Many	Limited	Yes	High
Single-locus nuclear							
Allozymes	No, protein	Yes	Yes	Moderate	Direct	Yes	Low–moderate
Microsatellites	Yes	Yes	Yes	Many	Indirect [b]	Some	High

[a] Variability depends on variation per marker and number of markers obtained readily. The assessment here approximates the outcome of a typical marker system. [b] Data from these markers are indirectly, but meaningfully, connectable given adequate models of molecular evolution.

2.2.5 Limitations of Using Microsatellites

Despite their well-documented popularity, microsatellites have a number of specific limitations. Microsatellite loci require initial development on a species-specific basis that can prove both costly and time consuming. As they are present in non-coding DNA regions, this means markers are only applicable for the species in question or other close relatives (Selkoe and Toonen 2006). Typically in non-model species, the development of up to 10 suitable polymorphic markers takes 3–9 months (problem dependent),

however population genetics approaches often warrant an array of 10–20 highly diverse markers (Luikart et al. 2003). In addition, the high diversity of alleles present at some microsatellite loci and increasing numbers of markers required to estimate allele frequencies, command large samples sizes, hampering sample processing speed (Guichoux et al. 2011). Technical difficulties including null alleles, stuttering and large allele dropout are also apparent with the use of microsatellites but are loci specific and may be more prevalent in some rather than others (see Selkoe and Toonen 2006, Guichoux et al. 2011 for full discussion of these problems). The software package MICROCHECKER (Van Oosterhout et al. 2004) goes some way towards addressing these potential artefacts.

2.2.6 Application of Microsatellites to the Study of Fish Populations

In fisheries biology, genetic markers are popular for the identification of stock structure (Carvalho and Hauser 1994), detecting levels of genetic variation among individuals (Hartl and Clark 1989) and to discern natural from hatchery-reared, introduced fish (Abdul-Muneer 2014). A common objective of more recent applications is to gain insights into the diverse array of reproductive behaviour displayed by fish species, including: parentage analysis, mating success, levels of inbreeding, pedigree analysis and natural/sexual selection (Figure 2.11, Ferguson and Danzmann 1998). Their versatility and applicability in detecting genetic variation make them a desirable alternative for yielding information, previously only attainable with extensive observations of natural populations (trait change over time). For example, Adams and Hutchings (2003) used five microsatellite genetic markers and mark–recapture studies to compare the population structure of brook charr, (*Salvelinus fontinalis)* inferred from the different approaches. Concluding that the agreement between tagging and genetic data means microsatellite markers can be useful tools for assessing biological endpoints in populations, requiring less intensive field studies.

Genotyping at multiple loci also allows quantification of genetic variations within and between populations of the same species, documenting migration patterns, panmixia or gene flow between local populations (Chistiakov et al. 2006). For example, the utilisation of microsatellites allowed investigation into spatiotemporal population structure of the endangered European eel (*Anguilla anguilla*, Maes and Volckaert 2002, Dannewitz et al. 2005) as well as a tool to examine the impact of heavy metal pollutants on the genetic constitution of eel populations (Maes et al. 2005). Additional applications of microsatellite loci for fish populations include indirect estimates of effective population

size, ecologically significant units (ESUs) for stock management, and the identification of historical population bottlenecks (Santos et al. 2013).

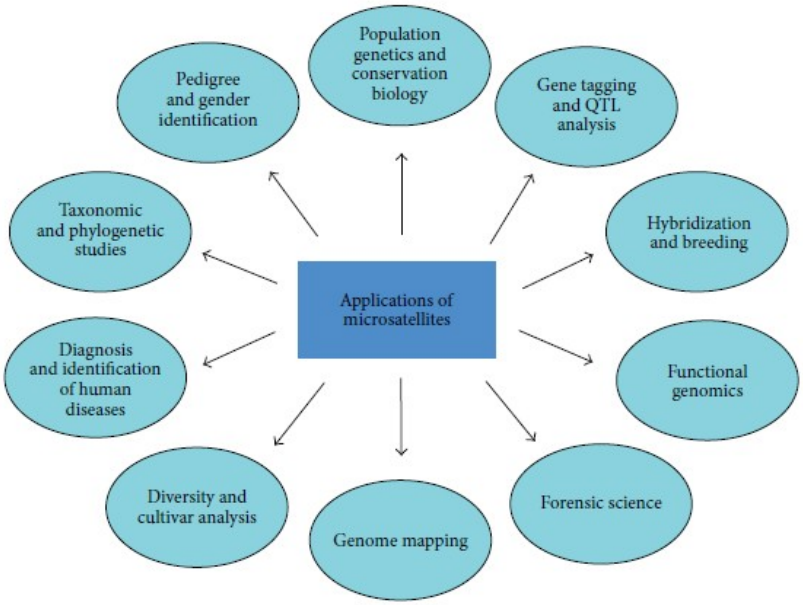


Figure 2.11 Common applications of microsatellite use across many scientific disciplines. Taken from Abdul-Muneer (2014)

2.3 Detailed Methodology of Microsatellite Use within this Thesis

Knowledge of breeding patterns, levels of genetic diversity, population size and structure of wild fish populations are critical for understanding how human activities impact populations. Application of DNA microsatellites to infer patterns of parentage has shown that EDC exposures in fish, for example, can modify dominance hierarchies (Coe et al. 2008b) and alter breeding behaviour (Coe et al. 2010). Thus, chemical exposure could potentially change the contributions of parental fish to the next generation. In turn, varying the genetic make-up of a population and/or reduce genetic diversity of populations, without necessarily affecting the size of fish populations. In most cases, however, the impacts of chemical exposure on natural patterns of reproductive success are unknown.

Recognising this void in our knowledge, the following chapters all use information gleaned from microsatellites to help evaluate population structure of roach in light of effluent contamination, trans-generational breeding success and additional abiotic population-level drivers. To do this, I examine population genetic structure of roach in the Thames region using DNA microsatellite analysis in Chapter 3 and associated this with additional environmental factors (Chapter 6). Microsatellite data were also used to calculate N_e, and estimate levels of gene flow to determine the effect of effluents on wild roach populations in the south east of England in Chapter 4. The same loci were

applied to a long-term, multigenerational exposure of second generation roach to determine parental contribution of exposed fish in a competitive breeding scenario (Chapter 5). The following details describe how samples were collected, processed and analysed to provide material for the results seen in the aforementioned experimental chapters.

2.3.1 Development of Microsatellites used in this Thesis (work conducted by P. Hamilton)

Polymorphic microsatellite markers are a powerful tool for population genetics and continue to increase in popularity and application. Previous work on *R. rutilus* by Hamilton and Tyler (2008) developed seven microsatellite loci for genotyping individuals, which were subsequently utilised to assign parentage in experimental breeding populations of roach (Harris et al. 2011). However, additional microsatellite loci were required for Chapter 5 of this thesis, in order to accurately match offspring and parents in breeding studies with increasing numbers of potential partners. When using microsatellite loci to detect population declines, previous studies have suggested that the addition of extra loci is interchangeable with increasing numbers of samples (Waples 1989; Waples & Do 2010). Antao et al. (2011) suggest that adding more individuals is more beneficial than adding loci and contrastingly, Guichoux et al. (2011) state accuracy in estimating genetic effective population size using a limited sample of wild individuals (Chapter 4) is improved with an increasing number of microsatellite loci. This unfortunate disparity of opinion meant ensuring approximately 50 wild individuals were genotyped from all field samples and additional loci were developed to more accurately characterise effective population size estimates, genetic diversity and possible bottleneck events. In order to increase the number of DNA microsatellites available for genotyping UK roach, PH undertook a literature search for primer sets that have been developed for roach or closely-related species (Barinova et al. 2004, Muenzel et al. 2007, Dubut et al. 2010 , Vyskocilova et al. 2007, Mesquita et al. 2003). DNA sequences from expressed sequence tags [ESTs short nucleotide sequence reads from cloned cDNA of any organism/tissue/cell (Nagaraj et al. 2007)] were also examined for roach for the presence of microsatellite repeats and designed primers flanking these regions. The ability of these primer sets to amplify microsatellites for UK roach was then assessed and size range and variability was also examined. To increase the speed of genotyping, combinations of primer sets for different microsatellites that could be amplified in a single PCR reaction were identified. This was facilitated by using the Type-it Microsatellite PCR Kit (Qiagen), which is specifically designed to enable

amplification of multiple fragments without loss of amplification sensitivity or accuracy for each microsatellite. From these results, 14 microsatellite markers were developed that could be run together on a DNA sequencer (details in Table 2.6 and 2.7).

2.3.2 Sample Collection

The biological material (fin clips and scales) for genetic analysis of the roach populations was derived from a combination of archived material from study sites where feminised fish have been previously reported (Jobling et al. 1998, 2002, Harris et al. 2011) and freshly collected material from fish captured via electrofishing (during 2010–2011 season). Fin clips were stored in 100% ethanol for DNA extraction and scales were kept in scale packets for subsequent DNA extraction. Electrofish sampling was conducted by experts from the Environment Agency and is a method used routinely to determine abundance, species richness, growth rate and population density of fish populations. This method of sampling anticipates that larger fish show a greater reaction to the electrical output and are also more likely to be caught in the nets, therefore resulting population data are for parr age classes and not juveniles/fry. For genetic analyses conducted in Chapters 3&4 this did not effect the interpretation of results, as the inclusion of parents and potential offspring fish in diversity and effective population size estimates can downwardly bias results. Chapter 6 would have benefitted from the inclusion of under-represented age classes, however sampling regime was kept uniform across all fish population surveys in this chapter to improve comparability of population density estimates and species richness. Fin clips and/or scales were collected from roach during terminal sampling (in 2012) for those used in parentage analysis in Chapter 5.

2.3.3 DNA Extraction

Many methods are available for extracting DNA from a wide range of samples such as blood, hair, bones, saliva, muscle and skin epithelial cells. Isolation method depends on the sample type and the quantity of starting material. These operate through the disruption of cellular membranes and ensuing cell lysis, denaturation of proteins, and the separation of DNA from other cellular components. The Chelex method uses a resin with a high affinity for polyvalent metal ions and effectively removes them from solution, allowing denatured DNA to remain in liquid phase above Chelex matrix (Walsh et al. 1991). Although this method produces a relatively crude DNA extract, the supernatant is sufficiently clean to be used in downstream applications such as PCR. Despite the alternative HOTSHOT extraction method being quicker and easier to apply, the Chelex method remains popular because it is inexpensive, avoids using harmful chemicals and

can be completed in one tube thus reducing the risk of contamination.

2.3.4 DNA Extraction using Chelex protocol

DNA was extracted from scale/fin tissue from adult fish using the following Chelex extraction method. A 10% Chelex stock solution was made up using 10 g of Chelex and 100 ml of HPLC water. This was incubated at 80°C to activate the Chelex, just before use. Each fin clip/scale was taken from its sampling tube, and placed into a new 1.5 ml eppendorf. Subsequently, 500 µl of activated 10% Chelex was added to each tube along with 10 µl of Proteinase K enzyme (PK20, 20 mg/ml) and then the samples were pulse-vortexed for 10 sec. Tubes were held in floating racks and incubated at 55°C for 75 min, removing them every 15 min to vortex (~5 sec). Samples were then incubated at 100°C for 15 min to deactivate the PK20 enzyme and to ensure that all the cells have ruptured and that the protein is denatured. Finally the tube was vortexed at maximum speed for 10 sec and centrifuged at 13,000 rpm for 5 min to pellet the Chelex resin and the denatured protein at the bottom of the tube, leaving the aqueous solution containing the DNA to be used in PCR. Supernatant was pipetted into a new sterile 1.5 ml eppendorf tube and stored at -20°C for future use, or in the fridge for immediate use (no longer than 2 days).

2.3.5 DNA Sample Preparations for PCR

The multiplex reaction mix, (containing microsatellite primers and dyes at the right concentrations, Sigma-Proligo), 500 µl of Type-it Multiplex PCR Master Mix (Qiagen) and 500 µl of nuclease-free water were pipetted into a tube which was then vortexed. Once completely mixed, 8.7 µl of the reaction mix was dispensed into each well of a 96-well PCR plate (Thermo Fisher). Following this, 1.2 µl of template DNA was loaded in to each well and a drop of mineral oil was placed on top. This process was repeated 3 times to produce replicates for each multiplex of loci (M2, M1a, M1b Table 2.7). After creating a plate document - a labelled matrix of the PCR plate layout containing a 3- letter river ID, 3-letter location site, 3 number for the sample ID - each well was checked for bubbles before the plate was sealed with foil and placed in the PCR instrument.

2.3.6 Microsatellite Amplification Using PCR

DNA samples were amplified using an Applied Biosystems instrument, on a cycle designed to enable genotyping from scales with degraded DNA. The following touchdown PCR cycle for was used for multiplex 1a, 1c and 2: 95^{o}C for 5 min followed by 37 cycles of 95^{o}C for 30 sec, annealing temperature for 90 sec and 72^{o}C for 3 min.

Annealing temperatures were 3 cycles at 62°C, 4 at 58°C, 5 at 55°C, 10 at 53°C, 5 at 51°C, 5 at 49°C and 5 at 47°C followed by a final extension of 72°C for 10 min and 60°C for 35 min. The following cycle was used for 1b: 95°C for 90 sec, followed by 35 cycles of 30 sec at 95°C, 45 sec at 55°C and 60 sec at 72°C and a final extension period of 72°C for 10 min and 60°C for 35 min. After the final extension step, the temperature was brought down to 4°C. The PCR plates were covered with foil and stored at -20°C until genotyping.

2.3.7 Running Samples on Sequencer

A master mix containing 4 µl of DNA size standard and 240 µl of sample loading solution (SLS) was prepared for each row (8 samples) in the sequence plate. Prior to the addition of the master mix, the volumes of each PCR product in Table 2.4 were pipetted into the 96-well sequencer plate. After vortexing, 28 µl of master mix (size standard and SLS) was added to each well and pipetted up and down several times slowly, to mix the liquid. Following agitation, a drop of mineral oil was deposited into each well. In a second 96-well buffer plate, separation buffer was loaded in to every well so that they were approximately ¾ full.

Table 2.4 Quantity of PCR product (M1a, b and M2) added to each sequencer plate (M1 and M2). Microsatellites were pooled for two runs on a Beckman Coulter DNA sequencer

	Multiplex 1	**Multiplex 2**
M1a	2.2 µl	-
M1b	1.2 µl	-
M2	-	1.2 µl

Prepared amplicons (DNA products of amplification) were run on two lanes on a Beckman Coulter DNA sequencer. Beckman software (CEQ system) was opened and a working database set up with each sample on the plate labelled using the code mentioned previously. Both buffer and sequence plate were loaded into the sequencer drawer. Microsatellite genotypes were determined using the Fragment Analysis program on CEQ 8000 (Beckman Coulter). STR locus tags were imported for the corresponding multiplex in question (either M1 or M2) and re-analysed. Unique locus tags allow the software to identify and apply names to the alleles identified during the automatic binning process; using expected fragment length range and repeat unit length criteria specified by the user. All different coloured peaks on the output electropherogram were examined individually, ensuring that all are ‘called’ correctly according to exact sizes. Following

this, fragment results were saved and exported into Microsoft Excel for further genetic analyses.

2.3.8 Data Analysis

All output data from the Fragment Analysis on CEQ 8000 (Beckman Coulter) was converted using a macro designed in Excel (by P. Hamilton). From here, data could subsequently be compiled in a master spreadsheet in GenAlEx 6 format (Peakall and Smouse 2006; as seen in Table 2.5). Individuals with substantial amounts of missing data were recognised and repeated in the first instance, and omitted immediately from further analyses if they were found to be problematic. Methods used to calculate genetic indices and examine population structure assume that loci are in Hardy–Weinberg equilibrium (HWE), are not in linkage (by being close together on the same chromosome), and are not under selection. As violations of these assumptions could result in inaccurate estimates, tests were undertaken (by P. Hamilton) to identify loci that do not conform to these expectations in order to exclude these loci from further analyses. Deviations from HWE for each locus in each population were calculated in GenAlEx 6 (Peakall and Smouse 2006). Possible linkage between microsatellite loci was investigated using GENEPOP on the web, version 4.0.10 (Raymond and Rousset 1995, Rousset 2008) with critical levels of significance adjusted for multiple tests using the Bonferroni correction procedure (Rice 1989, i.e. for a population sample with 14 microsatellites genotyped, $p = 0.05/14 = 0.0036$). For each locus in each population sample Micro-Checker v.2.2.3 (Van Oosterhout et al. 2004) was used to examine the presence of null alleles, allelic dropout and potential scoring errors caused by microsatellite stuttering. The program LOSITAN was used to identify loci that are potentially under selection (Antao et al. 2008).

Subsequently, the following loci were excluded from all analyses (see Hamilton et al. 2014 for full details): Lsou08 was excluded from N_e and genetic bottleneck analyses as analysis suggested it was under selection. Locus Ca3 was implicated in departure from HWE equilibrium in 5 of the 39 population samples, compared to a maximum of 3 for the other loci. Ca3 was retained for population genetic structure. There was no evidence of large allelic dropout or scoring errors caused by stuttering in any sample. Similarly, no locus consistently showed evidence for null alleles -point mutations in primer annealing sites- that mean microsatellites fail to amplify in PCR).

Less than 1.6% of all pairwise comparisons showed significant linkage disequilibrium after sequential Bonferroni corrections. No pairs of loci were found to be in linkage in

more than three of the population samples, so linkage disequilibrium was deemed to be negligible. In the LeeHyd'95 population, six of the 14 loci showed departure from HWE (compared to a maximum of three loci in other sites) and analysis in Microchecker (Van Oosterhout et al. 2004) suggested the presence of null alleles in these six loci. This may have resulted from allelic dropout, due to the poor quality of DNA obtained from this sample, which had 7% missing data, compared to an average of 1.52% for the complete data set. Once viability of the data was ensured after these checks, further relevant analyses were run for specific experimental chapters; the details of which can be found in the respective methods sections.

Table 2.5 Example layout of excel spreadsheet file for GenAlex 6.5, comprising information for every population and all microsatellite loci

	A	B	C	D	E	F
1	N° Loci	N° Samples	N° Pops	N° Samples Pop1	N° Samples Pop2	N° Samples Pop3
2	Name Data Set			Name Pop1	Name Pop2	Name Pop3
3	**CODE**	**SITE**	**Locus1**	**Locus1**	**Locus2**	**Locus2**
4	Sample Codes	Site codes	Allele size	Allele size	Allele size	Allele size
5	Sample Codes	Site codes	Allele size	Allele size	Allele size	Allele size
6	…	…	…	…	…	…

Table 2.6 Primer details of microsatellite loci used in this thesis

Primer codes (accession number)	Primer sequences D2–-D4 = Beckman Coulter code for fluorochrome for fluorescently-labelled primer	Reference
Rru2	D4 TTCCAGCTCAACTCTAAAGA GCACCATGCAGTAACAAT	(Barinova et al. 2004)
Z20908	D4 ATTGATTAGGTCATTGCCCG AGGAGTCATCGCTGGTGAGT	http://zfin.org
CypG27	D2 AAGGTATTCTCCAGCATTTAT GAGCCACCTGGAGACATTACT	(Baerwald and May 2004)
Ca3	D4 TCTAGCCCCCAAATTTTACGG GGACAGTGAGGGACGCAGAC	(Dimsoski et al. 2000)
Ca1	AAGACGATGCTGGATGTTTAC D3 CTATAGCTTATCCCGGCAGTA	(Dimsoski et al. 2000)
CypG24	D4 CTGCCGCATCAGAGATAAACACTT TGGCGGTAAGGGTAGACCAC	(Baerwald and May 2004)
Rru4	D4 TAAGCAGTGACCAGAATCCA CAAAGCCTCAAAAGCACAA	(Barinova et al. 2004)
Lsou34	D3 CCAGACAGGGTGATGATTCC GTAGCGACGTTCAGGTCTCG	(Muenzel et al. 2007), tested by (Dubut et al. 2010)
LC290	D3 CCCTAATGGCCCTCAATACA ACTTCGCTGGCTTGACAAAT	(Vyskocilova et al. 2007)
Llea029	D4 TTTACCAGCATCCCCCAT CATTTCACTCACTGAAGGAGAAC	(Dubut et al. 2010)
Llea131	D4 TGACCTTTGCTATTTCAACATAACT GTGTTTGAAATGTTTGTGCG	(Dubut et al. 2010)
N7K4	ACGAGCATCAGTATCCAGAGACAC D3 CATGTTTCCACATCTGAGCTAAAA	(Mesquita et al. 2003), tested by (Dubut et al. 2010)
Lsou08	GCGGTGAACAGGCTTAACTC D2 TAGGAACGAAGAGCCTGTGG	(Muenzel et al. 2007), tested by (Dubut et al. 2010)
Lid11	D4 CTCCTGATTCTTTGTCTGACT TTATTATTTCCTGTGGTGATTG	(Barinova et al. 2004)

Table 2.7 Summary statistics and PCR conditions for microsatellites used in this thesis

Primer codes (accession number)	PCR multiplex/ sequencer multiplex	Size range (bp)	N° of alleles	Expected hetrozygosity
Rru2	1a/1	88–120	13	0.7694
Z20908	1a/1	131–189	15	0.6787
CypG27	1a/1	206–394	41	0.8154
Ca3	1a/1	181–367	58	0.9526
Ca1	1b/1	99–147	25	0.7885
CypG24	1b/1	172–228	20	0.8302
Rru4	2/2	170–280	19	0.6617
Lsou34	2/2	219–235	8	0.5583
LC290	2/2	172–206	21	0.8111
Llea029	2/2	236–418	49	0.8846
Llea131	2/2	92–136	21	0.7847
N7K4	2/2	115–171	12	0.5575
Lsou08	2/2	171–223	18	0.6742
Lid11	2/2	178–386	25	0.8272

Chapter 3

Population Differentiation and Genetic Diversity of *R. rutilus* across England

Pages 67- 101

3.1 Statement of Contribution to this Work

This work was conducted in association with the University of Exeter, specifically Dr Patrick Hamilton. I planned and collected all fish samples from the field, aged all fish and was trained in genotyping and microsatellite analysis. I contributed to the writing, content and layout of the paper where these results are published (Hamilton et al. 2014). Contains Environment Agency information © Environment Agency and database right.

3.2 Introduction

3.2.1 Importance of Genetic Variation

Genetic variation is a trait of both individuals and populations; the origin, amount and distribution of which are the principle concerns of population genetics (Lacy 1997, Templeton 2006). As a result of normal cellular operations or gene–environment interactions, all organisms are subject to mutations in their genetic code. These genetic polymorphisms are the source of variation that can accumulate over time. The nature and fate of this variation across spatial and temporal timescales warrants understanding when seeking to quantify any biological consequence to natural populations. At the level of the organism, genetic variation within diploid individuals is primarily characterised by the percentage of heterozygous loci; as opposed to the inter-individual variation between populations, commonly quantified by gene diversity, number of alleles per locus, or the percentage of polymorphic loci (Nei 1973). Measures of between-individual variation encompass mean within-individual variation; enhancing the utility of population genetic theory to unravel the role of evolutionary forces of selection, migration, mutation and genetic drift in shaping individual and population fitness (Garant et al. 2000). Therefore, understanding the influence of evolutionary and ecological forces on population genetic architecture is imperative and is becoming increasingly accessible through the advancement of molecular genetic methods.

3.2.2 Application of Molecular Markers

DNA marker technology can be used to reveal patterns of genetic variation across any species. Early work in the 1970s discovered that allelic variation in structural genes can be examined using electrophoresis, deducing differences in the degree of fragment polymorphism between species and taxonomic groups (Clarke 1979). Further development with DNA markers such as allozymes, mitochondrial DNA, restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites, single nucleotide

polymorphisms (SNP), and expressed sequence tags (EST) have now made it possible to exploit genetic variation throughout the entire genome of any organism (Figure 3.1, Liu and Cordes 2004). Each of these markers detects genetic variation at the DNA level which can arise from: simple base substitutions or polymorphisms, insertions or deletions of nucleotide sequences within a locus, inversion of a segment of DNA within a locus, and rearrangement of DNA segments around a locus of interest (Liu and Cordes 2004).

Neutral marker loci have proved popular for deducing (recent) population genetic structure in aquatic organisms. The application of population genetic principles to management and conservation of fish populations (Allendorf and Luikart 2007) has paralleled the advancement of molecular techniques for analysing genetic differentiation between (Luikart and England 1999) and genetic diversity within populations (Hansen 2002). Inferring population structure using molecular techniques is reliant on estimating divergence in allele frequencies at marker loci (Heath et al. 2002, Andre et al. 2011). However, realised patterns of divergence and demography can be largely dependent on the resolution of different genetic markers (Avise 1994, Ryman et al. 2006). Larsson et al. (2009) show that organelle markers such as mitochondrial DNA (mtDNA), may offer superior utility in detecting recent signals of population divergence, compared to a single nuclear microsatellite locus. However, the combination of multiple polymorphic nuclear loci are expected to provide greater sensitivity in detecting population differentiation than mtDNA (Liu and Cordes 2004, discussed in Karl et al. 2012). The most appropriate molecular marker is therefore guided by the study aims, selected on a case-by-case basis.

Historically, the application of molecular markers to fish population research has been pursued principally in the economically important salmonid species (salmon and trout). This family of fish are anadromous (live at sea and return to rivers to spawn) and their characteristic natal homing ability has been found to produce significant population genetic structure, recognisable through the application of molecular markers. Early findings from isoenzyme studies on Atlantic Salmon (*S. salar*) identified a major discontinuity between populations found in the east and west Atlantic, from which, those fish from North America and Europe could be distinguished (Saunders 1981, Verspoor et al. 2005). More recent examination of salmonid population genetic structure, using microsatellite loci, has highlighted significant genetic differentiation between populations found in relatively close proximity (~1 km;—Dionne et al. 2008, Griffiths et al. 2009, Durrant et al. 2011). The utility of molecular markers therefore allows us to decipher very specific information relating known life-history traits to population structure and

connectivity in different fish species.

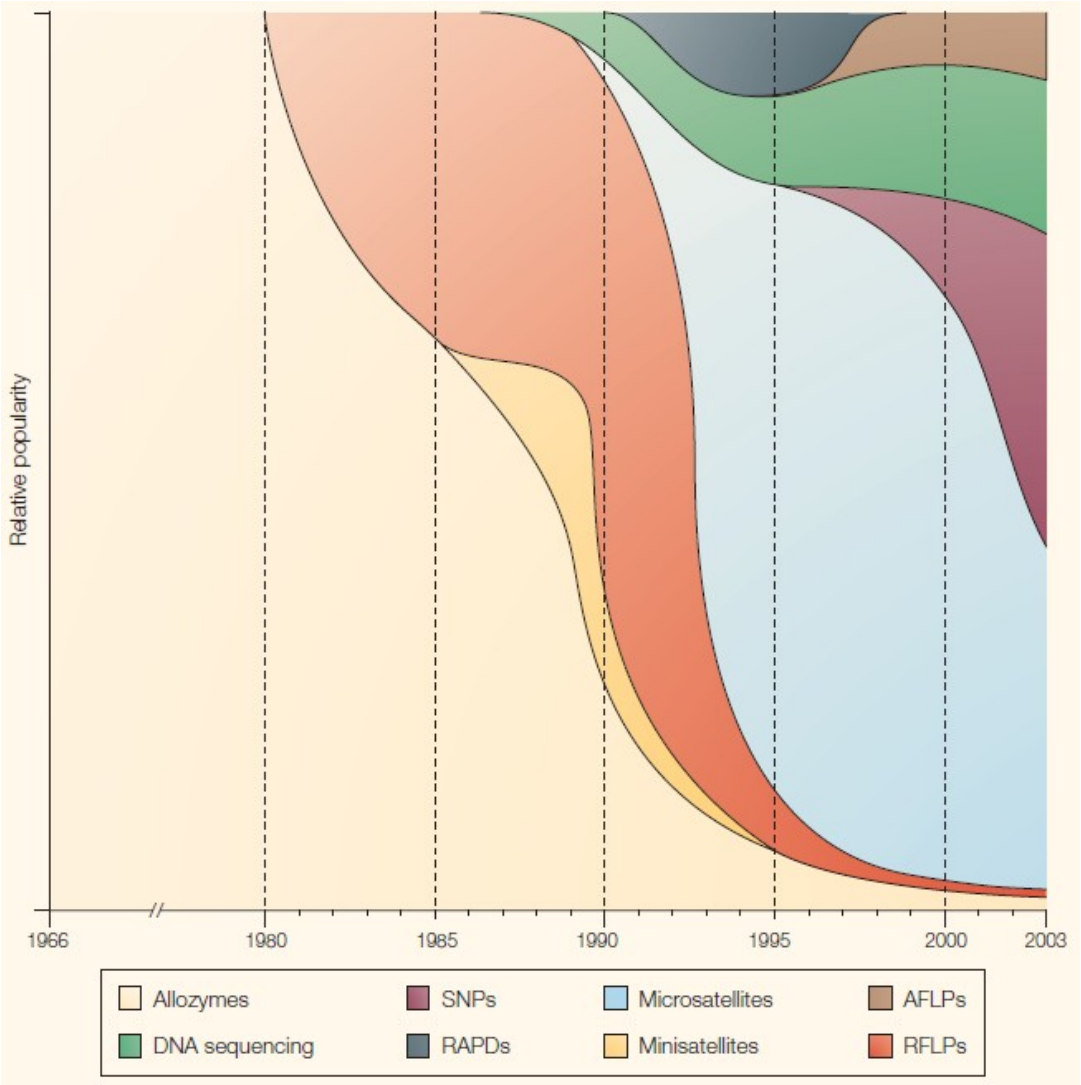


Figure 3.1 Subjective representation of the changing importance of different molecular markers over time. For each year represented on the x-axis, the y-axis corresponds to the total use of molecular markers, which is then separated by the proportion of different markers used at any given time point. Taken from Schlötterer (2004)

Fish species diversity requires distinct assessment of population structure, as little predictability can be cast between species. The biological significance of different life cycles and reproductive strategies can be uncovered by examining genetic structure, especially in marine fishes that have extensive habitat ranges and are not easily monitored. Recent application of microsatellites to marine fisheries has generated insights into significant genetic differentiation within oceanic basins, or even along contiguous coastal regions (e.g. zebra shark, *Stegostoma fasciatum* (Dudgeon et al. 2009) and longnose skate, *Dipturus oxyrinchus* (Griffiths et al. 2011). Previously unknown factors such as the size and connectivity of breeding populations have also been revealed through the application of neutral molecular markers (Ward et al. 1994). Recognising significant

population structure even in the open marine environment would be unattainable or take years to acquire using traditional methods of mark and recapture or tagging studies. Accurate predictions of demographic change require a deeper understanding of population genetic structure over space and time; comprising basic information on the roles of mutation, random genetic drift, gene flow and selection, alongside patterns of genetic differentiation cast by geological and geographic factors (Hansen 2002). Attaining this level of information lies within the purview of phylogeography.

3.2.3 Introduction to Phylogeography

Phylogeny is the evolutionary history within a group of organisms. Phylogeography is concerned with the principles and processes governing the geographical distributions of genealogical lineages, especially those within and among closely- related species (Avise 1994). Valuable insights can be gleaned from the innate connections between genealogy, population demography and the historical component of population genetics (Avise 2000). It is this integration that provides great practical application whereby contemporary spatial distributions of taxonomic units can be linked with their historic origins, using genetic relatedness. Gauging the relatedness of an individual in its geographic and spatiotemporal context is a powerful approach to studying natural variation within a gene, across genes within a species, or across multiple species (Kidd and Ritchie 2006). As a sub-discipline of biogeography, phylogeography is of huge importance for the effective conservation and management of a species. To conserve the evolutionary potential of any population warrants clarification of the genetic relationships in relation to demographic data, to provide an accurate characterisation of species population structure (Dunham et al. 1999). Modification of genetic processes through habitat alteration threatens many European freshwater fishes, such that large-scale phylogeographical analyses remain mandatory for developing appropriate conservation guidelines to protect individual species (Avise 2000, Larmuseau et al. 2009).

Valuable insights into concordance patterns of genetically controlled traits (such as morphology) have relied on assessing mtDNA across individuals and populations. The role of ecological pressures and environmental gradients in generating organismal adaptations through natural selection leaves evolutionary footprints on contemporary traits that can be unravelled by molecular phylogenetics (reviewed in Avise 2000). It is now well established that the Pleistocene glacial–interglacial cycles have had fundamental evolutionary consequences (Hewitt 1999, 2000), as repeated glaciations displaced and restricted many temperate species of both aquatic and terrestrial organisms

to glacial refugia (Kotlik et al. 2008). Isolated refugial populations act as the source populations during warmer interglacial periods of recolonisation, resulting in demographic divergence of individuals (Hewitt 2004).

Rapid post-glacial colonisation of the empty habitat in central and northern Europe, (left untouched after the retreat of the European ice sheet), was dominated by terrestrial animals from southern refugia (Larmuseau et al. 2009). However, European freshwater fish are hypothesised to have a different colonisation pattern due to their restriction to hydrographical basin networks and inability to disperse through seawater (Reyjol et al. 2007). Therefore, southern Europe and those rivers draining into the Mediterranean host strikingly different ichthyofaunas from the rest of Europe north of the Pyrenees (Slechtova et al. 2004). The ability of each fish species to successfully expand their range during favourable conditions is likely determined by their individual life-history traits, ecology, biology and physiology (Larmuseau et al. 2009). For example, mountainous regions within Europe represent largely impassable barriers to aquatic fauna, except in the instance of the cold-water adapted bullhead, *Cottus gobio*. Their successful ability to 'cross' high mountains was recently uncovered using phylogenetic relationships, suggesting that active transfers of haplotypes between the highest stretches of watercourses across the European mountains, have been taking place since glacial cycles (Slechtova et al. 2004). No other fish species is found to possess such extensive gene flow. In fact, many European freshwater fish are characterised by a unique colonisation history and phylogeographic distribution patterns, making extrapolation across species inaccurate.

Major components of the UK freshwater fauna invaded from mainland European rivers such as the Danube and the Dniepr (Hewitt 2004). The temperate fishes arrived via the North Sea river, before Britain became isolated by the influx of the Atlantic ocean into the English channel (Crookes and Shaw 2009, Dawnay et al. 2011). Levels of diversity within and among UK drainages should therefore reflect post-glacial colonisation routes and size/numeracy of refugial populations. To date, many common fish species are characterised by low haplotype diversity in species such as *Leuciscus cephalus* (Durand et al. 1999), *Barbus barbus* (Kotlik and Berrebi, 2001), *Perca fluviatilis* (Nesbo et al. 1999) and *Chondrostoma nasus* (Costedoat et al. 2004), supporting the theory that western European colonisation was relatively recent and that genetic variability would be lower in comparison to refugial populations (Costedoat et al. 2006). These studies provoked investigation of the nature and distribution of contemporary diversity among

the genera *Leuciscus*, *Chondrostoma*, *Barbus* and more recently *Scardinius* (Bianco and Ketmaier 2001, Ketmaier et al. 2003). Yet little knowledge has been accrued for other cyprinid species, to provide enough detail to understand phylogeography at a regional level, *Rutilus* included (Bianco et al. 2004). Such information is crucial when attempting to discern the causal mechanism driving genetic similarities/differences, by separating the influence of relatedness from those brought about by an external stressor or the environment (Oleksiak 2010).

3.2.4 Family Cyprinidae —- the Carps and Minnows

The Cyprinidae are the largest and most specious of the freshwater fish families throughout Eurasia, North America and Africa. With over 2400 species in 220 genera (Nelson 2006) within Europe, the Cyprinidae family represents more than 50% of the total number of fish species (Reyjol 2007). Similarly, around 16 of these species belong to the genus *Rutilus* (Ketmaier et al. 2008). One of the most common species within this genus is *R. rutilus*, which shows a clear preference for riverine and lacustrine waters throughout north and central Europe, eastwards into Siberia and as far south as the Arabian Peninsula (Kottelat and Freyhof 2007). Phylogenetic analyses, carried out recently by Larnuseau et al. (2009), demonstrated that the *R. rutilus* distribution is split into two clades: One covering Western Europe and the other (the Ponto-Caspian clade) spans from Greece to Siberia. These lineages are largely congruent with fossil evidence, mitochondrial lineage studies (Ketmaier et al. 2008) and the estimated divergence of most extant cyprinid species presently seen in Europe (Zardoya and Doadrio 1999). Divergence times are estimated to be less than 10,000 years and the success of this diverse family has provided a good model of phylogenetic comparisons, to explore evolutionary mechanisms driving the diversification of other freshwater fish species (Zardova and Doadrio 1999).

3.2.5 Cyprinid Population Structure

The current distribution of roach throughout the British Isles is ubiquitous, although substantially less exist in southwest England and northern Scotland (Figure 3.2). Characterised by their high abundance throughout the lowland lotic systems of the UK, *R. rutilus* serves as a good model organism to scrutinise the effects of habitat destruction, fragmentation and modification (Baade and Fredrich 1998, Hänfling et al. 2004), migration barriers (Knaepkens et al. 2006, Geeraerts et al. 2007), environmental oestrogens (Jobling et al. 1998, 2002, Beresford et al. 2004, Tyler et al. 2005), pollution (Karels et al. 2001, Reynders et al. 2008) and restocking for angling and river restoration

(Demandt and Björklund 2007). Regarded as both an economically and ecologically valuable coarse fish, the importance of maintaining large census size is a key management focus. Despite its widespread importance and popularity, less is known about the population genetics of roach in the UK, compared to the more commercially valuable salmon and trout (Crookes and Shaw 2009).

Seen as an ecologically plastic, fecund and widely tolerant species, the roach exhibits characteristic traits that suggest populations will show little genetic differentiation across interconnected environments. Drainage systems of the UK offer ideal environments for the successful proliferation of this species; however a long history of local translocations (as far back as the late Bronze Age) may suggest that not all populations recolonised naturally after the recession of the Great British Ice Sheet (Crookes and Shaw 2009). Natural repopulation of the UK from European refuges would have been followed by the isolation of the British Isles (~7500 yr ago) and the segregation of colonised drainages into distinct subunits. Thus giving rise to the relatively low levels of genetic differentiation in both mtDNA and nuclear DNA markers, exemplified by Crookes and Shaw (2009). These authors suggest that the patterns of genetic diversity and differentiation seen across UK rivers are consistent with a historical population bottleneck, thought to have arisen from isolated refugial populations, sourced from two major migratory pathways from the continent. Recent work underpins assumptions of colonisation routes similar to the majority of northern European freshwater fish, yet little is known about local adaptation and evolution in roach populations.

Rapid divergence in life history and morphological traits can ensue when populations separate. This has been found in a selection of other fish species: grayling *Thymallus thymallus* separated only 80–90 years ago, adapting early life-history traits in 13–18 generations (Haugen and Vøllestad 2001); sockeye salmon *Oncorhynchus nerka* stocked into Lake Washington diverged adaptively within 9–14 generations in a number of early life-history traits (Hendry et al. 1998); chinook salmon *O. tshawytscha* introduced to New Zealand indicate that genetic differences influencing early growth rate may evolve within approximately 28 generations (Kinnison et al. 1998); guppies *Poecilia reticulata* in Trinidad show that life-history traits may evolve as a response to changes in mortality schedules within relatively few generations (Reznick et al. 1997). Each of these events will promote genetic divergence between local subpopulations, which can be recognised using molecular markers.

Population subdivision will also cause populations to vary in space and time. Whether

this is genetically evident within roach populations remains unknown to date (and is not accessible with the use of neutral markers). However numerous physical obstructions and substantial restocking practices that have taken place over hundreds of years, may have altered recent demographic and population genetic structure in the Thames. Consequently, reproductively isolated populations with little concurrent gene flow between them may, constitute different genetic structures. Uncovering temporal variation in genetic markers provides essential information on past microevolutionary changes and can aid the interpretation of population structure, which often becomes complicated in situations of demographic stochasticity (Tessier and Bernatchez 1999).

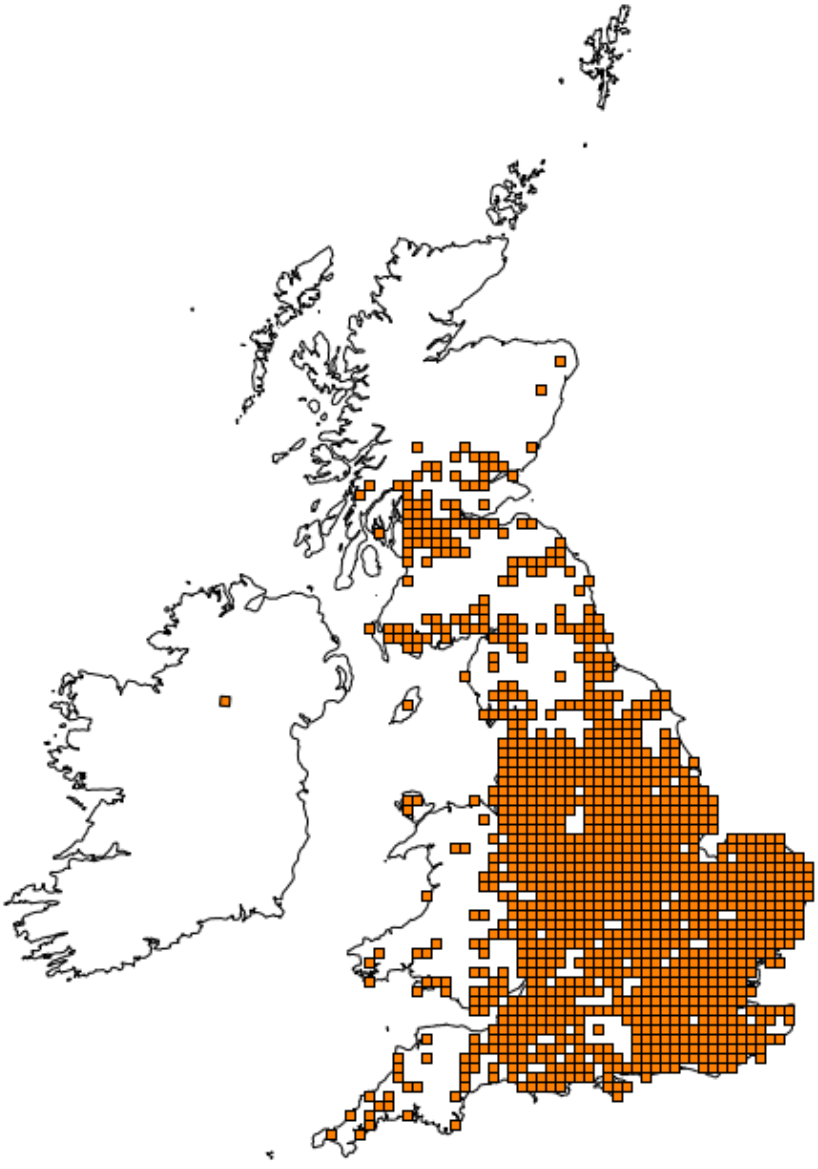


Figure 3.2 Map of recorded occurrences of *Rutilus rutilus* (represented by orange boxes) across the UK. Downloaded from https://data.nbn.org.uk

3.2.6 Temporal Stability of Fish Population Dynamics

Contextualising genetic population structure within both a spatial and temporal

framework is fundamental. Despite many studies assuming the paradigm of genetic stability over time (due to their reliance on point samples at different time points; Waples 1998, Tessier and Bernatchez 1999, Garant et al. 2000), recent studies on gastropods, shrimps and fish have focussed on emphasising temporal changes to genetic variation (He and Wang 2010, see references therein). Demographic stochasticity has the capacity to alter allele frequencies and divergence among distinct populations of a species, ultimately changing the extant population structure. However, inconsistencies within the literature on temporal stability of allele frequencies in fish species, limit any consensus being drawn. Some salmonid studies document stable patterns of genetic differentiation over time (Nielsen et al. 1999, Tessier and Bernatchez 1999, Hansen 2002), where as other marine species confirm genetic admixture — introduction of new genetic lineages through interbreeding of two or more separate populations (Hoarau et al. 2002, Barcia et al. 2005).

Mechanisms of biological forcing (life history and demographic parameters) can change genotypic frequencies in natural populations. For example, species such as roach, with high fecundity but also high mortality rates in early life stages, have a type III survival curve, making them susceptible to large fluctuations in reproductive success (Barcia et al. 2005). This can result in high variability in the number of progeny and small effective population sizes due to the contribution from a few potential parents (‘Hedgecock effect’, Waples 1998). The genetic consequences of this are expressed as significant temporal variation of allele frequencies, especially if different cohorts have been sampled (Hedgecock 1994). Unstable environments also provoke both temporary and permanent changes in species reproductive success and population size, equally likely to arise as a result of natural and anthropogenic disturbance (He and Wang 2010). Therefore profound bias can result from non-random single sampling occasions, suggesting that genetic differentiation should be investigated over longer timescales to ensure accurate estimates of population structure (Waples 1998).

Despite substantial recognition of the influence of genetic drift and microevolutionary processes on temporal genetic heterogeneity, studies are scarce within the literature. The paucity of information is largely down to practicality of obtaining suitable tissue samples that encompass multiple generations from the same spatial locations (Nielsen et al. 1999). However, the rise in popularity of PCR technology now opens up a previously untapped resource of archived tissue samples, from which genetic information can be extracted and compared (Heath 2002). Accessibility of historic genetic material has been widely

exploited for studying genetic differentiation in time and space for salmonid species, frequently concluding that temporal change within populations is minor in comparison to between population variations (Palm et al. 2003). For example, archived scales from Atlantic salmon *Salmo salar* show that genetic structure has remained relatively stable over periods of 80 years (Nielsen et al. 1999, Tessier and Bernatchez 1999), despite large changes in population sizes and the potential for allele frequency shifts due to overlapping generations (Jorde and Ryman 1995). Systematically following genetic changes through an extended period of time provides key information pertaining to population viability and evolutionary trajectories, summarising population characteristics that are directly related to the maintenance of genetic variability (Ryman et al. 1995).

3.2.7 Aims and Objectives

Before attempting to understand the influence of stochastic environmental drivers on roach populations (Chapters 4 and 6), I first needed to ascertain a baseline of information regarding their population structure, dispersal and gene flow within rivers. Knowledge of population structure can inform on the geographical scale at which changes in population size could occur. The aim of the present study was to explore the hypothesis that populations of roach in the UK represent different genetic stocks linked by gene flow, under a model of panmixia and Isolation By Distance (IBD). I first tested for population differentiation across 39 sites in the UK using allele frequencies at 14 polymorphic microsatellite loci. Sample sites were separated by in-stream barriers and had not been restocked over the last 10 years. A second line of research was to investigate the temporal population structure (using microsatellites) of roach populations in five southeastern UK rivers. I expect these restricted populations to show temporal genetic stability between years, which should denounce the relative role of drift and gene flow in shaping long-term genetic structure. Comparisons can then be drawn with other cyprinid species in the UK and discussion can ensue on appropriate conservation implications and the influence of obstructions, restocking and the size of management units.

3.3 Materials and Methods

3.3.1 Study Species and Site Selection

Cyprinid fishes commonly inhabit regions of northern Europe that have undergone alterations to the natural environment, frequently as a result of human activities. Ecologically diverse and widely abundant in lacustrine and lowland water bodies, the genus *Rutilus* play an important role in the function and structure of ecosystems, yet their

profound diversity leaves taxonomy largely unsettled and understudied. *R. rutilus* are the only species within the genus to occupy habitats north of the Pyrenees, perhaps in part due to their advantageous tolerance of habitat alteration, nutrient enrichment and slight organic pollution. Genetic differentiation or structuring between rivers will inform about gene flow among roach populations occupying different rivers and drainages.

Southern England, particularly the region within the Thames catchment, was chosen for this study for a number of reasons. Firstly, it is a densely populated region with a relatively long history of anthropogenic effects (restocking and canalisation), which could have effectively homogenised roach populations. Secondly, roach and other small cyprinids dominate fish catch in these rivers so I was able to obtain large sample sizes. Thirdly, many rivers in the region have locks, dams or weirs that could act to limit movement of fish species between stretches of river with different colonisation histories. In total, 39 sites throughout the UK were sampled between 1995 and 2011, five of which were re-visited on more than one occasion to obtain temporally separated samples. Sample sites were selected where no recorded introductions had occurred since 2000 (~3 generations for roach), based on information obtained for each of these sites from the England and Wales Environment Agency Live fish Movement Database (LFMD). Sampling locations chosen outside the Thames catchment were included based on the availability of temporal samples from scales taken historically.

Figure 3.3 Location of sample sites within the UK. The majority of roach were obtained from sites within the Thames catchment (black boundary line in A), with others sourced from populations in the River Aire, Trent, Nene and Arun.

3.3.2 Sample Collection

Biological material was derived from freshly collected fin clips and scales, obtained during routine sampling by the Environment Agency, UK. Sampling dates and geographic locations are shown in Table 3.1 and Figure 3.3 respectively. Electric fishing surveys were carried out using catch depletion electric-fishing techniques, using non-independently switched pulsed Directed Current (PDC) equipment via an Electracatch control box, with a frequency of 50 Hz (generating between 10 and 15 amps). Where the river depth was <1 m, electrofishing was conducted via wading, with a small boat to hold the generator equipment (see Figure 3.4A) and operated within enclosed sections of approximately 100 m length. At deeper sites or where catch depletion was not practical, electric-fishing was carried out using a purpose built 'boom boat' with two 2m diameter fixed anodes with concentric rings of stainless steel 'droppers' (Figure 3.4B). A single timed run of approximately 20 min was performed at each site in a downstream direction, where stunned fish were captured by EA staff in hand-held fishing nets. Captured individuals were retained in aerated holding tanks, until subsequent species identification and body length (as fork length, FL) was measured to the nearest millimetre (Figure 3.5). It was at this point that finclips (0.5 cm^2 of caudal/anal fin tissue) and 4/5 scales were obtained (Figure 3.5) from all roach for genetic analysis and retained in ethanol or scale packets, respectively. Fish were then released back into the river.

DNA analysis from archived scale samples (pre-2010) facilitated the assessment of genetic diversity over longer time periods. Dried scales had been routinely collected in fish monitoring surveys conducted by the Environment Agency and previous research projects undertaken to examine the influence of endocrine disruption on roach (Jobling et al.1998, 2002). Exact geographic locations (NGRs) were available for all historic samples, to ensure that they derived from the same locations as the contemporary collection sites. Historic samples were selected on the basis of availability of at least 30 individuals from the oldest and most proximal sample sites. Age of fish was determined by counting scale annuli (explained in more detail in Chapter 4).

Figure 3.4 Typical electrofishing procedure carried out by the Environment Agency, to obtain roach samples. Two methods are commonly employed where (A) in shallow waters, fish can be stunned using two hand-held anodes powered by a battery and transformer contained in the boat. Fish swim towards the anode, are stunned, and then caught in a net. In deeper water, (B) electrofishing is conducted using a 'boom boat'. The boat itself is the cathode, and the anodes are mounted off the bow. The stunned fish swim toward the anode, where they are then caught using a dip net

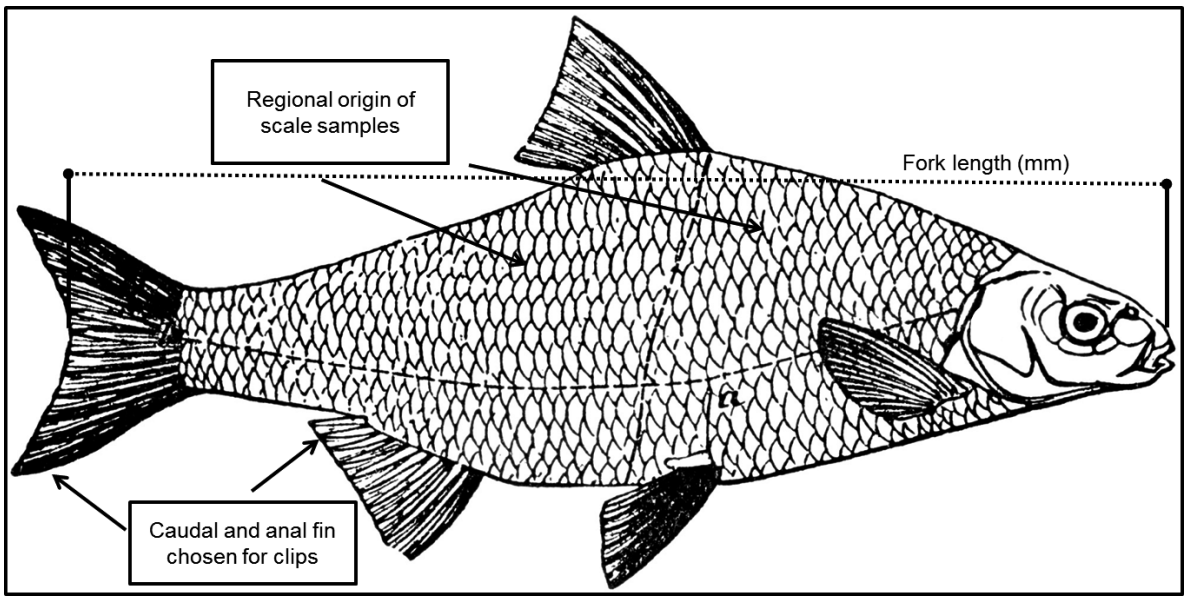


Figure 3.5 Diagrammatic representation of samples and measurements taken from each individual fish obtained from the wild. Duplicate finclips were taken to ensure against subsequent problems during processing. 4–5 fish scales were removed from behind the dorsal fin to minimise sampling of regenerated scales (as the EA routinely take theirs from in front of the dorsal fin)

3.3.3 Microsatellite Analysis

Protocols for DNA extraction from scale/fin clip tissues and details of amplification of the microsatellite loci are illustrated in general methods Chapter 2. As are details of tests performed on microsatellite loci to ensure they do not violate assumptions of further analysis undertaken in this chapter. I analysed microsatellite loci variation in 1769 fish sampled between 1995 and 2011. Each fish was genotyped at 14 microsatellite loci.

3.3.4 Population-Genetic Analyses

Data for 14 microsatellite loci were used to calculate three measures of genetic diversity: observed heterozygosity (H_o) and expected heterozygosity (H_e) using GenAlEx 6 (Peakall and Smouse 2006); allelic richness (*AR*) was calculated using Fstat v2.9.3 (Goudet 2002). To understand the extent to which roach populations are restricted to various stretches of river, several approaches were used to understand population genetic structure. Pairwise genetic differentiation between the sampled sites was estimated using $F_{ST,}$ calculated using Arlequin 3.5 (Excoffier et al. 2005). The significance of the F_{ST} estimates was assessed based on 10,000 permutations. Analysis of molecular variance (AMOVA) was performed using Arlequin. Secondly, in order to test whether fish are more likely to produce offspring with local mates, compared to mates in geographically distant locations within the Thames catchment, isolation by distance analysis was performed using the Mantel test (Mantel 1967) in GenAlEx 6 (Peakall and Smouse 2006). Differences in genetic diversity among sampled populations were tested using ANOVA. All statistical analyses were performed using the software SPSS v20.

3.3.5 Population Trees

Cavalli-Sforza and Edwards' chord distance measure D_C (Cavalli-Sforza and Edwards 1967) was used to construct trees, which has been shown to perform well in recovering the correct phylogeny in closely-related populations (Goldstein and Pollock 1997). The resulting tree was visualised using the Molecular Evolutionary Genetic Analysis (MEGA 5) program (Tamura et al. 2011).

3.3.6 Principal Component and STRUCTURE Analyses

Principal component analyses (PCA) based on Nei's D_A genetic distances (Nei et al. 1983), were carried out in GenAlEx (Peakall and Smouse 2006). To estimate the number of genetically differentiated clusters (*K*) within the data set, the program STRUCTURE v2.3.3 was used; this assigns individuals to probable clusters using a Bayesian approach (Pritchard et al. 2000). I used the standard model and a "locprior" model designed to detect weak population structure by using sampling location information. Log-likelihood values for each *K* (ranging from 1 to 39) were computed from multi-locus genotypes by running STRUCTURE three times with 125,000 repetitions each time (burn-in = 100,000 iterations). The most likely number of populations (*K*) was evaluated visually and with the method of Evanno et al. (2005).

3.4 Results

3.4.1 Genetic Diversity

In total, I genotyped 1769 roach from different geographic locations in the UK, most of which derived from the Thames catchment region (Figure 3.3). Data for 14 microsatellite loci revealed high genetic diversity in all 39 samples (Table 3.1). Allelic richness (*AR*) ranged from 6.8 to 8.9; and expected heterozygosity (H_e) ranged from 0.69 to 0.75 (Table 3.1, see Hamilton et al. 2014 for diversity statistics for each locus). Nevertheless, there were significant differences in *AR* (ANOVA, $F_{(38,532)} = 2.1398$, $p = 0.00014$) and observed heterozygosity (H_o) (ANOVA, $F_{(38,532)} = 1.8677$, $p = 0.0017$) among samples (Figure 3.6). Roach sampled at two distant sites (LamSha and LeeHUS'95) exhibited significantly low allelic richness and LeeHyd'95 exhibited low observed heterozygosity. No significant differences in H_e were found.

3.4.2 Genetic Analysis of Roach in English Rivers

Genetic differentiation, estimated by F_{ST} between sampling locations ranged from 0.000 to 0.09 and 0.000 to 0.19 respectively (LeeHyd sample from 1995 and LamSha in the tributary of the Kennet). Analysis of molecular variance (AMOVA; Table 3.2) indicated

the majority of variation was partitioned among individuals within river locations, with river location accounting for a small 2.27%, but highly significant proportion of the genetic variation. Average F_{ST} between roach samples from different catchments was 0.027 (Table 3.3). Within the Thames catchment itself, the average F_{ST} between samples was 0.022, only slightly lower than the average for between-catchment comparisons (0.027). Additionally, 262 of the 325 pairwise F_{ST} comparisons in the catchment were highly significant after sequential Bonferroni correction of *p*-values (adjusted $p = 0.000069$, Appendix Table 3.5). Significant genetic differentiation existed between samples obtained from neighbouring river stretches (Table 3.4), for example within all the upper Lee sites, between the Blackwater and main Thames, between the Lambourn and the Kennet and within the Anglian Blackwater. However, this divergence over short distances was not always present, as there is no significant genetic differentiation within neighbouring sites on the Stort, the main Thames, the Kennet, the Arun, the Nene and the Trent (F_{ST} Appendix Table 3.5). Notably, significant yet weak, isolation by distance was observed across the Thames catchment ($r^2 = 0.109$, $p < 0.01$; Figure 3.7).

Table 3.1 Sampling locations, genetic diversity statistics (allelic richness (*AR*) and expected heterozygosity (*He*) for each population sample).

Sample code	River	Year	Age range (yrs)	N° of fish genotyped	*AR*	H_e	Source of historic samples
	Blackwater						
BlaBlM	Blackwater	2010	2-8	55	7.9	0.71	
BlaSti	Blackwater	2010	2-7	55	8.2	0.72	
CheAbB	Chelmer	2010	2-8	50	7.7	0.75	
	Nene						
NenBro'95	Nene	1995	2-6	47	8.3	0.72	(Jobling et al. 1998)
NenBro'99	Nene	1999	2-7	47	8.3	0.73	(Jobling et al. 2002)
NenEct	Nene	2007	3-7	51	8.6	0.73	
Aire							
AirDar	Aire	2011	1-7	43	8.6	0.75	
Arun							
AruHor'95	Arun	1995	2-7	54	7.8	0.73	
AruHor'00	Arun	2000	2-7	34	7.6	0.73	(Jobling et al. 2002)
AruHor'08	Arun	2008	3-7	69	7.9	0.74	(Harris et al. 2011)
AruHUS	Arun	1995	2-6	48	7.4	0.73	(Jobling et al. 1998)
	Thames catchment						
BlaEvH'10	Blackwater	2010	2-5	41	8.5	0.72	
BlaEvH'00	Blackwater	2000	2-6	47	8.3	0.71	(Jobling et al. 2002)
BouChe'11	Bourne	2011	2-6	56	8.3	0.74	
BouChe'02	Bourne	2002	3-7	31	8.8	0.75	
BouChe'06	Bourne	2006	3-6	48	8.3	0.75	(Harris et al. 2011)
GadCas	Gade	2010	2-7	56	8.1	0.74	
KenBul	Kennet	2010	2-9	51	8.4	0.74	
KenFou	Kennet	2010	2-6	32	8.7	0.75	
KenNor	Kennet	2010	2-12	52	8.5	0.73	
LamSha	Lambourn	2011	4-7	41	6.8	0.72	
LeeEss	Lee	2010	1-5	56	8.3	0.73	
LeeHyd	Lee	2010	2-7	28	8.2	0.73	
LeeHyd	Lee	1995	1-6	44	7.7	0.70	(Jobling et al. 1998)
LeeHUS	Lee	1995	1-7	37	7.0	0.73	(Jobling et al. 1998)
LeeSta	Lee	2010	1-6	31	8.4	0.71	
Lee'00	Lee	2000	3-7	41	8.1	0.70	(Jobling et al. 2002)
LeeWhe	Lee	2010	1-6	55	8.5	0.75	
MolMea	Mole	2010	2-8	42	8.4	0.73	
RayRod	Ray	2003	2-6	30	7.7	0.72	
StoBri	Stort	2010	1-5	52	8.1	0.71	
StoTed	Stort	2010	2-7	30	8.2	0.69	
ThaCul	Thames	2010	1-8	44	8.8	0.74	
ThaHam	Thames	2010	2-6	44	8.2	0.74	
ThaWhi	Thames	2010	2-7	60	8.2	0.74	
ThaSha	Thame	2010	1-7	50	7.9	0.74	
WanMoh	Wandle	2011	1-7	48	8.2	0.75	
Trent							
TreWol	Trent	1995	1-9	45	8.0	0.72	
TreNot	Trent	2007	3-7	24	8.9	0.75	

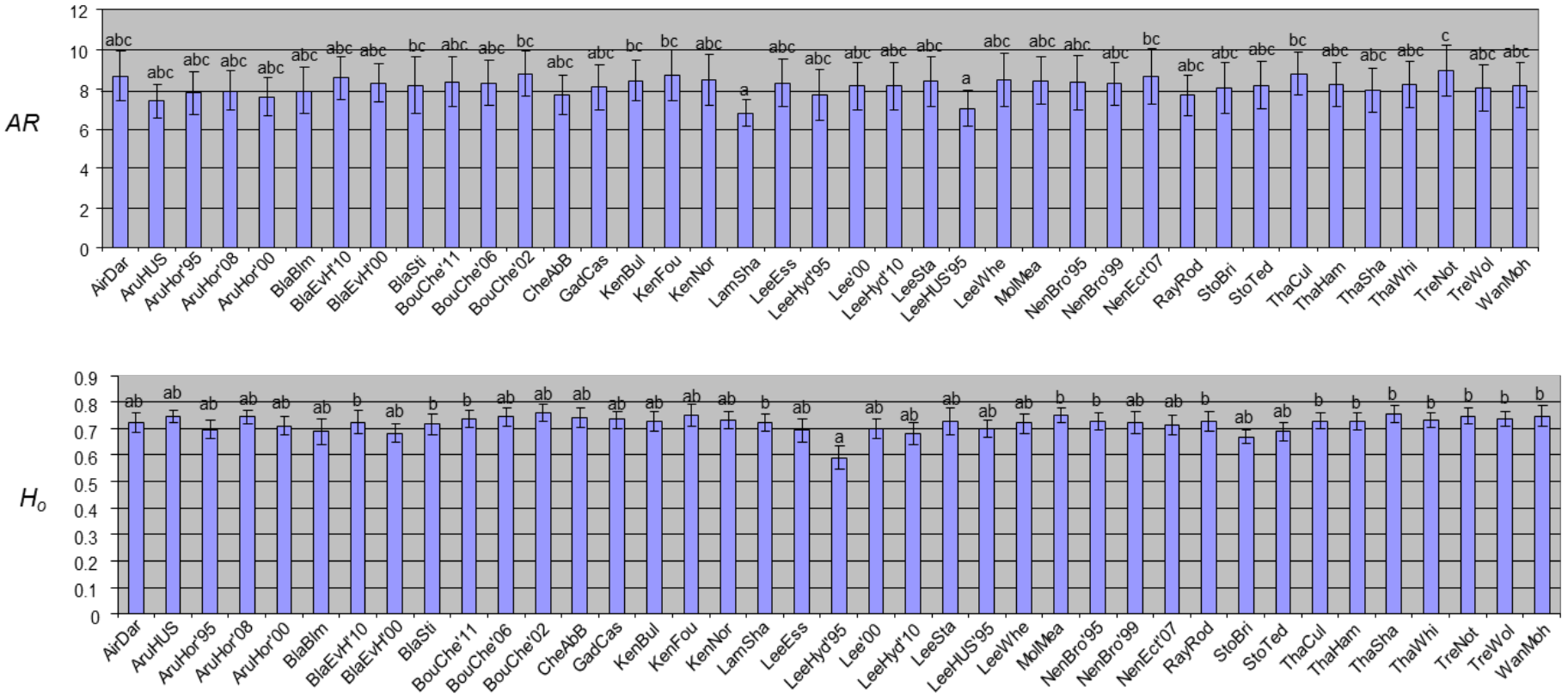


Figure 3.6 Genetic diversity among roach *Rutilus rutilus* populations. Two measures of genetic diversity, allelic richness and observed heterozygosity (*Ho*) were estimated from the genotype data for 14 microsatellite loci (mean ± SE). Levels not connected with the same letter are significantly different according to post-hoc Tukey-Kramer HSD

Table 3.2 Analysis of molecular variance (AMOVA) testing for partitioning of genetic variation among roach samples, grouped according to geography

Source of variation	d.f.	Sum of squares	Variance	% total	*p*-value
Geographical partition, location [1]					
Among locations	32	584.063	0.11631	2.27	< 0.00001
Among samples within locations	6	35.197	0.00953	0.19	0.03226
Within samples	3499	17,489.296	4.99837	97.54	< 0.00001
Total	3537	18,108.556	5.12421		
Geographical partition, catchment [2]					
Among groups	5	189.529	0.06309	1.22	< 0.00001
Among samples within groups	33	429.731	0.08909	1.73	< 0.00001
Within samples	3499	17,489.296	4.99837	97.05	< 0.00001
Total	3537	18,108.556	5.15055		

[1] Samples from same location caught in different years are grouped.

[2] Samples grouped by catchment (Aire, Arun, 'Blackwater and Chelmer', Nene, Thames and Trent).

Table 3.3 Summary of pairwise F_{ST} among roach samples from different catchments. [1] average, (range)

	F_{ST}
Between catchments	
Thames/Arun	0.033, (0.002–0.074) [1]
Thames/Nene	0.026, (0.007–0.076)
Thames/Trent	0.026, (0.008–0.069)
Thames/Chelmer, Blackwater	0.022, (0.005–0.057)
Within catchments	
Thames	0.022, (−0.004–0.090)
Arun	0.007, (0.002–0.014)
Nene	0.002, (0.000–0.003)
Blackwater/Chelmer	0.022, (0.005–0.057)

Table 3.4 Pairwise F_{ST} among neighbouring samples in the River Lee, Blackwater and Arun from 1995 and 2010

	F_{ST}	*p*-value [2]
LeeHUS '95/LeeHyd'95	−0.002	0.78
LeeHyd'10/LeeWhe	0.020	>0.00001
LeeWhe/LeeSta	0.009	>0.00001
LeeSta/LeeEss	0.009	>0.00001
Other neighbouring stretches		
BlaBlM/BlaSti	0.007	>0.00001
AruHUS '95/AruHor'95	0.002	0.22

[2] for F_{ST} estimate

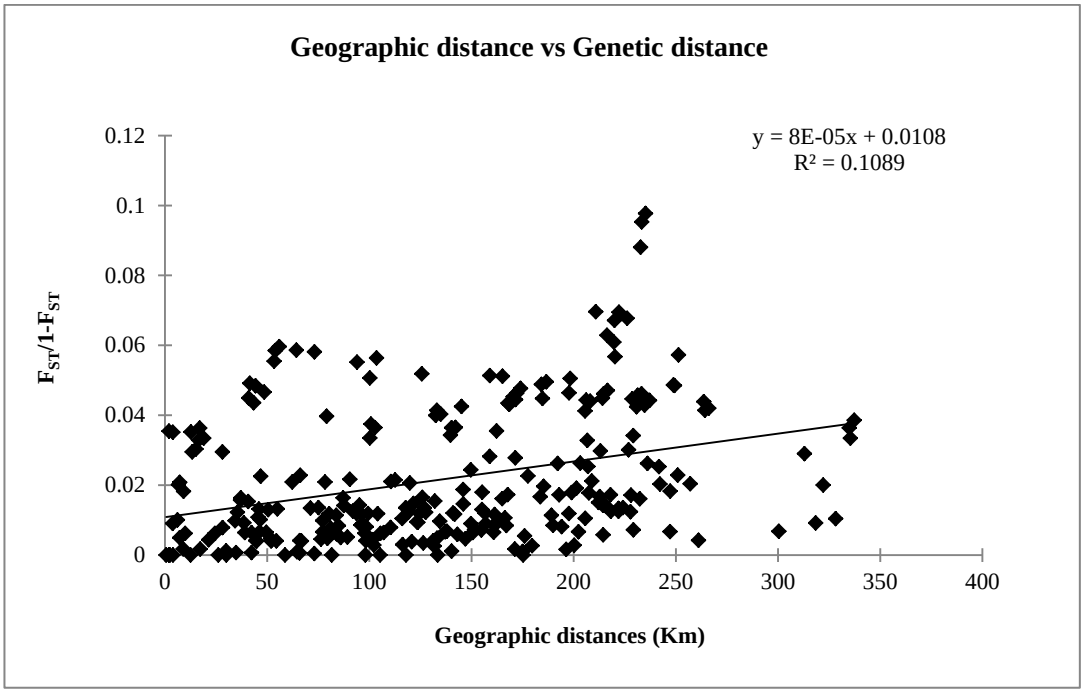


Figure 3.7 Correlation between genetic distance and geographic distance (km) between pairs of sites for 24 population samples from the Thames catchment

3.4.3 Population-Genetic Structure of Roach

The population tree (Figure 3.8) showed clusters of samples from rivers outside the Thames: the Arun, the Nene, the Anglian Blackwater and the Trent, supported with moderate-high bootstrap values (>64%). Some geographically distinct groups failed to unite, for example all sites in the River Thames failed to cluster, and the River Lee and River Stort failed to group, despite being geographically distant from the rest of the Thames. I found no evidence for a distinct genetic identity of roach in the Thames region compared to any other catchment, as samples failed to group together in the tree. However, within the Thames catchment, several of the populations in geographical proximity grouped, although not necessarily receiving bootstrap support. Including populations in the Wandle and Mole sites, and the three populations from Kennet and its tributary the Lambourn. Within the River Lee, populations from the two most upstream sites grouped with moderate bootstrap support (69%) and grouped with the next population downstream (LeeWhe). The two populations further downstream in the River Lee (LeeSta and LeeEss) clustered with each other, with high bootstrap support (77%). These downstream sites on the Lee (LeeEss, LeeSta) also group closely with those from the River Stort (80%), which is consistent with their geographical proximity.

The inclusion of temporal samples from a subset of locations provided additional insights into the short-term demographic temporal stability at some locations. The population samples from the River Blackwater (Thames) collected 10 years apart (~2–3

generations) clustered with 98% bootstrap, as did samples from an upstream location in the River Lee collected 15 years apart, (approximately 3–4 generations). The only river where multiple temporal samples were taken, that did not cluster together in the tree was the Bourne. Some disparity and genetic distance was evident between contemporary roach sampled (BouChe11) and those obtained from historic samples on the Bourne in 2002 and 2006. Overall, analysis of replicate samples suggested a strong signal of temporal stability in some river sections.

Despite inconsistencies in the genetic differentiation between samples from proximal river stretches, the PCA analysis demonstrated the distinctiveness of samples from the River Arun, the River Nene (Figure 3.9a) and the two uppermost sites in the River Lee. Sites from the River Aire and the River Trent however were not distinctive despite being geographically isolated. Restricting analyses to sites within the Thames catchment (Figure 3.9b) revealed greater structure; samples from the River Lee clustered and were close to those from the River Stort, a tributary of the River Lee. Figure 3.9b identifies LamSha as an outlier within the Thames catchment samples, which is also backed up by evidence from the tree (Figure. 3.8) and significantly different *AR* results (Figure 3.6) from this site.

Samples from the Arun and the Nene grouped in the PCA and the STRUCTURE analyses (Figure 3.9 and 3.10) demonstrating a distinct genetic identity of the roach at these sites and a concordance between these analytical approaches. Adopting the Evanno et al. method (2005), the inferred most likely number of genetically distinct clusters in the STRUCTURE showed K=3: the Arun, the two most upstream in the Lee, and the remaining sites. Nevertheless, visual examination of STRUCTURE plots at higher levels of K demonstrated the distinctiveness of several of the locations (three most upstream sites in the River Lee, LamSha, River Nene and River Arun), but failed to identify distinct clusters within the majority of the Thames catchment.

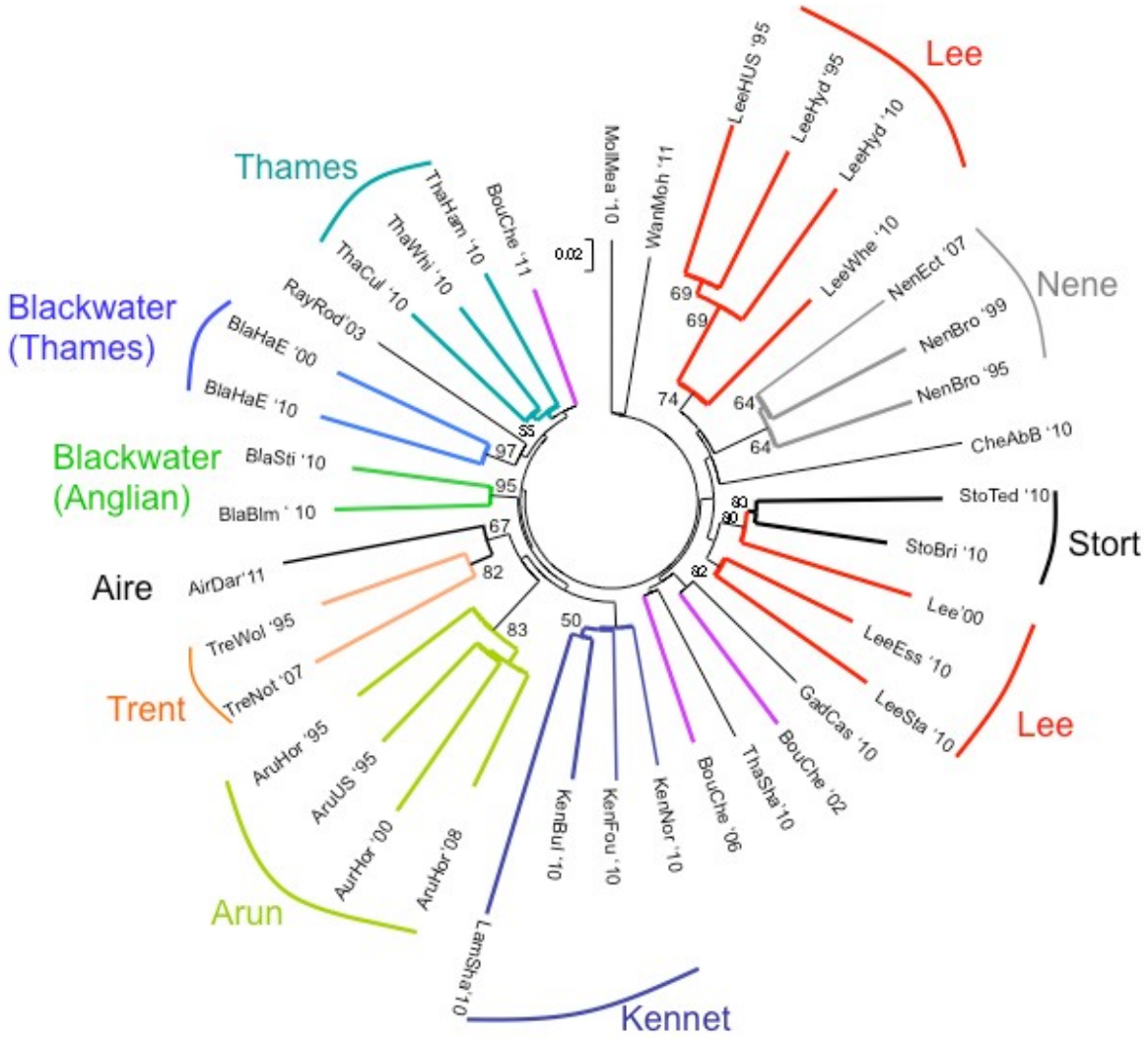


Figure 3.8 Neighbour-joining phylogenetic tree for the 39 roach population samples. The tree is based on the data from 14 microsatellite loci using chord distance (Cavalli-Sforza and Edwards 1967). Only bootstrap values above 50% are shown. Abbreviated names of sample sites are explained in Table 3.1. Numbers at the end of sample codes indicate years that populations were sampled and groups from the same river are highlighted by adjoining lines

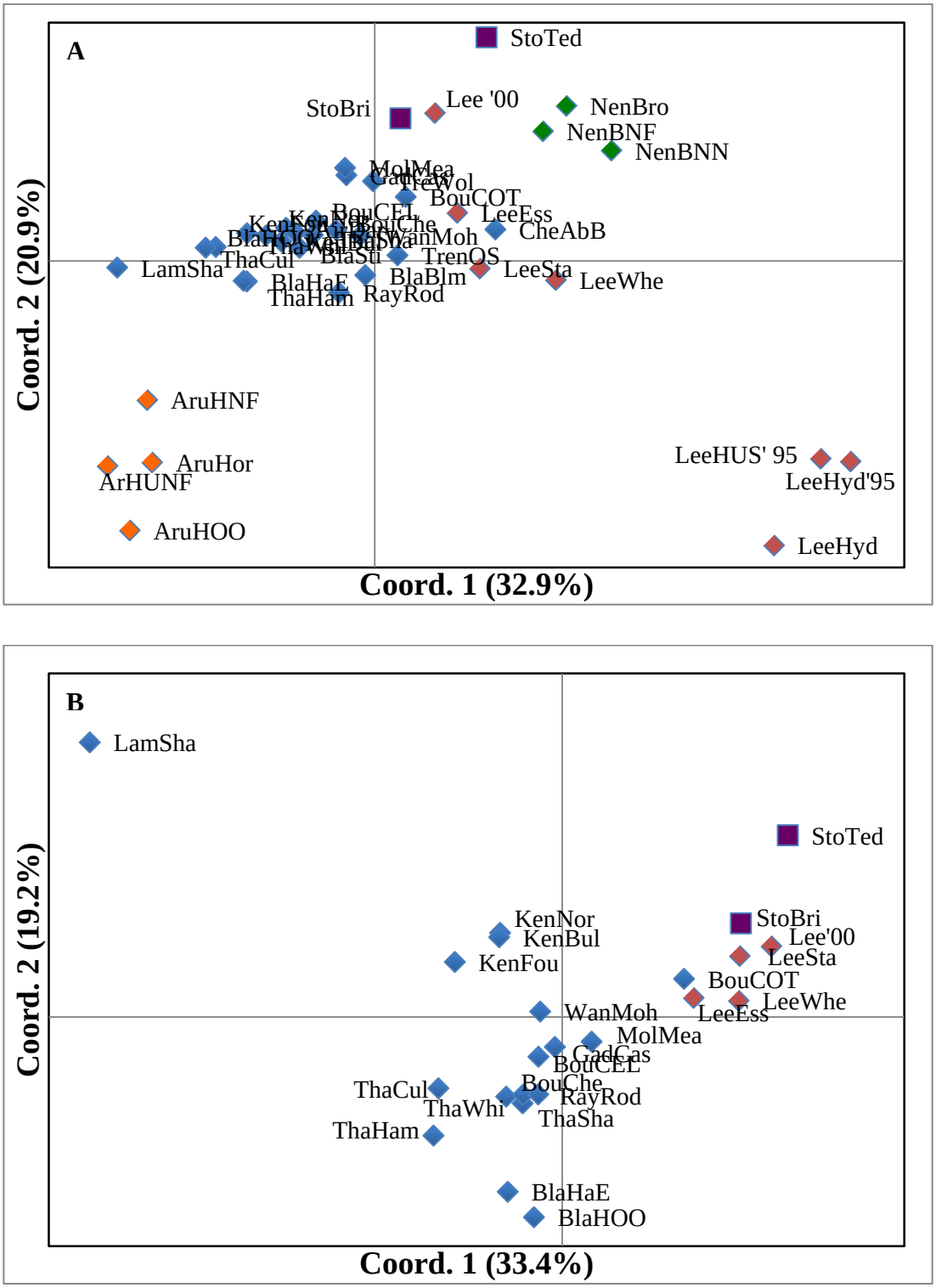
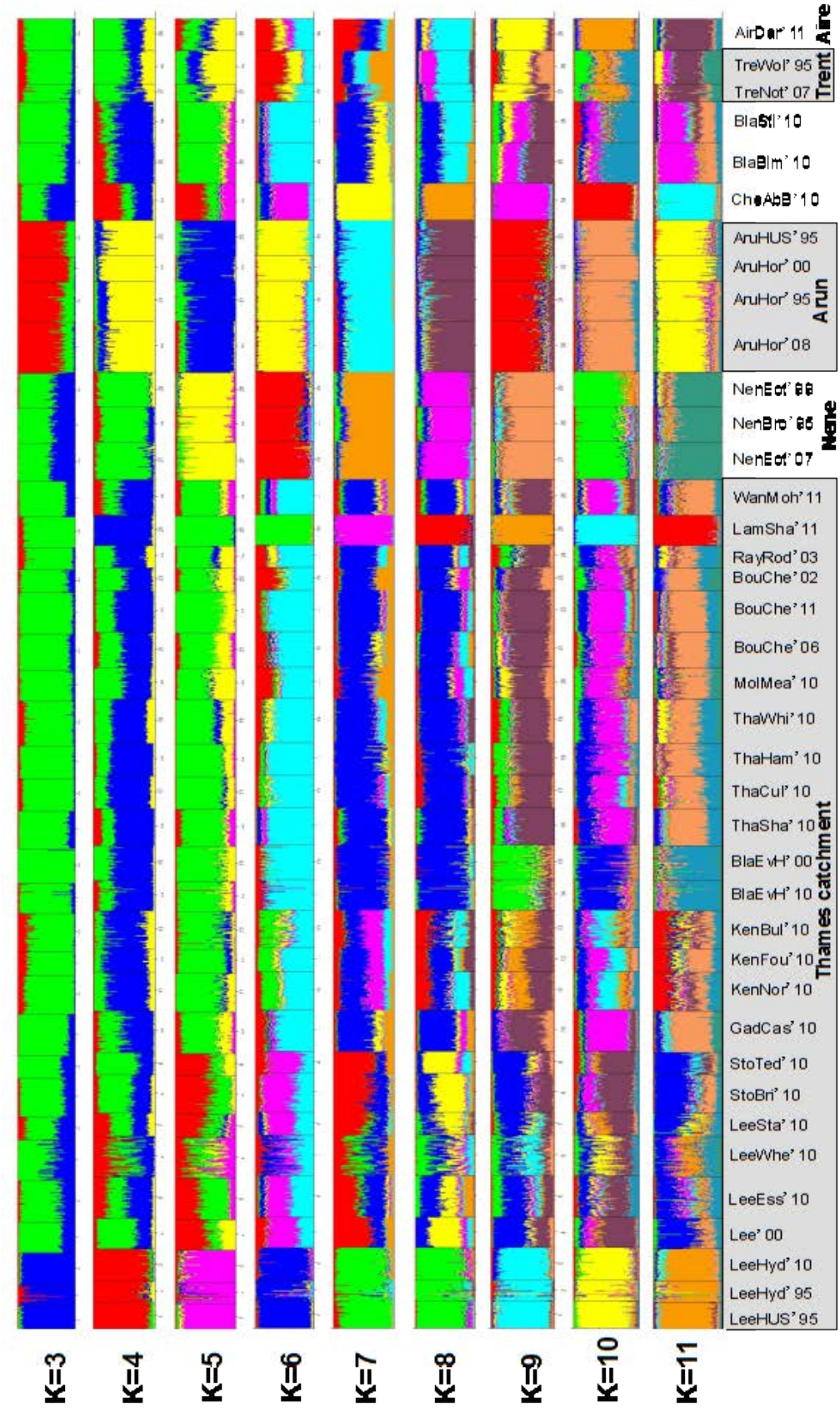


Figure 3.9 Multidimensional scaling plots of pairwise Nei genetic distances. Populations that are genetically similar group close to each other. The percentage of variation explained for each PCo axis is in parentheses. (A) Analysis including all 39 population samples. (B) Analysis of sites within the Thames catchment and excluding three population samples from the upper River Lee that are distinctive in Figure 3.9A

Figure 3.10 Structure analyses plots using the locprior model. Each individual is represented by a thin horizontal line which is partitioned into *K*-coloured segments representing an individual's estimated membership to fractions of *K* clusters. Analyses suggested the optimum number of genetic units to be 1 (indicating that all fish belong to a single population) using the standard admixture model and 3 using the location model



3.5 Discussion

The primary objective of this chapter was to map the genetic differences within and among roach populations from UK rivers. This was achieved by comparing genetic diversities across 32 locations using 39 contemporary population samples, employing 14 microsatellite loci. Analysis of population structure revealed inconsistencies in evidence of genetic structuring using microsatellite markers, which are understood to be selectively neutral.Roach populations throughout all sites had moderately high levels of genetic diversity at microsatellite DNA loci (for a northern European freshwater fish). In comparison to other fish species, roach seem to possess levels of genetic variation similar to marine fishes, which have large census population sizes and extensive habitat range (Dewoody and Avise 2000). High levels of genetic diversity may in part be due to their ubiquity, abundance and widespread distribution through the UK (Frankham 1996). However, statistically significant differentiation between and within all catchments indicates the presence of multiple genetically differentiated sub-populations of roach in UK rivers. This is consistent with previous work on roach (Crookes and Shaw 2009) and other freshwater fish species that used mtDNA markers to uncover the phylogeography of species such as barbel, chub and perch (Kotlík and Berrebi 2001, Durand et al. 1999**,** Nesbo et al. 1999).

The majority of genetic variation was partitioned among individuals within river locations and separate catchments (97.54 and 97.05% respectively). Although a small but significant proportion of genetic variation was also accounted for between catchments and locations (1.22 and 2.27%). Similar patterns of variation in roach across sites denote limited gene flow between sub-populations, repressing interbreeding and promoting divergence among sites and rivers through genetic drift (diverging allele frequencies until mutation–drift equilibrium; Crookes and Shaw 2009). Evidence of genetic structuring may also be a factor of scale; roach populations sampled here, although dominated by rivers within the Thames catchment, covered a relatively large expanse of the range of roach within the UK, from latitudes as far south as the Arun, to the Aire river system in the northeast of England. Ignoring the potential confounding influence of human translocations and restocking, physical segregation over these distances would be expected to result in genetic differences in roach population structure. As such, the results seen here are in agreement with common patterns arising from geographical segregation of populations.

No evidence from the tree, PCA or STRUCTURE analyses indicated that the Thames

catchment stands as a single genetic unit. Roach populations from this catchment are statistically indistinct from geographically distant drainages where mixing of individuals (except via human translocation) is highly improbable, due to lack of riverine connectivity. This may reflect a lack of distinctiveness of roach in the Thames catchment or may also result from the limited ability of microsatellites to detect population structure at such a fine scale in a common, abundant species. Within catchment differentiation of roach populations sourced from the Thames sites is close to that seen between catchments, along with >97% of the variation being between individuals. Such variety within a single catchment, and sometimes within proximal sites/river stretches may have meant that most Thames individuals will have mixed membership in multiple population clusters (Pritchard 2004).

Anecdotal visual examination of STRUCTURE plots run with higher levels of *K* (Figure 3.10) suggests the presence of multiple genetic groups but unfortunately these are not statistically supported. Conflicting results could arise from differing methods and parameters applied within the array of programs used to decipher genetic population structure across sample ranges. For example, it has been suggested that the underlying model upon which STRUCTURE is based, is poorly suited to situations where isolation by distance is apparent (as seen here; Pritchard et al. 2010). Accuracy of the correct number of clusters can also be compromised by limited sample sizes or reduced numbers of microsatellite loci (Waples and Gaggiotti 2006), perhaps eluding to the potential reasons behind the limited number of clusters statistically supported by STRUCTURE (3).

3.5.1 Roach Population Situation in the Thames Catchment

Despite inconsistencies in genetic identity outputs from various statistical tests, iterative analyses indicated significant structuring within roach populations from the Thames catchment. Local sub- populations, capable of exchanging small numbers of effective migrants (breeding individuals) over time are emergent, with river distance also playing a role in genetic divergence between populations (Mantel test, isolation-by-distance; r^2 = 0.1089, p = 0.010 Figure 3.7). IBD is not uncommon in European freshwater fish but has only been reported once previously for roach (Crooke and Shaw 2009). Testing relatedness of individual roach was beyond the remit of this study, however the weak but significant relationship between genetic and geographic distance provides evidence to reject panmixia (where all individuals are potential partners). Instead, these findings

indicate a tendency for individuals to produce offspring with fish from nearby rather than distant populations. This assumption was further supported by 262 of the 300 pairwise F_{ST} comparisons in the catchment being highly significant.

The level of differentiation among roach populations within the Thames catchment is low compared to other freshwater fish (Ward et al. 1994, DeWoody and Avise 2000) but is consistent when examined in context with previous studies on roach and other cyprinids (F_{ST} roach = 0.036 and 0.02, Hanfling et al. 2004; Tench = 0.024, Kohlman et al. 2007). Low spatial genetic structure may be because genetic differentiation is potentially continuous in mobile species, with a wide ranging distribution, and known migrations of up to 10 km (Baade and Fredrich 1998). This evidence shows commonalities with the typical metapopulation structure that appears to emerge from the sites sampled within the Thames catchment. Roach populations in this study consist of a group of spatially-separated entities, which interact but do not exchange a large number of individuals over few generations. Neighbouring populations however, do encounter rates of gene flow (migration) over longer timescales that mitigate reproductive isolation and strong divergence aided by the opportunistic and mobile nature of roach as a species. This emerging pattern appears consistent across the Thames catchment and within individual rivers themselves. For example, the population tree (Figure 3.8) and PCA analyses (Figure 3.9) showed groups comprising samples from neighbouring sites: three from the main Thames; four from the Kennet and its tributary (Lambourn); samples from the Stort and the Lee; and samples from the Wandle and Mole.

Biogeographic and historical processes are known to influence the genetic structure of fish populations across drainages (Carvalho 1993). Within the Thames catchment, the observed population structure likely results, in part, from the large number of physical barriers such as weirs and locks, which are major factors restricting movement (including downstream) of roach (Geeraerts et al. 2007). Temporally stable populations in a subset of sites sampled over multiple generations (Figure 3.9) also lends support to the hypothesis that roach populations are largely restricted to stretches of river, exchanging few effective migrants over time. However, this study was not systematically designed to test for potential causes of within-river genetic heterogeneity. Therefore it remains unclear whether the magnitude of apparent population subdivision is related to the number of in-stream barriers, the distribution of spawning beds within tributaries, and/or isolation-by-distance processes.

Microsatellite markers were effective in uncovering sub-population structure in Thames roach populations, which appear different between and within proximal river stretches. A notable conflict in results is apparent between some neighbouring stretches in the Thames drainage that do not exhibit any genetic differentiation, despite being separated by in-stream impoundments. Examples include sites within the River Stort, mainstream Thames, and the River Kennet. Characteristically, all of these large rivers are coupled with or form part of navigable stretches delineated by locks that are in regular operation. This would be conducive to fish movement and allow more opportunity for migration and mixing of individual fish between stretches. It must also be noted that the locks within the main R. Thames and the R. Kennet are equipped with fish passes, and although some passes can be used by roach, the effectiveness of these passes has not been studied (Knaepkens et al. 2006). Similar patterns are exhibited in the River Nene and Trent roach populations, where a low degree of differentiation between proximal sites may have been compounded by their relatively large size and navigable habitat. All these rivers clearly provide greater opportunities for movement between sites, thus increasing the population carrying capacity due to bigger habitat patches and minimising differentiation between locales.

3.5.2 Implications for Roach Populations

The value of conserving roach populations lies in their importance as a commodity species for angling enthusiasts. Conservation and management of stocks by the Environment Agency is funded through rod license fees, where efforts to maintain natural population biodiversity are based on knowledge of fish population dynamics However, roach also contribute significantly in numbers and biomass to the functioning of riverine ecosystems. Knowledge gaps, including roach population differentiation and the relative influence of human-induced modifications to riverine environments, can be better understood from information obtained through genetic analyses.

The population structure of roach in the UK, observed in this study, may have been influenced to a greater or lesser extent by historical biogeography, natural behaviour, human translocations and obstructions. With these processes in mind, interpretation of my findings leads us to explore commonalities of roach population structure with typical northern European freshwater fish. Post-glacial distribution of small isolated refugial populations of roach and other freshwater fish species are thought to have colonised the UK from the continent during range expansion (Hewitt 2000), giving rise to the low

levels of genetic variation seen across the UK (typical of post-colonisation bottleneck). Isolation of successful drainage populations prompts diversification and exacerbates genetic differentiation between river systems, such that mtDNA haplotype frequencies demonstrate significant differences among 15 UK drainages (Crookes and Shaw 2009). Significant genetic differentiation between catchments sampled here, appears consistent with historic biogeography and processes akin with physical separation/isolation (Hanfling et al. 2004), despite low levels of genetic differentiation apparent across the UK river network. Retaining high heterozygosity following a historical bottleneck suggests that roach populations in the UK possess adequate population sizes, temporal stability and some exchange of individuals to buffer demographic fluctuations.

Broad scale patterns seen are in line with expectations derived from typical life-history characteristics of a widespread and mobile freshwater cyprinid species. Local scale patterns of roach population genetics within river stretches suggest differences in population unit size (some of which may be smaller than a river). However, anthropogenic translocations do not appear to have homogenised population genetics of roach in the UK, nor do I see consistent effects of barriers on levels of genetic differentiation between proximal sites. Previous studies document ambiguity concerning the influence of locks and weirs on genetic structuring, proving to be more important in highly mobile species such as grayling *T. thymallus* (Meldgaard et al. 2003) as opposed to bottom-dwelling stone loach *B. barbatula (*Knapen et al. 2009). Roach can migrate over 10 km particularly in the spawning period April–June if migration is not obstructed (Baade and Fredrich 1998), which is consistent with previous observations of local deme sizes of approximately 10 km (Crookes and Shaw 2009).

Current distribution of many freshwater fish species in the UK has been affected by the sporadic introduction and translocation of individuals by artificial means (Wheeler and Maitland 1973). Direct impacts of roach restocking, (approximately 500,000 1+ roach have been introduced into the Thames catchment since 2000, from a facility where roach broodstock are sourced from the River Trent), may have weakened population structure throughout the Thames catchment. Studies have recommended that stocked individuals (as an intervention of last resort) should originate from local broodstocks, after first attempting to boost population numbers through ecological and environmental improvements (Crookes and Shaw 2009). However, the census size of roach populations in the Thames catchment and the success of the reintroduced fish are currently unknown, making it impossible to predict the influence of restocking on population structure,

prompting further examination in future studies.

Maintenance of population genetic diversity is reliant on a population's effective size. Reducing the number of breeding individuals increases the likelihood of inbreeding/genetic drift resulting in genetic diversity loss (Frankham 2002). Preservation of the genetic mosaic is therefore paramount to the viability of populations, and vice versa. My results, encompassing genetic variation and differentiation within and between populations, show that management should be conducted at limited geographic scales to protect evolutionary significant units. These genetic units define broad patterns comprising single river catchment populations, where demographic processes act over tens of kilometres (in line with previous studies Crookes and Shaw 2009). There is some evidence that roach show fidelity in migration and return to spawning sites they have used previously (Brodersen et al. 2011, Vollestad and Labeelund 1987) which may substantiate observations of temporal stability in genetic structure over generations. Additionally, the presence of consistent patterns of genetic structuring affirms the unlikeliness of sampling artefacts and assures us that the population structure seen is reliable and not a result of noise from non-random sampling of the global population (Garant et al. 2000). Nonetheless, species-specific characteristics are such that, I cannot exclude the possibility that populations have developed local adaptations to their lotic environment.

3.6 Conclusion

Currently in the UK, fisheries managers assume panmixia; estimating census population sizes from formulas that extrapolate catch from 100 m stretches to full river length. This form of monitoring is not suitable for UK roach populations and makes large assumptions that could produce misleading estimates of population abundance and changes over time. To this end, neutral markers offer an important approach for estimating genetic diversity and historic variation in fish populations (Carvalho et al. 2003, Reed and Frankham 2003), providing a valuable picture of population structure. Number of microsatellite loci and individuals sampled appear appropriate for discerning genetic differences even between sub-populations in close proximity. Without a baseline understanding of historic population fluctuations and patterns of dispersal, it is impossible to discriminate between the relative contributions of true population differentiation versus temporal and stochastic variation (Waples 1998, Garant et al. 2000). These results provide novel information on the genetic structure of roach populations within the UK, unveiling

additional knowledge on the temporal stability of genetics over multiple generations. However, implications of these findings require careful interpretation. Despite documenting high levels of genetic diversity and limited evidence for detrimental anthropogenic influences, these may be attributable to species' tolerance and plasticity as opposed to proof of infliction. For example, roach are well documented to be abundant in a wide variety of habitats, including slow moving lotic systems and lakes where waters are nutrient rich (Kottelat and Freyhof 2007). An inherent tolerance for pollution and such ecological flexibility may moderate signatures of genetic erosion brought about by anthropogenic influences, compared to other freshwater species that are more sensitive to environmental change. This study was not systematically designed to unravel the causes of genetic heterogeneity across the species range; it therefore remains unclear the role of the number of in-stream barriers, the distribution of spawning beds within tributaries, and/or isolation-by-distance processes being responsible for population subdivision in this species.

	LeeHUS	LeeHyd'10	LeaHyd'95	Lee'00	LeeEss	LeeWhe	LeeSta	StoBri	StoTed	GadCas	KenNor	KenFou	KenBul	BlaEvH'10	BlaEvH'00	ThaSha	ThaCul	ThaHam	ThaWhi
LeeHUS	*																		
LeeHyd'10	0.000	*																	
LeaHyd'95	-0.002	-0.003	*																
Lee'00	0.052	0.055	0.052	*															
LeeEss	0.032	0.035	0.034	0.014	*														
LeeWhe	0.020	0.020	0.019	0.017	0.006	*													
LeeSta	0.034	0.029	0.030	0.013	0.009	0.010	*												
StoBri	0.050	0.050	0.048	0.001	0.015	0.019	0.017	*											
StoTed	0.056	0.057	0.058	0.009	0.018	0.025	0.017	0.000	*										
GadCas	0.042	0.043	0.045	0.020	0.013	0.016	0.027	0.017	0.026	*									
KenNor	0.041	0.043	0.043	0.016	0.012	0.017	0.013	0.016	0.019	0.013	*								
KenFou	0.040	0.042	0.043	0.016	0.011	0.018	0.017	0.015	0.020	0.009	-0.004	*							
KenBul	0.041	0.044	0.045	0.016	0.014	0.013	0.017	0.015	0.024	0.010	-0.001	-0.001	*						
BlaEvH'10	0.051	0.045	0.055	0.024	0.021	0.022	0.020	0.028	0.040	0.018	0.019	0.015	0.017	*					
BlaEvH'00	0.062	0.055	0.068	0.027	0.025	0.031	0.024	0.032	0.040	0.022	0.022	0.017	0.025	0.001	*				
ThaSha	0.042	0.040	0.041	0.024	0.018	0.020	0.022	0.020	0.028	0.011	0.008	0.004	0.012	0.013	0.015	*			
ThaCul	0.046	0.046	0.055	0.022	0.016	0.020	0.026	0.022	0.032	0.012	0.008	0.004	0.007	0.009	0.011	0.009	*		
ThaHam	0.047	0.043	0.048	0.027	0.017	0.022	0.027	0.024	0.038	0.010	0.013	0.004	0.010	0.009	0.014	0.005	0.001	*	
ThaWhi	0.043	0.044	0.046	0.019	0.012	0.017	0.019	0.016	0.028	0.007	0.007	0.002	0.006	0.008	0.012	0.006	0.001	0.001	*
MolMea	0.042	0.042	0.043	0.011	0.007	0.012	0.018	0.009	0.017	0.006	0.007	0.003	0.009	0.014	0.016	0.009	0.001	0.008	0.001
BouChe'06	0.037	0.037	0.035	0.020	0.017	0.016	0.022	0.017	0.028	0.002	0.011	0.005	0.010	0.011	0.015	0.004	0.009	0.005	0.003
BouChe'11	0.038	0.040	0.040	0.016	0.010	0.016	0.020	0.013	0.024	0.007	0.003	0.000	0.004	0.014	0.018	0.004	0.003	0.005	-0.001
BouChe'02	0.033	0.035	0.036	0.009	0.009	0.005	0.013	0.007	0.016	0.001	0.006	0.005	0.004	0.017	0.026	0.010	0.012	0.013	0.008
RayRod	0.035	0.032	0.038	0.014	0.009	0.010	0.020	0.019	0.030	0.013	0.008	0.007	0.010	0.006	0.012	0.012	0.005	0.006	0.004
LamSha	0.081	0.087	0.090	0.067	0.059	0.063	0.063	0.063	0.068	0.050	0.034	0.029	0.034	0.055	0.066	0.052	0.038	0.045	0.046
WanMoh	0.032	0.036	0.036	0.015	0.011	0.012	0.014	0.013	0.024	0.006	0.005	-0.001	0.006	0.013	0.018	0.005	0.008	0.009	0.003
NenEct	0.035	0.048	0.040	0.019	0.020	0.017	0.026	0.022	0.022	0.021	0.028	0.027	0.022	0.035	0.042	0.033	0.027	0.030	0.022
NenBro'95	0.035	0.042	0.034	0.021	0.022	0.018	0.023	0.019	0.018	0.018	0.023	0.025	0.022	0.028	0.038	0.023	0.024	0.026	0.018
NenBro'99	0.030	0.039	0.030	0.019	0.022	0.016	0.025	0.020	0.019	0.019	0.025	0.027	0.021	0.036	0.043	0.031	0.029	0.032	0.025
AruHor'08	0.055	0.049	0.056	0.035	0.027	0.030	0.034	0.037	0.053	0.027	0.022	0.016	0.018	0.027	0.031	0.028	0.017	0.019	0.019
AruHor'95	0.062	0.051	0.069	0.040	0.028	0.027	0.027	0.028	0.046	0.021	0.011	0.010	0.013	0.025	0.030	0.017	0.012	0.017	0.014
AruHor'00	0.071	0.059	0.073	0.051	0.048	0.044	0.040	0.048	0.066	0.046	0.036	0.033	0.028	0.034	0.046	0.047	0.035	0.037	0.034
AruHUS	0.063	0.051	0.070	0.044	0.036	0.036	0.038	0.040	0.057	0.030	0.027	0.021	0.022	0.025	0.031	0.030	0.018	0.019	0.020
CheAbB	0.033	0.039	0.033	0.028	0.017	0.019	0.031	0.022	0.026	0.015	0.021	0.016	0.021	0.035	0.041	0.024	0.021	0.020	0.018
BlaBlm	0.046	0.038	0.044	0.029	0.020	0.018	0.025	0.027	0.037	0.016	0.016	0.018	0.017	0.016	0.026	0.019	0.018	0.019	0.019
BlaSti	0.044	0.042	0.049	0.024	0.015	0.019	0.018	0.023	0.031	0.012	0.010	0.008	0.015	0.012	0.012	0.008	0.010	0.013	0.007
TreNot	0.037	0.033	0.042	0.020	0.019	0.012	0.015	0.013	0.027	0.015	0.016	0.014	0.011	0.020	0.030	0.021	0.019	0.017	0.017
TreWol	0.056	0.057	0.057	0.024	0.024	0.022	0.029	0.017	0.024	0.025	0.029	0.023	0.021	0.033	0.042	0.035	0.030	0.026	0.025
AirDar	0.056	0.050	0.052	0.027	0.029	0.028	0.026	0.020	0.028	0.024	0.017	0.015	0.017	0.029	0.030	0.018	0.019	0.022	0.019

Appendix Table 3.5 Matrix of pairwise F_{ST} among all roach population samples (continued on next page). Values highlighted in blue are not significantly different at the p =0.000069 level (Bonferroni corrected p value)

	MolMea	BouChe'06	BouChe'11	BouChe'02	RayRod	LamSha	WanMoh	NenEct	NenBro'95	NenBro'99	AruHor'08	AruHor'95	AruHor'00	AruHUS	CheAbB	BlaBlm	BlaSti	TreNot	TreWol
LeeHUS																			
LeeHyd'10																			
LeaHyd'95																			
Lee'00																			
LeeEss																			
LeeWhe																			
LeeSta																			
StoBri																			
StoTed																			
GadCas																			
KenNor																			
KenFou																			
KenBul																			
BlaEvH'10																			
BlaEvH'00																			
ThaSha																			
ThaCul																			
ThaHam																			
ThaWhi																			
MolMea	*																		
BouChe'06	0.004	*																	
BouChe'11	0.000	0.003	*																
BouChe'02	0.004	**0.006**	0.005	*															
RayRod	0.005	0.009	**0.007**	**0.010**	*														
LamSha	**0.050**	**0.047**	**0.048**	**0.053**	**0.049**	*													
WanMoh	**0.006**	0.002	0.004	0.004	**0.007**	**0.041**	*												
NenEct	**0.015**	**0.026**	**0.024**	**0.014**	**0.023**	**0.068**	**0.022**	*											
NenBro'95	**0.012**	**0.014**	**0.019**	**0.011**	**0.022**	**0.064**	**0.016**	0.003	*										
NenBro'99	**0.016**	**0.020**	**0.022**	0.007	**0.025**	**0.076**	**0.022**	0.002	0.000	*									
AruHor'08	**0.023**	**0.025**	**0.022**	**0.020**	**0.019**	**0.052**	**0.023**	**0.046**	**0.046**	**0.042**	*								
AruHor'95	**0.018**	**0.015**	**0.015**	**0.013**	**0.019**	**0.052**	**0.018**	**0.040**	**0.033**	**0.037**	0.003	*							
AruHor'00	**0.043**	**0.040**	**0.036**	**0.032**	**0.037**	**0.074**	**0.038**	**0.061**	**0.056**	**0.053**	**0.009**	**0.014**	*						
AruHUS	**0.027**	**0.026**	**0.028**	**0.025**	**0.021**	**0.052**	**0.025**	**0.047**	**0.044**	**0.045**	0.003	0.002	**0.010**	*					
CheAbB	**0.014**	**0.015**	**0.018**	**0.013**	**0.020**	**0.046**	**0.015**	**0.023**	**0.022**	**0.027**	**0.027**	**0.031**	**0.051**	**0.036**	*				
BlaBlm	**0.015**	**0.013**	**0.017**	**0.016**	**0.010**	**0.057**	**0.014**	**0.035**	**0.025**	**0.033**	**0.035**	**0.021**	**0.052**	**0.031**	**0.030**	*			
BlaSti	**0.010**	**0.009**	**0.011**	**0.015**	0.007	**0.048**	0.005	**0.032**	**0.025**	**0.036**	**0.029**	**0.014**	**0.051**	**0.026**	**0.026**	**0.007**	*		
TreNot	**0.012**	**0.013**	**0.016**	0.008	**0.015**	**0.057**	0.008	**0.018**	**0.017**	**0.016**	**0.027**	**0.016**	**0.034**	**0.021**	**0.025**	**0.014**	**0.016**	*	
TreWol	**0.020**	**0.026**	**0.027**	**0.013**	**0.029**	**0.069**	**0.022**	**0.020**	**0.021**	**0.019**	**0.034**	**0.025**	**0.037**	**0.027**	**0.030**	**0.028**	**0.032**	0.004	*
AirDar	**0.015**	**0.017**	**0.015**	**0.018**	**0.023**	**0.065**	**0.016**	**0.037**	**0.031**	**0.030**	**0.032**	**0.018**	**0.037**	**0.030**	**0.029**	**0.031**	**0.023**	**0.010**	**0.025**

Chapter 4

Examining the Influence of Oestrogenic Effluent Contamination on Wild *Rutilus rutilus* Populations

Pages 102 – 138

4.1 Statement of Contribution to Work

This work was conducted in association with the University of Exeter, specifically Dr Patrick Hamilton. I planned and collected all fish samples from the field, aged all fish and undertook my own analyses of microsatellite data. I contributed to the writing, content and layout of the paper where these results are published (Hamilton et al. 2014). Contains Environment Agency information © Environment Agency and database right.

4.2 Introduction

A myriad of interacting physical, chemical and biological forces are present across all ecosystems. Cumulatively, these can threaten the fitness, survival, and reproduction of individuals through drivers of: habitat degradation, climate change, increasing levels of pollution, over-exploitation and the introduction of alien species. Acting on a variety of scales, each has been found to contribute to the decline or subsequent extinction in a number of local populations worldwide (Sala et al. 2000, Parmensan and Yohe 2003, Jenkins 2003, Mora et al. 2007). Notwithstanding this, the difficulty comes in isolating the precise influence involved especially in field conditions, as mechanisms often act in combination. For example, a healthy population of freshwater fish may be able to withstand the introduction of a novel diseased organism, but the same disease vector may have devastating consequences on another population of fish, compromised by pollutant exposure or over-exploitation. Rarely do we see a linear relationship between cause and effect when scrutinising driver and feedback processes in respect to population responses. More often, intricate linkages are evident through both direct and indirect effect mechanisms (Carpenter et al. 2009).

Pervasive factors are indiscriminate across both terrestrial and aquatic ecosystems; accelerating modification of niche architecture within the European landscape perhaps more so than anywhere else in the world (Freyhof and Brooks 2011). Alteration of natural habitats by human activities has proved influential in shaping the distribution and abundance of fish populations (Figure 4.1; Cowx 2002) with statistics suggesting at least

37% of Europe's freshwater fish are threatened on a continental scale (Freyhof and Brooks 2011). Historically, the more persistent factors were deemed to be over-exploitation and alien introductions; both biological influences which affect levels of density-dependent factors: predation, competition, population density and novel disease vectors. However, renewed focus has shifted towards the significance of habitat fragmentation (Keyghobadi 2007) and water quality problems such as eutrophication, which are fundamentally important in regulating riverine fish populations (Grenouillet et

al. 2001, Beardsley and Britton 2012). Underpinning all of these drivers is the influence of climatic change, which plays a well-studied regulatory role in density-independent population dynamics of cyprinid fishes throughout temperate regions (e.g. Mills and Mann 1985, Grenouillet et al. 2001, Nunn et al. 2003, 2007).

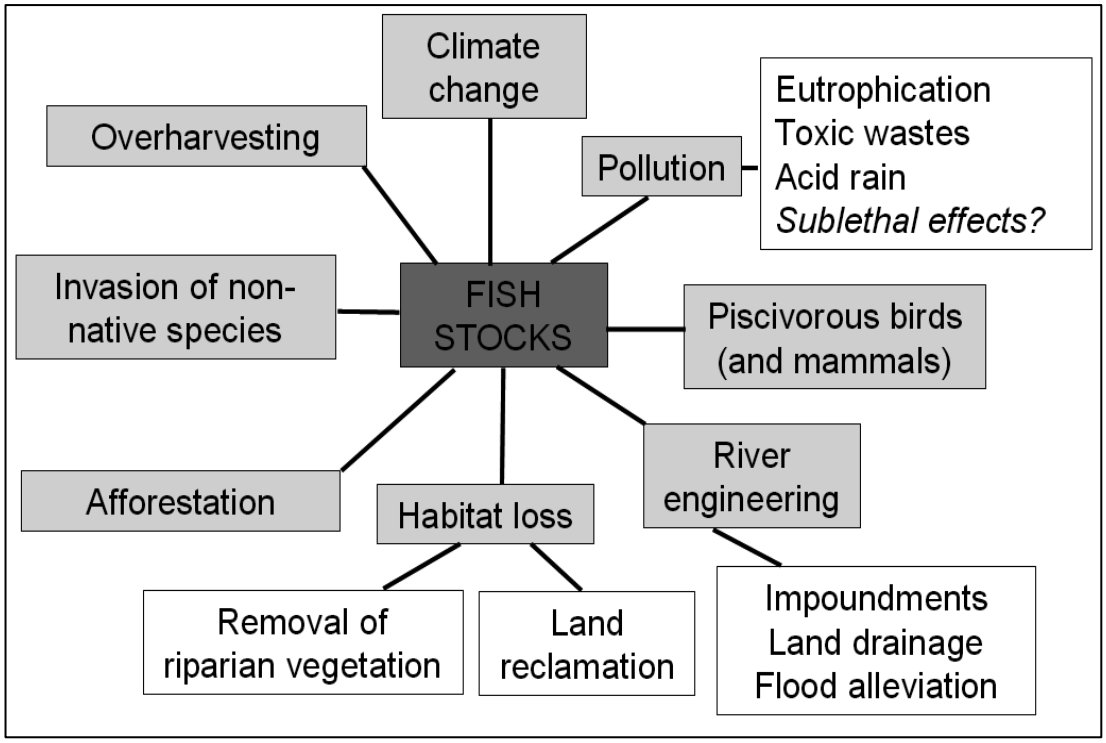


Figure 4.1 Key drivers typically affecting fisheries throughout inland waters (modified from Cowx 2002)

4.2.1 Pressures on Global Water Resources

With human population expansion comes the by-product of increasing waste and burgeoning demand for water (Figure 4.2). This demand is expected to rise by 70% by 2050 (Gilbert 2012) in line with projected population expansion and the growth of urban settlements (6.3 billion people by 2050). Urbanised environments are a primary source of pollution, discharging thousands of chemicals into the aquatic environment through the production of wastewater. In the developing world, more than 80% of this water is not treated following use (Gilbert 2012). In each instance, a wide variety of man-made chemicals derived from sources such as energy production, agriculture, manufacturing, mining, health care, and transport find their way into aquatic ecosystems through the release of treated effluents from point sources or surface runoff (Figure 4.3). Total numbers of chemicals in everyday use exceeds 60,000 (not including by-products and metabolites). These include pharmaceuticals and synthetic steroid oestrogens that derive from human consumption and are poorly removed in the sewage system (Ternes et al. 1999, Johnson and Sumpter 2001, Kanda and Churchley 2008).

4.2.2 UK Riverine Environment

A key aim of this thesis was to determine the influence of human waste on riverine fish populations in the UK. As the worldwide demand for water increases, so does the intensification of competition between natural and anthropogenic necessities, which often result in high environmental costs. Water requirements of agriculture and industry need to be fulfilled alongside necessary supplies of clean drinking water in dense areas of human inhabitation. Following use, all wastewater is eventually discharged as waste into the air, soil and watercourses. Unsurprisingly, pollution has been recognised as one of the key contributors to declines in fish populations since the 18th century (Clark 1992). Problems that have since been confounded by the industrial revolution and the growth of new industrial processes. Historically, many rivers in heavily populated areas of the UK and Europe have been vulnerable to poor water quality and subsequently endured habitats devoid of fish (Jones and Reynolds 1997). For example, prior to the 1980s, rivers such as the Thames, Trent, Mersey and Don were dominated by high organic loading (ammonia and heavy metals) from industrial and domestic effluents, severely impacting fish populations of both cyprinids and salmonids (Cowx and Broughton 1986, Lyons et al. 2007).

Heavily industrialised areas, prone to chronic and acute pollution, see frequent alterations in water chemistry and increased likelihood of eutrophication. Commonly, this results in reduced connectivity and heterogeneity in riverine environments, driving shifts in fish community composition to more tolerant species that prosper in stochastic conditions (Noble et al. 2007). Considerable work over the last 50 years has been focussed around improving water quality of many European rivers in an attempt to alleviate organic loading and improve water clarity. Biological and chemical water quality monitoring (undertaken by the Environment Agency) is now routinely implemented, in line with conserving freshwater fish species diversity. Improvements have been recognised in recent years through the recovery of coarse fisheries and the return of Atlantic salmon (*Salmo salar*) to larger rivers such as the Mersey and Trent (Lyons et al. 2007). However, no single factor is attributable to the documented fluctuations in coarse fish populations (Cowx 2001, Nunn et al. 2003). Frequent environmental perturbations in UK lotic environments have been found to associate with significant shifts in growth and recruitment rates of cyprinid fisheries (Nunn et al. 2003, 2007), which may ultimately affect the abundance and distribution of all fish species. Thus, fish populations fluctuate both spatially and temporally, sometimes in synchrony, but not always driven by common

ecological phenomena.

Recognition of coarse fish as an important indicator of ecological status (Water Framework Directive; Noble et al. 2007) emphasises the significance of long-term monitoring. Trends in abundance allow benchmarks of fish population quality to be established, as they occupy a high diversity of ecological niches across broad ecological scales (Cowx 2008). Despite emphasis on the importance of historical monitoring, few studies have documented the effects of water quality on population trends in cyprinid species. One example from the UK showed a shift in community composition from roach to chub following water quality improvement in the 1970s–80s on the River Trent (Cowx and Broughton 1986), although the role of additional environmental factors during this period was not considered in this work. Therefore, when ranking insidious impacts of human activities on UK freshwater fish populations, the placement of sub-lethal exposure to exogenous chemicals remains unknown and is complicated by a paucity of information on likely biotic/abiotic interactions.

4.2.3 Occurrence of Endocrine Disrupting Chemicals (EDCs) in the Aquatic Environment

An ever-increasing number of anthropogenic pollutants are being classified as EDCs, which are fast becoming a ubiquitous feature of receiving waters. Growing concern surrounds the occurrence and fate of these pharmaceutical compounds once they enter the aquatic environment (Figure 4.3), as they can be biologically active at low (ng/l) concentrations (Heberer 2002). Acting in a similar manner to hormones, these substances have the capacity to disrupt the mechanisms of the endocrine system by altering pathways and the physiological function of endogenous hormones. Despite this, their presence within the water column (or sediment) does not cause direct toxicity to fish but may evoke harmful health repercussions when accumulated or mixed with other chemicals, especially in the long term. Example effects of EDCs derive from a broad spectrum of wildlife including invertebrates (Depledge and Billinghurst, 1999, Gagnaire et al. 2009, Gibbs and Bryan 1986, Mensink et al. 1996, Segner et al. 2003), fish (Jobling et al. 1998, Saaristo et al. 2010, van Aerle et al. 2001, Wang et al. 2010), amphibians (Hayes et al. 2002, 2006), reptiles (Crain and Guillette 1998, Guillette et al. 2000, Rie et al. 2005), birds (Fry 1995, Lundholm 1997) and mammals (Hall et al. 2006, Jenssen 1996, Kjellqwist et al. 1995).

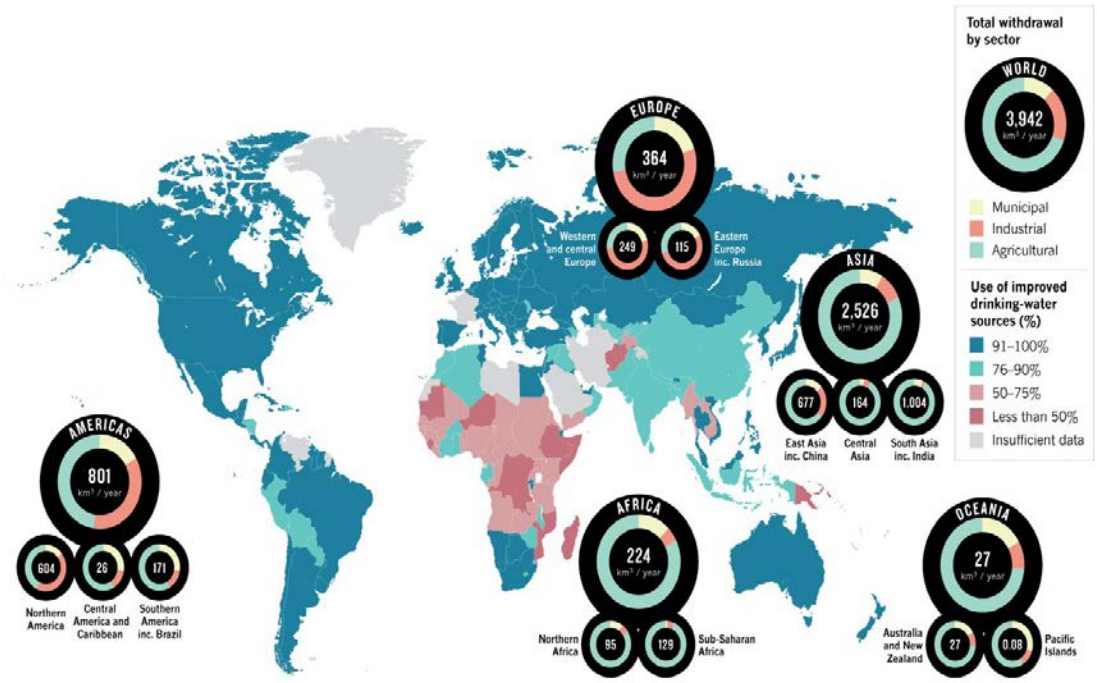


Figure 4.2 Access and use of water sources globally. Agriculture presents the highest demand for the world's extracted water however urban settlements are the main source of pollution— a problem that will only grow with an expanding urban population. Sourced from Gilbert (2012)

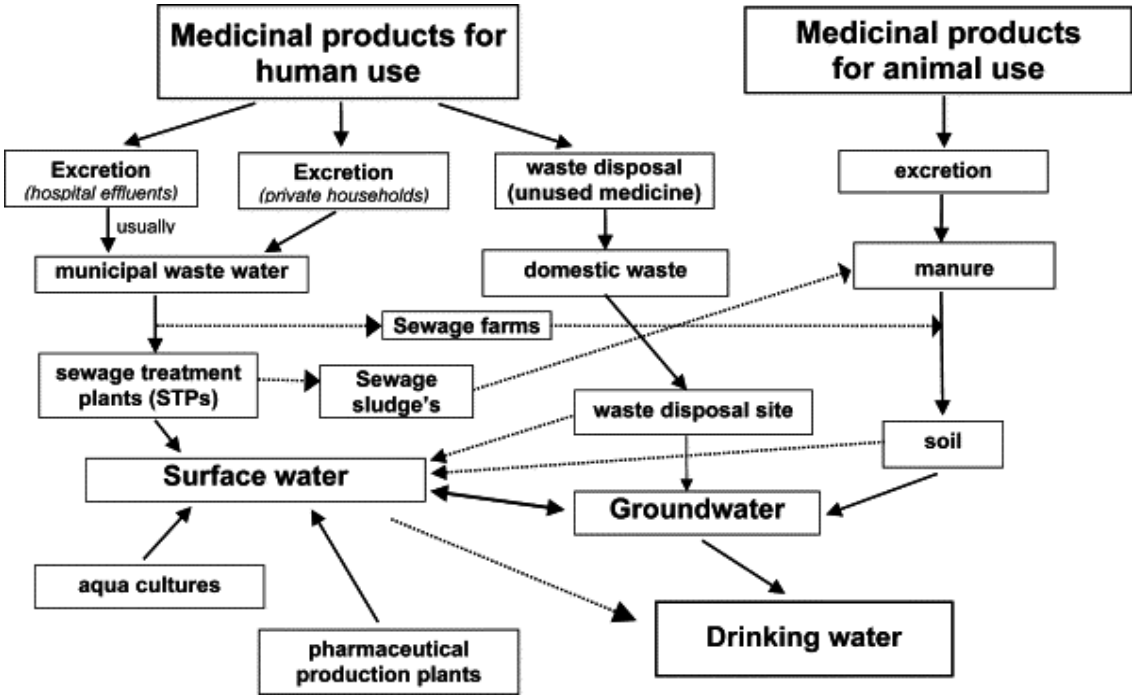


Figure 4.3 Schematic representation of the possible sources and routes of delivery for pharmaceutically-active compounds in the aquatic environment. Highlighted in the grey box is the final endpoint of concern; their presence in surface waters. Sourced from Herberer (2002)

4.2.4 Influence of Contaminants on Fish

Concentrations of contaminants in effluent may vary, as do the routes of exposure. In fish, these commonly include uptake across the gills and via the skin, as a result of ingestion, or even through maternal transfer into developing eggs (Ellis et al. 2005). A substantial body of literature has shown that exposing susceptible aquatic organisms to such pollutants contributes detrimentally to sexual development and disrupts reproductive function, especially during early life stages (Devlin and Nagahama 2002). Sex determination is very flexible but can strongly be influenced by exogenous sex steroids: altering sex ratios and gonad morphology, quality and quantity of gametes, as well as more subtle effects on reproductive behaviour (Jones and Reynolds 1997). In the wild, incidences of the 'intersex' condition (the simultaneous presence of both testicular and ovarian gonadal tissue) are well documented in numerous fish species (Barnhoorn et al. 2004, Gross- Sorokin et al. 2006, van Aerle et al. 2001, Vigano et al. 2001, Jobling et al. 1998, 2002, Woodling et al. 2006). These are typically associated with the presence of steroid oestrogens derived from effluent, implicated in the feminisation of male fish (Jobling et al. 2006). The presence of natural oestrogens [oestrone (E1) or 17β-oestradiol (E2)] and the synthetic oestrogen used in birth-control pills [17α-ethynylestradiol (EE2)] have also been linked with the production of vitellogenin (VTG — a blood protein normally synthesised by females during oocyte maturation) in male fish living downstream of wastewater outfalls (Jobling et al. 1998, Desbrow et al. 1998).

Recognising these effects on reproductive function provokes interest in subsequent detrimental impacts of endocrine disruption at the population level (Brown et al. 2003). The complexity by which pollutants may exert their effects on sexual phenotypes and function, means that changes at the population level are uncommon in the literature. Additional environmental pressures that also drive changes in fish populations make evaluations extremely difficult (see Chapter 6). Approaches to decipher changes in fish population parameters have previously attempted to use predictive models to address the ecological risk of chemicals on population dynamics, using standard laboratory toxicology data on fecundity of fathead minnow (*P. promelas*) to infer population growth rate (Miller and Ankley 2004). Additional approaches have attempted to re-enact large-scale exposures of fish populations in 'real-world' and laboratory settings, by dosing fish with the synthetic oestrogen EE2 over long time periods (Nash et al. 2004, Kidd et al. 2007). However, despite observations of adverse effects on individuals that have the

potential to perpetuate at the population level, evidence is still inconclusive when discerning measurable changes in population parameters of wild fish (An et al. 2009, Mills and Chichester 2005). Additionally, subtle changes in behavior and genetics of fish populations that could arise from exposure to EDCs remain relatively unexplored (Coe et al. 2008a, 2008b, 2009, 2010, Brown et al. 2009, Bickley et al. 2013).

The principle effects seen in the literature indicate the uncertainty of long-term ramifications of EDC exposure on fish populations. As a result, EDCs continue to enter the environment at acceptable concentrations (derived for each individual chemical pollutant) despite doubt in the level of risk posed to aquatic populations (Hutchinson et al. 2006). Understanding the impacts of long-tern exposure to EDCs from wastewater is therefore of paramount importance but has been limited to date by logistical challenges involved in measuring exposures and effects in wild populations.

4.2.5 Influence of Contaminants at the Genetic Level

Environmental contaminants are becoming increasingly prolific, having an unequivocal, but sometimes subtle, impact on natural populations. Exposure to chemicals has been implicated in the extirpation of a number of populations since the 1940s — when early research discovered that xenobiotics played an important role in population decline through disruption and impairment of reproduction (Bickham et al. 2000). Since then, the exposure of organisms to novel, man-made chemicals has been found to have the potential to induce chronic and acute toxicity in a whole host of organisms, distinctly recognisable at the molecular level (Brown et al. 2009). To this end, fish have been employed as important sentinels to aquatic pollution; engaged both in the regulatory and biomonitoring arena to demonstrate exposure levels, tissue burdens or short-term biomarker responses aligned with fish health. The direct interaction between fish tissues and water-borne chemicals make them increasingly susceptible to pollutant effects (Tyler et al. 2008) and valuable models for ecological, environmental and behavioural questions (Oleksiak 2010).

The multitude of environmental contaminants to which species are exposed, provoke an array of genetic repercussions at both the local population and species level (Figure 4.4). Few early studies were capable of studying the genetic consequences of such phenomena, however the advancement of allozyme techniques quickly resolved this knowledge gap (Avise 1994); directly documenting genetic changes in exposed and unexposed populations. Genetic effects emerge via a transformation in genetic patterning through the

direct mutagenic effect of exogenous substances or via population-mediated processes such as selection for pollutant tolerant genotypes, increased frequency of bottleneck events, and/or modification of migration pathways of genotypic exchange (see van Straalen and Timmermans 2002 for a detailed overview). Significant differences brought about by the above factors mean that allele and genotype frequencies of some loci can be rapidly altered in both tolerant and sensitive individuals (Chen et al. 2003), making the elucidation of any genetic effect hugely challenging. There is growing recognition however, that genetic ecotoxicology (the extent of contaminant induced changes in genetic composition of natural populations; Hebert and Luiker 1996) is becoming increasingly important in the establishment and quantification of anthropogenic impacts on species sustainability.

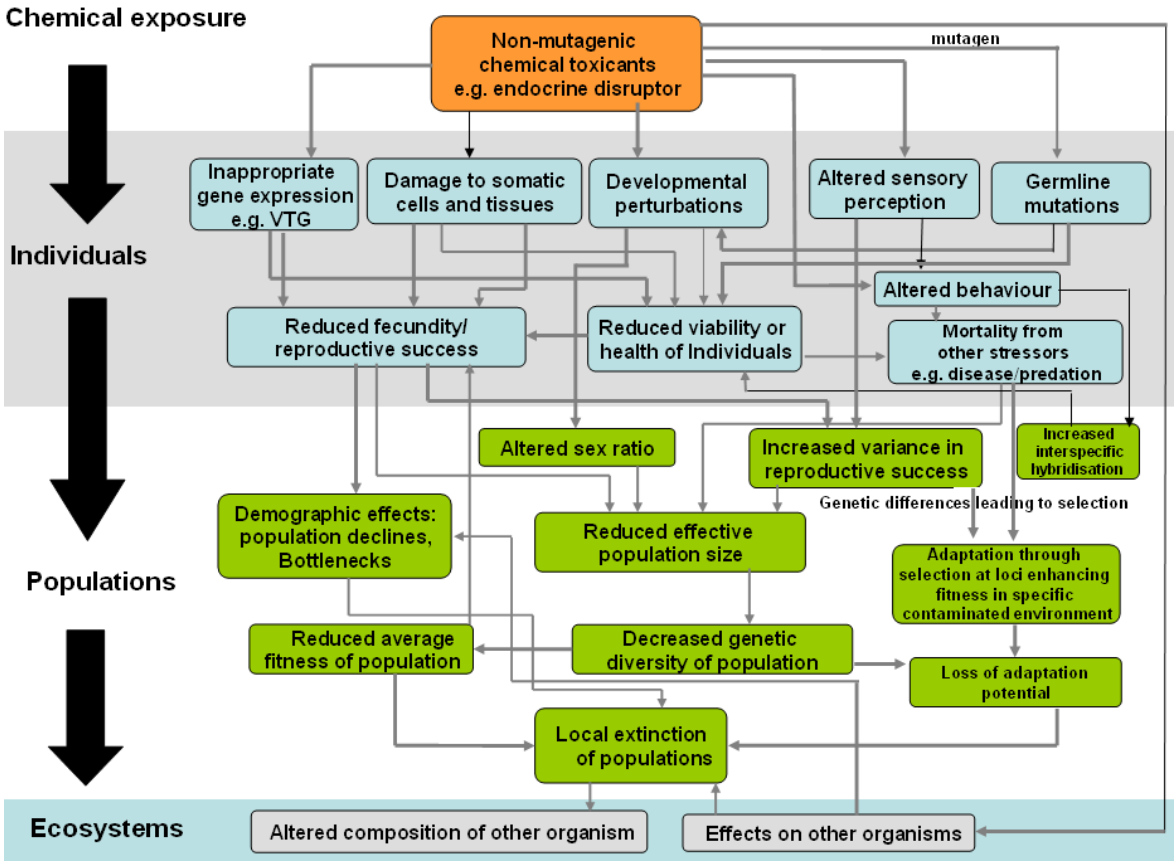


Figure 4.4 Schematic to show the amplification of impacts of sub-lethal contaminants on individuals to the population level. Links are hypothesised and have not all been demonstrated in the literature, similarly some chemicals can have an overall positive impact on species. Adapted from Bickham et al. (2000)

Chemical exposure has frequently been documented to have negative effects on genetic variation of natural populations (Bickham et al. 2000, van Straalen and Timmermans 2002, Whitehead et al. 2003). Erosion of reproductive fitness can be preceded by a decrease in genetic diversity, a reduction in gene flow and a heightened risk of a genetic bottleneck. Novel toxic pollutants can invoke all of these mechanisms in unison or in

combination, leading to genetically depauperate wild populations. Genetic erosion has been encountered in field populations of different taxa from polluted aquatic environments, such as fish (Kopp et al. 1992, Murdoch and Hebert 1994), crustaceans (Krane et al. 1999, Street et al. 1998) and gastropods (Kim et al. 2003). In these instances, erosion of genetic diversity renders these populations increasingly susceptible to extirpation through an irremediable loss of fitness (Bickham et al. 2000). One of the first crucial studies to document lower levels of mtDNA diversity in contaminant-exposed populations was performed using brown bullheads in the Great Lakes, North America. Murdoch and Hebert (1994) demonstrated this using RFLP analysis in individuals from four 'clean' sites and five 'Areas of Concern'. It was found that the populations from poor quality areas exhibited consistently lower genetic diversity estimates, indicative of environments where bottlenecks may have previously occurred. However, other studies in this field show little evidence for erosion of genetic variation due to pollution (see examples in Nowak 2008). Indeed, Theodorakis et al. (2006) found evidence of altered gene flow in response to pulp mill effluent in redbreast sunfish (*Lepomis auritus*) in several sites in Pigeon River, North Carolina. RAPD analysis revealed, contrary to expectations, higher levels of genetic diversity in contaminant-exposed populations. Through further analysis they were able to attribute much of this to the prevalence of immigration. Without supplementation through immigration, the population number would decline, inferring this as an important and perhaps more prevalent vehicle with which genetic diversity alterations/losses are dampened.

Inevitably, the ability to successfully or unsuccessfully adapt to toxicant challenge will ultimately have implications at the genetic level; however difficulties come when attempting to experimentally examine the occurrence of selection in wild populations in the absence of other mechanisms. Coors et al. (2009) were able to document findings that provide evidence for local adaptation of *Daphnia magna* populations to pesticide contamination in Belgian ponds. Here, egg masses of *D. magna* in ponds exposed to differing local levels of two model toxicants (carbaryl and potassium dichromate) were genotyped using polymorphic allozyme loci, establishing the genetic diversity and tolerance for each population once hatched. Differences in susceptibility and levels of genetic diversity indicated that the two contaminants were initiating significant genetic forcing on pond populations. Concluding remarks by the authors suggest that toxic tolerance was enhanced, whereas genetic diversity was depleted in ponds in high agricultural land use areas. Taken together, these two findings provide significant

evidence for selection pressures taking place within these *D. magna* populations. This study is not the only one to demonstrate such findings; in fact Nacci et al. (1999) found similar signatures of inherited tolerance of mummichog (*Fundulus heteroclitus*) to toxic PCBs in exposed populations. *F. heteroclitus* in the highly-contaminated New Bedford Harbour have adapted to PCBs through 'hard selection' and limited gene flow (McMillan et al. 2006).

4.2.6 Influence of EDCs on Population-level Endpoints

At the evolutionary level, reproductive alterations become significant and latent effects of chemicals can accumulate over time (Brown et al. 2009). EDCs (oestrogens, anti-androgens, androgens, progestogens, and many others) sourced from both domestic and industrial effluents may exert inconspicuous heritable reproductive effects on wildlife (Vos et al. 2000). To date, few studies have categorically examined the relationship between population genetic diversity and EDCs, yet inferences can be made as to their importance based on ancillary studies. Exposure to EDCs in both freshwater and marine environments has been shown to modify the reproductive physiology and morphology of fish (Kime 1999, Tyler et al. 1998) including alterations in the induction of female-specific proteins in male fish (Tyler and Routledge 1998), reduced sperm counts (Haubruge 2000), skewed sex ratios (Larsson et al. 2000) and an increased prevalence of inter-sexuality (fish containing gonads with both male and female structures/germ cells — Jobling et al. 1998). Collectively or in unison, these reproductive modifications could occur through exposure to EDCs, ultimately having implications on the genetic viability of individuals and populations.

Aside from these reproductive effects, indirect consequences of EDCs can manifest themselves at the population level through a reduction in N_e/N_c. Any change in population size will alter the genetic viability of a population, nevertheless documented examples of local extinctions and population decline due to EDC exposure are themselves scarce. The only exceptions being tri-butyl tin exposure resulting in the induction of imposex in *Nucella lapillus* (Bryan et al. 1988) and the eggshell thinning in raptors following bioaccumulation of DDT and its metabolites (Ratcliffe 1967). Therefore, considerably more work is needed in this area to explicitly elucidate the genetic and population effects of EDCs in wildlife, especially as implications on population fitness are potentially enormous. Not only is this true for EDCs but contaminants in general.

Much of the work addressing effects of EDCs contamination on fish has been demonstrated in laboratory studies, where environmental relevance and extrapolation to

real world scenarios, including the population level, are challenging. Laboratory test systems commonly consider acute or single-generation effects of chemical exposure on life-history traits of individuals (Vogt et al. 2007); contrastingly, natural populations endure chronic, life-long exposure, often in concert with numerous other environmental factors. Essentially the literature base accentuates the need for additional studies to clarify the repercussions of contaminant-induced physiological, morphological or genetic change at the level of the population. Fundamentally this is concerned with the impact of pollutants on the number of individuals.

4.2.7 Measuring Population Impacts using Genetic Approaches

Fluctuations in population size, and the driving forces behind any oscillations, warrant a level of understanding that is often unattainable through simple demographic indices. Important variables encompassing the conservation and evolution of species are now being explored through genetics, such as effective population size (N_e). Essentially, N_e is defined as 'the number of breeding individuals in a population' and elucidates a species evolutionary and demographic potential, as well as quantifying the risk of population extinction (Waples 2002). Given the relatively rapid rise in molecular marker application, a suite of methods are now at a scientists disposal to derive N_e from frequency changes in alleles over multiple generations (for reviews, see Schwartz et al. 1999, Leberg 2005, Wang 2005). Calculated N_e estimates are often smaller than the absolute population demographic, but are thought important due to their ability to indicate how a population may respond to selection pressures (Pujolar et al. 2011, Nikolic 2009). Small estimates of N_e can highlight populations at risk, with 'safe' thresholds of long-term viability deemed to be around 500 individuals (Franklin 1980, Lande 1988). However, these estimated values remain heavily debated and have rarely been tested in the wild (Frankham and Franklin 1998).

Census population sizes (N_c) of roach are uncommon, but where present, can give misleading impressions of genetic variability and health of populations. The high fecundity of female roach means that a few successful individuals can maintain large population sizes. This reproductive strategy is shared with numerous marine fish species like the exploited New Zealand snapper (*Pagrus auratus)*. Populations of this species have been found to display census sizes estimated in the millions, yet two populations had N_e less than 1000 (Hauser et al. 2002). These authors hypothesised that a few old fish contributed disproportionately to overall recruitment.

Critically, adopting N_e gives more clarity in linking population estimates (of breeding individuals) with genetic variation, inbreeding and a subsequent loss of fitness (Wright 1970). The value of N_e to this thesis is that it is influenced by the census population size, sex ratio and variance in individual breeding success of both males and females. All of which have the potential to be affected by oestrogenic contamination. For instance, compromised spawning ability or gamete quality in feminised male fish may skew the parental contribution to a few successful individuals. Surreptitiously, this would reduce N_e and the rate at which genetic diversity is lost from a population through genetic drift (Frankham 2005, Charlesworth 2009), ultimately altering population sustainability/persistence (Reed 2005). Therefore, a critical question is whether chronic exposure to effluents and the EDCs they contain, can negatively impact fish populations. However, few studies have examined the impact of contaminants on N_e in fish, and none focus on oestrogenic compounds. The closest example is from central stonerollers (*Campostoma anomalum*) in an urban catchment in Ohio, U.S.A. Waits et al. (2008) found no significant variation in genetic diversity with habitat quality, although N_e estimates suggested that the populations were likely sustained by colonisation from areas of relatively high habitat quality. A similar story may be apparent in roach at contaminated sites, persistence of which could be attributable to supplementation from less contaminated locations where roach reproduce successfully.

4.2.8 Hypothesis, Aims and Objectives

The work undertaken here follows on from the examination of population structure of UK roach explored in the previous chapter. Here, I investigate whether correlations exist between riverine effluent concentrations and the effective population size of roach (*R. rutilus*) inhabiting a broad spectrum of effluent-impacted rivers in the UK. The hypothesis is that highly contaminated riverine environments will exhibit smaller population sizes with less genetic diversity and more frequently encountered bottleneck events. My findings will provide a valuable contribution of novel information to the question of population level effects of EDCs for wild fish.

To achieve this, DNA microsatellite data was sought from barrier-confined roach populations in stretches of river that have variable levels of predicted effluent exposure. N_e was calculated from 14 microsatellite loci and related to modelled levels of oestrogen contamination (as a marker of domestic effluent) to establish any evidence of long-term effect at the population level.

4.3 Materials and Methods

4.3.1 Study Location

For full justification and explanation of sample site selection, please see Section 3.3 in Chapter 3. The same samples were utilised throughout this chapter and the location of these sites with reference to the predicted levels of oestrogen can be seen in Figure 4.5.

4.3.2 Choice of Sample Sites

As outlined in Chapter 3, sample sites were chosen based on ample sample sizes for genetic analyses and no recorded roach restocking since 2000 (from the England and Wales Environment Agency Live Fish Movement Database). Impoundments between study sites are crucial in restricting fish movements, although some unavoidable migration of individuals may take place downstream during river spate periods. Additionally, preferential selection of river sites spanning a wide range of very low and high effluent (and oestrogen) concentrations was undertaken using model predictions of oestradiol equivalents (E2Eq) obtained for all EA routine monitoring sites.

4.3.3 Effluent Mapping for Sample Site Predictions

In order to assess the risk posed by effluent contamination, the model described in detail in Chapter 2 was utilised for predicting effluent concentrations on a river reach scale. GIS based LF2000-WQX outputs of E2Eq and effluent concentration were calculated for each site location (NGR), based on data from each WWTW within a hydrometric area: the domestic population served (for an estimate of pharmaceutical loading), the dry weather flow, and the type of treatment utilised at that works (i.e primary, secondary, tertiary, see Chapter 2 for full explanation — Williams et al. 2003). Justification for using this modelling approach lies in its ability to predict E2 equivalents and an overall % effluent concentration. Verification of the GIS-based model has taken place on two rivers in England, where measured and modelled results were in good agreement in terms of oestradiol equivalents and predicting overall risk posed to fish populations (Williams et al. 2012). Similarly, modelled estimates of E2Eq have been shown to correlate with the incidence and severity of intersex in fish found downstream of WWTWs in the UK (Jobling et al. 2006); which should increase any chances of finding population-level effects of oestrogenic contamination, if they exist in the wild.

4.3.4 Roach as the Study Species

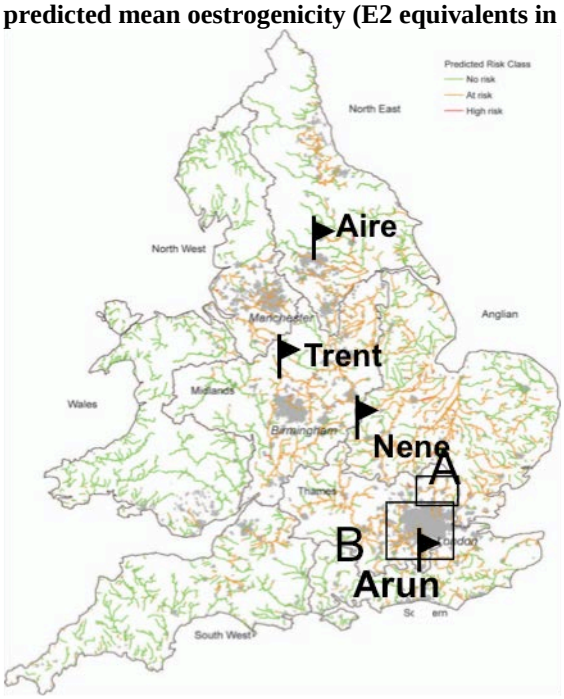
The rationale for choosing roach, for this chapter in particular, derives from their application to date regarding chemically induced endocrine disruption in UK roach

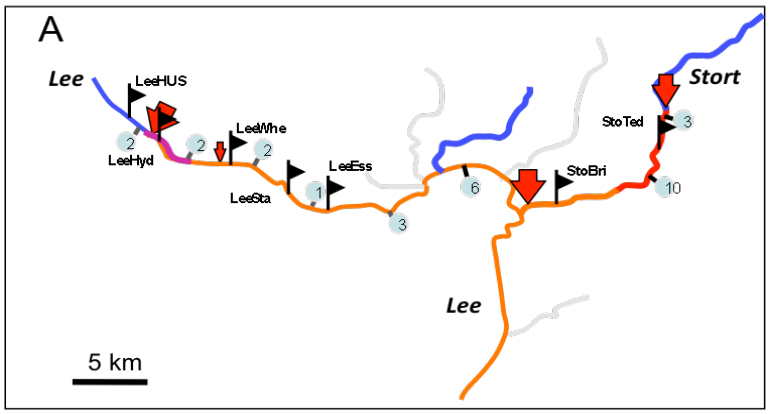
populations downstream of WWTW. This evidence of sexual disruption has realistic potential to perpetuate at the population level, provoking significant consequences for fish fauna. Historic samples from previous work (explored in Chapter 3) also provide a temporal perspective on the dynamics of a species that possesses high ecological importance (roach can comprise more than half the fish biomass in UK lowland rivers). Native and abundant in UK rivers, the roach is also widely tolerant of poor water and habitat quality therefore increasing the likelihood of occurrence in highly contaminated stretches of river. As a non-migratory species, which can complete their entire lifecycle in a single stretch of river (Geeraerts et al 2007), this means that roach populations can remain largely confined to highly polluted stretches over multiple generations. They spawn annually in the spring and recruit to the population around 2/3 years later for males and females respectively.

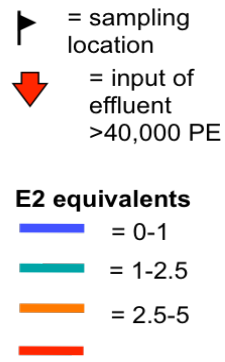
4.3.5 Sample Collection

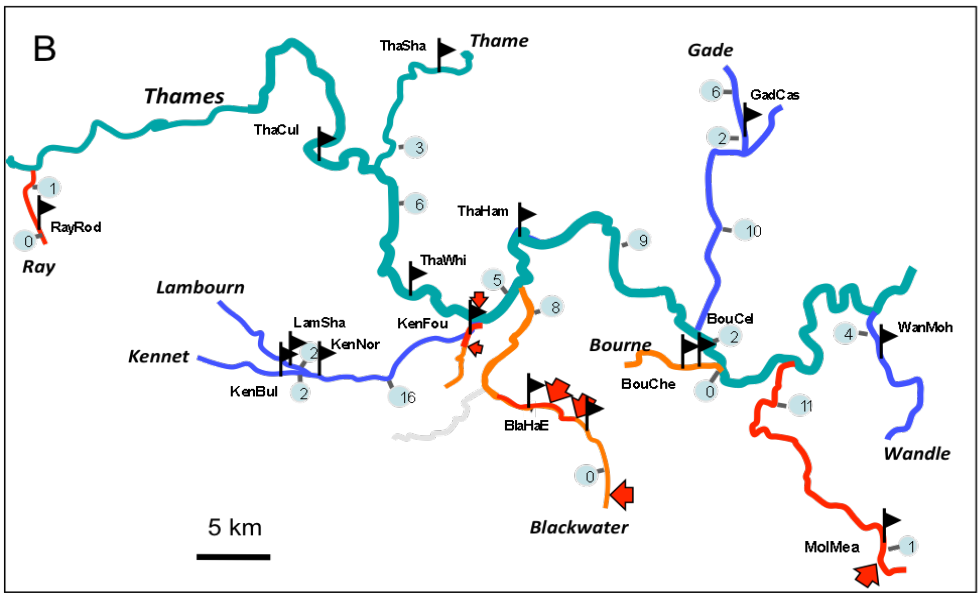
See Section 3.3.2.

Figure 4.5 Locations of sample sites in England modified from Williams et al. (2009) opposite. For (A) and (B), numbers in circles represent the number of obstructions to fish movement (weirs or locks). Only weirs over 1 m are shown. PE = population equivalents, which relates to the size of the population served by the WWTW. The different colours used to depict the rivers represent predicted mean oestrogenicity (E2 equivalents in ng/l)









4.3.6 Fish Scale Aging

Following capture, fish were measured (fork length, mm) and scales removed, to enable age determination. Scales were taken from the left flank between the lateral line and dorsal fin (Figure 3.3), approximately 4–7 scales taken in total. The scales were washed in water following return to the laboratory, dried with tissue, and then placed in individual paper envelopes for storage at room temperature. For aging, cleaned and dried scales (2–3) were arranged between two glass carrier sheets and examined on the projection screen of the Micron 150 portable reader (MicroImage Display, 1995, USA). Annual increments were recorded from the focus to the lateral margin as described by Kamilov (1984) which can be seen in Figure 4.6. Aging was integral for all fish in this chapter, as the information was incorporated into estimates of N_e using the Sibship Assignment (SA) method, to exclude the likelihood of entering genotypes from parents and offspring. Inaccuracies can arise through aging false-reads (Britton et al. 2004) but all samples were examined by one person and subsequently cross-checked with age- length distributions (published by the EA) for each river.

4.3.7 Genetic Sample Processing

Details of genotyping methods and microsatellites used can be found in Chapter 2. I analysed microsatellite loci variation in 1769 fish, sampled between 1995 and 2011. Each fish was genotyped using 14 microsatellite loci.

4.3.8 Population-Genetic Analyses

To understand the extent to which roach populations are restricted to various stretches of river, several approaches were used to investigate population genetic structure. Results from this work can be found in Chapter 3 and will be referred to where necessary, to aid interpretation.

4.3.9 Detecting Population Bottlenecks

As a result of prolonged exposure to EDCs, compromised reproductive success and reduced fecundity could have triggered demographic declines in roach. Population bottlenecks are, by definition, an event that drastically reduces the population size for at least one generation through the loss/death of organisms. Detecting recent declines in N_e or actual population size is possible through the loss of genetic variants or alleles that were present in the original population. The surviving population possesses characteristically low levels of genetic diversity, but more recognisable is the rapid reduction in allelic frequency compared to heterozygosity (Figure 4.7; Cornuet and

Luikart 1996). An excess of heterozygosity at polymorphic loci, compared to the expected reduction in allele numbers following a population crash, is a detectable consequence of a genetic/population bottleneck.

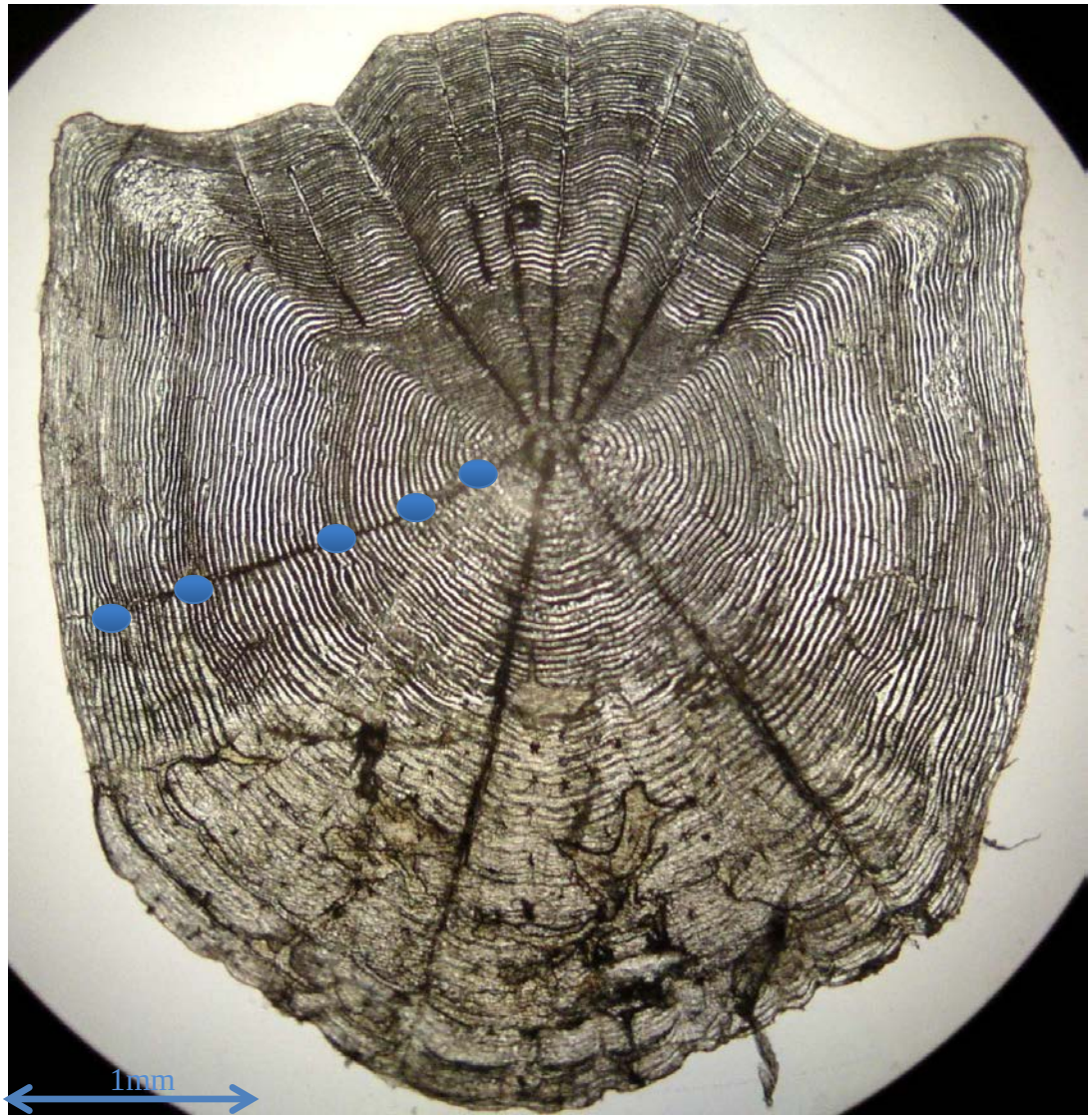


Figure 4.6 Micrograph of a roach scale typically examined for aging. This fish scale was taken in January, from a 130 mm fish. 5 years of growth can be derived from counting scale annuli (darkened concentrations of growth rings in times of slow growth, such as winter) marked here with blue circles

To detect the presence of historic bottleneck events in the roach population samples I used the program BOTTLENECK (Cornuet and Luikart 1996). This computes the expected heterozygosity from allele data frequencies (under the assumption of mutation–drift equilibrium) and compares it to the observed heterozygosity, to establish any disparity that may aid detection of past bottlenecks. Allele frequency distributions were compared to that of populations in mutation–drift equilibrium using the two-phase model (TPM, with a 70% stepwise mutation model) as microsatellite loci evolve under a model

that is more closely aligned with SMM than the IAM (Infinite Alleles Model; Luikart et al. 1998). BOTTLENECK was run for 10,000 iterations. Deviations between observed and expected frequency distributions were tested using Wilcoxon's signed rank test.

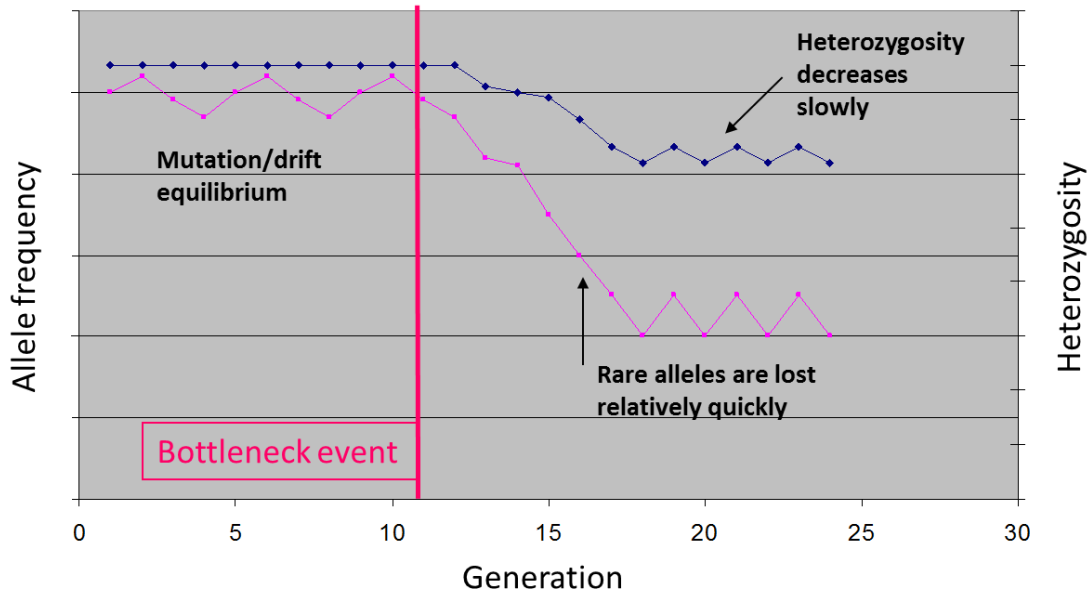


Figure 4.7 Graphical simulation of a population bottleneck and the corresponding change in allele frequency, which is recognisable through the use of genetic markers and relevant software programs

4.3.10 Estimating Effective Population Size (N_e)

To address the key aim of this chapter, N_e estimates of 39 roach populations were calculated using individual genotypes obtained from 14 microsatellite loci. Effective population sizes could then be compared across levels of predicted oestrogen exposure, to test whether WWTW effluent contamination compromises population size in this sentinel fish species. Numerous methods have been developed to calculate N_e from single samples or multiple temporal samples separated by at least one generation. Here I use two single sample approaches to estimate N_e (temporal changes in allele frequency), which utilise linkage disequilibrium and genetic relatedness to estimate the number of adults that were most likely to produce the sample (Palstra and Fraser 2012). Despite the two methods using different aspects of the data, N_e estimates produced from each have been found to be comparable, especially when estimating small population sizes (Barker 2011).

The first approach uses the method of Tallmon et al. (2008), implemented in the program ONeSAMP, to estimate N_e from a single sampling time-point. For each population, the program uses Approximate Bayesian Computation (ABC) and summary statistics to provide precise estimates of N_e. ABC assumes no migration and can perform well in species with overlapping generations (Barker 2011). Overlapping generations can bias N_e

due to complexities in allele frequency fluctuations when multiple cohorts exist in the same sample (Jorde and Ryman 1996). Upper and lower prior bounds for N_e were set at 2–500 for all runs, based on known double the estimated census population size.

As a second complementary approach, N_e was estimated using the Sibship Assignment (SA) method in the program COLONY v2.0 (Jones and Wang 2009). Commonly, this method is implemented using a sample of individuals from a single cohort but is deemed applicable to populations with overlapping generations, random and non-random mating, and those populations experiencing immigration (Wang 2009). Estimates of N_e using this method are based on sibship assignments, which quantify the probability of each pair of individuals in a population sample being half or full-sibs (sharing one or two parents), based on their genotype data. Applying this method to roach populations with overlapping generations, including randomly sampled individuals from multiple cohorts, characterises the effective number of breeders (N_b) that produced the cohort rather than N_e explicitly. However, both indices are influenced by changes in reproductive success so remain appropriate and will be referred to as N_e throughout. In the software itself, I used full likelihood analysis, no information on sexes and assumed random mating/polygamy. Estimates obtained using this method may be downwardly biased through the inclusion of parents as full-sibs- therefore fish that could be parents and offspring (>3 years age difference) were excluded. COLONY also requires error rates for each locus, so 136 fish were genotyped twice, giving error rates of: LC290 = 0.02; Lea029 = 0.03; Rru478 = 0.02; Ca3 = 0.02; CypG24 = 0.02, CypG27 = 0.02 and Z21908 = 0.02. No errors were observed for the other microsatellite loci, so these were set at a lower nominal value of 0.005.

4.3.11 Temporal Ne Estimates (calculated by P. Hamilton)

For a restricted number of sites with multiple sample points, temporal estimates of N_e were calculated using the change in allele frequencies between generations. Firstly, to allow comparisons to be drawn with other studies, the moment-based method of Waples (1989) was used in NeEstimator (Do et al. 2014). To explore the congruence between estimation methods, calculations of N_e using the temporal method were also undertaken in the software TempoFs (Jorde and Ryman 2007) following either ‘sample plan 1’, if individuals were sampled non-destructively, or ‘sample plan 2’, when individuals were sampled destructively before reproduction and were not returned to the population. This method generally performs better than other temporal methods when sample sizes ae smaller and if allele frequencies are skewed, as is common in microsatellite data (Jorde

and Ryman 2007). A mean generation time of 4 years was applied, although in practice generation times may vary between populations and locations.

4.3.12 Statistical Analysis

Further analysis focussed on testing for a relationship between N_e and predicted oestrogen exposure, along with other variables such as total fish/roach density, genetic diversity and the identification of bottlenecks (using GLM in SPSS). Utilisation of multiple genetic indices led us to explore effects seen over different timeframes, for example N_e is primarily influenced by the parental generation where-as genetic diversity is more indicative of long-term demographic processes across the entire population. Therefore, N_e would remain largely unaffected by any recorded restocking since 2000, however as a precautionary measure, those populations recently restocked (with 1+ fish) were excluded from further statistical analysis (AirDar and WanMoh). TreNot was excluded from any statistical analysis due to the fact that the sample size included <30 fish. E2Eq 90th percentile concentrations were used in all statistical analyses, to represent a worst case scenario for population-level effects.

Average flow rate (m^3/s) was included as a cofactor to control for river size (as it was found to be highly correlated). In addition, data for a subset of sample sites (19 sites) where average roach density (from 2000 onwards) had been estimated using the catch-depletion methodology, were included as an additional covariate in any analyses. Density of roach (numbers per m^2) were obtained from the National Fish Populations Database (NFPD) held by the Environment Agency, UK. All statistical analyses were performed using the software SPSS (Version 20.0. Armonk, NY: IBM Corp).

4.4 Results

4.4.1 Evidence for Population Bottlenecks

Population bottlenecks were expected to be more evident at highly-polluted sites, if roach populations were being detrimentally affected by WWTW effluents. These results suggest evidence for genetic bottlenecks at three sites (Table 4.1): two from relatively unpolluted sites sampled in 1995 on the River Arun (AruHUS) and Lee (LeeHUS), and the third from a polluted site sampled in 2010 on the Lee (LeeWhe). LeeHUS is the only one of these sites that has a significantly lower allelic richness (see Chapter 3) however no differences are seen between estimates of N_e using either method. Demographic data of census size for AruHUS and LeeHUS in 1995 are unavailable, so evidence of a historic population crash at these sites cannot be fully elucidated.

4.4.2 Relationship between Predicted Oestrogen Concentration and N_e

N_e estimates calculated from the microsatellite data, range from 29 to 301 for the N_e ABC method and 36–145 for the N_e SA method (Figure 4.8). Due to the methods of computation, smaller N_e estimates possess narrow confidence limits and improved precision compared to those populations with larger N_e. SA method also had wider confidence limits for the same samples but patterns between the two methods remain similar. Linear regression analysis found no correlation between N_e ABC and the predicted E2 equivalent (90^{th} percentile) at all sample sites where restocking had not occurred (ANOVA $r^2 = 0.011$, $p = 0.598$ for N_e ABC). However, the 95% confidence intervals (CI) for the model coefficient indicated N_e ABC could decrease by a maximum of 5.6% for each incremental increase in exposure of 1 ng/l E_2Eq, or 65% at 11.6 ng/l E_2Eq, equivalent to the most polluted river stretch included in this study. Despite the absence of a statistically significant relationship between N_e and predicted oestrogenic contamination of river water, a more detailed examination reveals that all sites with 90^{th} percentile oestrogen exposure >10 ng/l have N_e estimates of less than 100. Below a mean predicted oestradiol concentration of 6 ng/l, the largest N_e estimates are evident, but these also possess the greatest variation. These boundaries coincide with high risk classification (E2EQ>10 ng/l, >0.25EE2 mean) set out by Williams et al. (2009) based on 90^{th} percentiles.

No genetic diversity indices (as presented in Chapter 3) showed a significant relationship with the predicted E2 equivalent (mean and 90^{th} percentile) at all sample sites. Similarly, no correlations were found when removing the influence of year of sample or sample location, flow rate, or mean body size of fish sampled, using an interaction in GLM. The inclusion of roach density as an additional covariate within the model also produced a non-significant result (GLM, $F_{(1,16)} = 0.515$, $p = 0.483$), albeit for a reduced number of sites (19). N_e estimates at this subset of 19 sites are smaller than the number of roach caught in routine surveys by the EA, including KenFou, where mean N_e estimates equal 40, yet catch data states that 132 roach have been caught on average between 2000-2010.

4.4.3 Temporal Variability in N_e

Temporal estimates of N_e, calculated from allele frequency changes over several generations (Jorde and Ryman 2007), varied from 14 to 265. Classic temporal-based N_e estimates using Waples (1989) gave considerably larger N_e estimates, (often double that of TempoFS) ranging from 48 to 495 (Table 4.2). Upper confidence limits of infinity are

frequently encountered with both methods when N_e estimates are >100, thought to be an artifact of N_e modeling (Luikart et al. 2010, Wang 2005). The Nene exhibits the highest N_e estimates using both methods, despite samples only being separated by one generation. This river is almost double the length and width of the others, so may be expected to support a larger fish population. Both of the temporal methods of N_e estimation assume populations are isolated and do not encounter immigration (Wang 2005). However, significant genetic differentiation between some replicate samples taken in different years (Arun and Bourne) appear to minimally effect estimates of N_e, compared to temporal samples that exhibit no genetic differentiation (Table 4.2). For those rivers with available demographic data, comparable N_e estimates (using Jorde and Ryman 2007 method) to the average number of roach caught at the same location appear evident. However, this is less apparent using the method of Waples (1989) and on the River Lee, where catch data infers that roach numbers are very small (9) yet N_e estimates suggest breeding populations >100 individuals.

Table 4.1 Sampling location, year of sample, modeled mean E2Eq of river water at that site and bottleneck probability statistic for each roach population

Sample code [a]	River	Year	E_2Eq (ng/l) [b]	Bottleneck (TPM) [c]
Outside Thames				
BlaBlM	Anglian Blackwater	2010	4.1	0.40
BlaSti	Anglian Blackwater	2010	7.1	0.60
CheAbB	Chelmer	2010	1.2	0.06
NenBro'95	Nene	1995	1.2	0.69
NenBro'99	Nene	1999	1.2	0.80
NenEct	Nene	2007	4.2	0.79
AirDar	Aire	2011	2.7	0.73
AruHor'95	Arun	1995	4.1	0.90
AruHor'00	Arun	2000	4.1	0.29
AruHor'08	Arun	2008	4.1	0.73
AruHUS	Arun	1995	0.2	**0.01**
TreWol	Trent	1995	3.7	0.55
TreNot	Trent	2007	NM	0.50
Thames catchment				
BlaEvH'10	Blackwater	2010	4.2	0.98
BlaEvH'00	Blackwater	2000	8.8	0.98
BouChe'11	Bourne	2011	4.8	0.62
BouChe'02	Bourne	2002	5.8	0.55
BouChe'06	Bourne	2006	5.8	0.25
GadCas	Gade	2010	NM [f]	0.38
KenBul	Kennet	2010	0.6	0.82
KenFou	Kennet	2010	8.1	0.64
KenNor	Kennet	2010	0.2	0.62
LamSha	Lambourn	2011	0.03	0.10
LeeEss	Lee	2010	6.6	0.60
LeeHyd	Lee	2010	10.3	0.36
LeeHyd	Lee	1995	11.6	0.62
LeeHUS	Lee	1995	NM	**0.001**
LeeSta	Lee	2010	6.6	0.85
Lee'00	Lee	2000	NM	0.90
LeeWhe	Lee	2010	6.6	**0.05**
MolMea	Mole	2010	5.8	0.84
RayRod	Ray	2003	10.9	0.40
StoBri	Stort	2010	4.1	0.92
StoTed	Stort	2010	6.0	0.97
ThaCul	Thames	2010	1.6	0.45
ThaHam	Thames	2010	1.8	0.45
ThaWhi	Thames	2010	1.5	0.88
ThaSha	Thame	2010	1.9	0.38
WanMoh	Wandle	2011	3.3	0.33

[b] Oestradiol equivalents, the predicted average oestrogenicity at the sample site.

[c] TPM = Two-phase model, used for microsatellite evolution in this test.

[e] A composite sample of fish caught at BlaEve (51.354119, -0.8584853) and BlaHaw (51.324119, -0.7665606). Effluent concentrations are an average between the two sites for statistical analysis.

[f] NM = not modelled. For this site there are no major upstream discharges.

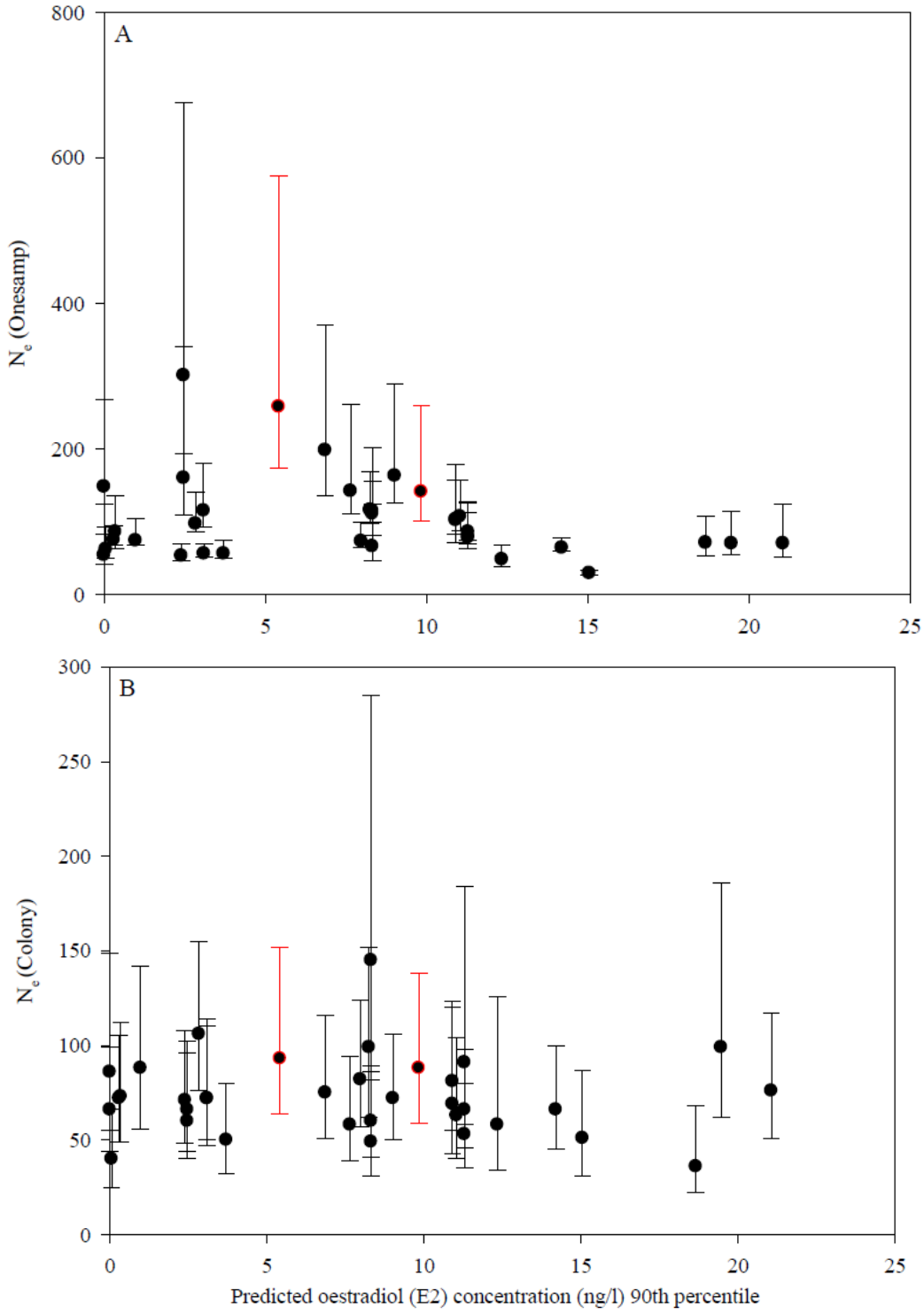


Figure 4.8 Effective population size (*Ne*) plotted against predicted oestrogen exposure for 37 population samples of *Rutilus rutilus*. (A) ***Ne*** **calculated using the Approximate Bayesian Computation (ABC) method in the program OneSAMP.** (B) ***Ne*** **calculated using the sibling assignment (SA) method in Colony. In A and B, error bars are 95% confidence intervals. In cases in which more than one population had similar values, data points overlie each other; thus, individual data points are not always visible. These plots include estimates from sample sites sampled in different years e.g. in the River Nene (which were averaged for statistical analysis) and sites where recent re-stocking had occurred [red markers, River Aire, River Wandle], which were excluded from the statistical analyses**

Table 4.2 Temporal estimates of effective population size (*Ne*) among roach samples in relation to the number of roach caught at the same sites in routine surveys and the genetic differentiation between samples taken in different years

River	Time interval	G [a]	Average No. of roach	N_e (95% CI) [b]	N_e (95% CI) [c]	F_{ST} [d]	*p-value*
Nene	1995–1999	1	*No data	265 (66–∞)	619 (82–∞)	0.00029	0.43
Arun	1995–2000	1	*No data	14 (8–79)	48 (26–137)	0.014	<0.0001
	2000–2008	2	63	60 (31–1733)	321 (106–∞)	0.009	<0.0001
	1995–2008	3	*63	73 (43–247)	232 (123–669)	0.003	0.054
Bourne	2002–2006	1	108	32 (17–219)	51 (25–151)	0.00622	<0.0001
	2006–2011	1	123	45 (23–9572)	63 (35–162)	0.00345	0.036
	2002–2011	2	104	87 (44–3897)	495 (295–1405)	0.00492	0.027
Lee	1995–2010	3	9	137 (81–∞)	346 (145–35,518)	0.003	0.88
Blackwater (Thames)	2000–2010	2	125	141 (68–∞)	206 (83–∞)	0.00071	0.38

[a] G assumed number of generations between sampling points.
[b] Calculated using the Jorde and Ryman method (2007) implemented in TempoFs.
[c] Calculated using the classical moment-based method of Waples (1989) implemented in Neestimator.
[d] F_{ST} between sampling years and respective *p*-values. Methods of calculation are described in Chapter 3.

4.5 Discussion

The need to discern population-level responses to environmental stressors is a key area of research. Crucial knowledge is lacking, due to the intricacy associated with higher levels of organization and biological responses to EDC exposure are no less complex. Most aquatic ecosystems are contaminated with man-made chemicals, which exert adverse impacts on individual health, with the potential to induce measurable changes in population dynamics. Our understanding of exposure effects derive almost exclusively through extrapolations from laboratory studies to wild populations. Nevertheless, uncertainties arise from the complex nature of wild populations that constrain the translation of individual response measures and markers, to adverse health outcomes at the population level. The work presented in this chapter applies a genetic approach to studying how the oestrogenic component of WWTW effluent contamination has affected the effective population size of wild roach, ultimately informing on fish population sustainability.

4.5.1 Understanding Population Bottlenecks using Demographic Information

A dramatic population crash triggers a loss in average heterozygosity, recognisable at the genetic level as a bottleneck event (Nei et al. 1975). Bottlenecks are typically detectable between 0.5 and 5 N_e generations after the population reduction, where N_e is taken at the time of the bottleneck (Cornuet and Luikart 1996). Only three out of 39 roach populations sampled presented evidence of genetic bottlenecks: two classified as relatively unpolluted

sites (upstream of STWs), and another polluted site further downstream on the River Lee. Evidence of genetic bottlenecks in wild roach is novel — Hanfling et al. (2004) did not find any in 22 *R. rutilus* populations around the Elbe. Historic records of population sizes from fish surveys are sporadic prior to 2000 so it remains implausible to associate demographic and genetic bottleneck events in the River Arun in 1995. However, N_e estimates for the Arun from 1995 until 2000 infer a breeding population size of only 14 (using Jorde and Ryman 2007) for this period; the lowest of all temporal estimates. This evidence, along with the larger N_e estimates recorded for subsequent samples taken in 2000 and 2008, substantiate the suggestion of a bottleneck having taken place at this site.

Demographic records indicating fish population dynamics are more complete for other rivers, especially on the Lee. Following severe fish kills in 1967 and 1991 from pesticide contamination (mecarbam) and sewage respectively, the upper Lee has been stocked extensively with over 1200 roach. Little is known about the survival and reproduction of hatchery-reared roach specifically, however, introduced populations are known to have a similar effect on the genetic constitution as that observed during a population crash; when few founding individuals present limited genetic diversity or are supplemented in small numbers with depauperate individuals (Demandt and Bjorklund 2007, Dawnay et al. 2011). Likewise, the bottleneck identified at LeeWhe (further downstream sampled in 2010) is supported by very low roach numbers (<10) recorded in 2003–2005, based on past catch records. This pattern of events and population survey data may therefore help explain the finding of genetic bottlenecks at these sites and support the results of the BOTTLENECK program. However, with this evidence and the documented fish kills on this river you would expect more sites on the Lee to demonstrate bottleneck events, so without historical samples and precise population records it remains impossible to determine the exact timed occurrence of a population crash or a loss of numbers followed by a recolonisation.

The unknown size of bottlenecks and rate of immigration, further limit the interpretation of the genetic bottlenecks observed at these three sites (Santos et al. 2013). Regardless, it is apparent that roach populations from highly polluted sites are not more susceptible to drastic reductions in size, nor have they undergone bottlenecks severe enough to leave a recent genetic footprint (detection depends on magnitude and duration; Cornuet and Luikart 1996). From these results, it also appears evident that a previous population bottleneck does not necessarily equate to reductions in measures of genetic diversity or N_e. Both of these outcomes are in line with previous studies on different fish species that

document no evidence of reduced genetic diversity or genetic bottlenecks in polluted locations (Whitehead et al. 2003, Roark et al. 2005, McMillan et al. 2006, Lind and Grahn 2011).

4.5.2 N_e Estimates of Roach in Context

The paucity of demographic data available for many wild freshwater fish species emphasises the importance of seeking alternative methods of estimation. Effective population size is an important parameter in conservation and evolutionary biology and has gained tremendous interest through its use in informing on natural population sizes across a wide variety of species (Luikart et al. 2010). Increasing availability of polymorphic genetic markers and the development of statistical software for calculating N_e (Wang 2005), overcome difficulties in collecting sufficient demographic information, allowing us to calculate novel stock estimates in many economically and ecologically valuable fish species.

Effective population sizes of roach across samples sites in the UK were generally small, with no contemporary estimates >300 and almost 60% of populations appear comprised of less than 100 breeding individuals (Figure 4.8). Despite the application of different single sample methods of estimation, the N_e predictions in this chapter are similar to others documented for roach populations in the UK (Crookes and Shaw 2009). However, temporal estimates of N_e from Demandt et al. (2010 — using the same TempoFs and Neestimator methods), show predominantly smaller N_e of roach across isolated populations. All roach populations studied appear to have large enough N_e to maintain genetic variation between generations, exceeding the suggested threshold value of 50 individuals (on all but three occasions) for short-term population persistence (<100 years, Soule 1980), between samples taken more than one generation apart. This discrepancy between studies could be due to Demandt et al. (2010) sampling isolated basin populations in Sweden with notably poor recruitment, in addition to using only five microsatellite markers. Indeed, estimates of N_e in a second common freshwater species, the perch (*Perca fluviatilis*), were undertaken in the same study and yielded similarly small N_e estimates across the same time intervals (Demandt et al. 2010). Both these species share similar life history and reproductive strategies, prompting speculation that the fish populations in this enclosed water body exhibit a limited carrying capacity and naturally smaller fish populations compared to those in this study. Overall, estimates of N_e from the roach populations sampled here appear consistent with other freshwater species (Palstra and Ruzzante 2008) and are in line with expectations from a species with

such a ubiquitous distribution and a wide tolerance to habitat alterations.

4.5.3 Limited Evidence of Effluents Impacting Ne of Roach Populations

Many ecosystems are adversely affected by human activities, commonly resulting in detrimental impacts on species abundance and sustainability. Riverine environments are no exception, with demographic repercussions altering the balance of birth/death rates and accelerating migration of individuals between habitat patches. The intuitive link between demographic and genetic processes means changes in population dynamics can be recognised through the use of molecular analytical tools (Avise 2000), allowing the impact of environmental changes on wild population persistence to be assessed. One approach is to evaluate genetically effective population size across an environmental gradient of interest, in this case, levels of oestrogenic contamination.

Contemporary $N_{e,}$ examined using DNA microsatellite loci, revealed no significant evidence of exposure to oestrogenic sewage effluents detrimentally impacting roach populations. Additionally, the inclusion of historic samples from sites with previously documented occurrence of gonadal feminisation in male roach, provided evidence of self-sustaining populations in highly-contaminated river stretches over multiple generations. This species exhibits gonochoristic sexes and a broadcast spawning reproductive strategy, known to be disrupted in the presence of EDCs, with recent studies carried out using wild populations. Roach populations with a history of exposure were placed in breeding colonies and allowed to spawn in controlled breeding experiments, with resulting parental contribution of feminised/intersex males being reduced by up to 76% in comparison to non-intersex males (all other variables being equal; Harris et al. 2011). However, despite this outcome, the prevalence of males with such moderate/severe intersex condition has been estimated to be less than 10% in English rivers (Jobling et al. 2006). Based on this evidence, the predominance of male roach in the wild can be expected to possess normal or mildly feminised gonads, where both gonadal phenotypes contribute similarly in competitive breeding situations (Harris et al. 2011). Therefore this outcome is largely in agreement with the findings presented here; reiterating that roach confined to highly-contaminated river stretches are able to maintain viable populations, independent of immigration and supplementation.

Long-term effects of effluent exposure remain poorly explored with reference to fish populations. One of the few studies conducted to simulate the risk of long-term oestrogen exposure on wild fish populations, demonstrates findings that are inconsistent with my remarks. Observations from chronic exposure (7 years of 5–6 ng/l EE2) of entire lake

populations of the cyprinid, *P. promelas*, showed near extinction of this species and detrimental alterations of reproductive development in both sexes after 7 years, across multiple generations (Kidd et al. 2007). Contrastingly, temporal estimates of N_e documented here suggest that barrier-restricted sub- populations of roach in the Lee and the Blackwater have high predicted exposure levels (4–10 ng/l E2Eq) but possess relatively consistent N_e over time, with temporal estimates of N_e exceeding 135 breeding individuals. The discrepancy seen between this study and that of Kidd et al. (2007) may be due to lower levels of oestrogen exposure experienced in the wild roach populations sampled. On average, rivers in the UK experience levels of effluent discharge constituting around 10–30% of total flow (Williams et al. 2009), which, based on the correlation between E2Eq and percentage effluent, is around 1–7 E2Eq or 0.05–0.2 ng/l of EE2. The highest site examined here (LeeHyd) has a mean predicted effluent concentration of ~70%, (reaching 86% in very low flow conditions) which equates to ~0.5 ng/l EE2 and E2EQ of ~12 ng/l. These percentage effluent estimates correlate well with work conducted on the same stretch of the Lee in 2003, where over a 14-day monitoring period, effluent made up 75% of the river flow near East Hyde (Williams et al. 2003). Concentrations of EE2 in effluent samples of R. Lee STW's equated to 0.4–1.1 ng/l, prior to discharge into the river. Therefore, given that both lake and laboratory studies demonstrating sexual disruption or reproductive failure (Kidd et al. 2007, Nash et al. 2004) have exposed fish to 5 ng/l EE2, it is perhaps unsurprising that effects seen in wild fish populations are different. E2 is 10 times less potent than EE2 in provoking gonadal feminisation, so even with a life time of exposure it is unlikely that wild fish experience oestrogenic concentrations near those used in the aforementioned studies. Limited numbers of sites in the high exposure scenarios hamper firm conclusions, but are poorly represented due to the fact that they are relatively rare in the wild (1–3% of effluent contaminated river reaches in the UK have E2Eq >10 ng/l: Williams et al. 2009). Therefore, the results presented here may be more indicative of real-world scenarios for fish populations in UK rivers.

Notwithstanding this, the wide 95% confidence limits of these N_e estimates (the summary statistics model ONESAMP), meant that significantly smaller population sizes cannot be ruled out at some highly polluted sites. This has important implications for interpretations regarding effects of effluents on roach populations. The possible reduction in N_e to lower 95% CI limits mean that population-level effects of effluents in highly contaminated regions could yield breeding population estimates 65% lower, which could

alter conclusions drawn. Likewise, concerns remain regarding the estimation of N_e in wild populations. Several factors are known to complicate temporal estimates of N_e (Palstra and Ruzzante 2008); overlapping generations, and fluctuating population size (Waples 1989) are known to bias N_e and are known characteristics of natural roach populations. Furthermore, methods of N_e estimation make assumptions which are commonly hard to control in nature and are likely violated to some degree (no migration, no selection and no mutation), making N_e notoriously difficult to compare across wild populations.

4.5.4 Comparing Findings with Laboratory Studies and Simulations

Despite the positive evidence presented here regarding roach population sizes, anecdotal evidence has reported severe declines in cyprinid fisheries throughout history. Whether these are the result of chronic exposure to oestrogenic effluents, remains uncertain. One of the few examples of cause and effect of EDCs was demonstrated in the eelpout (*Zoarces viviparous)* off the Swedish Baltic coast. Wild eelpout broods were significantly male biased in the vicinities polluted with pulp mill effluent. These alterations/shifts in sex ratio to male dominant broods coincided with periods of mill activity, whereas during a mill closure, sex ratios returned to normal (Larsson et al. 2000, Larsson and Forlin 2002). Despite such demonstrable evidence of chemicals being responsible in shifting sex ratios in the wild, these changes have not been linked to population declines even in afflicted fish populations, unlike those seen in simulated laboratory exposures.

Prior to this work, the elucidation of long-term effects of persistent exposure to oestrogens or oestrogenic effluents was undertaken primarily in laboratory studies using fathead minnow, zebrafish and roach. Effects such as sex reversal of male fish, reproductive failure at concentrations above typical environmental concentrations (undiluted effluent or ~5 ng/l EE_2) or reduced fecundity and skewed sex ratios at more realistic environmental concentrations (below 1 ng/l EE_2 — Hannah et al. 2009) have only been observed in persistent exposures that include the period of sexual differentiation (Nash et al. 2004, Lange et al. 2001, 2009, Kidd et al. 2007, Parrott and Blunt 2005). Here it is assumed that the predicted level of oestrogen/ effluent exposure has remained relatively constant throughout the life-cycle of individual roach, encompassing all developmental stages. However, had exposure levels been significantly reduced during critical windows of development (van Aerle et al. 2002 — due to high dilution or juvenile habitats being in less impacted stretches), then this may explain the discrepancy between the findings here and those demonstrated in life-long laboratory exposures. Impacts could have been lessened if oestrogenic exposure levels fluctuated in

the rivers studied here or were significantly lower during crucial periods of roach development.

Much of our knowledge about population-level effects has been accrued from controlled simulations, modelling, and extrapolations, to predict population risk (Grist 2003, Gutjahr-Gobell et al. 2006, An et al. 2009). However, it can be difficult to predict long-term effects of EDC exposure from this knowledge, highlighting the inherent danger of assuming mirror effects between laboratory and field studies. A likely reason for inconsistencies may in part be reflected in the inability to pinpoint *in-situ* exposure levels both temporally and spatially. In the laboratory this is closely monitored, however in the absence of extremely detailed and time-consuming water sampling I use modelled predictions of oestrogen exposure to assess risk for roach populations sampled. Exposure history is therefore broadly estimated, with inherent assumptions made regarding the movement of individuals between regions and uniformity of contamination within stretches. Consequently, differences between experimental findings and those seen here may be exacerbated by lower than expected predicted levels of oestrogenic contamination present across all sampled sites.

Closely related work on roach kept in 100% effluent for 3 years also yielded all female populations, demonstrating that complete feminisation is possible through prolonged exposure to water-borne chemicals (Lange et al. 2011). This study allows little potential to address the question of sex ratios in UK roach populations, as roach are not recognisably sexually dimorphic from their exterior, nor do we currently possess a genetic sex marker. Without this information it remains difficult to comment on the constitution of the wild roach populations or how many breeding males and females could potentially contribute to the N_e estimates. Therefore we cannot dismiss the possibility that oestrogenic effluents are altering sex ratios of wild roach populations (Cotton and Wedekind 2009), but given the high fecundity of female roach, even a very small amount of males would likely still sustain population viability.

Often laboratory exposures find adverse effects at higher concentrations than those found in the environment. These are suggested to be indicative of longer-term exposures at lower levels in ‘real-world’ scenarios. To date there is very little support for this conclusion. However, I do not dismiss the utility of laboratory exposures, nor discard their environmental relevance —especially in light of increased population projections and climate change. Riverine environments may be altered dramatically over the next 30–40 years, becoming increasingly afflicted by reduced river flows and increasing waste

from population growth. Projections based on population and climate change recently alluded to a possible two-fold rise in oestrogenic contamination of surface waters by 2050 (Green et al. 2013), posing greater risk for aquatic organisms (unless combatted by improved removal efficiencies at STWs). If encountered by natural populations, these would be moving towards the concentrations found to produce negative effects on reproduction and development in laboratory simulations of fish populations.

4.5.5 Caveats

I have highlighted the importance of careful extrapolation between laboratory and field studies; however caveats exist within the approach used here that warrant consideration for interpretation. Assumptions introduced for accurate calculation of N_e, (isolation between sites of differing levels of exposure and across generations), were addressed by selecting populations restricted by physical barriers impeding movement. Significant genetic differentiation between sample sites (concluded from Chapter 3) gave support to the assumption of local sub-populations exchanging few individuals, and strengthened the confidence in the sample sites selected here. These results emphasised the role of barriers in impeding movement, meaning that any violation of this assumption for the estimation of N_e was likely minor.

Migration or movement of individuals can also be associated with supplementation of fish populations with those from cultured brood stock facilities. Efforts were made to obtain samples from those rivers that had no recorded introductions since 2000 (when the LFMD was started by the EA). Little is known about the survival of cyprinid hatchery fish, their success, or the genetic consequences for wild stocks (Araki and Schmid 2010), making inferences regarding their effects on N_e more complex. Published information on re-introduced populations of roach in Swedish lakes, suggested that only a fraction of those introduced were likely to survive and adapt well enough to reproduce (Demandt and Bjorklund 2007). Additionally, introductions since 2000, albeit in nearby habitats, are unlikely to have confounded N_e estimates due to physical barriers delineating populations. Supplementation prior to 2000 remains largely anecdotal but would be more likely to have influenced population genetic parameters (as they encompass many generations). To address this potential problem, attempts to reveal evidence of introductions using molecular data were undertaken using assignment of individuals at known restocked sites. For instance, the River Wandle has received 3500 individual roach since 2007, sourced from the Calverton hatchery. Each year, wild roach are caught in the Trent, stripped and the offspring are raised in the hatchery until suitable for

supplementary or restoration restocking across many UK rivers. The microsatellite dataset presented here includes samples from both the Trent and the Wandle and assignment of individuals showed the majority coincided with the Thames region (73%) with two fish assigning to the Trent. Such small numbers may even be incorrect assignments, suggesting little influence of restocked fish.

4.5.6 Improvements for Further Work

Difficulties with obtaining suitable archived samples meant this study lacked historical emphasis, so comparisons between temporal and contemporary N_e estimates remain anecdotal. Therefore in order to establish specific correlations between N_e and the extent of exposure, any approach would require a large number of populations to distinguish a definite effect (if it exists). For these reasons, further improvements would include more samples from the most polluted roach populations in the UK and additional temporal/historical samples indicative of breeding sizes of past generations.

Sexual development in some fish species is relatively labile and is known to be affected by chemical exposure. Additionally, periods of depuration following laboratory exposures have demonstrated partial recovery in reproductively-compromised individuals (Nash et al. 2004). This would be unlikely to happen in roach as sexual development is less flexible and disruptions appear permanent (Beresford et al. 2004, Liney et al. 2005). This chapter would therefore benefit from a comparative analysis in a different species with a different life history and short generation time. Manifestation of reproductive disruptions at the population level may arise more rapidly in fish which breed several times a year (Kidd et al. 2007) compared to those with longer generation times and later age at maturity.

This work highlights the inherent danger of assuming that effects in laboratory exposures mirror those likely to be occurring in wild populations. Consideration of the variation in chemical impacts at the level of the individual also remains to be explored in the field of EDC research. Determining the population-level impacts of EDCs are still, to an extent, in their infancy, however new molecular technologies offer a powerful tool to examine ecotoxicological responses at both the individual and population level. For example, parentage analysis of offspring from wild populations would provide a useful assessment for evaluating reproductive fitness of individuals from river stretches experiencing differing levels of exposure.

4.6 Conclusion

Previous studies on the impacts of oestrogenic contaminants derive mostly from work conducted in a laboratory setting, exposing model fish species to WWTW effluent. Individual effects are then extrapolated to populations. Such studies have highlighted that wild populations of fish living downstream of effluent discharges are reproductively compromised (altering reproductive physiology and morphology) by the presence of persistent oestrogenic pollutants. Notwithstanding this, there is currently no evidence of population- level effects of endocrine-disrupting chemicals on wild fish, anywhere in the world.

To this end, I sought to understand the long-term impacts of oestrogenic contaminants, deriving from WWTW effluents, on population sustainability of wild roach. Importantly, the results reveal that roach populations restricted to river stretches comprising of high proportions of effluent over multiple generations are self-sustaining and reproduce successfully, not maintained by immigration. No conclusive evidence suggests that oestrogenic effluents reduce the N_e of roach populations or make them more susceptible to population bottlenecks. However, these results are tempered by a possible 65% reduction in N_e at the most highly contaminated sites, due to the confidence intervals associated with N_e predictions. Evidence of reproductively self-sustaining roach populations that have been present in these contaminated river stretches for multiple generations brings into question the possibility of adaptation. Adaptation to environmental change can be rapid, which may help species counter stressful conditions or realise opportunities arising from environmental change. Essentially nothing is known about adaptation to oestrogens in the environment, which highlights a critical future avenue of exploration in seeking to understand the population-level impacts of oestrogenic effluent exposure. Undeniably, this work is testament to the utility of molecular markers in informing on previously unattainable information regarding the genetic variation and number of breeding individuals in a wild, non-model, fish species. Aligning this knowledge with detailed information on exposure history and reproductive strategy of fish populations moves towards predicting the long-term impact of EDCs on population sustainability, demarcating insights for the future management and conservation of aquatic populations.

Chapter 5

Influence of Multigenerational WWTW Effluent Exposure on the Reproductive Success of *Rutilus rutilus*

Pages 137 - 173

5.1 Introduction

Higher levels of biological organisation can accumulate reproductive abnormalities from the insult of oestrogenic contaminants. Considerable interest therefore surrounds the risk posed by endocrine disrupting compounds to higher taxa and humans (Patisaul and Adewale 2009). For example, reproductive health in male populations of some western countries has declined, with reduced average sperm counts and a parallel increased incidence of testicular dysgenesis being reported (Skakkebaek et al. 2001). Evidence of declining reproductive health in both sexes across westernised countries points towards an environmental basis, however the cause is likely multifaceted and remains poorly defined.

The call to incorporate evolutionary timescales in assessing population risk to effluent exposure has recently come under renewed focus. Empirical evidence states that exogenous chemicals in the environment do not pose a novel challenge, however the acute discharge of such a diverse and persistent range of active chemicals is unparalleled in evolutionary history. Growing concern also arises from the heightened potency of these chemicals compared to naturally occurring hormones. For this reason, the continual presence of EDCs in the riverine environment provokes consideration of their non-lethal, transgenerational, effects on aquatic organisms. For instance, the consequences of disrupting reproductive processes can extend beyond the individual, with detrimental effects amplified at higher levels of organisation affecting population growth and persistence, with the potential to impinge on ecosystem structure and function (Figure 5.1).

5.1.1 Persistent Pollutants present in WWTW Effluents

Wastewater treatment work effluent contains a complex mixture of compounds influencing neural, immune, behavioural and endocrine systems (Norris and Lopez 2010). Among these are the steroid oestrogens, a set of compounds that play a critical regulatory role in developmental pathways of the hormonal/reproductive system. Key contributors to the oestrogenic loading of surface waters are urinary oestrogens (E1 and E2) and synthetic oestrogens (namely EE2) commonly used in the contraceptive pill. These sex hormones are generally viewed as posing greatest concern to wild fish health, due to profound negative effects on reproduction (Lange et al. 2001, Nash et al. 2004). For example, exposure to oestrogenic contaminants during critical periods of development has been associated with deleterious health effects such as decreased fertility, feminisation

and poor hatch success across a broad spectrum of wildlife (WHO Report 2013). Parallel evidence from mammalian studies also shows that early life exposure to oestrogenic chemicals can affect brain development and reproductive behaviour (Patisaul and Adewale 2009).

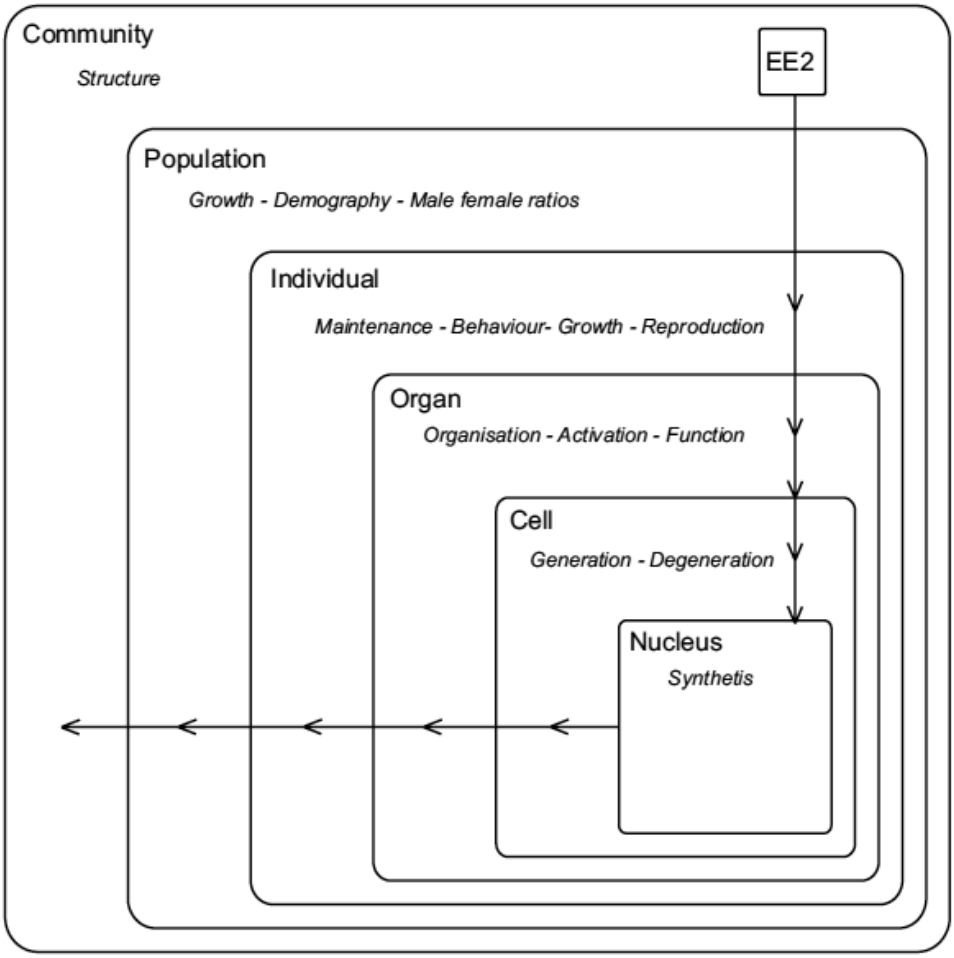


Figure 5.1 Diagrammatic representation of the hypothesised pathway of EE2 emission in surface waters from cells, to individuals, to populations. Once the chemical enters the individual, alterations to the activation of enzymes/proteins can provoke changes in the functioning of tissues, organs and eventually alter individual behaviour or fertility. Depending on the size of effect at lower levels of organisation, the repercussions can modify size and demography of populations and structure of the community. Sourced from Per Hallgren, unpublished

Wastewater treatment works are successful in lowering the overall organic load from humans reaching rivers, but they are not capable of removing all contaminants. Thus, in addition to oestrogens, in-stream fauna is exposed to a very diverse range of chemicals ranging from heavy metals and persistent organic pollutants, to more polar drugs and other EDCs associated with past, or present human activity. Studies linked with the UK Endocrine Disruption Demonstration Programme, have shown that although removal of steroid oestrogens from WWTW effluents reduced, or even removed biomarker responses

(vitellogenin induction); reproductive output was still adversely affected in exposed fish. This suggests that other non-oestrogenic pollutants contained in WWTW effluents can also cause effects on reproductive health (Filby et al. 2010).

5.1.2 Potential Consequences of Oestrogenic Exposure for Fish

As the oldest and most abundant group of vertebrates, fish species exhibit the greatest diversity of reproductive modes. Reproductive strategies range from all types of sexuality (gonochorism, sequential or simultaneous hermaphroditism) to breeding systems differing in their patterns and durations of paternal care, the number of mates, courtship behaviour, coercion and competition, mating recourses, and the extent of mate choice (Reynolds 1996). Results from several studies indicate that the most sensitive wildlife species to oestrogenic chemicals also belong to this group (Caldwell et al. 2008). This is perhaps unsurprising, given the conservation of oestrogen receptors through vertebrate evolution and the targeted design of pharmaceutical oestrogens to mimic natural processes in humans (Gunnarsson et al. 2008).

Some EDCs interfere with natural hormone action and can provoke dramatic changes in an organism's physiology. Compelling evidence from UK rivers suggest that oestrogenic contamination can elicit feminised responses in male fish, finding widespread occurrence in populations of wild roach (Jobling et al. 1998, 2002). Examples of reproductive disruptions include female biomarker responses (e.g. vitellogenin), feminised reproductive ducts and intersex (the presence of both male and female germ cells in the same gonad). Moreover, continuous exposure to effluent (which is possible in English rivers) may induce more significant effects with age. Critically, for this work, these responses in male fish have all been linked experimentally to exposure to WWTW effluent discharges (Liney et al. 2005) and EE2 (Lange et al. 2008, 2009). Further, Lange et al. (2009) found that the degree of feminisation in male fish was related to the concentration of effluent exposure, suggesting that a gradation in intersex severity may be an informative marker of contaminant levels.

Roach are group-spawning fish, a breeding behaviour commonly associated with cyprinids. This lek-like mating system means mate choice is not random and sexual selection of mates is based on individual preferences of females (Wedekind 1996). Observations made during spawning events suggest that females often select male partners with some kind of territory and dominant males with large breeding tubercles (induced by sex hormones) and more active courtship behavior (male quivering, butting or chasing) are thought to indicate a better quality male (Kortet et al. 2004). Dominance of males, through

ornamentation and the securing of a spawning site, may improve their spawning success in a roach lek however little is known about the influence of size or age on dominance/courting behavior in natural roach spawning aggregations. Multi-male fertilisations are common in simultaneous spawning events (Wedekind 1996) indicating that sperm competition may be an important additional selective force, yet these speculations have not been exhaustively tested in natural spawning scenarios nor have the considerations of how reproductive success relates to individual fitness or offspring survival. Therefore, dominant behaviour and the proliferation of breeding tubercles may be the key characteristics that promote fertilisation success of competing male roach.

Exposures to mixtures of environmental oestrogens, in WWTW effluent or to 17β-oestradiol have been shown to disrupt reproductive behaviour. For example, a 21-day exposure of male fathead minnows to sewage effluent resulted in exposed males suffering near total reproductive failure when they had to compete with control males (Martinovic et al. 2007). Suppression of reproductive behaviour renders exposed males less likely to reproduce successfully, altering patterns of gene flow in wild populations. Chemical exposure therefore has the potential to alter parental contributions through modifications to physiology or behavioural interactions that impart inferiority during sexual selection. Application of DNA microsatellites to infer patterns of parentage has shown that EDC exposure, can alter dominance hierarchies (Coe et al. 2008) and breeding behaviour in zebrafish in laboratory experiments (Coe et al. 2010). Modifications triggered by the presence of EDCs could subsequently cause abnormal and potentially detrimental changes in the genetic make-up of a population and/or reduce genetic diversity, without necessarily affecting the size of fish populations.

Fish population sustainability is largely governed by reproductive success and group social dynamics. Nonetheless, the impacts of chemical exposure on natural patterns of reproductive success remain poorly explored in wild populations. Innovative application of DNA markers to infer parental contributions demonstrated that reproductive success of moderately to severely intersex roach from WWTW effluent polluted rivers possess a reduced capability to reproduce under competitive breeding scenarios (Harris et al. 2011). Furthermore, compelling evidence has also demonstrated that oestrogenic contamination can disrupt reproduction to such an extent that a near-extinction ensued in a lake dosed with 5–6 ng/l EE2 (Kidd et al. 2007). In the latter experiment, the exposure dose was considerably higher than that likely to be experienced by fish living in UK surface waters. Therefore it is not known whether the complex mixture of oestrogenic compounds found

in WWTW effluent can compromise reproductive fitness or the breeding capability of roach in natural spawning scenarios (Lange et al. 2009).

5.1.3 Sensitive Windows of Development

Large or prolific effluent inputs into habitats utilised by aquatic organisms during vulnerable/critical windows of development enhance likely impacts on endocrine signalling networks that regulate reproduction in vertebrates. It is well established that the shaping of the vertebrate brain and reproductive organs during development are manipulated via hormonal control. In roach specifically, the ontogeny of sexual development within captive populations has been studied best and findings show complete sexual differentiation from as early as 74–102 dph and that male and female roach can reach sexual maturity from as early as 290 dph and 718 dph, respectively (Paull et al. 2008). Initiated within discrete windows, these developmental phases of the reproductive system present heightened sensitivity to steroidal hormones, rendering them more at risk of disruption (Ankley and Johnson 2004).

Often latent effects of EDCs on fecundity may not manifest themselves until subsequent generations, making detection challenging. Likewise, dosage and timing of exposure dictate the effects seen in individual fish (Figure 5.2). For example, the induction of intersex is believed to be triggered during a vulnerable window of gonadal development, likely to be encountered in early life (van Aerle et al. 2002). Additional support for this theory derives from multigenerational (Kidd et al. 2007) and full life cycle tests encompassing this critical window (Nash et al. 2004), which have observed intersex in male fish following exposure to EE2 during early life. Furthermore, reproductive alterations initiated during development remain largely irreversible, increasing the possibility of accumulative problems in aging organisms and long-term fitness repercussions in wildlife populations.

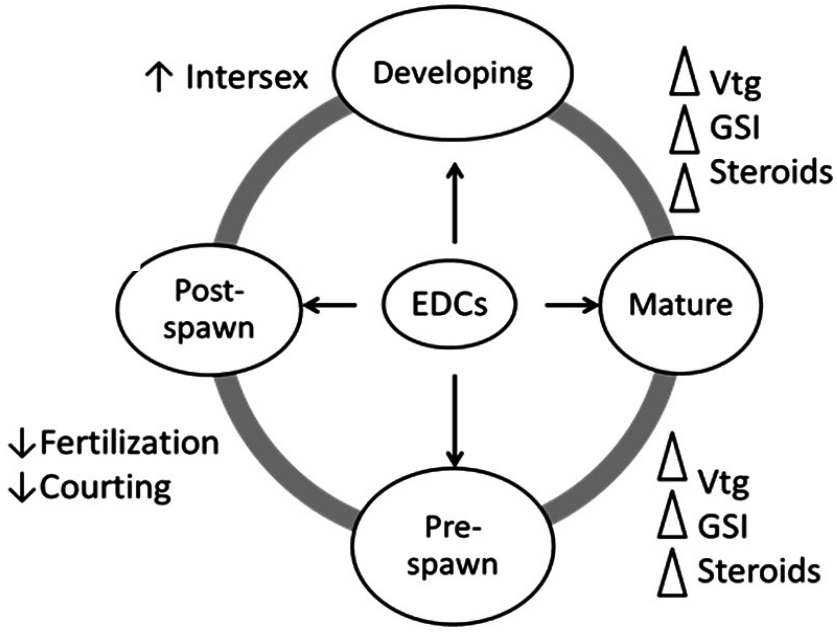


Figure 5.2 Influence of timing and length of EDC exposure on different stages of gonadal development in fish. Chemical factors interact with the gonad during development to change the size of the gonad, alter vitellogenin levels (with oestrogenic EDCs) and disrupt steroid hormones. Following development and maturation, EDC exposure during the pre-spawning window can negatively affect the fertilisation process as well as behaviour (e.g. courting) (Söffker and Tyler 2012). The post-spawning period is characterised by the prevalence of germ cells, and exposures during this time frame can affect sexual differentiation and is thought to result in the presence of oocytes within the testes. Adapted from Bahamonde et al. (2013)

5.1.4 Methods for Investigating Multigenerational Effects of Effluent Exposure

Laboratory studies investigating long-term effects of EDCs have found concerning long-term alterations in vertebrate development and reproduction. The predominance of representative experiments have included exposures initiated at the embryo stage (F0) and carried through to 30–40 dph of the F1 generation; for example, tributyltin (TBT) exposures initiated with sheepshead minnow (Manning et al. 1999), exposure to E2 and tamoxifen of adult zebrafish (*D. rerio* — van der Ven et al. 2007), and medaka (*Oryzias latipes*) exposed to the weak xenoestrogen 4-nonylphenol (NP) from F0 eggs to F1 maturation (Yokota et al. 2001). Histological examination based on the occurrence of testis–ova in the full life cycle test of medaka by Yokota et al. (2001) showed that the F1 fish appeared to be more sensitive to NP, with a higher incidence of testis–ova compared to the parental generation. These findings indicate potential greater sensitivity in successive generations; a finding which was mirrored in another full life cycle test using medaka and a weak oestrogen agonist (Seki et al. 2003).

Effects on fecundity, hatch success, and fertility that extend beyond the first generation are noted with EDCs (Colborn et al. 1993, Schwindt et al. 2014). This finding was exemplified by Nash and colleagues (2004), who reported that exposure of F1 zebrafish

to 5 ng EE2/l caused reproductive failure and a 56% reduction in fecundity, yet the same concentration yielded no observable effects in the F0 generation. Reductions in fecundity were also amplified between generations of the Chinese rare minnow (*Gobiocypris rarus*) exposed to EE2 at 0.2 and 1 ng/l, resulting in spawning failure in the exposed F1 generation (Zha et al. 2008). However, only a handful of studies are extended beyond the F1 generation. Recent research on the long-term effects of 17*β*-oestradiol (E2) on sheepshead minnow demonstrated compounding reductions in reproduction (in F1 and F2 generations) and survival of embryos in the F2 generation (Cripe et al. 2009). In most instances, the mechanism responsible for effects seen in offspring of exposed parents is unknown but has been associated with altered DNA methylation patterns (Liu et al. 2014, Aniagu et al. 2008). Unlike in mammals, where the majority of DNA methylation patterns are "wiped clean" during early embryonic development, studies using zebrafish (*Danio rerio*), have shown DNA methylation patterns are retained to a much greater extent as in mammals (Macleod *et al.* 1999). However as germ cells are exposed in these experiments, proof of multigenerational inheritance if induced DNA methylation patterns requires examination of the grandchildren of the exposed fish. Importantly, all these studies raise concern over the potential amplification of risk posed to populations exposed over multiple generations.

5.1.5 Transgenerational and Epigenetic Influence of EDCs

Subtle impacts of persistent contaminants may remain undetected or poorly captured by a single generation in an experimental setting. Likewise, mounting evidence emphasises the complexity of long term health consequences of EDC exposure, highlighting the alterations that can occur in the next generation (Skinner et al. 2010). Examples include, but are not limited to, altered developmental programming in offspring and the delayed expression of abnormalities until maturity/later life (Colborn et al. 1993). Essentially, exposure of aquatic organisms to a complex mixture of chemicals, that may exhibit identical or independent modes of action, can potentially disrupt any endocrine-mediated interaction regulating reproduction, physiology and behaviour across multiple generations (Norris and Lopez 2010).

The potential for oestrogenic contaminants to 'imprint' on the endocrine system of developing offspring (affecting subsequent phenotypes) is a relevant question because the action of certain hormones is modulated through epigenetic regulators. Molecular mechanisms of endocrine-active substances can also involve multiple pathways including interactions with nuclear hormone receptors (see mechanisms section; Greally and Jacobs

2013). Well-studied examples include Bisphenol A (BPA — a component of polycarbonate plastics and resins) with epigenetic effects on steroid pathways, resulting in increased VTG levels in F1 and F2 male zebrafish after parental exposure (Keiter et al. 2012). The relationship between prolonged contaminant exposure and reproductive endpoints was earlier explored by Matta et al. (2001), where exposure of the mummichog (*F. heteroclitus)* to methylmercury reduced male survival, ability of offspring to successfully reproduce, and altered sex ratios in offspring generations.

Transgenerational effects are those in which direct exposure of the parent results in an altered phenotype that is transmitted to multiple generations (at least F3), in the absence of their direct exposure (Skinner et al. 2010). Early work by Gray et al. (1999) denotes transgenerational effects of EDCs with the occurrence of various developmental problems seen in hatched offspring of octylphenol-exposed medaka (*O. latipes*), along with reduced reproductive success. Contrastingly, multiple generation effects of chemical contaminants are investigated using designs incorporating an F2 generation, where maternal transfer of contaminants is possible and exposure of successive generations is commonplace. The breeding experiment proposed here is better aligned with multigenerational exposure and much of the literature concerning fish employs a similar approach. To understand the implications of full life-cycle exposure to EE2, studies involving full life-cycle exposures found consequences for the filial (F2) generation including impaired growth, delayed onset of spawning and reduced fecundity and fertilisation success at 2.0 ng/l EE2 (Schafers et al. 2007). Whether these observations occur in the presence of WWTW effluents, where concentrations of single EDCs are typically lower than experimental situations, needs to be investigated.

Further dysfunctional effects in adults, as a result of fetal exposure to toxicants, have been demonstrated. Mammalian studies using rodents have provided some of the only evidence of effect transmission to later generations of offspring through epigenetic mechanisms. For instance, Nilsson et al. (2008) examined the long-term effects of vinclozilin (ED and anti-androgen fungicide) to rats and found that exposure during the embryonic phase was detectable in the F3 generation. F3 rats demonstrated reprogramming of the brain transcriptome and F3 males were less preferred by females in a mate choice test. This demonstrates transgenerational effects of EDCs and emphasises the importance of prolonged exposure durations of guideline testing.

Overall, it appears that multigenerational effects of EDC compounds, present in WWTW effluents, are capable of reducing survival and promoting abnormalities in subsequent

generations. However, high concentrations of natural oestrogens (e.g. oestrone) do not provoke transgenerational effects in fathead minnows, so adverse effects can differ among fish species depending on the concentration, duration and timing of exposure (Dietrich and Krieger 2009). Therefore, understanding the effects of WWTW effluent on a common freshwater cyprinid species over multiple generations will elucidate the involvement of multigenerational, changes in adverse health effects and their implications for population viability.

5.1.6 Experimental Aims and Hypothesis

Transient lifetime exposures to effluent-dominated systems may affect reproduction and survival, with the possibility that effects persist in subsequent generations. Simulating lifetime exposure with a view to understanding population consequences is a crucial requirement for realistic experiments exploring mechanisms of population failure. In a previous study conducted in a collaborating research group, roach were exposed for over three years to a treated WWTW effluent (Lange et al. 2011). In breeding scenarios that employed roach derived only from the exposure to 100% effluent there was reproductive failure due to the absence of males. In this chapter, offspring of the exposed females and control males from that study were exposed to effluent from the same WWTW for up to 3 years and 9 months. I aimed to investigate the multigenerational effects of oestrogenic WWTW effluent in roach, over two generations, by assessing whether parental exposure to WWTW effluent alone could impair offspring reproductive health. To sufficiently establish phenotypic change and reproductive success in male roach, an assessment of the reproductive ability of fish with exposed mothers was undertaken after three years of exposure using competitive breeding trials against fish with control mothers. DNA microsatellites were used to determine parentage and the outcomes of this multigenerational exposure are expected to align physiological endpoints associated with feminisation, with reproductive capabilities in competitive breeding scenarios.

Given the evidence published from the previous exposure experiment by Lange et al. (2011), the hypothesis for this experiment states that there are multigenerational effects of effluent exposure on roach populations, with reproductively compromised males being less successful in fathering offspring in competitive breeding scenarios.

5.2 Materials and Methods

5.2.1 Exposure Effluent

WWTW ‘A’ supplied the treated effluent to each of the exposed mesocosm systems

containing roach. Previous calculations suggest that industrial influent makes up approximately 6% of the total influent and the population equivalent (number of people served) of the works is approximately 137,000. The population equivalent is often an indicator of influent 'strength' and has also been linked to the severity of intersex roach found in the river downstream from STWs (Jobling et al. 1998). Domestic influents commonly contain urine, faeces, soap, paper, and synthetic detergents, and industrial trade influents can contain high loads of more varied chemical components.

The influent to WWTW A undergoes preliminary treatment consisting of coarse screening and grit removal, to eliminate large particulates. This is then followed by primary sedimentation to settle any sludge, and subsequent secondary treatment. The secondary treatments are far more effective than primary treatment at removing activity from steroid oestrogens (Baynes et al. 2012), with removal rates as high as 97.8% for oestrone (Kanda and Churchley 2008). WWTW A comprises of trickling filters, activated sludge treatment and re-circulation for secondary treatment, but there is no tertiary treatment at this site. Extensive studies on the effluent from this WWTW have shown it to be oestrogenic (discussed below). This is likely due to the poor removal rates of EE2, a higher potency oestrogen, during secondary treatment (Kanda and Churchley 2008). This level of exposure is higher than occurs in rivers, with Lange *et al.* (2011) (using data from Jobling *et al.* (2006) for 44 lowland rivers) calculating the average proportion of river flow comprising effluent was 27%, with the most polluted of these having an average of 50%. However, during the summer months and times of exceptionally low flow, rivers in the UK can comprise 50–100% of WWTW effluent (Jobling et al. 1998, 2006).

This and other studies have extensively demonstrated the oestrogenicity of this effluent using *in vitro* assays and biomarkers of feminisation in fish exposed *in vivo* (Liney et al. 2005, Rodgers-Gray et al. 2000, Thorpe et al. 2009). Previous quantification of treated effluent concentrations of the synthetic oestrogen, ethinylestradiol (EE2) range between 1.5–7.9 ng/l. Most recently in 2005, average concentrations of E1 were 42.1 ng/L, 17α E2 was 0.17 ng/L and 17 β E2 2.49 ng/L and EE2 was 0.57 ng/L and horse estrogens 17 β-Eqn and Eqn were measured at 0.10 and 0.43 ng/L respectively (Tyler *et al.* 2009). These concentrations are higher than those commonly found in the environment, however observations in surface waters have recorded levels up to 12 ng/l (Filali-Meknassi et al. 2007) with predicted maximum concentrations of EE2 of 6 ng/l in US waters (Kostich et al. 2014). A spot sample was taken for measurement of steroid estrogen content during exposure of the fish in this study in June 2012 for chemical analysis (Severn Trent

Services). Estrogens were quantified using LC-MS/MS, using an Agilent system for LC and AB SCIEX 5000- for MS/MS and nonylphenols were quantified using GCMS.

5.2.2 Fish Origin and Maintenance

Fertilised eggs and subsequent hatched fry from the previous breeding study by Lange et al. (2011) were maintained under flow though conditions in dechlorinated tap water until 35 days post-hatch (dph). During these early life stages, fry were fed flake and *Artemia* sp. nauplii. These fish originated from two breeding crosses conducted by Lange et al. (2011), both of which had control fathers and either mothers that had been kept in control conditions, or mothers that had been exposed to 100% effluent for 3 years. During the experiment, adult roach were fed to excess with crumbled flake food and supplemented twice weekly with frozen brine shrimp (*Artemia* sp.) or bloodworm (*Chironomus* sp).

5.2.3 Experimental Design

The experimental set-up for the entire experiment is summarised in Figure 5.3. All roach used in this study were derived from a previous breeding experiment, similarly designed to assess reproductive fitness of roach, chronically exposed to the same WWTW effluent for 3.5 years (Lange et al. 2011). After 35 days post-hatch, fry from Lange et al. (2011) were transferred (~500) into four 1 m^3 tanks that were supplied with either de-chlorinated tap water (filtered through granulated active charcoal (0% effluent, control conditions) or full strength WWTW effluent (100%). After 2 years (April 2010), the fish in each control tank were subdivided into three tanks and fish in each effluent tank were subdivided into two in order to reduce densities so to encourage growth (10 tanks in total, see Figure 5.3). These tanks were maintained continually outdoors for a period of 3 years under an ambient temperature and light regime, similar to that of roach found in UK rivers. Flow rates were maintained at 5 l/min in both control- and effluent-exposed tanks, along with additional constant aeration to guarantee sufficient oxygen.

5.2.4 Breeding Experiment

In order to compare the multigenerational effects of maternal WWTW effluent exposure on the reproductive competiveness of male offspring, a breeding study was carried out in April 2011 (after approximately 3 years) using a similar set-up as that used by Lange et al. (2011); a schematic summary of the experiment is shown in the breeding tanks section of Figure 5.3.

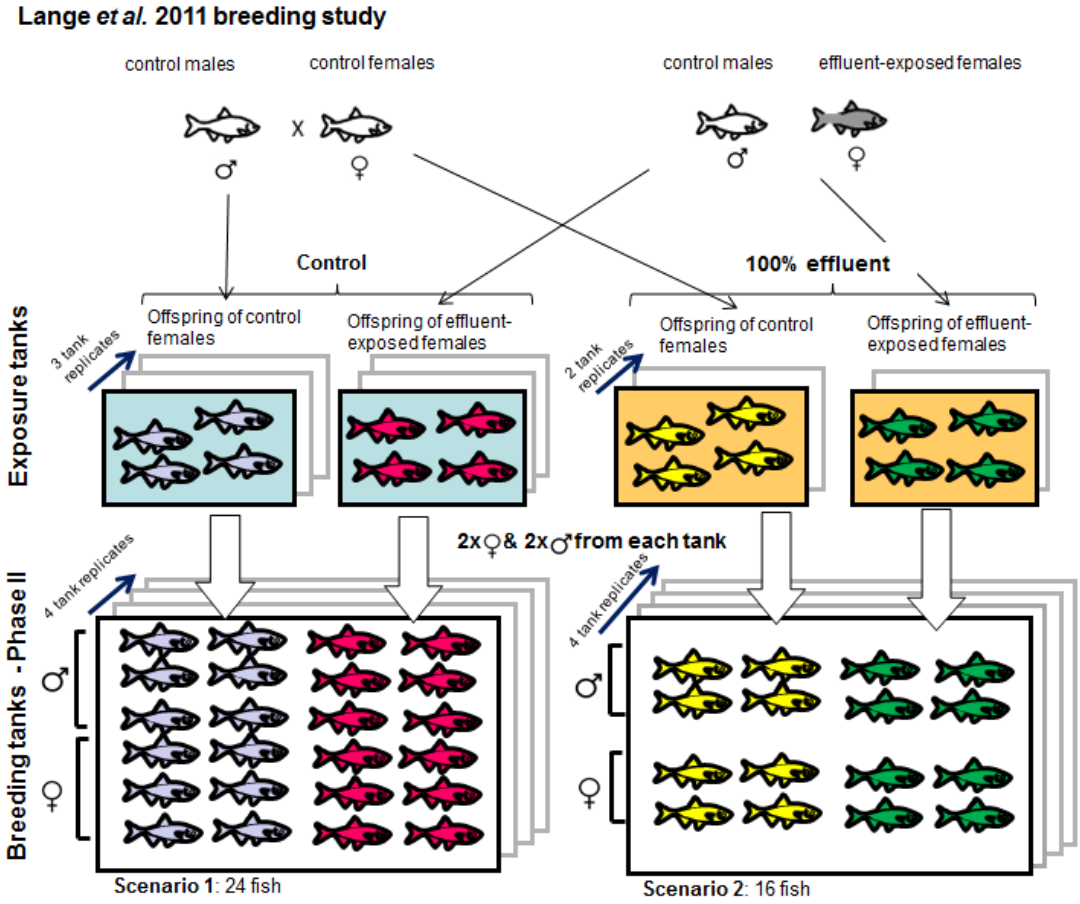


Figure 5.3 Experimental set-up for breeding study, using roach kept in exposure tanks in either control conditions (0% effluent), or exposed to 100% effluent for 3 years. Fish coloured purple and yellow are the offspring of control mothers and control fathers, whereas fish in red and green are the offspring of exposed mothers and control fathers (exposed fathers did not reproduce in the previous study (Phase I, Lange et al. 2011), due to complete feminisation, so no offspring of these fish were available). For the effluent-exposed group, some fish were not used in the breeding study. These were kept in water for 4 weeks during the breeding study, then were maintained in 100% effluent for a further year

Fish that had been kept in either 100% effluent or in control conditions for 3 years were separated equally into 8 x 1 m^3 breeding tanks, where they were then maintained in dechlorinated water for the period of the breeding experiment. This was to ensure that the number of fish with mothers exposed to 100% effluent for 3 years was equal to those with control mothers. Efforts were made to include equal numbers of males and females in each tank (50:50 sex ratio), although roach are not sexually diamorphic so this was determined based on secondary sex characteristics (roughness of skin caused by breeding tubercles in males, distended abdomen in females, the presence of an ovipositor in females and milt production in males). Preference was given to those larger fish when setting up the breeding tanks, as it is impossible to tell if roach are sexually mature at 3 years old from their external appearance. Fin clips were taken (and stored in 100%

ethanol) prior to the allocation of breeding tanks, so that the origin (exposure tank) of each fish could be traced after sampling by matching their microsatellite genotypes.

In summary, the following two scenarios were used for the breeding experiment (Figure 5.3):

***Scenario 1 (4 replicate tanks)*:** Each tank contained 24 fish, all kept in control conditions for 3 years. Six males and six females had effluent-exposed mothers and control fathers and the remaining six males and six females had mothers and fathers kept in control conditions.

***Scenario 2 (4 replicate tanks)*:** Each tank contained 16 fish, all kept in 100% effluent for 3 years. Of these, four males and four females had effluent-exposed mothers and four males and four females had both maternal and paternal parents kept in control conditions.

These tanks were the same as those used for exposure, with the exception of the inclusion of a layer of *Enkamat*® (a three dimensional synthetic mat consisting of randomly placed nylon filaments) to the bottom of each breeding tank as a spawning substrate, as used previously (Lange *et al.* 2011). Roach are broadcast spawners that commonly deposit their eggs on submerged vegetation or gravel beds, so this was deemed the most natural material upon which eggs could be collected and removed from tanks before predation by the adult roach. Fish were left to breed naturally and spawning occurred in all eight tanks over a period of 7 days. All fertilised eggs were removed from the tanks on the 15th and 20th April by cutting out the section of spawning substrate and transported to the aquarium facility at the University of Exeter, UK, where they remained in clean water until hatching. Fry hatched within 7–10 days and at 5 dph ~50 offspring were terminally sampled and placed in 100% ethanol for subsequent parentage analysis.

Parental fish were sampled ~2 weeks after spawning (5th May) at the WWTW to ensure that all spawning was complete. Fish were sacrificed by lethal anesthesia using benzocaine (ethyl-p-aminobenzoate), and according to UK Home Office procedures. Three adults from the unexposed fish died naturally during the experiment, but were no deaths in the effluent-exposed fish. Fork length and weight were recorded to the nearest millimeter and 0.01 g respectively, and a fin clip was taken from each fish and stored in 100% ethanol to identify reproductive success/parentage from DNA microsatellite analysis. Each fish body was then preserved in Bouins fixative (Sigma Aldrich), for subsequent histopathology and analysis of gonadal development.

During the breeding study, 36 fish from the effluent exposed tanks were kept in clean

water then returned to 100% effluent exposure for an additional year (until they were sampled in April 2012, 24.4.2012) for histological analysis. For these fish gonads were removed from the body cavity prior to preservation in Bouin's solution, so the presence of feminised ducts could not be determined. After fixation, all samples were processed for histological analyses and an assessment was made of alterations in germ cell development and/or to the structural organization of the gonad due to the exposure.

5.2.5 Histological Analyses of Gonadal Development

Gonadal histology was examined at 3 years (160 fish) for the control and effluent-exposed fish used in the breeding experiment, and again at 4 years (36 fish) for the remaining exposed fish. At 3 years, the whole body of each fish sampled was fixed in Bouins for histological analysis, so the presence of female-like ovarian cavities could be examined. For fish sampled after 4 years of exposure, the gonad was dissected out of the body, so the presence of an ovarian cavity could not be established as the points of attachment of the gonad to the body need to remain intact for this to be possible.

5.2.6 Fixation and Dissection Processing

At the 3-year sampling point, all roach from the breeding experiment were preserved via whole body perfusion in at least three times their volume of Bouins fixative solution. Bouins was preferentially chosen due to the good morphological preservation of gonadal tissue for light microscopy examination. All fish samples remained in this solution for 24 h to ensure adequate fixing and preservation of tissue morphology, then subsequently rinsed and stored in 70% Industrial Methylated Spirits (IMS; 70% IMS and 30% purite water) at room temperature until processing. To prepare the whole bodies for processing, a 3–5 mm transverse section was sliced from the central portion of the fish, using a microtome blade just in front of the dorsal fin. As the gonads of roach sit either side of the swim bladder and run along the majority of the body between the gills and the anal fin (see Figure 5.4), taking a representative section in the middle was consistent throughout sampling. Each tissue section was then placed in a single biopsy cassette and labelled with the individual fish reference number, which matched the fin clip taken previously.

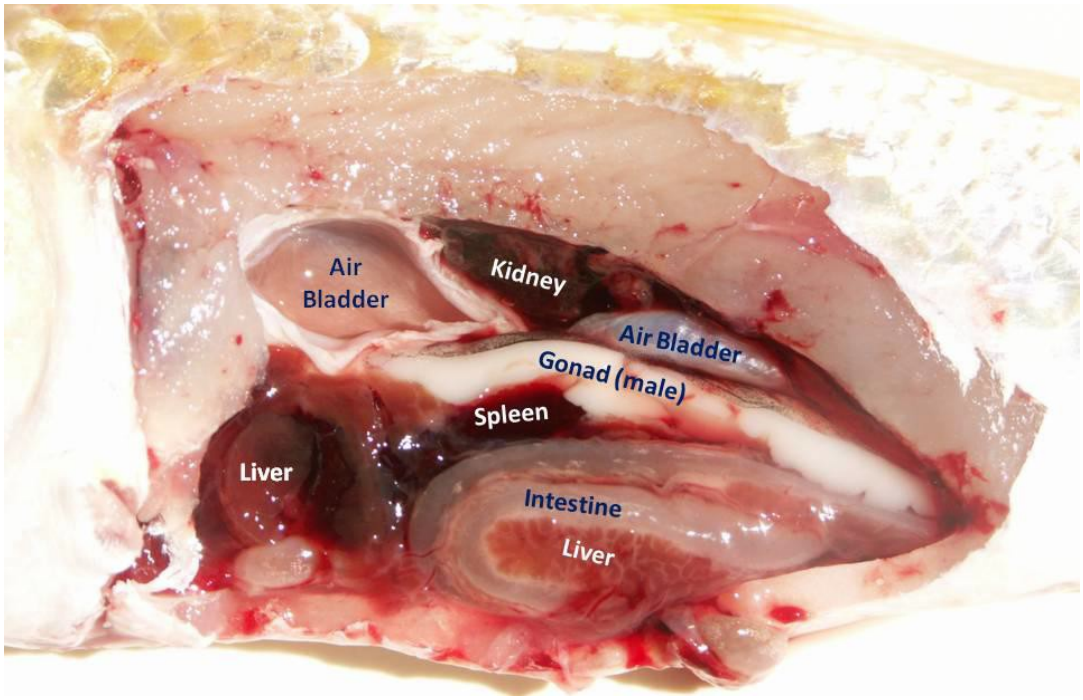


Figure 5.4 Typical lateral bauplan of the internal structure of a fish. Note the gonad sits just below the swimbladder and runs the length of the body. Source: http://www.koi-pond-guide.com/fish-anatomy.html

5.2.7 Tissue Processing

Following sample preparation and the collation of roach sections in a series of cassettes, these were batched processed in the tissue processor to remove any remaining water or fixative from the tissue samples. All cassettes were placed into a wire basket and automatically processed using a Shandon Citadel 1000 automatic carousel tissue processor (Thermo-Shanndon). During a single run, the tissue processor submerges the samples into a series of baths of increasing alcohol concentrations to gradually dehydrate the specimens and replace the water with alcohol. Once dehydrated, a clearing agent is introduced (Histoclear, RA Lamb) to remove all alcohol, which is then replaced by the infiltration of molten paraffin wax (Lamb wax W1) into the tissue. Table 5.1 provides an accurate schedule of the steps, timings and order of solutions used in the tissue processor. After completion of the tissue processor run, the samples remain submerged in molten wax until further embedding.

Table 5.1 Standard immersion time for each solution used during tissue processing of histology samples

Step	Solution	Immersion time (h)
1	70% IMS	3
2	90% IMS	2.5
3	95% IMS	1.5
4	100% IMS	1.5
5	100% IMS	1.5
6	100% IMS	1.5
7	100% IMS	1.5
8	Histoclear II	1.5
9	Histoclear II	1.5
10	Histoclear II	1.5
11	Hot paraffin wax	1.25
12	Hot paraffin wax (vacuum)	1.25

5.2.8 Embedding

Processed cassette samples, recovered from the automatic tissue processor, are then ready to be orientated into a paraffin block and subsequently sectioned. Firstly to embed tissues, biopsy cassettes were placed into a hopper of paraffin wax (at 65^{o}C) along with steel moulds. Cassettes were removed from the hopper and placed on a hot plate, along with a mould of an appropriate size. The base mould was then filled with hot molten wax from the hopper and replaced on the hot plate to ensure the wax remained liquid. Each tissue sample was then removed from the cassette using heated forceps and placed into the wax-filled mould, ensuring vertical orientation (Figure 5.5A). Dorsal–ventral orientation is easier for sectioning, as it minimises interpretation difficulties/artefacts and is important for the preservation of representative morphology. The labelled cassette (minus the lid) is then placed on top of the mould and further filled with molten wax. Each mould (including the tissue and cassette) are then transferred to a cold plate (-8^{o}C, RA Lamb) to solidify the wax, cassette and tissue in one block. Once cooled (after approximately 30 min), the wax blocks could be removed from the steel moulds (Figure 5.5B) and stored at room temperature indefinitely.

Figure 5.5 (A) Typical procedure of placing processed tissue into a mould filled with wax, obtaining the correct orientation for subsequent sectioning. Once the cassette case has been replaced on top of the sample, refilled with wax, and then cooled, it can be removed from the mould (B) and be ready for sectioning on a microtome

5.2.9 Sectioning

All tissue samples embedded in wax blocks were sectioned using a rotary microtome (Leica RM2235) and disposable microtome blades (Shanndon MB35 premier 35^{o}/ 80 mm), at a thickness of 5 µm. Wax blocks were kept on ice during sectioning to prevent wax warming during manipulation. Cut sections stick together to form a ribbon (Figure 5.6A) which can then be floated on a distilled water bath (at 42^{o}C) to flatten any creases. From here they could be floated on to a Histobond glass slide (RA Lamb, Figure 5.6B), which usually held 3–4 consecutive tissue sections. Each individual tissue block was cut at three depths, 50 µm apart, making six slides for each fish. This was to ensure that a representative sample of the gonad was taken, as the incidence of oocytes is unevenly distributed along the entire length of the gonad. All slides were labelled with the sample reference number, matching the number on the wax block (and original cassette) and the slide number, on the frosted end of the slide. Following this, all slides were left to dry on warming racks overnight prior to staining, to ensure sections remained attached.

For all fish sampled after 4 years, gonads were removed from the body and sampled separately. The same process was followed for processing, embedding and sectioning but tissue preparation involved taking the sections from the anterior, middle and posterior of the gonad length and fixing them in a single block.

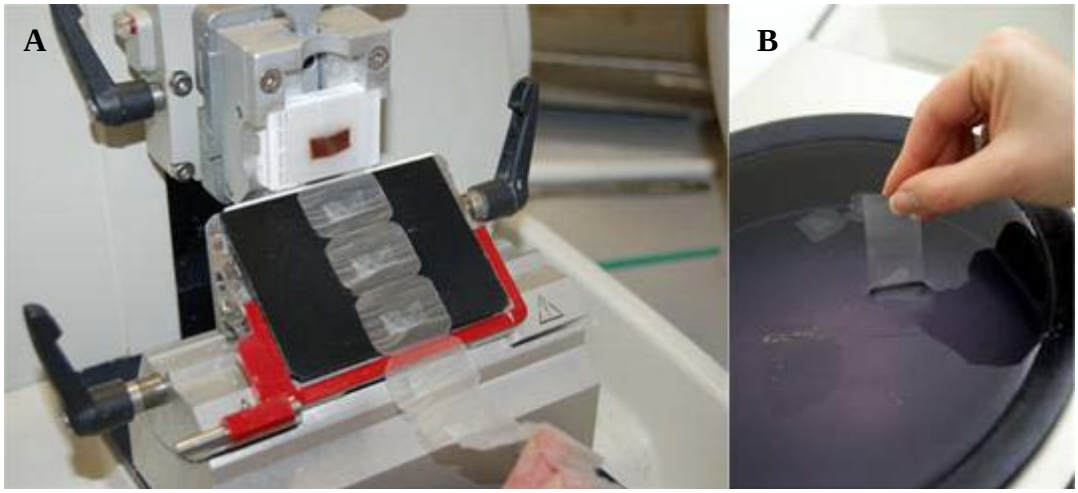


Figure 5.6 Procedure of sectioning was performed using a microtome, on a wax block containing the roach gonad. This produces a thin ribbon of wax containing thin slices of the gonadal tissue (A), which are then lifted off and floated in a water bath (B) prior to adherence on to a glass slide. Slides can then be stained to discern various tissues

5.2.10 Staining

The natural pigments of cells and structures are often colourless, so in order to examine them using light microscopy, staining is required. Slides were stained with universally common Haematoxylin and 1% Aqueous Eosin (RA Lamb) in batches of 25 slides per slide rack on an automated stainer (Stainmate). The slide rack is immersed in a step series of solution baths to firstly dissolve the excess wax, then hydrate, strain and dehydrate cells respectively (full details in Table 5.2). Using this routine method, cell nuclei are stained blue (haematoxylin) and extracellular components in shades of pink (eosin), which allows detailed examination and diagnosis of cell structure within the gonads. Following completion of staining, all slides were left to dry for a few minutes in a fume cupboard and then glass coverslips (22 x 50 mm) were affixed over each slide using Histomount glue. Any excess glue and bubbles were removed before drying slides in a fume cupboard overnight.

Table 5.2 Table of standard immersion times for each solution used on the Stainmate

Step	Chemical	In bath time (s)	In bath agitation	Out bath time (s)	Out bath agitation
0	Histoclear	900	N	8	Y
1	100% IMS	120	N	8	Y
2	90% IMS	120	N	8	Y
3	70% IMS	120	N	8	Y
4	Flowing tap water	120	N	8	Y
5	Haematoxylin	600	N	8	Y
6	Flowing tap water	600	N	8	Y
7	Acid alcohol[1]	20	N	8	Y
8	Flowing tap water	20	Y	8	Y
9	Saturated. Li_2CO_3	20	N	8	Y
10	Flowing tap water	20	Y	8	Y
11	Eosin	40	N	8	Y
12	Flowing tap water	300	N	8	Y
13	70% IMS	120	N	8	Y
14	90% IMS	120	N	8	Y
15	100% IMS	300	N	8	Y
16	Histoclear	300	N	0	N
17	Histoclear	0	N	0	N

[1]*Acid alcohol was produced by mixing 1% HCL with 70% IMS in purite water.*

5.2.11 Light Microscopy and Histopathological Analysis

Examination of gonadal disruption and sex determination of roach using slides was conducted under an Olympus (BX51) light microscope at multiple levels of magnification. The sex of an individual was determined by the presence of sex cells (spermatocytes or oocytes) and secondary sexual organs (sperm duct). Categorisation of phenotypes of sexual disruption seen in adult roach was based on the presence of gonadal structures associated with feminisation of male fish. Categories were as follows normal: ovarian cavity: presence of oocytes: oocytes and ovarian cavity for male fish. An index of feminisation (such as the intersex index) was not possible, as representative sections along the full length of the gonad were not taken. Female fish gonads were classified according to their stage of oogenesis based on the predominant oocytes present (see Box D): PN — perinucleolar stage; CA — cortical alveoli stage; VO — vitellogenic oocytes, respectively. Abnormalities in female gonads were recorded as seen, purely for reference and not used in any further analysis. A record of photomicrographs was taken using a digital camera (Q Imaging Micropublisher 5.0RTV) and Q Capture Pro 5.1 software to

capture and view images of gonadal structure of each fish, for future analysis.

5.2.12 Parentage Analyses

DNA microsatellites were again used in this chapter to undertake parentage analysis (as introduced in the general methods, Chapter 2). Because of their high variability and wide availability, microsatellites are preferential for studies of parentage assignment in wild populations (Hauser et al. 2011) and can be used successfully for the reconstruction of sibship families in the data (offspring sharing the same parents must be full sibs). Seven of these microsatellites were used successfully in a previous project to assign parentage in experimental breeding populations of roach, which contained a maximum of nine fish (Harris et al. 2011). Additional loci are used here to improve accuracy with increasing numbers of possible parents (a maximum of 24 in each tank), all of which are detailed in general methods Chapter 2, along with the precise genotyping methods. Matching alleles across multiple microsatellite loci was undertaken using offspring and their potential parents from the breeding study.

In short, DNA was extracted from the fin clips of parent fish and ~50 (except tank J where only 39 fry were available) fry offspring from each breeding tank using a HotSHOT-based protocol (Truett et al. 2000). PCR amplifications were performed as described in Chapter 2 and allele sizes were determined using a DNA Size Standard Kit (Beckman Coulter) with the CEQ Fragment Analysis software. Fourteen microsatellite loci were used to genotype each fish, all of which were used for parentage analysis. Additionally, the origin of each fish was determined by matching the microsatellite genotypes from fins taken at sampling to those from fins taken during the setting up of the breeding study using GenAlEx 6.5 (Peakall and Smouse 2006).

To determine the reproductive contribution of individuals from each exposure scenario I used COLONY (v2.0.5.0, Jones and Wang 2009) to infer parentage using multilocus genotypes and error rates of loci. For the COLONY analysis, microsatellite loci data for all parents and offspring were run with locus-specific error rates (estimated from individuals genotyped in duplicate, following Hoffman and Amos 2005), which make them perform better in assignment compared to programs such as Cervus3, that only consider average per genotype error rate (Hauser et al. 2011). Both sexes were assumed to be polygamous, and analyses were carried out without sex information for parents, to allow for the possibility that intersex fish may reproduce as both males and females. Assignment outputs from COLONY (using full likelihood long runs) were only considered if the probability was >95% for maternal and paternal pairs identified. If

parents were not identified initially, offspring samples were repeated again individually.

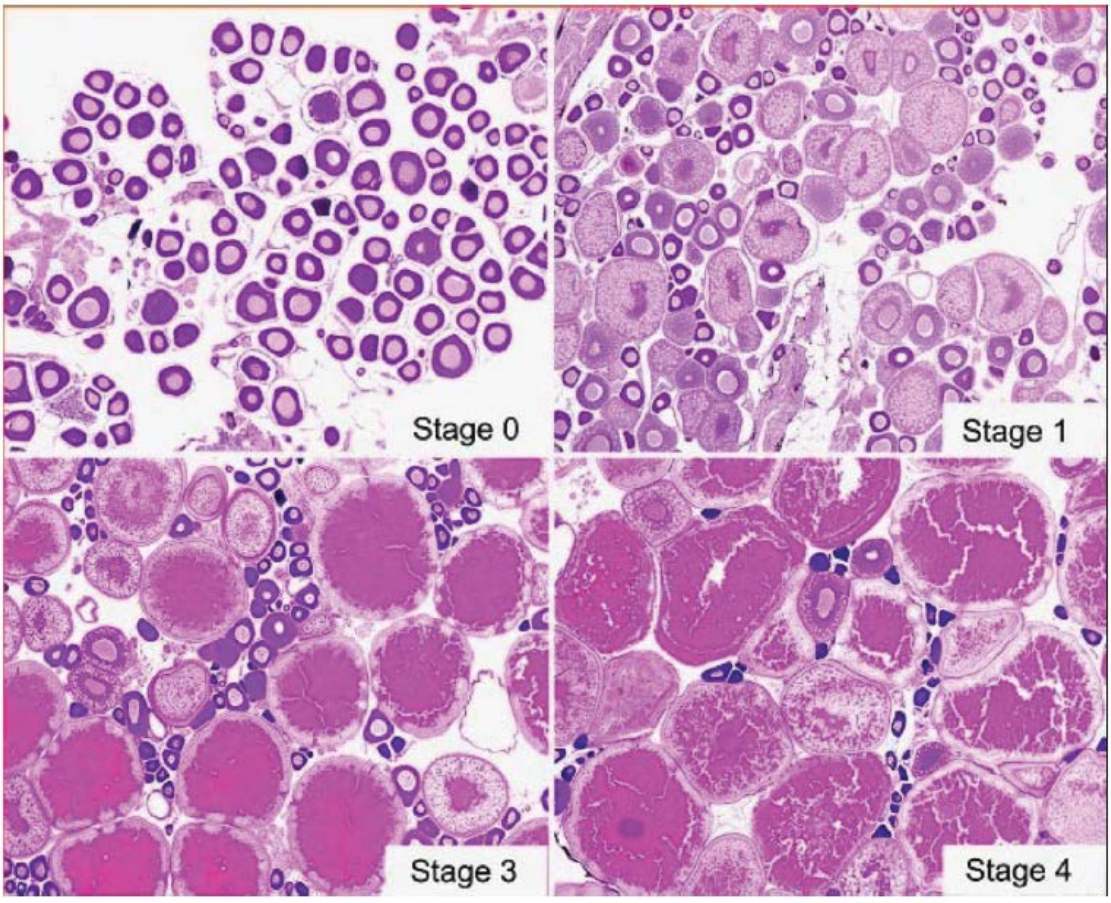
5.2.13 Statistical analysis

To deduce whether reproductive success significantly differed between fish with exposed mothers and control mothers, influences on reproductive performance were assessed by comparing between groups (separate sexes) using standard Students t-test in SPSS. Additional t-tests were conducted using factors suspected of influencing reproductive performance, including size, exposure tank and maternal exposure. For male fish in effluent, the presence of oocytes was also included as another factor in the analysis and compared to their individual reproductive success. Unequal variances were assumed between groups, as data did not adhere to normality when examined initially using Kolmogorov-Smirnov Test, also performed in SPSS. Deviation of sex ratios from 50:50 were examined across all tanks, exposure scenarios and in those fish kept in effluent for an additional year using Chi-squared Test conducted in SPSS. Statistical significance was accepted for values of $p < 0.05$.

Box D: Criteria for Staging Ovaries (sourced from US EPA, Diagnostic histopathology for screening EDCs in small fish)

The following are morphologic criteria for staging female fish:

- **Juvenile**: gonad consists of oogonia exclusively; it may be difficult or impossible to confirm the sex of these individuals.
- **Stage 0 – Undeveloped**: entirely immature phases (oogonia to perinucleolar oocytes); no cortical alveoli.
- **Stage 1 – Early development**: vast majority (e.g. >90%) are pre-vitellogenic follicles, predominantly perinucleolar through cortical alveolar.
- **Stage 2 (not shown) – Mid-development**: at least half of observed follicles are early and midvitellogenic.
- **Stage 3 – Late development**: majority of developing follicles are late vitellogenic.
- **Stage 4 – Late development/hydrated**: majority are late vitellogenic and mature / spawning follicles; follicles are larger as compared to Stage 3.
- **Stage 5 (not shown) – Post-ovulatory**: predominately spent follicles, remnants of theca externa and granulosa.



5.3 Results

5.3.1 Gonadal Development, Histological Examination and Sex Ratios in Roach

Gonadal development of both effluent-exposed and control roach were assessed after 3 years, following spawning (160 fish in total). All fish kept under control conditions for 3 years showed no evidence of sexual disruption or altered gonadal development (Figure 5.7A, B and E). Contrastingly, all male effluent-exposed roach showed feminised morphological responses, possessing female-like ovarian cavities (reproductive ducts) with oocytes seen in 85% of male roach. Male ovotestes occurred in all but four fish sampled from the 100% effluent-exposed regime, however each incidence of intersex was only mild and associated with low numbers of oocytes distributed randomly within each testicular section (Figure 5.7C). Analyses of effluent- exposed roach after 4 years (n=36) was conducted only on dissected gonads and therefore it was not possible to observe duct phenotypes or ovarian cavities. Nonetheless, the gonad of one fish was predominantly ovarian tissue (Figure 5.9K and L) and five others had moderate numbers of oocytes scattered throughout the testicular tissue (Figure 5.9I and J). Ovotestes were absent in five male roach after 4 years but the presence of feminised ducts cannot be ruled out, for reasons mentioned previously. Notably, roach exhibit no histopathological effects of maternal exposure to 100% effluent. Levels of disruption seen in fish with exposed and non-exposed mothers showed no significant difference in the incidence of feminised ducts or the presence of oocytes within the testicular tissue of male roach.

Sex ratios across all control and 100% effluent tanks did not differ significantly from 50:50 when examined after 3 years (Chi-Square Test p = 0.26, expected ratio 50:50; Figure 5.8). In the remaining 36 fish sampled, after 4 years of 100% effluent exposure, these demonstrated a significantly female-biased sex ratio (67%) using combined data for both exposure tanks (Chi-Square Test, p = 0.025, n=36, expected ratio 50:50, Figure 5.9). Importantly, as there is currently no genetic marker for sexing roach, it is impossible to ascertain if any of the histologically female fish were sex-reversed genetic males.

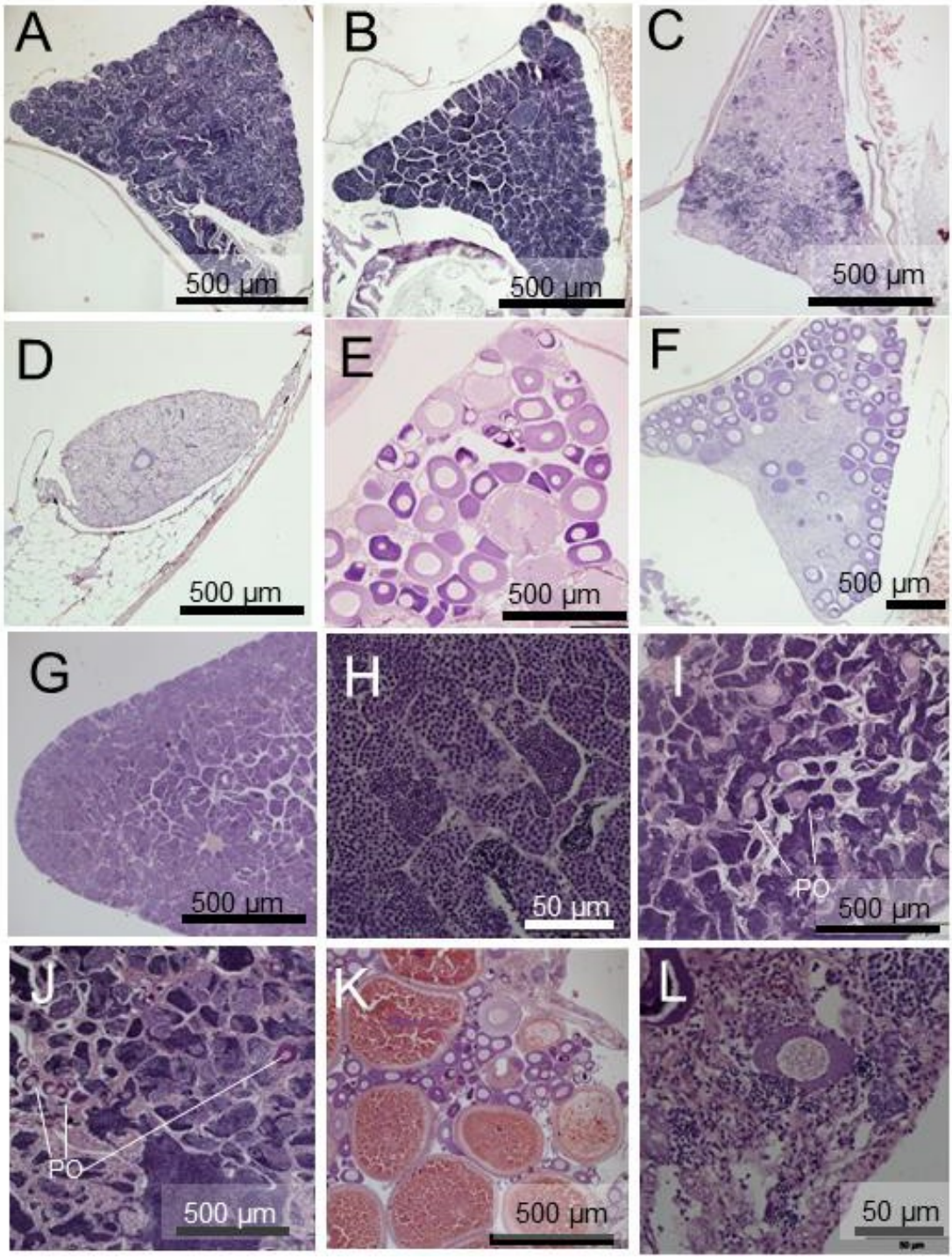


Figure 5.7 Gonadal histology from roach either exposed to 100% effluent or kept in control conditions for up to 4 years. Gonads from control (A, B) effluent-exposed (C,D) males and control (E) and exposed (F) females used in the breeding study and sampled at 3 years. Gonad of a normal (G,H) and moderately feminised (I, J) males sampled after 4 years of exposure to 100% effluent. (K, L) Gonad consisting of predominately ovarian tissue but had a small section with the appearance of testicular tissue after 4 years of exposure to 100% effluent. PO = primary oocytes.

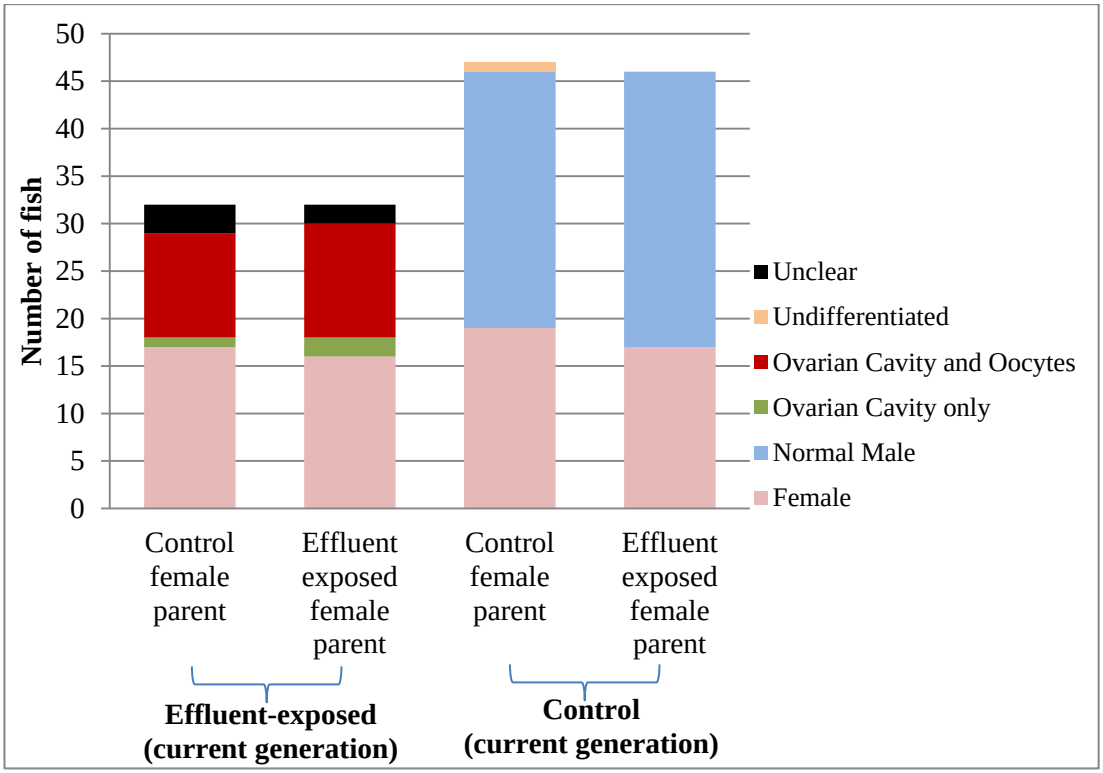


Figure 5.8 Observations on the effects of long-term exposure to WWTW effluent on gonadal sex of roach. Three from the control group died during the experiment, so no histology was done, two of these had an effluent-exposed maternal parent and the other had a control maternal parent.

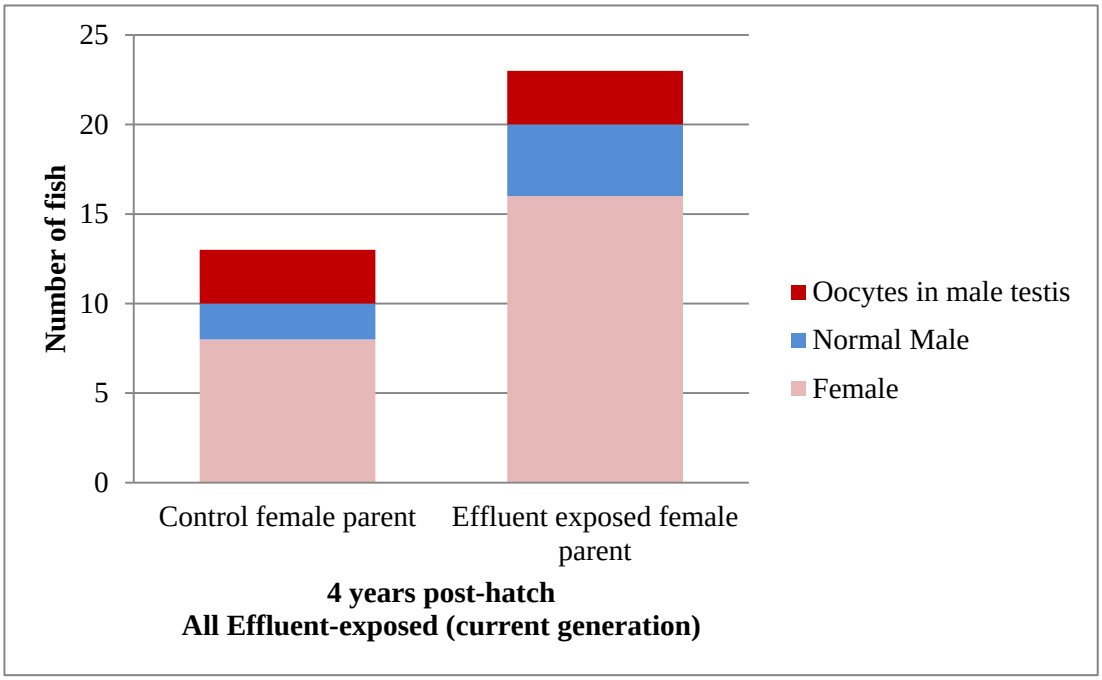


Figure 5.9 Analyses of fish at 4 years were done on dissected gonads, so it was not possible to score duct phenotypes

5.3.2 Impacts of WWTW Effluent Exposure on Breeding Capacity of Male Roach

Spawning occurred in all eight breeding tanks. In total, 69% of males (*n*=20) and 66% (*n*=23) of females were found to have reproduced from those kept in effluent, compared to 43% (*n*=24) of males and 32% (*n*=12) of females kept in control conditions. A factor in the increased success of the effluent fish may be due to their larger average size. Both mean length (cm) and weight (g) of fish that had been exposed to effluent for 3 years were significantly larger than those kept in control conditions (Table 5.3A *t*-test p = 0.024, 0.019). Interestingly, length and weight of fish were also significantly different when grouped based on their maternal exposure scenario (Table 5.3B). With fish from effluent- exposed mothers being significantly longer and heavier, than those from control mothers. Overall, all fish were smaller than those used in Lange et al. (2011) where body lengths ranged from 11.0 to 13.2 cm, which may also explain why a lower proportion of fish reproduced in this study.

Table 5.3 Mean length (cm) and weight (g) of fish from different current (A) and parental (B) generation exposure scenarios and the significance of any difference between these groups. Both sexes are included

A	**Current generation exposure scenario**	**Mean**	**Std. Dev**
Length	**Control**	8.5426	0.88482
	Effluent	8.8656	0.85379
Weight	**Control**	7.9730	2.74540
	Effluent	9.0581	2.94956
***t*-test for Equality of Means between groups**			
		t	**Sig. (2-tailed)**
Length	**Unequal variances assumed**	-2.285	0.024
Weight	**Unequal variances assumed**	-2.366	0.019

B	**Maternal generation exposure scenario**	**Mean**	**Std. Dev**
Length	**Control**	8.4862	0.88228
	Effluent	8.8654	0.84927
Weight	**Control**	7.6944	2.61460
	Effluent	9.1491	2.95036
***t*-test for Equality of Means between groups**			
		t	**Sig. (2-tailed)**
Length	**Unequal variances assumed**	-2.751	0.007
Weight	**Unequal variances assumed**	-3.282	0.001

5.3.3 Reproductive Success of Individuals Fish using Parentage Analysis

Parentage analysis revealed multi-male and multi-female fertilisation in each breeding tank. The individual reproductive success (proportion of offspring sired) for control fish ranged between 0 and 100% for females, and 0 and 42% for males. Similarly, individual success of effluent-exposed females ranged from 0 to 81% and males 0–55%.

Examining the influence of maternal generation exposure found that the average proportion of offspring sired by control fish with control mothers was significantly lower than that of fish with 100% effluent-exposed mothers (Figure 5.10). Roach derived from mothers kept in control conditions sired a similar proportion of offspring, regardless of their current life exposure history (Control*Control and Control*Effluent). Closer examination reveals this may have been largely due to the increased success of effluent-exposed females, more of which produced offspring compared to control females. In one tank, D, a single individual control female (from an effluent-exposed mother) produced all 49 offspring. When comparing reproductive success between sexes, based on a combination of maternal and current exposure history, female fish from control mothers (both Control*Control and Control*Effluent females) produced significantly less offspring than those females with effluent-exposed mothers who were also kept in effluent (Effluent*Effluent) (*t*-test assuming unequal variances C*C vs E*E $t = -2.777$ $p = 0.013$, C*E vs E*E $t = -2.603$ $p = 0.019$). Male reproductive success showed no significant differences between maternal or current generation exposure scenarios.

Consideration of reproductive success using just the two current generation exposure scenarios (100% effluent-exposed and control) yielded no significant difference in the number of offspring produced, when males and females were grouped ($t = -1.471$, $p = 0.143$). Likewise, no difference was found in the reproductive success of different sex fish that were kept in effluent-exposed or control conditions in the current generation. Reproductive success (proportion of total offspring sired) did not correlate with the presence of oocytes or ovarian cavities in the histological testis sections of male roach, indicating no significant detrimental impact of gonadal phenotypes. Similarly, ovarian development was not examined explicitly in this study, but staging of oocytes in female roach histopathology did not show any relationship with reproductive success of individual females in all exposure scenarios.

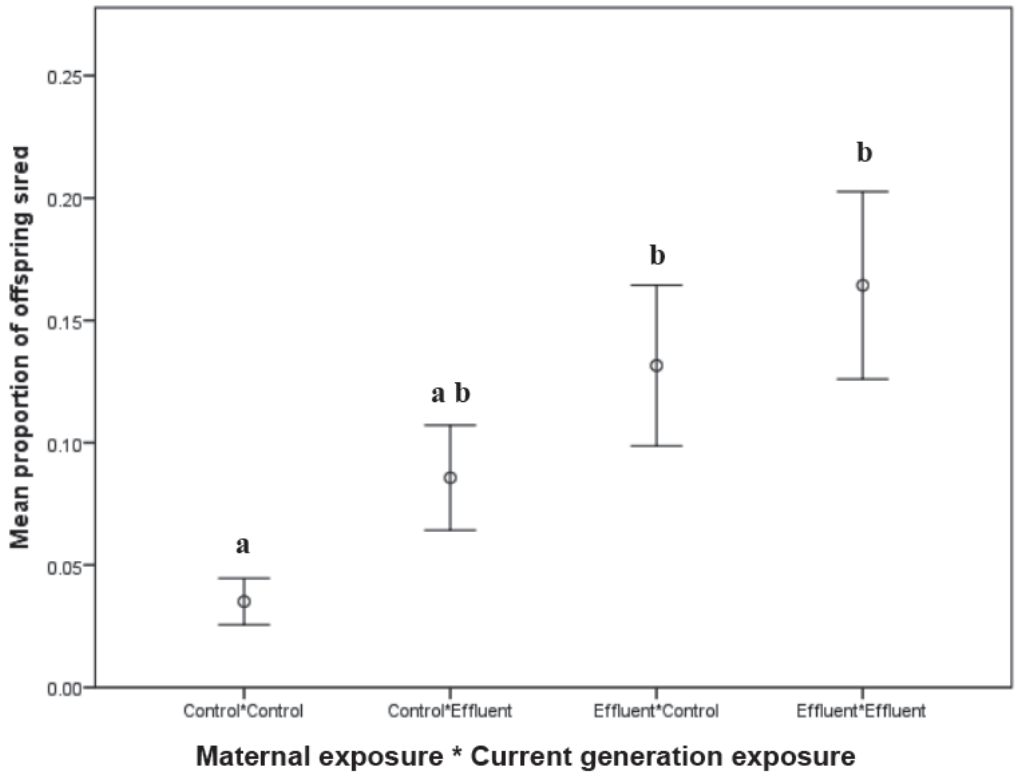


Figure 5.10 Reproductive success of male and female roach combined in breeding scenarios, based on their current and maternal exposure (±1SE). For fish kept in water (0% effluent) in the current generation, the results summarise the results of four breeding tanks, each containing approximately 12 males and 12 females. For those fish exposed to 100% effluent in the current generation, the results summarise data from four breeding tanks each containing approximately eight males and eight females. Each tank contained an equal number of fish where the mothers were exposed to an effluent to those in which the mothers were kept in control conditions). Levels not connected with the same letter are significantly different

5.4 Discussion

Exposure of roach to oestrogenic chemicals found in WWTW effluents has been reported to have significant reproductive consequences, including gonadal alterations, sex reversal, disruption to steroid hormone dynamics and reduced fecundity (reviewed in Tyler and Jobling 2008). Here I assessed the reproductive performance of roach populations chronically exposed to WWTW effluent through current and maternal generations; to my knowledge, the longest known exposure of any fish species to a WWTW effluent. Crucially, this breeding experiment demonstrates that offspring of WWTW effluent-exposed mothers, also exposed to 100% effluent from early life to sexual maturity, are able to father/mother offspring in a competitive breeding scenario. In addition, individuals with control mothers that were kept in control water in the current generation produced the smallest proportion of offspring, despite no evidence of feminisation in these control fish. This was likely a consequence of the smaller size of these fish. Overall, adult male roach exposed to WWTW effluent from 35 dpf showed

no impairment when siring offspring, despite demonstrations of gonadal feminisation in almost all male fish.

5.4.1 Gross Morphological Endpoints

Somatic indices of length and weight showed significant differences between control and effluent-exposed fish. All fish living in WWTW effluent for 3 years (regardless of their maternal origin) were larger and heavier, giving them a higher condition factor. Likewise, fish with effluent-exposed mothers were also significantly longer and heavier. Previous studies examining the influence of WWTW discharge on the body length and mass of wild three-spined sticklebacks (*G.aculeatus*) found both length and weight showed a significant downward trend with distance downstream from the River Ray WWTW outfall (Pottinger et al. 2011). Sticklebacks captured close to the point of discharge were significantly larger than those at the farthest downstream site. Additional evidence from experimental exposures to bleached sulphite mill effluent comes to the same conclusions. Increased growth of male and female fathead minnow, in long-term laboratory effluent exposures (Parrott and Wood 2002, Parrott et al. 2003) are also replicated in wild fish exposed to pulp mill effluents in many studies in North America and Europe (reviewed in Sandström 1996). These growth-enhancing effects are commonly attributed to increased water temperatures and nutrient loading from the effluent, which have broadly positive effects through the proliferation of algae and bacteria to feed invertebrates (the food source of most freshwater fish). In this experiment, increased length and weight of effluent-exposed roach was likely a result of nutrient enrichment from the effluent and subsequently increased food availability, promoting growth and development of these fish. The implications of increased size on fecundity and productivity of these effluent-exposed fish may have been a significant factor.

Growth is a physiological performance trait that has direct ecological implications. Wild roach reach sexual maturity after 2-3 years for males, and 3-4 years for females, where the rate of sexual development is often dependent on growth (Paull et al. 2008) and first spawning is linked to body size (Segner et al. 2013). Commonly observed changes in wild fish downstream of pulp/paper effluent outfalls include increased bodily growth and liver weight, but decreased gonadal size (Lowell et al. 2003). This suggests that less energy may be partitioned to gonadal growth as a result of exposure. Likewise, published studies have reported an inhibition of gonadal growth in intersex roach in contaminated UK rivers (Jobling et al. 1998) and nonylphenolic chemicals have been found to retard the growth of the testes and inhibit development in rainbow trout (Jobling et al. 1995). The

implications of restricted growth and development of gonads are profound for the fecundity and reproductive success of individual fish and may have ecological significance. Future recommendations to improve on this work would comprehensively include the measurement of Hepatosomatic and Gonadosomatic Indexes (HSI and GSI respectively). These endpoints can be difficult to interpret, yet they would help inform on the effects of WWTW effluent exposure on the metabolism and energy partitioning of individual fish, which could ultimately affect reproductive health.

5.4.2 Effluent Exposure Effects on Testicular Development

Altered sexual development (germ cells) in 100% effluent-exposed males, with both exposed and non-exposed mothers, was evident in this study through the presence of ovotestes and feminised ducts in male gonads. Crucially, this demonstrates no differences in response to exposure for roach where the mothers had been exposed to 100% effluent for 3 years, compared to roach with mothers kept in control conditions (0% effluent). Feminised phenotypes of male roach, such as ovarian cavities, ovotestes and VTG production, are consistent with several previous exposure studies, including ones using the same WWTW effluent (Liney et al. 2005). The earlier work (which provided fish for this study) by Lange et al. (2011) found that exposure to the same WWTW effluent, at 100% concentration for 3 years resulted in an all-female population where no roach contained any discernible testicular tissue. Additionally, exposure to 50% treated effluent for 3 years caused half of the exposed males to develop a mild intersex condition (few oocytes within predominantly testicular tissue). Results presented here conform with the mild intersex condition seen in the male fish exposed to 50% diluted effluent in Lange et al. (2011), where research regarding the quality of gametes produced in mildly intersex fish suggests they appear relatively normal (Jobling et al. 2002).

The marked difference between the rate of feminisation of fish exposed to 100% effluent in this study and previous work by Lange et al. (2011) indicates a possible change in the oestrogenic potency of the treated effluent. Lower concentrations of oestrogens in the effluent of this study may have prevented total feminisation of males, although limited chemistry data is available to substantiate this claim. Unfortunately, steroid oestrogens were not measured throughout either study. A chemical analysis on the effluent taken in June 2012, after the exposure period, measured the concentrations of steroidal oestrogens at 19 ng/l E1, 2.1 ng/l E2, and EE2 below the limit of detection (detection limit =0.1ng/l). This equates to an estrogenic potency of 8.4 E2 equivalents (E2eq) (Williams et al. 2009).This is substantially lower than measurements taken between September and

November of 2005 shortly before the (Lange et al. (2011) exposure study which gave equivalent to a potency 22.4 E2Eq (Tyler et al. 2009) with individual estrogens measured at 42.1 ng/L E1, 2.66 ng/L E2 and 0.57 ng/L EE2. A slight reduction in estrogen content may be all that is required to explain the differences in feminisation; roach exposed to 50% effluent in the previous study (Lange et al. (2011) for almost three years had ovotestes, but these were mildly intersex (a few oocytes per gonadal section) indicating that a halving of the oestrogen concentration would be sufficient to prevent complete feminisation for a three year exposure.

5.4.3 Sex Ratios in Exposed Roach Populations

In most fish species, sex determination is enveloped in a critical window of gonadal differentiation, directing sexual assignment toward male or female. Roach are typically gonochorists (single sexed). However, sexual differentiation and normal sexual development in this and other fish species is known to be disturbed by endocrine disruptive compounds commonly found in treated wastewater effluents. In addition, exposure of roach to effluent has been reported to have consequences including complete sex reversal (Lange et al. 2011). Therefore oestrogenic pollution can threaten wild roach populations through negative effects on family sex ratios (Wedekind 2014). Results presented here show that persistent exposure of roach populations to full strength effluent for 3 years did not produce a sex ratio that differed from control fish or 50:50, in all exposure scenarios, including those fish from exposed mothers in the parental generation. Similar findings suggest that transgenerational exposure of adult Japanese medaka to <4 ng/l of EE2 did not cause sex change in developing embryos or any significant deviation from 50% female population in offspring (Foran et al. 2002). The absence of sex change in the Foran et al. (2002) study is potentially explained by lack of exposure during critical periods of early gonadal development, the limited 2-week exposure and a period of depuration after exposure which recovered normal function. None of these instances were replicated in this study, so it remains likely that the lack of biased sex ratios in exposed roach (with and without exposed mothers), is a factor of reduced oestrogenic potency of the effluent as speculated by the lessened severity of the intersex condition in male roach (discussed previously).

Early exposure can provoke gonadal malformations, but extended exposure is necessary to see phenotypic changes. A further year of exposure of roach to a full strength effluent (4 years in total) resulted in a significantly female-biased sex ratio compared to the expected 50:50 ratio seen in controls. The apparent cumulative effect of feminisation and

prevalence of ovotestes with age (Jobling et al. 2006) aligns with this observation, and previous studies also document biased sex ratios as a result of prolonged oestrogenic effluent exposure in other fish species (Lange et al. 2001, 2009, 2011, Vajda et al. 2008, reviewed in Piferrer 2001). However, in both studies the authors are unable to confirm that biased sex ratios are a result of complete feminisation of male fish. The same conclusion is evident here, as there is/was no method available for genetic sex determination. This finding may also have been a result of a limited, non-random representation of fish. Only 36 roach were maintained for a further year in effluent and they were likely small immature females (as fish were selected on their size and maturity, with females taking longer to mature than males). Therefore, this limited sample size and a possible bias for females may explain the difference in sex ratio after 4 years of effluent exposure.

If sex reversal and biased sex ratios do occur in fish communities from effluent-polluted rivers, the repercussions still remain poorly explored. Indeed, reproduction in some fish species is relatively labile, so detrimental impacts of effluent on male fish could potentially be mitigated by female counter-adaptations (early maturity, increased brood size). Such alterations could limit the impacts at the population level, especially in fish species that produce many more offspring than will survive (Holman and Kokko 2013), characteristic of R-strategists. Future interest therefore lies in the plasticity of sexual behaviour of fish populations, particularly in contaminated environments where the ecological and evolutionary consequences of prolonged oestrogenic exposure could affect species persistence.

5.4.4 Breeding Success of Male and Female Roach

Wild native fish species within UK rivers have demonstrated limited evidence of low levels (1–4%) of intersex in unexposed locations. This gonadal phenotype is considered abnormal in gonochoristic species, but has been seen in populations of roach (Jobling et al. 1998) pike (*Esox lucius*), and gudgeon (*Gobio gobio*) (van Aerle et al. 2001). Structural changes in the gonad could have an effect on an individual's ability to reproduce, with particular concern over the permanence and severity of endocrine system disruption. In the present study, all but five (two of which were immature) males in effluent-exposed groups possessed feminised ducts and/or oocytes in the testis. However, these feminised gonadal phenotypes did not prove disadvantageous when competing with male roach reared in control conditions. Indeed, the majority of male and female fish in control and exposed scenarios were able to reproduce after 3 years, suggesting limited

detrimental impact of effluent exposure in both the current and maternal generation. The finding that fish derived from multigenerational exposure to full strength effluent reproduced successfully, is an important outcome regarding the impact of endocrine disruption on the ability of fish to compete and spawn successfully.

In this study, adult fish exposed from birth show no reproductive impairment. Of those individuals that reproduced successfully, the relative contribution to the number of offspring produced varied widely among individuals. Baseline data for quantifying variability in reproductive success is lacking in this species, however roach are known to spawn in large groups of mixed sex where multi-male/female fertilisations commonly occur (Wedekind 1996). Male–male competition and the role of mate choice (Rankin and Kokko 2007) by female fish renders those dominant, ornate males likely to sire more offspring (Jacob et al. 2009) influencing population processes. In this context, effluent-exposed/feminised males would be predicted subordinate as they frequently lack secondary sexual characteristics of breeding tubercles and have reduced sperm quality and quantity (Jobling et al. 2002). Fertilisation and mating success of effluent-impacted males would therefore be assumed compromised, however this appears unapparent here. Increases in body size within exposed fish may have been a contributing factor to their unexpected success, as it is likely that a higher proportion of the roach kept in effluent were sexually mature and assertive mating can be size dependent (Wedekind 1996). Other studies also found larger male fish to be more productive, out-competing smaller individuals (Nile tilapia — Fessehaye et al. 2006, minnow — Jacob et al. 2009), including studies on roach (Harris et al. 2011). The lek-like spawning strategy of roach (Wedekind 1996) can favour larger, dominant males. Indeed, other species which display a lek-like spawning strategy, such as the European minnow (*Phoxinus phoxinus*), suggest that larger males are better able to defend spawning territory and have greater reproductive success (Jacob et al. 2009). Reproducing females were also larger, which is consistent with the previous exposure to this effluent and with the finding that the majority of wild females from WWTW contaminated rivers bred successfully (Harris et al. 2011).

5.4.5 Implications for Roach Populations

Reproductive capacity and performance of male fish from control- and effluent-exposed mothers was not correlated with intersex severity, nor the presence of ovarian cavities. Recent demonstrations of complete feminisation of male fish as a result of WWTW effluent exposure, and population failure during multigenerational chemical challenge of

fathead minnows to a synthetic oestrogen (Kidd et al. 2007, Schwindt et al. 2014) are not replicated here. Additionally, roach demonstrate no heritable differences in susceptibility to effluent or degree of feminisation, in those fish that had effluent-exposed mothers compared to those with control mothers. This finding is important given the significance of the male contribution to drive population genetic variability and effective size of populations. Those populations constituting a larger number of breeding individuals can present increased genetic variability, rendering them better equipped to persist in light of predicted environmental change (Frankham 2005).

The results presented here demonstrate that intersex fish are able to reproduce in a realistic competitive breeding scenario, but sex ratios may be affected by long-term exposure to contaminants in WWTW effluent. Evaluation of ecological risk posed by chemical stressors remains well understood at the level of the individual; however it is difficult to isolate subtle factors that could influence population dynamics of wild fish. Recent modelling approaches have provided important information, rarely attainable from wild fish populations, suggesting that concentrations of EDCs found in the environment could lead to population declines, mainly resulting from reduced female fecundity (Grist et al. 2003, Gutjahr-Gobell et al. 2006, Miller et al. 2004). Additionally, population consequences of environmental sex reversal and female-biased sex ratios have also been simulated to become increasingly negative with progression of environmental feminisation from moderate to severe (Cotton and Wedekind 2009). Likewise, a paucity of males in the modelled scenario subsequently limits population growth, leading to potentially rapid population decline. Little is known, however, about the viability of offspring derived from sex-reversed roach, nor do we understand the spawning behaviour and the number of males and females required to sustain a population. There is a need to establish a robust means of extrapolating from standard test outcomes to wild fish populations.

Environmental feminisation and biased sex ratios still pose moderate concern, yet predicted overt changes in fish populations may not represent situations experienced in the wild. Whether such effects as a result of EDC exposure could occur in the wild is still unclear (An et al. 2009, Mills and Chichester 2005) where populations may also be locally adapted and resistant to chemical challenge. Evidently, transgenerational effects of effluent exposure through the maternal germ line do not impinge on ability of offspring to reproduce however further studies are needed to ascertain possible epigenetic effects or inherited tolerance to EDCs. For example, if susceptibility of fish to effluent exposure has

a genetic component, then the increased contribution of tolerant individuals could provide a mechanism for evolved tolerance to EDC exposure. Likewise, the identification of subtle factors of genetic variability and heritability of traits (Skinner et al. 2008) would prove informative and influential for understanding the population dynamics and viability of wild roach. To date, such information is lacking for wild roach populations, therefore it is difficult to extrapolate this data directly to the wild. Speculative assessment would suggest that if WWTW improvements do not match increasing pharmaceutical use and possible decreases in river flow, then fish populations may come under increasing pressure.

Establishing whether fish (here roach) have adapted to oestrogenic contaminants and how they do this (the mechanisms) are essential in understanding resilience (and thus sustainability) of fish populations living in polluted environments. These are questions that remain beyond the scope of this thesis, but the demonstration of resistance developed through rapid evolution of Atlantic tomcod (*Microgadus tomcod*) from highly contaminated sites (Wirgin et al. 2011) emphasises the possibility of tolerance to WWTW effluent over few generations. Although adaptation enhances population persistence, it also demonstrates that chemicals have impacted on survival or reproduction. The examination of these endpoints during a multigenerational exposure of roach should help determination of risk posed by WWTW effluents and indicate possible adaptation across longer durations of exposure (Tierney et al. 2013). However, it cannot be assumed that all species can and will adapt following multigenerational exposure to any harmful chemical cocktail; no evidence was found for pollutant-resistance or genetic differentiation in darter gobies (*Gobionellus boleosoma*) inhabiting a hydrocarbon-contaminated water discharge site (Klerks et al. 1997). Thus, adaptive mechanisms across generations are not yet understood and are not explored genetically here. A critical question is whether adaptive changes have other fitness consequences, rendering adapted or non-adapted fish more or less successful in siring offspring.

5.5 Conclusion

Empirical studies documenting the effects of WWTW effluent exposure on fish populations over multiple generations are limited. Sexual development, reproduction and behaviour all appear altered in the presence of oestrogenic contaminants; adverse effects that are well understood at the level of the individual but ramifications remain poorly addressed in exposed aquatic populations. Likewise, the population collapse of a closely-related species, the fathead minnow, following multiple generation exposure to EE2

(Kidd et al. 2007) provides emphasis on the paucity of knowledge regarding population-level studies.

The present study examines the reproductive capabilities of a common freshwater cyprinid during multigenerational exposure to a known oestrogenic WWTW effluent. Roach spawn annually, displaying lek-like group spawning behaviour, providing the opportunity to examine multigenerational effects on reproduction in fish from differing maternal exposure histories. Results herein confirm that prolonged exposure of roach to WWTW effluent can have negative sexual development implications for individuals (including feminisation of male gonads) yet the majority of both male and female fish were still able to reproduce successfully after 3 years. However, endpoints examined in this study were confounded by size differences of fish; number of replicates and importantly, the genetic fitness consequences of exposure were not examined. Similarly, additional information on the survivorship of offspring and their fitness would provide a crucial next step for this experimental design to evaluate realised success of effluent exposed male and female fish over multiple generations.

Overall, the findings presented are in line with other assessments of reproductive performance in effluent-exposed roach, where only in extreme cases denoting severe feminisation do we see impaired reproductive success (Harris et al. 2011). Crucially, despite WWTW effluent exposure impacting gonadal morphology in exposed fish, there is no indication that exposure of the parental generation directly impacts reproductive traits of the next generation. Significant population changes have only been predicted as a result of major alterations in sex ratios; an effect that will endanger the survival of the population irrespective of the fish species or effluent exposure duration. In view of this, and other evidence, one could conclude that only overt changes in gonadal histopathology, sex ratios or the inhibition of gamete production as a result of heightened chemical exposure would potentially provide detrimental long-term effects to fish populations, affecting population persistence. This information has implications for how hazard and risk posed by EDCs are determined in reference to wild fish populations.

Chapter 6

Environmental Influences of Stream Habitat and Proximal Land Use on Two Levels of Freshwater Fish Diversity

Pages 174 - 210

6.1 Introduction

6.1.1 Biodiversity and the Environment

For millennia, humans have relied on the natural environment for provision of biological resources. The impact of which is now becoming staggeringly apparent. Our continuous indiscriminate exploitation of species has accelerated environmental modification to such an extent that ecological niche architecture has been changed irreversibly, and once extant wildlife populations have been lost (Secretariat of the Convention on Biological Diversity 2005). The scale of the problem is becoming evident as current published statistics by the IUCN and the Millennium Ecosystem Assessment (MEA) catalogue 8782 species on the Red List as critically endangered, endangered or vulnerable (IUCN 2009). Additional research suggests that biodiversity is declining a thousand times faster now than at any other time in the fossil record (MEA 2005).

Concern for the accelerated reduction in global biodiversity — 'the variety of life and its processes' (Hughes and Noss 1992) — is universal. The diversity-stability hypothesis outlined this many years previously, emphasising the importance of species diversity in providing an insurance policy against ecosystem collapse in response to environmental perturbations (Chapin et al. 2000). Accurate monitoring of diversity levels are therefore of paramount importance in enhancing our understanding of which species are most at risk from anthropogenic pressures. Historically, research has utilised extinction rates as a means with which to quantify human-driven modifications of biodiversity (Garner et al. 2005). However, a multifaceted approach recognising the importance of assessing genetic diversity — which depicts the allelic and genotypic variation within a population (Frankham et al. 2002)— and species diversity, has now become widely publicised and accepted (McNeely et al. 1990). Neglecting any level of diversity when monitoring ecosystem function, could prove inherently detrimental to the preservation of biodiversity. Thus, conservation of all three levels of biodiversity, that is, ecosystems, species and genes is an impending challenge of ecological, economical and societal concern.

6.1.2 Ecological and Evolutionary Drivers of Species and Genetic Diversity

Understanding the interplay between ecological and evolutionary processes is integral in deciphering community dynamics; unifying biodiversity research in quantifying the impact of environmental alteration. Features of the biotic environment have been documented to drive ecological and morphological traits in exposed species, therefore it

would be likely to assume this legacy would perpetuate at the species and genetic level. Parallel drivers appear equally influential in shaping population genetics and community composition both within and between species, as a change in one is deemed likely to directly influence the other (Vellend 2005, Vellend and Geber 2005). Analogous responses of species richness and genetic variation to local processes are widely accepted, with co-variation being demonstrated in a variety of environments and species (butterflies — Cleary et al. 2006, bats — Struebig et al. 2011, and fish — Blum et al. 2012).

Similar species and genetic responses to characteristics such as habitat area, isolation (neutral processes) and spatial/ temporal heterogeneity are expected, given their intrinsic link through mechanisms of migration, drift and selection (Vellend and Geber 2005; see Figure 6.1). For instance, the level of drift will determine random changes in both species composition (community drift) and allele diversity (genetic drift). In environments where substantial levels of drift are occurring, we are likely to see erosion of genetic variation and increased likelihood of species extinctions (Taberlet et al. 2012). Conversely, gene flow and migration between poorly isolated localities will encourage movement of alleles, individuals and species (Blum et al. 2012) promoting both species and gene diversity. Equally influential are non-random forces of selection, competition and predation, whereby successful individuals or genotypes proliferate and alter both allelic and species composition of a population/community. Finally, mutation and speciation may be effective over long periods of time, generating new evolutionary material/trajectories. However, mutation and speciation are unlikely to explain variation in diversity at the regional level, and speciation would be uncommon in connected populations (Blum et al. 2012). Despite this coherent theoretical understanding of how corresponding ecological and evolutionary forces can shape both species and genetic diversity, studies document inconsistent outcomes especially when correlating them with environmental conditions at local, regional and global scales (Vellend and Geber 2005). Consequentially, few studies have examined the influence of environmental impairment on genetic and species diversity in parallel (Vellend 2004, Blum et al. 2012).

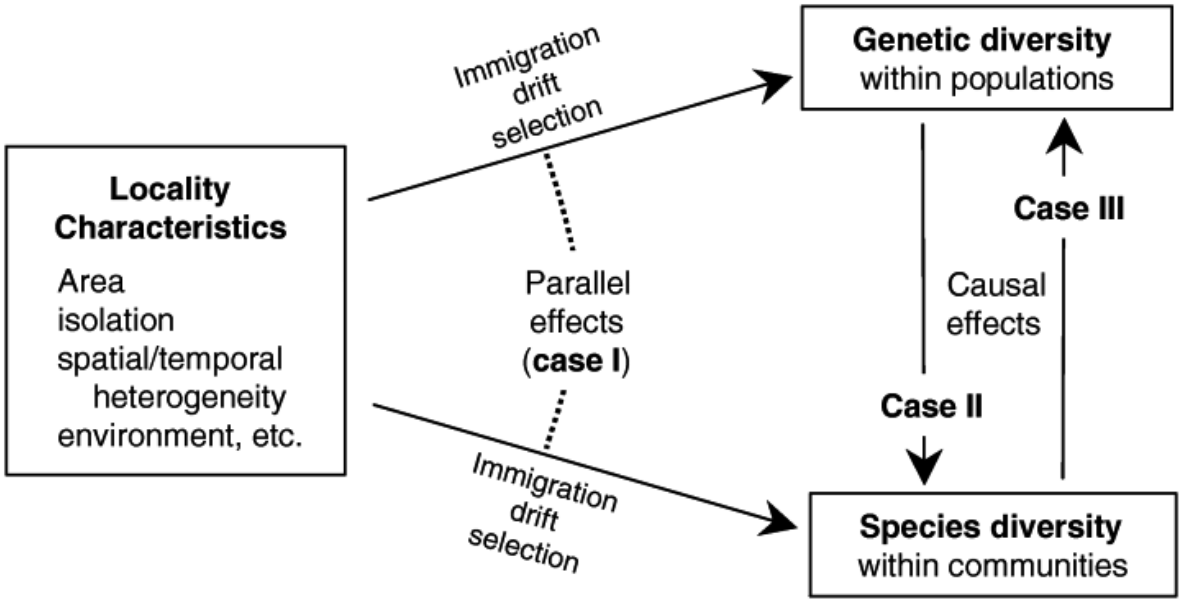


Figure 6.1 Schematic showing the relationship between species and genetic diversity and the way in which parallels or disparity may arise. Firstly, in case I, if locality characteristics influence the both levels of diversity in a similar manner, then a positive correlation between them may result. Secondly, case II, genetic diversity may influence species diversity, as genetic variation determines the viability of a population. Thirdly, if the species diversity of a community is changed due to the influence of a selection regime, then it is likely the genetic composition of the population is also altered by the relative abundances of co-existing species (case III). Sourced from Vellend and Geber (2005)

6.1.3 Genetic Diversity and Environmental Change

Genetic diversity becomes increasingly important in the light of rapid environmental change. Naturally, populations respond in equilibrium to the landscape mosaic, in which environmental fluctuations can impinge on the movement of both genes and individuals (Leclerc et al. 2008). Such that, changes in the dynamics of gene flow can have repercussions that exert a profound influence on ecosystem function. Genetic diversity has consequences at all levels of biodiversity; it influences organism fitness, population viability, the ability of individuals to respond to environmental change, the evolutionary trajectory of species, and therefore the entire structure of communities (Sax and Gaines 2003). Consequently, any environmental disturbance that impacts on genetic diversity is likely to trigger ecological and evolutionary ramifications at higher levels of organisation. Widely regarded as one of the most significant drivers, habitat fragmentation is thought to be a major cause of biodiversity decline worldwide (Keyghobadi 2007), eroding the genetic structure of species and becoming increasingly frequent with human expansion.

Landscape heterogeneity can invoke divergent selection in the wild, for example, individual dispersal in wild populations is frequently hampered by physical barriers or unsuitable habitat patches (ocean currents and habitat discontinuities) between locales enhancing divergence and differentiation between individuals (Ruzzante et al. 2006,

Leclerc et al. 2008, Bergek and Olsson 2009). Connectivity and dispersal are important between locally adapted populations, providing fluctuations in genetic material through the movement of individuals, altering genetic structure. For example, Perrier et al. (2011) discern the influence of natural environmental features and anthropogenic factors on the genetic structure of Atlantic salmon, indicating that river length is a key environmental factor influencing gene flow and potential local adaptation among populations. Accelerating patterns of gene flow among locally adapted populations during mating has been seen to result in higher offspring heterozygosity in fish (Garant et al. 2005, Dibattista et al. 2008). These observations highlight the importance of mitigating fragmentation and more importantly, the benefits of maintaining high genetic diversity given its association with individual fitness (Frankham et al. 2002).

Consideration of the interaction between environmental drivers and genomic footprint is lacking; pertaining to a crucial void in our understanding of ecological forcing on population adaptation, sustainability and evolutionary dynamics of wild organisms (Porlier et al. 2009). Large riverine catchments offer an exploratory arena within which some of these factors can be elucidated, as to their role in shaping genetic structure. In theory, those populations living in relative proximity should exhibit similar genotypes, although this is dependent on their connectivity and migratory habits. River networks offer a circuit-scape within which exchange of individuals occurs through the four normal processes (migration, gene flow, natural selection, genetic drift) however, movement of individuals is often restricted even in continuous environments. For instance, Leclerc et al. (2008) examined genetic variation of perch (*P. flavescens*) among 16 localities and distributed over 310 km in the freshwater section of the St Lawrence River. They found four genetically distinct biological units, which is in contrast to the current management basis for perch that is currently based on six fluvial sectors. Isolated populations can differ in traits and behaviour in response to different habitats, thus requiring different management strategies at appropriate scales.

In the short term, populations demonstrate plasticity in response to global change (Parmesan et al. 2000), promoting gene flow between populations and enhancing genetic differentiation within various taxa (Keyghobadi et al. 1999, 2005, Dionne et al. 2008). How landscape-level environmental features interact with spatial distribution of individuals, constraining or facilitating dispersal, is pivotal in our understanding of evolutionary processes and natural selection.

6.1.4 Factors Affecting Fish Assemblages

Rapid population growth, industrialisation and economic development over the last 50 years have inadvertently altered freshwater ecosystems more than ever before (Thomas et al. 2004). Consequently, unparalleled changes in aquatic habitats have promoted fish species vulnerability to 20% of the 10000 freshwater species in existence (CBD 2005). Substantial influences on in-stream physical, chemical and biological habitat have resulted from altered land use within catchments and along stream margins (Allan 2004). Indeed, Harding et al. (1998) demonstrate the relative influence of past and present day land use, on current diversity in stream communities. Finding that past land use activity, particularly agriculture, may result in long-term modifications to and reductions in aquatic diversity suggesting limited reversibility of human disturbance. Such unprecedented expansion and human development, alongside increasing agricultural intensity and the surrogate use of pesticides are not sustainable, and present themselves as major triggers of habitat degradation globally (Venter et al. 2006). Focussing purely on physical habitat (substrate, extent of pools versus riffles, vegetation, undercut banks, flow amount and variability) provides a snapshot of the features which are often degraded by changes in human activities, any of which can influence fish assemblage composition present in a stream (Diana et al. 2006).

In this context, the south-eastern UK is no exception. Substantial urbanisation creates intensive human-mediated pressure on floodplains and subsequent degradation on freshwater ecosystems. Much of the south-eastern UK has been transformed from landscapes previously dominated by woodland and open grassland/floodplains, into large urban conurbations or high-intensity agriculture. Such conversions of land use practices have been linked to deterioration of biodiversity (Acevedo-Whitehouse et al. 2009, Bickham et al. 2000, Frankham et al. 2002) and the declining prevalence of aquatic populations. Agriculture and urbanisation have both been reported to exert strong influences on fish assemblages (Allan 2004) and exhibit commonalties in stream impacts. Both urban and agricultural land use increase contaminated runoff delivered to the river/stream through the introduction of drainage systems and the clearing of riparian and bankside vegetation (to make way for impervious surfaces in the case of urbanisation); often this also encourages sediment transfer to the river. Increasing flow variability and sedimentation create unfavourable habitats for fish species, especially those who require sediment-free substrate for egg laying and nest building (Waters 1995).

Over-enrichment from both agriculture and effluent discharges has been a persistent and

direct repercussion of increasing population growth over the last 20 years, transforming pristine stream habitats into eutrophic waters dominated by extensive in-stream vegetation (Allan 2004). Furthermore, point source discharges of chemical pollutants from STW effluents can also impact reproductive parameters, sex ratios and immunocompetence of aquatic fauna (reviewed in Sumpter 2005). As a result, improvement of water quality for the protection of aquatic fauna has been addressed through stringent consents and the introduction of the WFD; however the rate of unprecedented modification of the catchment landscape remains unrelenting.

Although literature is extensive on the ecological impact of nutrient loading (and resulting eutrophication, Smith 2003) on species diversity (Seehausen et al. 1997, Vonlanthen et al. 2012) less is known about the diversity consequences of changing land use patterns within proximity of the aquatic environment. One certainty is that river catchments, such as the Thames, are strongly influenced by their surroundings (Allan 2004) and impacts of human-induced eutrophication constantly alter living conditions for aquatic organisms. A review of the relationship between watershed land use and biological integrity of 103 Wisconsin streams, demonstrates an obvious decline in index of biotic integrity (IBI scores) with high agricultural and urban land use (Wang et al. 1997). Clearly, watershed land use imparts significant effects on habitat quality through subtle effects on water chemistry (fluctuations in dissolved oxygen concentration and pH) along with more obvious changes in habitat structure, increased water turbidity and poor visibility for the inhabiting fauna. Nonetheless, predicting the effect of anthropogenic stress gradient on biological condition or ecological integrity of fish communities remains challenging. Overall, these observations strongly suggest that a transition from undisturbed to human-dominated landscapes are likely to impact the health of exposed fish populations, particularly when acting in synergy with other environmental stressors (Acevedo-Whitehouse et al. 2009).

6.1.5 Study Aims and Hypothesis

The overall aim of this study was to contextualise the impact of proximal land use patterns and influences of water quality, on the genetic diversity of wild roach populations and species diversity of fish assemblages, throughout the Thames catchment. The relative paucity of studies exploring parallel relationships of variation between species diversity and genetic diversity in relation to environmental condition is testament to the complexity of the task. Rarely do driving mechanisms act in isolation; however characterisation of the simultaneous impacts of environmental variation within and

surrounding stream networks may provide insights into their manifestation at several levels of diversity for inhabiting fish species.

Here I use both land use indices, alongside additional environmental and water quality parameters of N and P, to characterise human influence on the internal relatedness and genetic diversity of a ubiquitous cyprinid species, the roach (*R. rutilus*) in UK rivers. Focus is cast upon the genetic structure of roach in the UK, using 14 microsatellite markers and a variable scale of ecological/ landscape parameters sourced from habitat surveys and GIS data. Subsequently, species diversity of the entire stream fish assemblage is examined, along the same gradient of water chemistry and land use modifications. The influence of bankside land use and changes in water quality on aquatic fauna are undeniable, therefore I assess 19 fish populations to ascertain whether levels of diversity are negatively correlated with common water quality parameters of nitrate and phosphate, or environments dominated by intensively managed landscapes.

Key outcomes allows us to assess (i) to what extent the genetic and species structure of fish communities in the Thames catchment are correlated, and (ii) interpret the response of genetic and species diversity to in-stream conditions and watershed land use. Increasing presence of disturbed environmental conditions (due to urban/arable land use and nutrient loading) is hypothesised to reduce both species and genetic diversity of stream fish within the Thames catchment.

6.2 Materials and Methods

6.2.1 Study system and site selection

Fish sample sites were all located within the Thames catchment. This was to minimise the influence of underlying variation due to post-glacial colonisation and dispersal trajectories of roach among different UK river basins (see Chapter 3 for explanation). Collection from a single catchment can bring inherent problems concerning independence of samples, however the sites extend across an extensive area with a gradient of habitat and surrounding land use characteristics. The Thames catchment is located in south- eastern England and covers an area of approximately 16,000 km^2, much of which is dominated by urban conurbations (Figure 2.3). Inherently, this limits extensive grasslands and woodland areas in favour of modified landscapes, giving rise to an aquatic environment largely influenced by urbanisation and intensive agriculture practices. Farming varies from intensive fruit and vegetable, arable land, to dairy and beef farming on grassland. In 2004, 35% of the Thames Region was classified as arable, 19% grassland

and 11% woodland (EA 2009). Given the intensity of surrounding land use patterns, the riparian habitats of the Thames catchment present one of the most impacted regions in the UK, both through habitat modification and water quality fluctuations (Johnson et al. 2009). In 2009 28% of Thames catchment surface water bodies were at good biological status, projected to be 34% by 2015; considerably lower than the targets set by the Water Framework Directive.

All sites were chosen based on the likely presence of roach from historical records; however I aimed to sample roach from sites representing the full span of agricultural and urban intensification, calculated using Land Cover Maps. This increased the chance of finding a statistically significant outcome, if land use does indeed influence genetic parameters. In addition, sites were chosen where there was no recorded restocking within the last 10 years, as this would significantly alter the genetic structure of roach populations. Restocking information was obtained for each of these sites from the Environment Agency Live Fish Movement Database (LFMD), which records the translocation and supplementation of any English fishery with common freshwater fish species.

6.2.2 Species Selection

Quantifying environment-driven changes in genetic diversity and species diversity of fish populations likely varies, when considering different species and the genetic variants therein (Vellend and Geber 2005). Consequently detection and strength of these environment-driven relationships may be reliant on the choice of study organism. *R. rutilus* is ubiquitous throughout English rivers and often dominates freshwater communities both in biomass and numerically, making it a valuable recreational species. As a common cyprinid, abundant in most lowland streams, they are able to exploit a wide variety of habitats and food resources (benthic invertebrates, zooplankton, plant material and detritus), which along with their tolerance for habitat degradation and modification, fathers their success (Kottelat and Freyhof 2007). Short spawning migrations and a retained dominance within stream communities means *R. rutilus* can exert strong effects on stream biota and primary productivity, through competition and predation. Therefore, as a common, historically abundant and largely tolerant species, if genetic effects are seen in this species, then it is likely being felt by other sensitive species and may indicate a persistent driver/problem.

6.2.3 Specimen Collection for Genetic Data and Species Diversity Calculations

In total, 19 sample locations met the necessary requirements and constituted a gradient in land use that encompassed the environmental variation seen within the Thames catchment. An accessible sampling location was identified on focal river stretches where movement of individuals was restricted by the presence of in-stream obstructions or migratory barriers. Ensuring that the movement of individual roach was restricted to defined river stretches, was integral to allowing associations to be drawn between habitat differences and genetic diversity measures. Transient movement of fish between upstream and downstream environments would make genetic/species-environment patterns indiscernible as individuals would not be representative of the environmental conditions in one given location. Quantification of species and genetic diversity therefore present a snapshot of the composition of fish populations, as fish movements are a dynamic process. However sample reaches were kept consistent to ensure accurate representation of typical functional assemblages present at each sample site.

Fish were sampled from all sites in one season (2010), in an attempt to reduce inter-annual variability in fish communities. Catch depletion sampling using non-independently switched pulsed DC electrofishing equipment took place at each location over a 100m reach (beginning at the sample grid reference and progressing DS), restricted at both upstream and downstream extremities with large stop-nets. All fish captured were transferred to holding tanks between runs, identified to species level, measured to the nearest millimetre and then released. It is necessary to note that sampling via electrofishing favours larger fish and not juveniles, so counts used here are not representative of the entire fish population present but are comparable across sites. For later interpretation of results, it is necessary to be mindful that this sampling methodology was not exhaustive and does not account for small fish/ fry so catch data my underestimate total population numbers. Non-destructive fin-clip samples and fish scales were collected from all adult roach (869) from all rivers for genetic analysis. Fin clips were stored in 100% ethanol for DNA extraction and scales were kept in scale packets for subsequent DNA extraction and aging (see Chapter 2 for detailed method). All fish were aged by counting scale annuli but sex was not able to be determined just by examining external appearance.

6.2.4 Characterising Species Diversity Indices

All fish caught at each site were retained and recorded to calculate estimates of fish species diversity. Repeat-pass depletion sampling is believed to be one of the most

successful methods for estimating local abundance of riverine fishes (Sutherland 2006) so was undertaken at every sample location until all individuals were believed to have been caught (to provide fish population estimates). Simple measures of total fish abundance and species richness (number of different species caught) were recorded for every sample location. More complex indices of species diversity, including evenness (the relative abundance of rare and common species) and the Shannon (*H*) diversity index (which incorporates both species richness and evenness; Shannon and Weaver 1949), were calculated using the formulae below.

$$\text{Shannon diversity } (H) = -\sum p_i \ln (p_i)$$

$$\text{Evenness (E): } E = H / \ln (S)$$

Here p_i is the proportion of total number of species made up of the *ith* species and *S* is the total number of species in the community (i.e. species richness). Species richness was calculated simply using the number of species found at each location, for comparison to measures of allelic richness.

6.2.5 Genetic Analyses

For molecular analysis, see General Methods section on microsatellites (Chapter 2)

6.2.6 Calculating Genetic Diversity and Internal Relatedness Measures

All loci that did not significantly deviate from Hardy–Weinberg equilibrium after Bonferroni correction were used to estimate a variety of parameters for each population (See Hamilton et al. 2014, Chapter 3 & 4). Genetic diversity: Observed hetrozygosity ($H_{o,}$) was calculated in GENALEX 6 (Peakall and Smouse 2006) and allelic richness (*AR*) using F_{stat} (Goudet 1995, 2002). In addition, individual genetic diversity of each roach was assessed using internal relatedness (*IR*). *IR* is a multilocus measure of relatedness, which estimates the similarity between parental half-genotypes within an individual and weights the importance of each allele according to its frequency in the population (Amos et al. 2001). *IR* values have been used as proxy measures of 'how inbred an individual is' and are shown to negatively influence reproductive success in a wide range of aquatic organisms, such as the long-finned pilot whale (*Globicephala melas*), grey seal (*Halichoerus grypus*, Amos et al. 2001) and Atlantic salmon (*S. salar,* Garant et al. 2005). Mean internal relatedness was calculated and averaged for all individuals at each site, using a free open-source R extension package (Rhh) available at http://www.helsinki.fi/biosci/egru/research/software. Individuals with an *IR* near zero are

assumed to be a product of random mating, and those individuals with a high *IR* can demonstrate reduced fitness and survivorship (Bean et al. 2004). The Rhh package also creates outputs of homozygosity by loci (*HL*, Aparicio et al. 2006) and standardised heterozygosity (*SL*) but I present only the results pertaining to *IR*, since *IR*, *SL* and *HL* were highly correlated (r = 0.98, p = 0.001). Differences in genetic diversity endpoints among sampled populations were tested using ANOVA.

6.2.7 Characterisation of Environmental Variables- Including Land Use, Water Quality, Hydrology and Distance Endpoints

River ecosystems are strongly influenced by their surroundings at multiple scales. This warrants the inclusion of local and regional environmental conditions, which may manifest themselves at the level of the individual or the community. Here, I define regional environmental variation as conditions attributable to watershed geomorphology and watershed land cover. Generally, land use across the Thames watershed largely reflects the underlying geology and topography of the area, in association with the high demand for housing around the capital city. Despite this, large gradients in agriculture-grassland remain evident, owing to the sheer distance covered between source and mouth of the Thames.

Local (2 km) and broad scale (5 km) landscape composition was assessed at each sample site from a digital landcover map (LCM2007). This map is derived from categorised parcels of satellite images, projected in ArcGIS 10 (ESRI 2011, Redlands, CA: Environmental Systems Research Institute). The LCM2007 25 m raster dataset (distributed by CEH Information Gateway, Wallingford, UK) provides digital cartography of the UK, defining broad habitats akin to those seen in the UK Biodiversity Action Plan. Typical habitat classes, as used here, can be seen in Table 6.1 and are described in more detail by the Joint Nature Conservancy Committee (JNCC; Jackson 2000). Overall, the LCM2007 shows the UK is dominated equally by Arable/Horticulture and Improved Grassland (25% each), with urban areas making up just 6% of land cover.

For the purpose of this work, an assessment of landscape characteristics within a buffer zone of 2 or 5 km surrounding each sampling site was recorded as a proportion of arable, grass, wood and urban land. I used a script in ArcGIS10 to generate a circular buffer zone (of 2 or 5 km in total diameter) encompassing the immediate land use surrounding the river reach of each site. Subsequently, the Spatial Analyst tool in ArcGIS10 was used to return the relative percentage of each land use type within the associated buffer (see Figure 6.2). Log-transformed percentages were then used in subsequent analyses, as well

as categories of dominant land use seen at each site (3 = Arable, 4 = Grassland, 10 = Urban). These two buffer sizes were chosen on the basis of other published literature suggesting that within this distance most insecticides are adsorbed and therefore their toxicological potential will be greatly reduced (Sibley et al. 1991, Liess and Schulz 1999, Probst et al. 2005). Similar estimates of carrying distance or degradation of nutrients could not be found in the literature, however mean daily migrations of roach in fragmented rivers are known to be relatively short (< 2.5 km) so their lifecycle would likely be completed in that river reach, which is encompassed by the 5 km buffer. Using land use patterns may also act in surrogate for current pesticide pollution. Brown et al. (2006) showed that the consideration of agricultural land use data and insights into landscape geography can be indicative of pesticide contamination of water bodies. However, it must be noted that the land cover overlay used here was dated 2007 but only released in July 2011. Therefore it is appropriate to be mindful of changes to land use that may have occurred in the interim, which could alter river habitats on more recent timescales.

Riverine habitat modification and high nutrient loading are present throughout the Thames catchment to varying degrees. Therefore to quantify in-stream environmental variation, I consider 2 common water chemistry variables (N, P) at each site. Water quality profiles were obtained from Environment Agency databases, where average annual nitrate (mg/l), orthophosphate (mg/l) and dissolved oxygen (% saturation) are derived from river water samples taken once a month, at set reaches along the course of a river. Many of the selected sites appear to be moderately or highly impacted by nutrients, increasing the chances of seeing a relationship between water chemistry and inhabiting fish species, if it exists. All sample sites corresponded to routinely sampled reaches, therefore I utilised average nitrate, phosphate and dissolved oxygen data from 2010 in subsequent analyses.

At broader spatial scales, I consider additional physical riverine characteristics more commonly associated with driving changes in fish populations. Numerous studies have shown correspondence between species diversity, watershed area and discharge (Angermeier and Schlosser 1989) yet little is known about the involvement of these physical variables in shaping genetic diversity. Therefore I include additional over-land flow regime characteristics such as rainfall (mm), runoff (mm) and catchment area (m^2); all of which were obtained from the UK hydrometric register produced by CEH (available at http://www.ceh.ac.uk). Catchment summary information, as used here, is synthesised

from validated rainfall data provided by the Met Office alongside hydrogeological information of catchment topography and geology derived from the British Geological Survey 1:625,000 map. Mean annual runoff (mm) is the estimated depth of water over the catchment equivalent to the mean annual flow, calculated using the formula; Runoff = Mean Flow (m^3/s) x 86.4 x 365/ Catchment area (km^2). Mean long-term river flow (m^3/s) from proximal gauging stations associated with each sample site, was averaged from catalogued daily flow measurements obtained from the National River Flow Archive (NRFA). The Gauging Station Register consists of a UK network of around 1500 gauging stations, comprehensively recording near real-time water level changes using ancillary data loggers that transmit data from 15 min intervals for subsequent processing. Data was used from when the period of record began, until the month prior to sample collection, taking an average of all daily gauged flows.

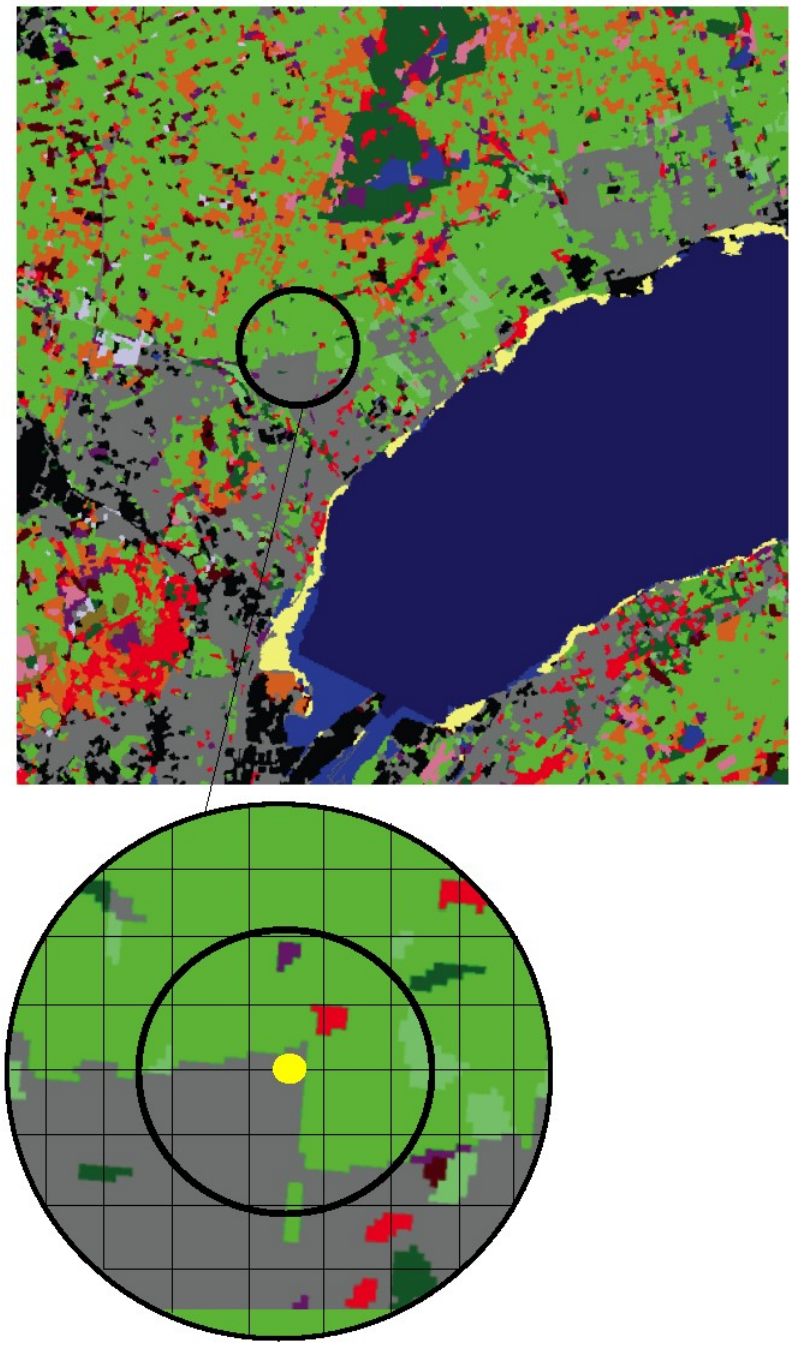


Figure 6.2 Example of how surrounding land use percentages were calculated from circular buffer zones around samples sites. Area (m 2 covered by each land use class (represented by different colours) within a 2 km (inner circle) /5 km (outer circle) buffer surrounding each sample site (central yellow dot) is quantified using a grid. This is then divided by the total buffer area, to return a percentage for each land use class

Table 6.1 Relationship between aggregate classes, broad habitat and LCM2007 classes. Green shading highlights broad habitats as documented by JNCC (Jackson, 2000). Aggregate class number was used here as a broad classification scheme, noting that only classes 3,4 and 10 (starred in red) were the only types present in the Thames catchment. Sourced from Morton et al. (2011)

Aggregate class	Aggregate class number[1]	Broad habitat	LCM2007 class	LCM2007 class number[2]
Broadleaf woodland	1	*'Broadleaved, Mixed and Yew Woodland'*	**Broadleaved woodland**	1
Coniferous woodland	2	*'Coniferous Woodland'*	*'Coniferous woodland'*	2
Arable	3	*'Arable and Horticulture'*	*'Arable and horticulture'*	3
Improved grassland	4	*'Improved Grassland'*	*'Improved grassland'*	4
Semi-natural grassland	5	**Rough Grassland**	**Rough grassland**	5
		'Neutral Grassland'	*'Neutral Grassland'*	6
		'Calcareous Grassland'	*'Calcareous Grassland'*	7
		'Acid Grassland'	**Acid grassland**	8
		'Fen, Marsh and Swamp'	*'Fen, Marsh and Swamp'*	9
Mountain, heath, bog	6	*'Dwarf Shrub Heath'*	**Heather**	10
			Heather grassland	11
		'Bog'	*'Bog'*	12
		'Montane Habitats'	*'Montane Habitats'*	13
		'Inland Rock'	*'Inland Rock'*	14
Saltwater	7	**Saltwater**	**Saltwater**	15
Freshwater	8	**Freshwater**	**Freshwater**	16
Coastal	9	*'Supra-littoral Rock'*	*'Supra-littoral Rock'*	17
		'Supra-littoral Sediment'	*'Supra-littoral Sediment'*	18
		'Littoral Rock'	*'Littoral Rock'*	19
		'Littoral Sediment'	**Littoral sediment**	20
			Saltmarsh	21
Built-up areas and gardens	10	*'Built-up Areas and Gardens'*	**Urban**	22
			Suburban	23

Accounting for connectivity between sample sites is of crucial importance when examining differences in species and genetic diversity. The movement of fish between sites and the physical carrying capacity of each location will largely determine the number of individuals and variety of fish species seen. Similarly, geographical isolation between locations within the dendritic structure of a river network is found to be largely deterministic in inter- and intra-specific genetic diversity patterns (Osbourne et al. 1992). Accordingly, I incorporated the log-transformed river width (m), measured using satellite imagery at each sample site, and the distance of each site from the main stream River Thames (km) as additional covariates when comparing species and genetic diversity

between sites. Measurement of pairwise geographic distance between sites was calculated via ArcMap 10 (ESRI, Redlands, CA, USA) using the Fastest Path routine in conjunction with Network Analyst. Shortest pairwise downstream/upstream distances (km) were estimated by following a stream network shapefile of the Thames catchment rivers. The same approach was used to calculate curvilinear distances from each sample location to the mainstream Thames. This would be used to examine the role of isolation from the historical migration corridor and to define the influence of watershed location within the Thames basin on genetic and fish assemblage similarity.

6.2.8 Statistical Analysis of Environmental Variation, Genetic Diversity and Species Diversity

Relationships between genetic diversity and species diversity were examined across all sites and populations. For both sets of analyses, Pearson correlation coefficients were calculated in preference to other correlation indices, as I was looking for a linear association in normally distributed continuous/interval data. Measures of mean inbreeding/genetic diversity (*IR*, *AR*, H_o) and species diversity (Shannon–Weiner diversity and species richness) were compared to assess the strength of pairwise association between indices using Pearson correlations. All test variables used were inspected for normality using the Kolmogorov–Smirnov (K–S) test and if requirements of a normal distribution were not met, the data was log-transformed. $\alpha = 0.05$ was chosen as the accepted significance level for all statistical tests. Unless otherwise specified, statistical analyses were carried out with SPSS, Version 20 (IBM Chicago, IL).

Pairwise Bray–Curtis dissimilarity in fish assemblages was estimated between sites using multidimensional scaling (MDS) of species richness and abundance data, as implemented in SAS 9.3 (SAS Institute Cary, NC). Mantel tests (with GenAlEx 6.5) were used to compare pairwise estimates of geographic distance to estimates of species assemblage dissimilarity between all 19 sites, to evaluate the strength of biogeographical influences and structuring. Mantel tests were also conducted to assess the strength of association between pairwise estimates of genetic differentiation (linearised F_{ST} /1- F_{ST}) and river distance among sites.

Nine environmental variables related to land use, in-stream water quality and river catchment characteristics were included in multivariate analyses. I used Principle Components Analysis (PCA) in SPSS to study the loading of different environmental factors and to transform multiple correlated variables to independent linear components.

For subsequent regression modelling, I took three principle components forward from the

PCA, to evaluate how measures of species and genetic diversity correspond to PCA summary statistics. It must be noted that these synthetic variables would not inform biological interpretation, but instead act as initial indicators of important relationships within the data. Following this, I could identify the variables that loaded strongly (r > 0.7) on to each principle component, to explore relationships further. Simple regressions in SPSS, using log-transformed environmental predictors (extracted from PCA components) as interacting factors, against species and genetic diversity measures followed.

6.3 Results

6.3.1 Population-Genetic Structure

I collected 869 individual roach from 19 sites within the Thames catchment, covering a riverine distance of 176 km and spanning a range of land-use characteristics. Genotype data was obtained for all individuals at 14 microsatellite loci and revealed high genetic diversity in all populations (Table 6.2). Both *AR* (range: 6.79–8.76) and H_o (range: 0.67–0.75) comparisons between populations revealed significant differences (See Chapter 3 Figure 3.5). Lamsha and BlaHaw show low *AR*, with LeaHyd and both the River Stort sites exemplifying low H_o. ThaSha (0.0024) and StoBri (0.1037) were the only two populations that exhibited significantly different mean *IR* when assuming unequal variances (p = 0.021). Mean *IR* and H_o indices for each population were found to be highly correlated with each other (ANOVA $F_{1,18}$ = 139.742, p < 0.001) however *AR* showed no such relationship with other genetic indices. Additionally, no significant difference was found between genetic endpoints (*IR*, *AR*, H_o) from fish of different ages, in regard to any of the genetic indices. Figure 6.3 demonstrates this just for *IR* (ANOVA $F_{1,867}$ = 0.488, p = 0.899).

Table 6.2 Summary of species and genetic diversity endpoints calculated for each of the 19 sample sites within the Thames catchment

Sample site	Shannon–Wiener diversity (*H*)	Species richness	Total fish abundance (Number of fish)	Evenness across species	Mean IR	Mean H_e	Average *AR*
BlaHaw	1.13	7	555	0.58	0.036	0.70	7.37
BouCeL	1.58	8	154	0.76	0.024	0.74	8.35
GadCas	1.65	9	530	0.75	0.031	0.73	8.11
KenBul	1.66	9	114	0.76	0.044	0.74	8.44
KenFou	1.49	8	119	0.72	0.015	0.75	8.71
KenNor	1.14	9	287	0.52	0.031	0.73	8.47
LamSha	1.06	4	77	0.76	0.063	0.72	6.79
LeaEss	0.93	8	409	0.44	0.080	0.69	8.29
LeaHyde	0.77	6	43	0.43	0.120	0.68	8.17
LeaSta	1.43	7	335	0.74	0.030	0.73	8.37
LeaWhe	1.48	6	723	0.83	0.056	0.72	8.46
MolMea	1.35	8	943	0.65	0.005	0.75	8.42
StoBri	1.63	9	204	0.74	0.104	0.67	8.07
StoTed	1.06	9	410	0.48	0.074	0.69	8.21
ThaCul	1.34	11	307	0.56	0.043	0.73	8.76
ThaHam	1.58	9	127	0.72	0.033	0.73	8.23
ThaSha	1.60	11	729	0.67	0.002	0.75	7.93
ThaWhi	0.98	9	298	0.45	0.027	0.73	8.22
WanMoh	1.36	6	229	0.76	0.022	0.75	8.20

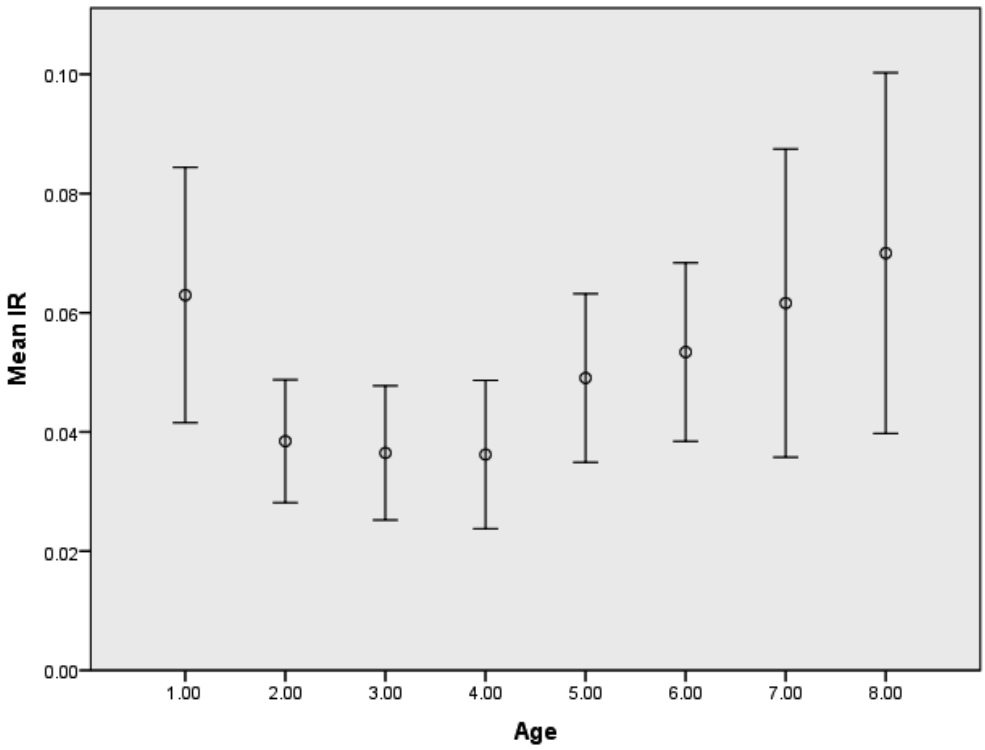


Figure 6.3 Simple scatter plot of fish age versus mean internal relatedness (IR) of all individuals sampled (n = 869). Open dots represent the mean IR value of fish of the same age with error bars ±1SE

6.3.2 Fish Species Patterns Across the Catchment

Catch data from routine surveys of 19 Thames catchment sites allowed analysis of fish population composition, to parallel genetic diversity of roach populations. Sixteen freshwater fish species were commonly recorded throughout the Thames catchment in various proportions at each site, with roach being the most abundant species encountered at all sites (Figure 6.4; total number = 2959, 43.9% of total catch of all species). Common cyprinids were ubiquitous throughout all rivers and made up over 80% of the total catch across all sites (roach > chub > gudgeon > dace), followed by the most prevalent non-cyprinid species, the perch (532 fish, 7.9% of the total catch). Total abundance of fish caught at each site varied by more than a factor of 10 across the catchment; the highest at MolMea and the lowest at LeaHyd (943 and 43 respectively).

Catch data was utilised in the subsequent calculation of species richness (number of species present) and Shannon diversity (*H*) index for each sample site, to allow a better inter-site comparison of community composition (Table 6.2). Species richness ranged between 4 and 11 species per location and Shannon diversity extended between 0.77 and 1.66 over all 19 sites. Community composition and the dominance of certain species was examined using evenness, whereby the most and least evenly distributed communities were found to be sites on the same river, LeaWhe (0.83) and LeaHyd (0.43) respectively.

A lower value of evenness suggests that the fish population is dominated by a few successful species, whereas individuals are more evenly distributed in line with diversity where values move closer to 1. It must also be noted that *H* and evenness are highly correlated ($r = 0.838$, $p < 0.001$), thus to avoid over complication, evenness was omitted from later regression analyses.

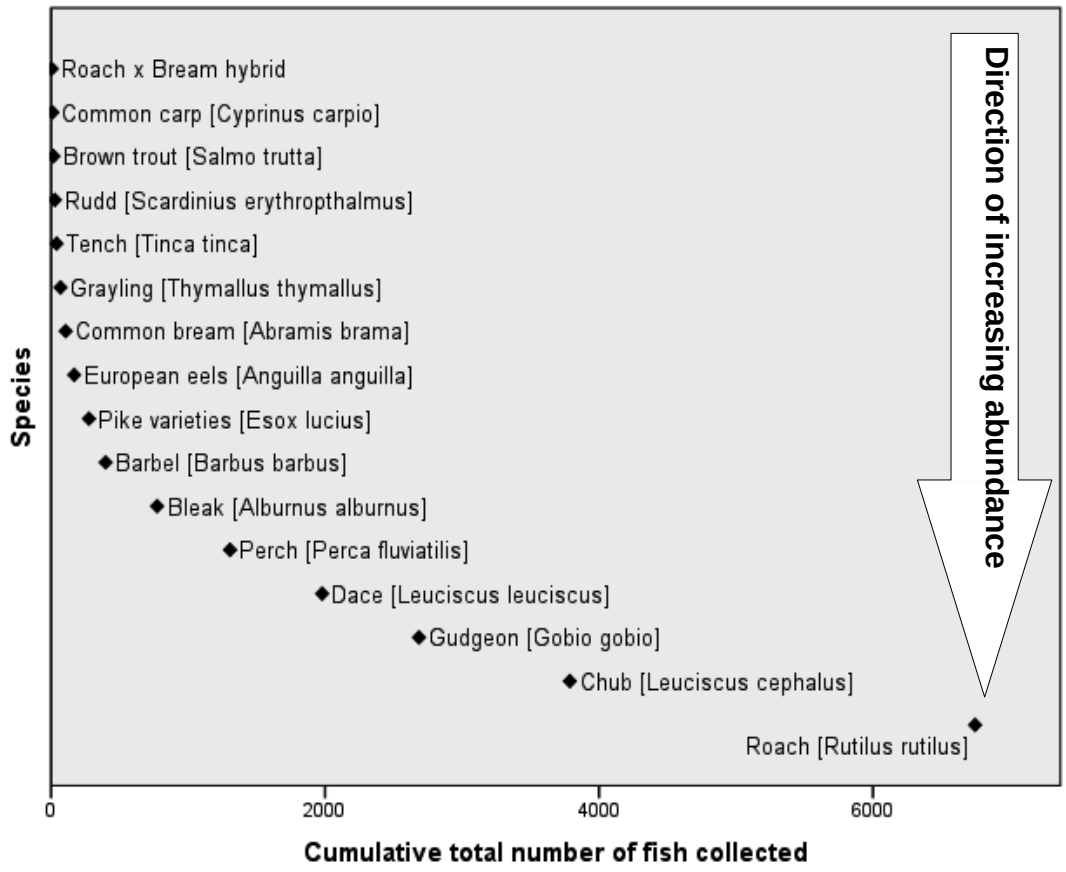


Figure 6.4 Typical fish species found across 19 sample sites within the Thames catchment in 2010, along with their cumulative total abundance

6.3.3 Relationships Between Species Diversity and Genetic Diversity

Significant correlations among measures of genetic diversity of roach and species diversity of stream fishes were found in some, but not all, pair-wise comparisons. *IR* showed a weak but significant linear increase with Shannon diversity (*H;* $r^2 = 0.467$, $p = 0.044$). Contrastingly, *H* was not significantly linearly related to AR ($r^2 = 0.071$, $p = 0.206$) or H_o ($r^2 = 0.406$. $p = 0.084$). Additional variables of total fish abundance, species richness and evenness at all sites were examined in relation to all genetic diversity indices but showed no relationship with any other data. I also tested the strength of pair-wise relationships between species and genetic diversity when accounting for downstream distance to the Thames. Partial Pearson correlation coefficients between diversity

measures changed when controlling for downstream distance to the mainstream Thames, with a significant correlation found between total abundance of fish in the community assemblage and *IR* (r = -0.731, p = 0.001). The same relationship was found between H_o and total fish abundance, albeit a slightly lower significance level (r = 0.560, p = 0.016).

6.3.4 Genetic Divergence Relationships with Biogeography and Assemblage Divergence

Chapter 3 found evidence for genetic structure within roach sampled from the Thames catchment, indicating the existence of local sub-populations rather than panmixia. Likewise, pairwise linearised F_{ST} (Slatkins 1985) of sites in the Thames catchment showed a weak, but significant, relationship with geographic waterway distance between sites (r^2= 0.057, p = 0.010). Assemblage divergence of stream fishes also covaries across the Thames catchment. A significant but weak correlation between Bray–Curtis dissimilarity values and geographic distances was recovered (Figure 6.5A r^2 = 0.02, p = 0.05), along with a comparable, but much stronger relationship between population genetic divergence (linearised F_{ST}) and assemblage divergence (Bray–Curtis distances, Figure 6.5B r^2 = 0.28, p = 0.01).

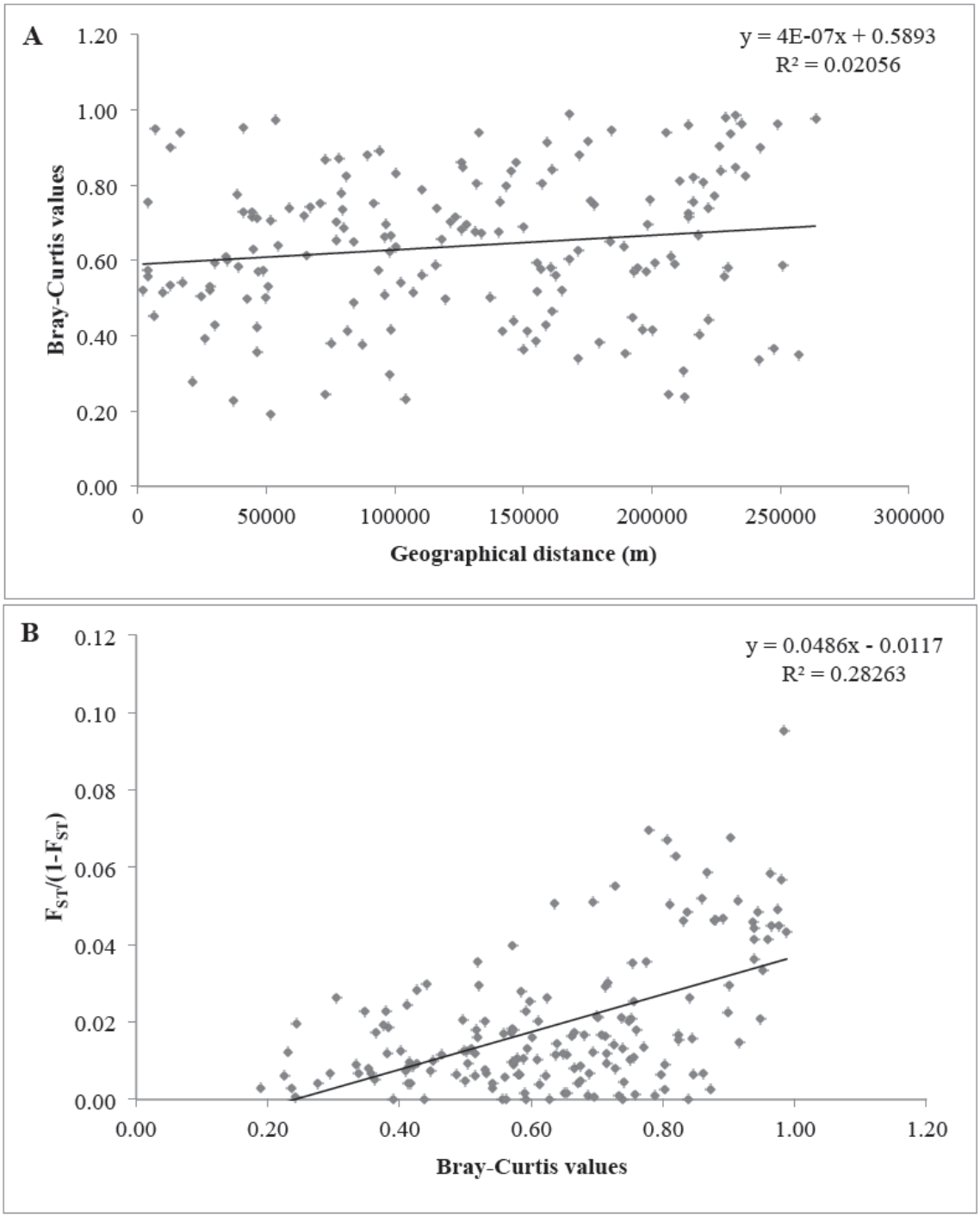


Figure 6.5 (A) Comparison of geographic distances to Bray–Curtis values across all sites, with regression line. (B) Comparison of Bray–Curtis values to linearised F_{ST} genetic distances across all sites, with regression line

6.3.5 Land Use Patterns Across the Catchment

The dominant land-use class within each 2 km buffer was positively correlated with that at 5 km, suggesting habitat characteristics remain relatively similar at both local and broad scales. Table 6.3 confirms the similarity between land-use percentages at the 2- and 5-km scale as being significant. The modal land use class commonly found across the Thames catchment sites was arable and horticulture (class 3 in Table 6.1) at the 5 km scale, whereas both urban (class 10) and improved grassland (class 4) represented a similar number of sites each. Land use within the 2 km buffers was relatively well distributed across all classes. Calculating percentage land use constituting the 2- and 5-km buffers, defined by four categories (arable, grassland, woodland and urban), provided a more continuous scale of land use seen at each sample location. Broadly speaking, at the 2 km scale WanMoh was the most urbanised; MolMea had most extensive improved grassland; arable land made up 90% at StoTed; woodland was uncommon at most sites, but highest at KenBul. Due to the strong correlation between land use at the 2- and 5 km scales, further statistical analyses were conducted using only the 2 km buffer data.

Table 6.3 Dominant land use class (a) and the percentage of each class constituting 2 and 5km buffer zones (b) surrounding each site across the Thames catchment. Pairwise Pearson correlation coefficients between percentage land use at 2 and 5km scales are shown.

a) Dominant land use class			**Arable & Horticulture**	**Improved Grassland**	**Urban**
2 km Buffer	Number of sites		6	6	7
5 km Buffer	Number of sites		10	4	5
b) % Land use		**Woodland**	**Arable & Horticulture**	**Improved Grassland**	**Urban**
2 km Buffer	Median	0.2	18.5	22.5	19
	Range	0–19	0–90	0–84	0–100
5 km Buffer	Median	6	31.5	21	25.5
	Range	0–18	0–80	3–63	2–94
2 km vs 5 km	Pearson corr. Coefficient	0.586	0.934	0.843	0.854
	Sig.	.007	.000	.000	.000

6.3.6 Environmental Predictors of Genetic and Species Diversity

To elucidate the key drivers of species and genetic diversity, an initial analysis using PCA was undertaken, as environmental variables comprised of different ranges and were

likely to be highly correlated. Nine variables were included in the PCA, which incorporated indicators of in-stream water chemistry, flow characteristics and surrounding land use within 2 km of the sample locations. Constructing the PCA allowed removal of redundant, highly correlated environmental variables. The first three components of the PCA explained >75% of the variation in the environmental variables for all sites within the Thames catchment. Percentage of arable and urban land use within a 2 km buffer, in-stream phosphate levels and mean annual rainfall (mm) were highly correlated ($r > \pm 0.7$) with the first PCA component, whereas percentage improved grassland within a 2 km buffer comprised much of the second PCA component (Table 6.4). These three component factors were taken forward as orthogonal predictors for further analyses, as they were found to summarise all habitat variables and exhibited initial eigenvalues >1. Newly derived principle component residuals were regressed against all species diversity and genetic diversity endpoints, yielding inconsistent results between diversity indices. Component 1 was the only significant predictor of genetic diversity indices including *IR* and H_o (see Table 6.5 for Pearson correlation coefficient statistics). No PCA components significantly correlated with *H* or *AR*, although species richness and component 2 did show a weak but significant relationship ($p = 0.022$). It must be noted that these synthetic variables from the PCA do not inform biological interpretation, but instead act as initial indicators of important relationships. Therefore, original variables were taken forward for further regression analyses.

Table 6.4 Component matrix derived from the PCA analysis, including the original variables entered and their loading on to the three extracted components. Three components extracted based on Eigenvalues > 1

Original variable	Component		
	1	2	3
%Woodland	0.452	-0.011	0.071
% Arable land	-0.874	-0.136	-0.261
% Grassland	-0.073	0.898	0.329
% Urban land	0.702	-0.640	-0.057
N	-0.502	-0.113	0.675
P	-0.776	-0.131	0.341
Mean ann. rain (mm)	0.864	0.090	0.053
Mean ann. runoff (mm)	0.575	0.296	0.620
Mean flow (m^3/s)	-0.007	0.670	-0.649

Table 6.5 Results of pairwise correlations between measures of genetic and species diversity, against three components derived from the PCA analysis of nine environmental variables. **Correlation is significant at the 0.01 level. * Correlation is significant at the 0.05 level

		Component 1	Component 2	Component 3
IR	Pearson Correlation	-0.560*	-0.426	-0.127
	Sig. (2-tailed)	0.013	0.069	0.603
H_O	Pearson Correlation	0.602**	0.391	0.068
	Sig. (2-tailed)	0.005	0.088	0.776
AR	Pearson Correlation	-0.104	0.329	0.038
	Sig. (2-tailed)	0.661	0.157	0.875
H	Pearson Correlation	0.220	0.215	0.102
	Sig. (2-tailed)	0.352	0.362	0.670
Species richness	Pearson Correlation	-0.141	0.508*	-0.225
	Sig. (2-tailed)	0.552	0.022	0.340

Significant correlations between genetic indices (*IR* and H_o) and orthogonal predictor C1 led us to explore the variables in this principle component in more detail. Principle component 1 consists predominantly of reach scale variables (arable and urban land use and river water phosphate concentration) that could be deemed indicative of habitat alteration and intensification of human activities. Therefore I chose to sum land use percentages to form surrogate variables of percentage 'disturbed' (arable + urban) and 'natural' (grassland + woodland) land at each sample site. This concept has been used previously, where a summary statistic of 'disturbance' was thought to be more indicative of degradation in stream ecosystems and a good proxy for human activity (Allan 2004).

Simple linear regressions between % disturbed land and genetic and species diversity endpoints showed negative relationships (although none were statistically significant). As percentage disturbed land increases we see increases in mean *IR* of each population (lower genetic diversity and increased inbreeding) and a decrease in Shannon diversity (*H*). However, once percentage disturbed land and distance from the mainstream Thames are included as interacting factors in a GLM, collectively they become a significant predictor of four key diversity indices (*H*, Species richness, *IR* and H_o) at the $p = 0.05$ level (Table 6.6). Those fish populations increasingly isolated from the mainstream Thames and surrounded by more disturbed land exhibit lower species richness/ diversity and roach populations present decreased genetic diversity (H_o) and become more inbred (increasing *IR*). Again, there is no significant relationship between natural or disturbed land use percentages and *AR*, or total abundance of the fish assemblage.

Table 6.6 GLM statistical outputs from SPSS, using multiple diversity endpoints and predictors of disturbed land use, distance downstream to the mainstream River Thames and both in combination. Significant values are highlighted in bold

Factor	Dependent variable	Test statistics	F	Sig.
Disturbed land	*H*	r^2 = 0.145 (Adjusted r^2 = .097)	3.042	0.098
	Species richness	r^2 = 0.196 (Adjusted r^2 = 0.151)	4.388	0.051
	Abundance	r^2 = 0.104 (Adjusted r^2 = 0.054)	2.090	0.165
	IR	r^2 = 0.180 (Adjusted r^2 = 0.134)	3.951	0.062
	H_o	r^2 = 0.166 (Adjusted r^2 = 0.120)	3.588	0.074
	AR	r^2 = 0.067 (Adjusted r^2 = 0.015)	1.293	0.270
Distance to mainstream Thames	*H*	r^2 = 0.099 (Adjusted r^2 = 0.049)	1.982	0.176
	Species richness	r^2 = 0.138 (Adjusted r^2 = 0.090)	2.882	0.107
	Abundance	r^2 = 0.153 (Adjusted r^2 = 0.106)	3.260	0.088
	IR	r^2 = 0.243 (Adjusted r^2 = 0.201)	5.768	**0.027**
	H_o	r^2 = 0.234 (Adjusted r^2 = 0.192)	5.509	**0.031**
	AR	r^2 = 0.013 (Adjusted r^2 = -0.042)	0.237	0.632
Disturbed land *Distance to mainstream Thames	*H*	r^2 = 0.255 (Adjusted r^2 = 0.214)	6.168	**0.023**
	Species richness	r^2 = 0.226 (Adjusted r^2 = 0.183)	5.256	**0.034**
	Abundance	r^2 = 0.000 (Adjusted r^2 = -0.055)	0.002	0.962
	IR	r^2 = 0.477 (Adjusted r^2 = 0.448)	16.434	**0.001**
	H_o	r^2 = 0.471 (Adjusted r^2 = 0.442)	16.040	**0.001**
	AR	r^2 = 0.068 (Adjusted r^2 = 0.017)	1.322	0.265

6.4 Discussion

Understanding the principle factors driving population size is fundamental to ecology, population genetics and conservation of wild resources (Frankham et al. 2002). Patterns of variation in species and genetic resources are rarely considered in parallel with respect to environmental conditions, however they are ultimately rooted at the same level; that of the individual. Every organism has the potential to represent a different genetic variant or a different species, imparting influence at the community and population level through commonalities of birth, death and movement (Vellend and Geber 2005). The objectives of this study were to examine whether genetic and species diversity patterns of stream fishes in the Thames catchment covary across a land use gradient. Additionally, I assess

the importance of watershed location, in addition to environmental conditions, to elucidate how isolation can influence biodiversity of freshwater fish at different levels of biological organisation.

These results demonstrate that parallel patterns of species and genetic diversity can present themselves in large river networks such as the Thames, although they are commonly moderated by strong spatial components including geographic isolation. Findings also suggest that genetic diversity and species diversity both decline with an increasing presence of disturbed land (arable and urban) within their catchment, when combined with isolation from the mainstream Thames (Table 6.6). Ultimately, it is apparent that landscape features and geographic distance affect both the abundance of freshwater fish species and the level of inbreeding/genetic diversity of roach across this river network.

6.4.1 Relationships between Fish Species Diversity and Genetic Diversity of R. rutilus

In many terrestrial studies, the amalgamation of population genetics and community ecology has been employed to understand the partitioning of species and genetic variation. The merit of this approach lies in the strong relationship between functional diversity and species richness (Cardinale et al. 2006), along with the likelihood that levels of diversity can vary in accordance to area and isolation, as well as environmental conditions (Vellend and Geber 2005). However, parallel processes acting *in situ* may not culminate in parallel effects at different levels of biodiversity. Varying responses to processes such as drift, immigration, and spatially variable selection pressures may manifest themselves differently at species and individual levels of organisation, affecting the strength of correlations between species and genetic diversity (Robinson et al. 2010) rather than direction.

Simultaneous responses of both species and genetic diversity appear to covary within the river network of the Thames catchment, but not for all endpoints considered. Individual genetic diversity of roach (*IR*) and species diversity appear significantly correlated (p = 0.04), as do levels of genetic and species assemblage divergence across the Thames catchment. Positive relationships between species and genetic diversity are not uncharacteristic in wild populations and have been found in a range of different taxa and environments (Vellend 2003, Vellend and Geber 2005). Examples of positive correlations have occurred in organisms such as butterflies (Cleary et al. 2006), bats (Struebig et al. 2011) and stream fishes (Blum et al. 2012). Previous work on stream fish assemblages and allelic richness of Central stonerollers in the US, found a similar linear increase

between indices, except this work by Blum et al. (2012) depicted a much stronger relationship (r = 0.53, p = 0.002). My results partially support the notion of Vellend (2004) who suggests that analogous processes should affect species and genetic diversity similarly, although the strength of this relationship is not presented consistently throughout all species and genetic diversity endpoints used. Parallel patterns of species and genetic diversity appear to be inconsistent, perhaps due to the examination of genetic diversity in only one common species (*R. rutilus*).

Species richness of stream fish assemblages and additional endpoints of genetic diversity (allelic richness and heterozygosity) show no correspondence. Inconsistencies in the strength and continuity of species and genetic diversity relationships have been demonstrated in the literature. For example, empirical data from plant species are contradictory- some of which confirm a relationship between species richness and genetic diversity (He et al. 2008) where other recent studies do not (Silvertown et al. 2009, Taberlet et al. 2012). Variability in the patterns of genetic and species diversity in response to environmental gradients can be seen in faunal patterns over multiple spatial scales. Indeed, Robinson et al. (2010) found that only when the entire dataset of eight saltmarsh invertebrate species was analysed, did they find a significant correlation between diversity endpoints. When all species were considered individually, this was not seen. This evidence suggests varying individual responses to geographical isolation or scale-dependent effects causes discontinuity in the findings of empirical studies, which is similar to those seen here. For instance, the diversity of fish species present across all sample sites exhibited significant differences in life histories, sensitivity to disturbance and habitat preferences. Ultimately, the response of different species to environmental fluctuations may be different to that of roach populations (and their genetic diversity).

Neutral theories explaining patterns of diversity in both species (community assembly) and genetic diversity (molecular evolution) show tight parallels (Wright 1940, Vellend 2005). However, weak correlations between Shannon diversity and *IR* are the only positive relationship seen. A plausible explanation for this lack of symmetry in diversity responses is that the genetic diversity of *R. rutilus* is strongly driven by population size, where as Shannon diversity is less affected by sporadic fish abundance modifications (Blum et al. 2012). Given that *R. rutilus* is ubiquitous throughout the Thames catchment, a slight reduction in their population size may allow another species to colonise. Abundance of roach may therefore exert greater effects on genetic diversity than Shannon diversity, because smaller roach populations may lead to greater overall evenness in

species diversity. Genetic diversity is likely to be more affected by large fluctuations in abundance observed across sampling sites, altering genetic variation through the presence/absence of different alleles under different population size scenarios (Frankham et al. 2002). Therefore, genetic diversity of roach populations may fall with declining population sizes (Frankham 1996), which could be paralleled by little change in species diversity because of the removal of a few individuals, which minimally affect assemblage structure (Blum et al. 2012). Vellend (2004) also note that if diversity measures are not analogous, then the likelihood of seeing strong relationships between indices is reduced. Shannon diversity and all genetic level information were calculated using different data sets, so this may provide additional explanations of the lack of continuity between pairwise relationships. Together these suggestions may go some way towards explaining the inconsistency seen here however this does not disentangle the influence of isolation or environment in driving patterns of diversity.

Overall, these results show that some parallels exist between species and genetic diversity measures, although not all. Vellend (2005) suggest that relationships between species and genetic diversity may attenuate as local environments become more patchy and heterogenous, causing a shift in mean fitness of genotypes and species. Increasingly extreme environmental conditions would shift patterns of selection towards tolerant species such as roach, increasing their population size and genetic diversity, but reducing the diversity of species assemblages through the eradication of less tolerant species. Therefore, the variability in relationships between diversity indices used in this study evokes questions surrounding the generality of the assumed covariance between diversity endpoints (Blum et al. 2012). The idea of using one diversity measure as a surrogate for another appears ill-advised. Therefore emphasis may be cast upon the inclusion of a multi-species approach, analysing genetic diversity in more than one species within an assemblage, which may alter the final outcome (Taberlet et al. 2012). Many of the cases reported in the literature which document positive patterns of diversity, have been conducted in isolated situations where the number of species and genetic diversity will be naturally reduced at small geographic scale. This study possesses a similar caveat and therefore may not be indicative of the situation experienced in open/continuous environments, where drift is less profoundly important and a response to environmental features (selection) may drive species/genetic patterns.

6.4.2 Environmental Predictors of Species and Genetic Diversity

Environmental impairment undermines neutral genetic diversity and species diversity in

populations disturbed by human activities (Blum et al. 2012, Bagley et al. 2004). Resolution of the key habitat characteristics that cause these shifts is of prime importance for both ecology and evolutionary biology, yet remains minimally explored. Heino et al. (2005) suggest that environmental gradients trigger variable responses from species in lotic systems. River networks therefore offer a good opportunity to study the influence of landscape and proximal environmental attributes on diversity, as species are intrinsically tied to their hydrological structure. Results on the population genetic structure of roach from Chapter 3 inform us that roach populations remain largely restricted within rivers and population diversity has not been homogenised by restocking events or high levels of migration between sites. These results utilise the dendritic structure of rivers in a single catchment to indicate that genetic and species diversity are lower in isolated riverine environments which are surrounded by disturbed land use patterns within the Thames catchment. The precise habitat characteristics driving these patterns at both levels of biodiversity appear more difficult to discern, but are elaborated in more detail hereafter.

Unprecedented rates of environmental change are provoking shifts in the composition and integrity of biological communities. Similar patterns of alterations to genetic diversity in stream fishes have been attributed to human alteration of habitat (Bagley et al. 2004), water quality and hydrology. Pairwise correlations of PCA-derived factors consistently identified C1 as the only predictor variable related to *IR* and heterozygosity indices of roach populations. Measures of percentage arable and urban land, riverine phosphate and annual rainfall all loaded positively on to C1. Intensification of agricultural land use has been linked with alterations in physical habitat and water chemistry in surrounding water bodies. Typical changes have been related to declines in species and genetic diversity in fish populations, with impairment arising from increasing levels of turbidity and nutrient loading, which promote the loss of both species and genetic diversity (Seehausen et al. 2008, Blum et al. 2012). Despite these published findings, responses observed in fish assemblages across the Thames catchment exhibit no consistent correlations with water quality determinants used here (N, P). Instead, patterns of species and genetic diversity reflect a suite of dynamic attributes that fluctuate under disturbance regimes driven by changing land use patterns across the catchment. This suggests a complex relationship between physical habitat changes and impairment of diversity.

Despite attempting to minimise the influence of watershed location within the Thames by using a single basin, connectivity between sample sites stood out as a significant driving factor in relation to fish assemblage and genetic differentiation. This suggests

connectivity between sites plays an important role in all fish species. Longitudinal distance between sample sites and the placement of sample sites across a drainage network has previously been found to influence patterns of genetic diversity in stream fishes (Costello et al. 2003). Therefore, robust correlations seen in Figure 6.5b, uphold theoretical expectations of genetic and assemblage divergence varying in parallel with geographic isolation (Vellend and Geber 2005). Cleary et al. (2006) also demonstrate analogous spatial and temporal associations between species and allelic richness across rainforest habitats affected by disturbance, using a species of butterfly with high dispersal ability. Likely explanations of this decay in assemblage and genetic similarity with distance are commonly associated with decreasing similarity in environmental landscapes or due to dispersal barriers between locales, limiting drift and migration (Soininen et al. 2007, Sei et al. 2008); analogous to the hypothesis of isolation by distance.

The conversion of natural grassland /woodland to intensive land use practices consistent with agriculture and urbanisation, have well documented effects on fish communities. Human alteration of catchment land use, has been found to exert a major influence on stream ecosystems, adversely affecting water quality and promoting degradation of the stream channel (Diana et al. 2006). A proxy of human activity is encapsulated here in the summary statistic of ‘disturbed’ land use, which is thought to be more indicative of degradation in stream ecosystems (Allan 2004). Increasing percentages of disturbed land use within a 2km buffer of each sample site led to decreases in species diversity and richness, likely reflecting an unfavourable habitat change as a result of altered land use patterns. Support for the findings documented here come from examination of other pollution-tolerant species, where flow modifications and poor habitat can lead to significant losses in diversity and persistence of stream fish (Silbiger et al. 2001, Roy et al. 2005). Additional causative factors of species diversity gradients include a historic legacy of basin-scale land use (Harding et al. 1998), modification of stream habitat, and water chemistry (Blum et al. 2012). Consistent themes throughout this body of literature support this work, indicating that a reduction in assemblage diversity is commonly associated with environmental impairment as a result of disturbance to the stream system. However, no single physical or chemical variable can be associated with promoting the loss of fish species in degraded habitats, therefore the responses observed are likely to be a metaoutcome of a suite of in-stream characteristics that change as a result of alterations to surrounding land use patterns.

Patterns of genetic diversity reported in the literature are inconsistent with respect to

habitat quality (Waits et al. 2008) and environmental heterogeneity (Bagley et al. 2004). In this instance, genetic diversity measures appear largely driven by physical isolation. Nonetheless, when combining isolation distance from the mainstream Thames and the amount of disturbed land in a 2 km proximity of the sample site, evidence indicates that measures of genetic diversity fall significantly in isolated and highly disturbed environments (H_o: p = 0.001). Similarly, levels of internal relatedness — an individual coefficient of inbreeding to rank individuals — increase with the combination of increasing isolation from the mainstream Thames and land-use disturbance (*IR*: p = 0.001). Previous declines in genetic diversity of stream fishes have been linked to intensive land use patterns, associated with increasing agricultural intensity, across a gradient of similarly sized watersheds in the US (Blum et al. 2012). This pattern has also been observed in other taxa, with genetic diversity of populations of *D. magna* found to be negatively impacted by agricultural land use intensity (Coors et al. 2009). The experimental design used here cannot deduce the possibility of physiological adaptation and susceptibility of roach population explicitly; however results demonstrate evidence of genetic erosion (although only slight) in natural populations that is likely related to land use intensification and isolation.

Patterns of genetic variation are known to be influenced by human activities, by altering levels of genetic drift, migration, and natural selection (Bickham et al. 2000, Theodorakis 2003). For example, habitat modification resulting in physical or ecological barriers (unsuitable habitat) that restrict gene flow (Hebert et al. 2000) can reduce levels of population diversity by increasing genetic drift within and divergence among populations (Shaw et al. 1994). Thus, degraded stream habitats likely to occur in disturbed landscapes compound lowered genetic diversity and heightened inbreeding in geographically-isolated populations of roach, which have been found to exchange a limited number of individuals. This could have implications for individual and population fitness of roach when facing future environmental change (Frankham 1995, Newman and Pilson 1997); however current estimates of population sustainability and roach abundance appear minimally affected. In conclusion, spatial patterns of genetic variation among roach from degraded freshwater systems bear signatures of population persistence and colonisation, in relation to habitat quality.

6.4.3 Issues and Improvements

Declining biodiversity has implications for ecosystem functioning. Importantly, the maintenance of genetic and species diversity in natural ecosystems may depend on similar

forces that act in concert, but not always in parallel. While relatively high levels of genetic diversity and allelic richness are present in *R. rutilus* populations, some sites within the Thames catchment exhibit low species richness and assemblage diversity. Therefore, despite documentation of parallel responses of genetic and species diversity to environmental disturbance and watershed location, any assessments made using a single scale of observation to quantify fish species diversity could yield misleading interpretations (Robinson et al. 2010). For instance, allelic richness seems ill affected by any environmental or geographical variables, so conclusions would be very different if analyses only focussed on this measure alone. The clarification of observed relationships likely benefitted from the use of multiple genetic and species diversity indices, although both should be verified by additional sampling where possible, as these results only present a single snapshot of events.

Inclusion of additional samples and exploring genetic diversity within a different focal species could prove beneficial. This would facilitate our understanding of the response of organisms with different life histories, to riverine habitat degradation (Lamy et al. 2013, Robinson et al. 2010, Wei and Zhang 2010). Likewise, comparisons of genetic diversity in fish species less tolerant or less vagile than *R. rutilus* may be more indicative of the risk posed by habitat disturbance or fragmentation (Struebig et al. 2011). A species with more restricted movement (Gobidae) or more sensitive to environmental degradation (Salmonidae), would further deduce whether connectivity or habitat are the most important deterministic features shaping species and genetic diversity in stream fish assemblages within the Thames.

From a conservation standpoint, the sustained presence of more inbred roach at disturbed/degraded sites in isolated stretches conveys their tolerance in comparison to other freshwater fish species. This provokes the question of local adaptation at sites where roach populations are able to persist, yet other species are impaired. Adaptation has been minimally explored in natural fish populations, however the theoretical implications of selection against less tolerant genotypes/phenotypes is known to alter the size, viability, and genetic diversity of populations (van Straalen and Timmermans 2002). Examples of local adaptation in fish populations have been studied in response to exposure to waterborne contaminants, reported for mosquito fish (*Gambusia affinis*) to insecticides (Andreasen 1985), brown trout (*S. trutta*) to metals (Durrant et al. 2011) and killifish (*F. heteroclitus*) and Atlantic tomcod (*M. tomcod*) to PCBs (Wirgin and Waldman, 2004). Likewise, increasing agricultural intensity and the proliferation of

pesticide residues in nearby ponds impose a significant selection pressure on natural populations of *D.magna*, with increasing levels of carbaryl tolerance seen in test animals from areas of more intensive agricultural land use (Coors et al. 2009). Together, this evidence suggests anthropogenic actions can evoke environmental gradient which drive physiological adaptation, recognisable through the examination of population genetic diversity. Therefore, natural populations provide an important ecological context to study the genomic responses of fish to changes in the aquatic environment. Molecular mechanisms of tolerance can be explored through next generation sequencing, with genome wide SNP genotyping being used to test the importance of candidate genes and also to identify new genes responsible for adaptation to particular environmental conditions. Utilisation of next generation approaches combined with phenotypic patterning across natural fish populations would provide crucial information on genetic variants and potential fitness consequences of future human-induced habitat alterations.

A caveat with this work is that the sites sampled within the Thames catchment may not represent demographically separated populations, despite efforts to select populations restricted by barriers. However, even with possible inflow between sites of both new alleles and new species, divergent diversity patterns are still evident and are likely maintained by the hypothesised processes of natural selection, limited dispersal, and competition (Robinson et al. 2010). Longer-term datasets are needed to address the temporal stability of species and genetic diversity of fish populations in the Thames, along with detailed consideration of the impedance posed by in-stream barriers. Further investigation of colonisation and dispersal routes of common fish species may establish whether contemporary or historical heterogeneous environmental conditions are primarily influencing biodiversity across the spatial scale (Blum et al. 2012) through recent evolutionary origin or divergent adaptation (Seehausen et al. 2008). One of the advantages of using genetic diversity is that it can encapsulate historic time frames, making measures well suited to multigenerational investigations with a long-term perspective (Bagley et al. 2004).

6.5 Conclusion

To my knowledge, this is the first study that provides evidence of parallel responses of species and genetic diversity to disturbed land use and isolation in UK river systems. Results from this study suggest that an increasing prevalence of arable and urban land use in a river basin, and reduced connectivity, act in concert to negatively impact species and genetic diversity of stream fishes. Consistent patterns of land use change occurring in

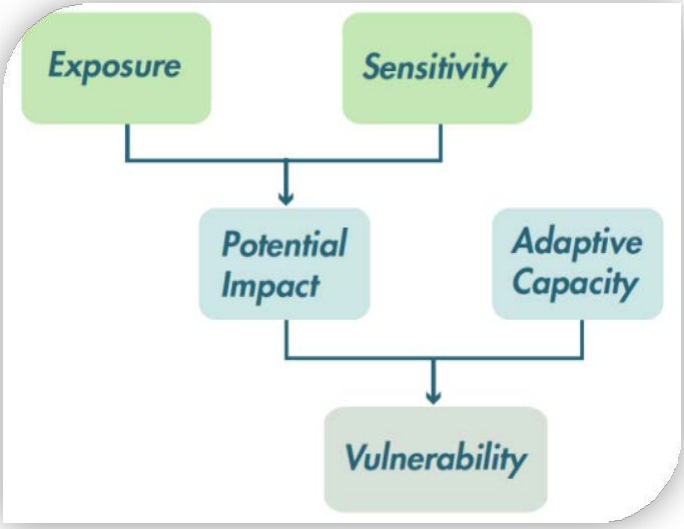
many lotic environments, not just the Thames, means that findings are relevant internationally in other river systems enduring increasing anthropogenic pressure. Important biodiversity repercussions are double threaded: reduced species diversity can directly influence ecosystem function, and genetically depauperate species may be at increased risk of extirpation, due to a limited capacity to adapt to future environmental challenge. However, this was not the case when using allelic richness as a measure of genetic diversity. Therefore this study cautions against the reliance on a single endpoint upon which generalisations are cast about the impact of disturbance on fish assemblages.

The ability to detect dominant environmental variables influencing both community and population composition of fish species is necessary for understanding drivers of biodiversity at different scales, especially in ecosystems where anthropogenic disturbance is accelerating. These results confirm the usefulness of integrating information from different scientific fields and underline the importance of incorporating the effects of biogeography and habitat/environmental factors in shaping two important levels of biodiversity. Further testing of the diversity pattern is recommended across alternate river systems and with different taxa; to resolve possible discrepancies arising from spatially varying responses to hierarchical environmental filtering and the role of adaptation in tempering species success. However acquisition of knowledge on species and genetic diversity remains integral from a conservation standpoint, for quantifying/predicting functional diversity of an ecosystem in response to changing land use practices. The results presented here offer potential insights and novel information regarding fish assemblage diversity in the UK, alongside a much needed baseline upon which to direct future management objectives.

Chapter 7

General Discussion

Pages 211 – 223



Overview

Historically, fisheries science and the surrounding body of published literature has been preferentially dominated by salmonid research; ensuing a lack of information regarding populations of coarse fish species. However, the profile of the coarse-fishing industry in the UK is rapidly expanding, and is now believed to be worth close to one billion pounds annually. Coarse fish populations, predominantly constituting cyprinids, have been historically and indiscriminately exposed to substantial levels of human-induced translocation, habitat modification, pollution and exploitation — to name a few dominant factors. Consequently, resulting opinion is that any prior population structure and diversity has been heavily compromised/eroded in these species, as a by-product of human proliferation.

Throughout this thesis I focus on the central theme of conservation biology; to explore whether and how natural populations are affected by anthropogenic changes in their habitat. A major challenge to aquatic ecosystems is the residual waste of humans, entering surface waters as chemical pollution (Sumpter 2005). Point sources of chemicals typically arise from the discharge of effluents from treatment works, which retain a cocktail of natural and synthetic compounds poorly removed during processing. Concern over the consequences of these persistent chemicals on wild fish populations, evoked the start of this work.

A research collective has focused on the effects of a profound group of chemicals, the steroid oestrogens, at the individual organism level. However, there remains a void in our understanding of the impacts of endocrine disruption on more complex populations and ecosystems. In order to address this knowledge gap, comprehensive, high quality data enquiries are necessary across fields of population genetics, species biology/life-history and predictive modelling. To date, these fields provide information that remains poorly integrated, limiting our capacity to derive population level implications of EDCs.

7.1 Roach as Sentinels for Studying Population Impacts of EDCs

As a vertebrate lineage, fish represent a conserved, simplified endocrine system similar to that seen in humans. The use of fish in ecotoxicology has been widely pursued through sampling wild populations or exposure studies of laboratory-reared individuals of ‘model’ fish species. Common small-bodied species such as zebrafish, fathead minnow and stickleback are preferentially used to inform on the general toxicology of EDCs and model behavioural interactions (Ankley et al. 2004). Yet in order to extrapolate from

laboratory species to real-world scenarios, a suitable native species is required to better understand commonalities of response throughout fish populations. Fundamentally, protection of fish species in the wild operates at the level of the population; therefore an improved understanding of the biological effects of chemicals and mixtures requires requisition of knowledge from wild fish populations.

Due to its ubiquitous distribution in freshwaters, the roach (*R. rutilus*) is an exemplary model to test whether continual, obligatory exposure to aquatic contamination influences the sustainability of wild fish populations. Moreover, roach represent the best-studied wild fish species with regard to documented effects of EDCs, more specifically the natural and synthetic oestrogens. Essentially, roach demonstrate reproductive traits of gonochoristic (genetic determinism of separate sexes) pelagic broadcast spawners with external fertilisation (Wedekind 1996, Kortet et al. 2004), similar to ~60% of other fish species. As such, any reproductive and breeding disruptions seen in roach are likely comparable to additional species, with similar group-spawning strategies. However, extension of findings to fish species in freshwater systems should be made with care. For example, other gonochoristic fish species do not develop the intersex condition even in contaminated environments (Bahamonde et al. 2013). Therefore, assumed effects based on one species should be tempered with theoretical and empirical investigations regarding differences in life-history of additional species of interest.

An additional demonstration of the disparity between life-history characteristics of roach and other fish species concerns the susceptibility of species to early-life exposure. Critical windows of heightened sensitivity to chemical disruption are demonstrated in roach (van Aerle et al. 2002), where early development takes a relatively fixed course and any alterations remain permanent. This is not the same in other model species such as zebrafish, which demonstrate developmental plasticity and reversibility of EDC effects (e.g zebrafish; Fenske et al. 2005) — a process that is not seen in roach (Beresford et al. 2004, Liney et al. 2005). The implications of this plasticity in other species may mean that any sexual disruption affects seen (Chapter 5) can be different in other species, or may not arise at all. In addition, the susceptibility of different fish species and their responsiveness to oestrogens are known to be different (Lange et al. 2012). As such, a comprehensive understanding of the ecology and genetic constitution of a population can be considered an integral precursor to investigating population level implications of chemical exposure (Brown et al. 2009).

7.2 Genotyping Application for Population Genetics of Roach

Natural species ranges and their evolution over time can be delineated by population genetics. In-stream obstructions, habitat modification and the popularity of restocking practices are factors that could initiate erosion of genetic variability and increase divergence in UK roach populations. Here, microsatellite markers were effective tools for deciphering previously unexplored population structure of UK roach populations, both spatially and temporally. Demographic indices of census population sizes show widespread abundance of roach populations across much of England, yet significant genetic differentiation and structuring was evident between proximal rivers and stretches on the same river. This is consistent with other freshwater fish species of both cyprinids and salmonids (Spruell et al. 1999) and shows that fish populations are often restricted to specific river stretches, unable to move readily to a different habitat if in-stream conditions become unfavourable.

Populations of roach across the UK have moderately high levels of genetic diversity for a freshwater fish species (Dewoody and Avise 2000). This was perhaps expected due to the significant proportion of the biomass of cyprinids fisheries constituting of roach (~40%) and the large census size evident within the River Thames. To marry with recorded demographic data, the contemporary genetic population structure of this species can be recognised from the results of this study. Modern genetic assemblages of roach in the UK exhibit low levels of genetic differentiation across the sampling range, indicating consistency with previous F_{ST} values for roach (0.036; Hanfling et al. 2004). Despite this, significant differentiation between study sites was evident and a typical pattern of isolation by distance was also adhered to, demonstrating that river distance played a significant role in the divergence between populations. Geographic distance between sites explains genetic distances seen in stone loach, *B. barbatula* and gudgeon, *G. gobio* assemblages in a Hungarian river system, but not chub, *L. cephalus* (Takacs et al. 2008). This emphasises difference in ecology (and resulting genetic structure) of some species, perhaps due to temporal and spatial variation in gene flow and habitat requirements (general or specific).

Widely-distributed species can still show strong site fidelity. The mosaic pattern of habitat modification and in-stream obstructions pose as ecological barriers, which may limit movement of individuals. However, some roach populations, despite separation by impoundments, displayed no significant genetic differentiation; suggesting that patterns of genetic structure differed between stretches, with predominant differences seen in

navigable tributaries of the River Thames. Roach are known to migrate during spawning seasons (L'Abe´e-Lund and Vøllestad 1985) but movements are moderate compared to anadromous species. The likely picture in the Thames is that navigable stretches of river denote easier routes of movement for roach and the regular opening of locks along river courses facilitates migration and gene flow. Overall, permanent in-stream obstructions present across the Thames catchment act to partition different roach populations, making them self-propagating but not reproductively isolated. For the purpose of conservation and management, this conveys more straightforward protocols which consider managing populations as separate units, for which supplementation (if necessary) should ideally occur from populations genetically similar to the recipient.

Roach are known to be capable of adapting their behaviour to fragmented habitats, completing their full life cycle in reaches shorter than 2 km (Geeraerts et al. 2007). Therefore it is plausible that low levels of (downstream) gene flow are balanced by the plasticity of this species to complete biological activities in limited stretches of rivers; retaining population viability and diversity despite physical separation. Intricacies of the role played by barriers in relation to localised gene flow and differentiation and the implications of possible life-cycle adaptation are all instances that require further study to unveil their importance for fish population biodiversity. Acquiring such knowledge should also provide useful data to define specific lines of enquiry, for the target of experimental studies attempting to assess risk, towards vulnerable species and life-history stages.

The ability of roach to survive in highly-disturbed environments parallels known traits of ecologically tolerant and generalist species, able to maintain viable population sizes in perturbed unstable environments. The resolution of microsatellite markers allowed us to understand processes acting over multiple generations which may produce subtle alterations in genetic structuring, perpetuating at a higher level of organisation over time. Calculation of N_e estimates is deemed to be more accurate with multiple samples separated by more than one generation (temporal method, Laikre et al. 1998); accordingly I observed increases in N_e estimates with longer timespans between samples. Nonetheless, no significant differences between allelic richness, heterozygosity or N_e (effective number of breeders in organisms with overlapping generations) were evident between samples taken from the same sites in different years. The biological significance of this finding is restricted by the limited number of sites sampled, however it provides a novel insight into the history of genetic change in roach population dynamics within UK rivers.

7.3 Investigating the Impacts of Effluent Exposure — Bridging the Gap between ndividuals, Populations and Multiple Generations

The insidious trespass of man-made chemicals into surface waters ameliorates their interaction with every organism's communication system, as well as modulating interactions between individuals, and between individuals and their environment. Aquatic populations subject to pollutant exposure are known to experience alterations to reproductive, immune and developmental programming which can impart effects on genetic structure of populations (Belfiore and Anderson 2001). Additionally, subtle effects of EDCs such as changes in breeding behaviour, social interaction and parentage dynamics (Coe et al. 2008) are becoming widely recognised and also have the potential to alter genetic integrity at the population level.

Population genetic theory indicates the maintenance of high genetic diversity and a large N_e are important attributes for ensuring population sustainability. The fate of fish populations in surface waters laced with the residues of WWTW effluents are best studied in the natural environment, where the damaging effect of oestrogenic pollutants can be ascertained in barrier-restricted populations (Wedekind 2014). Wild fish populations collected from impacted environments naturally incorporate individuals from a full life cycle of exposure in addition to mixture effects from multiple chemicals and other environmental stressors. These populations also benefit from encompassing normal social and behavioural interactions that occur in the wild (Ungerer et al. 2008). Chapter 4 presents a unique study that examines the genetic variation and effective population sizes of wild roach populations subject to varying levels of WWTW effluent exposure in rivers throughout the UK. Correlations between the modelled oestrogenic component of effluent and measures of population genetic variation, bottlenecks and N_e were examined but showed no consistent pattern across all sample sites. Thus indicating that effluent contamination of river water is not threatening wild populations of roach, despite negative effects documented in individual fish. Aside from this work, the use of fish in 'environmental genomics' has not been widely applied in relation to stochastic reductions from environmental degradation (Lind and Grahn 2011), so very little is known about the effects of dilute effluent on the genetic structure of other fish populations.

When considering potential risk posed by oestrogens, the importance of successful reproduction and any alterations to normal patterns of parentage become important drivers of population change. Analysis of the reproductive success of males originating from mothers exposed to life-long WWTW effluent showed that they were still able to breed. Importantly, there was no difference in reproductive success when placed in

competitive breeding scenarios with males kept in control conditions. In fact, those fish kept in effluent sired more offspring than those from control conditions, but this was likely a result of increased size/growth of those fish reared in effluent due to food availability. It is possible, given the results of the precursory Lange et al. (2011) study that the levels of EDCs in effluent experienced by the fish used in Chapter 5 was of insufficient magnitude to impact on reproductive success, despite evidence of feminisation in gonads of male roach used in breeding experiments. Perhaps these results simulate more accurately what is taking place in the wild (i.e. lower effluent/oestrogenic concentrations), as they coincide with the findings from Chapter 4 where there is limited evidence of oestrogenic effluents impacting the genetics of roach populations in the UK.

Previous long-term studies of EDC exposure on the reproductive capabilities of wild populations have been carried out with roach, finding that reproductive performance was compromised only in the most severely feminised fish (Harris et al. 2011). Similar findings have been demonstrated in more controlled exposure studies where no adverse effects of continual EE2 exposure were found in populations of longer-living fish species, pearl dace (*Margariscus margarita* Palace et al. 2006) and lake trout (*Salvelinus namaycush* Werner et al. 2006). These findings correspond with those culminating from Chapters 4 and 5, however the wider implications remain difficult to predict. For instance, species with shorter generation times, or those that breed more than once a year, may encounter population-level effects more rapidly (Kidd et al. 2007). This uncertainty in extrapolation across species is compounded by inadequate knowledge of the effects of low chronic exposure, in association with additional environmental stressors.

The results presented in Chapters 4 and 5 benefit from encompassing life-long exposure to EDCs, giving a better overall evaluation of adverse consequences of prolonged exposure on individuals, and across generations. Neither chapter derives a detrimental effect on the population, however both studies have caveats. If conducted again, Chapter 4 would benefit from more detailed knowledge on migration rates of individual roach, to ensure the results seen represent populations from different pollution profiles. Similarly, the influence of roach restocking was mitigated in part by selecting sites separated by major physical barriers; however the introduction of individual roach may have altered genetic signatures of wild populations. Chapter 5 would be greatly improved by additional numbers of individuals and the inclusion of roach derived from paternal males exposed to a lifetime of effluent contamination. Conclusions drawn from this study are limited by the number of replicate exposure scenarios and the lack of chemical analysis

on the effluent over the entire study period. These improvements would allow a more informed evaluation of the vulnerability of wild fish populations to multigenerational exposure, aiding derivation of the importance of male and female fecundity in driving population success.

7.4 EDCs in Context — the Role of Ecology and Geography Influencing Fish Population Patterns

A compendium of biotic and abiotic factors plays an important role in influencing species vulnerability and success. Freshwater fish populations are subject to a host of insidious impacts from human activities (Cowx and Portocarrero-Aya 2011, Figure 7.1); therefore to understand the detrimental impact of EDC exposure on fish population ecology requires interpretation in reference to additional interacting factors. Conclusions drawn in Chapter 6 emphasise the importance of additional environmental drivers of species and genetic diversity. Here I am able to explore current distribution of fish species diversity and genetic diversity of roach populations by integrating patterns of disturbed land use (arable and urban) and biogeographical history. Isolated fish populations appear more vulnerable to land use practices taking place within riverine catchments. This study also emphasised the importance of using multiple species and genetic diversity endpoints to assess the role of environmental factors driving fish population dynamics. Parallel effects at different levels of organisation cannot always be assumed and are therefore recommended to be included in future conservation and management considerations.

Accordingly, the lack of evidence relating to the significance of oestrogenic contamination shaping fish population dynamics, suggests that the importance of exposure appears relatively minor. Justification of this assumption is tempered by few detectable impacts of EDCs on wild fish populations, with evidence including mostly minor or subtle interactions (Mills and Chichester 2005, An et al. 2009, Coe et al. 2008, Lind and Grahn 2011). Endocrine disruption as an environmental phenomenon may therefore be a contributor to long-term deterioration of microevolutionary forces in aquatic animals, indistinctly altering population dynamics of various species (Wang and Zhou 2013). Examples of local adaptation to pollution across fish species specifically lend support for chemical contaminants exerting selection pressures (Whitehead et al. 2010, Wirgin et al. 2011, Lind and Grahn 2011, Williams and Olesiak 2011), allowing individuals to survive and reproduce successfully in polluted locations. Despite this, the role of EDC pollutants as a threat to the survival and fitness of aquatic species appears largely superseded by gross, overt pressures such as habitat loss and fragmentation, which are often of greater significance.

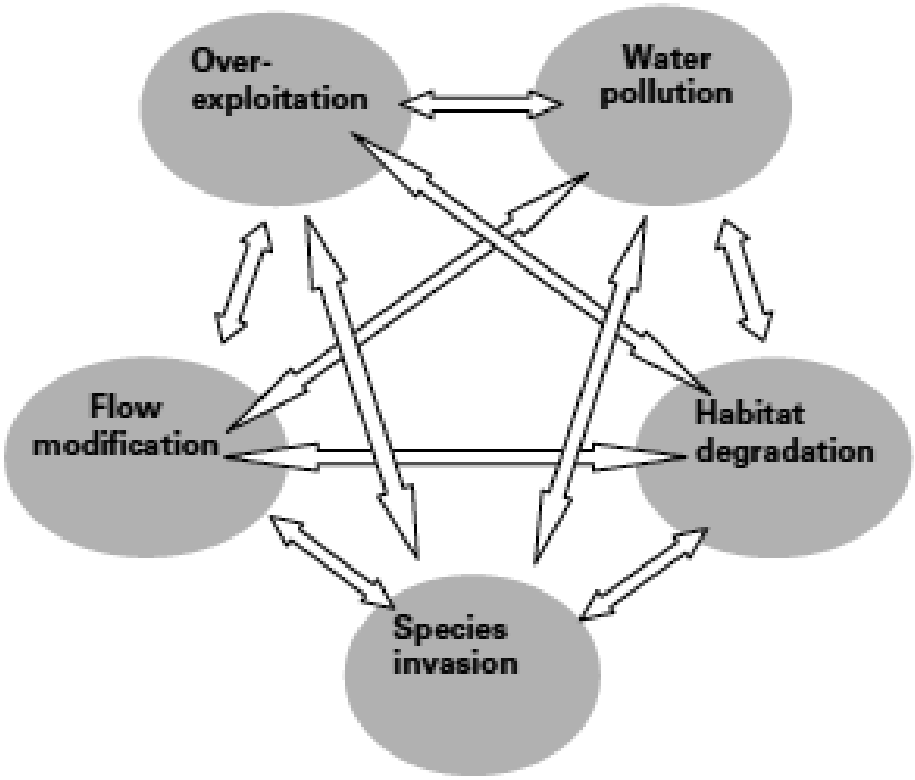


Figure 7.1 Five major threats to freshwater biodiversity and the interactions between them, which shape aquatic population success. From Dudgeon et al. (2006)

7.5 Contribution to Science

1) *I challenged the assumption that reproductive disruptions to individual fish as a result of oestrogenic effluent exposure have population-level implications. My research highlights the difficulty in extrapolating from laboratory/field studies on individuals, to real-world situations.*

2) *I produced novel maps of mean effluent concentration present in rivers across the Thames catchment.*

3) *I combined ecotoxicogenomic approaches with biogeographic principles to provide a systematic population genetic approach for addressing the complexities of potential population responses of fish to oestrogenic contaminant stress.*

4) *I utilised microsatellite markers to derive fine scale population structure of a common cyprinid species across the UK, unveiling little detrimental impact of historic restocking practices or in-stream obstructions to fish population viability.*

5) *I showed, for the first time, that roach populations in restricted stretches of river can be self-sustaining and maintain high genetic diversity, despite high levels of*

oestrogenic pollution (Published in Hamilton et al. 2014).

6) *I characterised the reproductive success of roach to a prolonged, multigenerational WWTW effluent challenge. Finding that impaired reproductive success in roach was not related to exposure history, instead it was predominantly governed by body size.*

7) *I showed that genetic diversity/internal relatedness of roach, and species diversity of the entire fish assemblage displayed an inconsistent pattern across the Thames catchment. Although both were moderated by disturbed land use in the proximal river habitat and watershed location.*

7.6 Recommendations and Future Avenues of Investigation

Spatial and temporal approaches hold great promise for addressing emerging issues in biodiversity management and species conservation. A long- term temporal record can provide important data for predicting future impacts under a changing environmental regime (Costedoat and Gilles 2009). Here, temporal samples of individual roach from the same river sites, over multiple years, gave us an insight into the changes in allele frequencies and thus the relative contributions of true population differentiation versus temporal and stochastic variation. Consequently, routine collection of useful historic DNA samples, for recreationally important species such as the roach should be pursued for tests of temporal contribution to observed genetic structure in natural populations. Fundamental conservation-orientated studies and management concerns would benefit from insights into population-specific temporal variation, uncovering insights into basic evolutionary biology and ecology (Tessier and Bernatchez 1999, Garant et al. 2000) especially in light of environmental change.

The importance of studying behaviour is an area of research that remains in its infancy in relation to EDCs. Previous work has demonstrated alterations in behaviour as a result of EDC exposure, which ultimately have the potential to shift parentage dynamics of group spawning fish (Coe et al. 2008, 2010). Various types of enrichment (environmental complexity and population structure —hierarchies, relatedness, sex ratio, group sizes) can also affect interaction between individuals. Subtle effects on behaviour can change the reproductive contribution of individual fish; a source of variation that may modify population dynamics and therefore needs to be further explored.

A natural progression of this work would be to examine the possibility of selection and adaptation to oestrogenic pollutants. Results from Chapters 4 and 5 bring into question

the reproductive resilience of fish populations exposed continually to effluent, genetically adapting over time. Adaptation to specific chemicals is evident in a range of other fish species (Durrant et al. 2011, Wirgin and Waldman 2004, Williams and Olelsiak 2011) suggesting that chemical contaminants can affect fish populations. These considerations are emphasised when related to longer-lived animals, in that fish have evolved adaptive responses relatively rapidly (~15 generations for killifish *F. heteroclitus,* Williams and Oleksiak 2011). However, demonstrating this process in nature remains a complicated task. I propose that for this to be investigated, adaptive regions of genes which influence susceptibility to pollutants need to be examined. Detecting adaptation is possible through recognised shifts in allele frequencies of genes known to be candidates for selection (due to prior knowledge regarding their function) compared to those not under selection (Wirgin et al. 2011). Linking genetic variants to polluted environments has shown that many loci can be altered during mechanisms of adaptation. However, resistance attributes may also affect the fitness of adapted individuals, so any life-history costs incurred will need to be investigated especially when individuals are challenged by additional environmental stressors (Meyer and Di Giuliuo 2003).

Alterations in the frequency of certain beneficial genes (Williams and Oleksiak 2008, 2011, Lind and Grahn 2011,Wirgin et al. 2011) may be influencing the conclusions drawn in this thesis. Similarly, the proportion of rare alleles within natural populations of roach from highly contaminated sites warrants further investigation. Rare alleles can be especially important in preserving a population's ability to persist in the face of environmental challenge, thus should be considered across environments. Accurately predicting retention of rare alleles and long term viability of roach populations will provide crucial knowledge for rescuing populations and will guide management options to mitigate diversity loss among differentially exposed fish populations in the future.

7.7 Final Conclusion

The component chapters of this thesis demonstrate the complexity apparent with aligning field and laboratory studies concerning the possible impacts of EDCs (specifically oestrogens), within the broader context of additional anthropogenic changes to habitat. Summation of the work presented here, demonstrates no evidence of significant long-term effects of oestrogens (derived from STW effluent) on roach populations. Despite expectancies regarding the negative effects of oestrogenic pollutants on development, reproduction and general viability in fish, I can conclude that: (i) wild populations of roach in the UK are able to maintain substantial genetic diversity in highly contaminated

environments; and (ii) the same populations do not show a reduction in effective population size. Nevertheless, a 65% reduction in N_e could not be ruled out in those roach populations encountering highest exposure (due to confidence limits of the N_e estimates).

Modelled expectations of detrimental effects of oestrogens in fish species, predict biased sex ratios, reduced population growth and subsequent extinctions. None of these impacts could be verified experimentally in roach populations exposed over multiple generations. Although we must stress that it would be inappropriate to suggest these findings would be congruent across the fish fauna. Species differ widely in life history and reproductive strategies, and with the ability to adapt to environmental stressors or groups of contaminants. Targeted experimental studies are therefore required to address some of this variation and complement any findings from wild populations.

I have also shown that the influence of in-stream obstructions and restocking practices have not homogenised the genetic population structure of roach throughout the Thames catchment. Instead, differentiation between populations is largely driven by geographic distance. From the exploration of possible additional drivers of fish population success, both species and genetic diversity are detrimentally impacted by a combination of surrounding arable and urban land use, and geographical isolation. It is not evident however, whether the increased presence of disturbed land creates unfavourable conditions for fish populations through habitat degradation/modification or alterations to in-stream water quality conditions. Nevertheless, diversity of the entire fish population and the genetic diversity of individual roach are eroded in disturbed, isolated river habitats; emphasising the importance of proximal land use on allied riverine environments. Whether these findings present a legacy consistent to all fish populations remains to be explored, however they direct future considerations for conservation research, relevant to the field of fish population sustainability.

Finally, expecting overt changes in wild fish populations as a result of long-term exposure to human-derived pollutants may be simplistic; neglecting the complexity of aquatic ecosystems. The results of this thesis emphasise the lack of sufficient understanding regarding the major threats to fish at the population level. Within a broader fisheries ecology context, the effect of EDCs may be minor in comparison to other anthropogenic impacts such as habitat alterations, overfishing, alien species and the pressures of a changing climate. However, such a myriad of drivers do not act alone; so subtleties of EDC exposure may work in concert or be exacerbated under changing environmental regimes. Similarly, genetic differences within or between populations could influence the

responsiveness of species in light of environmental change. Therefore, future studies should work to encompass multiple stressors, and multiple endpoints of species and genetic variation, in an effort to try and replicate a more realistic scenario for the wider sphere of fish population ecology and management.

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