Non-native Bullhead in Scotland

Molecular and Morphological Identification and Parasite Links with Native Fauna

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To the adventures to come.

Abstract

The arrival of a non-native species to has the potential to shape native communities by influencing ecological interactions such as predation, foraging, competition and disease transfer. A designation of invasive is applied to an introduced non-native species that has the potential to threaten the continued wellbeing of a native species, pose a risk to human health or negatively impact the economy. The European bullhead (*Cottus perifretum*) is a freshwater benthic-dwelling fish that is native to England but considered invasive in Scotland. The species was first reported in Scotland in the 1950's and thriving populations are now established in the waters of the Clyde, Forth and Tweed catchments. Bullhead presence is thought to negatively impact native stone loach (*Barbatula barbatula*) and brown trout (*Salmo trutta*) parr, due to shared preferences for habitat and prey resources. They are also thought to prey upon the eggs of native Atlantic salmon (*Salmo salar*) and brown trout, two species that are of high commercial value in Scotland. In other areas of introduction, bullheads have been found to increase parasite infection rates in native fishes. The species therefore has the capacity to incite competition and alter parasite/host interactions in areas of introduction, to the potential detriment of native fauna and the Scottish economy. The European bullhead has been the subject of considerable taxonomic scrutiny in recent years, resulting in its reclassification as a species complex. What was once considered a single species with a distribution encompassing Europe, Russia, Asia and Scandinavia, has been shown to consist of at least 15 distinct species. Genetic examination of bullheads from England confirmed the presence of *Cottus perifretum*, not *Cottus gobio* as traditionally listed in all UK literature and legislation. Native English bullhead is currently protected under Annex II of the European Commission Habitats Directive 92/43/EEC, based on the historic assumption that the species present is *C. gobio*. Analysis of the taxonomic identity of Scottish bullheads has remained outstanding.

In this study the invasive status of bullhead was explored by examining feeding and parasitological interactions between bullheads and native freshwater communities in south-east Scotland. An assessment of the feeding preferences of native brown trout and stone loach in the presence and absence of bullheads tested competition for prey resources. Parasitological interactions were investigated by examining the shared parasite fauna of bullheads and native fish and invertebrate species. Bullheads from the Clyde and Forth catchments were analysed to provide a molecular and morphological description of this introduced species.

Phylogenetic analysis of COX1 sequences obtained from Scottish bullheads, and a pair-wise distance calculation based on a Kimura 2-parameter model**,** showed that samples clustered in a distinct clade with English *C. perifretum*. Significant intraspecific variation was reported in all morphological features examined, but pooled data also revealed a resemblance to the published description provided for *C. perifretum*. Scottish bullhead is therefore confirmed to be an introduced pocket of the native English species, which is considered under threat in some areas due to habitat modifications and population decline. Comparisons between the dietary compositions of bullheads, brown trout and stone loach showed that the prey selection of brown trout and stone loach varied in the sample locations that contained co-occuring bullheads, when compared to locations where bullheads were absent. However, no direct evidence of trophic competition between bullheads and either brown trout or stone loach was reported. An examination of parasitological interactions recovered eight parasite species from four distinct taxonomic groups in total, of which four species (*Echinorhynchus* *truttae, Apatemon gracilis, Diplostomum volvens* and *Raphidascaris acus*) were shared between bullheads and one or more native fishes. *Echinorhynchus truttae* was also shared with gammarid crustaceans. Bullhead presence was found to coincide with *D. volvens* infections in European minnow (*Phoxinus phoxinus*) and a reduced prevalence of *E. truttae* in brown trout.

Diplostomum volvens was only reported from minnow found in sites with bullheads, strongly suggesting bullheads were responsible for introducing this parasite to native minnows. Bullheads functioned as an alternative host for *E. truttae,* diluting brown trout parasite loads and reducing overall infection rates in sites where bullhead and brown trout co-exist. The findings reported for both feeding preference and parasite burdens in the presence and absence of bullheads suggest that bullheads do have some effect on the ecology of native species, but these are considered minimal and unlikely to impact the long-term survival of native species. Eradication of Scottish bullhead may contradict the conservation effort that is currently in place. Given the current lack of evidence to validate bullhead's invasive qualities and the recent confirmation of its genetic lineage, revisiting the designation of the bullhead as invasive is warranted. Active eradication should be treated with caution until a significant negative impact can be proven.

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List of In-text Abbreviations

A	Abundance
AIC	Akaike information criterion
ANCOVA	Analysis of covariance
ANOSIM	Analysis of similarities
BCa	Bias-corrected and accelerated
BL	Body length
BLAST	Basic local alignment search tool
BW	Body width
CB	Copulatory bursa
CCB	Community composition by biomass
CCC	Community composition by count
CG	Cement gland
COI	Cytochrome c oxidase I
Cy	Cyst
CyW	Cyst wall
DLI	Distance between lappets (interior)
DLP	Distance between lappets (peaks)
DNA	Deoxyribonucleic acid
E	Eyespots
HB	Holdfast organ breadth
HL	Holdfast organ length
Ho	Holdfast organ
I	Intensity
L	Lips
Lb	Lobes
Le	Lemnisci
Ll	Lateral line
Lp	Lappets
M	Metacercariae

MCMC	-	Monte Carlo Markov chain
MDS	-	Non-metric multidimensional scaling
ML	-	Maximum likelihood
mtDNA	-	Mitochondrial Deoxyribonucleic acid
N	-	Nerve ring
NBN	-	National Biodiversity Network
Oe	-	Oesophagus
Os	-	Oral sucker
OSB	-	Oral sucker breadth
OSL	-	Oral sucker length
Ph	-	Pharynx
P	-	Prevalence
PCR	-	Polymerase chain reaction
Pr	-	Proboscis
PCR-RAPD	-	Random amplified polymorphic polymerase chain reaction
PERMANOVA	-	Permutational multivariate analysis of variance
Ps	-	Proboscis sheath
RNA	-	Ribonucleic acid
Sc	-	Scolex
SD	-	Standard deviation
SEM	-	Scanning electron microscopy
SEPA	-	Scottish Environmental Protection Agency
Sg	-	Segmentation
SIMPER	-	Similarity percentages analysis
Su	-	Suckers
SV	-	Seminal vesicle
T	-	Teeth
Te	-	Testis
Ti/Tv	-	Transition/transversion
TN	-	Tamura-Nei

UK - United Kingdom

USA - United States of America

Vs - Ventral sucker

VSA - Distance between ventral sucker and anterior

VSB - Ventral sucker breadth

VSL - Ventral sucker length

WFD - Water Framework Directive

Chapter 1. Introduction

1.1 Freshwater Fishes in the British Isles

Native (or indigenous) species are those that occur naturally within a given area and are thought to have done so since before the Neolithic period (Manchester and Bullock, 2000). Natural dispersal and migration of these species occur independently of human interference (Copp *et al.,* 2005). Non-native (also referred to as 'alien' or 'exotic') species are those that have been introduced outside of their natural range by direct or indirect anthropogenic actions (Jeschke *et al.,* 2014). The terms 'non-native' and 'introduced' can therefore be used interchangeably.

At the end of the last ice-age, some 13,000 to 15,000 years ago, an ice cap covered Scotland and the northern half of England and Wales. Two main theories have been put forth to explain the presence of obligate freshwater fishes in the British Isles after the ice cap began to retreat. Schindler (1957) proposed the existence of glacial refuges in areas of southern England that were not covered by the ice sheet. It was suggested that some freshwater species were able to survive in these locations, and therefore predate the last period of glaciation in the UK. This theory is contested by Wheeler (1977), who reasoned that deoxygenated waters and extreme temperatures during this period would have made essential processes such as gonad maturation, spawning and fry development extremely difficult. Wheeler (1977) put forth the widely accepted theory that a large number of freshwater species arrived in Great Britain by a land bridge that spanned the North Sea for 300 years and connected England to the continent. This region contained freshwater rivers that flowed to England from tributaries of the River Rhine (Maitland, 1994), and were the precursors for a number of major eastern English rivers including the Humber, the East Anglian and the Thames (Wheeler, 1977). A number of obligate freshwater species currently found in Britain, such as ruffe (*Gymnocephalus cernuus*), spined loach (*Cobitis taenia*), silver bream (*Blicca bjoerkna*) and barbel (*Barbus barbus*), are thought to have arrived by these means (Lucas *et al.,* 1998).

Truly native Scottish freshwater fish species are few in number. As the land bridge did not extend north to northern England and Scotland, colonization of Scottish waters by the same means was not possible. Fish that were found in the freshwater regions of Scotland at the time were those with marine-associated ancestors (e.g. Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*)), or the capacity to survive in the ice lakes and glacial rivers still present at the time (Maitland, 1994). Euryhaline fishes, the majority of which spawn in fresh water, found Scottish waters easily accessible and became established as resident Scottish fauna. Many Scottish estuarine species (e.g. common sturgeon (*Acipenser sturio*), sea bass (*Dicentrarchus labrax*), and grey mullet (*Chelon labrosus*)) are thought to have been present in Scottish estuaries for thousands of years (Maitland, 1974). As the ice sheet retreated, many of the freshwater fish species found in England gradually migrated north to colonize new river systems. Those that reached Scotland took up residency alongside the relatively small number of species that had recently colonized these waters, to establish the communities of native fish fauna still found in Scottish rivers and lochs today.

Other freshwater species that arrived more recently in Scotland's history did so either through natural dispersal, expanding their native range from other areas of the British Isles, or by human-mediated introductions. Natural dispersal between unconnected water bodies can result from fish egg adhesion and subsequent transport on the feet of waterfowl, or opportunistic migration during flooding events (Maitland, 1994). Although these events are uncommon, flooding between closely associated water bodies is a probable method of fish transport. Waterfowl transport of invertebrate eggs is considered an important means of invertebrate introduction in freshwater systems (e.g. Figuerola and Green, 2002), but the desiccation of fish eggs that would occur during transport, fish preference for the positioning of eggs under gravel and rocks, and the high number of individuals required to successfully establish a population make this a less likely method for the introduction of most new fish species. It is more likely that anthropogenic actions, whether intentional or accidental, have been responsible for the majority of fish introductions that have occurred in British waters (Campbell, 1971). Maitland (1987) gives a detailed historical account of Scottish fish stocks and the northward range expansion of fish from English waters. The Victorian era saw the import of a large number of non-native species from England, Asia and North America, as the fashion for housing collections of rare exotic species and stocking sport fish spread. A surge in the number of freshwater fishes resident in Scottish waters followed. Since the 19th century, anthropogenic actions have almost doubled the number of Scottish freshwater fish on record (Maitland, 1977).

It has since been estimated that invasive species constitute 38% of all freshwater fish species currently found in Scotland (Maitland, 2004). A species is considered invasive when its presence is found to be detrimental to the economy, pose a threat to public health or threaten the continued survival of native fauna. The European bullhead of the *Cottus* genus is one such fish, currently considered invasive in Scotland due to the overlap in feeding and habitat niche that exists between native stone loach (*Barbatula barbatula*) and brown trout fry, and its perceived capacity to displace native fishes.

1.2 Bullhead Dispersal in the British Isles

Bullheads are indigenous only to south-east England (Maitland and Campbell, 1992) and are now well distributed throughout England and Wales (Smyly, 1957; Tomlinson and Perrow, 2003; Carter *et al.,* 2004; Boon and Lee, 2005). A dense population of bullheads in the Clyde catchment in the west of Scotland was first documented by Patton (1951) and Gemmel (1962). Specimens were reported in the Gogar Burn just outside Edinburgh in the late 1960's (Morris, 1978). Maitland (1977) documented a Scottish distribution encompassing the Earn area of the Clyde, and the Gogar and Leith areas of the Forth catchment. The waters of the Clyde and Forth catchments still support the majority of established bullhead populations in Scotland, although a small population of bullheads have now also been reported in the Ale water of the Tweed catchment (J. Hunt 2017, pers. comm. 19 January 2017) (Figure 1.1). Anthropogenic introductions are considered the likely explanation for bullhead presence in Scotland (Maitland, 1972; Tomlinson and Perrow, 2003), although its lack of economic or angling value have lead some authors to question whether this was done with intent (Mills and Mann, 1983).

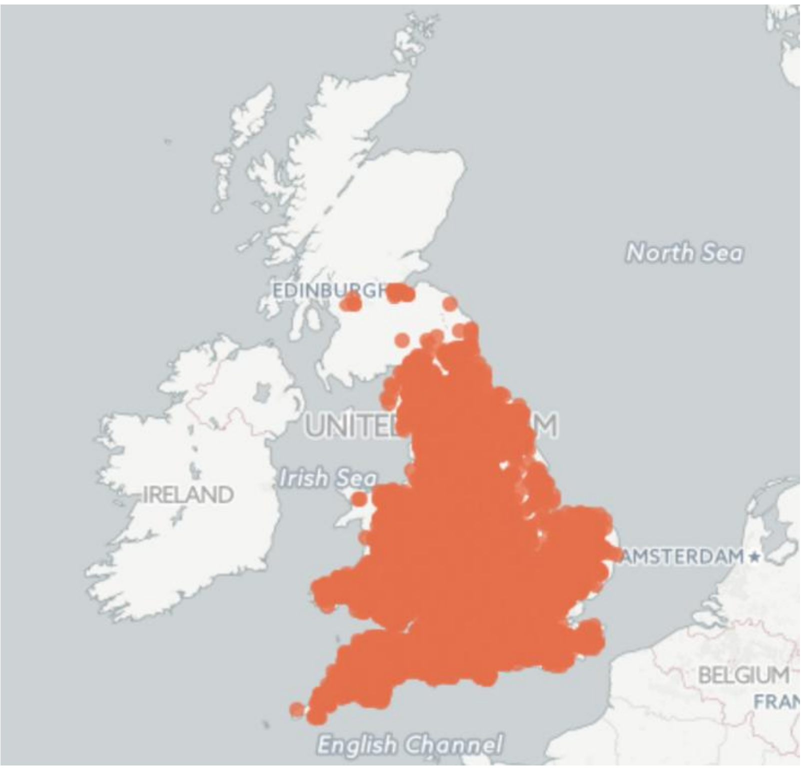

Figure 1.1. Distribution of non-native bullhead in the British Isles (NBN Atlas)

1.3 Bullhead Life History

Thriving populations of bullhead can be found in both lakes and rivers in the British Isles. Scottish populations are restricted to lowland rivers but English bullheads inhabit a range of water types, including the lowland chalk streams of southern England and the relatively acidic moorland streams of northern England (Mann and Orr, 1969; Fox, 1978). Rheophilic individuals are more common, and show a preference for fast flowing stony stretches of rivers and streams (Newdick, 1979). Fish are found closely associated with the stones and rocks of the river bed, surrounded by a substrate of firm sand or gravel. Mark recapture studies have shown that bullheads possess a strong homing instinct, with large numbers of recaptures recorded in the same area over repeated chronological sampling (Smyly, 1957). Males are territorial and spend the majority of their time around a home stone, which they will defend during mating time using croaking vocalisations and head-nodding threat displays (Tomlinson and Perrow, 2003).

Bullheads are generally inactive during the day, displaying short bursts of activity at dawn and dusk when chasing prey. They are considered opportunistic feeders and will prey upon a range of available invertebrates. Smyly (1957) compared the diets of river and lake dwelling bullheads in England and attributed the dietary variations observed to local and seasonal availability. Individuals were found to select moving prey that could be actively stalked, showing a strong preference for invertebrate bottom fauna such as the nymphs of mayfly and stonefly, caddisfly larvae; and representatives of the genera *Gammarus* and *Sialis.*

Physiologically, bullheads are highly adapted to the benthic environment they favour. The species is sexually dimorphic but both sexes exhibit dark mottled skin, flattened dorsoventral tapering and no swimbladder (Tomlinson and Perrow, 2003) (Figure 1.2). Males possess a wider head and larger mouth in comparison to the narrow head and pointed snout of the female (Morris, 1954). Growth rates vary between the sexes, with males generally growing more rapidly than females (Mann, 1971). Mature males also possess a triangular genital papilla which is absent in the female, and display darkened head colouration and a whitish-yellow trim along the anterior section of the dorsal fin during the breeding season (Smyly, 1957).

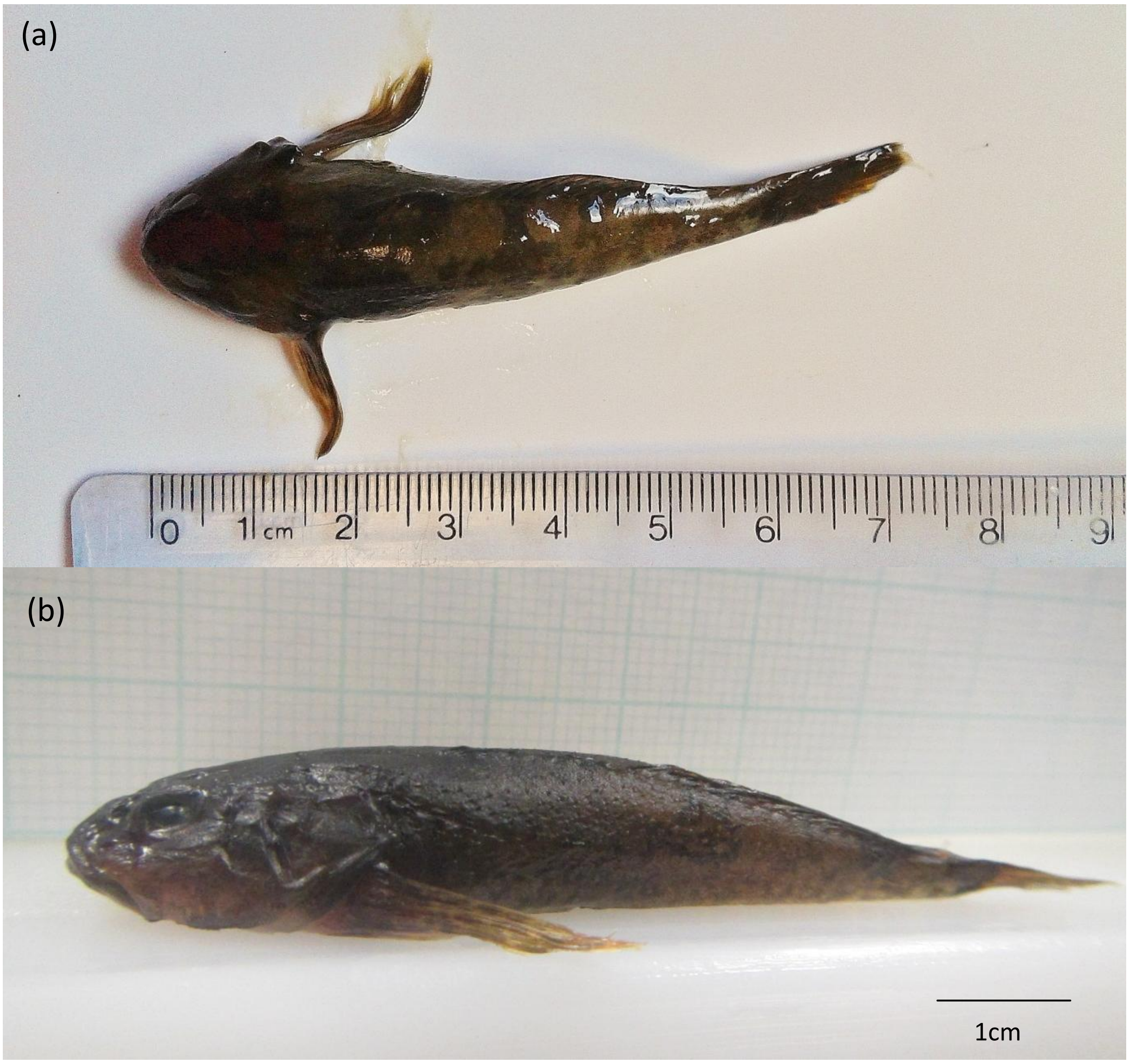

Figure 1.2. Scottish bullhead specimens. (a) Dorsal view showing lateral tapering of the body and dark mottled skin of adult bullhead from the River Almond. (b) Lateral view of tapering along the anteroposterior axis of adult bullhead from the River Almond

Breeding generally takes place between March and April, although slight regional and altitude variations occur. Male bullheads create a nest underneath their home stone in preparation for the arrival of a gravid female. The nest is defended against rivals by threat displays such as head nodding, raised gill covers and dorsal fins, and body undulations; all of which collectively function to make the defending male appear larger (Morris, 1954). Once a nest is complete, the male will remain sedentary inside, with its head protruding slightly from the entrance until the appearance of a gravid female. Morris (1954) gave a detailed account of the behaviours that precede spawning. The female predominantly remains upside down in the nest, near the roof where the eggs will be deposited. The male then positions himself alongside the female, twisting his body to bring their tails close together. A distinct mating display then follows, with the male nodding vigorously, erecting his dorsal fins and pushing himself up on his pelvic fins. This behaviour may continue for some time until spawning commences.

Females produce eggs approximately 2-2.4mm in diameter, with clutch size determined by the age and length of the female. Fecundity is positively correlated to female length, age and ambient water temperature, but negatively affected by local fish population density (Abdoli *et al.,* 2005). Smyly (1957) noted clutches of 50-250 eggs in English bullheads, but Abdoli *et al*. (2005) recorded some females in France that produced almost 900 eggs. Fertilization occurs externally. After reproduction the spent female leaves the nest and the male remains to protect the fertilised eggs. Smyly (1957) observed an instance in which a second female then entered the nest and produced a new batch of eggs with the same male. Morris (1954) also recorded males with two gravid females inside the nest simultaneously. Parental care of egg clutches is the sole responsibility of the male. Rare examples of paired parental formations have been documented but are considered exceptional (Morris, 1954). Throughout egg development, males move oxygen-rich water over the developing eggs by fanning their pectoral fins. Fanning frequency and intensity vary with ambient water temperature (Morris, 1954). Experimental removal of the male at this time quickly results in the development of fungi unless the eggs are placed in running water (Smyly, 1957). During this period of development, males can come under attack by other larger individuals, who seize the parental male by the head using their jaws (Smyly, 1957; Morris, 1954). Fighting will cease as soon as the smaller male moves away from the stone, thereby leaving his eggs to the mercy of the in-coming larger male (Smyly, 1957). Bisazza and Marconato (1988) noted a high number of incidences of unmated larger males displacing smaller egg-guarding males from their nests. Females show preference for males already guarding egg clutches, so this behaviour is thought to ultimately improve the male's chances of attracting a mate (Bisazza and Marconato, 1988).

Newly-hatched fry disperse into the surrounding substrate, to grow and develop in the same stretch of water in which they were spawned. The species exhibits a limited migratory capacity, despite the rapid spread throughout water bodies that has been reported within its Scottish range. Dispersal throughout a catchment is likely a consequence of forced movement due to high population densities, downstream flooding events and human-mediated translocations. High population densities and habitat dominance have facilitated bullhead's establishment in Scotland, lending weight to its invasive designation.

1.4 Taxonomic Considerations and Bullhead Legislation

The narrow home range and limited migratory capacity of bullheads make this an ideal fish to study in the context of speciation, as different populations rarely have a chance to mix and share genetic material. Speciation events occur when different evolutionary linneages are formed by reproductive isolation (de Queiroz, 1998), usually driven by sexual selection (Panhuis *et al*., 2001), habitat specialisation (Rice and Salt, 1990) or an extrinsic barrier (Palumbi, 1994). In the context of bullhead speciation, species groups are defined by the phylogenetic species concept (Mishler, 1985), wherein species are represented by an irreducible group of monophyletic organisms that share a set of derived and inherited characteristics.

It has historically been considered that a single species, *Cottus gobio*, spans Europe, with different countries maintaining isolated populations of the same species. Early studies used morphological characteristics to attempt to distinguish specimens from different populations (e.g. Koli, 1969). The regional variations uncovered led Kottelat (1997) to propose the *C. gobio* species complex, a grouping of multiple distinct species that have previously been considered a single species with a European-wide distribution. Advances in molecular techniques have subsequently shaped our understanding of *Cottus* taxonomy, confirming the existence of fifteen distinct species all previously misidentified under the *C. gobio* species complex (Freyhof *et al*., 2005) (Table 1.1). Of these, a single species, *Cottus perifretum,* has been described for the British Isles. The tissue used for this analysis was from a limited range of locations in England, and failed to take into account the introduced populations of unknown origin in Scotland. As such, classification of Scottish bullheads remains unresolved.

Despite these advances in *Cottus* taxonomy, all publications and legislation concerning bullheads in the British Isles continue to reference the species *C. gobio*. This is of particular relevance to environmental management decisions, as *C. gobio* is presently listed under Annex II of the European Commission Habitats Directive 92/43/EEC. As *C. gobio* has historically been considered a single species with a European-wide distribution, declining populations of bullheads in other areas of Europe prompted a continent-wide protection order on the species, in an attempt to help threatened stocks recover. Despite thriving numbers of bullheads across England and established populations in Scotland, current UK legislation therefore protects bullheads within their native English range. Revisitation of this legislative order seems warranted given the recent updates in *Cottus* taxonomy. Classification of Scottish bullheads will facilitate this process, by establishing whether individuals resident in Scottish waters are genetically similar to English *C. perifretum*, and confirming the current distribution of *C. perifretum* in the UK.

Table 1.1. Taxonomic classification and range description of fifteen species of the genus *Cottus* historically confused under the *C. gobio* species complex (Freyhof *et al.,* 2005)

Species	Distribution
C. gobio	Northern Baltic basin in Scandinavia to stream Maurine in south-western German Baltic basin; coastal Sweden, Finland and Russia southwest to Estonia; River Danube (excl. upper tributaries of Save and Arges); Rivers Elbe, Rhone and Weser; tributaries of upper Rhine north to Mainz (Germany); uppermost tributaries of the River Tevere (Italy); Adriatic drainages from River Potenza (Italy) to River Zrmanja (Croatia) (excl. Timavo spring); and Steenputbeek stream in Scheldt drainage (likely introduced).
C. poecilopus	Carpathian streams draining to the Danube, Dniestr, Vistula and Odra; Baltic Sea basin; central Finnish lakes; several streams in southern Finland; several lakes in northern Germany, Lake Hańcza (Poland), River Skjernaa drainage (Denmark); southern portion of Lake Onega drainage; and River Orb east to River Lena (Siberia).
C. ferrugineus	Tributaries of the Adriatic Sea
C. petiti	Streams from the River Lez (France)
C. hispaniolensis	Southern Pyrenean drainage of the River Garonne (Northern Spain)
C. koshewnikowi	Drainages of the Upper River Volga, River Ural and River Dniepr (Russia), northern and eastern Gulf of Bosnia (Finland), northern Sweden and Baltic tributaries west of Estonia
C. pellegrini	Upper Tisza basin (Ukraine)
C. microstomus	Dniestr drainage of Black Sea basin (Ukraine and Moldova), River Odra and Vistula (Poland) in the Southern Baltic drainage
C. aturi	Drainages of River Adour (France) and River Nivelle (Spain)
C. duranii	Upper Dordogne, Lot and Loire drainages (France)
C. rondelati	Hérault drainage (France)
C. perifretum	**River Scheldt (Belgium), Seine and lower Loire (France), Rhine (Germany), Garonne drainage (France and northern Spain) and all rivers of Great Britain**
C. rhenanus	Lower Rhine (Germany) and Meuse drainages (Belgium and the Netherlands)
C. scaturigo	Timavo spring (northeastern Italy)
C. metae	Upper Save (Slovenia, Croatia, Bosnia and Herzegovina and Serbia) in the Danube drainage
C. transsilvaniae	Upper Arges (southern Romania)

1.5 Ecological Considerations of Introduced Bullheads in Scotland

In order to be considered invasive on ecological grounds, a species must threaten the continued health and survival of native biota. These threats can be unpredictable and result from either direct biological interactions or indirect secondary influences. Direct biological interactions are termed biotic impacts, and encompass ecologica processes such as competitive displacement due to prey and habitat availability, hybridisation and disease transfer. Indirect influences are factors such as habitat modification, wherein the presence or actions of an introduced species alter the existing habitat to make it inhospitable to resident natives.

The biological impacts of invaders are perhaps the most documented in invasive studies. Hybridisation is considered a common and significant problem in fish conservation (Allendorf *et al.,* 2001). The consequential loss of genetic variability and potential for hybrid offspring to outcompete parental lineages can have severe consequences for long-term species survival. Hybridisation has been uncovered between several freshwater fishes both in the UK and abroad (e.g. in Scotland, Maitland, 1987; USA, Muhlfeld *et al.,* 2017). Hybrids have also been recorded between distinct *Cottus* species that were previously grouped under the *C. gobio* species complex (e.g. Mavárez and Linares, 2008; Stemshorn *et al.,* 2011; Vítek *et al*., 2014). Competition for food resources is a common consequence of invasions and can impact several interconnected ecological processes. The introduction of non-native rainbow trout (*Oncorhynchus mykiss*) in Northern Japan prompted a shift in prey selection by the native Dolly Varden charr (*Salvelinus malma*), and was found to coincide with the restructuring of stream and riparian food-webs (Baxter *et al*., 2004). Chinese mitten crab (*Eriocheir sinensi*), invasive in some regions of the UK, show a preference for *Gammarus* *pulex* amphipod prey, which forms a significant portion of the diet of native white-clawed crayfish (*Austropotamobius pallipes*). As a result, their presence was shown to influence productivity and energy transference throughout native communities (Rosewarne *et al.,* 2016). The interconnected nature of such interactions highlights the need for a community-based approach when evaluating the impact of an invasive species.

Indirect impacts are often more difficult to detect, as they usually occur alongside more direct biological interactions. The introduction and subsequent spread of North American signal crayfish (*Pacifastacus leniusculus*) in the UK has restricted habitat availability and habitat quality for native benthic fishes (Guan and Wiles, 1997), displacing local populations of stone loach (*Barbatula barbatula*) and bullhead in English waters. Their role as vectors for the crayfish plague disease agent *Aphanomyces astaci* has also been well documented (e.g. James *et al.,* 2017). This disease is fatal to native white-clawed crayfish (*Austropotamobius pallipes*), despite causing limited pathological affects in its original *P. leniusculus* host. Strauss *et al.* (2012) introduced the term 'disease mediated invasion' to define such cases, wherein invasive species transfer an invasive pathogen to a native population. Invasive species can bring with them novel parasites and disease agents that can spread rapidly to native species and threaten the survival of native fauna. Once introduced, these parasites and pathogens can remain in native communities even without the continued presence of their original host. The introduction of *Anguillicoloides crassus*, a nematode accidentally introduced to the UK by the Japanese eel (*Anguilla japonica*), has had a catastrophic effect on European eel stocks by significantly compromising swimbladder function, despite no current occurrence of *A. japonica* in UK waters (Barry *et al.,* 2014). The role of invasive species as vectors for parasite and disease transfer is often overlooked in invasion ecology, but is potentially the most significant consequence of non-native species establishment. The introduction of non-native parasites to a native community may drive some of the changes in ecological processes that are commonly reported. Amundsen *et al.* (2013) noted that parasites played a key role in driving the changes in food web structure that resulted from the introduction of invasive fishes in a Norwegian lake. Prenter *et al.* (2004) reviewed the literature documenting the role parasites play in invasion success in their concise review. Parasites have ultimately been shown to both influence a host's chance of initial invasion success, and provoke long-term impacts on the native communities to which they are introduced. Their potential involvement in community dynamics should therefore not be overlooked in invasive studies.

The Scottish bullhead is thriving in areas of introduction, and is considered a highly successful invader due to the high population densities found in these regions. The ecological factors that have led to its successful establishment, and the impacts of its presence in Scottish waters, have not been examined in any published works. Bullheads are known to share macroinvertebrate prey with native brown trout (*Salmo trutta*) and stone loach, with which they also share similar habitat preferences. Such a close association in both feeding and habitat requirements implies competitive interactions with native species are highly likely. In other areas of introduction, where bullheads from the *C. gobio* species complex are considered invasive, the species has been shown to spread parasites and increase infection rates in native fishes (e.g. in Finland, Ieshko *et al.,* 2013; and Czech Republic, Moravec, 2001). A study into the parasite fauna of bullheads and co-habiting Scottish fishes would allow this relationship to be explored, and determine if host/parasite interactions have been influenced by the arrival of non-native bullheads to Scottish waters.

1.6 Scope of This Project and Research Aims

Developments in our understanding of the *C. gobio* species complex have resulted in the re-classification of the British fish species traditionally considered to be *C. gobio* as *C. perifretum,* based on material analysed from three English rivers (Freyhof *et al.,* 2005). To date, no genetic analysis has been conducted on resident populations of Scottish bullhead. Given that current legislation and management protocols within Scotland and the rest of the British Isles are based on the presence of *C. gobio*, the need to establish the true identity of the Scottish bullhead species is clear. Taxonomic classification of Scottish bullheads, through morphological and molecular examination, is the first aim of this study and will form the basis of the first part of this thesis.

A level of ambiguity also exists concerning the invasive status of bullheads in Scotland. In order to put forth a case for their invasive or neutral status, and hence a proposed change in the legislation relating to the management of this species, it is necessary to determine if there is a case for considering Scottish bullhead invasive. The second aim of this thesis is to investigate the ecological interactions between introduced bullheads and native Scottish fauna in this context. Competition from feeding niche overlap between bullheads and other native fishes, and parasitological interaction with native fauna, are potential sources of conflict that have been documented in other areas where bullheads have been introduced. Investigating the feeding preferences of bullheads, stone loach and brown trout will shed light on the prospective feeding niche overlap between these species, and hence the level of interspecific competition that exists for prey resources. The close association and similar dietary preferences between bullheads and native benthic fishes suggests that the sharing of parasites is also likely. Previous studies of introduced bullheads across Europe have demonstrated that bullheads have the capacity to facilitate the spread of parasites to native fish communities. An analysis of the parasite links between introduced bullheads and native fishes will therefore help to determine whether introduced bullheads pose a significant risk to the survival of native Scottish fauna. An examination of feeding niche overlap and shared parasite fauna will form the second part of this thesis. Results will help to clarify if conditions for an invasive designation on the basis of ecological grounds are met.

Once the taxonomic classification for Scottish bullheads has been determined and several of the parameters for invasiveness have been tested, evidence for the revisitation of UK legislation pertaining to all UK bullheads is put forth. Recommendations for an updated management strategy in the context of future policy changes are explored in the final chapter, in order to provide scientific outputs that contribute to the continued preservation of native freshwater communities in the UK.

1.7 References

Abdoli A., Pont D. and Sagnes P. (2005) Influence of female age, body size and environmental conditions on annual egg production of the bullhead. *Journal of Fish Biology* **67**, 1327–1341.

Allendorf F. W., Leary R. F., Spruell P. and Wenburg J. K. (2001) The problems with hybrids: Setting conservation guidelines. *Trends in Ecology and Evolution* **16**, 613–622.

Amundsen P-A., Lafferty K. D., Knudsen R., Primicerio R., Kristoffersen R., Klemetsen A. and Kuris A. M. (2013) New parasites and predators follow the introduction of two fish species to a subarctic lake: implications for food-web structure and functioning. *Oecologia* **171**, 993–1002.

Barry J., McLeish J., Dodd J. A., Turnbull J. F., Boylan P. and Adams C. E. (2014) Introduced parasite *Anguillicola crassus* infection significantly impedes swim bladder function in the European eel *Anguilla anguilla* (L.). *Journal of Fish Diseases*, **37** (10), 921-924.

Baxter C., Fausch K., Murakami M. and Chapman P. (2004) Fish invasion restructures stream and forest food webs by interrupting reciprocal prey subsidies. *Ecology* **85**, 2656–2663.

Bisazza A. and Marconato A. (1988) Female mate choice, male-male competition and parental care in the river bullhead, *Cottus gobio* L. (Pisces, Cottidae). *Animal Behaviour* **36**, 1352–1360.

Boon P. and Lee A. (2005) Falling through the cracks: are European directives and international conventions the panacea for freshwater nature conservation? *Freshwater Forum* **24**, 24–37.

Campbell R. N. (1971) The growth of brown trout *Salmo trutta* L. in northern Scottish lochs with special reference to the improvement of fisheries. *Journal of Fish Biology* **3**, 1–28.

Carter M. G., Copp G. H. and Szomlai V. (2004) Seasonal abundance and microhabitat use of bullhead *Cottus gobio* and accompanying fish species in the River Avon (Hampshire), and implications for conservation. *Aquatic Conservation: Marine and Freshwater Ecosystems* **14**, 395–412.

Copp G. H., Bianco P. G., Bogutskaya N. G., Eros T., Falka I., Ferreira M. T., Fox M. G., Freyhof J., Gozlan R. E., Grabowska J., Kovac V., Moreno-Amich R., Naseka A. M., Penaz M., Povz M., Przybylski M., Robillard M., Russell I. C., Stakenas S., Sumer S., Vila-Gispert A. and Wiesner C. (2005) To be, or not to be, a non-native freshwater fish? *Journal of Applied Ichthyology*, **21** (4), 242–262.

de Queiroz K. (1998) The general lineage concept of species, species criteria, and the process of speciation: a conceptual unification and terminological recommendations. in *Endless Forms: Species and Speciation*, ed Howard D. J. and Berlocher S. H. Oxford University Press, Oxford.

Figuerola J. and Green A. J. (2002) Dispersal of aquatic organisms by waterbirds: a review of past research and priorities for future studies. *Freshwater Biology* **47**, 483–494.

Fox P. (1978) Preliminary observations on different reproduction strategies in the bullhead (*Cottus gobio* L.) in northern and southern England. *Journal of Fish Biology* **12**, 5–11.

Freyhof J., Kottelat M. and Nolte A. (2005) Taxonomic diversity of European *Cottus* with description of eight new species (Teleostei: Cottidae). *Ichthyological Exploration of Freshwaters* **16**, 107–172.

Gemmel H. (1962) Miller's thumb bullhead in Renfrewshire. *Glasgow Naturalist* **18**, 213-214.

Guan R. Z. and Wiles P. R. (1997) Ecological impact of introduced crayfish on benthic fishes in a British Lowland River. *Conservation Biology* **11**, 641–647.

Ieshko E. P., Shul'man B. S., Lebedeva D. I., Barskaya Y. Y. and Niemela E. (2013) Bullhead (*Cottus gobio* L.) invasion in the Utsjoki River (Northern Finland): Parasitological aspects. *Russian Journal of Biological Invasions* **4**, 17–23.

James J., Nutbeam-Tuffs S., Cable J., Mrugala A., Viñuela-Rodriguez N., Petrusek A. and Oidtmann B. (2017) The prevalence of *Aphanomyces astaci* in invasive signal crayfish from the UK and implications for native crayfish conservation. *Parasitology* **144**, 411–418.

Jeschke J. M., Bacher S., Blackburn T. M., Dick J. T., Essl F., Evans T., Gaertner M., Hulme P. E., Kühn I., Mrugała A. and Pergl J. (2014) Defining the impact of non-native species. *Conservation Biology* **28** (5), 1188-1194.

Koli L. (1969). Geographical variation of *Cottus gobio* L. (Pisces, Cottidae) in Northern Europe. *Annales Zoologici Fennici* ***6***, 353–390.

Kottelat M. (1997) European freshwater fishes. An heuristic checklist of the freshwater fishes of Europe (exclusive of former USSR), with an introduction for non-systematists and comments on nomenclature and conservation. *Biologia Bratislava* **52**, 1–271.

Lucas M. C., Mercer T., Batley E., Frear P. A., Peirson G., Duncan A. and Kubecka J. (1998) Spatio-temporal variations in the distribution and abundance of fish in the Yorkshire Ouse system. *Science of the Total Environment* **210**/**211**, 437–455.

Maitland P. S. (1972) A key to the freshwater fishes of the British Isles: with notes on their distribution and ecology. *Freshwater Biological Association Scientific Publication* **27**, 1-139.

Maitland P. S. (1974) The conservation of freshwater fishes in the British Isles. *Biological Conservation **6*** (1), 7–14.

Maitland P. S. (1977) Freshwater fish in Scotland in the 18th, 19th and 20th centuries. *Biological Conservation*, **12** (4), 265-278.

Maitland P. S. (1987) Fish introductions and translocations - their impact on the British Isles. In: Maitland P. S.; Turner A. K., (eds.). Angling and wildlife in fresh waters. Grange-over-Sands, NERC/ITE, 57-65. (ITE Symposium, 19).

Maitland P. S., Boon P. J. and McLusky D. S. (1994) *The Fresh Waters of Scotland.* John Wiley & Sons, Great Britain.

Maitland P. S. (2004). Evaluating the ecological and conservation status of freshwater fish communities in the United Kingdom. Scottish Natural Heritage Commissioned Report No. 001 (ROAME No. F01AC6).

Maitland P. S. and Campbell R. N. (1992) *Freshwater Fishes*. Harper Collins Publishers, U.K.

Manchester S. J. and Bullock J. M. (2000) The impacts of non-native species on UK biodiversity and the effectiveness of control. *Journal of Applied Ecology* **37**, 845–864.

Mann R. H. K. (1971) The populations, growth and production of fish in four small streams in Southern England. *Journal of Animal Ecology* **40**, 155–190.

Mann R. H. K. and Orr D. R. O. (1969) A preliminary study of the feeding relationships of fish in a hard-water and a soft-water stream in southern England. *Journal of Fish Biology* **1**, 31-44.

Mavárez J. and Linares M. (2008) Homoploid hybrid speciation in action. *Molecular Ecology* **17**, 4181–185.

Mills C. A. and Mann R. H. K. (1983) The bullhead *Cottus gobio,* a versatile and successful fish. *Annnual Report of the Freshwater Biological Association,* Ambleside, Cumbria, 76–88.

Mishler B. D. (1985) The morphological, developmental, and phylogenetic basis of species concepts in bryophytes. *Bryologist* **88**, 207-214.

Moravec F. (2001) Common sculpin *Cottus gobio* as a natural paratenic host of *Proteocephalus longicollis* (Cestoda: Proteocephalidae), a parasite of salmonids, in Europe. *Diseases of Aquatic Organisms* **45**, 155–8.

Morris D. (1954) The reproductive behaviour of the river bullhead (*Cottus gobio* L.), with special reference to the fanning activity. *Behaviour* **7**, 1–32.

Morris K. H. (1978) The food of the bullhead (*Cottus gobio* L.) in the Gogar Burn, Lothian, Scotland. *Forth Naturalist and Historian* **7**, 31-44.

Muhlfeld C. C., Kovach R. P., Al-Chokhachy R., Amish S. J., Kershner J. L., Leary R. F., Lowe W. H., Luikart G., Matson P., Schmetterling D. A., Shepard B. B., Westley P. A .H., Whited D., Whiteley A. and Allendorf F. W. (2017) Legacy introductions and climatic variation explain spatiotemporal patterns of invasive hybridization in a native trout. *Global Change Biology* **23**, 1–12.

NBN Atlas (2017) *Cottus gobio* https://species.nbnatlas.org/species/NHMSYS0000544660#overview. Accessed 02 May, 2017.

Newdick J. (1979) *The Complete Freshwater Fishes of the British Isles*. A & C Black Publishers Ltd, Great Britain.

Palumbi S. R. (1994) Genetic divergence, reproductive isolation and, and marine speciation. *Annual Review of Ecology and Systematics* **25**, 547-572.

Panhuis T. M., Butlin R., Zuk M. and Tregenza T. (2001) Sexual selection and Speciation. *Trends in Ecology and Evolution* **16**, 364-371.

Patton D. (1951) Miller's Thumb *Cottus gobio* in Renfrewshire. *Scottish Naturalist* **63**, 185.

Prenter J., MacNeil C., Dick J. T. A. and Dunn A. M. (2004) Roles of parasites in animal invasions. *Trends in Ecology and Evolution* **19**, 385–390.

Rice W. R. and Salt G. W. (1990) The evolution of reproductive isolation as a correlated character under sympatric conditions: experimental evidence. *Evolution* **44**, 1140-1152.

Rosewarne P. J., Mortimer R. J. G., Newton R. J., Grocock C., Wing C. D. and Dunn A. M. (2016) Feeding behaviour, predatory functional responses and trophic interactions of the invasive Chinese mitten crab (*Eriocheir sinensis*) and signal crayfish (*Pacifastacus leniusculus*). *Freshwater Biology* **61**, 426–443.

Schindler O. (1957) *Freshwater Fishes*. Translated by P. A. Orkin. Thames & Hudson, London.

Smyly W. (1957) The life-history of the bullhead or miller's thumb (*Cottus gobio* L.). *Proceedings of the Zoological Society of London* **128**, 431–454.

Stemshorn K. C., Reed F. A., Nolte A. W. and Tautz D. (2011) Rapid formation of distinct hybrid lineages after secondary contact of two fish species (*Cottus* sp.). *Molecular Ecology* **20**, 1475–1491.

Strauss A., White A. and Boots M. (2012) Invading with biological weapons: the importance of disease-mediated invasions. *Functional Ecology* **26**, 1249–1261.

Tomlinson M. L. and Perrow M. R. (2003). Ecology of the Bullhead. Conserving Natura 2000 Rivers Ecology Series No. 4. English Nature, Peterborough.

Vítek T., Halačka K., Bartoňová-Marešová E., Vetešník L. and Spurný P. (2014) Identification of natural hybrids between *Cottus poecilopus*, Heckel, 1837, and *Cottus gobio*, Linnaeus, 1758, at a hybrid zone on the Svratka River (Czech Republic). *Journal of Applied Ichthyology* **30**, 102–108.

Wheeler A. (1977) The origin and distribution of the freshwater fishes of the British Isles. *Journal of Biogeography* ***4*** (1), 1–24.

Chapter 2. Study Location and Sample Collection

2.1 Location

This study focused primarily on the river systems of Edinburgh, Midlothian and West Lothian. Material was also collected from the Clyde catchment for use in Chapter 3, in which the taxonomic identity of Scottish bullhead was investigated. No material was collected from the Ale Water in the Tweed catchment, as this recently established population was unknown to the author at the time. Sampling locations were selected on the basis of communities known to contain bullheads *Cottus perifretum* and bullhead-free control sites. Multiple unconnected water bodies were examined, in order to maximise community and genetic diversity.

Thirteen sample sites were selected in total, encompassing four separate river systems across Edinburgh and the Lothians, and one location in the Clyde catchment (Table 2.1). The overall Water Framework Directive (WFD) 2015 classification of each location ranged from moderate (Earn Water) and moderate ecological potential (Burdiehouse Burn), to bad ecological potential (Braid Burn) and poor (all others) (SEPA, 2015) (Table 2.2). It should be noted, however, that overall WFD classifications are based on a "one-out, all-out" system, resulting in sites receiving the lowest designation of all parameters considered (Hering *et al*., 2010). The biological parameters measured showed a greater range of values and more sites receiving moderate or high status when invertebrates and fish were considered independently.

Table 2.1. Information on the 13 sampling locations used throughout the study. Locations are listed by site name, river system, geographical location, chapters in which specimens from the location were used; and the presence and absence of bullheads within the fish community

Site	Water Body	Location	Relevant Chapter(s)	Bullhead Present/Absent
Brox Burn	River Almond	55° 55' 51.58" N 3° 29' 53.42" W	3	Present
Earn Water	White Cart Water (River Clyde)	55° 45' 10.85'' N 4° 19' 42.19" W	3	Present
Breich Water	River Almond	55° 52' 09.51'' N 3° 34' 22.84'' W	5	Absent
Carlops	River North Esk	55° 47' 30.34'' N 3° 03' 54.57'' W	4, 5	Absent
Happy Valley	River Almond	55° 52' 07.88'' N 3° 36' 31.36'' W	4, 5	Absent
Killandean Burn	River Almond	55° 52' 33.48'' N 3° 33' 03.82'' W	4, 5	Present
Kirkliston	Niddry Burn (River Almond)	55° 57' 01.05'' N 3° 24' 25.49'' W	4, 5	Present
Murieston Water	River Almond	55° 51' 39.82'' N 3° 30' 45.53'' W	4, 5	Absent
Oatridge College	Ecclesmachan Burn (River Almond)	55° 56' 50.86'' N 3° 30' 42.38'' W	4, 5	Absent
Braid Burn	Braid Burn	55° 57' 22.04" N 3° 07' 01.39" W	3, 4, 5	Present
Burdiehouse Burn	Burdiehouse Burn	55° 56' 44.55'' N 3° 04' 43.06" W	3, 4, 5	Present
Livingston/River Almond*	River Almond	55° 53' 21.69'' N 3° 30' 05.69'' W	3, 4, 5	Present
Newbattle Abbey/River North Esk*	River Esk	55° 52' 57.24'' N 3° 03' 54.57'' W	3, 4, 5	Present

* Sites named by water body in Chapter 3 and a site-specific name in Chapters 4 and 5

Table 2.2. 2015 WFD classification parameters for the 13 sampling locations used throughout this study (SEPA, 2015). Parameters are either given a pass/fail designation (specific pollutants category and each of the metal contaminant parameters) or ranking from poor to high (all other parameters). All parameters are colour coded based on their designation

	Brox Burn	**Earn Water**	**Breich Water**	**Carlops**	**Happy Valley**	**Killandean Burn**	**Kirkliston**	**Murieston Water**	**Oatridge College**	**Braid Burn**	**Burdiehouse Burn**	**Livingston**	**Newbattle Abbey**
Overall status	**Poor**	**Moderate**	**Poor**	**Poor**	**Poor**	**Poor**	**Poor**	**Poor**	**Poor**	**Bad ecological potential**	**Moderate ecological potential**	**Poor**	**Poor**
Overall ecology	Poor	Moderate	Poor	High	Poor	Poor	Poor	Poor	Poor	Poor	Moderate	Poor	Poor
Physico-chemical parameters	-	High	Moderate	High	Good	Good	Poor	High	Poor	Good	Good	Moderate	Good
Soluble reactive phosphorus	High	High	Moderate	High	Good	Good	Poor	High	Poor	Good	Good	Moderate	Good
Dissolved oxygen	High	High	High	High	High	High	High	High	High	High	High	High	High
Acidity	-	High	High	High	High	High	High	High	High	High	High	High	High
pH	High	High	High	High	High	High	High	High	High	High	High	High	High
Biological elements	Poor	Moderate	Poor	Poor	Poor	Poor	Poor	Poor	Poor	Moderate	Moderate	Poor	Poor
Invertebrate animals	Good	-	-	-	-	High	-	-	-	-	-	Good	-
Macroinvertebrates (RICT/WHPT)	Good	-	-	-	-	High	-	-	-	-	-	Good	-
Macroinvertebrates (ASPT)	Good	Good	Good	High	Moderate	High	Good	High	Good	Moderate	Moderate	Good	Good
Macroinvertebrates (NTAXA)	High	High	High	High	High	High	High	High	High	High	High	High	Good
Fish	Poor	High	Poor	Poor	Poor	Poor	Poor	Poor	Poor	High	High	Poor	Poor
Fish barrier	Poor	High	Poor	Poor	Poor	Poor	Poor	Poor	Poor	High	High	Poor	Poor
Aquatic plants	-	Moderate	-	-	Moderate	-	-	-	-	Moderate	Good	Moderate	Moderate
Macrophytes	-	Good	-	-	Good	-	-	-	-	Good	Good	Good	High
Phytobenthos (diatoms)	-	Moderate	Moderate	-	Moderate	-	Good	High	Good	Moderate	High	Moderate	Moderate
Specific pollutants	-	Pass	Fail	Pass	Fail	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Fail
Iron	-	-	Pass	Pass	Pass	Pass	-	Pass	-	-	-	Pass	Pass
Ammonium	Pass	Pass	Fail	Pass	Fail	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Manganese	-	Pass	Pass	Pass	Fail	Pass	-	Pass	-	-	-	Pass	Fail
Morphology	Good	Good	Good	High	Good	Good	Good	High	Good	Bad	Moderate	Good	Good
Overall hydrology	Good	Moderate	Moderate	High	High	High	High	High	High	High	Good	High	Good
Modelled hydrology	Good	Moderate	Moderate	High	High	High	High	High	High	High	Good	High	Good
Hydrology (medium/high flows)	High	Moderate	High	High	High	High	High	High	High	High	High	High	Good
Hydrology (low flows)	Good	High	Moderate	High	High	High	High	High	High	High	Good	High	High
Hydromorphology	Good	Moderate	Moderate	High	Good	Good	Good	High	Good	Bad	Moderate	Good	Good

Fishes from multiple locations were used in Chapters 3, 4 and 5 of this study. Where possible, an attempt was made to maximise the amount of information that could be extracted from each individual, in order to prevent unnecessary fish removals from native communities. Some locations did not yield sufficiently high numbers of sympatric native fishes or identifiable stomach contents, and have therefore been excluded from the relevant chapters of this study. The sampling locations utilised in each chapter are specified in the individual methods sections.

2.2 Specimen Collection

2.2.1 Fish Communities

Bullheads from the Earn Water were donated by Dr. William Yeomans of the Clyde River Foundation. Specimens were collected in the summer of 2016 and frozen immediately after sampling. Individuals were then transported on ice and stored in a -20°C freezer at Edinburgh Napier University until further investigation.

Fishes collected from the east coast sites were collected under the license held by Dr Robert Briers at Edinburgh Napier University. Sampling took place during the summer of 2014 and 2015, (for all sites except Murieston and Carlops) and 2015 and 2016 (Murieston and Carlops). Fishes were collected by electro-fishing using a Smith-Root LR-24 backpack electrofisher, with voltage automatically adjusted to reflect the conductivity at each site. Three successive ten minute runs were completed, and the captured fish population assessed (Figure 2.1). Thirty-five bullheads were selected from locations containing bullheads, along with a representative sample of the sympatric fish community, relative to the population density and availability at each site. Across all communities, the fish fauna included bullheads, stone loach (*Barbatula barbatula*), brown trout (*Salmo trutta*), European minnow (*Phoxinus phoxinus*) and European flounder (*Platichthys flesus*) (Figure 2.2). With the exclusion of bullheads, individuals were only taken when populations were considered robust enough to support their removal. This was determined by electrofishing success at the end of the three successive electrofishing runs, based on the number of individuals recovered and the age classes represented in each population (based on fish body lengths). Maintaining stable bullhead populations was not a concern due to the extremely high population densities evident across Scotland and the invasive designation of the species. The brown trout population at Carlops was deemed too small in 2016 to allow for the removal of any fish, resulting in only one year of samples available for this location. Specimens of minnow and stone loach were restricted to adult sizes, and a mixture of parr and adult brown trout were selected. A mixture of adult and juvenile life stages of bullheads and flounder were also taken. Fishes that were retained for the study were exposed to a lethal dose of 100mgL^{-1} benzocaine solution by trained personnel, placed in individual plastic bags and transferred immediately to a laboratory freezer (maintained at -20 °C). All other fishes were returned to the river. Sites were revisited the following year for another sampling session.

Figure 2.1. Fish capturing and sorting during fieldwork (a) banner and hand-net trapping of fish captured by electrofishing, and (b) recording of fish numbers after three successive runs. (a) Courtesy of the Forth Fisheries Trust

Figure 2.2. Fish species sampled from across Edinburgh and the Lothians that were used in the study (a) bullhead b) stone loach (c) brown trout (d) minnow and (e) flounder. (c) Courtesy of Dr Rob Briers

2.2.2 Invertebrate Communities

Invertebrate sampling was conducted in May of 2014 and 2015 (all sites except Murieston and Carlops) and 2015 and 2016 (Murieston and Carlops). Freshwater invertebrates were collected with standard fine-mesh sweep nets using a kick-sampling method (Figure 2.3a) (Barbour *et al.*, 1999; AQEM, 2002). At each site the river was divided equally between two samplers. A simultaneous three minute kick-sample was conducted across each half of the river, with sampling effort distributed evenly across the river to cover all available habitats equally. Rocks and large pieces of vegetation that were inadvertently collected during the process were examined in-situ at the end of the three minutes and discarded after any clinging invertebrates had been removed (Figure 2.3b). Invertebrates from each sample site were then placed in an individual 3 litre collection jar containing a sample of river water, and transported to the laboratory for immediate processing (see Chapter 4). This process was repeated over two consecutive years.

Figure 2.3. Invertebrate collection methods (a) kick sampling technique (b) volunteers checking contents of nets to remove large stones and woody debris after sampling

2.3 References

AQEM (2002). Manual for the application of the AQEM method. *A Comprehensive Method to Assess European Streams Using Benthic Macroinvertebrates, Developed for the Purpose of the Water Framework Directive. Version 1.0*, February 2002, 1-89.

Barbour M. T., Gerritsen J., Snyder B. D., and Stribling J. B. (1999). *Rapid Bioassessment Protocols for Use in Streams and Wadeable Rivers: Periphyton, Benthic Macroinvertebrates and Fish*. Second Edition. EPA/841-B-99-002. U.S. EPA, Office of Water, Washington, D.C, 1-197.

Hering D., Borja A., Carstensen J., Carvalho L., Elliott M., Feld C. K., Heiskanen A. S., Johnson R. K., Moe J., Pont D., Solheim A. L. and Van De Bund W. (2010) The European Water Framework Directive at the age of 10: A critical review of the achievements with recommendations for the future. *Science of the Total Environment* **408**, 4007–4019.

Scottish Environmental Protection Agency (2015) Water Classification Hub https://www.sepa.org.uk/data-visualisation/water-classification-hub/. Accessed on 23rd July, 2017.

Part I: Identification of Introduced Scottish Bullhead

Newbattle Abbey sample site on the River North Esk

Chapter 3. Identification of Invasive Bullhead *Cottus perifretum* (Cottidae, Scorpaeniformes) in Scotland based on Morphology and Molecular Phylogenetic Analysis

3.1 Introduction

The genus *Cottus* (Cottidae, Scorpaeniformes) is widely distributed throughout Europe, Siberia, North America and Asia, and represents the most speciose taxonomic group of freshwater sculpins (Goto *et al.,* 2015). Within Europe, a number of distinct species have been historically misidentified under the species name *Cottus gobio*, commonly known as the European bullhead. What was once thought to be a single species with a continent-wide distribution is now considered a species complex (Kottelat, 1997), and at least 15 species have subsequently been described across Europe (Freyhof *et al.,* 2005).

Morphological descriptors were historically used to distinguish between similarly related species. Variations in *Cottus* morphological characteristics both between and within populations across Europe have been well documented. Nybelin (1958) divided European *Cottus* species into two groups based on the number of pores present on the chin. Those within the *C. gobio* group possess one median chin pore whilst two pores are present in specimens of *Cottus poecilopus*. Koli (1969) examined a selection of northern European bullheads to determine geographical variations in external morphology. Three morphological features showed considerable variation: the distribution and presence/absence of skin prickling, the number of pores found along the lateral line, and the overall length of the lateral line. The density and distribution of skin prickling was found to best distinguish bullheads from different regions, albeit with considerable variation within populations. Prickling was most evident in specimens from northern Sweden, the inland waters of Finland and the former eastern Soviet Union. Bullheads originating from

Britain, the majority of Sweden and the region extending from the Pyrenees to the Carpathian mountains showed a lower density of prickling overall, with prickles completely absent in some specimens. These results led Koli (1969) to propose the existence of two morphological strains of bullheads that originated from separate post-glacial migratory pathways, with a secondary zone of intergradation in regions where these pathways historically overlap. Molecular studies have subsequently exposed an intergradation zone in the Rhine that contains two distinct species which also interbreed, resulting in hybrids (Englbrecht *et al.,* 2000).

Traditional morphological descriptors were unable to satisfactorily resolve the taxonomic uncertainty present within the European bullhead species complex, but through combination with molecular markers a more detailed picture of European *Cottus* phylogeny has been uncovered. The early examination of allozyme markers from different populations across Europe provided a measure of heterozygosity that had not previously been observed. Studies were conducted across south-western Germany and the connecting water bodies of France (Riffel and Schreiber, 1995; 1998); north-east Bavaria (Hänfling and Brandl, 1998a; 1998b), and southern France (Eppe, 1999), and showed significant allozyme diversity between populations of bullheads from within the same drainage basin. Mitochondrial DNA (mtDNA) and microsatellite loci have also been used to examine genetic relationships between populations in Finland (Kontula and Väinölä, 2004) and the drainage waters of the Adriatic (Šlechtová *et al.,* 2004). On a larger scale, a comprehensive phylogeographical analysis across the majority of the *C. gobio* European range was conducted by Englbrecht *et al.* (2000), based on 12 new microsatellite loci that were published in an earlier study (Englbrecht *et al.,* 1999). Analysis of molecular distances between haplotypes of different populations identified six distinct clades within the *C. gobio* species complex. The results were consistent with the genetic variability within the Rhine basin documented in earlier allozyme studies (Riffel and Schreiber 1995; 1998). Volckaert *et al.* (2002) identified a seventh clade in the Brittany-Loire region of France and described a larger geographical range for the North Sea and Lower Rhine clades than previously recognised. Subsequent studies have been able to assign populations to these clades based on mtDNA from closely associated populations (Knapen *et al*., 2003) and microsatellite markers from populations distributed across Europe (Hänfling *et al*., 2002). After analysing all available publications describing the genetic diversity within European cottids, Freyhof *et al.* (2005) ultimately confirmed the existence of nine genetic lineages across Europe, containing fifteen distinct species.

A detailed summery of the molecular advances that have helped to restructure the *C. gobio* species complex is presented by Freyhof *et al.* (2005). The authors assimilated this knowledge and combined it with their own detailed analyses of morphometric and morphological characteristics of individuals from across Europe, in order to resolve the taxonomic uncertainty surrounding the European bullhead. Of the fifteen confirmed European *Cottus* species, a single species, *Cottus perifretum,* was described for Great Britain (Freyhof *et al*., 2005). The material examined from the British Isles was taken from a limited number of location in England, where bullheads are a native species. Within the British Isles, bullheads are widely distributed across their native range in England and Wales (Smyly, 1957; Wheeler, 1977; Tomlinson and Perrow, 2003; Carter *et al.,* 2004; Boon and Lee, 2005), and found in four catchments in Scotland, where the species is considered invasive. An invasive designation is in place owing to a perceived threat to native salmonids and other fishes, due to competition and displacement from niche overlap (e.g. Pihlaja *et al.,* 1998; Carter *et al.,* 2004; Elliott, 2006), and bullhead consumption of salmonid eggs (e.g. Smyly, 1957; Gaudin and Caillere, 2000; Palm *et al.,* 2009). Bullheads are thought to have been introduced to Scotland in the 1950's (Smyly, 1957; Maitland, 1972, Maitland, 1977; Tomlinson and Perrow, 2003), although a lack of economic or angling value has led some to suggest the introduction was accidental (Mills and Mann, 1983). The waters draining into the Clyde and Forth estuaries and a small area of the Tweed catchment constitute the only known occurrences of bullheads in Scotland.

Differences in morphological characteristics exhibited by bullheads from across England were noted by Wheeler (1977), who reported two distinct morphologies in eastern England; a smooth skinned form with a complete lateral line extending across the length of the body wall, and a second morph with spinulose (prickled) skin and an incomplete lateral line. The finding of two separate spawning tactics in bullheads from the north and south of England by Fox (1978) appeared to support the idea of two separate genetic lineages in the British Isles. Hänfling *et al.* (2002) examined microsatellite markers in specimens from a range of locations across the described range of *C. perifretum*, and found a close association between specimens from the north of England and Wales. A noticeable divergence was found when comparing these specimens against those of southern England and the Scheldt. An isolation by distance analysis of the Northern England and Wales grouping showed that the high levels of association observed were not simply a result of the close geographic proximity of the sampled populations. Significant genetic variability is therefore evident between bullhead populations in southern England and those found in northern England and Wales.

Freyhof *et al.* (2005) were limited in the material they were able to examine from Britain. In their analysis, samples from the River Wensum and Great Ouse in Southeast England followed the general species description given for *C. perifretum*, but individuals examined from the River Wharfe in Yorkshire showed some differing morphological features, with an elongated body shape and a decrease in the amount of prickling present under the pectoral fin. No examination was conducted on specimens of bullhead originating from the novel populations that have established in Scotland. As the origin of Scottish bullhead populations is unknown, further investigation into the morphological and molecular characteristics of individuals from the northernmost limits of the British range will further our understanding of the level of speciation present within the British Isles. Confirmation of taxonomic identity is of particular importance owing to the emphasis placed on *C. gobio* conservation across Europe and throughout England. Detailed molecular and morphological analysis will also provide evidence to support the species' designation as invasive in Scottish waters.

3.1.1 Aims and Objectives

This study aims to provide a taxonomic identity for Scottish bullhead by conducting molecular and morphological analysis of bullheads from the Forth and Clyde catchments. The results of these analyses are compared to the descriptions provided for English *C. perifretum* and those of the other European *Cottus* species previously grouped under the *C. gobio* species complex. Results are discussed in the context of taxonomic revisions within the genus *Cottus*, and their contribution to our understanding of bullhead's invasive designation in Scotland. Although the exact timing and location of bullhead introduction is unknown, it is hypthesised that Scottish populations have originated from specimens that have been translocated from England; and that Scottish bullheads are therefore a genetic match to the English bullhead *C. perifretum*.

3.2 Methods

Specimens were collected from five sites on the east coast of Scotland and one location on the west coast (Figure 3.1). Samples from the east coast covered four separate rivers (the River North Esk, Burdiehouse Burn, Braid Burn and River Almond) and two locations on the same river that were separated by impassable barriers (Brox Burn and River Almond).

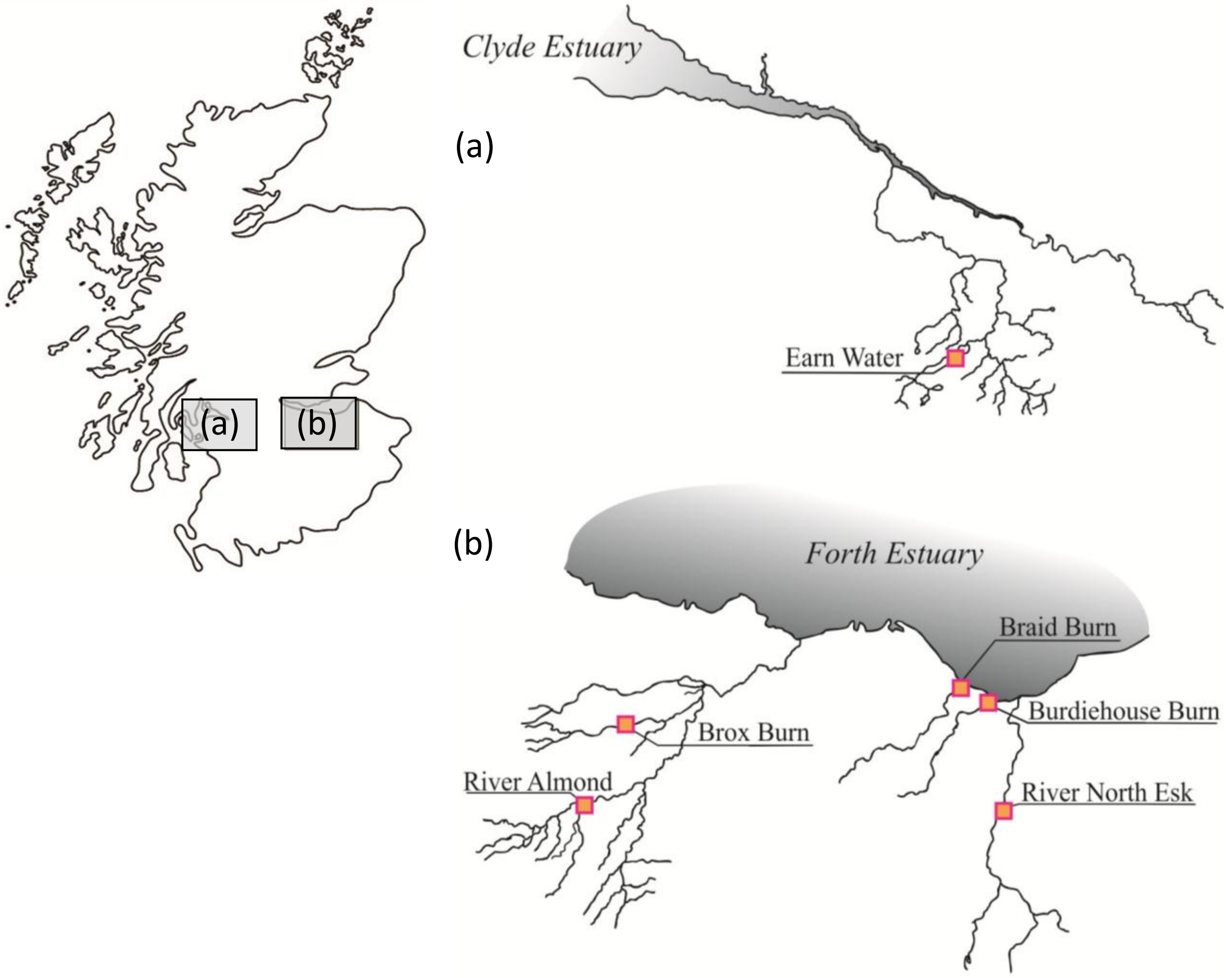

Figure 3.1. Location of sampling sites used for molecular and morphological analysis (a) Earn Water sample site within the Clyde catchment in the west of Scotland (b) five sampling sites within the Forth catchment on the Scottish east coast

3.2.1 DNA Isolation, PCR and Sequencing

Fish were thawed at room temperature for 30 minutes in preparation for morphological examination and tissue sampling for DNA extraction. Specimens were examined under an Olympus SZ51 dissection microscope and the organs exposed using a cranio-caudal incision from the gill covers to the vent region. Approximately 25mg of muscle tissue and 10mg of liver were then extracted from each individual and placed in individual sterile epindorf tubes. DNA was extracted from liver and muscle tissue using Qiagen® DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), as per the protocol provided. A 30 minute RNA digestion step using 1µl RNAseA (5µg/ml) was added to the end of the lysation process to ensure a purified end product. The mitochondrial gene Cytochrome c oxidase I (COI) was PCR amplified using the combination of primers COI FW 5'-TTCTCGACTAATCACAAAGACATT-3' and COI REV 5'-TAGACTTCAGGGTGACCAAAGAATCA-3' (Sonnenberg *et al.,* 2007), and puReTaq Ready-to-go PCR beads (GE Healthcare). The total reaction mix comprised 2.5µl DNA, 1µl COI FW primer, 1µl COI REV primer, 20.5 µl dH_2O and a PCR bead. PCR amplifications consisted of a 15 minute initial denaturation phase at 95°C; followed by 45 cycles of: 20 seconds at 94°C (denaturing), 90 seconds at 52°C (annealing) and 90 seconds at 72°C (extension); and a final extension phase at 72°C for 8 minutes. PCR products corresponding to the expected size were then gel isolated using a 25 minute electrophoresis process, and extracted from the gel using an Ultraclean® 15 DNA purification kit (Mo BIO, California, USA). The DNA content of each purified product was measured using a Nanovue spectrophotometer. A final sequencing mixture composed of 2µl of either COI FW or COI REV primer, and 75ng/15µl DNA (with the discrepancy in volume made up with dH_2O), was obtained. Forward and reverse reactions were sequenced by Eurofins Genomics in Germany.

3.2.2 Morphological Analysis

Bullhead specimens from the River Almond (n=33), River North Esk (n=35), Burdiehouse Burn (n=55), Braid Burn (n=35), Brox Burn (n=34) and Earn Water (n=37) were examined for both quantitative and descriptive morphological features. Morphological characters were selected based on their capacity to discriminate between bullhead populations in earlier publications (Koli, 1969; Riffel and Schreiber, 1998; Freyhof *et al*., 2005). These included meristic traits commonly observed in fish studies and bullhead-specific factors concerning the distribution and density of modified dermal skin prickles (Figure 3.2 and 3.3). The coverage and extent of prickling was reported as a visual estimate of the percentage of the posterior body wall covered in prickles. When conducting fin ray counts, rays extending from the same pterygiophore were counted as 1.5 rays, as in the work of Freyhof *et al*. (2005). Body weight, standard length (from tip of the snout to end of hypural complex) and full length (from tip of the snout to tip of caudal fin) were also recorded for each specimen. All examinations were completed with the assistance of an Olympic SZ51 dissection microscope using magnifications ranging from 0.8x - 4x.

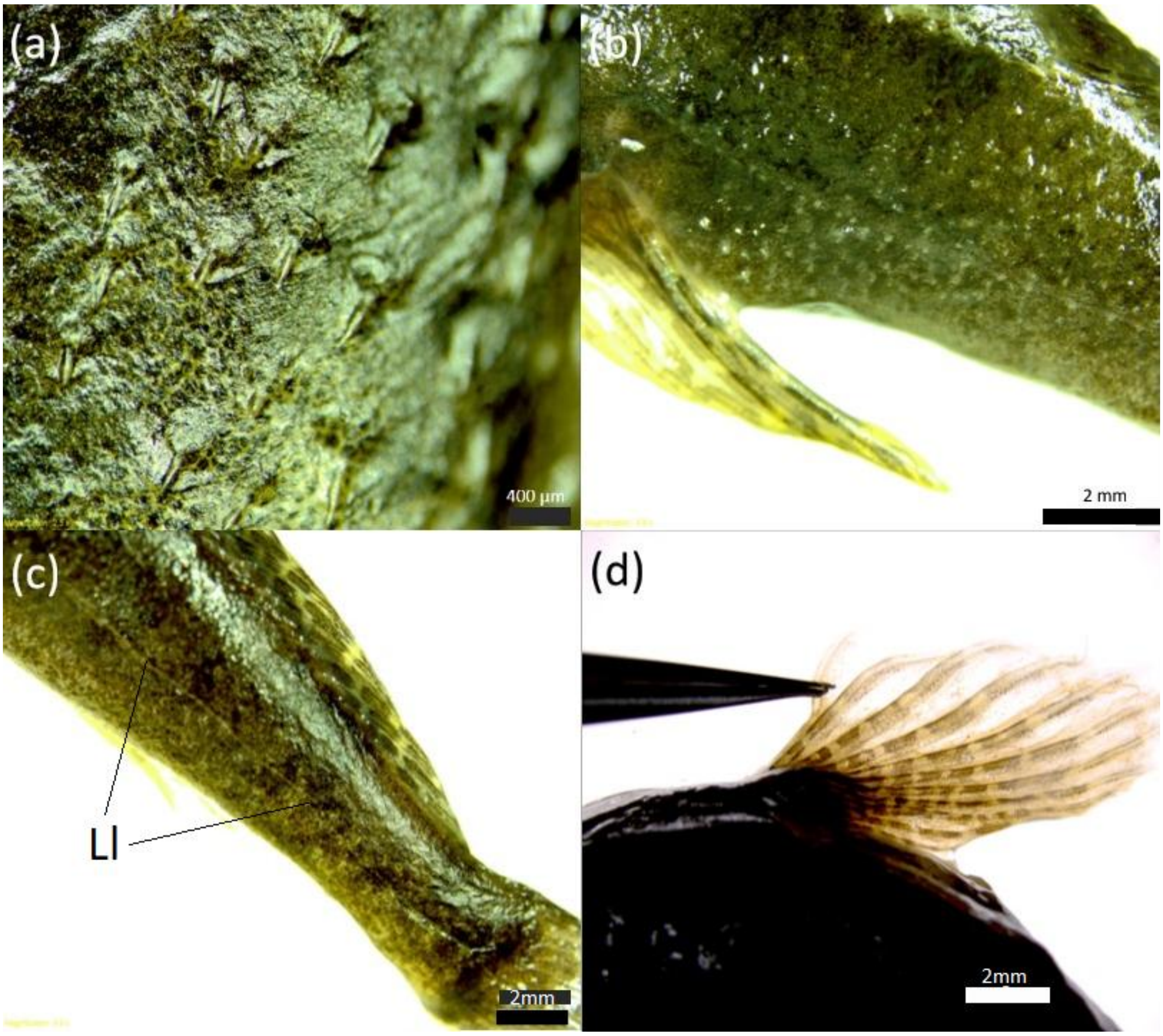

Figure 3.2. Bullhead morphological features of interest. (a) Skin prickles on dermal surface, (b) prickling dorsal and ventral to the lateral line, (c) complete lateral line (Ll) extending down the body wall, (d) pectoral fin displaying fin rays

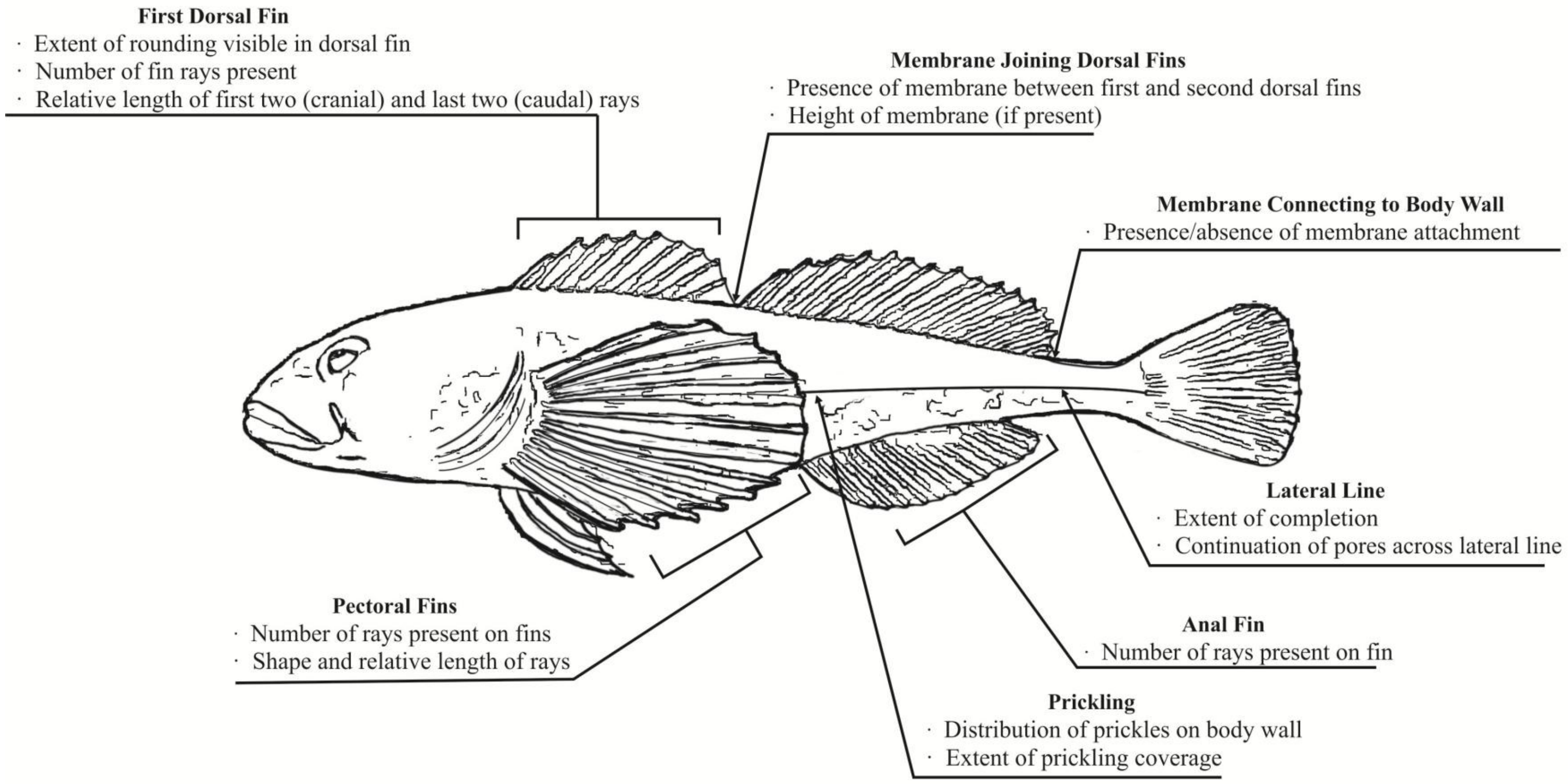

Figure 3.3. Schematic representation of morphological characteristics examined in Scottish bullhead specimens

Meristic traits were compared across each population. Statistical analysis was conducted using Minitab Statistical Software version 17. As assumptions for parametric analysis were not met for meristic trait data, non-parametric analysis was undertaken using Kruskal-Wallis testing with post-hoc Mann-Whitney U tests between selected variables. A Holm-Bonferroni correction was applied to all results (Holm, 1979). Variability in standard length and counts for second dorsal fin, anal fin and pectoral fin rays for bullheads examined from all locations were explored using box plots. As the distribution of prickling was normally distributed, a Pearson correlation analysis was applied to the grouped bullhead data to determine if the proportion of the body covered in prickles was related to standard body length. A one-way ANCOVA was conducted to compare skin prickling distribution with standard length, whilst controlling for location variability.

3.2.3 Molecular Phylogenetic Analysis

The obtained COI sequences were identified by BLAST analysis. Phylogenetic analysis was undertaken using the six mtDNA COI sequences of Scottish bullhead amplified in this study and those of 32 other species within the genus *Cottus* published in Genbank. Sequences from Atlantic salmon (*Salmo salar*) and European ruffe (*Gymnocephalus cernuus*) were added to the alignment as an outgroup. Sequences were aligned using Geneious (http://www.geneious.com, Kearse *et al*., 2012), with visual editing to confirm placement accuracy. An alignment of 475 base pairs was obtained after all gaps had been excluded. The alignment was analysed with Maximum Likelihood (ML) and Bayesian methods.

ML and bootstrap analyses were undertaken using the programme PhyML (Guindon and Gascuel, 2003; Guindon *et al*., 2010) with the Tamura-Nei (TN) model of nucleotide substitutions (Posada and Crandall, 1998) and a γ -distribution with a fixed proportion of invariable sites and a transition/transversion ratio (Ti/Tv) estimated from each data set (40-taxon alignment: six rate categories, $\gamma = 0.156$). The computation was completed under the TN93+G+F substitution model, using Akaike information criterion (AIC).

Bayesian analysis was performed using MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The program was set to operate with a GTR substitution model, γ - distribution and four Monte Carlo Markov chains (MCMC; default temperature = 0.2). A total of 10,000,000 generations were calculated based on trees sampled every 100 generations, with a prior burn-in of 25,000 generations (2,500 sampled trees were discarded). The 75,000 post-burn-in trees obtained were used to construct a majority-rule consensus tree, where reported posterior probabilities correspond to the frequency at which a given node occurred in a post-burn-in tree.

A pair-wise distance calculation based on Kimura's two-parameter model (Kimura, 1980) was performed on the same alignment, using MEGA 7.0 (Kumar *et al*., 2016).

3.3 Results

3.3.1 Molecular Phylogenetic Analysis

Analysis of the six Scottish bullhead sequences described in the study, along with sequences of other *Cottus* species from across North America, Asia and Europe, showed separation within the *Cottus* genus based on broad geographic groupings (Figure 2.4 and 2.5). ML support for the deepest branches of the phylogenetic tree was fairly low, at less than 50%. Higher support was found with Bayesian posterior probabilities, which strongly supported the first initial grouping of the North American *Cottus* species (with values ranging from 0.91 to 1), but were less supportive of the sister branch, which grouped *Cottus* species from a mix of geographical locations (Bayesian posterior probability value of 0.54). Weakly supported deep branches in this phylogenetic tree suggests that the separation between members of the *Cottus* genus is not well defined in this study.

Clustering is evident within the European *Cottus* species analysed in this study. Support for the European grouping is high (Bayesian posterior probability of 0.9871 and ML output 85%). Three distinct clades are visible within the European *Cottus* group, along with the separation of four distinct species that do not cluster well with any of the other species groupings, or each other. Scottish bullheads form a clade with English *C. perifretum*, which is well supported by Bayesian posterior probability analysis (0.98) and moderately supported by our ML bootstrapping model (73%). The direct sister clade to *C. perifretum* is comprised of *C. hispaniolensis* and *C. duranii*, of northern Spain and France respectively. *Cottus gobio* and *C. aturi* formed a third clade as sister to the former two. The species *C. rhenanus*, *C. rondeleti, C. scaturigo* and *C. microstomus* come off the same node independently, and do not cluster with any of the other European *Cottus* species. There are three distinct clades within Europe, but the overall support for these groupings within the European species is relatively low based on ML analysis (Figure 3.4). Support is higher based on Bayesian analysis.

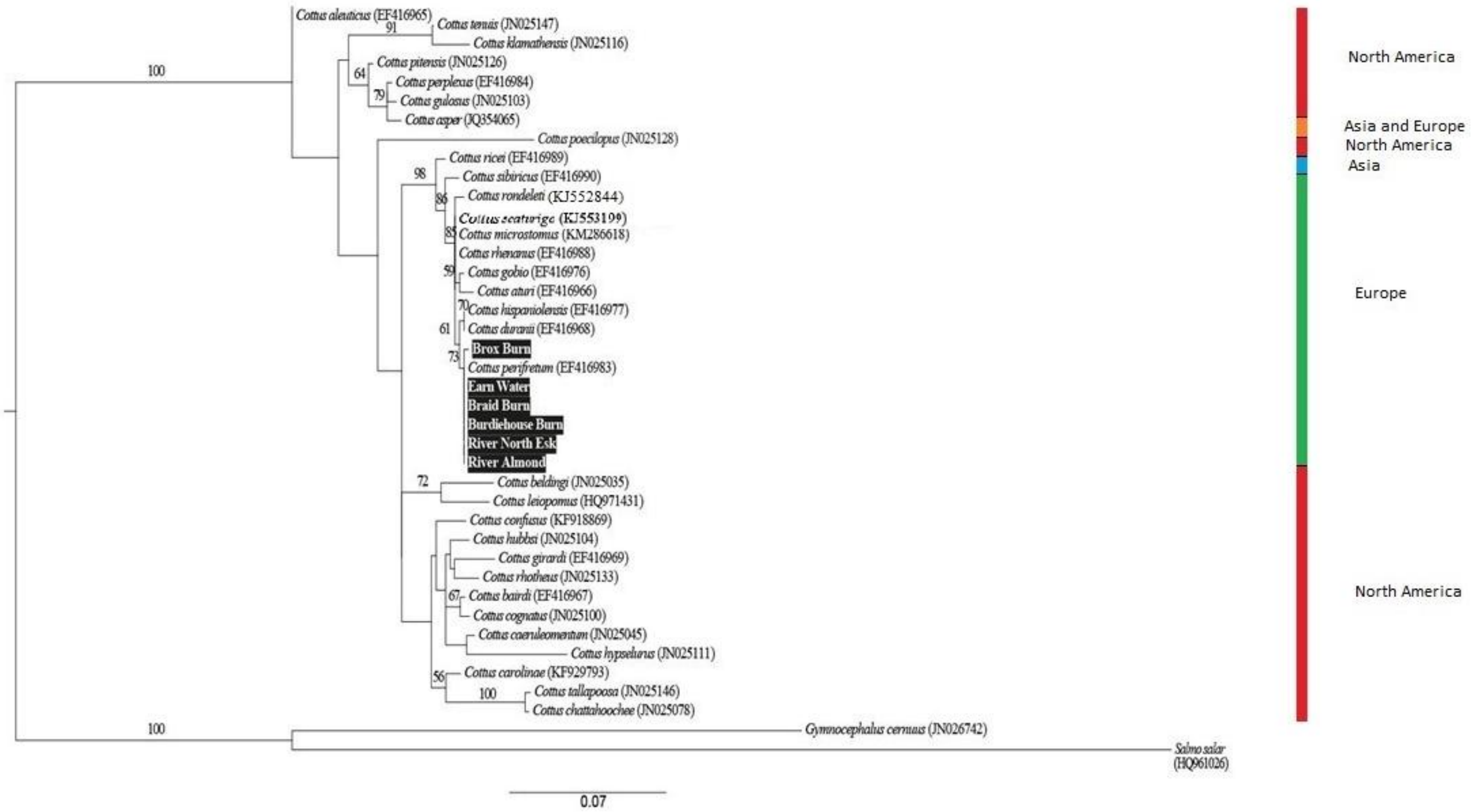

Figure 3.4. ML tree of an alignment of 40 *Cottus* COI mtDNA gene sequences (40-taxon alignment: six rate categories, γ = 0.156), produced using the TN model of nucleotide substitutions, a gamma distribution and invariable sites. Numbers denote ML bootstrap percentages. Bootstrap values of less than 50 % are excluded. Sequences of Scottish bullhead derived from this study are shown in black boxes. All other *Cottus* species are listed along with their NCBI accession numbers. Species geographical ranges are shown by coloured bars, separating specimens from North America (red), Asia and Europe (orange), Asia (blue) and Europe (green)

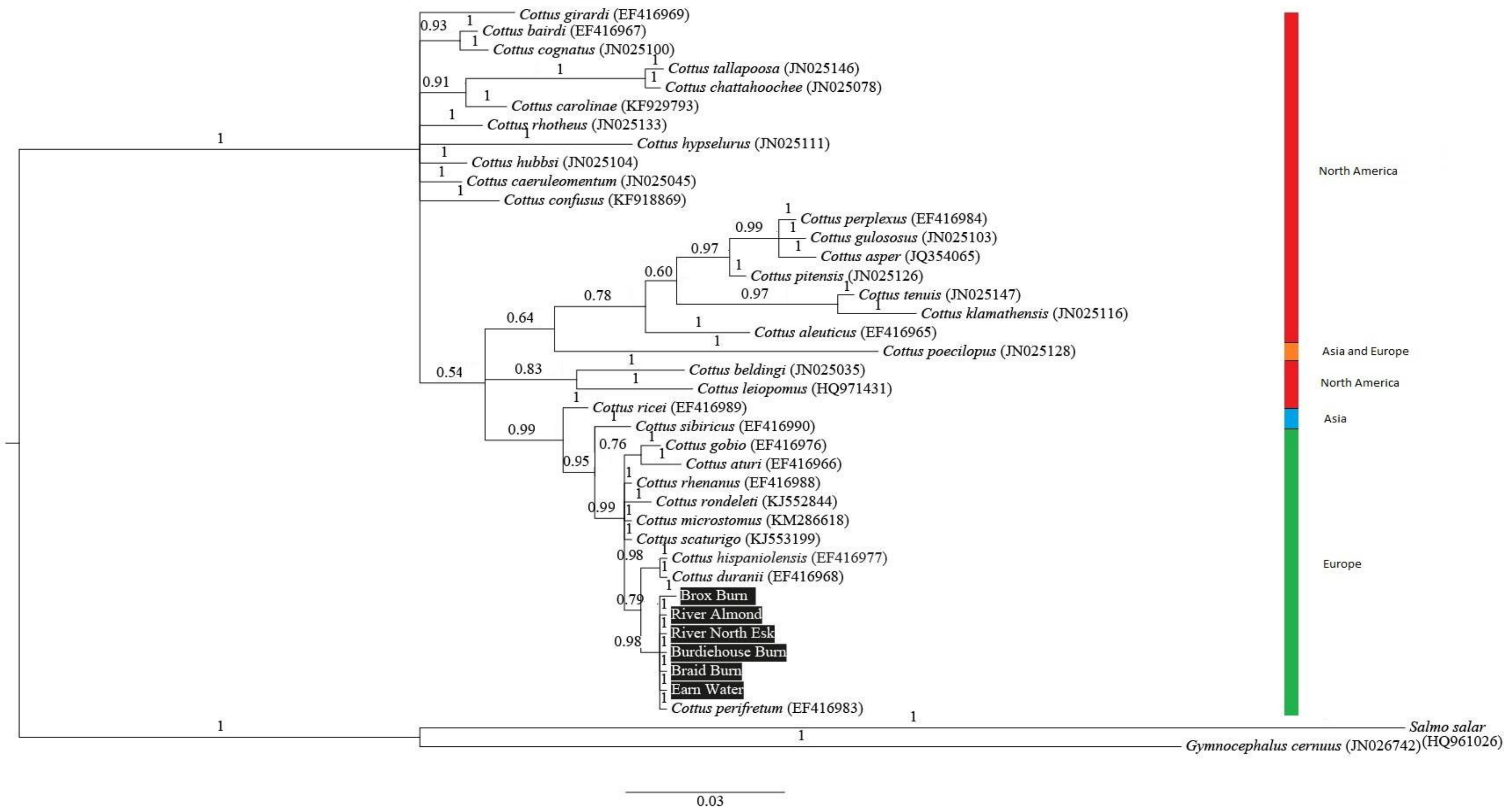

Figure 3.5. Bayesian tree output of phylogenetic analysis of 40 *Cottus* COI sequences. Values are inferred using a GTR substitution model, γ - distribution and four Monte Carlo Markov chains (MCMC; default temperature = 0.2). Numbers represent Bayesian posterior probabilities. Bayesian values less than 0.50 are excluded. Sequences of Scottish bullhead derived from this study are shown in black boxes. All other *Cottus* species are listed along with their NCBI accession numbers Species geographical ranges are shown by coloured bars, separating specimens from North America (red), Asia and Europe (orange), Asia (blue) and Europe (green)

A pair-wise distance calculation performed on 38 *Cottus* COI mtDNA sequences showed an extremely high degree of similarity between Scottish bullhead and English *C. perifretum*. No differentiation was found within the British sequences except the Brox Burn, which was only marginally distinct, yielding a 0.21% divergence from the other British specimens (Table 3.1). Comparisons between the Scottish bullhead sequences derived from this study and all other *Cottus* species examined showed higher divergence, although low interspecific divergence was shown across Europe. All sequences originating from specimens previously confused under the *C. gobio* species complex yielded interspecific divergence values of 1.50 % or lower. Similarly low values within this threshold were found between European cottids and *C. sibiricus*, originating from Siberia, and between *C. sibiricus* and *C. ricei* of Alaskan/north-west American origin. *Cottus* species originating from North America showed substantially higher divergence from the sequences derived in this study (ranging from 1.72-8.87%).

Table 3.1. Pair-wise distance calculations of COI mtDNA sequences across 38 *Cottus* species, based on the Kimura 2-parameter model (Kimura, 1980). Values represent percentage sequence divergence. Sequences from Atlantic salmon and European ruffe are also included as an outgroup. Values in bold represent comparisons of British bullhead. The box contains comparisons across European bullhead species all previously confused under the *C. gobio* species complex

Sequence	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
1. Brox Burn	-																																							
2. River Almond	**0.212**	-																																						
3. River North Esk	**0.212**	**0.000**	-																																					
4. Burdiehouse Burn	**0.212**	**0.000**	**0.000**	-																																				
5. Braid Burn	**0.212**	**0.000**	**0.000**	**0.000**	-																																			
6. Earn Water	**0.212**	**0.000**	**0.000**	**0.000**	**0.000**	-																																		
7. *Cottus perifretum*	**0.212**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	-																																	
8. *Cottus gobio*	1.070	0.855	0.855	0.855	0.855	0.855	0.855	-																																
9. *Cottus duranii*	0.640	0.426	0.426	0.426	0.426	0.426	0.426	0.857	-																															
10. *Cottus hispaniolensis*	0.640	0.426	0.426	0.426	0.426	0.426	0.426	0.857	0.000	-																														
11. *Cottus aturi*	1.501	1.285	1.285	1.285	1.285	1.285	1.285	0.854	1.286	1.286	-																													
12. *Cottus rhenanus*	0.640	0.426	0.426	0.426	0.426	0.426	0.426	0.426	0.426	0.426	0.854	-																												
13. *Cottus microstomus*	0.640	0.426	0.426	0.426	0.426	0.426	0.426	0.426	0.426	0.426	0.854	0.000	-																											
14. *Cottus scaturigo*	0.640	0.426	0.426	0.426	0.426	0.426	0.426	0.426	0.426	0.426	0.854	0.000	0.000	-																										
15. *Cottus rondeleti*	1.070	0.855	0.855	0.855	0.855	0.855	0.855	0.857	0.857	0.857	1.286	0.426	0.426	0.426	-																									
16. *Cottus poecilopus*	9.602	9.366	9.366	9.366	9.366	9.366	9.366	9.631	9.631	9.631	9.602	9.130	9.130	9.130	8.633	-																								
17. *Cottus sibiricus*	1.718	1.501	1.501	1.501	1.501	1.501	1.501	1.502	1.502	1.502	1.936	1.069	1.069	1.069	1.502	8.868	-																							
18. *Cottus ricei*	1.939	1.722	1.722	1.722	1.722	1.722	1.722	1.725	1.725	1.725	1.720	1.288	1.288	1.288	1.725	8.154	1.504	-																						
19. *Cottus perplexus*	6.696	6.467	6.467	6.467	6.467	6.467	6.467	6.953	6.475	6.475	7.414	6.475	6.475	6.475	6.002	8.633	6.230	6.011	-																					
20. *Cottus girardi*	6.485	6.256	6.256	6.256	6.256	6.256	6.256	6.267	6.267	6.267	6.485	5.793	5.793	5.793	6.267	8.706	5.549	5.332	6.029	-																				
21. *Cottus bairdi*	5.090	4.866	4.866	4.866	4.866	4.866	4.866	4.874	4.874	4.874	4.627	4.411	4.411	4.411	4.874	8.180	4.621	3.946	6.723	3.052	-																			
22. *Cottus aleuticus*	6.696	6.467	6.467	6.467	6.467	6.467	6.467	6.475	6.475	6.475	6.934	6.002	6.002	6.002	5.534	8.121	5.761	5.541	4.404	6.247	6.963	-																		
23. *Cottus tenuis*	8.375	8.142	8.142	8.142	8.142	8.142	8.142	8.154	8.154	8.154	8.621	7.666	7.666	7.666	7.666	9.900	7.898	7.677	4.642	7.458	7.677	5.323	-																	
24. *Cottus tallapoosa*	6.953	6.723	6.723	6.723	6.723	6.723	6.723	7.216	6.734	6.734	7.677	6.734	6.734	6.734	7.216	11.205	6.723	6.267	7.485	6.289	5.341	8.690	8.924	-																
25. *Cottus rhotheus*	6.256	6.029	6.029	6.029	6.029	6.029	6.029	5.567	6.040	6.040	5.315	5.567	5.567	5.567	6.040	8.940	5.323	4.642	7.458	2.833	2.387	7.702	8.924	5.588	-															
26. *Cottus pitensis*	6.467	6.238	6.238	6.238	6.238	6.238	6.238	6.723	6.247	6.247	6.704	6.247	6.247	6.247	5.775	7.424	6.002	5.315	1.288	5.567	5.784	3.498	4.419	7.228	6.505	-														
27. *Cottus klamathensis*	8.868	8.633	8.633	8.633	8.633	8.633	8.633	8.646	8.646	8.646	9.116	8.154	8.154	8.154	8.646	10.925	8.387	8.166	6.051	8.441	8.660	6.505	1.728	9.934	9.934	5.824	-													
28. *Cottus hypselurus*	7.888	7.655	7.655	7.655	7.655	7.655	7.655	7.666	7.666	7.666	7.405	7.183	7.183	7.183	7.666	8.646	6.934	6.238	7.909	6.040	4.621	8.868	9.631	7.446	5.557	6.953	10.649	-												
29. *Cottus hubbsi*	5.315	5.090	5.090	5.090	5.090	5.090	5.090	5.098	5.098	5.098	4.851	4.634	4.634	4.634	5.098	8.690	4.397	4.181	6.734	2.610	1.944	7.216	8.180	5.588	2.170	5.793	8.675	5.323	-											
30. *Cottus gulosus*	6.943	6.713	6.713	6.713	6.713	6.713	6.713	6.723	6.723	6.723	7.183	6.247	6.247	6.247	5.775	8.399	6.002	5.784	0.640	5.803	6.495	4.181	4.651	7.962	7.228	1.509	6.063	7.677	6.505	-										
31. *Cottus cognatus*	5.775	5.549	5.549	5.549	5.549	5.549	5.549	5.557	5.557	5.557	5.308	5.090	5.090	5.090	5.557	8.427	4.851	3.719	6.963	3.278	0.640	7.204	7.921	5.577	2.610	6.019	8.909	4.851	2.165	6.734	-									
32. *Cottus chattahoochee*	6.953	6.723	6.723	6.723	6.723	6.723	6.723	7.216	6.734	6.734	7.677	6.734	6.734	6.734	7.216	10.686	6.723	6.267	7.485	6.289	5.341	8.690	8.924	0.426	5.588	7.228	9.934	6.963	5.588	7.962	5.577	-								
33. *Cottus carolinae*	5.083	4.858	4.858	4.858	4.858	4.858	4.858	4.866	4.866	4.866	5.315	4.404	4.404	4.404	4.866	8.441	4.627	3.952	5.549	3.284	2.833	6.734	6.963	4.189	3.518	5.557	7.934	5.557	3.064	5.323	3.058	4.189	-							
34. *Cottus caeruleomentum*	5.549	5.323	5.323	5.323	5.323	5.323	5.323	5.332	5.332	5.332	5.083	4.866	4.866	4.866	5.332	9.191	5.090	3.952	6.256	3.511	2.165	7.216	7.689	4.883	2.392	5.323	8.675	4.397	2.392	6.029	2.387	4.883	2.392	-						
35. *Cottus beldingi*	6.926	6.696	6.696	6.696	6.696	6.696	6.696	6.704	6.704	6.704	6.689	6.230	6.230	6.230	6.704	9.340	5.768	5.302	8.131	6.475	5.768	7.666	7.636	7.666	6.485	7.173	7.878	7.878	5.076	7.898	6.002	7.666	6.011	5.541	-					
36. *Cottus leiopomus*	6.943	6.713	6.713	6.713	6.713	6.713	6.713	6.247	6.723	6.723	6.943	6.247	6.247	6.247	6.723	10.631	6.002	5.784	7.424	5.784	5.784	7.204	8.387	7.934	6.029	6.953	8.633	6.696	5.557	7.193	6.019	7.446	5.557	5.090	4.419	-				
37. *Cottus confusus*	6.011	5.784	5.784	5.784	5.784	5.784	5.784	5.793	5.793	5.793	6.011	5.323	5.323	5.323	5.793	9.462	5.083	4.866	6.267	3.291	2.605	6.723	6.974	5.107	3.284	6.040	8.441	5.567	2.610	6.278	2.828	5.107	2.833	2.610	5.768	5.549	-			
38. *Cottus asper*	7.173	6.943	6.943	6.943	6.943	6.943	6.943	7.435	6.953	6.953	7.898	6.953	6.953	6.953	6.475	8.633	6.704	6.485	0.854	6.505	7.204	4.866	5.341	8.194	7.947	1.725	6.770	8.399	7.216	1.071	7.446	8.194	6.019	6.734	8.621	7.909	6.986	-		
39. *Salmo salar*	27.928	27.632	27.632	27.632	27.632	27.632	27.632	26.999	27.679	27.679	27.247	26.999	26.999	26.999	26.663	26.165	26.707	26.372	26.576	24.012	24.012	26.328	26.372	24.615	24.048	25.954	27.045	25.954	23.730	26.285	24.651	24.615	24.689	24.367	23.449	25.954	24.974	26.244	-	
40. *Gymnocephalus cernuus*	22.544	22.511	22.511	22.511	22.511	22.511	22.511	22.855	22.855	22.855	23.101	22.234	22.234	22.234	21.927	22.790	22.758	23.449	23.484	22.822	21.044	22.060	22.267	24.443	22.234	23.204	22.577	23.696	21.621	23.204	21.896	24.443	21.621	21.621	24.240	24.565	22.822	22.544	22.036	-

3.3.2 Morphological Analysis

A substantial amount of intraspecific variation was exhibited in all morphological traits examined (Figures 3.6 to 3.8). Significant differences in second dorsal fin ray counts (Kruskal-Wallis test; $H = 21.69$, $df = 5$, $p < 0.05$), anal fin ray counts (Kruskal-Wallis test; $H = 22.03$, $df = 5$, $p < 0.05$) and pectoral fin ray counts (Kruskal-Wallis test; $H = 67.11$, $df = 5$, $p < 0.05$) were found between populations (Figure 3.9). Values obtained were compared with those published for native English bullheads (Maitland and Campbell, 1992; Kottelat and Freyhof, 2007). Results showed a larger variation in pectoral fin rays counts, anal fin ray counts and second dorsal fin ray counts than has been previously described for bullheads in Britain (Figures 3.6 to 3.8).

Bullheads sampled from the Earn Water had a higher average number of pectoral fin rays than bullheads from all other locations (Figures 3.6 and 3.9). Values for the Earn Water differed significantly from all other groups (Mann-Whitney U test; Earn Water and River Almond $W = 811.5$, $N1 =33$, $N2 = 38$, $p < 0.001$; Earn Water and River North Esk $W = 875.5$, $N1 = 35$, $N2 = 38$, $p < 0.001$; Earn Water and Burdiehouse Burn $W = 1739$, $N1 = 55$, $N2 = 38$, $p < 0.001$; Earn Water and Braid Burn $W = 880.5$, $N1 = 35$, $N2 = 38$, $p < 0.001$; Earn Water and Brox Burn $W = 749$, $N1 = 34$, $N2 = 38$, $p < 0.001$). Significant differences in pectoral fin ray counts were also found when comparing the River Almond and Burdiehouse Burn (Mann-Whitney U test, $W = 1788$, $N1 = 33$ $N2 = 55$, $P = 0.002$).

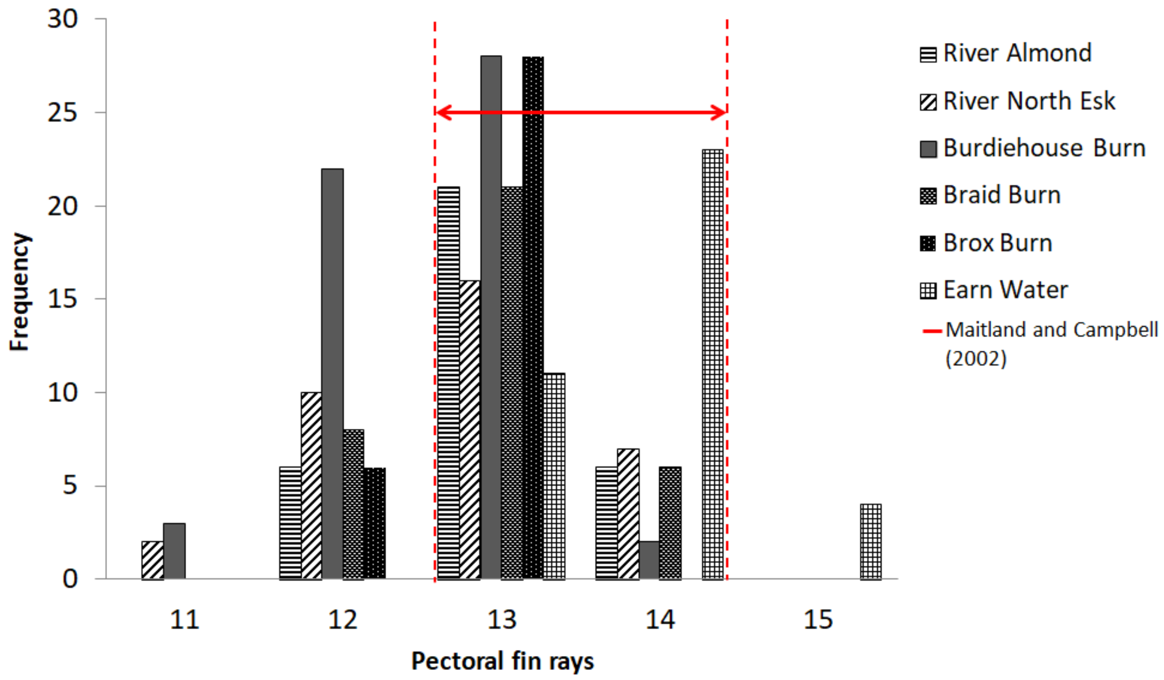

Figure 3.6. Pectoral fin ray count frequencies for examined bullheads across six sample sites in Scotland. Arrows and corresponding dashed lines indicate the range of values reported for bullhead in Britain by Maitland and Campbell (1992). For Figures 3.6 to 3.9, River Almond N=33, River North Esk N=35, Burdiehouse Burn N=55, Braid Burn N=35, Brox Burn N=34, Earn Water N=37

Anal fin ray counts differed between pairings of the River Almond with the Braid Burn (Mann-Whitney U test W = 898.5, N1 = 33, N2 = 35, p = 0.0021) and the River Earn (Mann-Whitney U test W = 931.5, N1 = 33, N2 = 38, p = 0.002). Significant differences were also found in the pairings of the Brox Burn with the Braid Burn (Mann-Whitney U test W = 1485, N1 = 35, N2 = 34, p = 0.001) and the River Earn (Mann-Whitney U test W = 965, N1 = 34, N2 = 38, p = 0.001) (Figures 3.7 and 3.9).

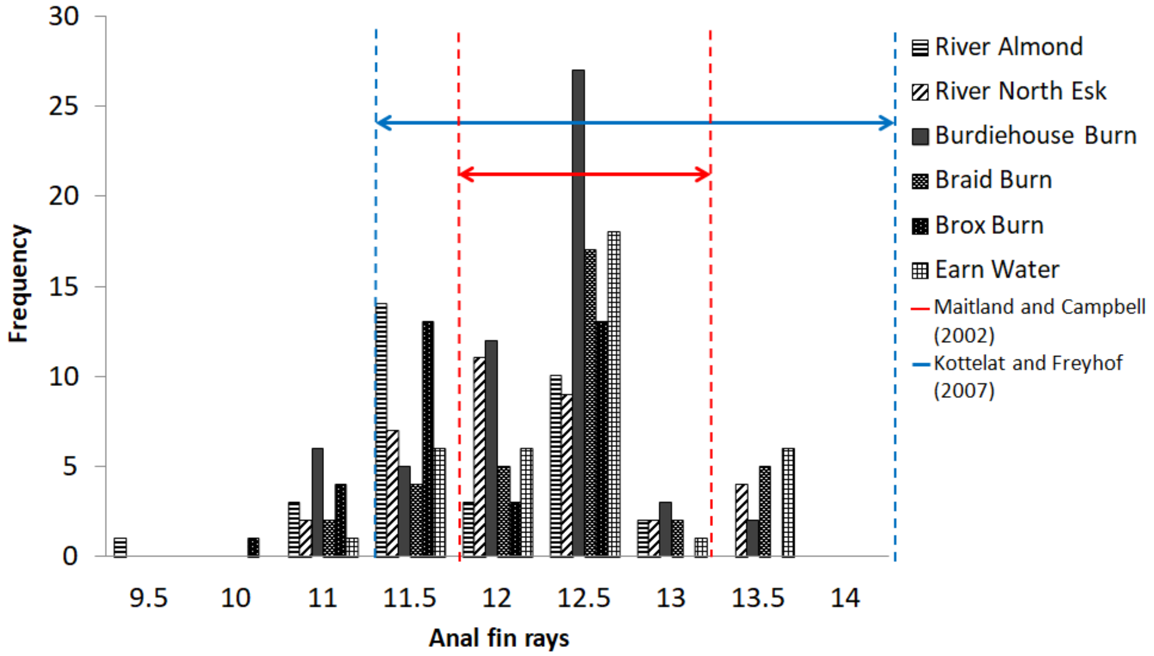

Figure 3.7. Anal fin ray count frequencies for examined bullheads across six sample sites in Scotland. Arrows and corresponding dashed lines indicate the range of values reported for bullhead in Britain by Maitland and Campbell (1992) and Kottelat and Freyhof (2007) (blue)

The second dorsal fin rays of the River Almond samples differed significantly from those of the River North Esk (Mann-Whitney U test W = 909.5, N1 = 33, N2 = 35, p = 0.0015), the Burdiehouse Burn (Mann-Whitney U test W = 1129, N1 = 33, N2 = 55, p = 0.0009) and Braid Burn (Mann-Whitney U test W = 897, N1 = 33, N2 = 35, p = 0.0012) (Figures 3.8 and 3.9).

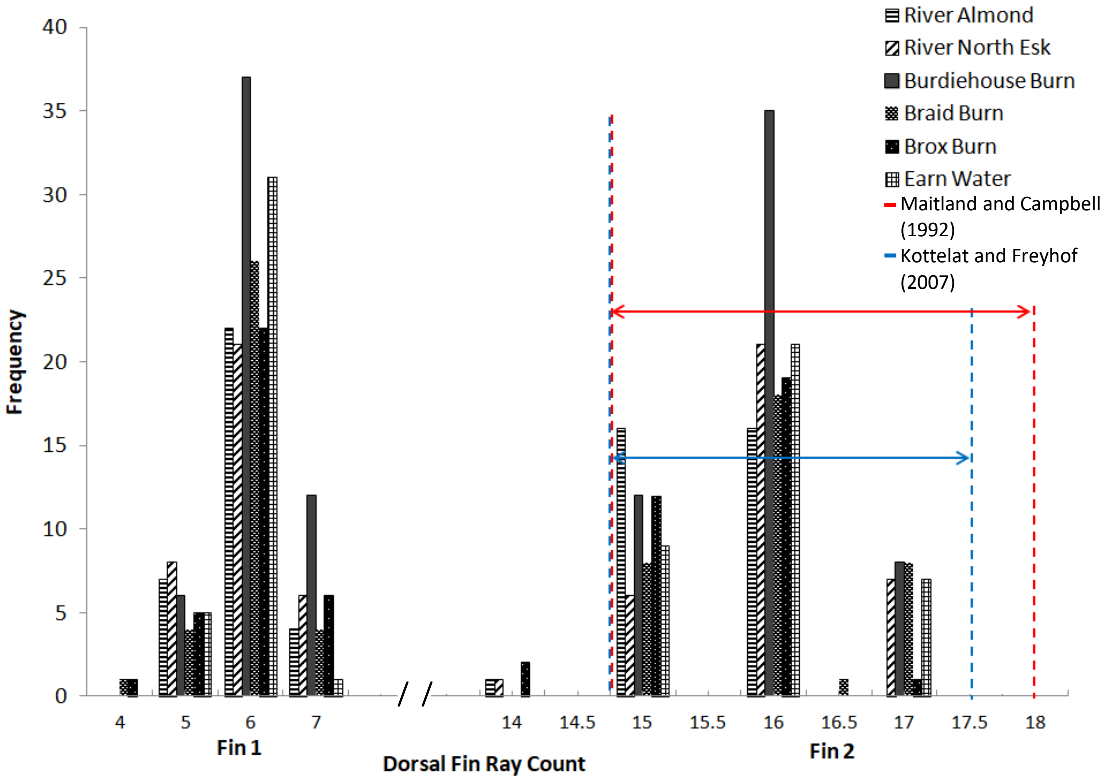

Figure 3.8. Dorsal fin ray count frequencies for examined bullheads across six sample sites in Scotland. Arrows and corresponding dashed lines indicate the range of values reported for bullhead second dorsal fins in Britain by Maitland and Campbell (1992) and Kottelat and Freyhof (2007) (blue)

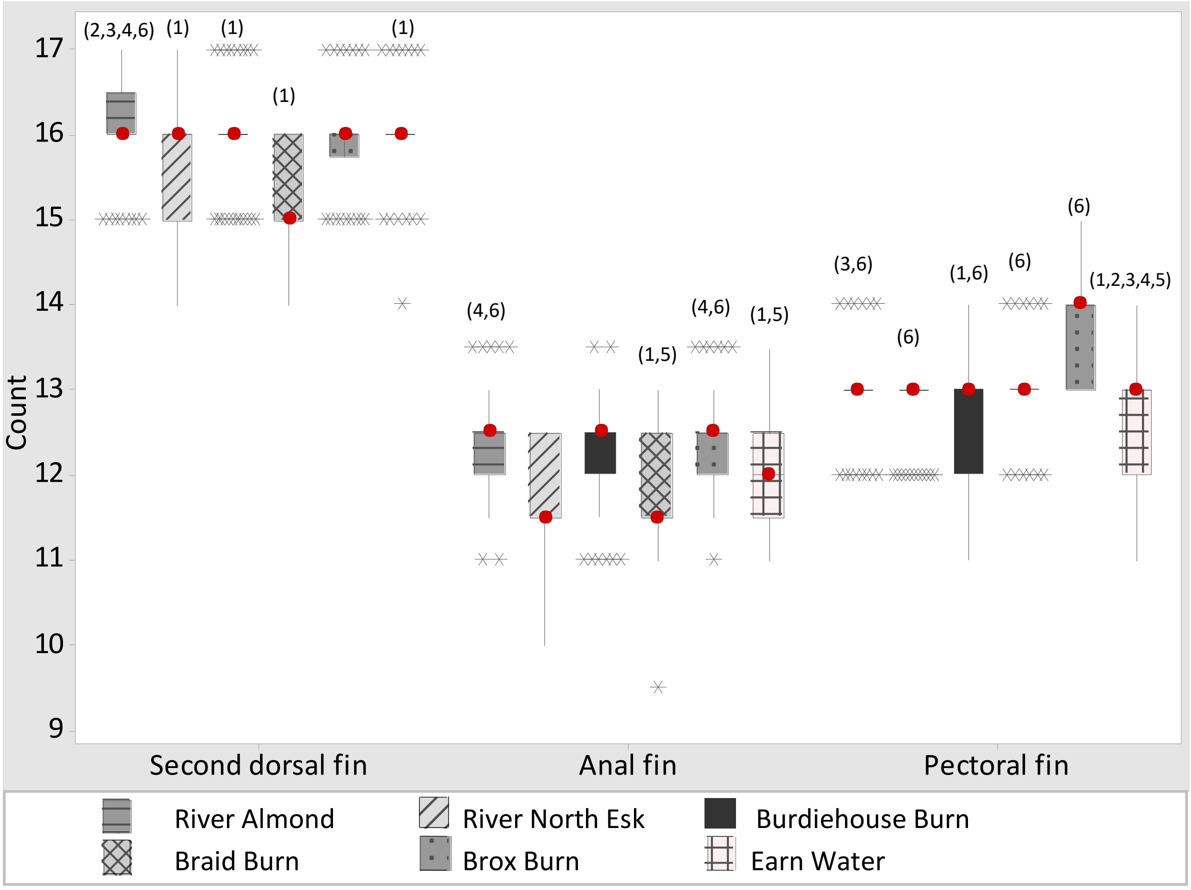

Figure 3.9. Comparison of ray counts from the second dorsal fin, anal fin and pectoral fin of bullheads collected from six sample sites in Scotland. The inter-quartile range, median values (●) and outliers (✳) are shown for each population. Statistically significant comparisons of group medians based on Mann-Whitney U tests (Holm-Bonferroni adjusted p < 0.0023) are given in parenthesis above each data series, where the presence of a number indicates a significant difference between the data series and that group. 1 = River Almond, 2 = River North Esk, 3 = Burdiehouse Burn, 4 = Braid Burn, 5 = Brox Burn, 6 = Earn Water

In terms of general morphology, an oval-shaped dorsal fin, rounded pectoral fin and four pelvic fin rays were found on all bullhead examined, as is consistent with features described for specimens of *C. perifretum* (Freyhof *et al.,* 2005). A membrane attaching the first and second dorsal fin and a second connecting the caudal dorsal fin with the body wall were also present in all specimens, regardless of their origin. All fish examined showed dense skin prickling around and inferior to the pectoral fin, although the coverage of prickles across the body wall varied greatly between specimens of all sizes. A Pearson correlation analysis showed that standard body length was not an effective predictor of prickling coverage in the

Scottish bullhead examined when all samples were grouped (r = - 0.086, n=225, p = 0.2). The results of ANCOVA showed that there was not a significant difference in prickling coverage for fish of different length, when location influences were controlled (F = 0.07; df = 1, 218; p = 0.795).

An attempt was made to identify Scottish specimens using the key to genera of European Cottidae provided in Kottelat and Freyhof (2007). The key could only be applied to juvenile individuals (considered to be those with standard length ≤ 50mm), greatly reducing the number of specimens that could be identified using this means (n=21 Burdiehouse Burn, n=26 Brox Burn, n=30 Earn Water) (Figure 3.10), but those that were of an appropriate size were confirmed as *C. perifretum.*

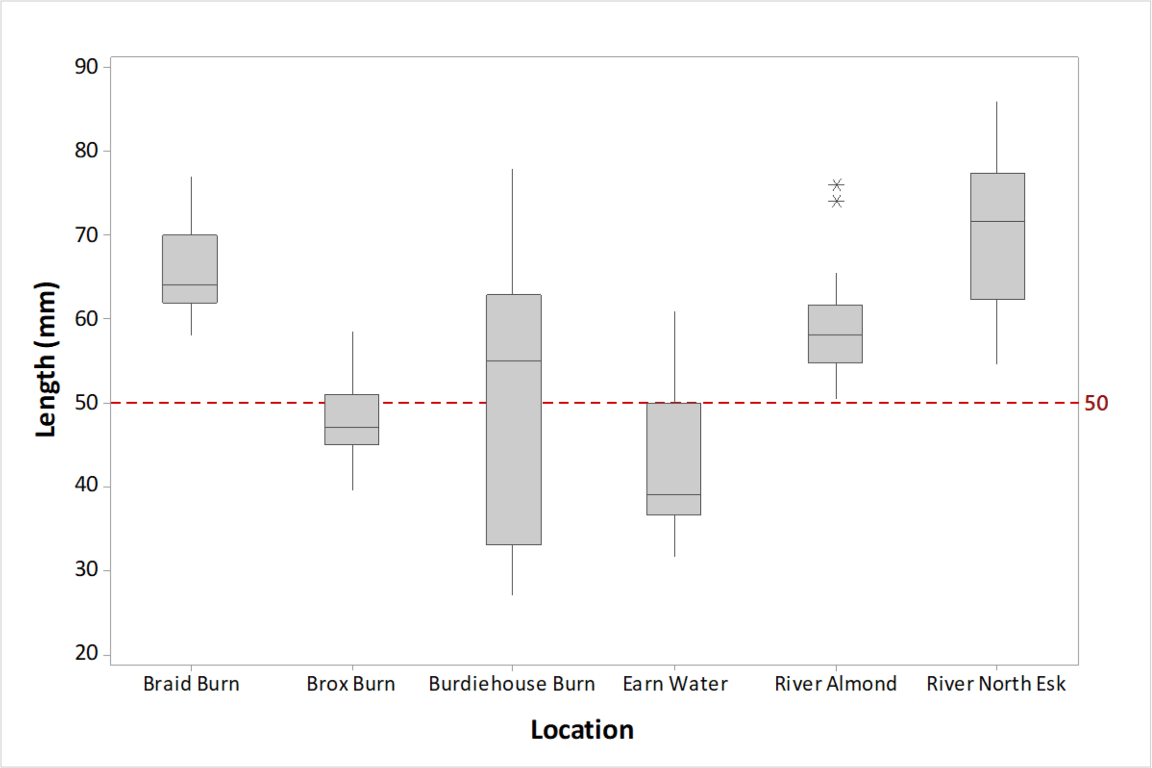

Figure 3.10. Variability within standard length (mm) measurements of examined bullheads from six sample sites in Scotland. The dashed line indicates the 50mm length threshold for specimens that were identified using the key supplied in Kottelat and Freyhof (2007). Data outliers (✳) are shown for the River Almond

3.4 Discussion

Phylogenetic analyses of mtDNA sequences obtained from Scottish bullhead, in conjunction with those reported for other *Cottus* species, provided evidence of three distinct clades within Europe. In addition, four other European species were found to be closely related but not strongly associated with any of these clades. The combined results of Englbrecht *et al.* (2000) and Volckaert *et al.* (2002) revealed seven clades within Europe. The description of species ranges and cladistics given in Freyhof *et al.* (2005) supported six of these clades, including a clade containing *C. perifretum* confirmed by specimens from both Great Britain and the continent. The results of this study verify the presence of a clade containing *C. gobio* (clade I of Englbrecht *et al*., 2000); one containing *C. duranii* (clade VII of Volkaert *et al.,* 2002), and a clade containing *C. perifretum* (clade IV of Volkaert *et al.,* 2002). In addition, results of this study grouped *C. hispaniolensis* alongside *C. duranii,* and *C. aturi* with *C. gobio*. The clade consisting of *C. gobio* and *C. aturi* was not well supported (59% bootstrap support and 0.76 Bayesian posterior probability). Englbrecht *et al*. (2000) and Freyhof *et al.* (2005) positioned *C. aturi* into a different clade, and the low support exhibited by ML and Bayesian analysis in the results section of this chapter implies an alternative position within the phylogenetic tree is possible. The clade consisting of *C. hispaniolensis* and *C. duranii* was moderately well supported (70% ML and 1 Bayesian posterior probability output), and would suggest that the two species are closely related. *Cottus scaturigo*, *C. microstomus*, *C. rhenanus* and *C. rondeleti* were all found among the European *Cottus* species, but did not form a strong association with any particular group. Šlechtová *et al.* (2004) were unable to discriminate between *C. rondeleti* and other closely associated *Cottus* species using mtDNA, but a morphological description that described the new species *C. rondeleti* was given by Freyhof *et al.* (2005). Similarly, Šlechtová *et al.* (2004) and Englbrecht *et al.* (2000) were unable to distinguish *C. scaturigo* from neighbouring *C. gobio* using molecular methods, although Freyhof *et al.* (2005) used morphological evidence to describe the new species *C. scaturigo*.

The ML tree produced through bootstrap analysis showed that, with the exception of *C. poecilopus*, all European cottids grouped closely together. *Cottus poecilopus* was distinguished from other European *Cottus* species by Koli (1969), who noted morphological differences in chin pore arrangement (one pore is found in all species grouped under the *C. gobio* species complex and two pores are found on *C. poecilopus*). The genetic dissimilarity of *C. poecilopus* from other European *Cottus* species agrees with the morphological differences. Bayesian support for the divergence of the European cottids was high (0.99), as was the support for the British clade containing *C. perifretum* and the Scottish bullhead samples of this study (0.98). Bootstrap values were somewhat lower, giving moderate support at 85% and 73% respectively. Hillis and Bull (1993) found that bootstrap values ≥70% correspond to at least a 95% probability that the simulated clade obtained is real. The combined results of Bayesian and ML analysis are therefore deemed complimentary. This is further verified by the results of pairwise distance calculations conducted across all *Cottus* sequences. Five of the six Scottish bullhead sequences and the sequence given for *C. perifretum* showed no divergence when compared against each other, and the sixth sequence showed only 0.21% divergence from the other British bullhead samples. Interestingly, there was also no divergence found when comparing the two European sequences *C. hispaniolensis* and *C. duranii*; and the collective grouping of *C. microstomus*, *C. rhenanus* and *C. scaturigo,* despite each being considered an independent species. The ML tree produced in this study showed that the sequences within these two groupings were found within the same clade, with very little distinction between sequences. A high degree of genetic similarity is therefore expected, and the determination of species-level distinctions between these populations has already been the subject of other, more detailed, studies (e.g. Freyhof *et al*., 2005). Although the comparison between the newly sequenced Scottish bullheads and the sequence already published for *C. perifretum* has produced a similar result showing minimal divergence, nothing in the analyses suggest that any of the compared British sequences differ on a molecular level. Whilst mtDNA typically evolves faster than single-copy nuclear DNA (Avise, 2000), mtDNA genes are integral to the speciation process (Gershoni *et al*., 2009; Lane, 2009) and have been described as an unambiguous measure of species identity in a recent publication (Hill, 2016). COX1 genotypes have been integral to phylogenetic studies for decades and are highly effective at defining species boundaries (Hill, 2016). The results obtained from COI mtDNA analysis of Scottish bullhead therefore demonstrate that Scottish bullhead are taxonomically similar to *C. perifretum,* representing an isolated branch of the same species. The published *C. perifretum* sequence used in this study originated from Belgium (Sonnenberg *et al.* 2007), and is therefore within the southern England and Belgium group described by Hänfling *et al.* (2002). The documented similarity between this sequence and those obtained from Scottish bullheads in this study does not support the distinctions shown in northern and southern British bullheads, or British bullheads and those from Belgium, that were discussed in Hänfling *et al.* (2002). However, the application of microsatellite loci, as employed by Hänfling *et al.* (2002), is known to provide a robust assessment of genetic relatedness (Chistiakov *et al.,* 2006), and could explain the distinctions provided through microsatellite analysis that were not reproduced in this study.

A combined approach is considered optimal when examining taxonomic identity, due to the long-standing belief that misinference can occur when mtDNA results are studied in isolation (e.g. Rognon and Guyomard, 2003; Hurst and Jiggins, 2005). Combined morphological and molecular characteristics have been successfully applied in other studies of European bullhead to discriminate between populations, hybrids and species (Riffel and Schreiber, 1995; Riffel and Schreiber 1998; Kontula and Väinölä, 2004; Freyhof *et al*., 2005). The examination of morphological traits in Scottish bullhead uncovered a greater variation in meristic factors than had previously been described for *C. perifretum* (Maitland and Campbell, 1992; Freyhof *et al*., 2005; Kottelat and Freyhof, 2007). When data from each of the six sampling locations were compared, significant differences were found between populations in median ray counts from the second dorsal fin, pectoral fin and anal fin, although the ranges overlapped. Differences were also very slight, with significant results being obtained from one ray differences in median counts, as was the case with pectoral fin ray counts in the Earn water (median value of 14 for the Earn Water and 13 for all other sites). Across these analyses no one location was differentiated for all three of the morphological traits, nor found to be uniquely different from the other Scottish populations. The River Almond and Brox Burn are the only two locations used in this study that are from the same river system. Counts of anal fin rays proved to be similar for both locations, and distinctly different from the Braid Burn and Earn Water populations. As bullhead are a relatively sedentary and territorial species (Tomlinson and Perrow, 2003), mixing between populations in restricted to high flow, downstream flooding events or rare density-mediated migration. It is therefore probable that populations within the same river system would exhibit morphological differentiation. The variation exhibited by our specimens is considered a result of natural variation and can be attributed to the higher quantity of specimens examined in this study compared to the small number available in reference material (e.g. Freyhof *et al.,* 2005). Adaptive radiation may be a contributing factor, due to the limited migratory capacity of the species and lack of genetic mixing, but is unlikely given the species was only introduced to Scotland in the 1950's.

Skin prickling has proven a useful measure of identifying different bullhead populations in Northern Europe (Koli, 1969; Kontula and Väinölä, 2004), but was applied with less success in Eastern Europe (Oliva and Hensel, 1962). Kottelat and Freyhof (2007) described dense prickling in juvenile *C. perifretum* but stated that prickling is greatly reduced or absent in mature males. Koli (1969) also noted that larger specimens possess less prickles than smaller individuals due to resorption of spines after the onset of maturity. The influence of age on prickling density in Scottish specimens was examined by considering the relationship between standard body length (as a proxy for age) and prickling coverage. Results showed no significant relationship between these factors in the east coast locations of the River Almond, River North Esk, Burdiehouse Burn, Braid Burn and Brox Burn. A positive relationship between standard body length and prickling coverage was produced in the Earn Water analysis. This is unexpected given that prickling density is thought to decrease with age. The relationship is moderate, reflecting the high variability of the data. The lack of relationship shown across other sites could also suggest that standard body length is a poor proxy for age, such that age-dependant prickling coverage is not examined fully in this analysis.

All bullhead examined in this study had some degree of skin prickling and a complete lateral line. This is contrary to the smooth-skinned form with a complete lateral line, and prickled-skin form with incomplete lateral line described in England by Wheeler (1977). However, results are in keeping with the description given for British bullhead in other studies (Maitland and Campbell, 1992; Freyhof *et al*., 2005; Kottelat and Freyhof, 2007). Of the morphological traits examined, the presence of dermal prickling, complete lateral line and presence of a membrane connecting the last anal fin to the body wall were attributes found in Scottish bullhead that have been previously assigned to *C. perifretum* (Freyhof *et al*., 2005; Kottelat and Freyhof, 2007). The key to genera of Cottidae in European freshwaters provided by Kottelat and Freyhof (2007) can only be applied to species under 50mm in length, due to the perceived change in prickling coverage with age. The specimens of Scottish bullhead meeting these criteria were examined using this key and were all identified as *C. perifretum*. This classic form of identification is a practical non-invasive approach, but the size restrictions in place requires additional methods of identification to be applied to adult specimens. The combined morphological and molecular approach utilised in this study has provided a more reliable means of identification. This method has shown that Scottish bullhead and *C. perifretum* are taxonomically equivalent, as proposed in the original hypothesis, thereby confirming the presence of the native English bullhead, *C. perifretum,* in Scotland.

3.5 References

Avise J. C. (2000) *Phylogeography: the history and formation of species*. Harvard University Press, Cambridge.

Boon P. and Lee A. (2005) Falling through the cracks: are European directives and international conventions the panacea for freshwater nature conservation? *Freshwater Forum* **24**, 24–37.

Carter M. G., Copp G. H. and Szomlai V. (2004) Seasonal abundance and microhabitat use of bullhead *Cottus gobio* and accompanying fish species in the River Avon (Hampshire), and implications for conservation. *Aquatic Conservation: Marine and Freshwater Ecosystems* **14**, 395–412.

Chistiakov D. A., Hellemans B. and Volckaert F. A. M. (2006) Microsatellites and their genomic distribution, evolution, function and applications: A review with special reference to fish genetics. *Aquaculture* **255**, 1–29.

Elliott J. M. (2006) Periodic habitat loss alters the competitive coexistence between brown trout and bullheads in a small stream over 34 years. *Journal of Animal Ecology* **75**, 54–63.

Englbrecht C. C., Largiadèr C. R., Hänfling B. and Tautz D. (1999) Isolation and characterization of polymorphic microsatellite loci in the European bullhead *Cottus gobio* L. (Osteichthyes) and their applicability to related taxa. *Molecular Ecology* **8**, 1966–1969.

Englbrecht C. C., Freyhof J., Nolte A., Rassmann K., Schliewen U. and Tautz D. (2000) Phylogeography of the bullhead *Cottus gobio* (Pisces: Teleostei: Cottidae) suggests a pre-pleistocene origin of the major central European populations. *Molecular Ecology* **9**, 709–22.

Eppe R., Persat H., Beaudou D. and Berrebi P. (1999) Genetic variability in sculpin (genus *Cottus*) from southern France, with reference to the taxonomic status of an endemic species, *C. petiti*. *Heredity* **83**, 533–540.

Freyhof J., Kottelat M. and Nolte A. (2005) Taxonomic diversity of European *Cottus* with description of eight new species (Teleostei: Cottidae). *Ichthyological Exploration of Freshwaters* **16**, 107–172.

Fox P. (1978) Preliminary observations on different reproduction strategies in the bullhead (*Cottus gobio* L.) in northern and southern England. *Journal of Fish Biology* **12**, 5–11.

Gaudin P. and Caillere L. (2000) Experimental study of the influence of presence and predation by sculpin, *Cottus gobio* L., on the drift of emergent brown trout, *Salmo trutta* L. *Archiv für Hydrobiologie* **147**, 257–271.

Gershoni M., Templeton A. R. and Mishmar D. (2009) Mitochondrial bioenergetics as a major motive force of speciation. *BioEssays* **31**, 642–650.

Goto A., Yokoyama R. and Sideleva V. G. (2015) Evolutionary diversification in freshwater sculpins (Cottoidea): a review of two major adaptive radiations. *Environmental Biology of Fishes* **98**, 307–335.

Guindon S. and Gascuel O. (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**, 696–704.

Guindon S., Dufayard J. F., Lefort V., Anisimova M., Hordijk W. and Gascuel O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology* **59**, 307–321.

Hänfling B. and Brandl R. (1998a) Genetic differentiation of the bullhead *Cottus gobio* L. across watersheds in Central Europe: evidence for two taxa. *Heredity* **80**, 110–117.

Hänfling B. and Brandl R. (1998b) Genetic variability, population size and isolation of distinct populations in the freshwater fish *Cottus gobio* L. *Molecular Ecology* **7**, 1625–1632.

Hänfling B., Hellemans B., Volckaert F. A. M. and Carvalho G. R. (2002) Late glacial history of the cold-adapted freshwater fish *Cottus gobio*, revealed by microsatellites. *Molecular Ecology* **11**, 1717–1729.

Hill G. E. (2016) Mitonuclear coevolution as the genesis of speciation and the mitochondrial DNA barcode gap. *Ecology and Evolution* **6**, 5831–5842.

Hillis D. M. and Bull J. J. (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* **42**, 182–192.

Holm S. (1979) A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics* **6**, 65–70.

Huelsenbeck J. P. and Ronquist F. (2001) MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17**, 754–755.

Hurst G. D. D. and Jiggins F. M. (2005) Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. *Proceedings of the Royal Society of London B: Biological Sciences* **272**, 1525–1534.

Kearse M., Moir R., Wilson A., Stones-Havas S., Cheung M., Sturrock S., Buxton S., Cooper A., Markowitz S., Duran C., Thierer T., Ashton B., Mentjies P. and Drummond A. (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**, 1647–1649.

Kimura M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**, 111–120.

Koli L. (1969) Geographical variation of *Cottus gobio* L. (Pisces, Cottidae) in Northern Europe. *Annales Zoologici Fennici* **6**, 353–390.

Kontula T. and Väinölä R. (2004) Molecular and morphological analysis of secondary contact zones of *Cottus gobio* in Fennoscandia: geographical discordance of character transitions. *Biological Journal of the Linnean Society* **81**, 535–552.

Kottelat M. (1997) European freshwater fishes. An heuristic checklist of the freshwater fishes of Europe (exclusive of former USSR), with an introduction for non-systematists and comments on nomenclature and conservation. *Biologia Bratislava* **52**, 1–271.

Kottelat M. and Freyhof J. *Handbook of European freshwater fishes*. Publications Kottelat, 2007.

Knapen D., Knaepkens G., Bervoets L., Taylor M. I., Eens M. and Verheyen E. (2003) Conservation units based on mitochondrial and nuclear DNA variation among European bullhead populations (*Cottus gobio* L., 1758) from Flanders, Belgium. *Conservation Genetics* **4**, 129–140.

Kumar S., Stecher G., and Tamura K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular biology and evolution* ***33***, 1870–1874.

Lane N. (2009) Biodiversity: On the origin of bar codes. *Nature* **462**, 272–274.

Maitland P. S. (1972) A key to the freshwater fishes of the British Isles: with notes on their distribution and ecology. *Freshwater Biological Association Scientific Publication* **27**, 1–139.

Maitland P. S. (1977) Freshwater fish in Scotland in the 18th, 19th and 20th centuries. *Biological Conservation* **12**, 265–278.

Maitland P. S. and Campbell R. N. (1992) *Freshwater fishes*. 368 pp. London, Harper Collins.

Mills C. A. and Mann R. H. K. (1983) The bullhead *Cottus gobio,* a versatile and successful fish. *Annnual Report of the Freshwater Biological Association,* Ambleside, Cumbria, 76–88.

Minitab 17 Statistical Software (2010) [Computer software]. State College, PA: Minitab, Inc. (www.minitab.com).

Nybelin O. (1958) Några resultat av naturhistoriska museets undersökningar över svenska sötvattensimpor. *Göteborgs Musei Årstryck* **1958**, 32–56.

Oliva O. and Hensel K. (1962) Studies on sculpins (*Cottus gobio* L.) from the river Pruth. *Vestnik. Cechoslov. Spolecnosti Zool* **36,** 244–249.

Palm D., Lindberg M., Brännäs E., Lundqvist H., Östergren J. and Carlsson U. (2009) Influence of European sculpin, *Cottus gobio*, on Atlantic salmon *Salmo salar*, recruitment and the effect of gravel size on egg predation - implications for spawning habitat restoration. *Fisheries Management and Ecology* **16**, 501–507.

Pihlaja O., Niemelä E. and Erkinaro J. (1998) Introduction and dispersion of the bullhead, *Cottus gobio* L., in a sub-Arctic salmon river in northern Finland. *Fisheries Management and Ecology* **5**, 139–146.

Posada D. and Crandall K. A. (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.

Riffel M. and Schreiber A. (1995) Coarse-grained population structure in Central European sculpin (*Cottus gobio* L.): secondary contact or ongoing genetic drift? *Journal of Zoological Systematics and Evolutionary Research* **33**, 173–184.

Riffel M. and Schreiber A. (1998) Morphometric differentiation in populations of the Central European sculpin *Cottus gobio* L., a fish with deeply divergent genetic lineages. *Canadian Journal of Zoology* **76**, 876–885.

Rognon X. and Guyomard R. (2003) Large extent of mitochondrial DNA transfer from *Oreochromis aureus* to *O. niloticus* in West Africa. *Molecular Ecology* **12**, 435–445.

Ronquist F. and Huelsenbeck J. P. (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574.

Šlechtová V., Bohlen J., Freyhof J., Persat H. and Delmastro G. B. (2004) The Alps as barrier to dispersal in cold-adapted freshwater fishes? Phylogeographic history and taxonomic status of the bullhead in the Adriatic freshwater drainage. *Molecular Phylogenetics and Evolution* **33**, 225–239.

Sonnenberg R., Nolte A. W. and Tautz D. (2007) An evaluation of LSU rDNA D1-D2 sequences for their use in species identification. *Frontiers in Zoology* **4**, 6.

Smyly W. (1957) The life-history of the bullhead or miller's thumb (*Cottus gobio* L.). *Proceedings of the Zoological Society of London* **128**, 431–454.

Tomlinson M. and Perrow M. (2003) Ecology of the Bullhead. *Conserving Natura 2000 Rivers Ecology Series No. 4*. English Nature, Peterborough.

Wheeler A. (1977) The origin and distribution of the freshwater fishes of the British Isles. *Journal of Biogeography* **4**, 1–24.

Volckaert F. A. M., Hänfling B., Hellemans B. and Carvalho G. R. (2002) Timing of the population dynamics of bullhead *Cottus gobio* (Teleostei: Cottidae) during the Pleistocene. *Journal of Evolutionary Biology* **15**, 930–944.

Part II: Exploring the Ecological Interactions of Introduced Scottish Bullheads with Native Fauna, to Address the Criteria for Invasive Designation

Carlops sampling site on the River North Esk

Chapter 4. Dietary Overlap Between Introduced Bullhead *Cottus perifretum* and Native Freshwater Fishes in Scotland

4.1 Introduction

The successful establishment of an invasive species can have significant ecological consequences for native biota. Impacts can be seen at a range of biological levels, from genetic and behavioural changes in individuals, to community and ecosystem-level impacts (Parker *et al.*, 1999; Simon and Townsend, 2003). In communities where an invasive species shares a similar ecological niche to a native species, direct competition for habitat and prey can occur. Such circumstances can potentially lead to behavioural and morphological adaptations in native species, competitive exclusion or extinction events (Mooney and Cleland, 2001).

Behavioural and ecological changes resulting from the arrival of an invasive species have been well documented in the aquatic environment. The introduction of Nile perch (*Lates niloticus*) to Lake Victoria for sports fishing is thought to be responsible for the loss of 200-300 species of native cichlid (Smout, 2003). Miller *et al.* (1989) looked at all known contributing factors in the extinction of North American freshwater fishes throughout the twentieth century and cited the introduction of invasive species as the second most significant factor, superseded only by physical habitat alteration. Changes in habitat complexity within benthic communities have been reported as a result of the establishment of the invasive zebra mussel (*Dreissena polymorpha*) and golden mussel (*Limnoperna fortunei*) across multiple continents (Burlakova *et al.*, 2012). Competitive exclusion by introduced brown trout (*Salmo trutta*) is thought to have fragmented the distribution of native fish in Australasia (Crowl *et al.*, 1992; McIntosh, 2000). Alterations in the feeding habits of native species are a common consequence of invasions, as are changes in the foraging activity of other trophic levels (e.g. Simon and Townsend, 2003). The disruption of trophic cascades has also been demonstrated in studies investigating the establishment of invasive carp (*Cyprinus carpio*) in a number of continents (e.g. Khan *et al.,* 2003; Wahl *et al.,* 2011), and the introduction of Arctic charr (*Salvelinus alpinus*) and three-spined stickleback (*Gasterosteus aculeatus*) in Norway (Amundsen *et al.*, 2013). The relative connectivity of aquatic systems, significant human activity associated with these regions and the mobility of piscivorous predators between unconnected regions render freshwater systems vulnerable to the spread of invasives. Early identification and careful monitoring of communities at risk from invasion-mediated changes to ecological processes is of fundamental importance to maintaining biodiversity and preventing the loss of native species.

In Scotland, competition between introduced bullheads (*Cottus perifretum*), native stone loach (*Barbatula barbatula*) and native brown trout (*Salmo trutta*) is likely, due to an overlap in their ecological niche. All three species exploit similar invertebrate prey sources, and have a shared habitat preference. A convoluted food web exists in mixed communities, in which brown trout adults can prey upon bullheads and stone loach (Mann and Orr, 1969; Tomlinson and Perrow, 2003), but adult bullheads can consume brown trout fry (Gaudin and Heland, 1884; Gaudin, 1985). Competitive displacement of native fishes by bullheads and their potential to prey upon the eggs and young of salmonids are largely responsible for the species' invasive designation in Scotland. Despite this, both interactions remain untested in Scottish communities containing novel bullhead populations.

Shared habitat requirements have been documented both in general descriptions of habitat preference for bullheads, stone loach and brown trout fry, and in studies examining competition for habitat resources. Bullheads and stone loach have a relatively small home range, residing in shallow crevices produced by stony substrates in slow flowing, well-oxygenated waters (Smyly, 1955; 1957). In rivers supporting populations of brown trout, these habitats also provide refuge for fry during late spring (Armstrong *et al*., 2003). Rapid development is essential for brown trout fry at this critical life stage and the selection of optimal habitat has a marked influence on growth (Höjesjö *et al*., 2004). Brown trout fry and stone loach are known to cohabit the same crevices under stones (Smyly, 1955), demonstrating peaceful coexistence between these species, however there is also significant overlap in their invertebrate prey choices. The relationship between brown trout fry and bullheads is less clear, with different studies providing contradictory results. Brown trout fry have been found to utilise less optimum habitat in the presence of adult bullheads under experimental conditions, showing a preference for margins of an artificial channel and maintaining cryptic colouration, indicating they are under stress (Roussel and Bardonnet, 1999). These findings were contradicted by Louhi *et al*. (2014), who investigated dietary variations and competition for habitat between Alpine bullheads (*Cottus poecilopus*) and brown trout juveniles (0+ and 1+ age classes) in an artificial set-up, and found that brown trout juveniles forced co-occurring bullheads into sub-optimal habitats. These conflicting results suggest that bullhead and brown trout interactions vary between populations, and are likely related to community dynamics and the availability of suitable shelter. As brown trout develop, they will migrate downstream and seek out deeper habitat with more vegetative cover (Armstrong *et al*., 2003). Competition for habitat between brown trout fry and bullheads in Scotland is therefore temporary, due to the transient nature of this developmental stage. In contrast, feeding niche overlap between bullheads, stone loach and brown trout of all age classes is potentially a consistent source of competition.

Bullheads, stone loach and brown trout of all age classes share a preference for macroinvertebrate prey, although terrestrial invertebrates make up a large component of the diet of adult brown trout (Bridcut and Giller, 1995) (Table 4.1). All species have been shown to vary their diet according to local availability, resulting in a high degree of dietary similarity in mixed communities (Hesthagen *et al*., 2004; Vlach *et al*., 2013). Some resource partitioning is evident in the foraging behaviour of each species. Adult brown trout are predominantly drift feeders (Horton, 1961; Chaston, 1968) and are most active during dusk, dawn and periods of darkness (Giroux *et al.,* 2000). Brown trout are relatively unselective in their feeding choices, consuming a range of prey species based on availability (Michel and Oberdorff, 1995) and habitat constraints (Bridcutt and Giller, 1993; Lehane *et al.,* 2001). Bullheads and stone loach are benthic feeders (Andreasson, 1971; Glova *et al*., 1992; Smyly, 1955; Hyslop, 1982), opportunistically foraging for prey within the benthos (Morris, 1978). Bateson (1890) gave a description of foraging activity of stone loach, which pick up the scent of a food source and feel around the surrounding area with their barbels to locate their prey source. Bullheads feed on mobile prey, stalking swimming invertebrates before striking and swallowing prey whole (Smyly, 1957).

Table 4.1. Diet composition of juvenile and adult life stages of brown trout, bullheads and stone loach. Adapted from Nunn *et al*. (2012). Shared prey items are shown in bold.

Species	Life Stage	Prey	Selected References
Brown trout	Juvenile	**Chironomid (larvae)**, **Coleoptera**, **Diptera**, **Ephemeroptera (larvae)**, **Plecoptera (larvae)**, **Simulidae (larvae)**, **Trichoptera (larvae)**	Frost (1950), Horton (1961), Zimmerman and Mosegaard (1992), Rincón and Lobon-Cervia (1999), Sanchez-Hernandez *et al.* (2011)
	Adult	**Chironomid (larvae and pupae)**, **Coleoptera**, **Diptera**, **Ephemeroptera (larvae)**, Hymenoptera, Lepidoptera, Limnaeidae, Oligochaeta, **Plecoptera (larvae)**, **Trichoptera (larvae)**; aerial and terrestrial insects; minnows, bullheads and stone loach	Horton (1961), Mann and Orr (1969), Lien (1981), Michel and Oberdorff (1995), Lehane *et al*. (2001), Shustov *et al.* (2014)
Bullhead	Juvenile	Asellidae, **Chironomid (larvae)**, **Ephemeroptera (larvae)**, Gastropoda, **Simulidae**, **Trichoptera (larvae)**	Hyslop (1982), Welton *et al.* (1983), Nunn *et al.* (2007)
	Adult	Asellidae, **Chironomid (larvae)**, **Ephemeroptera (larvae)**, **Diptera (larvae)**, Gammaridae, **Plecoptera**, **Trichoptera**; small sticklebacks and brown trout juveniles	Mann and Orr (1969), Andreasson (1971), Morris (1978), Crisp *et al.* (1978), Shustov *et al.* (2014)
Stone loach	Juvenile	**Chironomid (larvae)**, Chydoridae, Cyclopoida, **Ephemeroptera (larvae)**, Oligochaeta, Ostracoda, **Simulidae**, **Trichoptera (larvae)**	Hyslop (1982); Welton *et al.* (1983)
	Adult	Cladocera, **Chironomid (larvae and pupae)**, **Ephemeroptera (larvae)**, Gammaridae, Mollusca, **Plecoptera (larvae)**, **Simulidae** (**larvae** and pupae), **Trichoptera (larvae)**, zooplankton	Smyly (1955), Shustov *et al.* (2014)

Despite a clear overlap in feeding preferences, studies examining feeding interactions between bullheads, stone loach and salmonids have produced conflicting results as to the presence of direct competition. No intensive competition was found between sympatric Carpathian bullheads (*Cottus poecilopus*), stone loach and brown trout in Poland, largely due to variations in trophic niche exhibited by the trout and bullheads examined (Straskraba *et al.*, 1966). Other authors have similarly concluded that bullheads have no significant effect on densities of trout or juvenile salmon (*Salmo salar*) (Carter *et al.*, 2004), and cause no significant feeding competition within mixed freshwater communities (Straskraba *et al.*, 1966). Resource partitioning between bullhead and brown trout has been documented in a comparative dietary study on bullheads and brown trout in Sweden, where more than 80% of the prey consumed by brown trout was terrestrial in origin, compared to the entirely aquatic diet of bullheads (Dahl, 1998). In contrast, dietary-mediated competition between the two species has been reported in some regions, and was found to result in lower densities of trout in areas containing resident bullheads (Gaudin and Heland, 1984; Gabler and Amundsen, 1999; Gaudin and Caillere, 2000).

The results of studies conducted within the UK and Scandinavia also largely contradict the perception that bullheads prey upon the eggs and juveniles of Atlantic salmon and brown trout. In Finland, Pihlaja *et al*. (1998) reported that bullhead presence had no effect on juvenile salmon densities in the river catchment studied. Within the bullhead's native English range, Mills and Mann (1983) failed to show that the species is a significant predator of salmonid eggs or larvae. This was confirmed by Mann and Orr (1969), who recovered only one trout fry and no eggs from the stomach contents of 1125 bullheads examined. A recent study in Sweden found that Atlantic salmon recruitment was reduced in the presence of introduced bullheads (Palm *et al*., 2009), although the majority of other studies that verify this behaviour are focused on other members of the *Cottus* genus interaction with salmonids in North America (Foote and Brown, 1998; Biga *et al*., 1998; Tabor *et al.,* 2004).

Despite a strongly overlapping ecological niche, foraging habitat segregation between sympatric bullheads and stone loach has been reported. Under controlled laboratory conditions, Welton *et al*. (1991) examined feeding rates of both species under different light and substrate conditions and recorded higher incidences of bullhead foraging under high light exposure for both substrata examined, and more active stone loach foraging in darker laboratory conditions on a silt substratum. In a study investigating cohabiting wild populations in the UK, stone loach were found to select smaller invertebrate prey relative to stream availability, whereas bullheads actively sought out larger prey individuals (Welton *et al*., 1983). Contradictory results were published by Copp *et al.* (1994), who examined the dietary composition of bullheads and stone loaches in three English rivers and found no significant differences in the percentage dietary composition or overall diet of the two species. Vlach *et al*. (2013) also looked at food competition between a number of freshwater fish in Central Bohemia and found a substantial overlap between the prey selection of bullheads and stone loach.

There is a high degree of seasonality in the availability of prey and fish predation rates. In general, more food is consumed during summer months when invertebrate prey is plentiful (Thomas, 1962). Mann and Orr (1969) attributed this behaviour to a seasonal raise in ambient temperatures causing an increasing in fish digestion rates. In mixed fish communities in Russia, Shustov *et al.* (2014) suggested that competition for food resources during summer months was unlikely, due to high rates of consumption of aerial insects exhibited by brown trout. Autumn saw an overlap in the diets of Atlantic salmon parr and stone loach, but a reduction in the feeding activity of bullheads. In winter, the feeding activity of all species was considered low, resulting in no feeding competition. It was concluded that feeding competition was seasonal and likely to only be evident in autumn, when salmon parr and stone loach show a high degree of overlap in prey preference. Seasonal variations in competition are likely to be similar across freshwater systems in most temperate climates.

The contradictory results of dietary studies focusing on mixed communities of bullheads, stone loach and brown trout make it difficult to predict the extent of feeding niche overlap in the Scottish freshwater communities that contain this introduced species. Despite a number of studies examining competition between bullheads and native fishes within the species' native English range (e.g. Mann and Orr, 1969; Hyslop, 1982; Elliot, 2006), no published study has directly looked at feeding competition between introduced bullheads and native fishes in Scotland. Morris (1978) examined the diets of Scottish fishes and used the results in conjunction with other published works to conclude that, despite an overlap in macroinvertebrate prey preferences, there was no clear evidence of competition between bullheads, brown trout and Atlantic salmon in Scottish rivers. However, no attempt was made to study the stomach contents of sympatric bullheads and native fishes simultaneously. A multi-species investigation of stomach contents would build upon the knowledge obtained from the work of Morris (1978), and provide a comparative data-set some thirty-six years after the completion of the original study.

Due to the invasive status of Scottish bullheads, an investigation into competition between bullheads and native fishes in an important step in determining the full extent of the species' influence on Scottish freshwater communities. The capacity to determine levels of salmonid egg cannibalisation exhibited by bullheads is constrained by the seasonal availability of salmonid eggs. This study was conducted outside this window and, as such, an examination of this behaviour is not within the scope of this project. Based on the results of other studies, competition for habitats is also thought to be transient and not as significant a source of conflict as competition for prey.

4.1.1 Aims and Objectives

Competition for prey resources is the primary focus of this chapter, which aims to determine whether the introduction of non-native bullheads to Scottish freshwater communities has led to a shift in the dietary selection of co-occurring native fishes. Due to the similarity in macroinvertebrate prey preferences described in the literature, it is hypothesised that bullhead presence will influence the diets of native stone loach and brown trout, potentially resulting in these species preying upon a wider range of macroinvertebrates in the presence of bullheads. The feeding preferences of bullheads, stone loach and brown trout are explored in order to test this hypothesis, and determine if this relationship is a contributing factor in the invasive classification of bullheads in Scotland.

4.2 Methods

Fish were sampled from ten locations across Edinburgh and the Lothians (Figure 4.1). Four locations were bullhead-free control sites, containing either a mixed community of stone loaches and brown trout or a resident population of one of the species. The other six sites contained established bullhead populations and either co-occurring brown trout or stone loach, or a mixed community of all three species.

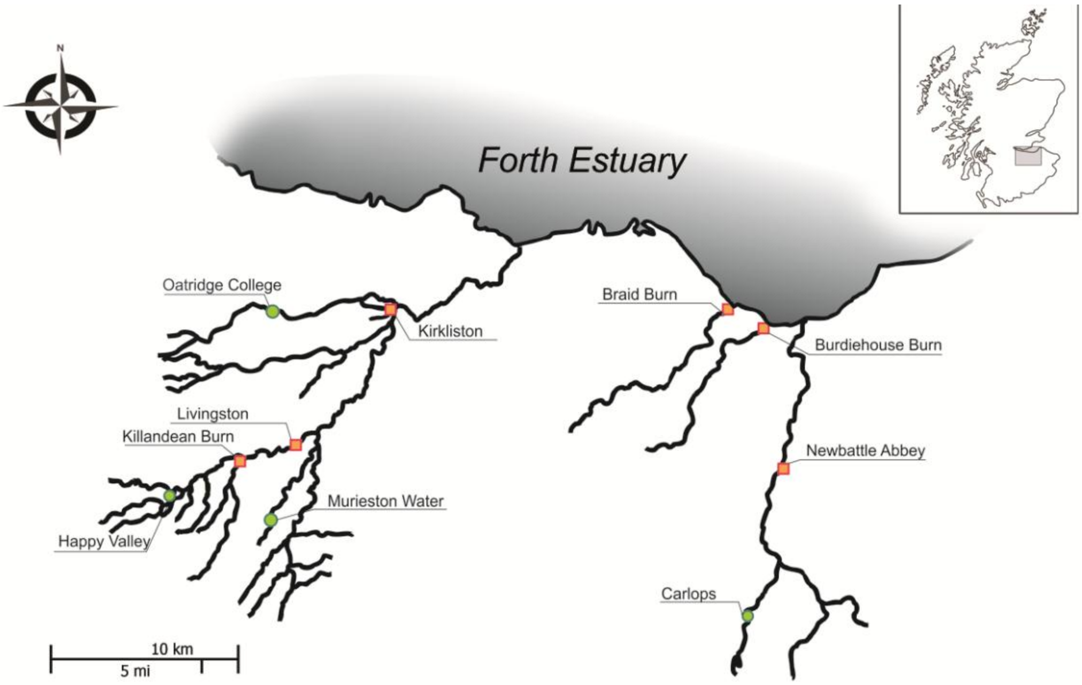

Figure 4.1. Location of the ten sampling sites used in the study. Locations are marked as either bullhead present (🟧) or bullhead absent (🟢) sites

4.2.1 Laboratory Examination

4.2.1.1 Invertebrate Communities

Kick sample contents of the storage jars were run through a 500µm sieve and then placed in individual sorting trays. Macroinvertebrates were systematically removed and identified to family level, before being placed in a 30ml vial of 70% ethanol with other members of the same family. Samples were later quality checked under an Olympic SZ51 dissection microscope to ensure accurate identification. Individuals from each family were counted to provide a measure of community composition.

An average weight per individual (g) was obtained for each family group by selecting five members of the same family at random and allowing them to air dry for thirty minutes to remove residual ethanol. Outer casings (cased caddis flies) and shells (bivalves and gastropods) were removed prior to air drying, as per Horton (1961). Each dried individual was weighed and an average weight across all five specimens calculated for each family group. For families where only a few individuals were recovered, all individuals were dried and weighed and an average taken across the number of available specimens. This was the case for Corixidae, Tipulidae (detritovore) and Psychomyiidae (one individual available); Lepidostomatidae, Curculionidae, Scirtidae, Ecnomidae, Ephemeridae, Leptoceridae, Hydrophilidae (an average of two individuals calculated); Perlidae and Philopotomidae (an average of three individuals calculated); and Chloroperlidae and Odontoceridae (an average of four individuals calculated).

4.2.1.2 Fish Stomach Contents

Fish were thawed in a refrigerated room (ambient temperature 8°C) for one hour (bullheads and stone loach) or overnight (brown trout). Prior to dissection, standard body length (to the nearest mm), fork length (to the nearest mm) and thawed body weight (g) were recorded. To remove the stomach, an incision was made along the ventral surface from the vent region to the operculum, exposing the internal organs. All organs were removed from the visceral cavity, and the stomach isolated from the rest of the digestive tract by an incision at the lower oesophageal sphincter and the caudal margin of the pyloric canal. The stomach was immersed in 0.9% saline solution and opened by a continuous incision covering the length of the stomach wall. An examination of stomach contents was carried out under an Olympic SZ51 dissection microscope at varying magnifications, ranging from 6.7x to 45x. Organisms were extracted from the stomach contents, then identified to the highest possible taxonomic resolution (up to family-level) and results recorded in the relevant prey category. Some prey items could only be identified to order level so prey categories were created to cover each invertebrate family, incidences of juvenile bullheads, unidentified fish and additional order-level categories covering unknown fly larvae (Diptera), mayflies (Ephemeroptera), stoneflies (Plecoptera) and leeches (Hirudinea). Partially digested organisms that could not be conclusively identified were not recorded.

4.2.2 Statistical Analysis

4.2.2.1 Invertebrate Community Composition

Invertebrate community data were examined to give an indication of the relative densities of each family within a sampling location. The community composition, based on counts of individuals, was calculated by working out the percentage of the total invertebrate count that belonged to a particular family. This factor was designated the community composition by count (CCC). Community composition based on biomass was also determined by multiplying the number of individuals of a family group by the group's average weight measure, summing the biomass of all family groups from that sample site, and reporting each family as a percentage of the total biomass. This factor was designated the community composition by biomass (CCB). The percentage of each fish species consuming an invertebrate family was then compared to the proportion of the invertebrate community comprising that prey group, based on the CCC and CCB. The different age classes of each fish species were considered separately, and comparisons made for each location and sample year (Figures 4.3 to 4.17). For simplicity, prey categories for which the values obtained for CCC or CCB were less than 5% were removed from the tabulated results.

4.2.2.2 Stomach Contents

Comparisons Between Invertebrate Community Composition and Fish Prey Selection

All fish species were analysed separately and data segregated by sample year and location. Brown trout and bullheads were also separated by adult and juvenile (or parr, in the case of brown trout) life stages, on the basis of body length (bullheads; juveniles <50mm (Tomlinson and Perrow, 2003)), or the presence of parr marks (brown trout). The freezing and thawing stages of sample processing caused significant degradation of the stomach contents of a number of individuals. Fish with empty stomachs or those containing exclusively degraded contents that could not be identified were excluded from the statistical analysis. As a result, some sample sites only had data for one species or one year of sampling. Stomach contents were reported using the occurrence method, based on the presence and absence of a given prey category (Hyslop, 1980; Lehane *et al.,* 2001). For each individual, identification of a prey item from one of the prey categories resulted in that category receiving a score of one. All prey categories that were not represented within that individual's stomach were assigned a zero value. Subsequent incidences of the same prey category were not noted. After all fish of the same species from a sample site had been processed, the proportion of individuals consuming each prey category was calculated. This was done by dividing the number of individuals eating a prey category by the total number of fish of that species that were examined. The process was repeated for each combination of species, sampling year and location for which data were available.

The proportion of individuals from each fish species and age class consuming a given prey category was then compared to the invertebrate CCC and CCB for each location and sample year, using stacked bar graphs. For simplicity, any component of fish dietary composition or the invertebrate community that was under 5% of the total proportion was removed. A decision was made not to merge data for age classes and sampling years at each location in order to give a fuller picture of the feeding ecology of each species, relative to prey availability. Data were not obtained for all combinations of fish species, age class, location and sampling year, either due to a lack of identifiable contents in the fish examined or a particular species or age class missing from that sampling location. In these incidences, only available data for CCC, CCB and the available fish/age class combinations were reported. Graphs were not reported for location and sampling year combinations for which no fish diet data were available.

Comparisons Between Fish Species

In order to determine the effects of bullhead presence on the dietary composition of each fish species, it was necessary to merge data for sampling years from each location, and age classes for bullheads and brown trout. This created a balanced study design that could examine the variation in dietary composition of each fish species, and the direct influence of bullhead presence on the dietary composition of stone loach and brown trout. All data were square-root transformed to down-weigh the influences of the large proportional data obtained for some prey categories in the presence of several categories containing zero proportionality.

Comparisons Between the Dietary Composition of Bullheads, Brown Trout and Stone Loach

Analyses of the diets of each species were performed using PRIMER version 6 (PRIMER-E LTD) and PAST version 3.15 (Hammer *et al*., 2001). Similarities in the dietary composition of bullheads, brown trout and stone loach were examined using non-metric multidimensional scaling (MDS) on resemblance data obtained with Bray-Curtis dissimilarity measures. The separation of these groups was further explored by performing a one-way analysis of similarities (ANOSIM), based on 9999 permutations and data grouped by fish species. The potential influence of multiple testing was addressed by adjusting obtained P-values using an integrated sequential Bonferroni significance correction (Holm, 1979).

Comparisons Between Dietary Composition in the Presence and Absence of Bullheads

The influence of sympatric bullheads on the dietary composition of brown trout and stone loach was analysed using PAST software version 3.15 (Hammer *et al*., 2001). A two-way permutational multivariate analysis of variance (PERMANOVA) was conducted on brown trout and stone loach data, using 9999 iterations and Bray-Curtis similarities. Fish species and the presence and absence of bullheads were used as grouping factors. To determine where dissimilarities existed between the bullhead present and bullhead absent groups of fish, Similarity Percentage Analysis (SIMPER) was conducted on the stone loach and brown trout data, using bullhead presence or absence as grouping categories.

4.3 Results

4.3.1 Comparing Invertebrate Community Composition and Fish Prey Selection

The selection of prey categories for each of the fish species and age classes examined was shown to vary with location and sample year (Figures 4.4 to 4.13). This was commonly disproportional to availability within the invertebrate community, as determined by the breakdown of the invertebrate community composition, based on numbers of individuals (CCC) or dry biomass (CCB). A common key was produced for all figures to facilitate the accurate viewing and interpretation of graphs (Figure 4.3).

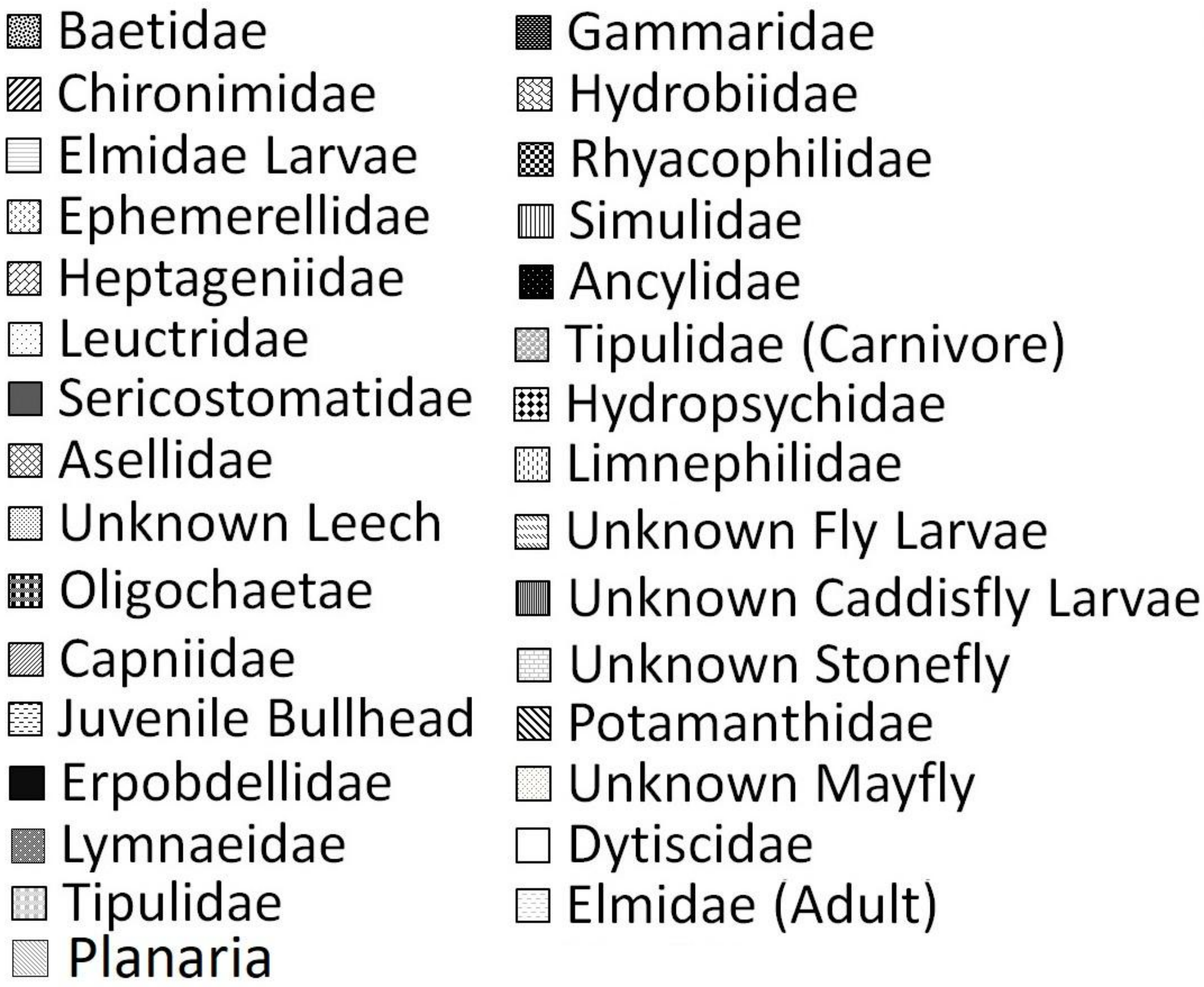

Figure 4.3. Key to prey categories used in stomach content and invertebrate community analyses

Kirkliston

At Kirkliston, Rhyacophilidae made up 50% of the total invertebrate community composition by biomass (Figure 4.4). Baetidae and Ephemerellidae collectively comprised a large portion of the invertebrate abundance based on count data. The most commonly consumed prey for adult bullhead was the fly larvae Simuliidae, which formed over 40% of the dietary composition. Rhyacophilidae and juvenile bullhead each contributed about 15% of the overall bullhead prey. Despite the large proportion of Baetidae, Rhyacophilidae and Ephemerellidae found at this location, adult brown trout most commonly consumed the beetle family group Hydrobiidae (over 20% of prey consumed), and large proportions of unidentified caddisfly larvae and Simuliidae.

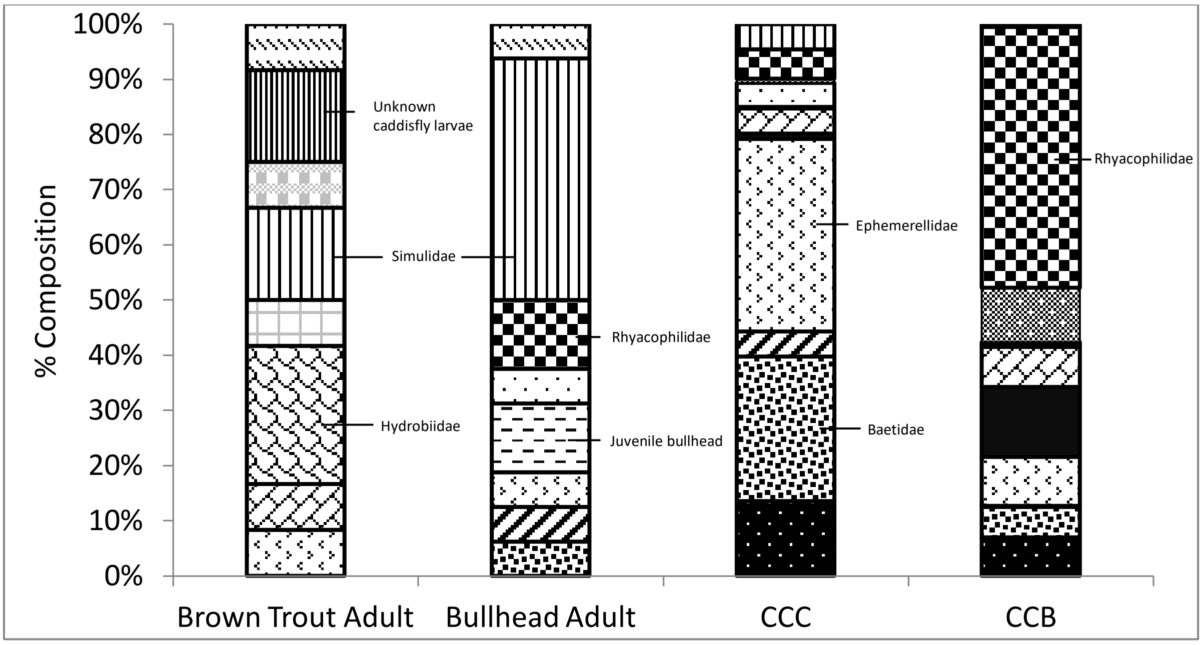

Figure 4.4. Invertebrate community composition based on count and biomass data (CCC and CCB, respectively), compared with fish dietary composition data for adult brown trout (N=4) and bullheads (N= 8), sampled from Kirkliston in 2015

Killandean Burn

At Killandean burn, the caddisfly larvae Limnephilidae and Rhyacophilidae formed the largest proportion of the invertebrate community based on biomass, and the stoneflies Baetidae and Leuctridae from the largest proportion by count (Figure 4.5). Consumption of fly larvae from the Chironomidae family was common in both adult bullhead and brown trout, and formed a substantial portion of the bullhead diet (over 40%). The remainder of the dietary composition of both species was a varied mix of invertebrate families. Feeding rates of either species were not related to invertebrate availability, as demonstrated by the disproportionate consumption of chironomids in relation to availability within the invertebrate community.

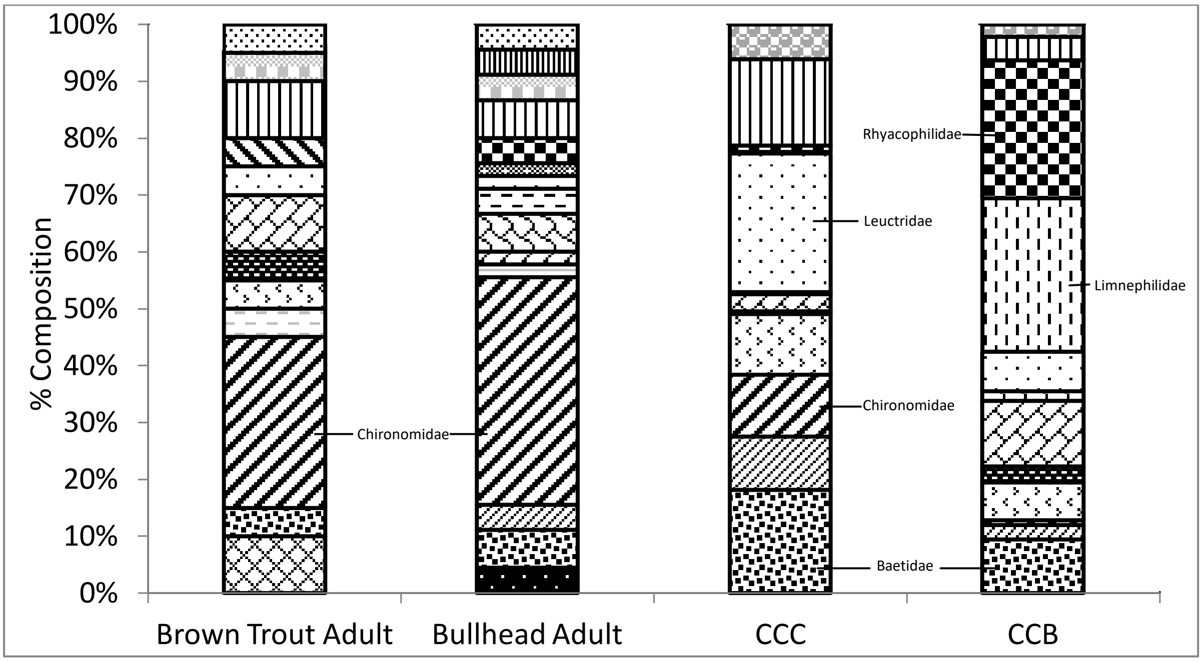

Figure 4.5. Invertebrate community composition based on count and biomass data (CCC and CCB, respectively), compared with fish dietary composition data for adult brown trout (N=6) and bullheads (N=20), sampled from Killandean Burn in 2015

Newbattle Abbey

Heptageniidae, Baetidae and Gammaridae dominated the invertebrate community at Newbattle Abbey in the 2014 samples (Figure 4.6a). Heptageniidae was an important food source for juvenile bullheads and adult brown trout in this location, forming approximately 30% and 20% of the diets of each fish respectively. Baetidae was the most abundant invertebrate based on CCC, constituting over half of the total sample. Feeding preferences in brown trout adult and parr reflected this high abundance, although the family group was proportionally less significant in the diets of adult bullheads, and absent entirely from the diets of juvenile bullheads.

In the 2015 sample, Simuliidae was the most abundant prey based on CCC information, but none were found in the stomach contents of fish. Rhyacophilidae, which was a common food source based on CCB, formed about 20% adult bullhead diet and 15% of the total prey consumed by adult brown trout. Gammaridae was equally as abundant as Rhyacophilidae based on CCB information, and constituted about 15% of bullhead diet and 10% of the diet of brown trout, but was absent completely in the diet of stone loach at this location. Almost 70% of all food selected by stone loach was the stonefly family Baetidae, which was highly disproportionate to the availability of Baetidae, as shown by the low values for CCB and CCC found for this prey category (under 10% for each composition parameter). The stonefly family Leuctridae was an important component of the diets of all fish sampled from this location in 2015, and comprised just under 25% and 10% of the CCC and CCB respectively.

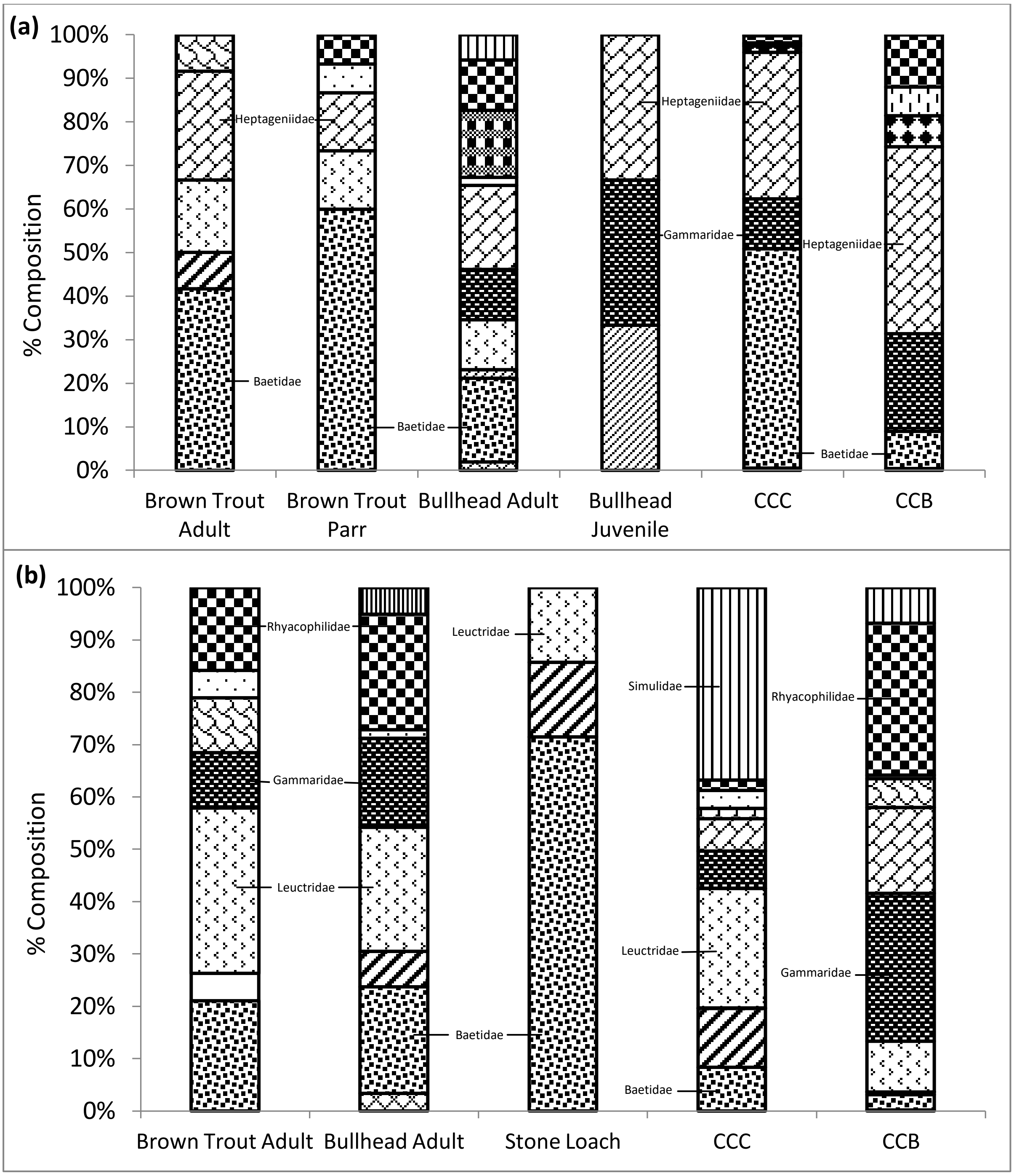

Figure 4.6. Invertebrate community composition based on count and biomass data (CCC and CCB, respectively), compared with fish dietary composition data. (a) Adult brown trout (N=5), brown trout parr (N=6), adult bullheads (N=19) and juvenile bullheads (N=2) sampled from Newbattle Abbey in 2014, (b) adult brown trout (N=6), bullheads (N=26) and stone loach (N=5), sampled from Newbattle Abbey in 2015

Burdiehouse Burn

Chironomidae larvae, which formed over 20% of the CCC at Burdiehouse Burn in 2014, formed a significant portion of the prey consumed by brown trout, bullheads and stone loach (Figure 4.7a). Rhyacophilidae contributed over 30% of the total CCB and constituted about 20% of adult bullhead diet. Tipulidae larvae and Elmidae, which form approximately 20% of stone loach (Tipulidae) and brown trout (Capniidae) diet comprise such small proportions of the invertebrate community that they are missing from the CCB and CCC measures, and must therefore be under 5% of the total count and biomass.

Analysis of the invertebrate community composition in 2015 showed a similar CCB composition to the one found in 2014, although the CCC showed more variation (Figure 4.7b). Rhyacophilidae and Lymnaeidae were the most abundant prey source based on biomass, and Elmidae larvae and Ephemerellidae the most abundant based on count. Sampled bullhead showed a preference for Asellidae, Gammaridae and chironomid larvae.

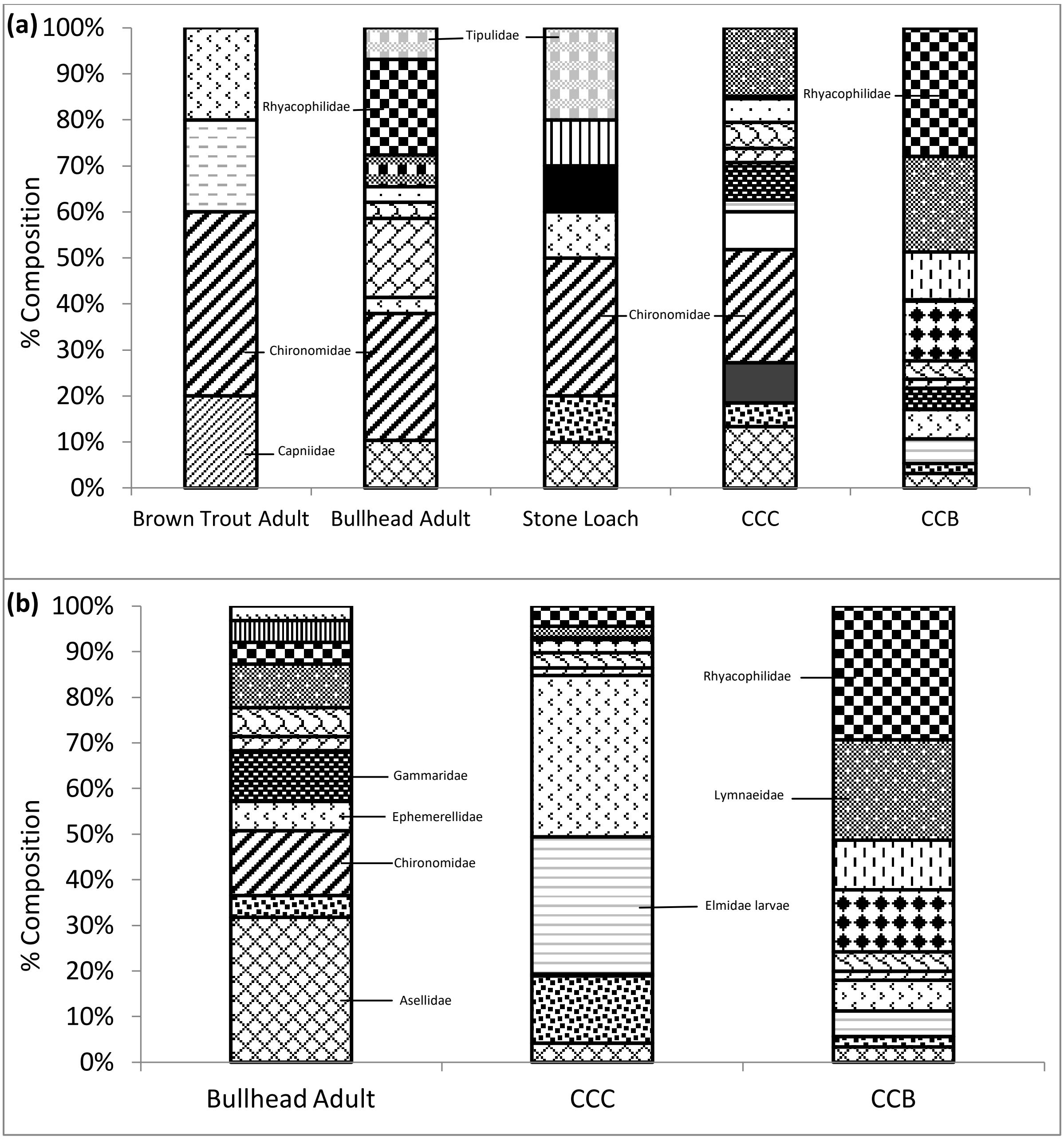

Figure 4.7. Invertebrate community composition based on count and biomass data (CCC and CCB, respectively), compared with fish dietary composition data. (a) Bullhead adult (N =20), brown trout adult (N=2) and stone loach (N=3) at Burdiehouse Burn 2014, (b) Bullhead adult (N=31) at Burdiehouse Burn in 2015

Braid Burn

Adult bullheads examined from the Braid Burn in 2014 relied heavily on Gammaridae as prey, which formed 60% of the total diet (Figure 4.8a). Asellidae and the flatworm Planaria were the only other prey categories consumed in large quantities. The most abundant prey sources, based on invertebrate count data, were Baetidae and Elmidae larvae, which were not found in the stomachs of any of the bullheads examined. Lymnaeidae, which was also missing from stomach contents, had the highest CCB score.

A range of prey sources were observed for adult bullhead at Braid Burn in 2015 (Figure 4.8b). The most common food categories, each contributing about 20% of all food consumed, were Chironomidae and Hydrobiidae. Both of these categories were equally proportional in the diets of sympatric adult brown trout. Asellidae, Ephemerellidae and Sericostomatidae were also consumed in higher abundances by the brown trout examined. Sericostomatidae had the highest biomass proportion and CCC abundance of all prey categories.

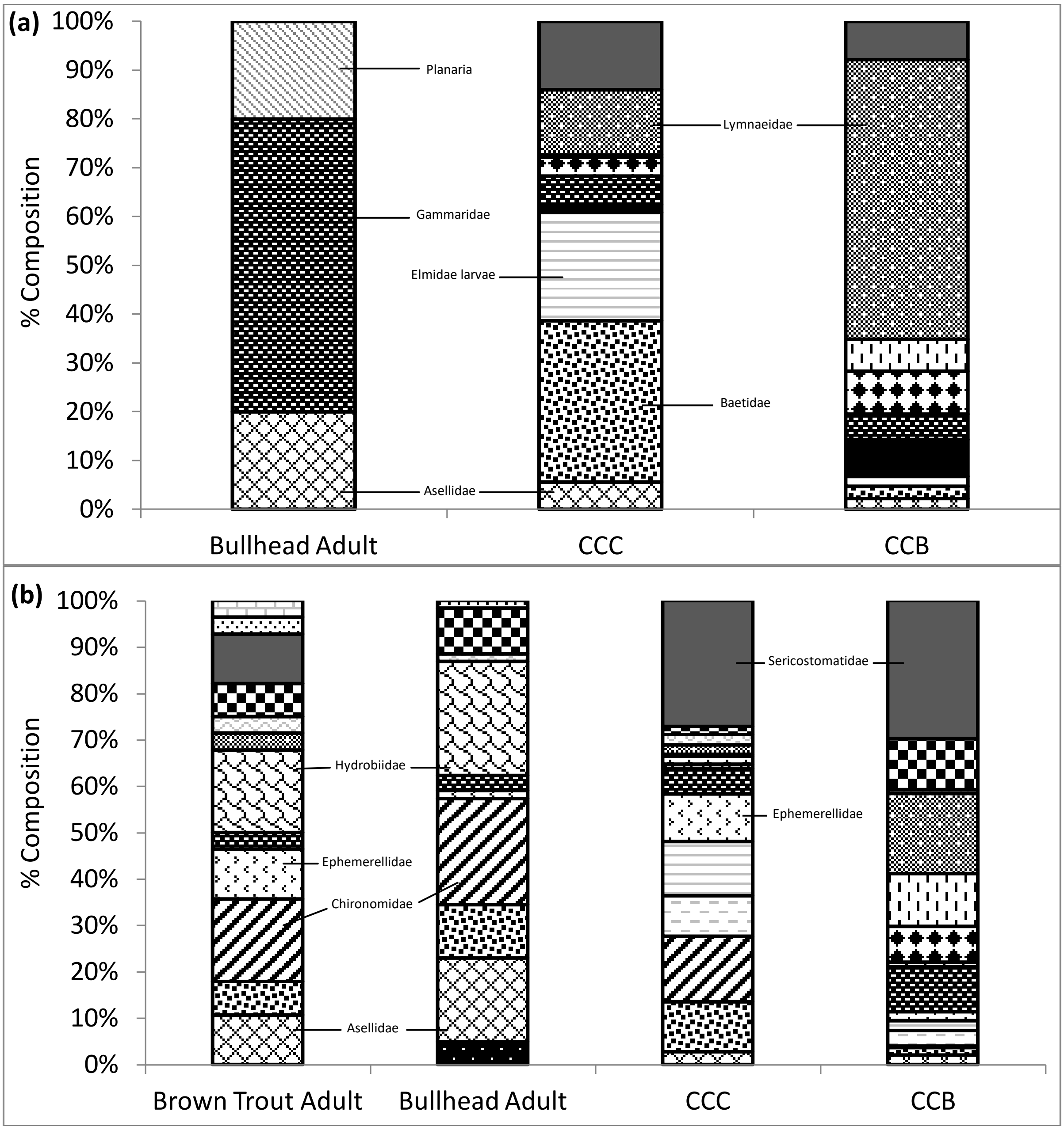

Figure 4.8. Invertebrate community composition based on count and biomass data (CCC and CCB, respectively), compared with fish dietary composition data. (a) Bullhead adult (N=30) at Braid Burn in 2014, (b) Bullhead adult (N=30) and brown trout adult (N=6) at Braid Burn in 2015

Oatridge College

Ephemerellidae dominated the CCB and CCC at Oatridge College in 2014 (Figure 4.9a). This was reflected in the diets of stone loach and brown trout, which each dedicated about 30% of their diets to this prey category. Heptageniidae and Baetide were also commonly eaten by both species. The consumption of Rhyacophilidae was also reported in both species, and seemed to reflect its availability within the community, based on CCB data.

Ephemerellidae (40%) comprised the most abundant prey available at Oatridge College in 2015, followed by Leuctridae (25%) and Baetidae (15%) (Figure 4.9b). The CCB showed relatively similar proportions of Ephemerellidae, Heptageniidae, Limnephilidae, Rhyacophilidae and Gammaridae. The presence of Ephemerellidae, Heptageniidae, Baetidae and Gammaridae in the diets of stone loach and brown trout appeared to reflect the availability of these prey categories. Adult brown trout selected the caddisfly family Rhyacophilidae and additional unidentifiable caddisfly families in almost 20% of their diet. In comparison, caddisflies provided approximately 5% of the stone loach diet.

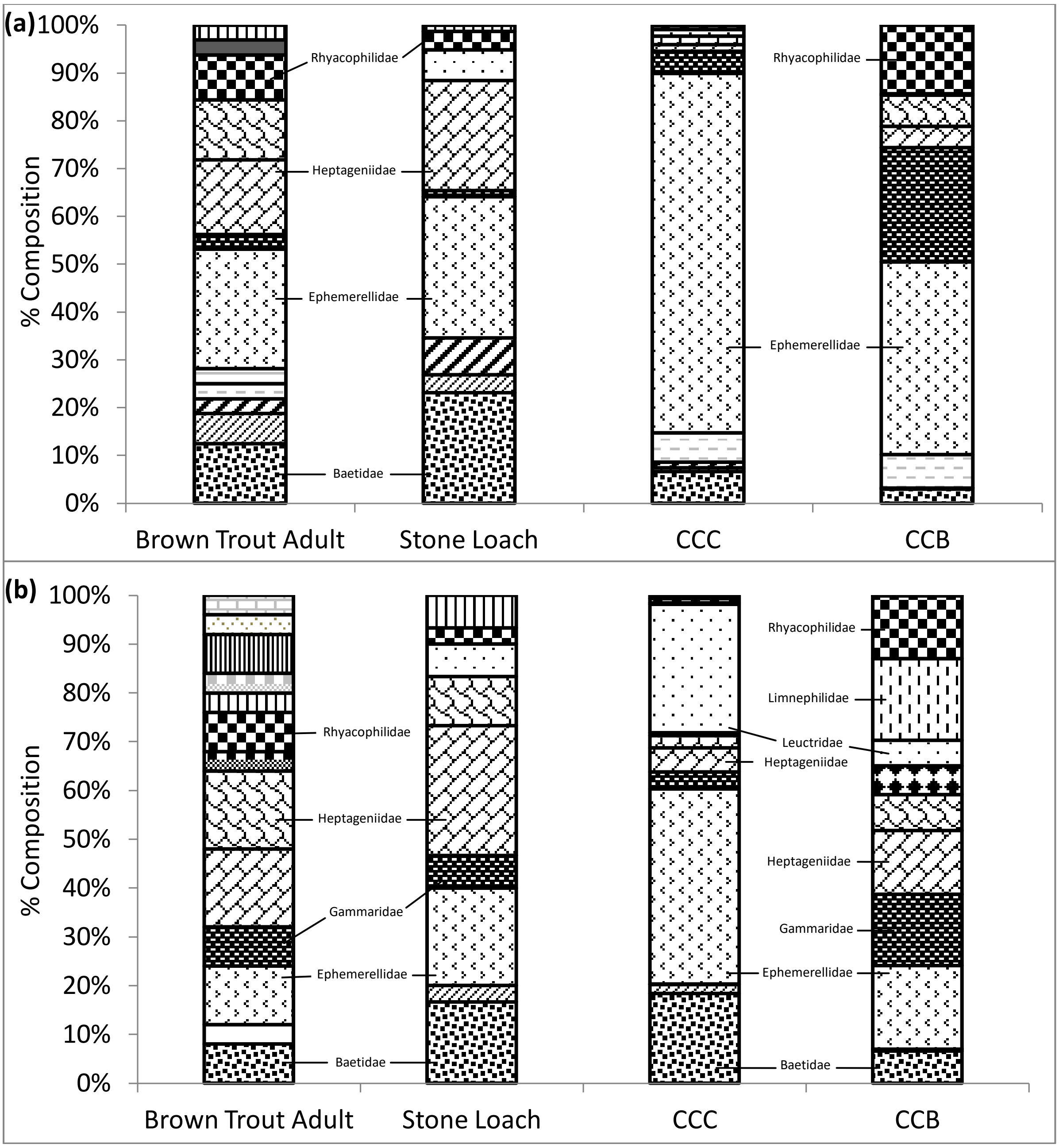

Figure 4.9. Invertebrate community composition based on count and biomass data (CCC and CCB, respectively), compared with fish dietary composition data. (a) Brown trout adult (N=8) and stone loach (N=31) at Oatridge College 2014, (b) Brown trout adult (N=7) and stone loach (N=14) at Oatridge College 2015

Livingston

Rhyacophilidae was highly abundant (>70%) at Livingston in 2015, based on CCB data (Figure 4.10). Baetidae formed about 60% of the prey available based on CCC. Rhyacophilidae was present in the diets of adult bullheads (20%) and stone loach (15%), but did not form the highest proportion of prey consumed by either species. Baetidae was the second largest component of stone loach diet (25%), and was also present in the diets of brown trout (15%). The most prominent prey category selected by stone loach was found to be Chironomidae (40%), which is absent from the CCB and only forms a small proportion of the CCC. Hydrobiidae was an additional significant prey source for bullheads, forming about 20% of the total prey consumed.

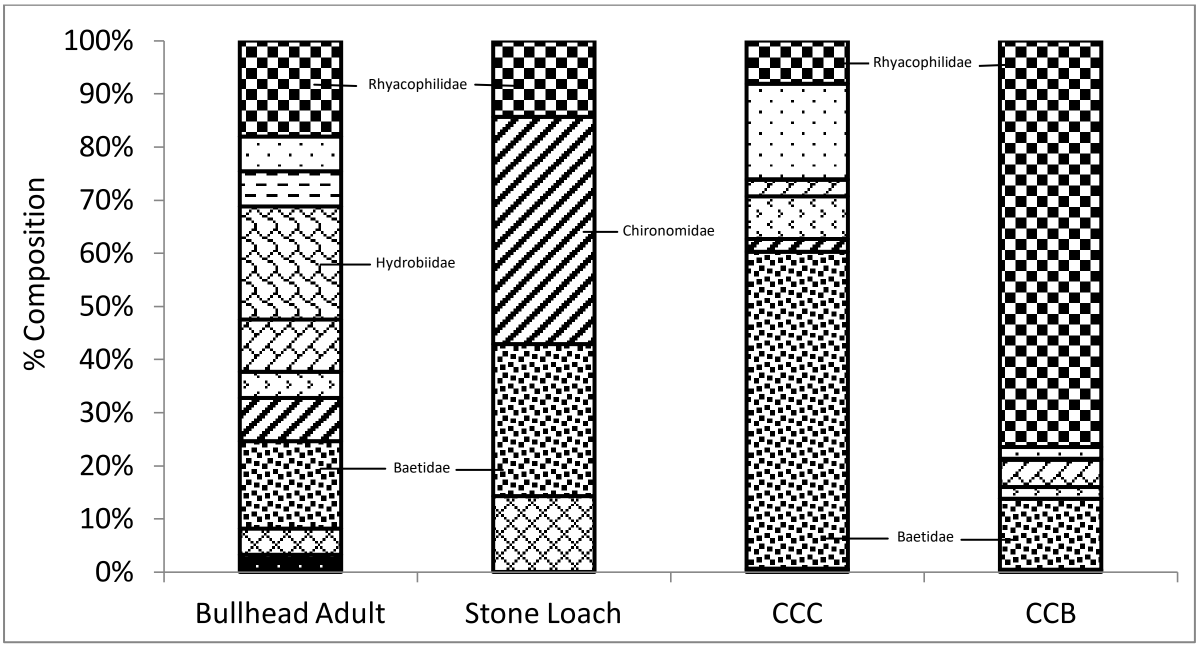

Figure 4.10. Invertebrate community composition based on count and biomass data (CCC and CCB, respectively), compared with fish dietary composition data at Livingston in 2015. Bullhead adult (N=29) and stone loach (N=4)

Happy Valley

Gammaridae was the most abundant prey source at Happy Valley based on CCB and CCC information, although Limnephilidae (40%) and Chironomidae (25%) made up a large proportion of CCB and CCC respectively (Figure 4.11). Stone loach present at this location consumed Baetidae and Heptageniidae in equal proportions (approximately 25%), with Chironomidae also providing a significant prey source. Despite significant representation in the CCC and CCB, Gammaridae was not a common source of prey for stone loach.

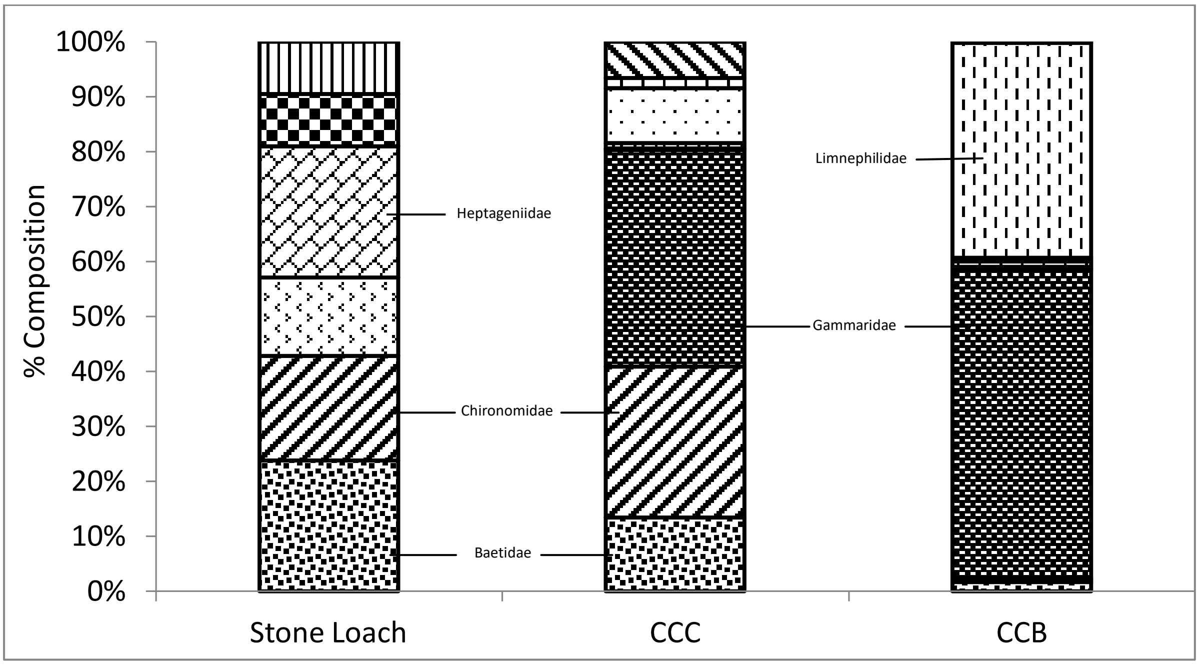

Figure 4.11. Invertebrate community composition based on count and biomass data (CCC and CCB, respectively), compared with fish dietary composition data at Happy Valley in 2015. Stone loach (N=7)

Murieston Water

Heptageniidae and Gammaridae dominated the invertebrate community at Murieston Water in 2014, based on the CCC and CCB (Figure 4.12a). Baetidae also formed just under 10% of the CCC. The availability of these prey categories was reflected in the feeding preferences of stone loach and brown trout parr. Stone loach consumed Gammaridae in extremely high proportions, with any other prey categories contributing less than 5% of the prey selected. About 60% of brown trout parr diet was formed of Heptageniidae. Chironomidae and Baetidae each provided about 10% of the prey selected. The diet of sympatric brown trout adults was highly variable. Heptageniidae and Baetidae were consumed in the highest proportions, each contributing about 20% of the total prey consumption.

Heptageniidae, Gammaridae and Hydropsychidae formed the majority of the CCB at Murieston Water in 2015 (Figure 4.12b). Baetidae and Leuctridae were also readily available in the community, based on the CCC. The diets of stone loach and adult brown trout were highly mixed. Baetidae and Chironomidae formed the highest proportion of stone loach diet, with each contributing about 20% of all prey consumed. Heptageniidae was the most significant contributor to brown trout diet, forming about 20% of the prey composition. Multiple other prey categories were consumed at this location.

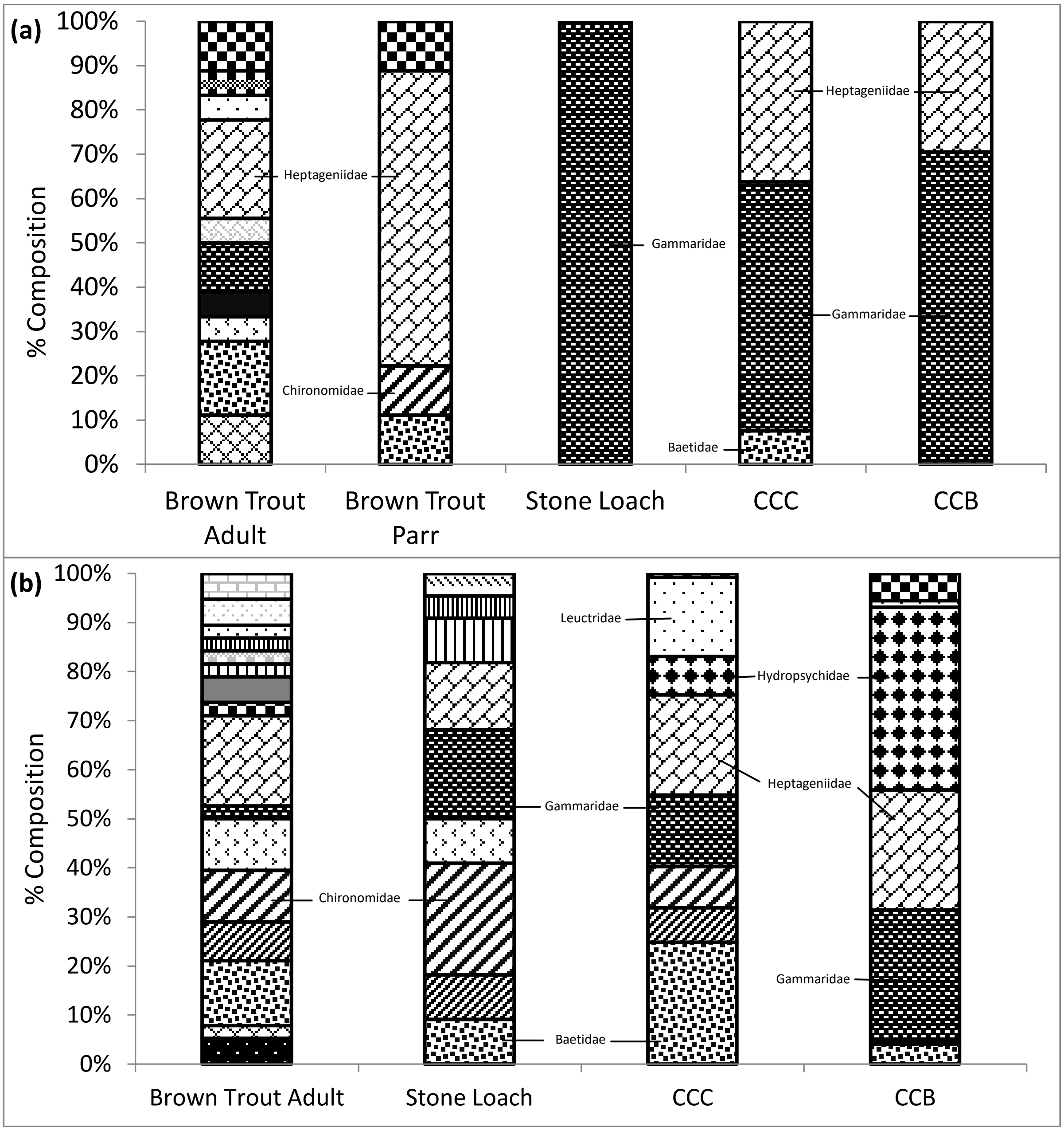

Figure 4.12. Invertebrate community composition based on count and biomass data (CCC and CCB, respectively), compared with fish dietary composition data. (a) Brown trout adult (N=7), brown trout parr (N=7) and stone loach (N=5) at Murieston Water in 2014, (b) brown trout adult (N=9) and stone loach (N=7)

Carlops

Gammaridae and Rhyacophilidae were abundant prey sources at Carlops, based on the CCB (Figure 4.13). The CCC data also showed that Baetidae and Chironomid larvae were also highly accessible. Brown trout parr diets relied heavily on Simuliidae larvae, Heptageniidae, Ephemerellidae and Capniidae, and with the former three categories each comprising about 25% of the prey composition. The diets of adult brown trout were far more varied. Baetidae and Ephemerellidae each contributed to about 15% of the total prey composition, with Gammaridae and Heptageniidae each providing an additional 10%.

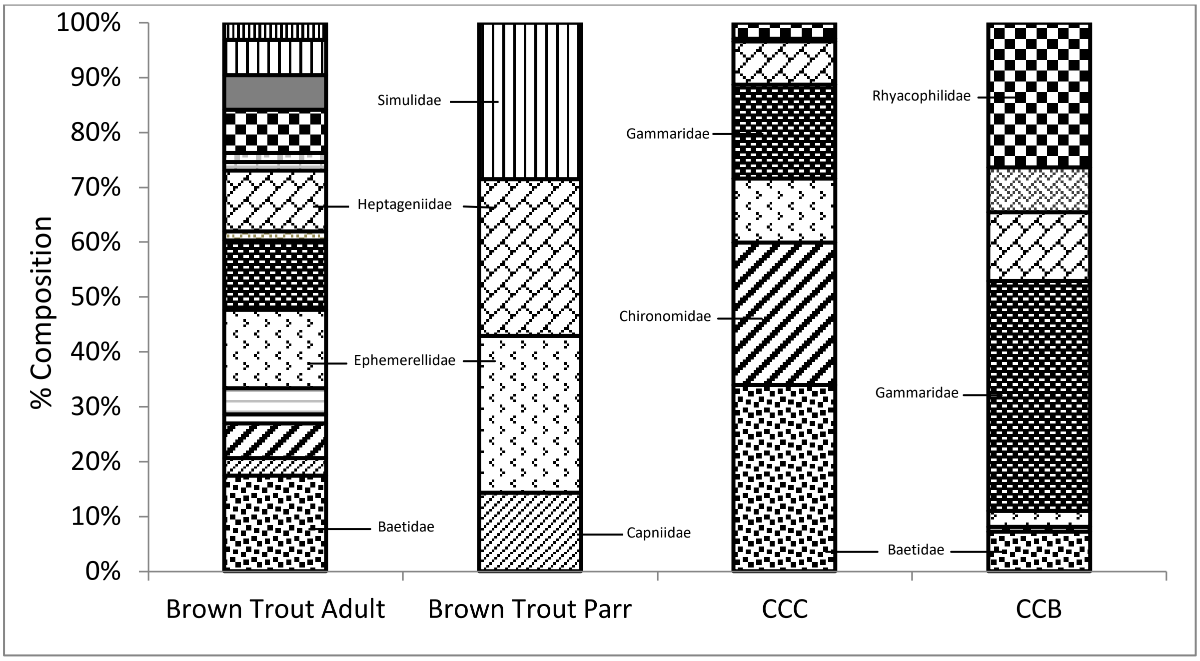

Figure 4.13. Invertebrate community composition based on count and biomass data (CCC and CCB, respectively), compared with fish dietary composition data at Carlops in 2015. Brown trout adult (N=14) and brown trout parr (N=2)

4.3.2 <u>Comparing the Dietary Composition of Bullheads, Brown Trout and Stone Loach</u>

The MDS plot of the dietary composition of bullheads, stone loach and brown trout shows some clustering of species data, particularly in the stone loach and bullhead groups (Figure 4.14). Several of the brown trout plots are interspersed among the bullhead and stone loach clusters, suggesting a higher degree of commonality between the dietary compositions of these brown trout and the stone loach and bullhead grouping than is evident between the brown trout individuals and the other conspecifics in the analysis.

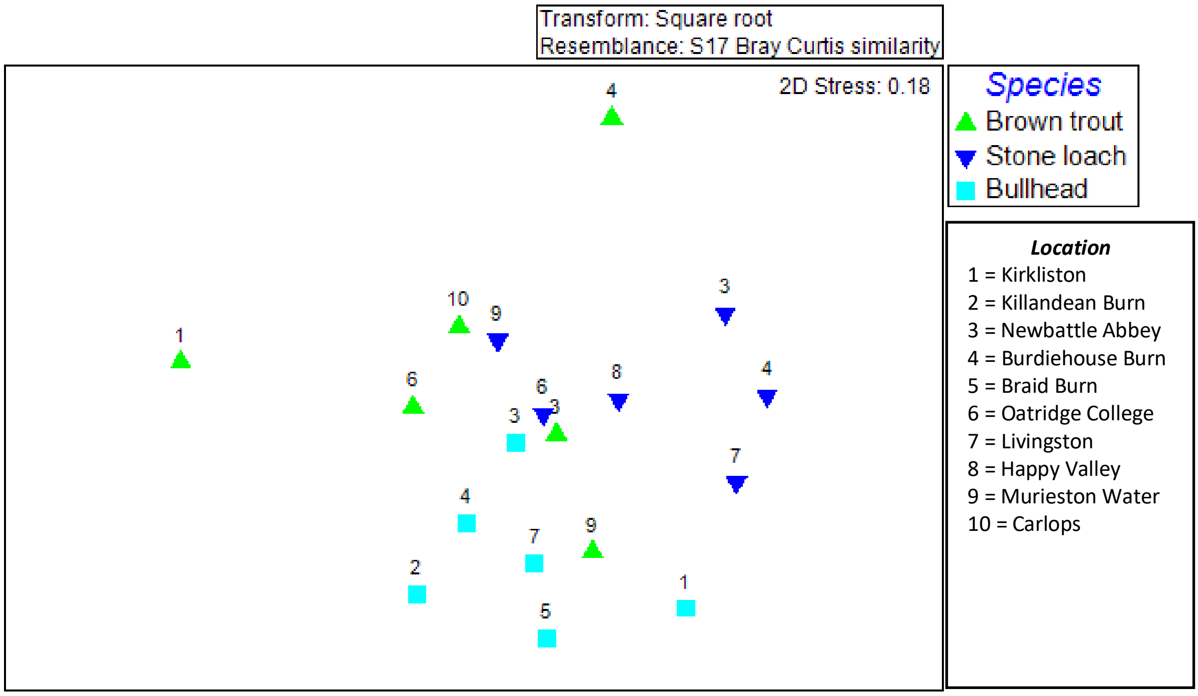

Figure 4.14. Similarities in brown trout, bullhead and stone loach dietary composition. Labels that are closer together show more similarity between the dietary compositions of those individuals. The further apart records are on the plot, the less resemblance there is between the data sets. Numbers correspond to sample locations, based on the key provided

Results of the one-way ANOSIM showed that fish species had a significant but moderate effect on the grouping of data in this analysis (P = 0.0066; R = 0.2049). Examination of the breakdown of results obtained showed significant differences between the combinations of brown trout and bullhead (P = 0.0438, R = 0.1593) and stone loach and bullhead (P = 0.0438 and R = 0.3519), but no significant separation of the brown trout and stone loach data (P = 0.113, R = 0.1315). The R value obtained for comparisons between the dietary composition of bullhead and stone loach suggests that separation of diet composition based on species alone accounted for a higher level of segregation than was evident in the other comparisons examined. Species is therefore shown to have a moderate influence on the differences exhibited in the diet composition of all three fish species, when examined collectively and for the combinations of bullhead and brown trout and bullhead and stone loach.

4.3.3 Comparing Dietary Composition in the Presence and Absence of Bullheads

Results of the two-way PERMANOVA showed that the diets of brown trout and stone loach differed in the presence and absence of bullheads, at a statistically significant level (P = 0.0449, F = 2.05, df = 1). Data were not shown to separate out on the basis of species (P = 0.1768, F = 1.4679, df = 1) and there was no interacting effect of fish species on the result obtained (P = 0.7236, F = 0.69525, df = 1). As fish species has no significant effect on the results, further examination of the differences in brown trout and stone loach diets based on grouping of bullhead present and bullhead absent locations was possible. SIMPER analysis gave an average overall dissimilarity value of 65.19, when comparing brown trout and stone loach in the presence and absence of bullheads. The prey categories Goeridae, Chironomidae , Baetidae and Ephemerellidae collectively accounted for over half of the total dissimilarity shown between groups (Table 4.2). Goeridae and Ephemerellidae were selected for prey in much higher proportions in the sampling locations that did not contain bullheads. In the presence of sympatric bullheads, chironomids formed a much higher proportion of fish diets.

Table 4.2. Result of SIMPER analysis showing the contribution of all prey categories to the overall dissimilarity found between combined brown trout and stone loach data, when data were grouped by the presence and absence of bullhead. Only prey categories that contributed to the total dissimilarity were included. % contribution shows the input of a particular prey category to the dissimilarity, and cumulative % shows the running total of contributions up to 100%. Mean abundance data for each prey category in fishes in the absence and presence of bullheads are also shown

Prey Category	Contribution %	Cumulative %	Mean abundance bullhead absent	Mean abundance bullhead present
Goeridae	14.22	14.22	0.523	0.0833
Chironimidae	14.13	28.35	0.247	0.5
Baetidae	13.31	41.66	0.442	0.456
Ephemerellidae	9.693	51.36	0.451	0.297
Gammaridae	6.405	57.76	0.212	0.0167
Hydrobiidae	6.184	63.94	0.1	0.15
Potamanthidae	5.179	69.12	0.192	0.075
Capniidae	4.62	73.74	0.0961	0.0833
Simulidae	4.587	78.33	0.0148	0.153
Unknown Fly Larvae	3.697	82.03	0.0569	0.0833
Asellidae	3.353	85.38	0.0145	0.0972
Ancylidae	2.976	88.36	0.0215	0.0833
Erpobdellidae	1.813	90.17	0.00725	0.0556
Unknown Leech	1.662	91.83	0.0139	0.0417
Leuctridae	1.462	93.29	0.0332	0.0167
Lymnaeidae	1.46	94.75	0.0104	0.0417
Rhyacophilidae	1.32	96.07	0.0528	0
Elmidae (Adult)	1.063	97.14	0.0424	0
Sericostomatidae	0.6235	97.76	0.0184	0
Dytiscidae	0.4907	98.25	0.0111	0.00833
Elmidae (Larvae)	0.3331	98.58	0.00725	0
Unknown Fish	0.321	98.9	0	0.0104
Unknown Stonefly	0.2904	99.19	0.0111	0
Unknown Caddisfly Larvae	0.2904	99.48	0.0111	0
Nemouridae	0.2575	99.74	0.0104	0
Glossiphonidae	0.2575	100	0.0104	0

4.4 Discussion

An examination of feeding niche overlap between sympatric populations of introduced bullhead, native brown trout and native stone loach showed a substantial overlap in the range of prey items utilised by all fish. Despite frequent reports of aerial and terrestrial prey in brown trout diets (e.g. Bridcut and Giller, 1995; Lehane *et al*., 2001), no evidence of this was found in this study. All fish species analysed shared a fairly similar dietary composition, with several invertebrate families commonly exploited by all species. These were larvae of the Chironomidae and Simuliidae fly families; nymphs of the most common mayfly and stonefly families Heptageniidae, Baetidae and Ephemerellidae; and the crustacean family Asellidae. Consumption of these families by all three species is commonly reported throughout the literature (Table 4.1). Copp *et al.* (2005) found that chironomid larvae were a preferred food source for cohabiting bullheads and stone loach, despite relatively low densities within the invertebrate community. An examination into the feeding preferences of brown trout fry in Norway showed high levels of consumption of chironomids in fry diets (Skoglund and Barlaup, 2006), which was also found by Sánchez-Hernández *et al*. (2011). The latter also reported active selection of Simuliidae larvae relative to local abundances. Consumption of mayfly and stone fly nymphs is also commonly reported for all species (e.g. Nunn *et al.,* 2012).

A previous study into the dietary composition of bullheads in the River Almond by Morris (1978) provides the only published account of bullhead diet in Scotland. The crustacean family Asellidae and the ephemeropteran family Baetidae formed a large component of bullhead diet in the account published by Morris (1978). Bullheads were also found to consume the most plentiful prey source relative to local availability. Asellidae and Baetidae were common components of bullhead diet, but Chironomidae, Heptageniidae and Rhyacophilidae were also significant prey sources. Six incidences of filial cannibalism were noted in bullheads examined in this study (two from Kirkliston and four from Livingston), but no predation of brown trout fry was observed. Morris (1978) did not record any incidences of cannibalism in Scottish bullheads. Consumption of unrelated conspecifics is logical from a survival perspective. It provides a nutritious prey source and minimises future competition (FitzGerald, 1992). Filial cannibalism is less easy to rationalise, but is particularly prevalent in fishes with parental care (FitzGerald, 1992), such as bullheads. Kondoh and Okuda (2002) suggested that filial cannibalism is favoured in conditions where the overall population density is high, the population is female dominated, the male care period is long or the male's energy reserves are low. Bullhead populations in the areas surveyed were extremely dense, and it certainly holds true that male bullheads are heavily invested in nest building and guarding during the reproductive period (Morris, 1954; Smyly, 1957). These conditions may, therefore, promote an environment in which some males will consume young bullheads, as was evidenced in this study.

Certain invertebrates were more common in the diets of some species over others. There was an obvious abundance of adult beetle groups within the dietary composition of brown trout, whereas fewer incidences were seen in bullheads and stone loach. This was particularly evident at Burdiehouse Burn in 2014 and Kirkliston in 2015. The caddisfly larvae Rhyacophilidae was common in the diets of brown trout and bullheads, but less common in stone loach (e.g. Burdiehouse Burn in 2015). Separation within the diets of adult and juvenile brown trout was also noted, with Gammaridae forming an important component of adult brown trout diets but being absent in the diet of juvenile individuals. All fish species examined are considered generalists (Morris, 1978; Michel and Oberdoff, 1995), and therefore show a large number of prey categories in their respective dietary composition. The variation in proportional consumption of certain prey organisms in brown trout, bullhead and stone loach diet may suggest a degree of resource partitioning in these mixed populations. Resource partitioning has previously been reported between cohabiting bullheads, brown trout and stone loach (e.g. Straskraba *et al.*, 1966; Welton *et al.,* 1983; Dahl, 1998), and has primarily been attributed to differences in prey choice due to size (in relation to stone loach and bullhead competition) or prey location in the water column (in relation to brown trout and bullhead competition). As this study did not take prey size or biomass into account, separation by size may have occurred within the diets of examined bullheads and stone loach. Segregation based on prey location in the water column was demonstrated in this study, as evidenced by the higher proportion of fast-swimming adult beetles in the diets of brown trout (such as Braid Burn, Newbattle Abbey and Livingston) compared to values reported for bullheads and stone loach.

Statistical analyses of the dietary composition data obtained for brown trout, bullheads and stone loach suggests that a degree of resource partitioning may be evidence in the communities observed. ANOSIM results showed significant separation of feeding data between the pairings of bullheads and stone loach, and bullheads and brown trout. No such separation was observed in the pairings of brown trout and stone loach, based on the ANOSIM results and the output of a subsequent PERMANOVA analysis. Despite strong similarities in the type of macroinvertebrate prey consumed, the bullheads examined in this study did not show a direct overlap in dietary composition with native brown trout or stone loach, based on the proportion of individuals observed to be consuming a given prey category. A high degree of similarity, and therefore potential competition, was found when brown trout proportional feeding choices were compared with those of examined stone loach. It should be noted, however, that both brown trout and stone loach are native species that frequently occur in the same freshwater communities.

Whilst direct comparisons of dietary composition did not provide evidence of feeding competition between bullheads and native fishes, differences in dietary selection were observed in native fishes when comparing locations with and without resident bullheads. A PERMANOVA analysis comparing the feeding preferences of brown trout and stone loach in the presence and absence of bullheads showed that fishes in locations containing sympatric bullheads had a different dietary composition from those in mixed communities. Local variations in the availability of prey would likely have an influence on dietary composition, and fluctuations in observed stomach contents between locations would therefore be expected. However, the breakdown in prey choices exhibited by each species in each location shows that individuals are frequently selecting specific prey categories, rather than consuming the most accessible or available option (Figures 4.4 to 4.13). Due to the similarity in brown trout and stone loach diets confirmed by the ANOSIM and PERMANOVA results, the diets of brown trout and stone loach were pooled to provide a comparison of feeding preferences in the locations where bullhead were present, and those where bullheads have not been introduced. SIMPER analysis showed that the most significant differences in prey consumption were shown in the Goeridae, Ephemerellidae and Gammaridae categories (more common when bullheads were absent); and the Chironomidae and Baetidae categories (more commonly consumed when bullheads were present). Goeridae are not a frequently observed prey resource for any of the species examined and the variation observed in the SIMPER analysis is a result of unusually high predation frequencies by brown trout at Carlops, where bullheads are absent, relative to the other locations where bullhead are present. This result is therefore attributed to local availability as opposed to the influence of bullheads. Direct observations of the dietary composition of each species at different sites (shown in Figures 4.4 to 4.13) showed that Ephemerellidae and Gammaridae are frequently consumed prey sources for bullheads, brown trout and stone loach, albeit in varying proportions. The variation highlighted in the SIMPER analysis is therefore potentially a result of bullheads preventing access to these preferred prey species, either through predation by bullheads, or the displacement of stone loach and brown trout from optimal foraging locations in the water column.

The presence of resource partitioning implies that each fish species would likely be consuming a specific type of prey, whether based on the prey's location within the water column or a species' preference for a particular food source. If this were the case, active selection of specific prey organisms would be demonstrated. This was tested by comparing the invertebrate community composition of each location with the dietary composition of each of the fish species. Data obtained from kick samples were used to catalogue the invertebrate community and provide a measure of community composition on the basis of counts of individuals and biomass. This proportional structuring of the community was then directly compared with the proportion of individuals from each the fish species consuming a prey category, to ascertain whether each species was foraging relative to community availability or was actively seeking a particular prey source. Both bullheads and stone loach regularly prey upon the most abundant organisms in some locations, although this was not consistently observed. At Murieston Water for both the 2014 and 2015 fish samples, the highest proportion of prey consumed by stone loach was Gammaridae. This prey category represented the highest proportion of prey available by biomass and count in 2014, and the second highest proportion observed in 2015. Results for Livingston in 2015 showed that the consumption of Baetidae and Rhyacophilidae in stone loach and bullheads coincided with each of these prey categories representing the highest proportion of the invertebrate community by count and biomass respectively. Rhyacophilidae comprised over 70% of the available invertebrate prey biomass and was consumed in moderate proportions by stone loach and bullheads. In contrast, the caddisfly larvae families Limnephilidae and Hydropsychidae represented the second highest and highest proportion of invertebrates by biomass at Happy Valley and Murieston Water in 2015, and were missing from the stomach contents of all fish examined at those sites. All fish species appear to be selecting for specific prey types and, within this grouping, will select the more accessible prey sources relative to individual preferences. Clearly some degree of dietary segregation is present, whether in relation to the size of prey versus the relative size of the consumer, or the prey's location within the water column. This further confirms the separation observed between the proportional dietary preferences of bullheads when compared with those of stone loach and brown trout, shown by ANOSIM analysis.

There are several limitations to determining the percentage composition of the macroinvertebrate community based on individual counts. The kick sampling method is appropriate for sampling aquatic-dwelling organisms, particularly those in the benthos. As noted by Horton (1961), attempts to correlate bottom fauna with the amount of food available in a given area would be difficult if brown trout predominantly feed on drift organisms. The consumption of drift organisms has been commonly reported in both brown trout and bullhead. Community composition based on count data does not take into account the relative size of each individual, which has a marked effect on the energy it provides for a consumer. Several individuals of the smaller family groups, such as chironomid larvae or Elmidae larvae, would need to be consumed to give the energy equivalent of one large *Gammarus*. This was compensated for by obtaining average weight values for each family group to allow for the determination of composition on the basis of biomass. Variations in the quality of each prey source will also be evident between prey categories and cannot be accounted for on the basis of biomass. Invertebrate nutrients and elemental composition vary between taxa (Evans-White *et al.*, 2005) and in relation to the invertebrate's diet (Wissing and Hasler, 1971). Selection on the basis of these factors is therefore hard to evidence by the community composition methods utilised in this study. It should also be noted that, due to sampling restrictions, kick samples were conducted slightly earlier in the season than fish samples were taken. Results obtained from the kick samples are therefore not indicative of the invertebrate community structure that was present at the same time fish feeding occurred. However, both samples were conducted during the peak of invertebrate seasonal occurrence and fish consumption rates (Thomas, 1962; Klemetsen *et al*., 2003), so all available invertebrate classes that would be components of fish diets should be represented in the samples.

Seasonality was found to be a key factor in determining trophic competition between bullheads, stone loach, juvenile brown trout and juvenile Atlantic salmon co-habiting in Russia (Shustov *et al*., 2014). The authors found that competition between both salmonids with bullheads and stone loach was high in autumn, due to high fish consumption rates and reduction in available prey, and low in summer and winter, due to an abundance of prey (summer) and reduced feeding activities in fish (winter). Seasonal variance seems likely in the fish communities examined in Scotland, and may have influenced the results of this study. Results are in agreement with the findings of Welton *et al.* (1983) and Welton *et al.* (1991), who demonstrated a degree of resource partitioning between cohabiting bullheads and stone loach. An established bullhead population has been present in some of these regions for over sixty years, so a degree of trophic competition is likely and may have resulted in resource partitioning among the different species present. This is evidenced by the selection of specific prey categories exhibited by each species at different locations, and potentially shown in the difference in prey resources utilised by brown trout and stone loaches in the presence and absence of bullheads. The results of this study also complement those of Vlach *et al.* (2013), who reported an overlap in prey types selected by sympatric bullheads, stone loach and brown trout. However, in contrast to Copp *et al.* (2013), a difference in the percentage composition of the diets of bullhead and stone loach (and examined brown trout) was demonstrated.

The generalist nature of bullhead, brown trout and stone loach predation makes it difficult to categorise specific prey preferences, particularly when the prey community also varies between sample sites. The results of this study suggest that some degree of resource partitioning is evident in communities hosting introduced bullheads. Although general prey preferences are similar between all three species, direct competition for prey has not been evidenced. Each species has been shown to select specific prey items that are influenced by, but not restricted to, the most abundance and available prey within a community. Sympatric bullheads were found to coincide with variations in prey selection in brown trout and stone loach, which is a potential consequence of resource partitioning between the three species. This provides some evident in support of the tested hypothesis; that the presence of introduced bullheads influences the feeding preferences of native brown trout and stone loach in Scotland.

4.5 References

Amundsen P. A., Lafferty K. D., Knudsen R., Primicerio R., Kristoffersen R., Klemetsen A. and Kuris A. M. (2013) New parasites and predators follow the introduction of two fish species to a subarctic lake: implications for food-web structure and functioning. *Oecologia* **171**, 993–1002.

Andreasson S. (1971) Feeding habits of a sculpin (*Cottus gobio* L. Pisces) population. *Report - Institute of Freshwater Research, Drottingholm* **51**, 5–30.

Armstrong J. D., Kemp P. S., Kennedy G. J. A., Ladle M. and Milner N. J. (2003) Habitat requirements of Atlantic salmon and brown trout in rivers and streams. *Fisheries Research* **62**, 143–170.

Bateson W. (1890) The sense organs and perceptions of fishes: with remarks on the supply of bait. *Journal of the Marine Biological Association of the United Kingdom (New Series)* **1**, 225–256.

Biga H., Janssen J. and Mersden J. E. (1998) Effect of substrate size on lake trout egg predation by mottled sculpin. *Journal of Great Lakes Research* **24**, 464–473.

Bridcut E. E. and Giller P. S. (1995) Diet variability and foraging strategies in brown trout (*Salmo trutta*): an analysis from subpopulations to individuals. *Canadian Journal of Fisheries and Aquatic Sciences* **52**, 2543–2552.

Burlakova L. E., Karatayev A. Y. and Karatayev V. A. (2012) Invasive mussels induce community changes by increasing habitat complexity. *Hydrobiologia* **685**, 121–134.

Carter M. G., Copp G. H. and Szomlai V. (2004) Seasonal abundance and microhabitat use of bullhead *Cottus gobio* and accompanying fish species in the River Avon (Hampshire), and implications for conservation. *Aquatic Conservation: Marine and Freshwater Ecosystems* **14**, 395–412.

Chaston I. (1968) A study on the exploitation of invertebrate drift by brown trout (*Salmo trutta* L.) in a Dartmoor stream. *Journal of Applied Ecology* **5**, 721–729.

Copp G. H., Spathari S. and Turmel M. (2005) Consistency of diel behaviour and interactions of stream fishes and invertebrates during summer. *River Research and Applications* **21**, 75–90.

Copp G. H., Warrington S. and de Bruine Q. (1994) Comparison of diet in bullhead, *Cottus gobio* and stone loach, *Barbatula barbatula* in a small English lowland river. *Folia Zoologica* **43**, 171–176.

Crisp D. T., Mann R. H. K. and McCormack J. C. (1978) The effects of impoundment and regulation upon the stomach contents of fish at Cow Green, Upper Teesdale. *The Journal of Fish Biology* **12**, 287–301.

Crowl T. A., Townsend C. R. and McIntosh A. R. (1992) The impact of introduced brown and rainbow trout on native fish: the case of Australasia. *Reviews in Fish Biology and Fisheries* **2**, 217–241.

Dahl J. (1998) Effects of a benthivorous and a drift-feeding fish on a benthic stream assemblage. *Oecologia* **116**, 426–432.

Elliott J. M. (2006) Periodic habitat loss alters the competitive coexistence between brown trout and bullheads in a small stream over 34 years. *Journal of Animal Ecology* **75**, 54–63.

Evans-White M. A., Stelzer R. S. and Lamberti G. A. (2005) Taxonomic and regional patterns in benthic macroinvertebrate elemental composition in streams. *Freshwater Biology* **50**, 1786–1799.

FitzGerald G. J. (1992) Filial cannibalism in fishes: Why do parents eat their offspring? *Trends in Ecology and Evolution* **7**, 7–10.

Foote C. J. and Brown G. S. (1998) Ecological relationship between freshwater sculpins (genus *Cottus*) and beach-spawning sockeye salmon (*Oncorhynchus nerka*) in Iliamna Lake, Alaska. *Canadian Journal of Fisheries and Aquatic Sciences* **55**, 1524–1533.

Frost W. E (1950) The growth and food of young salmon (*Salmo salar*) and trout (*S. trutta*) in the River Forss, Caithness. *Journal of Animal Ecology* **19**, 147–158

Gabler H. M. and Amundsen P. A. (1999) Resource partitioning between Siberian sculpin (*Cottus poecilopus* Heckel) and Atlantic salmon parr (*Salmo salar* L.) in a sub-Arctic river, northern Norway. *Ecology of Freshwater Fish* **8**, 201–208.

Gaudin P. (1985) Predation by the sculpin (*Cottus gobio* L.) on brown trout fry (*Salmo trutta* L.): total length of prey and predator. *Hydrobiologia* **122**, 267–270.

Gaudin P. and Cailliere L. (2000) Experimental study of the influence of presence and predation by sculpin, *Cottus gobio* L., on the drift of emergent brown trout, *Salmo trutta* L. *Archiv für Hydrobiologie* **147**, 257–271.

Gaudin P. and Heland M. (1984) Influence of sculpin adults (*Cottus gobio* L.) on brown trout (*Salmo trutta* L.) fry – Experimental study in semi-natural environments. *Acta Oecologica* **5**, 71–83.

Giroux F., Ovidio M., Philippart J. C. and Baras E. (2000) Relationship between the drift of macroinvertebrates and the activity of brown trout in a small stream. *Journal of Fish Biology* **56**, 1248–1257.

Glova G. J., Sagar P. M. and Näslund I. (1992) Interaction for food and space between populations of *Galaxias vulgaris* stokell and juvenile *Salmo trutta* L. in a New Zealand stream. *Journal of Fish Biology* **41**, 909–925.

Hammer Ø., Harper, D. A. T. and Ryan, P. D. (2001) PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* **4** (9).

Hesthagen T., Saksgård R., Hegge O., Dervo B. K. and Skurdal J. (2004) Niche overlap between young brown trout (*Salmo trutta)* and Siberian sculpin (*Cottus poecilopus*) in a subalpine Norwegian river. *Hydrobiologia* **521**, 117–125.

Höjesjö J., Johnsson J. and Bohlin T. (2004) Habitat complexity reduces the growth of aggressive and dominant brown trout (*Salmo trutta*) relative to subordinates. *Behavioral Ecology and Sociobiology* **56**, 286–289.

Holm S. (1979) A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics* **6**, 65–70.

Horton P. A. (1961) The bionomics of brown trout in a Dartmoor stream. *Journal of Animal Ecology* **30**, 311–338.

Hyslop E. J. (1980) Stomach contents analysis-a review of methods and their application. *Journal of Fish Biology* **17 (4)**, 1–429.

Hyslop E. J. (1982) The feeding habits of 0+ stone loach, *Noemacheilus bavbatulus* (L.), and bullhead, *Cottus gobio* L. *Journal of Fish Biology* **21**, 187–196.

Khan T. A., Wilson M. E. and Khan M. T. (2003) Evidence for invasive carp mediated trophic cascade in shallow lakes of western Victoria, Australia. *Hydrobiologia* **506** (1-3), 465-472.

Klemetsen A., Amundsen P. A., Dempson J., Jonsson B., Jonsson N., O'Connell M. and Mortensen E. (2003) Atlantic salmon *Salmo salar* L., brown trout *Salmo trutta* L. and Arctic charr *Salvelinus alpinus* (L.): a review of aspects of their life histories. *Ecology of Freshwater Fish* **12**, 1–59.

Kondoh M. and Okuda N. (2002) Mate availability influences filial cannibalism in fish with paternal care. *Animal Behaviour* **63**, 227–233.

Lehane B. M., Walsh B., Giller P. S. and O'Halloran J. (2001) The influence of small-scale variation in habitat on winter trout distribution and diet in an afforested catchment. *Aquatic Ecology* **35**, 61–71.

Lien L. (1981) Biology of the minnow *Phoxinus phoxinus* and its interactions with brown trout *Salmo trutta* in Øvre Heimdalsvatn, Norway. *Holarctic Ecology* **4**, 191–200.

Louhi P., Mäki-Petäys A., Huusko A. and Muotka T. (2014) Resource use by juvenile brown trout and Alpine bullhead: Influence of interspecific versus intraspecific competition. *Ecology of Freshwater Fish* **23**, 234–243.

Mann R. H. K. and Orr D. R. O. (1969) A preliminary study of the feeding relationships of fish in a hard-water and a soft-water stream in southern England. *Journal of Fish Biology* **1**, 31–44.

McIntosh A. R. (2000) Habitat and size-related variations in exotic trout impacts on native galaxiid fishes in New Zealand streams. *Canadian Journal of Fisheries and Aquatic Sciences* **57**, 2140–2151.

Michel P. and Oberdorff T. (1995) Feeding habits of fourteen European freshwater fish species. *Cybium* **19**, 5–46.

Miller R., Williams J. and Williams J. (1989) Extinctions of North American fishes during the past century. *Fisheries* **14**, 22–38.

Mills C. A. and Mann R. H. K. (1983) The bullhead *Cottus gobio*, a versatile and successful fish. *Annual Report of the Freshwater Biological Association,* Ambleside, Cumbria, 76–88.

Mooney H. A. and Cleland E. E. (2001) The evolutionary impact of invasive species. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 5446–5451.

Morris K. H. (1978) The food of the bullhead (*Cottus gobio* L.) in the Gogar Burn, Lothian, Scotland. *Forth Naturalist and Historian* **7**, 31–44.

Morris D. (1954) The reproductive behaviour of the river bullhead (*Cottus gobio* L.), with special reference to the fanning activity. *Behaviour* **7**, 1–32.

Moyle P. and Light T. (1996) Biological invasions of fresh water: empirical rules and assembly theory. *Biological conservation* **78**, 149–161.

Nunn A. D., Harvey J. P., Britton J. R., Frear P. A., Cowx I. G. (2007) Fish, climate and the Gulf Stream: the influence of abiotic factors on the recruitment success of cyprinid fishes in lowland rivers. Freshwater Biology **52**, 1576–1586

Nunn A. D., Tewson L. H. and Cowx I. G. (2012) The foraging ecology of larval and juvenile fishes. *Reviews in Fish Biology and Fisheries* **22**, 377–408.

Palm D., Lindberg M., Brännäs E., Lundqvist H., ÖStergren J. and Carlsson U. (2009) Influence of European sculpin, *Cottus gobio*, on Atlantic salmon *Salmo salar*, recruitment and the effect of gravel size on egg predation - implications for spawning habitat restoration. *Fisheries Management and Ecology* **16**, 501–507.

Parker I. M., Simberloff D., Lonsdale W. M., Goodell K., Wonham M., Kareiva P. M., Williamson M. H., Von Holle B., Moyle P. B., Byers J. E., Goldwasser L. (1999) Toward a framework for understanding the ecological effects of invader. *Biological Invasions* **1**, 3–19.

Pihlaja O., Julkunen M., Niemela E. and Erkinaro J. (1998) Changes in the density of introduced bullhead, *Cottus gobio* L., and its impact on juvenile Atlantic salmon, *Salmo salar* L., densities in a sub-Arctic salmon river in northern Finland. *Fisheries Management and Ecology* **5**, 189–199.

Rincón P. A and Lobón-Cerviá J. (1999) Prey size selection by brown trout (*Salmo trutta* L.) in a stream in northern Spain. *Canadian Journal of Zoology* **77**, 755–765

Roussel J. M. and Bardonnet A. (1999) Ontogeny of diel pattern of stream-margin habitat use by emerging brown trout, *Salmo trutta*, in experimental channels: influence of food and predator presence. *Environmental Biology of Fishes* **56**, 253–262.

Sánchez-Hernández J., Vieira-Lanero R., Servia M. J. and Cobo F. (2011) First feeding diet of young brown trout fry in a temperate area: disentangling constraints and food selection. *Hydrobiologia* **663**, 109–119.

Shustov Y. A., Tyrkin I. A. and Rasputina Y. N. (2014) Trophic competition of common sculpin and stone loach with juvenile salmond of Gen. *Salmo* in tributaries of the Onega Lake. *Hydrobiological Journal* **50**, 32–40.

Simon K. S. and Townsend C. R. (2003) Impacts of freshwater invaders at different levels of ecological organisation, with emphasis on salmonids and ecosystem consequences. *Freshwater Biology* **48**, 982–994.

Skoglund H. and Barlaup B. T. (2006) Feeding pattern and diet of first feeding brown trout fry under natural conditions. *Journal of Fish Biology* **68**, 507–521.

Smout T. C. (2003) The Alien Species in 20th-century Britain: Constructing a new vermin. *Landscape Research* **28**, 11–20.

Smyly W. J. P. (1955) On the biology of the stone-loach *Nemacheilus barbatula* (L.). *Journal of Animal Ecology* **24**, 167–186.

Smyly W. J. P. (1957) The life-history of the bullhead or miller's thumb (*Cottus gobio* L.). *Proceedings of the Zoological Society of London* **128**, 431–454.

Straskraba M., Chiar J., Frank S. and Hruska V. (1966) Contribution to problem of food competition among sculpin, minnow and brown-trout. *Journal of Animal Ecology* **35**, 303–311.

Tabor R. A., Brown G. S. and Luiting V. T. (2004) The effect of light intensity on sockeye salmon fry migratory behaviour and predation by cottids in the Cedar River, Washington. *North American Journal of Fisheries Management* **24**, 128–145.

Thomas J. D. (1962) The food and growth of brown trout (*Salmo trutta* L.) and its feeding relationships with the salmon parr (*Salmo salar* L.) and the eel (*Anguilla anguilla* (L.)) in the River Teify, West Wales. *Journal of Animal Ecology* **31**, 175–205.

Tomlinson M. and Perrow M. (2003) Ecology of the bullhead. *Conserving Natura 2000 Rivers Ecology Series* **No. 4**. English Nature, Peterborough.

Vlach P., Švátora M. and Dušek J. (2013) The food niche overlap of five fish species in the Úpoř brook (Central Bohemia). *Knowledge and Management of Aquatic Ecosystems* **411**, 4.

Wahl D. H., Wolfe M. D., Santucci V. J. and Freedman J. A. (2011) Invasive carp and prey community composition disrupt trophic cascades in eutrophic ponds. *Hydrobiologia* **678**, 49–63.

Welton J., Mill C. and Pygott J. (1991) The effect of interaction between the stone loach *Noemacheilus barbatulus* (L.) and the bullhead *Cottus gobio* (L.) on prey and habitat selection. *Hydrobiologia* **220**, 1–7.

Welton J. S., Mills C. A. and Rendle E. L. (1983) Food and habitat partitioning in two small benthic fishes, *Noemacheilus barbatulus* (L.) and *Cottus gobio* L. *Archiv für Hydrobiologie* **97**, 434–454.

Wissing T. E. and Hasler A. D. (1971) Intraseasonal change in caloric content of some freshwater invertebrates. *Ecology* **52**, 371–373.

Zimmerman C. and Mosegaard H. (1992) Initial feeding in migratory brown trout (*Salmo trutta* L.) alevins. *Journal of Fish Biology* **40**, 647–650.

Chapter 5. Parasite Links Between Introduced Bullheads and Native Fishes in South-East Scotland

5.1 Introduction

The close association between parasites, host fitness and host behaviour means that parasites can play a key role in the invasion process. They have the potential to modify predator/prey interactions between native and invading species and indirectly influence interspecific competition (Prenter *et al*., 2004). Parasites can also be independent invaders, producing novel host/parasite interactions that can have catastrophic consequences to the survival of native populations (e.g. Meeus *et al*., 2011). As with the establishment of any other non-native species, parasite colonization success is not guaranteed. Factors such as host invasion history, parasite life history and host-parasite geography influence the potential success of a parasite introduction (Blakeslee *et al*., 2012). Changes in host/parasite dynamics are a common occurrence in biological invasions and can have significant impacts on native communities (e.g. Emde *et al*., 2012).

There are several possible establishment and transmission processes that can occur between introduced species, native species and their respective parasites (Figure 5.1). Translocated non-native species commonly escape a large number of parasites from their native range when colonizing a new region (Mitchell and Power, 2003; Torchin and Mitchell, 2004). The enemy release hypothesis suggests that invasive species may be more successful in areas of introduction than their native communities, due to a lack of co-evolved natural enemies and the loss of parasites during the translocation process (Keane and Crawley, 2002; Wolfe, 2002). This allows invasive species to achieve higher growth rates and larger population densities in novel ecosystems (Drake, 2003), increasing their impacts on native communities. A study comparing the parasites of an extensive range of species in both their native and introduced ranges found that individuals generally contained twice the number of parasite species in their native range than were recorded in non-native locations (Torchin *et al.,* 2003). The loss of parasites when hosts become established in a new community is an example of the enemy release hypothesis, where invading individuals can thrive in new communities due to a loss of natural enemies; in this case their parasites. An investigation into parasite release in the invasive green crab (*Carcinus maenas*) suggested that a loss of parasites before establishment might afford individuals a more rapid growth rate and an overall increase in biomass (Torchin *et al.,* 2001). In contrast, Drake (2003) proposed that parasite release does not significantly affect invasion success, although the extent to which this held true was dependent on host virulence and the proportion of introduced individuals that were infected. The relative importance of parasite release is therefore population, species and host-specific.

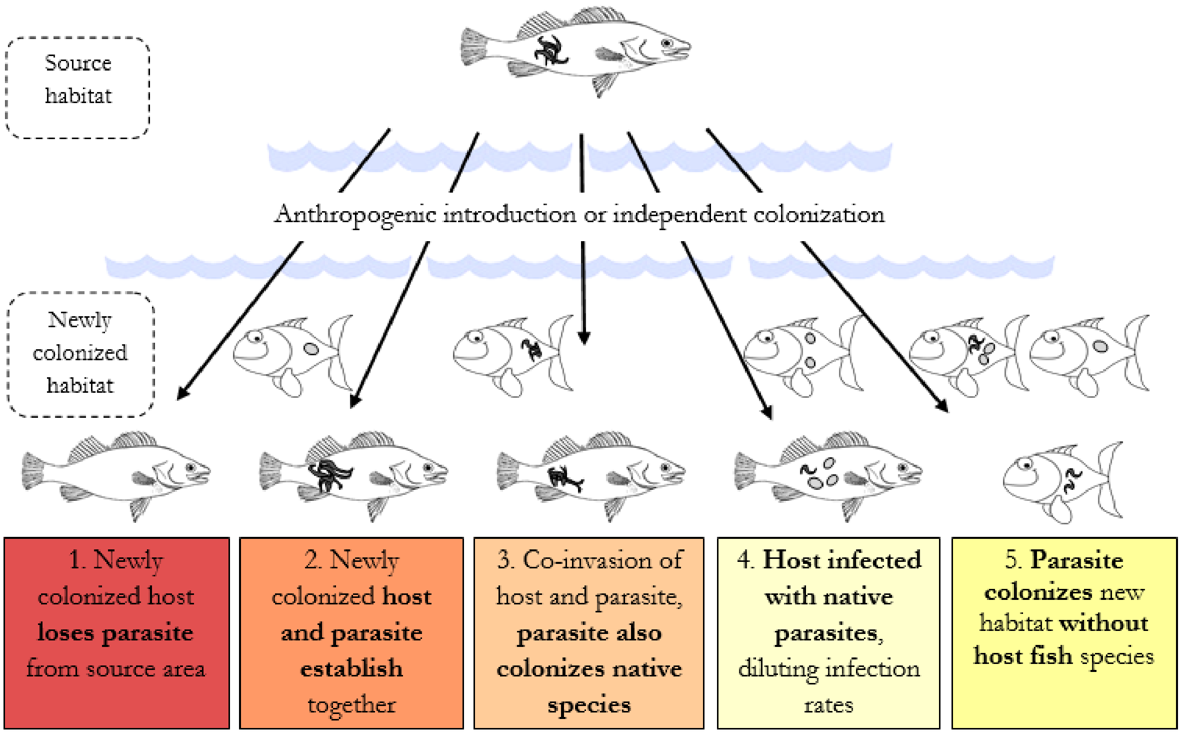

Figure 5.1. Potential scenarios for parasite/host interactions as a consequence of the introduction of a parasitised non-native species

Parasites that do survive translocation will only thrive in a habitat that is sufficiently rich in the obligate hosts required to sustain the parasite's life cycle (Torchin and Mitchell, 2004). Strauss *et al.* (2012) theorised that non-native species infected with virulent parasites are unlikely to survive the invasion process, resulting in only avirulent parasites or highly tolerant hosts arriving in new locations. Parasites that spread by vertical transmission (those that can be passed from parent to offspring) are usually less virulent than those that require contact between infected and uninfected individuals, so are more likely to successfully invade with their hosts (Prenter *et al*., 2004). Parasites that are avirulent in one host can still cause significant pathogenic responses if successfully transmitted to a novel host, which is of particular relevance in invasion studies. Non-native species can transmit their parasites to evolutionarily-naive native individuals in a process known as parasite 'spill-over' (Power and Mitchell, 2004). The reciprocal of this process is also possible, wherein non-native individuals can act as a reservoir for native parasites, increasing parasite spread and infection prevalence in native hosts. This process is termed 'spill-back,' and is generally the more prominent mechanism of parasite transfer in newly established communities (Kelly *et al.,* 2009). Invasive species can reduce infection rates in native species by either functioning as an alternative or paratenic host for native parasites, termed the dilution effect, or fulfilling the role of dead-end host, wherein parasites infect invasive species but cannot continue their life cycle. Introduced parasites may also have the ability to thrive in their new community without their original host. The invasive nematode *Anguillicoloides crassus*, for example, is thought to have played a considerable role in the recent decimation of global European eel stocks by compromising swimbladder function (Barry *et al*., 2014). It was introduced during the transportation of its native host *Anguilla japonica* throughout Europe, and has flourished due to its ability to infect a vast range of novel hosts (e.g. Moravec and Skoríková, 1998). Novel parasite-host relationships that result from such interactions are particularly harmful due to a lack of co-evolution between the parasite and host, leaving the host vulnerable to physiological and behavioural exploitation. These changes could result in knock-on effects at a community level.

The relationship between a parasite and its host is complex. Parasites can induce behavioural and physiological changes within a host to improve parasite transmission success. The consequences of parasite infection vary greatly depending on the host and parasite in question, and are influenced by host fitness and the presence of other co-occurring parasites. Changes to host behaviour that increase susceptibility to predation by a parasite's final host have been well documented in fish (Mikheev *et al.,* 2010; Seppälä *et al.,* 2004; 2005) and invertebrate hosts (e.g. Macneil *et al*., 2003; Seppälä *et al.*, 2008). The acanthocephalan parasite *Pomphorynchus laevis* has the capacity to influence the olfactory system of its amphipod host by removing its ability to distinguish the olfactory cues that indicate the presence of a fish predator (Baldauf *et al.,* 2007). Medoc *et al.* (2009) studied infection by the acanthocephala *Polymorphus minutus* and found that the parasite could manipulate its amphipod host to hide in refuges at the water's surface, thereby decreasing its host's vulnerability to predation by an undesirable non-host predator. In a recent study by Gopko *et al.* (2017) infections of the digenean parasite *Diplostomum pseudospathaceum* in fish eyes were found to modify their host's behaviour in opposite ways, depending on the age of the fish host. When *D. pseudospathaceum* are at a younger uninfectious stage, fish hosts were less active in the water column and therefore less vulnerable to predation by aquatic birds. The reciprocal was found in hosts containing mature infectious *D. pseudospathaceum*, who were found to swim more actively and recover from a simulated predation attempt more quickly than a control group, making them ultimately more susceptible to predation by piscivorous birds. In modifying the behaviour of host species, parasites are able to ensure their continued survival or improve their capacity to spread to their definitive host. These modifications can indirectly determine invasion success by influencing the competitive fitness, and potentially the survival, of a host.

Freshwater fish typically host a diverse range of parasite fauna (Poulin, 2016). Within the aquatic environment parasite infections can be autogenic, where the life cycle of the parasite is completed locally within the aquatic community, or allogenic, where intermediary stages are completed in the aquatic environment but further stages of the parasite's life cycle are completed on land. Autogenic parasites will typically complete their life cycle using a freshwater invertebrate as an intermediate host and one or more fish species as subsequent intermediate or final hosts. Allogenic parasites will similarly exploit freshwater invertebrates and fishes as first and second intermediate host, but will likely have a final adult stage that requires a piscivorous bird or mammal as a host. Allogenic parasites have a greater colonization potential than their autogenic equivalents due to the increased mobility of their terrestrial final hosts increasing dispersal capacity (Esch *et al.,* 1988). Typical life cycles of freshwater fish parasites involve the exploitation of amphipods or gastropods as intermediate hosts, although numerous other invertebrate species can also be utilised depending on the parasite taxa in question. Parasite occurrence in invertebrates and fish from within the same community are therefore closely related, validating a community-based approach to invasion studies.

Bullheads have been introduced to a number of different countries across Europe and Scandinavia. In other areas of introduction, their presence has been shown to influence parasitological interactions within native communities. A recent investigation into the introduction of bullhead in Finland showed limited changes to the overall parasite diversity of bullheads, but provided a clear example of parasite spill-over (Ieshko *et al.,* 2013). More than 20 years after bullhead were introduced, high densities of the trematode parasite *Apatemon gracilis* were reported in previously uninfected Atlantic salmon parr; a finding attributed directly to the establishment and dispersal of bullheads within the river system. There has also been documented evidence of parasite spill-back, where invasive bullheads have acquired novel parasite infections from native fish. Moravec (2001) recorded incidences of bullhead infection with the salmonid cestode parasite *Proteocephalus longicollis* in the Czech Republic, where their role as both paratenic parasite hosts and prey for the brown trout final host facilitated the spread of *P. longicollis*. The similar habitat and prey preferences of the two cohabiting species indicate the potential for parasite transfer.

Despite evidence of parasitological interactions in other areas of introduction, no study has been conducted into the parasitological interactions between introduced Scottish bullhead and native fish species. Introduced bullhead in Scotland are found in communities containing brown trout (*Salmo trutta*), a species that is of particular importance to the recreational fishing industry and the economy (Butler *et al.,* 2009). Bullheads are found in a similar habitat to brown trout fry and large adult bullheads will prey upon vulnerable fry (Gaudin, 1985). A reciprocal relationship also exists, in which adult brown trout actively prey upon smaller bullheads. Bullheads are also closely associated with native European minnow (*Phoxinus phoxinus*) and stone loach (*Barbatula barbatula*), sharing similar habitat and prey preferences. Close parasitological interactions between these species are therefore likely. An investigation into parasite links between introduced bullheads and co-occurring fauna will highlight any significant changes in host/parasite relationships that have resulted from bullhead arrival in Scotland.

5.1.1 Aims and Objectives

The aim of this chapter is to investigate parasitological interactions between bullheads and native fauna, to establish whether current host/parasite relationships relate to the arrival and establishment of non-native bullheads. As bullheads are responsible for the introdcution and spread of parasites in other areas of introdcution, it is hypothesised that the bullheads that have been introduced to Scottish freshwater communities will have influenced the parasite fauna of native Scottish fishes. Results are used to clarify whether invasive criteria for bullheads are met through bullhead-mediated spread of potentially harmful parasites.

5.2 Methods

Eleven sample sites were selected, encompassing four separate river systems across Edinburgh and the Lothians (Figure 5.2). Six sampling locations contained established bullhead populations and five sites were bullhead-free control sites. Depending on availability, fish samples consisted of bullheads, stone loach, brown trout, minnows and founder (*Platichthys flesus*) (Table 5.1).

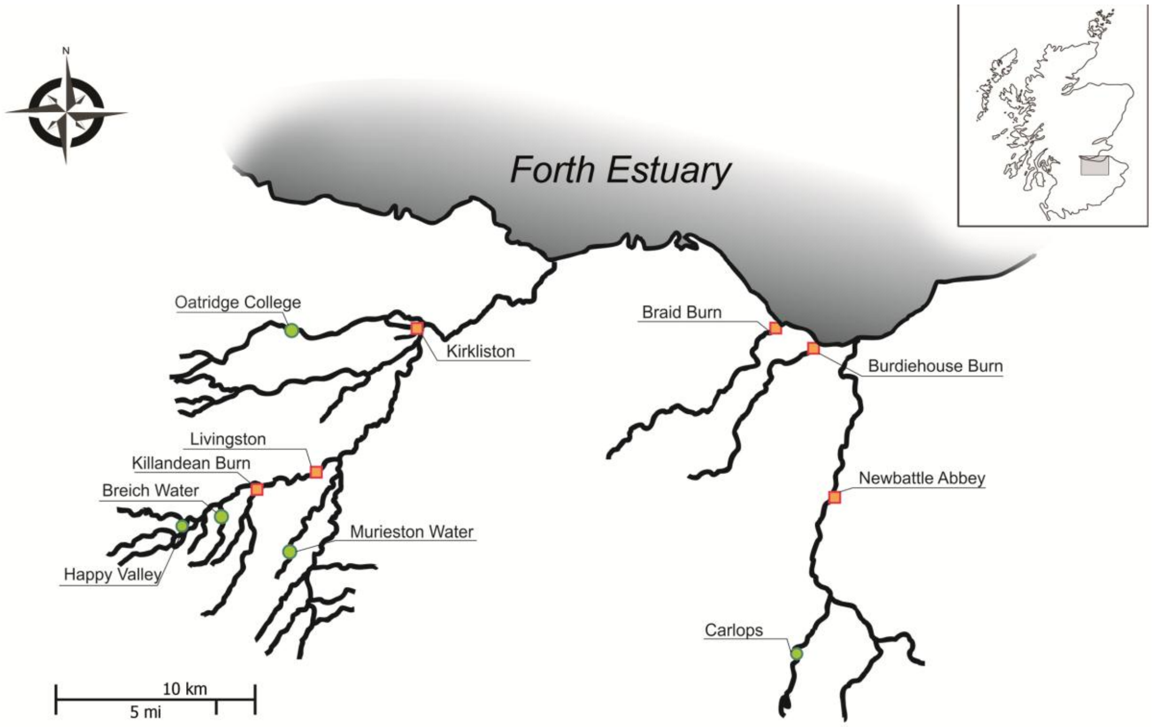

Figure 5.2. Locations of sample sites used in parasitological studies. Bullheads were present in six locations (🟧), while five locations were bullhead free (🟢)

Table 5.1. Fish species and sample sizes (*n*) collected from each location in 2014 and 2015

		Kirkliston	Killandean Burn	Newbattle Abbey	Burdiehouse Burn	Braid Burn	Oatridge	Livingston	Breich Water	Happy Valley	Murieston	Carlops
	Bullhead	*n* = 34	*n* = 35	*n* = 21	*n* = 31	*n* = 35	-	*n* = 35	-	-	-	-
	Brown trout	-	*n* = 13	*n* = 14	*n* = 3	*n* = 5	*n* = 8	-	-	-	*n* = 18	*n* = 16
2014	Stone loach	*n* = 14	*n* = 2	-	*n* = 3	-	*n* = 35	*n* = 14	*n* = 20	*n* = 20	*n* = 5	-
	Minnow	-	-	-	-	-	-	*n* = 9	n= 1	n= 7	-	-
	Flounder	-	-	-	-	-	-	-	-	-	-	-
	Bullhead	*n* = 32	*n* = 33	*n* = 35	*n* = 35	*n* = 35	-	*n* = 35	-	-	-	-
	Brown trout	*n* = 6	*n* = 6	*n* = 6	-	*n* = 6	*n* = 7	-	-	-	*n* = 9	-
2015	Stone loach	*n* = 2	*n* = 1	*n* = 6	-	-	*n* = 24	*n* = 5	*n* = 15	*n* = 8	*n* = 8	-
	Minnow	-	*n* = 8	-	-	-	-	*n* = 25	*n* = 13	*n* = 24	-	-
	Flounder	-	-	-	*n* = 7	*n* = 10	-	-	-	-	-	-

5.2.1 Laboratory Analyses

5.2.1.1 Fish Dissections

Fish samples were thawed in a refrigerated room (ambient temperature 8°C) for one hour (bullhead, stone loach, flounder and minnow) or overnight (brown trout). Prior to dissection, an initial external examination of the body surface, operculum, outer gills, eyes, oral cavity and fins was conducted under an Olympic SZ51 dissection microscope, to determine the presence of ectoparasites or growth abnormalities. Standard body length (to the nearest mm), fork length (to the nearest mm) and thawed body weight (g) were also recorded. Each fish was manually dissected using the following protocol: An incision was made along the ventral surface from the vent region to the operculum, exposing the internal organs. Any visible parasites were removed and their origin recorded. All organs were then removed from the visceral cavity, isolated from each other and immersed in 0.9% saline solution. Liver weight (g) and body weight (g) after the removal of all organs from the visceral cavity were recorded. The heart was removed from the thoracic cavity, followed by each of the gill rakers. The cranial cavity was exposed and the eyes removed, whilst the brain and surrounding tissues were examined for any signs of parasites. Finally, muscle tissue was isolated from the body wall by a bilateral anteroposterior incision from the caudal tip of the opercular cavity to the caudal peduncle, following the line of the vertebral column. Dissections and examination of organs and isolated tissues were carried out under an Olympic SZ51 dissection microscope at varying magnifications, ranging from 6.7x to 45x. The organs of the digestive tract and each of the eyes were opened and the contents rinsed into the surrounding saline solution. The tissue of all organs was squashed between two Petri dishes and carefully examined for the presence of parasites. Saline solution that was used to store each organ and (where relevant) its contents was also inspected. Gill rakers were separated and examined independently in a Petri dish containing 0.9% saline solution. Recovered parasites were fixed in Formalin-acetic acid-alcohol (FAA) for three hours, before long-term storage in 70% ethanol. A few drops of glycerol were also added to vials containing nematodes.

5.2.1.2 Parasite Clearing, Staining and Microscopic Examination

Fish parasites were subjected to a range of staining, clearing and mounting methods depending on the taxa and life stage of each parasite, following the procedures laid out in Kruse and Pritchard (1982).

Digenea

Digenean specimens were subjected to a clearing and staining process before microscopic examination. Each specimen was placed in acetocarmine stain for approximately three minutes, then returned to a 70% ethanol solution. Immersion in 80%, 90% and 100% ethanol solutions for 30 minutes per solution was then undertaken. Specimens were then placed in a solution of equal parts clove oil and 100% ethanol, and left in the solution until all ethanol had evaporated (usually approximately 3 hours). The cleared individuals were then mounted on microscope slides using Canada balsam, which was left to partially harden for several days before specimens were examine using an Olympus BH-2 compound microscope.

Species-level identification of Digenea recovered from the eyes required measurements of morphometric and morphological characters. Specimens were subjected to the procedure described above, before cellSens software (Olympus Corporation) was used to visualise and accurately measure the following features: body length (BL), body width at the holdfast organ (BW), holdfast organ breadth (HB) and length (HL), ventral sucker breadth (VSB) and length (VSL), oral sucker breadth (OSB) and length (OSL), distance between lappet peaks (DLP) and interior (DLI) and the distance between the ventral sucker and the anterior (VSA) (Figure 5.3). Selection of measured characters was based on the work of Shigin (1976), Brady (1989) and Cavaleiro *et al*. (2012). Specimens were photographed using an Olympus BH-2 compound microscope.

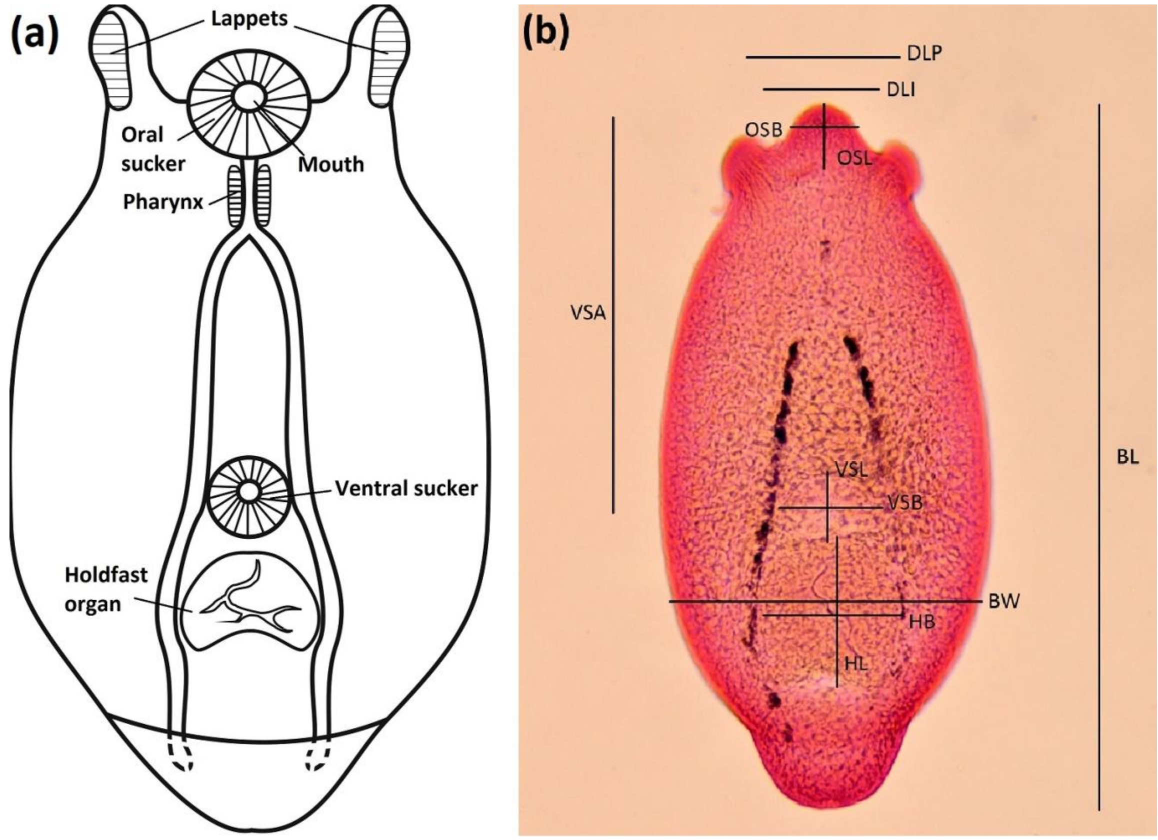

Figure 5.3. ***Diplostomum*** **morphology (a) Schematic drawing of observed digenean; (b) morphological and morphometric features measured in digeneans extracted from the eyes of fish hosts (abbreviations explained in the text)**

Cestoda

The recovered cestode specimen was identifiable without the use of staining and clearing methods. It was examined and photographed under an Olympus SZ51 dissecting microscope whilst immersed in a 70% ethanol solution.

Nematoda

Nematoda required clearing before microscopic examination. Each specimen was immersed in a solution of equal parts 70% ethanol and glycerol. Clearing occurred as the ethanol evaporated out of the solution. After approximately four hours, cleared specimens were removed and mounted in glycerol jelly to allow for examination and photography using an Olympus BH-2 compound microscope.

Acanthocephala

Acanthocephalan specimens were subjected to a dehydration and clearing process before microscopic examination. The body wall of all individuals was punctured with the head of a dissecting needle just after the proboscis, to facilitate mixing of different solutions with internal structures. They were then immersed in 80%, 90% and 100% ethanol solutions for one hour per solution. Individuals were then placed in a watch glass containing a 1:1 ratio of xylene and 100 % ethanol, and left sitting at the interface between the two immiscible liquids. Once the specimens fell through the interface of the two liquids and were immersed completely in the xylene bottom layer (usually after about 12 hours), they were removed and rinsed in a fresh solution of 100% xylene for 30 minutes. Several drops of Canada balsam were then added to the xylene and mixed through to form a solution, in which specimens were left for a further hour. All Acanthocephala were then mounted on microscope slides using Canada balsam, which was left to harden overnight before slides were examined under a compound microscope. Slides mounted with Canada balsam required up to two weeks to completely harden. Specimens were examined and photographed using an Olympus BH-2 compound microscope.

Cysts

Cysts were not stained, as their thick protective wall prevented any stain uptake. Individuals were examined and photographed under an Olympic SZ51 dissection microscope at 45 x magnification whilst immersed in 70% ethanol, and also squashed between microscope slides and viewed under a compound microscope at 40x - 400 x magnification.

5.2.1.3 Scanning Electron Microscopy

Reference specimens of acanthocephala from each host fish species, samples of unidentified nematodes and examples of the two types of digenean metacercariae were observed and photographed using scanning electron microscopy (SEM). Samples were dehydrated by placing them in a series of increasing concentrations of ethanol (in 10% increments from 70% to 100%) for one hour at each concentration. Each specimen was then critical point dried with liquid CO_2, using a Leica EM CPD300. Samples were then attached to a specimen stub and sputter coated with gold, before being viewed with a Hitachi S-4800 scanning electron microscope at an acceleration voltage of 3 kV.

5.2.2 Statistical Analysis

5.2.2.1 Fish Parasites

Data across replicate years were merged for each fish host (where appropriate), due to the limited sample sizes obtained for some species/location combinations in a given year. Different life stages of the same species were treated separately for the purposes of statistical analysis. All univariate analysis was undertaken using QPweb (version 1.0.13, Reiczigel and Rózsa, 2005) and multivariate testing was conducted with PRIMER (version 6, PRIMER-E LTD) software.

Fish parasites - *Diplostomum* identification

To allow direct comparisons of each *Diplostomum* individual based on allometric growth alone, the geometric size effect was removed from the results of the morphological and morphometric measurements. This was done by logging all data, calculating the mean log value for each measured feature and subtracting the mean from each individual. A similarity matrix was then constructed based on Euclidean distances (PRIMER, version 6, PRIMER-E LTD), and the similarity between *Diplostomum* recovered from all hosts visualised using a multi-dimensional scaling (MDS) plot. A two-way ANOSIM was then conducted to determine if significant differences were found between the results when grouped by host species or sample location.

5.2.2.2 Comparisons Across Fish Hosts/Sample Locations

For each fish host, the prevalence, mean infection intensity and mean infection abundance of all recorded parasites at each site were calculated; wherein infection prevalence (P) is the proportion of infected hosts in a sampled population among all hosts examined, mean intensity (I) represents the mean number of parasite individuals of the same species per infected host, and mean abundance (A) is the mean number of parasite individuals per all potential hosts examined (Bush *et al.*, 1997; Margolis *et al.,* 1982; Rózsa *et al.*, 2000). Confidence intervals, set at 95%, were calculated for each factor. Confidence intervals were selected over standard deviations due to the skewed nature of parasite infections, in which a small number of individuals will generally host a large proportion of all conspecific parasites, creating a high degree of variability in infection parameters. As per the recommendations provided in Rózsa *et al.* (2000), 95% confidence intervals were established for all three infection parameters, using Clopper-Pearson confidence intervals (Clopper and Pearson, 1934) for prevalence; and bootstrap BCa confidence intervals (Efron and Tibshrani, 1993), based on 2000 bootstrap replications, for mean intensity and mean abundance.

Overall mean intensity values were calculated for each fish species at a given location and Log(x+1) transformed to approach normal distribution. A similarity matrix was constructed using a Bray-Curtis resemblance matrix (PRIMER, version 6, PRIMER-E LTD), and the similarity between species and locations visualised using a multi-dimensional scaling (MDS) plot. A two-way ANOSIM was also conducted to determine if significant differences were found between the results when grouped by host species or sample location. Records where no parasites were reported for a given species in any particular sample location were considered outliers and were removed from the MDS plot to improve visual clarity.

5.2.2.3 Comparisons Between Locations With/Without Bullheads

Using stone loach and brown trout data, a comparison was made between the prevalence and mean intensity of parasite infections in sample locations with and without bullheads. This was undertaken for each parasite species using Fisher's exact test (prevalence), and a bootstrapped *t*-test with 2000 bootstrap replicates (mean intensity). A Holm-Bonferroni correction (Holm, 1979) was applied to all results to limit the probability of incurring a Type I error when carrying out multiple statistical tests in succession (Abdi, 2010).

5.3 Results

In total, eight parasite species from four distinct taxonomic groups were identified from all fish examined (Table 5.2). Two unidentified cyst types were also recorded. Of the species observed, four different parasites (*Echinorhynchus truttae, Apatemon gracilis, Diplostomum volvens and Raphidascaris acus*) were shared between bullheads and one or more native fishes.

Table 5.2. Observed parasites and their fish hosts. Shaded boxes containing an X represent incidences of infection

	Echinoryhnchus truttae	*Proteocephalus sagittus*	*Apatemon gracilis cyst*	*Apatemon gracilis metacercariae*	*Crepidostomum metoecus*	*Diplostomum volvens*	*Cystidicola farionis*	*Cystidicoloides tenuissima*	*Raphidascaris acus*
Bullhead	X		X	X		X			X
Stone loach	X	X	X	X					X
Brown trout	X		X		X		X	X	
Minnow						X			
Flounder			X	X					

5.3.1 Parasite Descriptions and Identification

Digenea

Species: *Apatemon gracilis* (Rudolphi, 1819)

Host: Bullhead, stone loach, brown trout, flounder

Location: Cranial cavity, ocular cavity, thoracic cavity, body cavity, muscle tissue, heart, liver, kidneys, gonads, mesenteries, intestinal wall (bullhead and stone loach). Mesenteries (flounder and brown trout).

Life stage: Cyst (second intermediate host)

First intermediate host: Freshwater molluscs (Palmieri, 1973)

Final host: Piscivorous birds, particularly anatids (Burton-Beverley, 1972)

Description: Found embedded in tissues or loose in the thoracic and body cavities. Internal structures are visible within some cyst contents but the majority lack obvious internal features. All cysts appear to be encapsulated, containing a fluid-filled cavity between the outer capsule wall and the internal cyst wall. The cyst wall is of uniform width. The strong outer capsule wall is able to withstand significant pressure and manipulation. Attempts to penetrate the cyst wall resulted in the brisk expulsion of lipid-rich internal contents. Cyst appear to be under significant pressure. Some cysts appear to be in different stages of development, with the majority appearing completely transparent and others housing opaque centres. Oral and ventral suckers are evident in some specimens (Figure 5.4). Total cyst length (including surrounding capsule) is approximately 1.2mm, and width is 0.5mm. All cysts are ovoid. Identification, based on the aforementioned features, was aided by the description of cyst characteristics given in Blair (1976). Specimens were also found embedded in tissues alongside the metacercarial form of the same species, further confirming their identity.

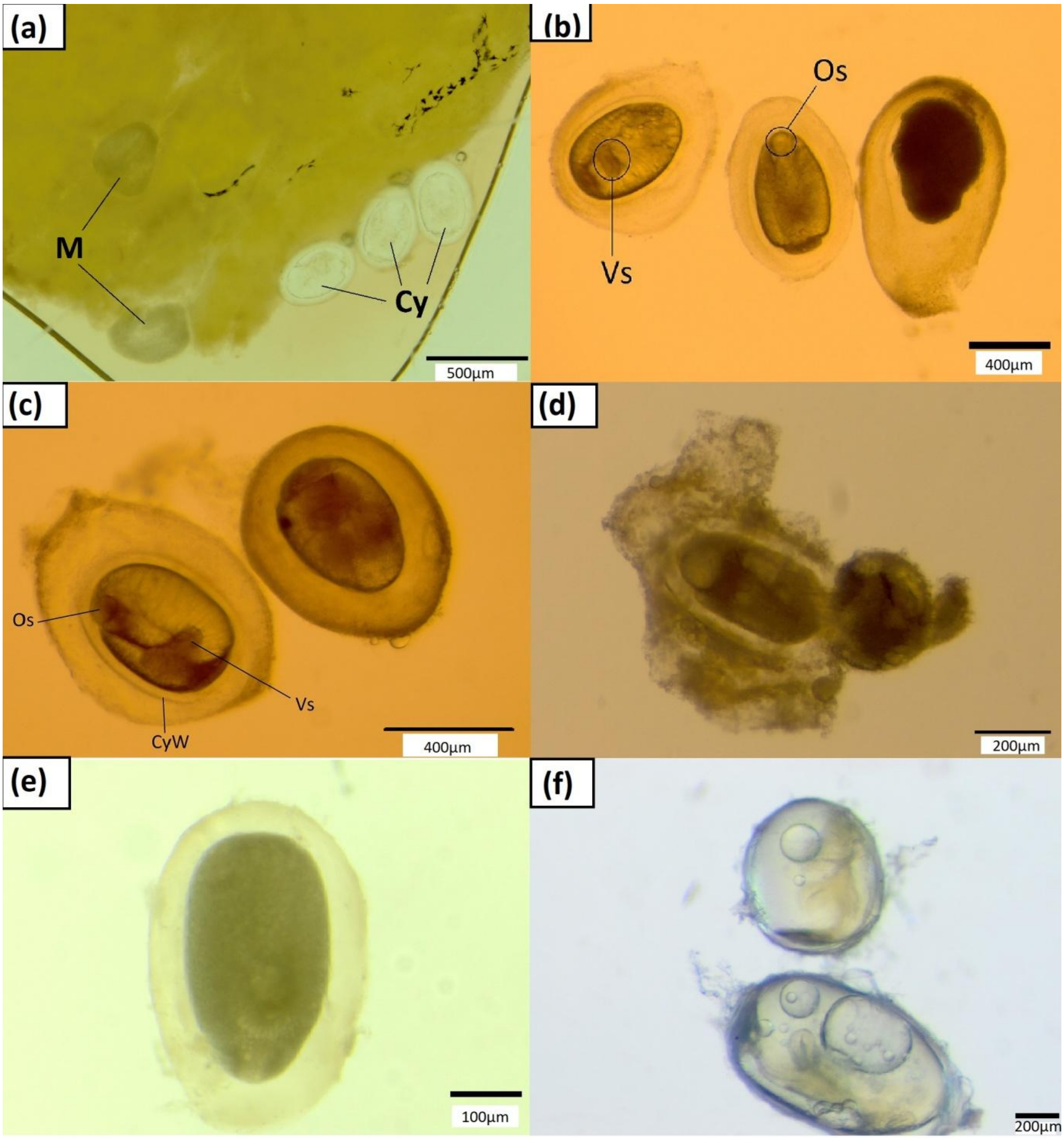

Figure 5.4. ***Apatemon gracilis*** **cysts recovered from stone loach (a-c) and bullhead (d-f). (a) Cyst (Cy) are shown attached to liver tissue and closely associated with** ***A. gracilis*** **metacercariae (M). (b and c) Cysts are shown contained within a secondary capsule, separated by the cyst wall (CyW). The oral sucker (Os) and ventral sucker (Vs) are visible in some specimens. (d) Attempted extraction of cyst contents results in the rapid expulsion of all internal tissue. (e) Completely opaque bullhead cysts and (f) transparent bullhead cysts with no visible internal structures**

Species: *Apatemon gracilis* (Rudolphi, 1819)

Host: Bullhead, stone loach, flounder

Location: Thoracic cavity, ocular cavity, body cavity, mesenteries, intestinal tissue, muscle tissue, gonads, heart (bullhead and stone loach). Body cavity (flounder).

Life stage: metacercariae (second intermediate host)

First intermediate host: Freshwater molluscs (Palmieri, 1973)

Final host: Piscivorous birds, particularly anatids (Burton-Beverley, 1972)

Description: Found loose in body and thoracic cavities, and embedded in organs and connective tissues. Metacercariae had a mean length of 1142.29µm (range 556.57-1972.62) and width of 670.75 (range 252.75-1181.09) (n = 20). A lobed holdfast organ projecting from the body surface and a small tail structure are visible in all samples (Figure 5.5). The ventral hindbody is noticeably depressed and wider than the ventral forebody. Bilateral lappets framing the oral sucker are visible in some individuals.

Metacercariae were often found co-occurring alongside the encysted form of *A. gracilis* and are presumed about to encyst (Blair, 1976). Identification, based on the aforementioned features, was confirmed using the key provided in Gibson (1996).

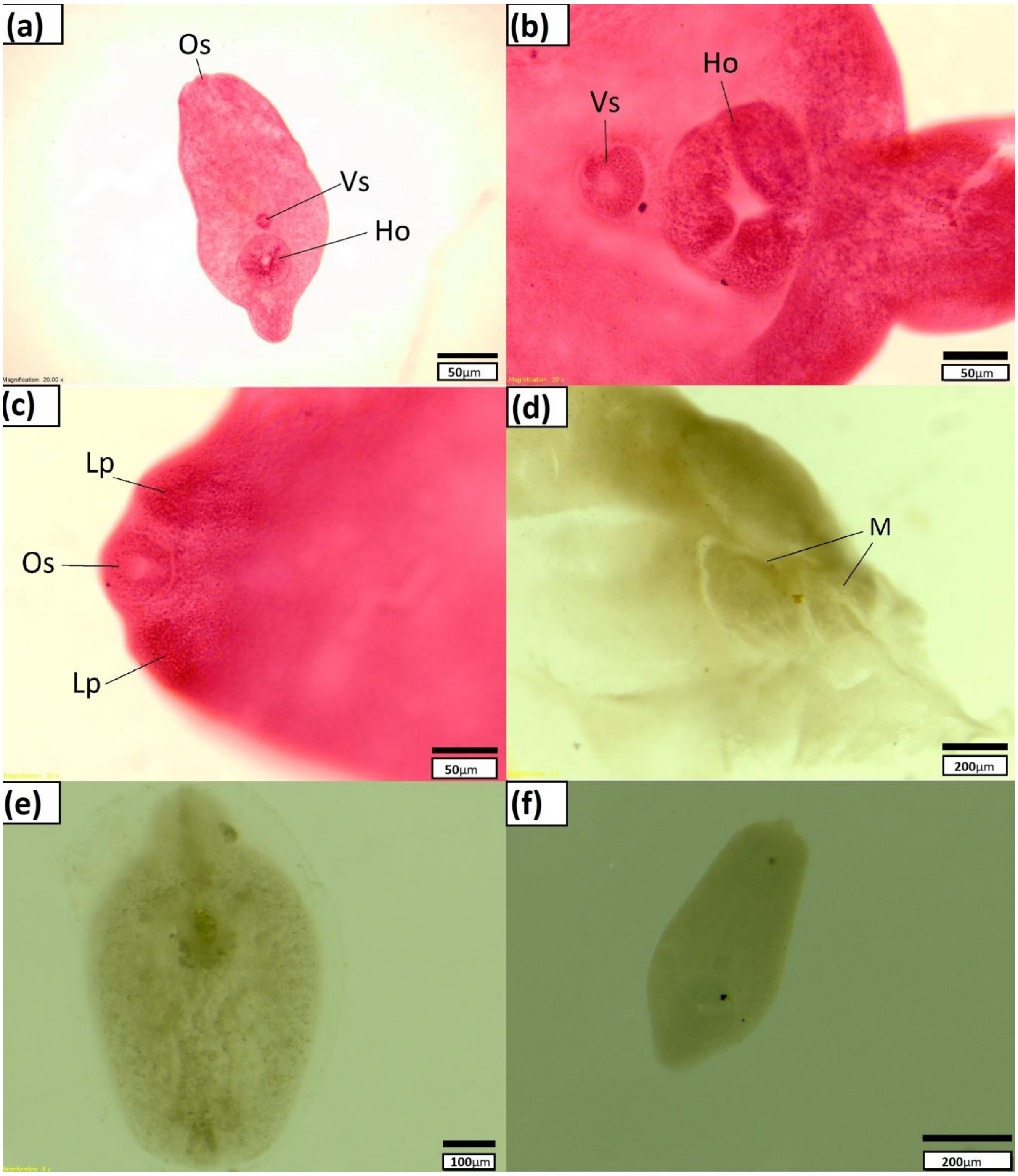

Figure 5.5. ***Apatemon gracilis*** **metacercariae recovered from bullheads (a-d), stone loach (e) and flounder (f). Ventral sucker (Vs), holdfast organ (Ho), lappets (Lp) and oral sucker (Os) are labelled on stained specimens. (d) 2 x metacercariae (M) embedded in intestinal wall**

Species: *Diplostomum volvens* (Nordmann, 1832)

Host: Bullhead and minnow

Location: Within the vitreous humour of the eye

Life stage: Metacercariae (second intermediate host)

First intermediate host: Aquatic snails, usually *Lymnaeidae* (Voutilainen *et al.,* 2009)

Final host: Piscivorous birds, primarily gulls (Wootten, 1974)

Description: Small digenean species with a maximum body length of 570µm (mean = 412 ± 99) and width of 240 µm (mean = 167 ± 34). The maximum body width is found in roughly the centre of the body. Two prominent lappets visible on either side of the oral sucker (Figure 5.6). Ventral sucker positioned just caudal to the midpoint of the body. The holdfast organ is composed of two separate folds and has a prominent concavity in the posterior margin. Specimens are found loose within the vitreous humour of the eye.

The similarity between the measurements of *Diplostomum* recovered from each fish hosts was shown in an MDS plot (Figure 5.7). A two-way ANOSIM testing for differences between host species and sample location showed that there was no significant separation of the data by host species ($p > 0.05$, $R = 0.183$) or location ($p > 0.05$, $R = -0.081$). The morphological features reported from both bullhead and minnow were a clear match with descriptions given for *D. baeri* (Niewiadomska and Laskowski, 2002; Faltýnková *et al.,* 2014) and *D. volvens* (McKeown and Irwin, 1995). Following the custom applied in Shigin (1993) and Niewiadomska (2010), *D. volvens* and *D. baeri* are considered synonyms. Each fish species is therefore concluded to be a host for *D. volvens*.

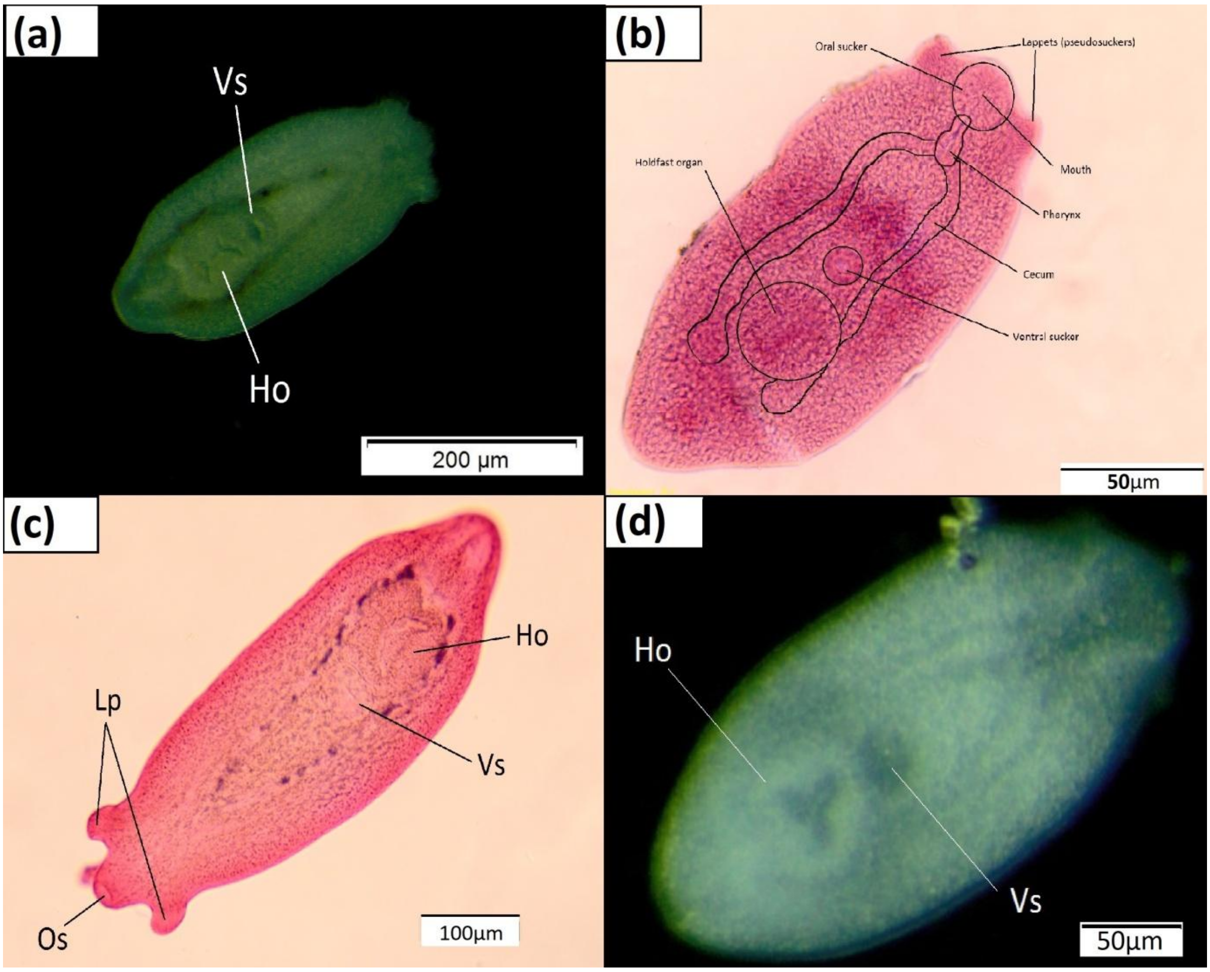

Figure 5.6. ***Diplostomum volvens*** **recovered from bullhead (a-c) and minnow (d). The holdfast organ (Ho), ventral sucker (Vs), paired lappets (Lp) and oral sucker (Os) are labelled. The position of the mouth, pharynx and caecum are shown in Figure 5.6 (b)**

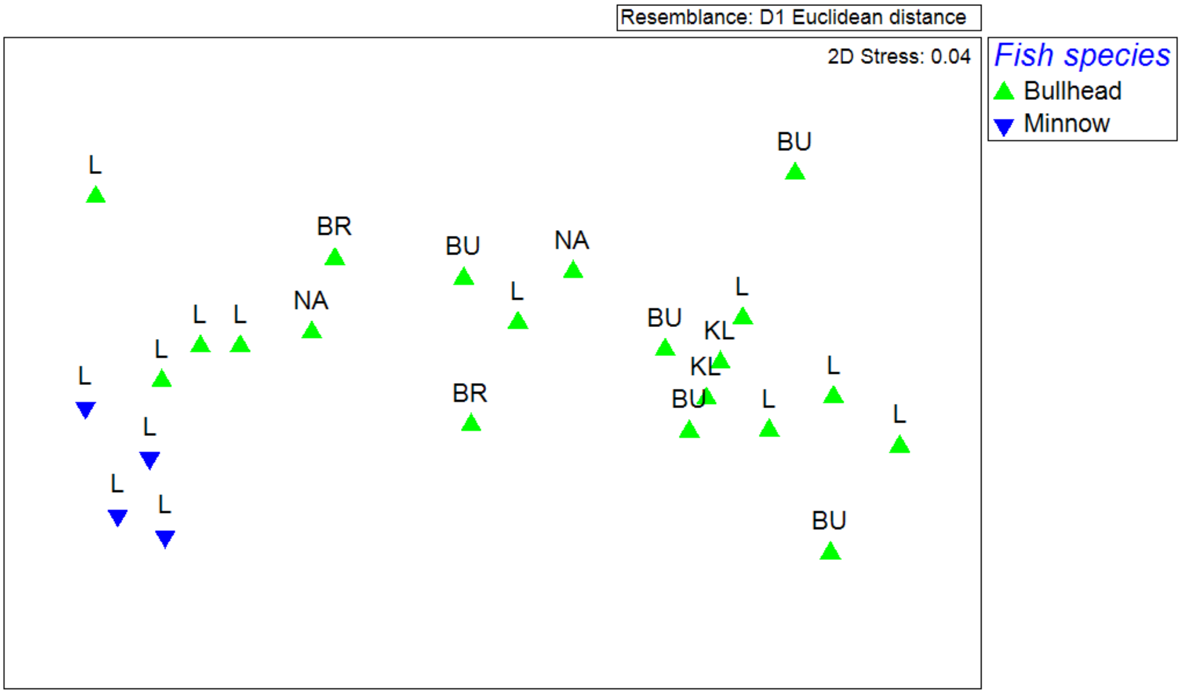

Figure 5.7. Similarities in morphometric features recorded from bullhead and minnow *Diplostomum*, based on Euclidean distances. Samples are labelled by fish species and sample location: KL = Kirkliston, NA = Newbattle, BU = Burdiehouse Burn, BR = Braid Burn, L = Livingston

Species: *Crepidostomum metoecus* (Braun, 1900)

Host: Brown trout

Location: Intestines and Pyloric caeca

Life stage: Adult

First intermediate host: Aquatic molluscs (Awachie, 1968)

Second intermediate host: Arthropods and amphipods (Awachie, 1968)

Description: Small specimens of average 668µm in length (minimum 333.28 µm, maximum 1258.12µm, n = 10). Individuals possess an elongated body, with maximum body width roughly in line with the ventral sucker. The ventral sucker is raised from the body surface and appears similar in size to the oral sucker. Two distinct eye spots are visible on the dorsal surface (Figure 5.8). Six muscular lobes are arranged in the three pairs around the oral cavity, distinguishing *C. metoecus* from the closely-related *C. farionis*. Identification, based on these features, is confirmed from the descriptions and images given in Thomas (1958), Gibson (1996) and Moravec (2002), although the size of specimens recovered were smaller in length than values reported in the literature.

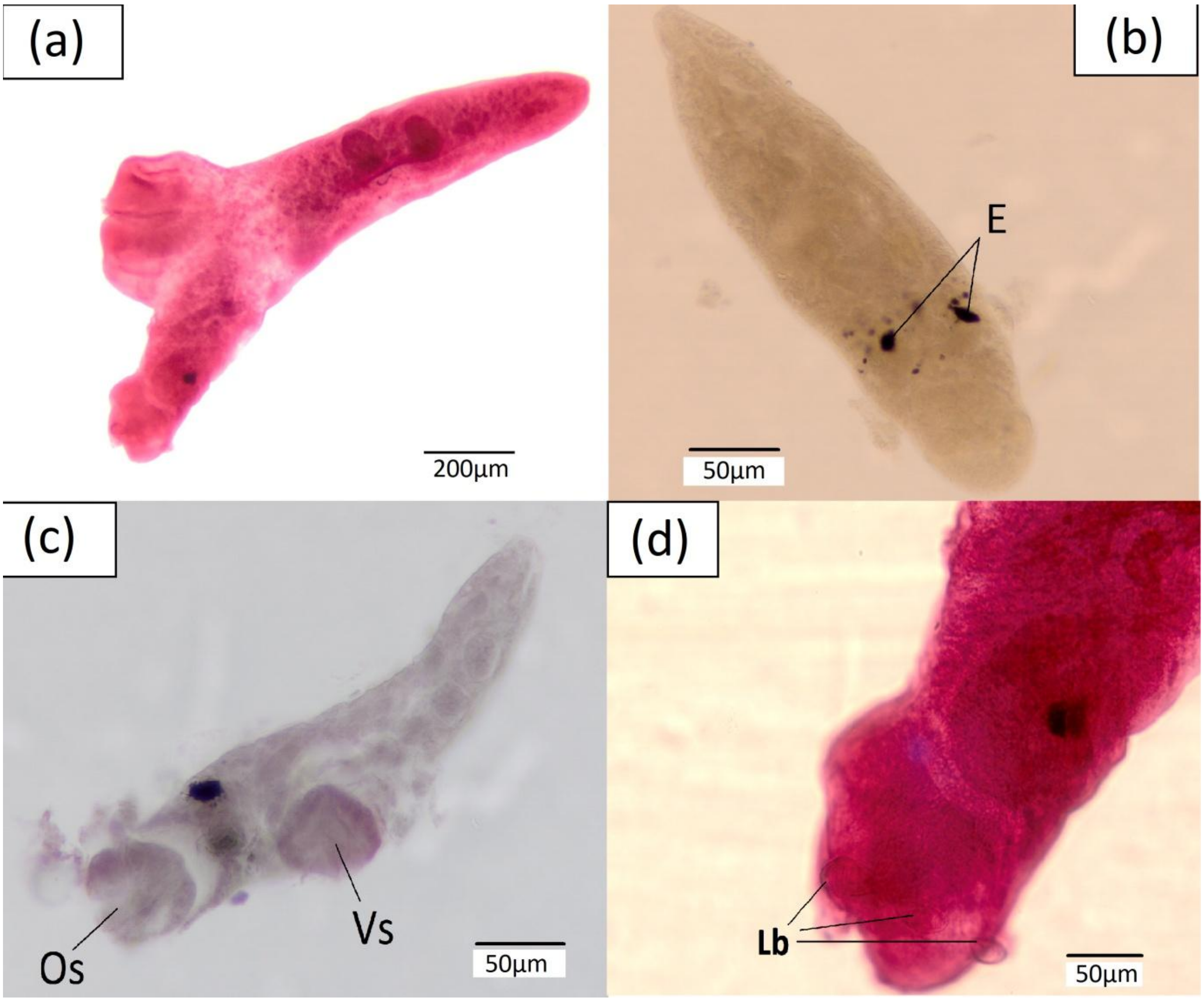

Figure 5.8. Specimens of *C. metoecus* isolated from the intestine and pyloric caeca of brown trout (a-d). The ventral sucker (VS) and oral sucker (OS) are similar in size. Eyespots (E) and six muscular lobes (Lb) encircling the oral cavity (three visible and labelled) facilitate species-level identification

Cestoda

Species*: Proteocephalus sagittus* (Grimm, 1872)

Host: Stone loach

Location: Intestines

Life stage: Juvenile

Intermediate host: Freshwater copepods (Hanzelová and Gerdeaux, 2003)

Final host: Stone loach

Description: Found loose within the lumen of the intestines. Total body length is approximately 5cm and the body (strobila) is clearly segmented into individual proglottids. The scolex contains four suckers, arranged symmetrically around the head (Figure 5.9). On the basis of the features described above, the recovered cestode fits clearly into the genus *Proteocephalus*, using the descriptions provided in (Chubb *et al.*, 1987). Subsequent identification provided in this publication is dependent upon the fish host of a gravid adult worm, since juvenile *Proteocephalus* can be found within the intestinal contents of fish in which they cannot mature (Chubb *et al.*, 1987). On this basis, the species could therefore be either *P. sagittus* or *P. filicollis.* Reproductive organs are not clearly visible in this specimen and, as such, species-level identification using this key is not possible.

The width of the scolex in the recovered specimen is about 500µm, and each sucker approximately 200 µm in diameter, giving a scolex to sucker width ration of 40%. Using the aforementioned features, the recovered cestode can be identified as *Proteocephalus sagittus*, using the descriptions provided in Scholz *et al.* (2003) and Scholz *et al.* (2017). Observed measurements provided in Scholz *et al.* (2017) gave a scolex width range of 324-

675mm, a sucker diameter of 122-244 µm and ratio of sucker width to scolex width of 32-50%. Values obtained in this study were directly comparable to these figures.

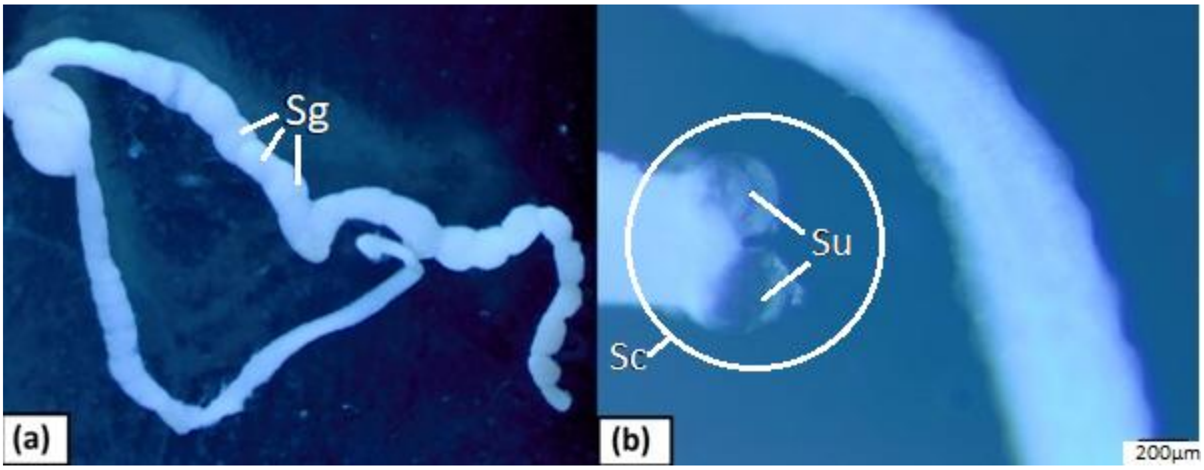

Figure 5.9. *Proteocephalus sagittus* specimen recovered from adult stone loach (a) Body shows segmentation (Sg) and (b) four suckers (Su) on the scolex (Sc), used to identify the *Proteocephalus* genus

<u>Nematoda</u>

Species: *Cystidicola farionis* (Fischer, 1798)

Host: Brown trout

Location: Stomach, pyloric caeca and swimbladder

Life stage: L3 larval stage (stomach and pyloric caeca) and adult (swimbladder)

Intermediate host: *Gammarus pulex*

Description: The body is tapered at both ends and has a smooth cuticle. Specimens possess an elongated pharynx and numerous teeth around the buccal cavity (Figure 5.10). The oesophagus is also long, and clearly divided between the narrow muscular region and a substantially longer glandular section. The excretory pore is visible roughly where these two areas meet. The nerve ring is found mid-way down the extended oesophagus. No appendix is visible.

L3 larvae were found in both the stomach and pyloric caeca, showing the pathway of larval migration within brow trout towards the swimbladder, where larvae develop into their final adult stage. Identification was confirmed using the descriptions provided in Smith and Lankester (1979) and Moravec (1994).

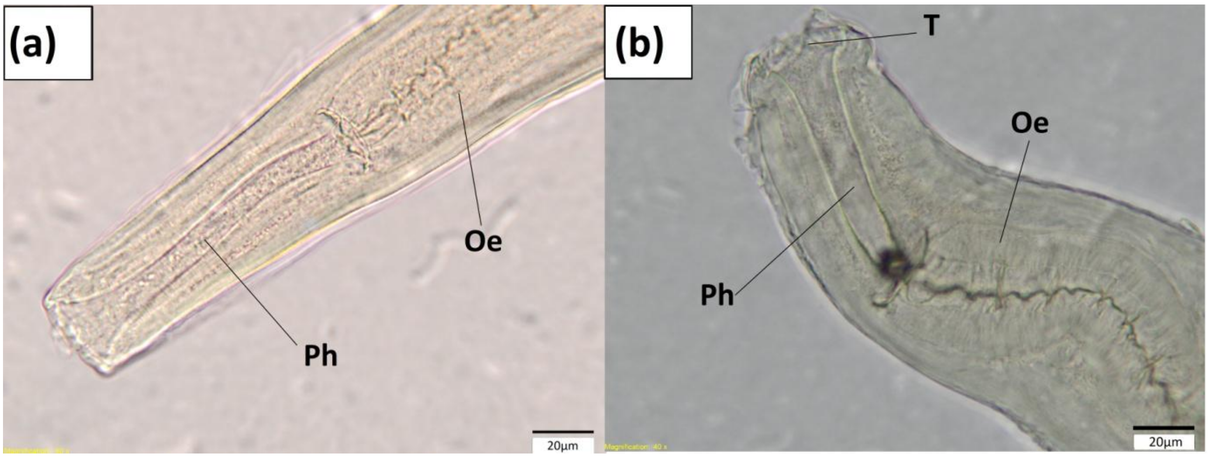

Figure 5.10. Lateral view of the cephalic region of *C. farionis* (a-b). Specimens show elongated pharynx (Ph), external teeth (T) and muscular portion of the oesophagus (Oe)

Species: *Cystidicoloides tenuissima* (Zeder, 1800)

Host: Brown trout

Location: Intestines and stomach

Life stage: Adult

Intermediate host: Several aquatic invertebrates, predominantly mayflies (Aho and Kennedy, 1984)

Description: Individuals possess a thin cuticle with dense transverse striations. The mouth is dorsoventrally flattened and contains two subventral sclerotized plates, which are widest at the dorsal and ventral tips and taper towards the well-developed lateral labia. The labia contain dorsal and ventral lobes. The oesophagus is clearly divided into a shortened and narrow muscular portion and a wider, elongated posterior glandular region. The nerve ring is positioned in the anterior portion of the muscular oesophagus, with the excretory pore exiting in the same area (Figure 5.11). Identification was confirmed by the descriptions provided in Moravec (1967) and De and Moravec (1979).

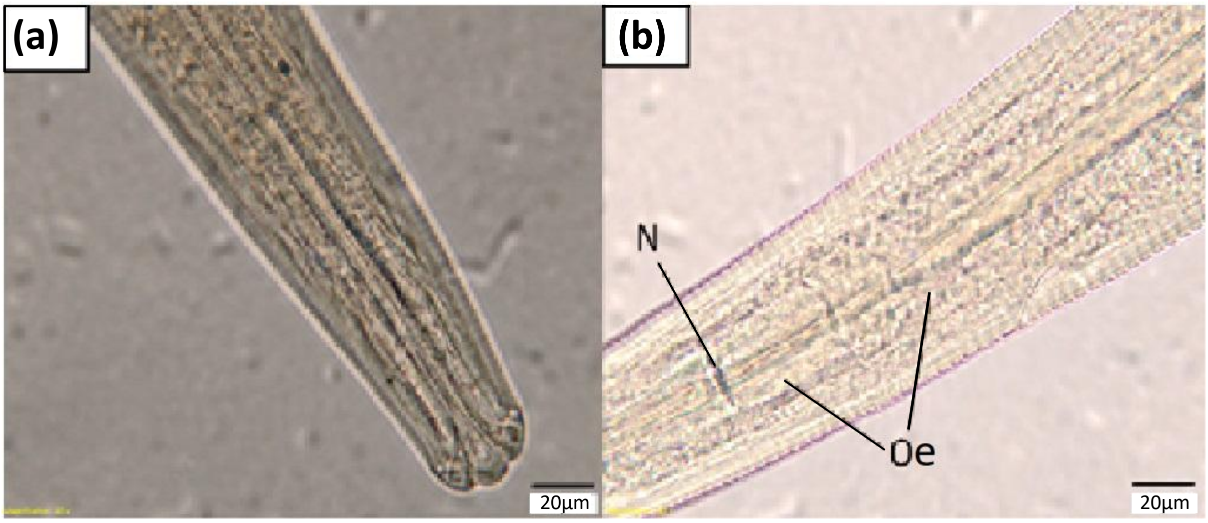

Figure 5.11. *Cystidicoloides tenuissima* extracted from brown trout (a) lateral view of the cephalic region and (b) nerve ring section (N) visible in the narrower muscular region of the oesophagus (Oe)

Species: *Raphidascaris acus* (Bloch, 1779)

Host: Bullhead and stone loach

Location: Liver

Life stage: L3 larvae (intermediate host)

Intermediate host: Numerous piscivorous fish species (Koubková *et al.,* 2004)

Definitive host: Piscivorous fish species such as pike (*Esox lucius*), burbot (*Lota lota*) and brown trout

Description: Specimens were found loose or encapsulated in liver tissue. Body is tapered at both ends with a smooth cuticle and two deep lateral folds (Figure 5.12f). The head is rounded and the buccal cavity terminates in three lips (one dorsal, two ventro-lateral) (Figure 5.12a and e). The oesophagus is cylindrical and muscular. The excretory pore is posterior to the nerve ring, which is found about a third of the way down the oesophagus. No caecum is visible. Identification is confirmed by the description provided in Moravec (1994) and Jahantab *et al.* (2014).

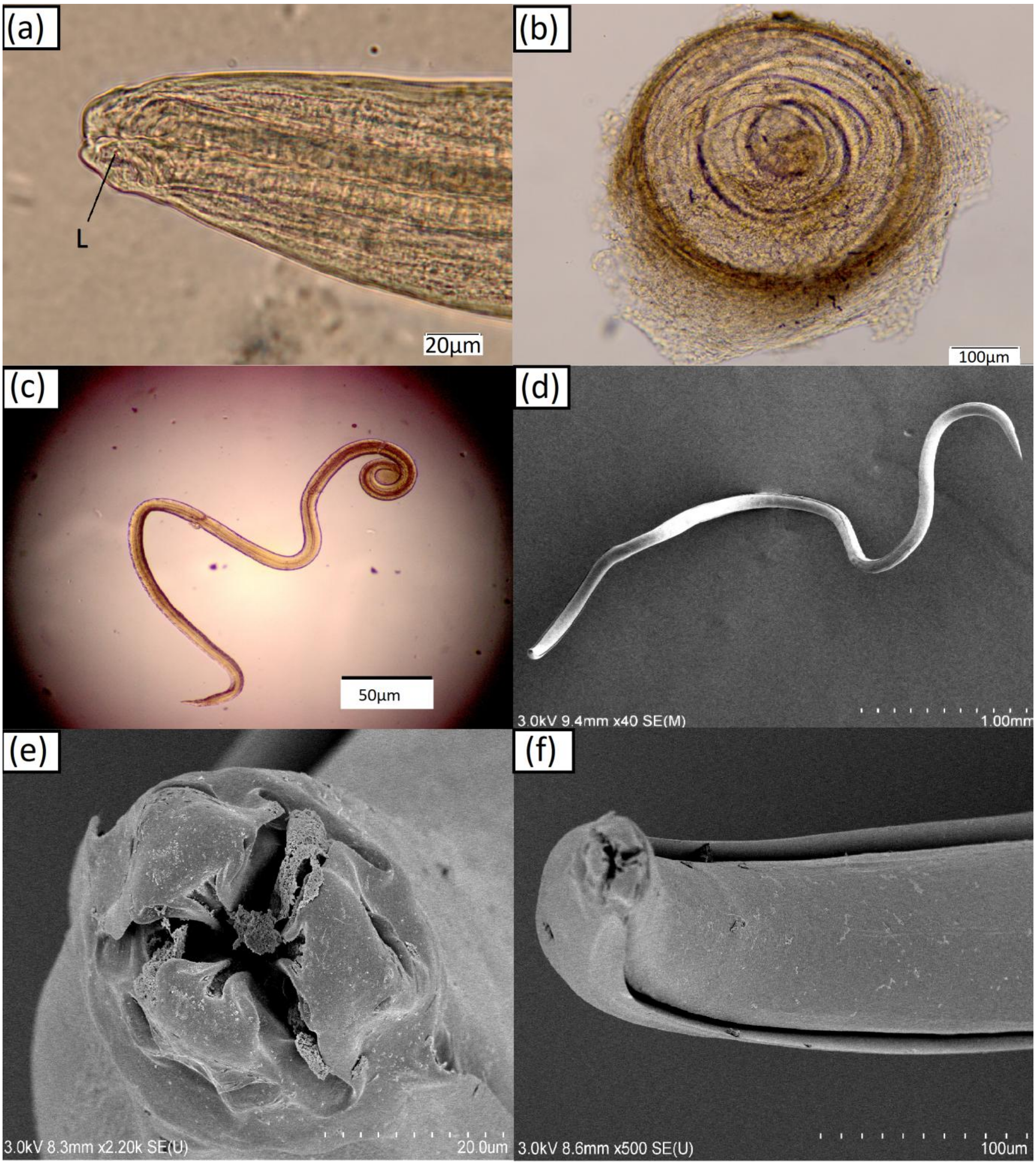

Figure 5.12. ***Raphidascaris acus*** **photographed under a compound microscope (a-c) and scanning electron microscope (d-f). (a, e, f) Lips (L) are visible at the termination of the buccal cavity. (b) Some specimens found encapsulated in tight coils. (c-d) Body is tapered at both ends**

Acanthocephala

Species: *Echinorhynchus truttae* (Schrank, 1788)

Host: Bullhead, stone loach and brown trout

Location in host: intestines (brown trout, stone loach and bullhead), stomach (bullhead and stone loach)

Life stage: Adult

Intermediate host: *Gammarus pulex* (Awachie, 1965)

Description: Individuals are found loose in the intestines or attached to the intestinal wall by proboscis hooks. The body is a characteristic orange/brown colour when recovered from fish hosts, but the colour is lost over time when specimens are stored in ethanol. Body length can reach up to 10mm, with a full-extended proboscis measuring an additional 4mm. The proboscis contains 13-15 hooks arranged in 20-21 rows. Several caudal rows were held within the body cavity in a number of specimens, due to the proboscis remaining partially inverted. Visualisation of internal morphology shows relatively long, paired lemnisci and 6 cement glands, both of which are feature known to characterise acanthocephala of the genus *Echinorhynchus* (Golvan, 1969) (Figure 5.13). Identification was confirmed using the descriptions and images given in Brown *et al.* (1986) and Amin and Christison (2005).

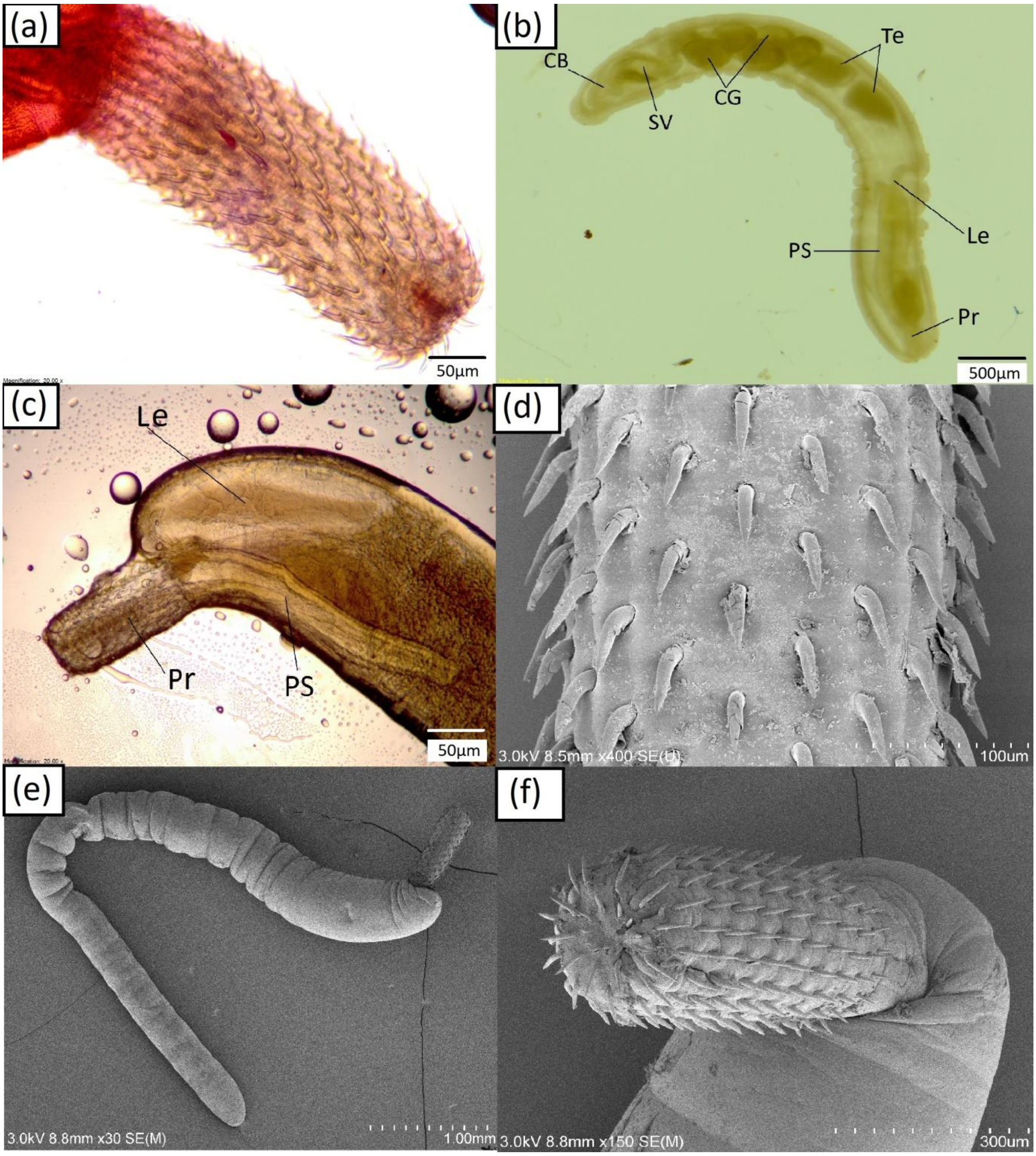

Figure 5.13. ***Echinorhynchus truttae*** **specimens photographed using compound microscope (a-c) and scanning electron microscope (d-f). (a) 11 of 13 hooks found per row on the partially-inverted proboscis. (b and c) Cleared specimens showing the proboscis (Pr) inside a proboscis sheath (PS); two lemnisci (L); testes (Te); 6 cement glands (CG); the seminal vesicle (SV) and copulatory bursa (CB). Hooks are shown to be arranged in 21 rows (a and f) and uniform in size (d)**

Unidentified cyst

Species: Unidentified cyst 1

Host: Minnow

Location: Within the vitreous humour of the eyeball

Life stage: Cyst

Description: Small kidney-shaped cyst, approximately 100 µm in length and 50 µm wide. The widest portion of the cyst is found in the mid section (Figure 5.14). Cyst appears to be fluid filled, with air bubbles visible within the liquid/gelatinous centre. The whole cyst is yellow in colour.

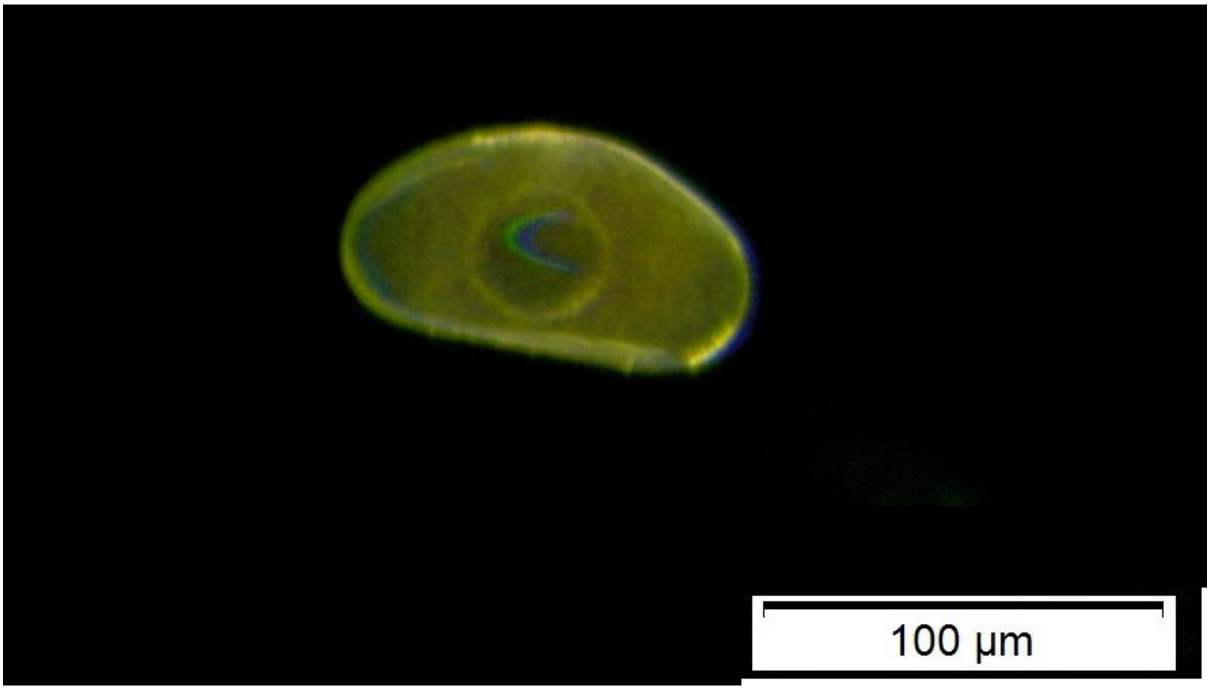

Figure 5.14. Kidney-shaped unidentified cyst from the vitreous humour of a minnow eye

Species: Unidentified cyst 2

Host: Bullhead

Location: Within the vitreous humour of the eyeball

Life stage: Cyst

Description: Small ovoid cysts, approximately 200 µm long and 150 µm wide. The cysts are a true oval shape, with the widest portion of the cyst directly in the centre. Each cysts possesses a feathery 'tail.' Numerous bubbles are visible in the liquid/gelatinous centre (Figure 5.15).

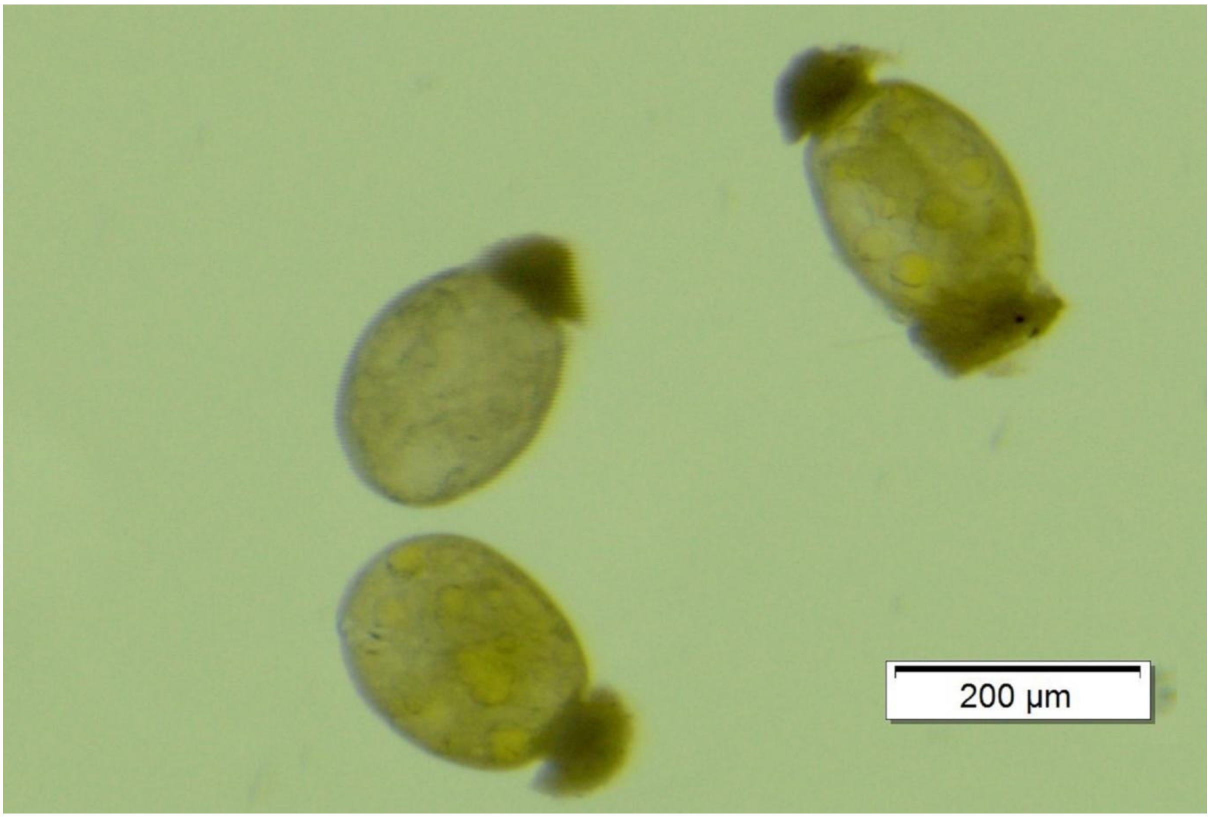

Figure 5.15. Oval-shaped unidentified cyst from the vitreous humour of a bullhead eye

As there were no obvious morphological features visible within the recovered cysts, identification is impossible. Their location within the vitreous humour of the eye suggests that they are an encysted digenean species, but no further identification is possible.

5.3.2 Comparisons Across Fish Hosts/Sample Locations

Apatemon gracilis cysts and metacercariae dominated the bullhead parasite fauna in all locations (Table 5.3). The encysted stage was the most common, with prevalence ranging from 57.1% to 84.1%. Infection intensity was relatively low, ranging from 2.06 to a maximum of 44.1 in the Killandean Burn. *Diplostomum volvens* was found in all but one location, but prevalence, mean infection intensity and abundance were all low (prevalence 3-10.6%, mean intensity 1-1.14 and mean abundance 0.03-0.15). *Raphidascaris acus* was reported in three out of the six sample sites but infection rates were low, with prevalence values ranging from 1.4% to 7.1% and infection intensities never reaching more than one parasite per infected individual. *Echinorhynchus truttae* infections were also relatively rare, with a peak prevalence of 32.1% recorded at Newbattle Abbey where the infection intensity was low (1.83 parasites per infected individual). A single incidence of infection was reported at Burdiehouse Burn.

Table 5.3. Prevalence, intensity and mean abundance records for each parasite species recovered from examined bullheads, sorted by sample site. Results are summed across two sampling years. Values are listed alongside 95% confidence intervals. n = number of individuals examined

		Digenea			Nematoda	Acanthocephala	Cyst
Location		*A. gracilis* cyst	*A. gracilis* metacercariae	*D. volvens* metacercariae	*R. acus*	*E. truttae*	Unidentified type 1
Kirkliston (n = 66)	P	77.3 (65.3-86.7)	15.2 (7.5-26.1)	3 (0.4-10.5)	0	0	0
	I	11.3 (8.1-15.7)	3.1 (1.2-5.1)	1	0	0	0
	A	8.71 (6.11-12.7)	0.47 (0.18-1.06)	0.03 (0-0.08)	0	0	0
Killandean Burn (n = 68)	P	77.3 (65.3-86.7)	2.9 (0.4-10.2)	0	0	0	0
	I	44.1 (32.1-56.7)	1	0	0	0	0
	A	2.33 (1.77-3.27)	0.03 (0-0.07)	0	0	0	0
Newbattle Abbey (n = 56)	P	57.1 (43.2-70.3)	5.4 (1.1-14.9)	7.1 (2-17.3)	1.8 (0-0.96)	32.1 (20.3-46)	7.1 (2-17.3)
	I	3.22 (2.5-4.09)	1.33 (1-1.67)	1	1	1.83 (1.28-2.91)	2 (1-3)
	A	1.84 (1.27-2.5)	0.07 (0-0.2)	0.07 (0-0.14)	0.02 (0-0.05)	0.59 (0.34-1.04)	0.14 (0.04-0.52)
Burdiehouse Burn (n = 66)	P	84.8 (73.9-92.5)	56.1 (43.3-68.3)	10.6 (4.4-20.6)	9.1 (3.4-18.7)	1.5 (0-8.2)	1.5 (0-8.2)
	I	3.5 (2.8-4.84)	2 (1.68-2.32)	1.14 (1-1.43)	1	1	1
	A	2.97 (2.3-4.12)	1.12 (0.83-1.42)	0.12 (0.05-0.23)	0.09 (0.03-0.17)	0.02 (0-0.05)	0.02 (0-0.05)
Braid Burn (n = 70)	P	70 (57.9-80.4)	28.6 (18.4-40.6)	4.3 (0.9-12)	1.4 (0-7.7)	0	0
	I	2.06 (1.71-2.47)	1.55 (1.25-1.8)	1	1	0	0
	A	1.44 (1.14-1.76)	0.44 (0.27-0.64)	0.04 (0-0.01)	0.01 (0-0.04)	0	0
Livingston (n = 70)	P	84.1 (73.3-91.8)	23.2 (13.9-34.9)	13 (6.1-23.3)	0	0	1.4 (0-7.8)
	I	7.16 (5.86-8.74)	1.81 (1.31-2.94)	1.11 (1-1.33)	0	0	1
	A	6.01 (4.71-7.5)	0.42 (0.23-0.77)	0.15 (0.06-0.25)	0	0	0.01 (0-0.04)

Apatemon gracilis was also the dominant species in the parasite fauna of stone loach (Table 5.4). As with bullheads, the encysted form was more common than the metacercarial life stage. Prevalence of the encysted form was high in all locations except Burdiehouse Burn, where *A. gracilis* was absent in the three examined stone loach. Prevalence ranged from 15.4% to 100%, with all hosts being infected with between 5.33 and 51.6 parasite individuals in five out of seven of the sampling areas examined. *Echinorhynchus truttae* was the second most common parasite infection in stone loach, with infections occurring in Newbattle Abbey and the bullhead-free areas of Oatridge College and Murieston Water. Prevalence was moderate, ranging from 11.9% at Oatridge College to 66.7% in Newbattle Abbey (compared to 32.1% prevalence in bullhead at this site). Overall infection intensity of *E.*

truttae was low, ranging from an average of 1.5 to 3 parasites per infected individual. *Raphidascaris acus* and *P. sagittus* infections were restricted to one location each. Prevalence of *R. acus* infection was higher than the values reported for bullhead, will 18.8% of the population at Kirkliston hosting *R. acus* parasites.

Table 5.4. Prevalence, intensity and mean abundance records for each parasite species recovered from examined stone loach, sorted by sample site. Results are summed across two sampling years. Values are listed alongside 95% confidence intervals. n = number of individuals examined

		Cestoda	Digenea		Nematoda	Acanthocephala	Cyst
Location		*P. sagittus*	*A. gracilis* cyst	*A. gracilis* metacercariae	*R. acus*	*E. truttae*	Unidentified type 1
Kirkliston (n = 16)	P	0	100 (79.4-100)	25 (7.3-52.4)	18.8 (4-45.6)	0	0
	I	0	22.9 (14.2-36.3)	2 (1-3.25)	1.33 (1-1.67)	0	0
	A	0	22.9 (14.7-36.9)	0.5 (0.13-1.31)	0.25 (0-0.56)	0	0
Killandean Burn (n = 3)	P	0	100 (29.2-100)	33.3 (0.8-90.6)	0	0	0
	I	0	5.33 (3-7)	1	0	0	0
	A	0	5.33 (3-7)	0.33(0-0.67)	0	0	0
Newbattle Abbey (n = 6)	P	0	100 (54.1-100)	50 (11.8-88.2)	0	66.7 (22.3-95.7)	7.1 (2-17.3)
	I	0	33.7 (19.3-67.5)	1	0	1.5 (1-1.75)	2 (1-3)
	A	0	33.7 (19.2-64.5)	0.5 (0-0.67)	0	1 (0.17-1.5)	0.14 (0.04-0.52)
Burdiehouse Burn (n = 3)	P	0	0	0	0	0	1.5 (0-8.2)
	I	0	0	0	0	0	1
	A	0	0	0	0	0	0.02 (0-0.05)
Oatridge College (n = 59)	P	0	96.6 (88.3-99.6)	49.2 (35.9-62.5)	0	11.9 (4.9-22.9)	0
	I	0	33.2 (26.8-41.5)	4.14 (3.07-5.31)	0	2 (1.29-2.71)	0
	A	0	32.1 (25.5-40.1)	2.03 (1.34-2.92)	0	0.24 (0.08-0.49)	0
Livingston (n = 19)	P	5.3 (0.1-26)	10 (82.4-100)	68.4 (43.4-87.4)	0	0	1.4 (0-7.8)
	I	1	43.5 (31.9-62.6)	3 (1.85-5.23)	0	0	1
	A	0.05 (0-0.19)	43.5 (31.6-65.3)	2.05 (1.11-3.84)	0	0	0.01 (0-0.04)
Breich Water (n = 35)	P	0	100 (90-100)	40 (23.9-57.9)	0	0	
	I	0	22.5 (17.8-27.7)	3.64 (2.29-6.14)	0	0	
	A	0	22.5 (17.8-27.6)	1.46 (0.77-2.73)	0	0	
Happy Valley (n = 28)	P	0	100 (87.7-100)	46.4 (27.5-66.1)	0	0	
	I	0	51.6 (39.4-74.9)	10.9 (5.14-18.5)	0	0	
	A	0	51.6 (38.8-74.4)	5.07 (2.25-9.81)	0	0	
Murieston Water (n = 13)	P	0	15.4 (1.9-45.4)	0	0	53.8 (25.1-80.8)	
	I	0	1.5 (1-1.5)	0	0	3 (1.57-5)	
	A	0	0.23 (0-0.62)	0	0	1.62 (0.62-3.31)	

Brown trout parasite fauna was characterised by the shared acanthocephalan parasite *E. truttae*, for which it is the preferred host, and salmonid-specific parasites *C. metoecus*, *C. farionis* and *C. tenuissima* (Table 5.5). Levels of *E. truttae* infection were highly variable, with prevalence ranging from 5.3% in the Killandean Burn (with an infection intensity of one parasite per infected individual) to 81.2% at Carlops (where the infection intensity reached 22.6 parasites per host). *Echinorhynchus truttae* was present in fish from all sample locations except Braid Burn. *Crepidostomum metoecus* was the second most dominant brown trout parasite, closely followed by *C. farionis* and *C. tenuissima*. Out of these parasite infections, *C. metoecus* was the most variable, with only one host carrying just one individual at Oatridge College versus five individuals carrying an average of 58.2 parasites each at Braid Burn. A single incidence of *A. gracilis* infection was reported at Burdiehouse Burn, where one individual was found to be a carrier of a single *A. gracilis* cyst.

Table 5.5. Prevalence, intensity and mean abundance records for each parasite species recovered from examined brown trout, sorted by sample site. Results are summed across two sampling years. Values are listed alongside 95% confidence intervals. n = number of individuals examined

		Digenea		Nematoda		Acanthocephala
Location		*A. gracilis* cyst	*C. metoecus*	*C. farionis*	*C. tenuissima*	*E. truttae*
Kirkliston (n = 6)	P	0	0	0	0	16.7 (0.4-64.1)
	I	0	0	0	0	7
	A	0	0	0	0	1.17 (0-2.33)
Killandean Burn (n = 19)	P	0	0	0	0	5.3 (0.1-26)
	I	0	0	0	0	1
	A	0	0	0	0	0.05 (0-0.16)
Newbattle Abbey (n = 20)	P	0	25 (8.7-49.1)	20 (5.7-43.7)	10 (1.2-31.7)	55 (31.5-76.9)
	I	0	45.2 (21-56)	7.5 (1-19.5)	4 (2-6)	4.36 (2.45-6.55)
	A	0	11.3 (3-23.1)	1.5 (0.15-7.68)	0.4 (0-1.28)	2.4 (1.15-4.41)
Burdiehouse Burn (n = 3)	P	33.3 (0.8-90.6)	0	0	0	66.7 (9.4-99.2)
	I	1	0	0	0	2.5 (2-2.5)
	A	0.33 (0-0.67)	0	0	0	1.67 (0-2.67)
Braid Burn (n = 11)	P	0	45.5 (16.7-76.6)	0	0	0
	I	0	58.2 (44-70.6)	0	0	0
	A	0	26.5 (10-47.8)	0	0	0
Oatridge College (n = 15)	P	0	6.7 (0.2-31.9)	0	20 (4.3-48.1)	80 (51.9-95.7)
	I	0	1	0	11 (10-11.7)	10.8 (4.34-22.6)
	A	0	0.07 (0-0.2)	0	2.2 (0-4.74)	8.67 (3.47-19.4)
Murieston Water (n = 27)	P	0	22.2 (8.6-42.3)	18.5 (06.3-38.1)	0	70.4 (49.8-86.2)
	I	0	17.7 (1.33-41)	3.4 (1.6-6.2)	0	11.6 (5.95-30.6)
	A	0	3.93 (0.26-13.3)	0.63 (0.19-1.81)	0	8.15 (3.89-22.6)
Carlops (n = 16)	P	0	0	25 (7.3-52.4)	0	81.2 (54.4-96)
	I	0	0	1.5 (1-1.75)	0	22.6 (10.6-38.9)
	A	0	0	0.38 (0.06-0.75)	0	18.4 (8.18-34.1)

The minnow examined contained a much less diverse range of parasites (Table 5.6). Only *D. volvens* was identifiable, and infections were only reported at the Livingston sample site. The parasite was found in moderate infection levels, with 38.2% of individuals infected, containing an average of 4.69 parasites each. These values are higher than those reported for *D. volvens* infections in bullhead. The only other parasite recovered from the examined minnow was an unidentifiable cyst, also only found in fish from Livingston. Similarly, flounder had a very limited range of parasites, with only a single incidence of an *A. gracilis* cyst and a metacercaria reported (Table 5.7).

Table 5.6. Prevalence, intensity and mean abundance records for each parasite species recovered from examined minnows, sorted by sample site. Results are summed across two sampling years. Values are listed alongside 95% confidence intervals. n = number of individuals examined

		Digenea	Cyst
Location		*D. volvens* metacercariae	Unidentified type 2
Killandean Burn (n = 8)	P	0	0
	I	0	0
	A	0	0
Livingston (n = 34)	P	38.2 (22.2-56.4)	5.9 (0.7-19.7)
	I	4.69 (2.23-14.3)	1.5 (1-1.5)
	A	1.79 (0.71-6.84)	0.09 (0-0.27)
Breich Water (n = 14)	P	0	0
	I	0	0
	A	0	0
Happy Valley (n = 31)	P	0	0
	I	0	0
	A	0	0

Table 5.7. Prevalence, intensity and mean abundance records for each parasite species recovered from examined flounder, sorted by sample site. Results are summed across two sampling years. Values are listed alongside 95% confidence intervals. n = number of individuals examined

		Digenea	
		A. gracilis cyst	*A. gracilis* metacercariae
Burdiehouse Burn (n = 7)	P	14.3 (0.4-57.9)	14.3 (0.4-57.9)
	I	1	1
	A	0.14 (0-0.43)	0.14 (0-0.43)
Braid Burn (n = 10)	P	0	0
	I	0	0
	A	0	0

Variability in parasite communities was tested using a resemblance matrix and two-way ANOSIM to compare mean infection intensities between fish species and locations. The results were visualised on an MDS plot (Figure 5.16). There was no significant separation of the data by sample location ($p > 0.05$, $R = 0.256$) or fish species ($p > 0.05$, $R = 0.911$).

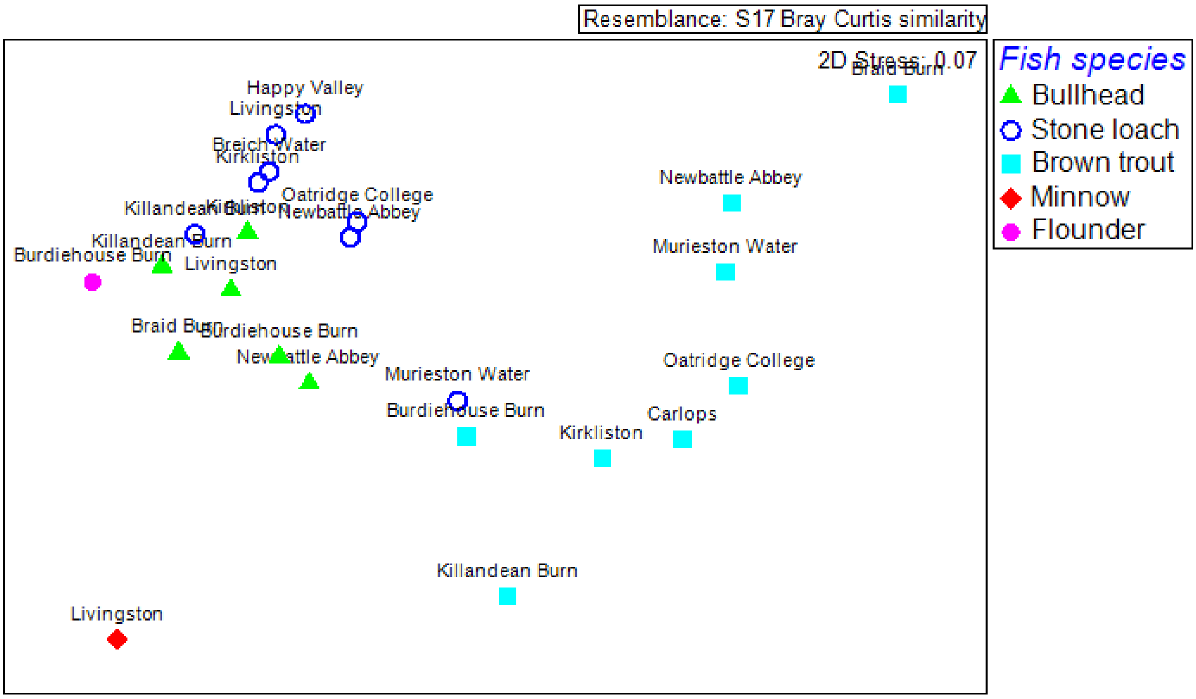

Figure 5.16. Log(x+1) transformed mean intensity parasite burden values, separated by fish species and labelled by sample location. Outliers representing species/location combinations where no parasites were recorded have been removed

5.3.3 Comparisons Between Locations With/Without Bullhead

Of all parasite infections examined, *E. truttae* infection in brown trout was found to be closely associated with the absence of bullhead (Fisher's exact test $p < 0.001$). Overall prevalence rates were higher in bullhead-free control sites when bullhead presence/absence was used as a grouping factor, even in the presence of the stone loach alternative host (Figure 5.17). Minnow were also heavily influenced by the presence of bullhead, as *D. volvens* infections were only reported at the Livingston site, where minnow and bullheads were sympatric (Table 5.6).

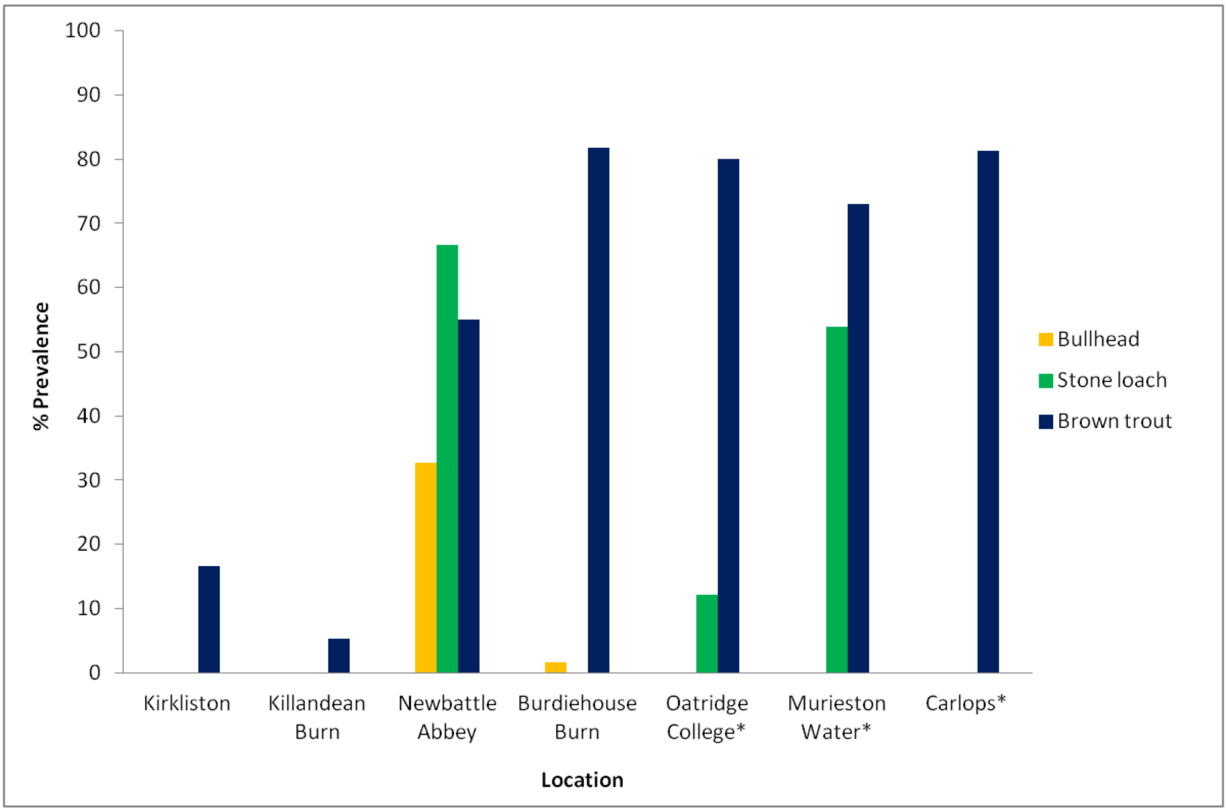

Figure 5.17. Prevalence (% infected individuals) of *E. truttae* recovered from bullheads, brown trout and stone loach in seven sample locations. Locations marked with an asterisk (*) are bullhead-free control sites

5.4 Discussion

Parasite infection intensity varies greatly between hosts, populations and locations (Crowden and Broom, 1980). Individual host attributes, such as immunity and antiparasite behaviour, can influence the infection success of a parasite (Johnson and Hoverman, 2014), however the large sample sizes and number of locations examined in this study should have exposed any significant host-parasite relationship that exists within the communities examined.

Parasite cross-overs were found between bullhead and all other fish species examined (Table 5.2). The cestode *Proteocephalus* was only found in one stone loach individual and is therefore not considered a wide-spread threat to species survival. Similarly, *C. farionis*, *C.*

tenuissima and *C. metoecus* were restricted to brown trout hosts and are not directly influenced by bullhead presence. The acanthocaphalan species *E. truttae*, the digenean parasites *A. gracilis* and *D. volvens*, and the nematode *R. acus* do, however, utilise bullheads as a host and require closer examination.

Parasites Infecting Native Scottish Fish

The recovered cestode species was not a fully developed adult, making identification to species level using morphological features impossible. This has been overcome in other *Proteocephalus* studies by exploiting molecular markers (Scholz *et al.,* 2003, 2007 and 2017). The application of molecular techniques requires specimens to be stored under specific conditions, but this single specimen was stored in lower grade 70% ethanol. As such, further molecular analyses were outside the scope of this project. Traditional identification of *Proteocephalus* requires confirmation of the definitive host identity. The fish community within Livingston, the location in which the specimen was found, is relatively stable. Electrofishing results suggest that only bullheads, stone loach, minnow and the three-spined stickleback (*Gasterosteus aculeatus*) are found in this location. Using the information supplied in Chubb *et al*. (1987), Moravec (2001) and Scholz *et al.* (2007), the only species of *Proteocephalus* that would parasitise any fish species in this community are *P. sagittus* (where the adult form is specific to stone loach), *P. filicollis* (specific to three-spined stickleback) and *P. longicollis* (a parasite of brown trout that can use bullheads as a paratenic host). There are no brown trout in this location and no cestodes were recovered from any of the bullheads examined, so the presence of *P. longicollis* is unlikely. Three-spined stickleback were not examined in this study, so it is unknown if *P. filicollis* is present in this community. Subsequent studies examining both stone loach and three-spined stickleback from Livingston may confirm if this is the case. The low recovery rate of the *Proteocephalus* species that was found (a single cestode found in one individual out of 19 stone loach examined, giving a prevalence of 5.3%) suggests that incidences of infection within the stone loach are low. A comparable prevalence value of 7.69% was recorded for *P. sagittus* parasitising stone loach in Russia (Shershneva and Zhokhov, 2013), although a substantially higher prevalence of 27% has been reported in stone loach examined from the Czech Republic (Jarkovský *et al.,* 2004). The low infection rate and the species' preference for non-bullhead hosts make this species a low priority within the confines of this study.

The nematode parasites *C. farionis* and *C. tenuissima* were only recovered from their preferred brown trout host, as was the digenean parasite *C. metoecus*. *Cystidicola farionis* has previously been reported in brown trout in Glasgow and East Lothian (Dorucu *et al.,* 1995), and *C. tenuissima* in England (Aho and Kennedy, 1987). Awachie (1968) studied the parasites of co-occurring brown trout and bullheads in Wales and observed *C. metoecus* only within brown trout. All of these parasite species are therefore known to the UK and are not related to the presence or absence of non-native bullheads in Scotland.

Parasites That Co-infect Bullheads and Native Fishes

Apatemon gracilis dominated the parasite fauna of bullheads and stone loach in this study. Incidences of infection were also reported in flounder and brown trout, although only one parasite was recovered in each host, suggesting these were irregular occurrences. Both metacercariae and cysts were found in high densities in bullheads and stone loach. Different life stages were analysed independently, due to the capacity for parasites to encyst in suboptimal conditions, for example when found in a paratenic host. It was initially hoped that differences in cyst/metacercariae proportions in each fish host may provide insight into the suitability of each host, although high levels of both cysts and metacercariae were recovered from both fish species, so no host studied appeared to provide more optimal conditions for development. Infections were also recorded in the bullhead-free control sites of Murieston, Happy Valley, Breich Water and Oatridge College. This implies that bullhead is not a preferred host for the parasite and strongly suggests that the species is a component of the natural parasite fauna of Scottish stone loach.

Free-living and encysted metacercariae have been previously recorded in wild stone loach, three-spined stickleback, brown trout, rainbow trout and bullheads in Scotland (Blair, 1976; Bell, 1996). *Apatemon gracilis* metacercariae are the pre-encysted form of the highly prevalent *A. gracilis* cysts. Metacercariae will encyst in the second intermediate host and develop into adults once they are ingested by the final host, piscivorous aquatic birds (Stunkard *et al.*, 1941). Within the UK, anatids are a known carrier of *A. gracilis* (Burton-Beverley, 1972). The parasite has a global distribution, with published studies on cercarial and metacercarial developmental stages from specimens in Australasia, Europe, Scandinavia, Japan and North America (Antal *et al.,* 2015; Blair, 1976; Blasco-Costa *et al*., 2016; Ieshko *et al*., 2013; Hoffman, 1959; Smith and Hickman 1983; Vojtek, 1964; Yamaguti, 1933). Prevalence varies greatly by study, with values as low as 17% recorded in invasive fish in Hungary (Antal *et al.*, 2015) and as high as 100% reported for metacercariae in Canadian brook stickleback during summer and autumn (Gordon and Rau, 1982). In the latter case, parasite-induced mortality was also evident during peak periods of infection. Values obtained in this study for metacercariae recovered from bullheads (2.9%-56.1%) and stone loach (33.3%-68.4%) are also highly variable. If we consider cysts and metacercariae together and analyse prevalence purely based on the presence of *A. gracilis,* prevalence values of 100% are found in stone loach from six out of the eight sites analysed (with a minimum of 15.3% reported for Murieston), and prevalence values for bullhead increase to reach a maximum close to 100% (47%-93.9%). This would suggest that that *A. gracilis* has the potential to impact the long-term survival of infected fish in these areas of high prevalence, as reported by Gordon and Rau (1982). As *A. gracilis* was found in stone loach recovered from bullhead-free control sites and sites containing bullheads in extremely high densities, it is unlikely that it was introduced by bullhead. The parasite could potentially have spread on the feet or in the excrement of mobile piscivorous birds, but the high prevalence and intensity levels found in stone loach from control sites implies incidental infections are not the likely cause of parasite spread. As a result, *A. gracilis* is not considered of relevance to the invasive status of bullheads in Scotland.

Raphidascaris acus was recovered from both bullheads and stone loach. Incidences of infection were restricted to areas where bullhead and stone loach coexisted with brown trout. This is understandable given brown trout is a common final host for the parasite and there are no other large piscivorous fish species in the chosen sample locations. Interestingly, no *R. acus* infection was found in any of the brown trout examined. Prevalence is therefore thought to be low within the trout population. There was also no cross-over between the location where *R. acus* was reported in bullhead and stone loach. The distribution of infection was higher in bullhead than stone loach, with bullhead infections occurring in three locations compared to the single area recorded for stone loach, although in each case only one bullhead individual was infected. Overall prevalence is therefore also thought to be low in the bullhead and stone loach intermediate host. *Raphidascaris acus* has previously been reported in brown trout and rainbow trout in Scotland (Dorucu *et al.,* 1995). Bullhead and stone loach are known carriers of the L3 larval stage (Moravec, 1994), and stone loach infections have been reported in other countries Koubková *et al*., (2004). The low prevalence observed and previous reports of *R. acus* in Scotland mean this parasite species is not likely to be influenced by the arrival and spread of bullheads in Scotland.

Diplostomum volvens was found in both bullhead and minnow at the Livingston sample site. It was also recorded in bullhead from four other sample sites, one of which contained minnows that were examined and found to be infection free. No *D. volvens* infections were found in minnows from bullhead free control sites. This suggests that bullheads are the primary carrier and transmitter of *D. volvens* in this region, and could be responsible for introducing the parasite to minnows in Scotland. An accurate identification of this species is therefore a high priority. Identification of *Diplostomum* species by morphological features can be difficult. Traditionally, the most accurate method of species identification would be to extract live metacercariae from fish intermediate hosts and feed them to final stage piscivorous birds. The adult digenean stage could then be removed from the digestive tract of the bird host and identified using standard morphological features. This controlled laboratory technique was successfully employed by McKeown and Irwin (1995) and used to give detailed morphological descriptions of *D. spathaceum*, *D. parviventosum* and *D. volvens*. Due to the difficulty in distinguishing *Diplostomum* metacercarial stages using classical morphological techniques, more recent studies have found success using molecular techniques. Awad and Manhel (2016) were able to distinguish between two metacercarial *Diplostomum* removed from the lens and aqueous humor of European carp (Cyprinus carpio), redbelly tilapia (Tilapia zillii) and Himri barbel (Barbus lutes) using Random Amplified Polymorphic PCR (PCR-RAPD). Attempts to identify recovered *Diplostomum* by molecular analysis were unsuccessful in this study, and traditional morphological features had to be relied on for accurate identification. The key developed by Shigin (1976) was utilised, although the author specifically mentions the need to follow his method of preservation and staining for accurate use of this method, which was not possible due to the initial sampling technique applied. As such, the size and prominence of necessary anatomical features may have been exaggerated or lost during slide staining and fixation. The holdfast organ was often difficult to see in *Diplostomum* originating from minnow specimens and it is suspected that the width and length of the holdfast organ may have been underestimated in some specimens. Similarly, the lappets of several bullhead specimens appeared more rounded and less pronounced than in others. This discrepancy could indicate intraspecific variation, or distinguishing characteristics of different species. Further statistical analysis was conducted to ensure the same species was present in both bullhead and minnows. No distinct separation was found between the morphometric characteristics measured from *Diplostomum* parasitising bullheads or minnows. Due to the comparatively smaller size of the minnow host (average weight 2.3g ± 0.6) relative to the bullhead host (average weight 7.7g ± 3.1), the differences observed in the MDS plot are thought to be a host-specific size effect exemplifying Harrison's Rule (Harrison, 1915; Johnson *et al*., 2005), wherein large-bodied hosts have a tendency to harbour large-bodied parasites. Parasite life-history traits such as longevity are also positively correlated with size, and host body size relates to parasite body size in these more specialised parasite species (Sasal *et al*., 1999). If bullheads are a preferred host and minnow are parasitised as a paratenic host (i.e. one that can be used as a host, but is not necessary to complete the parasite's life cycle), *Diplostomum* could attain a greater size within its preferred bullhead host. Both factors could explain the size difference seen in the *Diplostomum* recovered from bullhead and minnow.

The transmission of *Diplostomum* species and position they maintain within a host makes pathological responses to their presence likely. Cercariae of several *Diplostomum* species penetrate the eye of fishes and develop into metacercariae in situ. Infections create cataractous lenses and impaired vision which handicap the fish host and increase the possibility of predation by piscivorous birds, which constitute the final hosts in the parasite life cycle (Bush *et al.*, 2001). Bullhead-mediated introduction of *Diplostomum* could therefore cause visual impairment in population of native minnow. It should be noted that no pathological effects were seen in any of the specimens examined and generally low intensity values were recorded for infected minnows. If this is a relatively new introduction of a developing host-parasite relationship it may be something that requires monitoring in the future. Historic records do exist for *D. volvens* infection in minnow in other areas of the UK (Nicoll, 1924), although the original method for identifying a *Diplostomum* species through its location in the eye makes older records of *Diplostomum* infections potentially unreliable. Further monitoring of *Diplostomum* infections in areas of bullhead introduction and visual inspection of lenses in situ may help to determine if *D. volvens* infection rates are increasing in native minnow populations. This would help to establish whether *D. volvens* in Scottish European minnow populations constitutes a novel host-parasite relationship, brokered by the introduction of non-native bullhead, or whether minnows are a natural host for this species. At this time, this association is not thought to be a concerning consequence of the arrival of bullheads in Scotland.

The acanthocephalan parasite *E. truttae* was found in specimens of bullhead, brown trout and stone loach. All individuals recovered from bullhead and stone loach had a proboscis that was either partially or completely inverted. Attempts to manually extrude the proboscis using a dissection needle or to passively induce eversion using cold distilled water were unsuccessful. Identification therefore relied on counting the number of rows visible in the exposed portion of the proboscis, where applicable, and observing the structure and position of internal organs in cleared specimens. The *E. truttae* recovered in this study were shorter in length than those reported from Russia (Amin and Christison, 2015) and documented in French literature (Golvan, 1969), where specimens reached 20mm in both cases. Studies in the British Isles have found specimens of up to 16mm in fully developed females (Awachie, 1966). Even in the brown trout preferred host, the maximum length of *E. truttae* observed in this study was 10mm. It should be noted, however, that the brown trout examined were relatively small individuals, with an average fork length of 132.5mm (±39.5). It could therefore be the case that reduced host sizes in this study have influenced the length of acanthocephala recovered from different hosts, particularly in the smaller bullhead and stone loach samples. *E. truttae* has previously been reported as a common intestinal parasite of brown trout and rainbow trout throughout Scotland, with intensity values reaching 31 (±34 SD) parasites per individual and 93.3% prevalence in a reservoir near the Scottish borders (Dorucu *et al.,* 1995). Higher rates of accumulation would be expected in an isolated body of water where hosts are limited and repeated infection likely, so values obtained in this study are not necessarily comparable.

A comparison of *E. truttae* prevalence in brown trout from areas that are bullhead free and areas that contain resident bullhead show that infection prevalence is lower in brown trout in sample areas that also contain bullheads. This relationship appears to support the dilution effect, wherein bullheads act as an alternative host for *E. truttae* and reduce infections in brown trout. This relationship does not extend to stone loach. Brown trout is considered the preferred host for *E. truttae* and its presence in stone loach and bullhead in locations where these species co-occur is likely a result of high population densities of the parasite. Bullhead-driven decreases in *E. truttae* infection levels are unlikely to have a significant impact on brown trout survival and is therefore not considered of relevance to an invasive designation for Scottish bullhead.

The Designation of Scottish Bullhead as Invasive From a Parasitological Perspective

Detailed studies of parasitological interactions between introduced bullhead and native fishes are limited. In a similar study into parasite sharing between invasive bullheads and native fish species, Ieshko *et al.* (2013) described a similar parasite fauna to the species recovered in this study, in bullheads and salmonids in Finland. Recovered species included *C. farionis*, *R. acus*, *A. gracilis* and a species of *Diplostomum*. In this case, however, the arrival of bullheads was thought to be responsible for introducing *A. gracilis* to native communities, which had a significant impact on the mortality rates of native fishes. Bullhead have also been shown to spread the cestode parasite *Schistocephalus cotti* through a catchment as they colonize new locations (Chubb *et al.*, 2006; Seppälä *et al.*, 2007). No such relationship has been found in this study.

The present study provided evidence of parasite sharing between introduced bullheads and native stone loach, brown trout, flounder and minnows, across a range of parasite taxa. Due to frequent occurrences of the majority of these parasites in areas without bullheads and reports of infections in other regions of the UK, Only *D. volvens* is thought to have been introduced by bullheads in this region. Low prevalence rates and infection intensities were exhibited in examined European minnows and no pathological changes resulting from the presence of *D. volvens* within the eye were noted. The current infection levels in Scotland, and historical reports of *D. volvens* infections in minnows in other regions of the UK (e.g. Nicoll (1924), suggest that *D. volvens* does not currently pose a threat to Scottish minnow populations in the River Almond, Braid Burn, Burdiehosue Burn and River North Esk. The presence of bullheads was found to reduce infection levels of *E. truttae* in native brown trout, which may ultimately benefit the species. Bullheads are therefore not thought to be responsible for the introduction or passive spread of parasites that could threaten the health of native fish populations. The proposed introduction of *D. volvens* and the reduction of brown trout *E. truttae* infection rates in the presence of bullheads observed in this study do, however, support the hypothesis that introduced bullheads influence the parasite fauna of native fishes. Scottish bullheads currently pose a minimal risk to host/parasite equilibria within native aquatic communities, and invasive designation on the basis of disease or parasite transfer cannot be justified at this time.

5.5 References

Abdi H. (2010) Holm's sequential Bonferroni procedure. In N. J. Salkind (Ed.), *Encyclopaedia of Research Design* (pp574-578). Thousand Oaks, CA: Sage.

Aho J. M. and Kennedy C. R. (1984) Seasonal population dynamics of the nematode *Cystidicoloides tenuissima* (Zeder) from the River Swincombe, England. *Journal of Fish Biology* **25** (4), 473–489.

Aho J. M. and Kennedy C. R. (1987) Circulation pattern and transmission dynamics of the supra-population of the nematode *Cystidicoloides tenuissima* (Zeder) in the River Swincombe, England. *Journal of Fish Biology* **31** (1), 123–141.

Amin O. M. and Christison K. W. (2005) *Neoechinorhynchus* (Neoechinorhynchus*) dorsovaginatus* n. sp. (Acanthocephala: Neoechinorhynchidae) from the dusky kob *Argyrosomus japonicus* (Sciaenidae) on the southern coast of South Africa. *Systematic Parasitology*, **61** (3), 173-179.

Antal L., Székely C. and Molnár K. (2015) Parasitic infections of two invasive fish species, the Caucasian dwarf goby and the Amur sleeper, in Hungary. *Acta Veterinaria Hungarica* **63** (4), 472–484.

Awad A. H. H. and Manhel H. R. (2016) Diagnosis of larval stage (Metacercaria) of two eye trematodes from some local fishes by using polymorphic random amplified DNA (PCR-RAPD). *International Journal of Current Microbiology and Applied Sciences* **5** (9), 282–289.

Awachie J. B. E. (1965) The ecology of *Echinorhynchus truttae* Schrank, 1788 (Acanthocephala) in a trout stream in North Wales. *Parasitology* **55** (4), 747–762.

Awachie J. B. E. (1966) The development and life history of *Echinorhynchus truttae* Schrank, 1788 (Acanthocephala). *Journal of Helminthology* **40** (1-2), 11–32.

Awachie J. B. E. (1968) On the bionomics of *Crepidostomum metoecus* (Braun, 1900) and (Müller, 1784) (Trematoda: Allocreadiidae). *Parasitology* **58** (2), 307–324.

Baldauf S. A., Thünken T., Frommen J. G., Bakker T. C. M., Heupel O. and Kullmann H. (2007) Infection with an acanthocephalan manipulates an amphipod's reaction to a fish predator's odours. *International Journal for Parasitology* **37** (1), 61–65.

Barry J., McLeish J., Dodd J. A, Turnbull J. F., Boylan P. and Adams C. E. (2014) Introduced parasite *Anguillicola crassus* infection significantly impedes swim bladder function in the European eel *Anguilla anguilla* (L.). *Journal of Fish Diseases* **37** (10), 921-924.

Bell A. S. (1996) Studies on the biosystematics and biology of strigeids (Digenea) parasitic in freshwater fish. 402 pp. PhD Thesis, University of Stirling.

Blair D. (1976) Observations on the life-cycle of the strigeoid trematode, *Apatemon* (*Apatemon*) *gracilis* (Rudolphi, 1819) Szidat, 1928. *Journal of Helminthology* **50** (2), 125–131.

Blakeslee A. M. H., Altman I., Miller A. W., Byers J. E., Hamer C. E. and Ruiz G. M. (2012) Parasites and invasions: a biogeographic examination of parasites and hosts in native and introduced ranges. *Journal of Biogeography* **39** (3), 609–622.

Blasco-Costa I., Poulin R. and Presswell B. (2016) Species of *Apatemon* Szidat, 1928 and *Australapatemon* Sudarikov, 1959 (Trematoda: Strigeidae) from New Zealand: linking and characterising life cycle stages with morphology and molecules. *Parasitology Research* **115**, 271–289.

Brady A. (1989) Studies on the taxonomy and the biology of *Diplostomum* species (Digenea). PhD thesis, The University of Stirling, UK.

Brown A. F., Chubb J. C. and Veltkamp C. J. (1986) A key to the species of Acanthocephala parasitic in British freshwater fishes. *Journal of Fish Biology* **28** (3), 327–334.

Burton-Beverley M. (1972) Helminths from wild anatids in Great Britain. *Journal of Helminthology* **46** (4), 345.

Bush A., Fernández J. C., Esch G. and Seed R. J. (2001) Parasitism, the diversity and ecology of animal parasites. Cambridge University Press, Cambridge, UK. p125, 106-107.

Bush A. O., Lafferty K. D., Lotz J. M. and Shostak A. W. (1997) Parasitology meets ecology on its own terms: Margolis *et al.* revisited. *The Journal of parasitology* **83**, 575–583.

Butler J. R. A., Radford A., Riddington G. and Laughton R. (2009) Evaluating an ecosystem service provided by Atlantic salmon, sea trout and other fish species in the River Spey, Scotland: The economic impact of recreational rod fisheries. *Fisheries Research* **96** (2), 259–266.

Cavaleiro F. I., Pina S., Russell-Pinto F., Rodrigues P., Formigo N. E., Gibson D. I. and Santos M. J. (2012) Morphology, ultrastructure, genetics, and morphometrics of *Diplostomum* sp. (Digenea: Diplostomidae) metacercariae infecting the European flounder, *Platichthys flesus* (L.) (Teleostei: Pleuronectidae), off the northwest coast of Portugal. *Parasitology Research* **110**, 81–93.

Chubb J. C., Pool D. W. and Veltkamp C. J. (1987) A key to the species of cestodes (tapeworms) parasitic in British and Irish freshwater fishes. *Journal of Fish Biology* **31** (4), 517-543.

Chubb J. C., Seppälä T., Lüscher A., Milinski M. and Valtonen E. T. (2006) *Schistocephalus cotti* n. sp. (Cestoda: Pseudophyllidea) plerocercoids from bullheads *Cottus gobio* L. in an Arctic river in Finland, with a key to the plerocercoids of the Palaearctic species of the genus. *Systematic Parasitology* **65** (2), 161–70.

Clopper C. and Pearson E. S. (1934) The use of confidence or fiducial limits illustrated in the case of the binomial. *Biometrika* **26** (4), 404–413.

Crowden A. E. and Broom D. M. (1980) Effects of the eyefluke, *Diplostomum spathaceum*, on the behaviour of dace (*Leuciscus leuciscus*). *Animal Behaviour* **28**, 287–294.

De N. C. and Moravec F. (1979) Some new data on the morphology and development of the nematode *Cystidicoloides tenuissima* (Zeder, 1800). *Folia Parasitologica* **26** (3), 231–238.

Dorucu M., Crompton D. W. T., Huntingford F. A. and Walters D. E. (1995) The ecology of endoparasitic helminth infections of brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) in Scotland. *Folia Parasitologica* **42** (1), 29–35.

Drake J. M. (2003) The paradox of the parasites: implications for biological invasion. *Proceedings of the Royal Society of London B: Biological Sciences* **270** (Suppl. 2), S133-5.

Efron B., and Tibshirani R. (1993) *An introduction to the bootstrap*. Chapman & Hall, London, U.K., p 436

Emde S., Rueckert S., Palm H. W. and Klimpel S. (2012) Invasive Ponto-Caspian amphipods and fish increase the distribution range of the acanthocephalan *Pomphorhynchus tereticollis* in the River Rhine. *PloS one* **7** (12), e53218.

Esch G. W., Kennedy C. R., Bush A. O. and Aho J. M. (1988) Patterns in helminth communities in freshwater fish in Great Britain: alternative strategies for colonization. *Parasitology* **96 (Pt 3)**, 519–32.

Faltýnková A., Georgieva S., Kostadinova A., Blasco-Costa I., Scholz T. and Skírnisson K. (2014) *Diplostomum* von Nordmann, 1832 (Digenea: Diplostomidae) in the sub-Arctic: descriptions of the larval stages of six species discovered recently in Iceland. *Systematic Parasitology* **89** (3), 195–213.

Gaudin P. (1985) Predation by the sculpin (*Cottus gobio* L.) on brown trout fry (*Salmo trutta* L.): total length of prey and predator. *Hydrobiologia* **122** (3), 267–270.

Gibson D. I. (1996). Trematoda. In: *Guide to the Parasites of Fishes of Canada*, part IV (ed. Margolis, L. and Kabata, Z.), pp. 1–373. Canadian Special Publication of Fisheries and Aquatic Sciences No. 124. NRC Press, Ottawa, Canada.

Golvan Y. J. (1969) *Systématique des Acanthocéphales* (Acanthocephala Rudolphi 1801): L'ordre des Palæacanthocephala Meyer 1931. la super-famille des *Echinorhynchoidea* (Cobbold 1876) Golvan et Houin 1963. Éditions du Muséum.

Gopko M., Mikheev V. N. and Taskinen J. (2017) Deterioration of basic components of the anti-predator behaviour in fish harboring eye fluke larvae. *Behavioural Ecology and Sociobiology* **71** (4), 68.

Gordon D. and Rau M. (1982) Possible evidence for mortality induced by the parasite *Apatemon gracilis* in a population of brook sticklebacks (*Culaea inconstans*). *Parasitology* **84** (1), 41–47.

Hanzelová V. and Gerdeaux D. (2003) Seasonal occurrence of the tapeworm *Proteocephalus longicollis* and its transmission from copepod intermediate host to fish. *Parasitology Research* **91** (2), 130–136.

Harrison L. (1915) *Mallophaga* from *Apteryx*, and their significance; with a note on the genus *Rallicola*. *Parasitology* **8** (1), 88–100.

Hoffman G. L. (1959) Studies on the life-cycle of *Apatemon gracilis* pellucidus (Yamag) (Trematoda; Strigeoidea). *Transactions of the American Fisheries Society* **88** (2), 96–99.

Holm S. (1979) A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics* **6**, 65–70.

Ieshko E. P., Shul'man B. S., Lebedeva D. I., Barskaya Y. Y. and Niemela E. (2013) Bullhead (*Cottus gobio* L.) invasion in the Utsjoki River (Northern Finland): Parasitological aspects. *Russian Journal of Biological Invasions* **4** (1), 17–23.

Jahantab M., Haseli M. and Salehi Z. (2014) Morphological and genetic characteristics of the anisakid nematode *Raphidascaris acus* from the southwest Caspian Sea: evidence for the existence of sibling species within a species complex. *Parasitology Research* **113** (9), 3419–3425.

Jarkovský J., Jarkovský J., Koubková B., Scholz T., Prokeš M. and Baruš V. (2004) Seasonal dynamics of *Proteocephalus sagittus* in the stone loach *Barbatula barbatula* from the Haná River, Czech Republic. *Journal of Helminthology* **78** (3), 225–229.

Johnson K. P., Bush S. E. and Clayton D. H. (2005) Correlated evolution of host and parasite body size: tests of Harrison's rule using birds and lice. *Evolution* **59** (8), 1744–1753.

Johnson P. T. J. and Hoverman J. T. (2014) Heterogeneous hosts: How variation in host size, behaviour and immunity affects parasite aggregation. *Journal of Animal Ecology* **83** (5), 1103–1112.

Keane R. M. and Crawley M. J. (2002) Exotic plant invasions and the enemy release hypothesis. *Trends in Ecology and Evolution* **17**, 164–170.

Kelly D. W., Paterson R. A., Townsend C. R., Poulin R. and Tompkins D. M. (2009) Parasite spillback: A neglected concept in invasion ecology. *Ecology* **90** (8), 2047–2056.

Koubková B., Baruš V., Prokeš M. and Dyková I. (2004) *Raphidascaris acus* (Bloch, 1779) larvae infections of the stone loach*, Barbatula barbatula* (L.), from the River Haná, Czech Republic. *Journal of Fish Diseases* **27** (2), 65–71.

Kruse G. O. W. and Pritchard M. H. (1982) *The Collection and Preservation of Animal Parasites*. University of Nebraska Press, USA.

Macneil C., Fielding N. J., Hume K. D., Dick J. T. A., Elwood R. W., Hatcher M. J. and Dunn A. M. (2003) Parasite altered micro-distribution of *Gammarus pulex* (Crustacea: Amphipoda). *International Journal for Parasitology* **33** (1), 57–64.

Margolis L., Esch G. W., Holmes J. C., Kuris A. M. and Schad G. A. (1982) The use of ecological terms in parasitology (report of an ad hoc committee of the American Society of Parasitologists). *The Journal of Parasitology* **68** (1), 131–133.

Mckeown C. A. and Irwin S. W. B. (1995) The life cycle stages of three *Diplostomum* species maintained in the laboratory. *International Journal for Parasitology* **25** (8), 897–906.

Médoc V., Rigaud T., Bollache L. and Beisel J. N. (2009) A manipulative parasite increasing an antipredator response decreases its vulnerability to a nonhost predator. *Animal Behaviour* **77** (5), 1235–1241.

Meeus I., Brown M. J. F., De Graaf D. C. and Smagghe G. (2011) Effects of invasive parasites on bumble bee declines. *Conservation Biology* **25** (4), 662–671.

Mikheev V. N., Pasternak A. F., Taskinen J. and Valtonen E. T. (2010) Parasite-induced aggression and impaired contest ability in a fish host. *Parasites & Vectors* **3** (1), 17.

Mitchell C. E. and Power A. G. (2003) Release of invasive plants from fungal and viral pathogens. *Nature* **421** (6923), 625–7.

Moravec F. (1967) The systematic status of the genus *Sterliadochona* Skrjabin, 1946 (Nematoda: Rhabdochonidae). *Folia Parasitologica* **14** (4), 371–376.

Moravec F. (1994) *Parasitic Nematodes of Freshwater Fishes of Europe*. K Luwer Academic Publishers, Czech Republic.

Moravec F. (2001) Common sculpin *Cottus gobio* as a natural paratenic host of *Proteocephalus longicollis* (Cestoda: Proteocephalidae), a parasite of salmonids, in Europe. *Diseases of aquatic organisms* **45** (2), 155–8.

Moravec F. (2002) External morphological differences between *Crepidostomum farionis* and *Crepidostomum metoecus* (Trematoda: Allocreadiidae), parasites of salmonids, as revealed by SEM. *Folia Parasitologica* **49** (3), 211–217.

Moravec F. and Skoríková B. (1998) Amphibians and larvae of aquatic insects as new paratenic hosts of *Anguillicola crassus* (Nematoda: Dracunculoidea), a swimbladder parasite of eels. *Diseases of Aquatic Organisms* **34** (3), 217–22.

Nicoll W. (1924) A reference list of the trematode parasites of British freshwater fishes. *Parasitology* **16** (2), 127–144.

Niewiadomska, K. (2010) Fauna słodkowodna polski. 34A. Przywry (Trematoda). Częc´ ogólna; Częc´ systematyczna – Aspidogastrea, Digenea: Strigeida. Wydawnictwo Uniwersytetu Łódzkiego, Łódz´ (PhD Thesis. In Polish).

Niewiadomska K. and Laskowski Z. (2002) Systematic relationships among six species of *Diplostomum Nordmann,* 1832 (Digenea) based on morphological and molecular data. *Acta Parasitologica* **47** (1), 20–28.

Palmieri J. (1973) New definitive and intermediate hosts and host localities for *Apatemon gracilis* (Rud ., 1919) Szidat, 1928. *The Journal of Parasitology* **59** (6), 17–18.

Poulin R. (2016) Greater diversification of freshwater than marine parasites of fish. *International Journal for Parasitology* **46** (4), 275–279.

Power A. G. and Mitchell C. E. (2004) Pathogen spillover in disease epidemics. *The American Naturalist* **164** (S5), S79–89.

Prenter J., MacNeil C., Dick J. T. A. and Dunn A. M. (2004) Roles of parasites in animal invasions. *Trends in Ecology and Evolution* **19** (7), 385–390.

Reiczigel J. and Rózsa L. (2005) Quantitative Parasitology 3.0. Budapest. Distributed by the authors.

Rózsa L., Reiczigel J. and Majoros G. (2000) Quantifying parasites in samples of hosts. *The Journal of Parasitology* **86** (2), 228–232.

Sasal P., Trouve S., Muller-Graf C. and Morand S. (1999) Specificity and host predictability: a comparative analysis among monogenean parasites of fish. *Journal of Animal Ecology* **68** (3), 437–444.

Scholz T., de Chambrier A., Shimazu T., Ermolenko A. and Waeschenbach A. (2017) Proteocephalid tapeworms (Cestoda: Onchoproteocephalidea) of loaches (Cobitoidea):

Evidence for monophyly and high endemism of parasites in the Far East. *Parasitology International* **66** (1), 871–883.

Scholz T., Hanzelová V., Škeříková A., Shimazu T. and Rolbiecki L. (2007) An annotated list of species of the Proteocephalus Weinland, 1858 aggregate sensu de Chambrier *et al*. (2004) (Cestoda: Proteocephalidea), parasites of fishes in the Palaearctic Region, their phylogenetic relationships and a key to their identification. *Systematic Parasitology* **67** (2), 139–156.

Scholz T., Škeříková A., Hanzelová V., Koubková B. and Baruš V. (2003) Resurrection of *Proteocephalus sagittus* (Grimm, 1872) (Cestoda: Proteocephalidea) based on morphological and molecular data. *Systematic Parasitology* **56** (3), 173–181.

Seppälä O., Karvonen A. and Valtonen E. T. (2004) Parasite-induced change in host behaviour and susceptibility to predation in an eye fluke–fish interaction. *Animal Behaviour* **68** (2), 257–263.

Seppälä O., Karvonen A. and Valtonen E. T. (2005) Impaired crypsis of fish infected with a trophically transmitted parasite. *Animal Behaviour* **70** (4), 895–900.

Seppälä O., Valtonen E. T. and Benesh D. P. (2008) Host manipulation by parasites in the world of dead-end predators: adaptation to enhance transmission? *Proceedings of the Royal Society of London B: Biological Sciences* **275** (1643), 1611–1615.

Seppälä T., Chubb J. C., Niemelä E. and Valtonen E. T. (2007) Introduced bullheads *Cottus gobio* and infection with plerocercoids of *Schistocephalus cotti* in the Utsjoki, an Arctic river in Finland. *Journal of Fish Biology* **70** (6), 1865–1876.

Shershneva A. V. and Zhokhov A. E. (2013) Parasites of stone loach *Barbatula barbatula* (L., 1758) (Balitoridae) in small rivers of the upper and middle Volga basin. *Inland Water Biology* **6** (3), 236–245.

Shigin A. A. (1976) Metacercariae of the genus *Diplostomum* in the fauna of the USSR. *Parazitologiia* **10** (4), 346–351.

Shigin, A. A. (1993) *Trematodes of the Fauna of Russia and Neighbouring Regions*. Genus *Diplostomum*. Adults. Nauka, Moscow (In Russian).

Smith J. and Lankester M. (1979) Development of swim bladder nematodes (*Cystidicola* spp.) in their intermediate hosts. *Canadian Journal of Zoology* **57** (9), 1736–1744.

Smith S. and Hickman J. (1983) Two strigeoid trematodes*, Apatemon* (*Apatemon*) *gracilis* (Rudolphi, 1819) and *Diplostomum* (*Dolichorchis) galaxiae* n. sp., which encyst in the freshwater fish Galaxias auratus Johnston in Lake Crescent, Tasmania. *Papers and Proceedings of the Royal Society of Tasmania* **117**, 21–39.

Strauss A., White A. and Boots M. (2012) Invading with biological weapons: the importance of disease-mediated invasions. *Functional Ecology* **26** (6), 1249–1261.

Stunkard H. W., Willey C. H. and Rabinowitz Y. (1941) *Cercaria burti* Miller ,1923, a larval stage of *Apatemon gracilis* (Rudolphi , 1819) Szidat , 1928. *Transactions of the American Microscopical Society* **60**, 485–497.

Thomas J. D. (1958). Studies on *Crepidostomum metoecus* (Braun) and *C. farionis* (Muller), parasitic in *Salmo trutta* L. and *S. salar* L. in Britain. *Parasitology* **48** (3-4), 336–352.

Torchin M. E., Lafferty K. D., Dobson A. P., McKenzie V. J. and Kuris A. M. (2003) Introduced species and their missing parasites. *Nature* **421** (6923), 628–630.

Torchin M. E., Lafferty K. D. and Kuris A. M. (2001) Release from parasites as natural enemies: Increased performance of a globally introduced marine crab. *Biological Invasions* **3** (4), 333–345.

Torchin M. and Mitchell C. (2004) Parasites, pathogens, and invasions by plants and animals. *Frontiers in Ecology and the Environment* **2** (4), 183–190.

Vojtek J. (1964) Zur Kenntnis des Entwicklungszyklus von *Apatemon cobitidis* (Linstow, 1890). *Parasitology Research* **24** (6), 578-599.

Voutilainen A., Van Ooik T., Puurtinen M., Kortet R. and Taskinen J. (2009) Relationship between prevalence of trematode parasite *Diplostomum* sp. and population density of its snail host *Lymnaea stagnalis* in lakes and ponds in Finland. *Aquatic Ecology* **43**, 351–357.

Wolfe L. M. (2002) Why alien invaders succeed: support for the escape-from-enemy hypothesis. *The American Naturalist* **160**, 705–711.

Wootten R. (1974) Studies on the life history and development of *Proteocephalus percae* (Muller) (Cestoda: Proteocephalidae). *Journal of Helminthology* **48** (4), 269–281.

Yamaguti S. (1933) Studies on the helminth fauna of Japan. Part I. Trematodes of birds, reptiles and mammals. *Japanese Journal of Zoology* **5**, 1–134.

Chapter 6. Conclusion and Final Remarks

The reclassification of *Cottus gobio* as a species complex (Kottelat, 1997) and subsequent discovery of at least 15 new *Cottus* species (Freyhof *et al*., 2005) brings with it a challenging set of issues relating to the historical management of *C. gobio*, and the legislative legacy still in place in a number of European countries. The results produced in Chapter 2 of this study have confirmed that a single species, *Cottus perifretum,* resides in Great Britain, with a native range spanning England and Wales, along with sites of introduction in Scotland. Current UK legislation is yet to recognise this taxonomic shift, and all legislation and management plans pertaining to bullheads in Great Britain continue to list *C. gobio* as the native species. *Cottus gobio* was considered to have a distribution throughout the European continent and established populations in Russia and Scandinavia. *Cottus perifretum*, on the other hand, is limited to the British Isles and small catchments in Belgium, France, Germany and northern Spain (Freyhof *et al*., 2005). The species therefore has a more restricted distribution than was originally considered for *C. gobio*. *Cottus gobio* is protected under Annex II of the EC Habitat's Directive due to the considerable decline of bullheads throughout some regions in Europe (Knaepkens *et al.,* 2005). This law was put in place before the confirmation of the *C. gobio* species complex, resulting in the protection of bullheads in regions where their population levels are robust. In England and Wales, bullheads are abundant in some catchments and considered non-threatened in the UK as a whole (Carter *et al*., 2004). At present, eleven locations in England are listed as Special Areas of Conservations (SACs) with bullheads listed as the primary feature responsible for their designation (Boon and Lee, 2005). These areas receive enhanced protection measures, with the purpose of protecting the vulnerable species recognised in the area. The revisitation of the EC Habitat's Directive components of UK law that will likely result from the UK's departure from Europe will provide a window of opportunity for updating legislation concerning bullheads in the UK. Changing a scientific name should not, in itself, initiate any major legislative changes, however the opportunity exists to consider the implications of this taxonomic shift in terms of the population densities of *C. perifretum* in the UK and its designation as invasive in Scotland.

The second part of this study explored several ecological interaction that tested the validity of bullhead's designation as invasive in Scotland. Results from Chapter 4 showed that there was no direct feeding competition between introduced bullheads and native brown trout and stone loach at the time the study was carried out. However, variations in brown trout and stone loach diet were found in the presence and absence of bullheads. This was attributed to resource partitioning within communities containing all three species. The purpose of this investigation was to test whether an overlap in prey preference could produce competitive exclusion in communities containing bullheads and native stone loach and brown trout. Competition for prey resources is therefore not considered a significant factor in the establishment of bullhead populations in Scotland. Chapter 5 examined whether the introduction of bullheads has led to the transfer of parasites that could potentially harm native fish populations. Results confirmed that bullheads were unlikely to be responsible for the introduction or spread of parasites to native fishes, as incidences of parasite sharing involved species that were already known in Scotland. In the case of the acanthocephalan parasite *Echinorhynchus truttae*, the presence of bullheads was found to reduce infection levels in native brown trout. Thus, two conditions for invasiveness, competitive exclusion through feeding competition and the introduction of a parasite that would threaten the survival of native species, have been tested and not met. The potential for bullheads to outcompete native brown trout under certain environmental conditions has been documented in other areas of the UK (Elliott, 2006), and there is certainly habitat niche overlap between bullheads and stone loach (e.g. Smyly 1955; Smyly 1957). Feeding competition in mixed populations of bullheads, brown trout and stone loach has also been shown to be seasonally dependent in other regions (Shustov *et al.,* 2014). A more detailed investigation into the co-habitation of these species, integrating seasonal changes in resource availability, would potentially be of benefit and could clarify the extent to which resource partitioning or competition affect native fishes. The final condition for invasiveness concerns bullhead's capacity to negatively impact the economy. It's interactions with native Atlantic salmon and brown trout are of key importance, given the substantial recreation fishing industry that is centred around these two salmonid species in Scotland. Despite the perception that bullheads prey upon salmonid eggs (e.g. Mann and Orr, 1969), there is little evidence that this happens to a significant level in wild populations. In areas where bullheads co-habit with populations of brown trout and Atlantic salmon, it is the opinion of most that the salmonid species simply move up-river to a location where bullhead densities are lower (Dr. Joanna Girvan, pers comm. June 2014). Indeed, communities containing bullheads may also be of benefit to native species given brown trout adults will prey upon smaller bullhead individuals (Tomlinson and Perrow, 2003). It is therefore unlikely that the presence of bullhead has a significant impact on the Scottish economy, by way of interactions with salmonid species. The fundamental problem in communities containing introduced bullheads seems to be the significant population densities that are rapidly reached. Whilst undertaking fieldwork for this study, it was noted that a site visited in the first sampling year from which over 300 mixed age-class bullheads were removed, contained equally high numbers when surveyed the following year. Such large population densities must have a localised impact on the freshwater community, although this study has been unable to show this in practice. This is considered an area of concern although it appears, certainly in the locations examined in this study, that native communities adjust to these high population densities and a natural equilibrium is reached in which native species are able to thrive.

The lack of evidence to support an invasive designation for bullheads in Scotland, despite large population densities in areas of introduction, poses an interesting management conundrum. This is particularly interesting when considered alongside the current stable population levels but reduced distribution range of the native English species, *C. perifretum,* relative to the European-wide distribution of *C. gobio* considered in current UK legislation. Bullhead population levels in England have commonly been considered robust (Wheeler, 1977; Mills and Mann, 1983; Copp, 1992), leading Carter *et al.* (2004) to suggest an exemption of bullhead from the requirements of the Habitat's Directive in the UK. This practice has already been undertaken in Finland, where bullhead is an introduced non-native species (Pihlaja *et al.*, 1998; Carter *et al.,* 2004). However, emerging issues stemming from habitat loss and non-native species introduction could threaten species survival in some areas. Should English populations become reduced due to habitat depletion from introduced North American Signal Crayfish *Pacifastacus leniusculus* (e.g. Guan and Wiles, 1997), agricultural or developmental pressures (Tomlinson and Perrow, 2003), Scottish locations could provide a vital source of genetic material. Providing there is no clear detrimental effect on native species, maintaining ark sites in Scotland, where established populations are thriving, could be beneficial. The culling of Scottish bullhead would be extremely costly and difficult to achieve. The practice is unlikely to reduce population levels in the long term, unless a full fish removal is exercised. It would be beneficial to prevent further introduction and continue to monitor any sites where bullheads are present to detect early signs of changes to community dynamics. Current management efforts would be best placed in providing biosecurity education to prevent the unnecessary spread of bullheads, and monitoring communities containing established bullhead populations to ensure native fishes continue to thrive. Revisitation of UK legislation relating to the classification and management of bullheads within the UK is certainly required, and the evidence supplied in this study suggests that the invasive designation in place for bullhead populations in Scotland is potentially unjustified.

6.2 References

Boon P. and Lee A. (2005) Falling through the cracks: are European directives and international conventions the panacea for freshwater nature conservation? *Freshwater Forum* **24**, 24–37.

Carter M. G., Copp G. H. and Szomlai V. (2004) Seasonal abundance and microhabitat use of bullhead *Cottus gobio* and accompanying fish species in the River Avon (Hampshire), and implications for conservation. *Aquatic Conservation: Marine and Freshwater Ecosystems* **14**, 395–412.

Copp G. H. (1992) An empirical model for predicting microhabitat of 0+ juvenile fishes in a lowland river catchment. *Oecologia* **91**, 338–345.

Elliott J. M. (2006) Periodic habitat loss alters the competitive coexistence between brown trout and bullheads in a small stream over 34 years. *Journal of Animal Ecology* **75**, 54–63.

Freyhof J., Kottelat M. and Nolte A. (2005) Taxonomic diversity of European *Cottus* with description of eight new species (Teleostei: Cottidae). *Ichthyological Exploration of Freshwaters* **16**, 107–172.

Guan R. And Wiles P. R. (1997) Ecological Impact of Introduced Crayfish on Benthic Fishes in a British Lowland River. *Conservation Biology* **11**, 641–647.

Knaepkens G., Baekelandt K. and Eens M. (2005) Assessment of the movement behaviour of the bullhead (*Cottus gobio*), an endangered European freshwater fish. *Animal Biology* **55**, 219–226.

Kottelat M. (1997) European freshwater fishes. An heuristic checklist of the freshwater fishes of Europe (exclusive of former USSR), with an introduction for non-systematists and comments on nomenclature and conservation. *Biologia Bratislava* **52**, 1–271.

Mann R. H. K. and Orr D. R. O. (1969) A preliminary study of the feeding relationships of fish in a hard-water and a soft-water stream in southern England. *Journal of Fish Biology* **1**, 31–44.

Mills C. A. and Mann R. H. K. (1983) The bullhead *Cottus gobio*, a versatile and successful fish. *Annnual Report of the Freshwater Biological Association.* Ambleside, Cumbria, 76–88.

Pihlaja O. Niemelä E. and Erkinaro J. (1998) Introduction and dispersion of the bullhead, *Cottus gobio* L., in a sub-Arctic salmon river in northern Finland. *Fisheries Management and Ecology* **5**, 139–146.

Shustov Y. A., Tyrkin I. A. and Rasputina Y. N. (2014) Trophic competition of common sculpin and stone loach with juvenile salmonid of Gen. Salmo in tributaries of the Onega Lake. *Hydrobiological Journal* **50**, 32–40.

Smyly W. J. P. (1955) On the biology of the stone loach *Nemacheilus barbatula* (L.). *Journal of Animal Ecology* **24**, 167–186.

Smyly W. (1957) The life-history of the bullhead or miller’s thumb (*Cottus gobio* L.). *Proceedings of the Zoological Society of London* **128**, 431–454.

Tomlinson M. and Perrow M. (2003) Ecology of the Bullhead. *Conserving Natura 2000 Rivers Ecology Series* **No. 4**. English Nature, Peterborough.

Wheeler A. (1977) The origin and distribution of the freshwater fishes of the British Isles. *Journal of Biogeography* **4**, 1–24.

Appendix I. Described parasites of bullheads, brown trout, stone loach, minnows and flounder

Table AI.1. Described parasite of European bullhead species (encompassing historical publications listing those within the *C. gobio* species complex), listing parasite species, phyla, select publications documenting infections and geographical locations

Species	Phylum	Geographical location	Reference
Allocreadium angusticolle	Platyhelminth	UK	Nicoll (1924)
Apatemon gracilis	Platyhelminth	Finland	Ieshko *et al.* (2013)
Apiosoma cotti	Ciliophora	Finland	Ieshko *et al.* (2013)
Dermocystidium sp.	Ciliophora	England	Feist *et al.* (2004)
Diplostomum volvens	Platyhelminth	Finland	Ieshko *et al.* (2013)
Diplozoon paradoxum	Platyhelminth	UK	Nicoll (1924)
Distomum sp.	Platyhelminth	UK	Nicoll (1924)
Echinorynchus truttae	Acanthocephala	UK	Kennedy (1974)
Monostomum cotti	Platyhelminth	UK	Nicoll (1924)
Myxobilatus fragilicaudus	Cnidaria	Finland	Ieshko *et al.* (2013)
Nicolla gallica	Platyhelminth	UK	Kennedy (1974); Rumpus (1975)
Phyllodistomum folium	Platyhelminth	UK	Nicoll (1924); Kennedy (1974)
Pomphorhynchus laevis	Acanthocephala	England and Wales	Kennedy (1974); Kennedy (1996)
Pomphorynchus laevis	Acanthocephala	England	Rumpus (1975)
Proteocephalus longicollis	Platyhelminth	Czech Republic	Moravec (2001)
Raphidascaris acus	Nematoda	Finland	Ieshko *et al.* (2013)
Schistocephalus cotti	Platyhelminth	Finland	Chubb et al. (2006); Seppula *et al.* (2007)
Schistocephalus solidus	Platyhelminth	Finland	Ieshko *et al.* (2013)
Tetracotyle percafluviatilis	Platyhelminth	UK	Kennedy (1974)
Triaenophorus nodulosus	Platyhelminth	UK	Kennedy (1974); Rumpus (1975)
Trypanosoma sp.	Ciliophora	UK	Kennedy (1974)
Zschokkella nova	Cnidaria	Austria	Busher *et al.* (1992)

Table AI.2. Described parasites of brown trout, listing parasite species, phyla, select publications documenting infections and geographical locations

Species	Phylum	Geographical location	Reference
Acanthocephalus anguillae	Acanthocephala	UK	Kennedy (1974)
Acanthocephalus clavula	Acanthocephala	Ireland, UK and Norway	Kennedy (1974); Kennedy and Hartvigsen (2000); Byrne *et al.* (2003)
Acanthocephalus lucii	Acanthocephala	British Isles and Norway	Kennedy (1974); Kennedy and Hartvigsen (2000)
Anisakis sp.	Nematoda	Ireland and UK	Kennedy (1974); Byrne *et al.* (2003)
Argulus coregoni	Arthropoda	UK	Kennedy (1974)
Argulus foliaceus	Arthropoda	UK	Kennedy (1974); McPherson *et al.* (2012)
Bunodera luciopercae	Platyhelminth	UK and Norway	Kennedy (1974); Kennedy and Hartvigsen (2000)
Cacullanus truttae	Nematoda	UK and Norway	Kennedy and Hartvigsen (2000)
Camallanus lacustris	Nematoda	UK and Norway	Kennedy (1974); Kennedy and Hartvigsen (2000)
Capillaria salvelini	Nematoda	UK	Kennedy (1974); Dorocu *et al.* (1995)
Capillaria sp.	Nematoda	UK	Kennedy (1974)
Chloromyxum truttae	Cnidaria	UK	Kennedy (1974)
Costia necatrix	Ciliophora	UK	Kennedy (1974)
Cotylurus erraticus	Platyhelminth	UK	Betterton (1974)
Crepidostomum cooperi	Platyhelminth	USA	Muzzall, 1986
Crepidostomum farionis	Platyhelminth	UK and Ireland	Kennedy (1974); Kennedy *et al.* (1991); Dorocu *et al.* (1995); Kennedy and Hartvigsen (2000); Byrne *et al.* (2003)
Crepidostomum metoecus	Platyhelminth	British Isles and Norway	Kennedy (1974); Kennedy *et al.* (1991); Kennedy and Hartvigsen (2000)
Crepidostomum sp.	Platyhelminth	UK	Kennedy (1974)
Cucullanus truttae	Nematoda	UK	Kennedy (1974)
Cyathocephalus truncatus	Platyhelminth	British Isles and Norway	Kennedy (1974); Dorocu *et al.* (1995); Kennedy and Hartvigsen (2000)
Cystidicola farionis	Nematoda	Ireland and UK	Kennedy (1974); Dorocu *et al.* (1995); Byrne *et al.* (2003)
Cystidicoloides tenuissima	Nematoda	UK, Norway, Ireland and USA	Kennedy (1974); Muzzall (1986); Aho and Kennedy (1987); Kennedy and Hartvigsen (2000); Byrne *et al.* (2003)
Dendriticum ditremum	Platyhelminth	Scotland	Dorocu *et al* . (1995)
Dermocystidium sp.	Ciliophora	UK	Kennedy (1974)
Diphyllobothrium dendriticum	Platyhelminth	Ireland and UK	Kennedy (1974); Kennedy *et al.* (1991); Dorocu *et al.* (1995); Byrne *et al.* (2003)
Diphyllobothrium ditremum	Platyhelminth	Ireland and UK	Kennedy (1974); Kennedy *et al.* (1991); Byrne *et al.* (2003)
Diphyllobothrium latum	Platyhelminth	UK	Kennedy (1974)
Diphyllobothrium medium	Platyhelminth	UK	Kennedy (1974)
Diphyllobothrium norvegicum	Platyhelminth	UK	Kennedy (1974)
Diphyllobothrium sp.	Platyhelminth	UK	Kennedy (1974)
Diplostomum sp.	Platyhelminth	UK	Kennedy et al. (1991)
Diplostomum spathaceum	Platyhelminth	UK	Betterton (1974); Kennedy (1974); Wooten (1974)
Discocotyle sagittara	Platyhelminth	UK and Ireland	Kennedy (1974); Kennedy *et al.* (1991); Byrne *et al.* (2003)
Echinorhynchus salmonis	Acanthocephala	UK	Kennedy (1974)
Echinorhynchus sp.	Acanthocephala	UK	Kennedy (1974)
Echinorhynchus truttae	Acanthocephala	UK and Norway	Awachie (1965); Kennedy (1974); Kennedy *et al.* (1991); Dorocu *et al.* (1995); Kennedy and Hartvigsen (2000)
Epistylis sp.	Ciliophora	UK	Kennedy (1974)
Ergasilus sieboldi	Arthropoda	UK	Kennedy (1974)
Eubothrium crassum	Platyhelminth	UK and Ireland	Kennedy (1974); Kennedy *et al.* (1991); Dorocu *et al.* (1995); Kennedy (1996); Byrne *et al.* (2003)

Eubothrium sp.	Platyhelminth	UK	Kennedy (1974)
Eustrongylides sp.	Nematoda	Scotland and England	Kennedy (1974); Kennedy *et al.* (1991); Dorocu *et al.* (1995)
Glossatella sp.	Ciliophora	UK	Kennedy (1974)
Gyrodactylus salaris	Platyhelminth	UK	Kennedy (1974)
Gyrodactylus sp.	Platyhelminth	UK	Kennedy (1974)
Henneguya sp.	Cnidaria	UK	Kennedy (1974)
Ichthyocotylurus erraticus	Platyhelminth	UK	Kennedy (1974)
Ichthyophthirius multifiliis	Ciliophora	UK	Kennedy (1974)
Ligula intestinalis	Platyhelminth	UK	Kennedy (1974)
Myxidium truttae	Cnidaria	UK	Kennedy (1974)
Myxobolus nearobius	Cnidaria	UK	Kennedy (1974)
Neochinorhynchus rutili	Acanthocephala	British Isles and Norway	Kennedy (1974); Kennedy *et al.* (1991); Dorocu *et al.* (1995); Kennedy and Hartvigsen (2000)
Octomitus truttae	Ciliophora	UK	Kennedy (1974)
Phyllodistomum folium	Platyhelminth	UK	Kennedy (1974)
Phyllodistomum simile	Platyhelminth	UK	Kennedy (1974)
Pomphorhynchus laevis	Acanthocephala	British Isles, Norway and Ireland	Kennedy (1974); Kennedy (1996); Kennedy and Hartvigsen (2000); Byrne *et al.* (2003)
Procapillaria salvelini	Nematoda	British Isles and Norway	Kennedy and Hartvigsen (2000)
Proteocephalus neglectus	Platyhelminth	UK and Norway	Kennedy (1974); Kennedy and Hartvigsen (2000)
Proteocephalus percae	Platyhelminth	UK	Kennedy (1974)
Proteocephalus sp.	Platyhelminth	UK and USA	Kennedy (1974); Muzzall (1986)
Pseudocapillaria salvelini	Nematoda	UK	Kennedy *et al.* (1991)
Raphidascaris acus	Nematoda	British Isles, Czech Republic and Norway	Moravec (1970); Kennedy (1974); Dorocu *et al.* (1995); Kennedy and Hartvigsen (2000)
Raphidascaris cristata	Nematoda	UK	Kennedy (1974)
Rhabdias sp.	Nematoda	UK	Kennedy (1974)
Rhabdochona canadensis	Nematoda	USA	Muzzall, 1986
Rhabdochona Sp.	Nematoda	UK and Ireland	Kenedy (1974); Byrne *et al.* (2003)
Salmincola salmonea	Arthropoda	UK and Ireland	Kenedy (1974), Byrne *et al.* (2003)
Salminicola thymalli	Arthropoda	UK	Kennedy (1974)
Scyphidia sp.	Ciliophora	UK	Kennedy (1974)
Sphaerostoma bramae	Platyhelminth	British Isles and Norway	Kennedy and Hartvigsen (2000)
Spinitectus gracilis	Nematoda	USA	Muzzall, 1986
Tetracapsuloides bryosalmonae	Cnidaria	Scotland	Morris and Adams (2008)
Trichodina acuta	Ciliophora	Scotland	Gaze and Wootten 1998
Trichodina nigra	Ciliophora	Scotland	Gaze and Wootten (1999); Gaze and Wootten (2000)
Trichodina sp.	Ciliophora	USA	Muzzall, 1986
Truttaedacnittis sp.	Nematoda	USA	Muzzall, 1986
Tylodelphys clavata	Platyhelminth	UK	Kennedy (1974); Wooten (1974)

Table AI.3. Described parasites of stone loach, listing parasite species, phyla, select publications documenting infections and geographical locations

Species	Phylum	Geographical location	Reference
Acanthocephalan anguillae	Acanthocephala	Russia	Shershneva and Zhokhov (2013)
Acanthocephalan lucii	Acanthocephala	Russia	Shershneva and Zhokhov (2013)
Allocreadium isoporum	Platyhelminth	Russia	Shershneva and Zhokhov (2013)
Apatemon cobitidis	Platyhelminth	Russia	Shershneva and Zhokhov (2013)
Camallanus truncatus	Nematoda	Russia	Shershneva and Zhokhov (2013)
Crepidostomum oschmarini	Platyhelminth	Russia	Shershneva and Zhokhov (2013)
Diplostomum chromatophorum	Platyhelminth	Russia	Shershneva and Zhokhov (2013)
Diplostomum commutatum	Platyhelminth	Russia	Shershneva and Zhokhov (2013)
Diplostomum helveticum	Platyhelminth	Russia	Shershneva and Zhokhov (2013)
Diplostomum spathaceum	Platyhelminth	UK and Russia	Kennedy (1974); Wootten (1974); Shershneva and Zhokhov (2013)
Echinorhynchus truttae	Acanthocephala	Ireland	Kane (1966)
Gyrodactylus barbatuli	Platyhelminth	Russia	Shershneva and Zhokhov (2013)
Gyrodactylus cobitis	Platyhelminth	Russia	Shershneva and Zhokhov (2013)
Gyrodactylus jiroveci	Platyhelminth	Russia	Shershneva and Zhokhov (2013)
Gyrodactylus nemachili	Platyhelminth	Russia	Shershneva and Zhokhov (2013)
Gyrodactylus sedelnikowi	Platyhelminth	Russia	Shershneva and Zhokhov (2013)
Neoechinorhynchus crassus	Nematoda	Russia	Shershneva and Zhokhov (2013)
Neogryporynchus cheilancristrotus	Platyhelminth	Russia	Shershneva and Zhokhov (2013)
Nicolla gallica	Platyhelminth	UK	Kennedy (1974; Rumpus (1975)
Paracaryophylaeus gotoi	Platyhelminth	Russia	Shershneva and Zhokhov (2013)
Paracoenogonimus ovatus	Platyhelminth	Russia	Shershneva and Zhokhov (2013)
Phyllodistomum folium	Platyhelminth	Russia	Shershneva and Zhokhov (2013)
Pomphorynchus laevis	Acanthocephala	England and Wales	Kennedy (1974); Rumpus (1975)
Poroteocephalus torulosus	Platyhelminth	UK	Rumpus (1975)
Proteocephalus sagittus	Platyhelminth	Russia	Shershneva and Zhokhov (2013)
Pseudocapillaria totentosa	Nematoda	Russia	Shershneva and Zhokhov (2013)
Raphidascaris acus	Nematoda	Russia	Shershneva and Zhokhov (2013)
Raphidascaris sp.	Nematoda	UK	Kennedy (1974)
Rhabdochona ergensi	Nematoda	Russia	Shershneva and Zhokhov (2013)
Tetracotyle percafluviatilis	Platyhelminth	UK	Kennedy (1974)
Triaenophorus nodulosus	Platyhelminth	UK and Russia	Kennedy (1974); Rumpus (1975); Shershneva and Zhokhov (2013)
Unionidae gen. Sp.	Mollusca	Russia	Shershneva and Zhokhov (2013)

Table AI.4. Described parasites of minnows, listing parasite species, phyla, select publications documenting infections and geographical locations

Species	Phylum	Geographical location	Reference
Allocreadium isoporum	Platyhelminth	UK	Nicoll (1924); Kennedy (1974)
Caryophyllaeides fennica	Platyhelminth	UK	Kennedy (1974)
Chloromyxum phoxini	Cnidaria	UK	Kennedy (1974)
Cystidicoloides sp.	Nematoda	UK	Kennedy (1974)
Dactylogyrus auricularis	Platyhelminth	UK	Nicoll (1924)
Dactylogyrus phoxini	Platyhelminth	UK	Kennedy (1974)
Dactylogyrus sp.	Platyhelminth	UK	Kennedy (1974)
Diplostomum cuticola	Platyhelminth	UK	Nicoll (1924)
Diplostomum Phoxini	Platyhelminth	UK	Kennedy (1974); Barber and Crompton (1997); Dezfuli *et al.* 2007
Diplostomum sp.	Platyhelminth	UK	Kennedy (1974)
Diplostomum spathaceum sp.	Platyhelminth	UK	Kennedy (1974)
Diplostomum volvens	Platyhelminth	UK	Nicoll (1924)
Diplozoon paradoxum	Platyhelminth	UK	Nicoll (1924); Kennedy (1974)
Distomum phoxini	Platyhelminth	UK	Nicoll (1924)
Gyrodactylus aphyae	Platyhelminth	UK	Kennedy (1974)
Gyrodactylus elegans	Platyhelminth	UK	Nicoll (1924); Kennedy (1974)
Gyrodactylus laevis	Platyhelminth	UK	Kennedy (1974)
Gyrodactylus limneus	Platyhelminth	UK	Kennedy (1974)
Gyrodactylus macronychus	Platyhelminth	UK	Kennedy (1974)
Gyrodactylus medius	Platyhelminth	UK	Kennedy (1974)
Gyrodactylus sp.	Platyhelminth	UK	Kennedy (1974)
Ichthyophthirius multifiliis	Ciliophora	UK	Kennedy (1974)
Ligula intestinalus	Platyhelminth	UK	Kennedy (1974); Barber and Huntingford (1996)
Myxobolus sp.	Cnidaria	UK	Kennedy (1974)
Neoechinorhynchus rutili	Acanthocephala	UK	Kennedy (1974)
Phyllodistomum cuticola	Platyhelminth	UK	Kennedy (1974)
Phyllodistomum folium	Platyhelminth	UK	Kennedy (1974)
Pomphorhynchus laevis	Acanthocephala	England and Wales	Kennedy (1974)
Raphidascaris cristata	Nematoda	UK	Kennedy (1974)
Rhabdochona denudata	Nematoda	UK	Kennedy (1974)
Rhipidocotyle illense	Nematoda	UK	Kennedy (1974)
Sphaerostoma bramae	Platyhelminth	UK	Nicoll (1924); Kennedy (1974)
Tetracotyle phoxini	Platyhelminth	UK	Nicoll (1924)
Trichodina acuta	Ciliophora	Scotland	Gaze and Wootten (1998)
Trichodina intermedia	Ciliophora	Scotland and Wales	Gaze and Wootten (1999, 2000, 2001 and 2002)
Trichodina reticulata	Ciliophora	UK	Kennedy (1974)
Trichodina sp.	Ciliophora	UK	Kennedy (1974)
Trypanosoma sp.	Ciliophora	UK	Kennedy (1974)
Tylodelphys clavata	Platyhelminth	UK	Nicoll (1924)

Table AI.5. Described parasites of flounder, listing parasite species, phyla, select publications documenting infections and geographical locations

Species	Phylum	Geographical location	Reference
Acanthochondria depressa	Arthropoda	Scotland	Mackenzie and Gibson (1969)
Anisakis sp.	Nematoda	Scotland	Mackenzie and Gibson (1969)
Bothriocephalus scorpii	Platyhelminth	Scotland	Mackenzie and Gibson (1969)
Hysterothylacium aduncum	Nematoda	Scotland	Mackenzie and Gibson (1969)
Cucullanus heterochrous	Nematoda	Scotland	Mackenzie and Gibson (1969); Gibson (1972)
Capillaria sp.	Nematoda	Scotland	Mackenzie and Gibson (1969)
Contracaecum aduncum	Nematoda	Scotland	Mackenzie and Gibson (1969)
Corynosoma strumosum	Acanthocephala	Scotland	Mackenzie and Gibson (1969)
Cryptocotyle lingua	Platyhelminth	Scotland	Mackenzie and Gibson (1969)
Cucullanus heterochrous	Nematoda	Scotland	Mackenzie and Gibson (1969)
Cucullanus minutus	Nematoda	Scotland	Mackenzie and Gibson (1969)
Derogenes varicus	Platyhelminth	Scotland	Mackenzie and Gibson (1969)
Echinorhynchus gadi	Acanthocephala	Scotland	Mackenzie and Gibson (1969)
Hemiurus communis	Platyhelminth	Scotland	Mackenzie and Gibson (1969)
Lecithaster gibbosus	Platyhelminth	Scotland	Mackenzie and Gibson (1969)
Lepeophtheirus pectoralis	Arthropoda	Scotland	Mackenzie and Gibson (1969)
Lernaeocera branchialis	Arthropoda	Scotland	Mackenzie and Gibson (1969)
Phocascaris sp.	Nematoda	Scotland	Mackenzie and Gibson (1969)
Plagioporus varius	Platyhelminth	Scotland	Mackenzie and Gibson (1969)
Podocotyle atomon	Platyhelminth	Scotland	Mackenzie and Gibson (1969)
Podocotyle sp.	Platyhelminth	Scotland	Mackenzie and Gibson (1969)
Pomphorhynchus sp.	Acanthocephala	UK	Kennedy (1984); Mackenzie and Gibson (1969)
Pomphorynchus laevis	Acanthocephala	England	Kennedy (1984)
Porrocaecum sp.	Nematoda	Scotland	Mackenzie and Gibson (1969)
Raphidascaris sp.	Nematoda	Scotland	Mackenzie and Gibson (1969)
Spiruroid	Nematoda	Scotland	Mackenzie and Gibson (1969)
Tetraphyllidean larve	Platyhelminth	Scotland	Mackenzie and Gibson (1969)
Trypanorhynch	Platyhelminth	Scotland	Mackenzie and Gibson (1969)
Zoogonoides viviparus	Platyhelminth	Scotland	Mackenzie and Gibson (1969)

Appendix II. Parasite species with the potential to co-infect bullheads, brown trout, stone loach, minnow and flounder

Table AII.1.: Parasite species that have the potential to infection more than one of the species of fish selected in the study

Parasite	Bullhead	Brown trout	Flounder	Minnow	Stone loach
Protists					
Dermocystidium sp.	X	X			
Ichthyophthirius multifiliis		X		X	
Trichodina acuta		X		X	
Trypanosoma sp.	X			X	
Digenea					
Diplostomum sp.		X		X	
Diplostomum spathaceum		X			X
Diplostomum volvens	X			X	
Nicolla gallica	X				X
Phyllodistomum folium	X	X		X	X
Sphaerostoma bramae		X		X	
Tetracotyle percafluviatilis	X				X
Tylodelphys clavata		X		X	
Monogenea					
Diplozoon paradoxum	X			X	
Gyrodactylus sp.		X		X	
Cestoda					
Allocreadium isoporum				X	X
Triaenophorus nodulosus	X				X
Nematoda					
Capillaria sp.		X	X		
Raphidascaris acus	X	X			X
Raphidascaris cristata		X		X	
Acanthocephala					
Acanthocephalus anguillae		X			X
Acanthocephalus lucii		X			X
Echinorhynchus truttae	X	X			
Pomphorhynchus laevis	X	X	X	X	X