Assessment of Trophic Polymorphism in the Himalayan Fish *Tor putitora*

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Abstract

Assessment of Trophic Polymorphism in the Himalayan Fish *Tor putitora*

An investigation into intra-specific morphological variation in *Tor putitora* fish from the Mahakali River in western Nepal was carried out. Three main hypotheses were tested: i) two distinct variants, one with thin lips and a forward-facing mouth (Type A), another with thick lips, an extended protrusion known as a mental lobe and more ventrally oriented mouth (Type B), can be distinguished by meristic, allometric or landmark-based morphometric analyses; ii) differences in ecological conditions in spatially separated breeding streams, potentially linked to environmentally influenced morphological variation, can be observed; iii) differences in stable isotope ratios of carbon and nitrogen obtained from fish scales, and therefore evidence of dietary divergence, are evident between the two morphotypes.

Meristic and allometric analyses provided no evidence of distinguishing features between the morphotypes, however the landmark-based morphometric analysis produced very strong evidence that the two morphotypes clearly differ in shape and features of the head and mouthparts. Analysis of the physico-chemical conditions and aquatic invertebrate communities in the spatially separated breeding streams produced results which refuted the second hypothesis but suggested strongly that differences between seasons (snow-melt and post-monsoon) were statistically significant and suggested an alternative hypothesis that breeding events may be segregated temporally, rather than spatially. Stable isotope analysis uncovered statistically significant differences in nitrogen isotope ratios which supported the hypothesis that the two morphotypes display divergence in diet, very likely linked to the morphological variation in the head and mouthparts.

This examination provides evidence of trophic polymorphism in *T. putitora* and illuminates the path to further studies of this phenomenon which has been observed in several other species of the same genus across south and southeast Asia.

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Trophic Polymorphism in the Himalayan Fish *Tor putitora*

Introduction

The Himalayan mahseer fish *Tor putitora* (Hamilton, 1822) belongs to the *Tor* genus of cyprinids distributed across the Indian subcontinent and Southeast Asia. The species is regarded as potamodromous, signifying that it is migratory entirely within freshwater habitats (Shrestha, 1997) as shown in ***Fig. 1.1***. *Tor putitora* constitutes a highly important seasonal source of protein for the small-scale subsistence farmers who populate the Nepali and Indian hill catchments (Rai, 2008) and is a popular target for local fishermen who supply village markets in the mid-range of the Himalayas. *Tor putitora* was first described scientifically by Dr Francis Buchanan-Hamilton of the Bengal Medical Service after his 1807-1814 survey of areas under the control of the British East India Company as *Cyprinus putitora*. The species, along with other members of the genus *Tor*, had been recognised as an expedient alternative target for game anglers amongst the British expatriate community who more usually sought the Atlantic Salmon, *Salmo salar* Linnaeus, 1758, and as such, became the focus of various books concerning sport fishing in the British Empire (Lang, 1859, Wilson, 1860, Thomas, 1881). In earlier times several references to the mahseer are found recorded in ancient Indian literature known as the Vedas, as reported by Hora (1951), describing its privileged position within the complex regulations regarding which foods are permissible for certain castes of Hindus to consume. During the period of British administration in India (1858-1947), the species' range was reported to extend from Iran eastward to Thailand across the Himalayan belt and throughout the Indian subcontinent (Hora, 1939). The founding of the Journal of the Bombay Natural History Society in 1886 saw the first scientific articles published in India, and the first article on the species concerning breeding habits 30 years later (Neville, 1916), followed by papers on the difficulties in taxonomic classification (Hora, 1941), and feeding behaviour (Codrington, 1946).

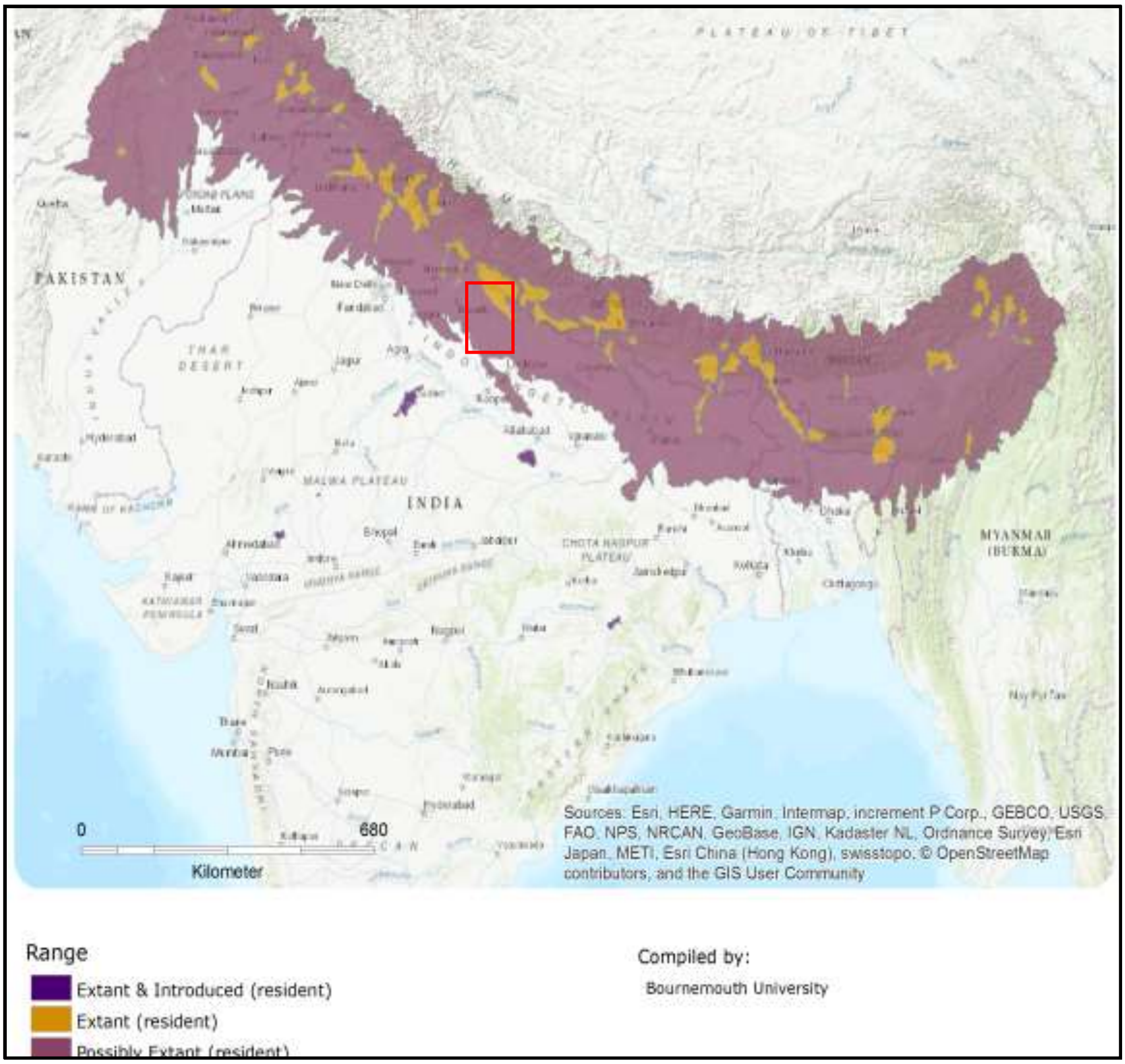

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Figure 1.1: *Distribution map of* Tor putitora *from IUCN Red List of Threatened Species, (*adapted from *Jha,* et al, *2018). Study area outlined in red.*

As early as 1940, reports of declines in some local populations appeared in the scientific literature (Hora, 1940, Raj, 1945) and it is now classified by the IUCN as Endangered (Jha and Rajamahji, 2010), due to a combination of overfishing and habitat degradation (Kulkarni, 1988, Singh *et al.*, 1993), including the impacts of hydroelectric dams on its migratory patterns (Bhatt *et al.*, 2004). An important consideration for the future will be the reduction of meltwaters entering the rivers on the southern slopes of the Himalayas (**Fig. 1.1**) from the shrinking of glaciers due to global heating. As was the case with salmon in North Atlantic countries when population declines became apparent, *T. putitora* gradually became the focus of attention of many aquatic biologists on the Indian sub-continent. The abundance and status of *T. putitora* within fish assemblages has been investigated in several Himalayan river systems (***Fig. 1.1***) including: the Ganges (Singh *et al.*, 1993, Singh and Sharma, 1998, Malik, 2011); Mahakali (Negi and Negi, 2010); and Ramganga rivers (Atkore *et al.*, 2011). The main focus of much of the early research was to quantify remaining stocks and to suggest methods of enhancement (stocking, ranching, engineering of spawning areas), rather than to investigate the migratory habits and population composition of those remaining stocks. Efforts to cultivate domesticated stocks for food and reintroduction to depleted habitats also produced a number of studies on physiology (Pathani, 1981, Basade *et al.*, 2000, Gupta *et al.*, 2013, Akhtar *et al.*, 2013a, b), breeding techniques (Pandey *et al.*, 1998, Patil and Lakra, 2005), and nutrition (Hossain *et al.*, 2002, Islam and Tanaka, 2004, Sawhney and Gandotra, 2010). Clarifying the taxonomy of the *Tor* genus through the use of morphometric and genetic techniques has been the focus of recent research in the Indian subcontinent and Southeast Asia. This has been the subject of long-standing debate and disagreement from the 19th Century until the present (Ullah *et al.*, 2018).

The Taxonomic Debate

During previous expeditions conducted by the author from 1994 to the commencement of this project, informal pilot studies on *T. putitora* in the Mahakali River have suggested that two morphological variants (see cover photo) exist in two of the Mahakali's sub catchments: one with wide, terminally positioned mouthparts with thin lips (Type A), and another with an underslung, protrusible aperture framed by thick hypertrophied lips (Type B). During the informal studies, Type B morphotypes were not observed or reported from any other sub catchments. Such morphological variation has been reported in angling (McDonald, 1948) and scientific (Hora, 1936, Desai, 2003) literature as occurring in rivers across the Himalayan range. Informal discussions with local fishermen conducted during the aforementioned expeditions suggested that the variation may be linked to exploitation of different food resources in separate portions of the water column (the thin-lipped variety mainly being caught using fish for bait in the mid to upper levels and the thick lipped variety only being caught with cereal-based paste baits presented on the riverbed). This phenomenon has triggered some of the controversy over species identification, as specimens displaying the characteristics of the Type B variant have been described as several separate species: *Tor macrolepis* (Heckel, 1838), *Tor progenius* (McClelland, 1839) and *Tor mosal mahanadicus* (David, 1953). The polymorphic variation noted above has also been observed in sympatric populations of *Tor tambra* (Valenciennes, 1842) and related *Neolissochilus* (McClelland, 1839) species in Malaysia and described as an ancient evolutionary heritage of barbin cyprinids, a group which includes *Tor* species, that has played an important role in the evolution, speciation and adaptive radiation of this fish group (Roberts and Khaironizam, 2008).

The current status of *Tor* species in the Indian subcontinent and Burma is recognised by the IUCN (Jha *et al*., 2019) and includes nine species: *T. putitora, T. khudree* (Sykes, 1839)*, T. tor* (Hamilton, 1822)*, T. remadevii* (Kurup and Radhakrishnan, 2007)*, T. mosal* (Hamilton, 1822), *T. kulkarni* (Menon, 1992), *T. yingjiangensis* (Chen and Yang, 2004), *T. malabaricus* (Jerdon, 1849) *and T. barakae* (Arunkumar and Basudha, 2003). Three of these species, *T. putitora*, *T*. *tor* and *T. mosal* have been recorded in the Mahakali catchment (Shrestha, 2008). The traditional taxonomic methodology utilised to distinguish between species included meristic (fin ray and scale counts) and allometric (dimensions of head and body parts) comparisons and the limited historical information available has been summarised by Nautiyal (2014). ***Table 1.1*** is derived from Nautiyal's review for the three species found in the Mahakali. As can be seen from the descriptions of the lips in column 4, there is still ambiguity about the thickness of the lips and presence of the mental lobe, here described as an adipose extension, and recorded as observed only in larger specimens. The fin ray and scale count formulas also contain overlaps in the ranges of several counts. Moreover, the descriptions of colouration and barbel and fin configurations in columns 3, 5 and 6, respectively could hardly be considered to be of scientific exactitude.

The difficulties faced in fish taxonomy can also be compounded by relatively common issues such as sexual dimorphism, phenotypic polymorphism and regional behavioural divergence, leading to incorrect species designations (Waugh, 2007). The limitations of using the traditional methods of taxonomy resulted in researchers from the subcontinent moving towards molecular genetic techniques in an attempt to disentangle the identities of *Tor* species in order to evaluate the status of each with respect to conservation initiatives.

Table 1.1: *Taxonomic characteristics of three* Tor *species from Mahakali River - adapted from Nautiyal (2014). Key to Fin/Scale Formula: D=Dorsal, P=Pectoral, V=Ventral, A=Anal. C=Caudal, L.l. =Lateral Line, L.tr. = Lateral Transverse.*

Species	Fin / Scale Formula	Colouration	Lips / Jaws / Interorbital	Barbels	Fins
Tor putitora (Hamilton, 1822)	D. 12 (3/9); P. 19; V. 9; A. 7-8 (2–3/5); C. 19; L. l. 25–28; L.tr. 3½/2½	Usually greenish above with light pinkish and silvery white below, a broad light greyish blue or purplish lateral line found generally in live specimens, fin yellowish with lower ones tinged with red, extreme redness of the lower fins sometimes associated with thick lips	Thick with a continuous labial groove, pronounced adipose extension of lips in larger fish present	Two pairs which are more or less equal and as long as eye	Originates midway between tip snout and base of caudal
Tor tor (Hamilton, 1822)	D. 12 (3/9); P. 19; V. 9; A. 7–8 (2–3/5); C. 19; L. l. 22–27; L. tr. 4¼/2½	Silvery green or greyish green dorsally with pinkish sides replaced by greenish gold above and light olive green below, lower fin reddish yellow	Thick fleshy with continuous labial folds across lower jaw. Snout, pointed. Jaw is of about same length. Interorbital space flat	Two pairs, maxillary slightly longer than rostral but shorter than eye	Opposite to or slightly in advance of ventral fin. Pectoral fin reaching pelvic fins as long as head excluding snout. Pelvic- shorter
Tor mosal (Hamilton, 1822)	D. 4/8–9; P. 17; V. 8–9; A. 3–5; C. 19; L. l. 23–26; L. tr.3½/3½	Delicate yellowish shade below, more yellowish colour than gold; deep blue fins except caudal fin with a red tinge; dorsal fin reddish orange and other fins yellowish orange	Lips with continuous labial fold, pronounced adipose extension of lips in larger fish	Two pairs	Dorsal fin almost opposite to pelvic fins but slightly towards caudal base

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Mohindra *et al.* (2007) utilised random amplified polymorphic DNA (RAPD) to distinguish three species of the *Tor* genus (*T. putitora, T. tor, T. khudree*) and the closely related species *Neolissochilus hexagonolepis* (McClelland, 1839) from five distinct river catchments across the Indian peninsula. They concluded that the putative species *T. mosal mahanadicus*, from the Mahanadi catchment in eastern India, was a synonym of *T. putitora*. Ghosh and Alam (2008) differentiated between *T. tor* and *T. putitora* using RAPD analysis in order to distinguish these two species, both regarded as critically endangered in Bangladesh, to improve conservation efforts by correctly identifying stocks. More recently, Arora and Julka (2013) utilised the same technique (in conjunction with morphometric characters) to investigate differentiation in two populations of *T. putitora* from Himachal Pradesh, India: one from a river system and another from a hydroelectric reservoir. Their analysis failed to distinguish discrete populations and they suggested microsatellite markers might be more appropriate for studying intra-specific variation. A more recent study by Yadav, *et al*, (2020) has been more successful in distinguishing stocks within the Ganges system using mitochondrial cytochrome *b* gene (cyt *b*), plus microsatellite genotyping finding clear differentiation between stocks from the two tributaries Bhagirathi and Alaknanda.

Inter- and intra-specific differences in *Tor* species have also been studied using 18S and 5S ribosomal DNA (rDNA) (Singh *et al.*, 2009a, b). The technique was fairly successful in distinguishing between species (see also Kumar *et al.*, 2013), but less so in terms of developing discrete population-specific markers. Nguyen *et al.* (2008) published a wide-ranging study aiming to elucidate mahseer phylogeny using mitochondrial DNA (mtDNA) analysis for a range of *Tor* species across Southeast Asia including the Indian subcontinent: *T. tambroides* (Bleeker, 1854) (Thailand and Malaysia), *T. khudree* (Sri Lanka and India), *T. tor* (Nepal), *T. douronensis* (Valenciennes, 1842) (Malaysia, Vietnam and China), *T. putitora* and its synonym *T. macrolepis* (Nepal, Pakistan and Bangladesh) and

Neolissochilus hexagonolepis (Nepal, Malaysia, Thailand, China). Utilising nucleotide sequences of three mitochondrial gene regions: cytochrome *b* (cyt *b*), ribosomal RNA (16S rRNA) and adenosine triphosphate subunits six and eight (ATPase), they constructed a phylogenetic tree, which differentiated the species considered. More importantly in terms of the current study, they also established *T. macrolepis* (a morphotype resembling Type B) as a synonym of *T. putitora*. Other studies (Esa *et al.,* 2008, Sati *et al.*, 2013) have used cytochrome c oxidase subunit I (COI) sequences to attempt to differentiate species and derive phylogenetic information. However, Esa *et al.*, (2008) detailed the limitations of using a single, maternally inherited gene (COI) and again recommended the use of microsatellites to further progress for population-level investigations. Mohindra *et al.*, (2004) conducted an early study on the feasibility of identifying polymorphic microsatellite DNA loci in *T. putitora*. Using 32 primers from three other cyprinid species, they successfully identified seven loci. The technique of cross species amplification was also followed in other studies on species found in Thailand (Nguyen *et al.*, 2007) and Malaysia (Esa *et al.*, 2011), which observed ten and nine polymorphic loci in *T. douronensis* and *T. tambroides*, respectively. Developments in the field of primer design include the publication of 12 polymorphic microsatellite markers derived from *T. putitora* specimens from the Indian rivers Ravi, Anji and Kosi (Sahoo, *et al.*, 2013). Nguyen (2008) has also demonstrated the utility of the primers developed in his 2007 paper (above) by defining two separate clusters of *T. douronensis* in the state of Sarawak on the island of Borneo, Malaysia.

Of particular relevance to the current work are two relatively recent papers, which used COI mtDNA barcoding to assign the previously as *T. macrolepis* and *T. progenius* described species as synonyms of *T. putitora* (Khare *et al.*, 2014; Laskar *et al.*, 2013). Both papers acknowledge that the distinguishing morphological features, which heavily influenced the original scientific descriptions of these species, were the thick lips and mental lobe, the same features apparent on the Type B morphotypes of *T. putitora* considered in the present work. These studies provided molecular evidence that points to the common specific identity of the two morphs, but there is still a significant gap in knowledge regarding the influence of genetic or environmental factors in the development of this phenomenon. Indeed, Khare and colleagues acknowledged shortcomings in the use of monoparental mitochondrial DNA and recommended the future use of biparental conserved nuclear genome sequences to further refine the systematics of mahseer taxonomy.

Moving forward with the taxonomic clarification of the *Tor* genus, research groups such as The Mahseer Trust are working with scientists from across the habitat range of the genus and are promoting an integrated approach using traditional taxonomic methods such as morphology, meristics and allometry, alongside genetic techniques (Pinder *et al*, 2018). They have achieved some early success in clarifying the identity of the species *Tor remadevii* or hump backed mahseer from South India and have recently published the most comprehensive review to date of the of the current state of knowledge on 16 species of *Tor*, their population status and known distributions (Pinder *et al.,* 2019). While this integrative approach to resolving an agreed methodology to the taxonomic question is still underway, and different opinions still exist as to the validity of many *Tor* species, it was decided that due to the lack of any evidence to the contrary, for the purposes of this investigation the work will proceed under the assumption that the two morphological variations called A and B are morphotypes of *T. putitora,* and the necessary genetic exploration of the morphotypes observed would be better served by waiting for a final consensus on the molecular methodology most suitable to employ.

Trophic Niche Utilisation

Morphometric analysis often focuses on differences relating to feeding mode or preferences. Differences in diet can be assessed by direct observations (such as stomach content analysis), but indirect techniques of evaluating dietary preferences can also provide evidence of trophic status. Elements such as carbon and nitrogen can have different numbers of neutrons and are referred to as different isotopes of the same element. Isotopes with extra neutrons are heavier and generally far less abundant in nature and their chemical bonds and attractive forces are stronger than their lighter counterparts. As a result, the rates of reaction differ and lead to fractionation within biological reactions. The resultant ratios of light to heavy isotope can then be used to investigate biological processes. Stable isotopes are an increasingly widely used indirect method to provide information on aspects of species ecology including intra-specific niche variation (Bearhop *et al.*, 2004, Post, 2002) and providing information on the location of breeding sites and migratory habits of populations (Campana and Thorrold, 2001, Gillanders, 2002, Huxham *et al.*, 2007, Ruttenburg *et al.*, 2005, Veinott and Porter, 2005). Differences in trophic level in aquatic ecosystems are generally reflected in relative depletion or enrichment of both carbon and nitrogen isotope ratios (Peterson and Fry, 1987, Vander Zanden and Rasmussen, 2001). Any differences recorded in these stable isotope ratios can provide insight into trophic specialisation and niche position. While the use of stable isotopes to determine trophic levels of *Tor* species does not currently appear in the literature, its use has become widespread in other species. Dixon *et al.* (2009) demonstrated how Arctic Char (*Salvelinus alpinus* (Linnaeus, 1758)) individuals could be assigned to profundal and littoral foraging groups within one lake habitat by analysis of $\delta^{13}C$ and $\delta^{15}N$ values. Rybczynski *et al* (2008) examined an assemblage of stream fishes from Twelvemile creek in South Carolina, U.S.A and managed to classify species into omnivores (yellowfin shiner, *Notropis lutipinnus* (Jordan and Brayton, 1878), bluehead chub, *Nocomis leptocephalus* (Girard, 1856)) and predators (blackbanded darter, *Percina nigrofasciata* (Agassiz, 1854), turquoise darter, *Etheostoma inscriptum* (Jordan and Brayton, 1876) and northern hogsucker, *Hypentelium nigricans* (Lesueur, 1817)) by comparison of $\delta^{15}N$ ratios. The trophic position analysis was based on methods described by Cabana and Rasmussen (1996) using the equation TPsia (trophic position stable isotope analysis) = [(fish $\delta^{15}N$ - primary consumer $\delta^{15}N$)/3.4] + 2 for each species, which produces the highest scores for predators (3.6) and reduced values incrementally for omnivores, herbivores down to plant material with the lowest TP (1). They also cross-referenced their findings against gut content analysis of each species and found consistent similarities in the results. Grey (2001) examined the ontogenetic shifts in diet through stable isotopic signatures of brown trout (*Salmo trutta* (Linnaeus, 1758)) in Loch Ness, Scotland from the early insectivorous parr stage up to the adult (>300mm) piscivorous stage. He noted that the larger individuals displayed elevated $\delta^{15}N$ ratios compared to all the previous life stages. Concurrent examination of gut contents from each individual supported the stable isotope results, with the overall conclusion that adults over 300mm had switched almost entirely to a piscivorous diet. The study detailed the correlation between fish length and dominant prey item for the three main life stages with associated habitats: parr (natal river), intermediate (littoral and open water) and piscivorous (pelagic). This study eloquently demonstrated how stable isotopes can be used to distinguish dietary variation within one species of fish.

Aims

The main overall aims are to uncover evidence relating to the possible existence of *T. putitora* subtypes, their morphological variation and possible differences in trophic position, as well as examining any potential differences in the physico-chemical profiles and aquatic invertebrate community composition of their breeding and nursery tributaries. Relatively few studies have combined information across multiple characteristics to compare consistency across different techniques. By combining multiple lines of evidence this study aims to comprehensively determine the extent, nature and significance of variation within *T. putitora* populations of the Mahakali River.

Objectives

The fundamental lines of enquiry will be to determine:

- If individual specimens of *T. putitora* can be allocated into two distinct morphotypes A and B by morphological characteristics using allometric, meristic and landmark-based morphological analyses.
- If physico-chemical conditions and aquatic invertebrate abundance and diversity vary between each separate breeding tributary and how the habitats compare in terms of food type and availability for juvenile *T. putitora*.
- If stable isotope analysis of *T. putitora* scale samples produce any evidence of dietary partition between the putative Type A and B morphotypes and demonstrate a link between head/mouthpart morphology and foraging behaviour.
- If individual specimens of *T. putitora* encountered in the vicinity of junctions of spawning tributaries with the Mahakali display any meristic or allometric indicators, which can be used to identify tributary-linked stocks in relation to potential management?

The general structure of thesis is presented in the following format:

Chapter 2 presents the background of the study system and the details of the methods.

Chapter 3 addresses the question whether there is evidence to clearly distinguish two morphological variants of *T. putitora* within the Mahakali catchment.

Chapter 4 examines the ecological and physico-chemical attributes of the spawning tributaries' habitat by investigating aquatic invertebrate community composition and stream physico-chemistry.

Chapter 5 examines stable isotope ratios in *T. putitora* scales in an attempt to provide evidence of dietary divergence and trophic position.

Chapter 6 presents the conclusions derived from the study and makes recommendations for future lines of research.

Chapter Two

The Study System

Introduction

The Mahakali catchment extends from the permanent snow and glacier zone of the Himalayas down to the Gangetic plain of northern India, encompassing three major belts of terrain: Himal (above snow line), Pahad (mid range mountains) and Terai (plains). The dramatic changes in elevation support five climatic zones: tropical, subtropical, temperate, subalpine and alpine. *Tor putitora* is generally confined to altitudes of 600m to 1200m in the main snow-fed rivers, known to ichthyologists of the subcontinent as the Tor zone (Sarma *et al.,* 2010) and ascends the warmer, mainly spring and rain-fed tributaries during the breeding season.

Study System Characteristics

The Mahakali (***Fig. 1.2***) is a large (mean flow rate 508 cumecs [cubic metres per second], catchment area 12,100 km^2) snow- and glacier-fed river, which carries considerable amounts of sediment (annual sediment load 29 million cubic metres) during the annual March–July period of snow-melt. From July to October it is subjected to the monsoon rains, which massively increase its volume and turbidity (Probable Maximum Flood (PMF) 23,500 cumecs) with input from the rain and groundwater-fed tributaries (Anon., 2015). The main trunk itself remains turbid from the commencement of snow-melt in March until shortly after the cessation of the monsoon rains in late October (***Fig. 1.3***). The hydrological conditions (turbidity, low temperatures) in the main trunk of the Mahakali limit the potential for autochthonous algal growth for much of the year (Ayoade *et al.,* 2009) and may be linked to the migratory habits of various fish species within the catchment (Bhatt and Pandit, 2016). From October to April there is little rain and the Himalayan snow line advances to lower altitudes, limiting the input of melting snow and glacial water into the catchment.

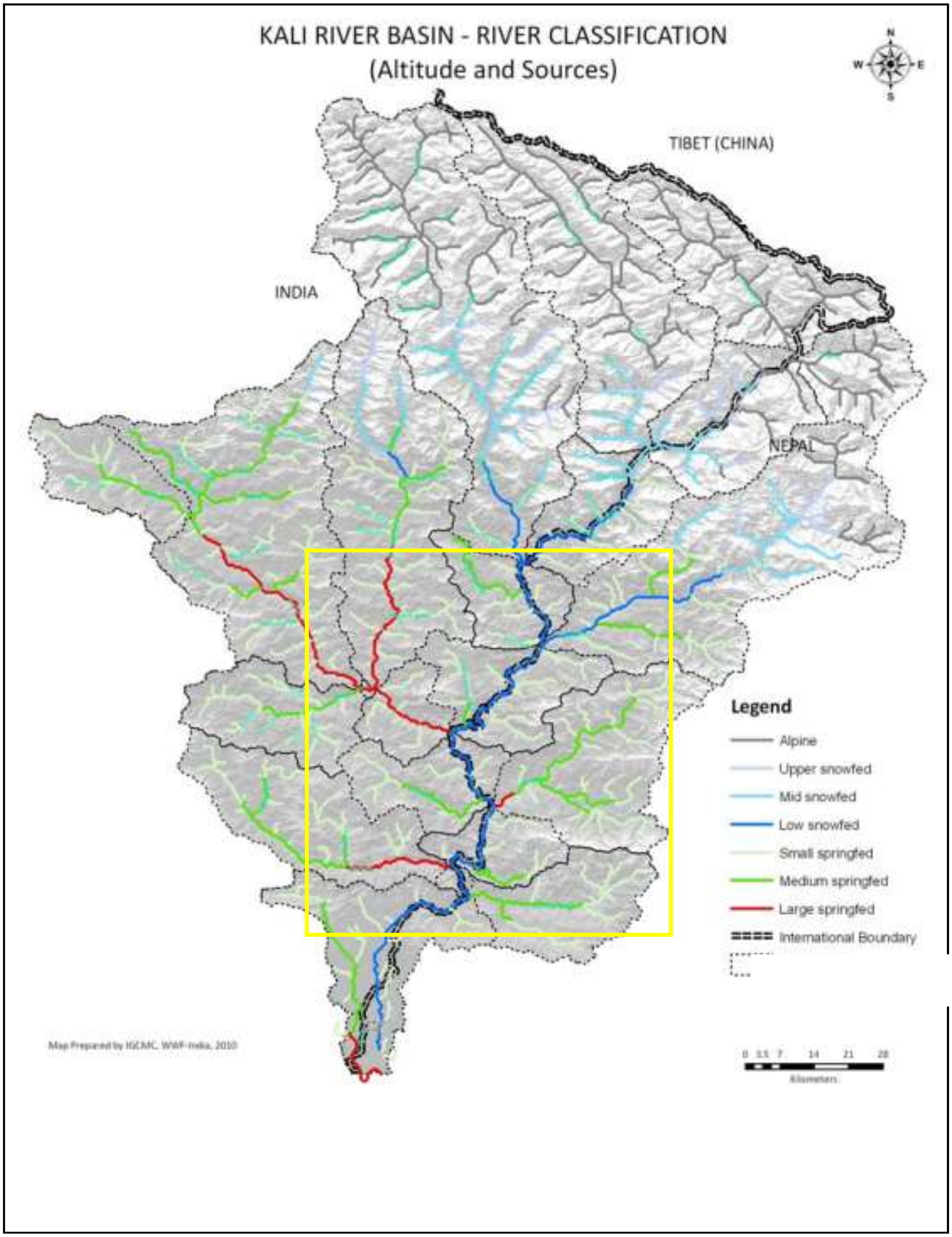


Figure 1.2: *Map of Mahakali River basin - sources (spring-fed or snow-fed), altitudes range from 255m-5500m SSW to NNE. Study area* (see ***Fig: 1.4***) *enclosed in yellow box* (adapted from IGCNC WWF India 2010).

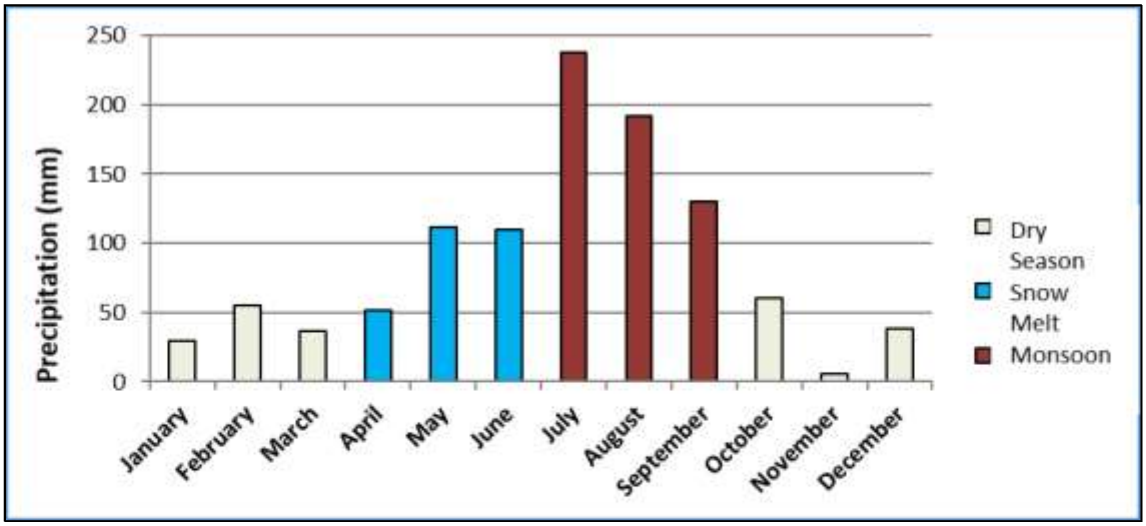


Figure 1.3: *Mean monthly precipitation (mm) in Mahakali catchment including main hydrological seasons (dry, snow-melt and monsoon), data from Government of India Water Resources Study - Anon. (2015).*

The rain and groundwater-fed tributaries flowing from the surrounding hills provide suitable habitats for nursery areas for *Tor* species (temperatures above 20°C are required for egg development; Ogale (1997)). Members of this genus are known to migrate during the breeding season (Bhatt *et al.*, 2004, Petr, 2003) when tributary water levels and turbidity are high. The tributaries remain turbid only for the duration of the monsoon rains and high temperatures and enhanced light penetration during the rest of the year create conditions, which are ideal for the growth of algal species that have evolved to resist swift currents. This algal growth, including Bacillariophyceae, Chlorophyceae, Cyanophyceae, Xanthophyceae (Mahaseth, 2016) on the stones and boulders of the river serves as an important food source for many fish species in the catchment, which have specially adapted algae-grazing mouthparts (e.g. *Labeo dero* (Hamilton, 1822), *Schizothorax richardsonii* (Gray, 1832), *Garra gotyla* (Gray, 1830), *Noemacheilus* spp.). These algae have already been identified as an occasional component of the diet of different *Tor* species (Desai, 2003). The same tributaries are also responsible for the annual allochthonous nutrient input during the monsoon rains when drainage of areas comprised of jungle vegetation and run off from cultivated areas swell them significantly, depositing the accumulated matter in the main trunk of the river system. As many of the large snow-fed rivers of the Himalayas have already been modified with hydroelectric dam projects (Grumbine and Pandit, 2013), an understanding of the migratory behaviour of *T. putitora* is essential for development of mitigation strategies prior to commencement of new projects on the few remaining free flowing rivers in the area. Any information obtained may have implications for stock conservation in the light of further proposed hydro-electric dam projects, which may interrupt migration routes. For example, if separate stocks are found to be tightly associated with each tributary, then placement of dams across the Mahakali itself may have less impact on migratory routes than if one homogenous stock is utilising the whole river as habitat. To collect information on the physico-chemical and ecological conditions in the breeding tributaries of the Mahakali catchment, eight aquatic invertebrate and water sampling sites were chosen, primarily for relatively convenient access by road or track, and only after confirmation of their functionality as spawning sites for *T. putitora* in discussions with local fishermen. The fish sampling sites presented a greater problem, owing to the fact that adult fish are only present in the spawning areas during the monsoon season when climatic conditions hamper access and river levels and velocities are unsafe for sampling procedures. The decision was taken to carry out sampling trips during the low-water snow-melt and post-monsoon seasons in conjunction with the aquatic invertebrate and water sampling. In order to collect fish samples, six sites located at the junctions of breeding tributaries with the main river and one site located on the main river itself were accessed, while travelling down the Mahakali by raft (***Fig. 2.1***). Samples were collected by cast netting, angling and negotiation with local fishermen, with the first two methods allowing the safe handling, rehabilitation and release of the fish, while the third relied on a small number of recently deceased examples which were destined for the locals’ subsistence.

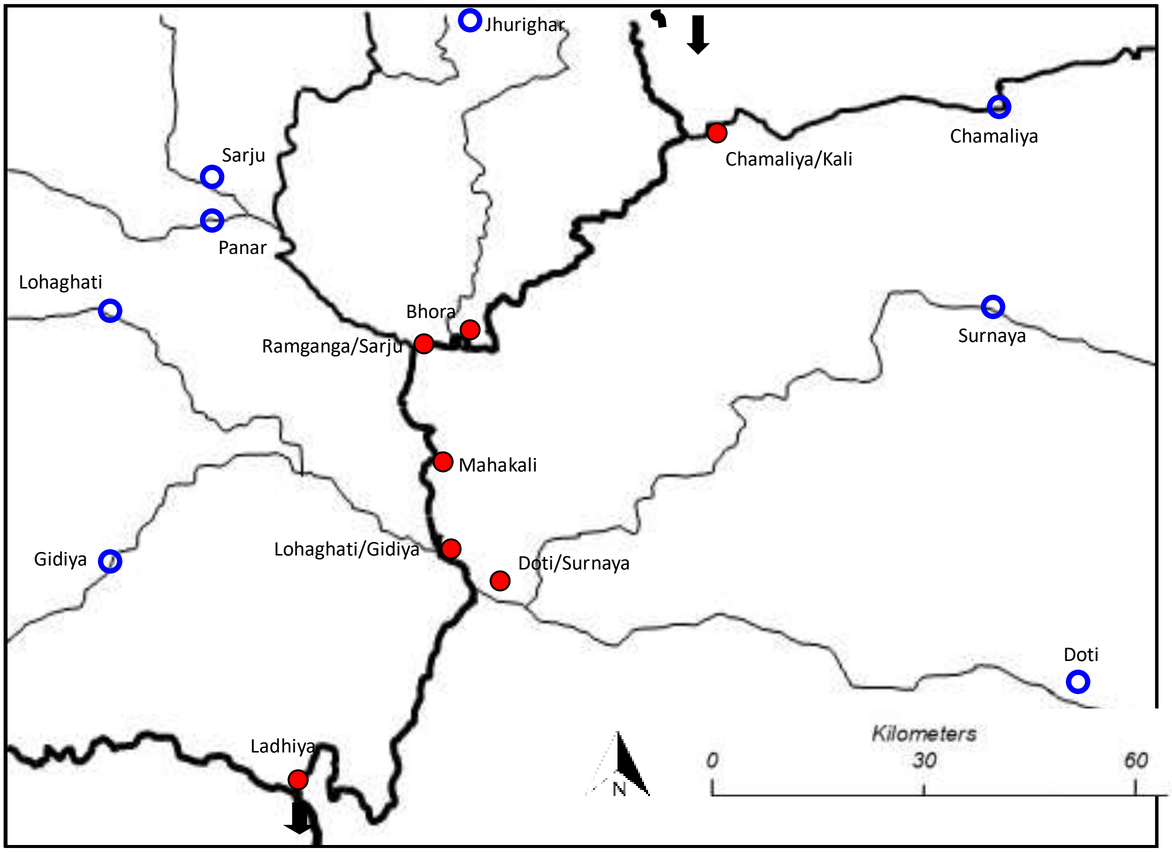


Figure 2.1: *The study area: Central river with flow direction arrows is the Mahakali, with seven* T. putitora *sampling sites (solid red dots). Peripheral rivers are breeding tributaries with eight aquatic invertebrate and physico-chemistry sample sites (hollow blue dots).*

The study area is subject to the annual monsoon weather patterns of the Indian subcontinent and as such displays wide variation in river levels, velocities, chemical composition and sediment load. Variation in biogeography and land use of river catchments was also apparent on field trips and in studies of satellite imagery of the Mahakali catchment. In general terms, the rivers which flow in deeper gorges with steep, uncultivable land, remain heavily forested, sparsely populated and relatively unaffected by anthropogenic influences. The rivers which flow through wide, flat, cultivated valleys or large towns linked by an adjacent road network, are subject to inputs of nitrogen (fertiliser, industrial waste, refuse tips) and phosphorus (human and animal waste, detergent) as well as other industrial and agricultural pollutants (Seth *et al.*, 2016).

The sub-catchments of most interest in an examination of the variation between the two morphotypes are the Lohaghati and Gidiya, which originate in the foothills well below the snow line and join to flow together for several kilometres into the Mahakali from the west bank; and the Surnaya and Doti, of similar description but entering from the east bank. Of all the fish sample sites, only those associated with these two sub-catchments produced both Type A and B samples in what is assumed to be sympatric populations of the same species, *T. putitora*. The sampling scheme is summarised in ***Table 2.1***. The post-monsoon and snow-melt season invertebrate sampling took place in Sept - Nov 2012 and Mar - Apr 2014, respectively. Fish sampling was carried out concurrently and during Sep - Oct 2013 and Oct 2014.

Table 2.1: *Breakdown of fish, aquatic invertebrate and physico-chemical sampling scheme for different sites.* ***AI*** *(aquatic invertebrates)* ***PC*** *(physico-chemical-water samples)* ***SI*** *(stable isotopes-fish scale samples)* ***MOR*** *(morphology-photographs of fish head areas)* ***MER*** *(meristics-fin ray and scale counts)* ***ALL*** *(allometry-body measurements). Fish sampling seasons- *=post-monsoon 2012, **=post-monsoon 2013, ***=snow-melt 2014, ****=post-monsoon 2014.*

Site Name	Type of Sampling	Number and Type of Fish Samples	Sampling Season: Post-monsoon (PM) Snow-melt (SM)
Chamaliya	AI/PC		PM,SM
Jhurighar	AI/PC		PM,SM
Sarju	AI/PC		PM,SM
Panar	AI/PC		PM,SM
Lohaghati	AI/PC		PM,SM
Gidiya	AI/PC		PM,SM
Doti	AI/PC		PM,SM
Surnaya	AI/PC		PM,SM
Mahakali	MOR/MER/SI/ALL	13 A (10*, 3****)	PM,SM
Lohaghati/Gidiya	MOR/MER/SI/ALL	20 A (8***, 4*, 8***), 11 B (8***, 1*, 2**)	PM,SM
Doti/Surnaya	MOR/MER/SI/ALL	23 A (4*, 12**, 7****), 8 B (1*, 7**)	PM,SM
Ramganga/Sarju	MOR/MER/SI/ALL	4 A (4***)	PM,SM

Chamaliya/Kali	**MOR/MER/SI/ALL**	**1 A (1****)**	**PM**
Bhora	**MOR/MER/SI/ALL**	**2 A (2****)**	**PM**
Ladhiya	**MOR/MER/SI/ALL**	**1 A (1***)**	**PM**

Chapter Three

Characterisation of Morphological Variants of *Tor putitora* from a Himalayan River in Far Western Nepal in relation to morphotype identification

Introduction

Variations in the shape of fishes are widespread in nature and usually reflect adaptations to their environment, such as current velocity, substrate composition, protection from predation and foraging behaviour (Lindsey, 1979). Species which inhabit fast flowing turbulent rivers such as the snow- and glacier-fed rivers that flow down the southern slopes of the Himalayas have evolved fusiform (streamlined, torpedo shaped) bodies to deal with the high current velocity and various adaptations to the head (flattened/equipped with suctorial pads or pointed/streamlined) and mouthparts (Shrestha, 2008). Bottom dwelling algae and invertebrate feeders such as *Garra gotyla*, *Labeo dero*, *Botia almorhae* (Gray, 1831) and *Schizothorax richardsonii* all possess, to varying degrees, mouthparts adapted to cling to the algae encrusted rocks where they forage. In contrast, species which forage mainly in the mid or upper water column such as *Neolissochilus hexagonolepis*, *Salmostoma acinaces* (Valenciennes, 1844), *Barilius bendelisis* (Hamilton, 1807) and *Raiamas bola* (Hamilton, 1822) possess forward facing mouthparts with wide gapes adapted to ingest fast moving prey in open spaces. Cases of intra-specific phenotypic plasticity, when individuals of a single species demonstrate variation of shape in the head and mouthparts in response to availability of different food resources, have been well documented. Keeley *et al.* (2007) observed that piscivorous rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792)) from three different lakes in British Colombia, Canada, had larger heads and mouthparts than their non-piscivorous conspecifics in sympatric populations. Hjelm *et al.* (2000) studied functional morphology in benthivorous and piscivorous perch (*Perca fluviatilis* (Linnaeus, 1758)) from eight lakes in central Sweden, concluding that piscivorous perch developed more fusiform body shapes and a wider gape as a result of their predatory behaviour. Adams and Huntingford (2004) and Adams *et al.* (2006) have investigated phenotypic plasticity in Arctic charr (*Salvelinus alpinus*) from Scotland (particularly in head shapes) and its link to foraging behaviour, breeding site fidelity and genetic sub structuring. They concluded that breeding site fidelity, *i.e.* separate populations returning to the same spawning sites, creating spatially isolated breeding groups, can be a significant factor in maintaining strong genetic and phenotypic population sub structuring in fish stocks living in sympatry. Ruehl and de Witt (2007) conducted controlled laboratory experiments on red drum fish (*Sciaenops ocellatus* (Linnaeus, 1766)) involving substitution of food items in juveniles and managed to induce morphological change in head structure, suggesting that dietary partition alone can influence morphological variation. In another study involving translocation of populations of Eurasian perch (*Perca fluviatilis*), Hjelm *et al.* (2001) recorded diet-influenced morphological change, independent of genetic influence, which included alterations to head shape produced by substitution of food items. Since morphological variation in relation to these factors is relatively common, it is possible that *T. putitora* also demonstrates the ability to exploit the entire water column by expressing different phenotypes, a benthivorous and a piscivorous form.

Meristics is a method of ichthyological taxonomy, which relies on counting quantitative features of a fish. A measurable trait or meristic, such as the number of scales or fin rays, is utilised to describe or identify fish species. This technique has previously been used in studies of *Tor* species in attempts to identify genetic and environmental influences in development (Ujjania *et al*., 2012), species identification (Zafar *et al*., 2002) and to describe a potentially new species (Chen and Yang, 2004). McDowall (1972) examined the difficulties associated with using meristics alone to determine the taxonomic identity of fish species highlighting the problems of allopatric speciation and the status of geographical isolates. Isolated populations have a tendency to evolve phyletically, thus demonstrating divergence in morphological and ecological traits leading to misidentification of species in some cases (e.g. northern hemisphere salmonids). The extent of reproductive isolation of populations in the current study is unknown, but the connectivity between the sub-catchments could certainly allow gene flow between them. Another factor, which can create uncertainty in species identification by meristics, is the issue of hybridisation (Mourao *et al.*, 2017). Three species of *Tor* genus are encountered in the studied section of the Mahakali catchment: *T. putitora*, *T. tor* and *T. mosal* (Shrestha, 2008) and as the occurrence or extent of hybridisation between them has never been examined. Therefore, it cannot be ruled out that hybrids, displaying mixed meristic characteristics, are present in the system. The chief difficulty presented by the use of meristic characteristics in this catchment, however, is the duplication and overlap in the original fin formulas and scale counts used by Hamilton (1822) in his classification of the three species as reviewed by Nautiyal (2014) and summarised in Chapter one.

Allometry is the study of the relationships between the size of an organism and the size of distinct parts of its body. The traditional taxonomic methodology followed by Hamilton, (1822) and Hora, (1939) and many researchers since used a combination of meristic and allometric techniques and have recognised two important characteristics: length of head relative to body, i.e. short and long gilled variants (Bhatt *et al.*, 1998, Dasgupta, 1991); and depth of body before dorsal fin, i.e. deep bodied variants as a distinguishing factor in determining *Tor* species. More recent examination of allometric techniques, across a broad range of organisms has cast doubt on the validity of this method due to the effects of ontogenetic development and phenotypic plasticity, which can alter relationships between the parameters measured (Pelabon *et al.,* 2013). For this reason, allometric studies must be regarded as complementary and not a stand alone method.

As mentioned in Chapter 1, there remains considerable uncertainty over the taxonomic status of *T. putitora* and its relation to at least three other variants, *T.*

macrolepis (Heckel, 1838)*, T. progeneius* (McClelland, 1839) and *T. mosal mahanadicus* (David, 1953). The differences in the descriptions of these species are generally confined to variations in the morphology in the head/mouth region, specifically, the orientation of the mouth – terminally positioned or underslung, and the thickness of the lips – including the existence of a mental lobe on the lower lip.

The utilisation of a landmark-based geometric morphometric analysis, a powerful method of quantifying biological shape, allows variation in shape, and covariation of other variables or factors with shape, such as features of the head, to be translated into shape variables. These variables can then be analysed statistically to determine if significant differences in head shape exist between two perceived morphotypes. This type of analysis is becoming increasingly common in studies of fish species and populations to examine different aspects of fish biology including: ontogenetic growth (Bravi *et al.,* 2013), phenotypic plasticity (Bouton *et al.*, 2002) and population discrimination (Maderbacher *et al.*, 2008). Landmarks are points added to two or three-dimensional representations of an object to be studied. Each landmark corresponds to a particular anatomical trait *e.g.* the tip of the mental lobe, a fleshy protuberance extending from the lower lip, which is visible in the ventral view of the head area. When the landmarks are combined for each sample, the resulting shapes are scaled and rotated to allow direct comparisons between them. This permits the examination of changes in multiple measurements simultaneously and highlights those characteristics that influence differences/variations between morphotypes the most. At the outset of this study, it was assumed that the two morphotypes A and B are *T. putitora* and that the morphological variation between the two types is linked in varying degrees to population identity and food resource partitioning.

Aims

This chapter aims to investigate the extent to which the two morphotypes of *T. putitora* can be distinguished by meristic, allometric and morphometric techniques or if the perceived differences vary along a continuum from one extreme to the other.

Objectives

Test morphotypes A and B for differences in fin ray and scale counts (meristics).

Analyse morphotypes A and B for relationships between several body measurements (allometry).

Utilise landmark-based morphometric shape analysis of lateral and ventral views of the head area of morphotypes A and B (morphometrics).

Methods

Fish samples were obtained and photographed from seven sites on the main stem of the Mahakali River, far western Nepal during 2012, 2013 and 2014 (***Fig. 3.1***) Data on allometric measurements, fin ray counts and scale counts were collected from 64 Type A and 19 Type B specimens (***Table 3.1***). The sites were located on the Mahakali itself as previous informal trips showed that fish sampling on the upstream sections of the spawning tributaries was unlikely to provide sufficient samples during the seasons outwith the monsoon, after which the majority of the fish descend to areas around the junctions and in the Mahakali itself. In effect, samples obtained at the Ramganga/Sarju site may be from the Sarju, Jhurighar, Panar or other unknown tributaries. Samples from the Mahakali site are also of unknown origin, but fish collected at Lohaghati/Gidiya, Doti/Surnaya, Ladhiya, Bhora and Chamaliya are assumed to be linked to the adjacent tributaries.

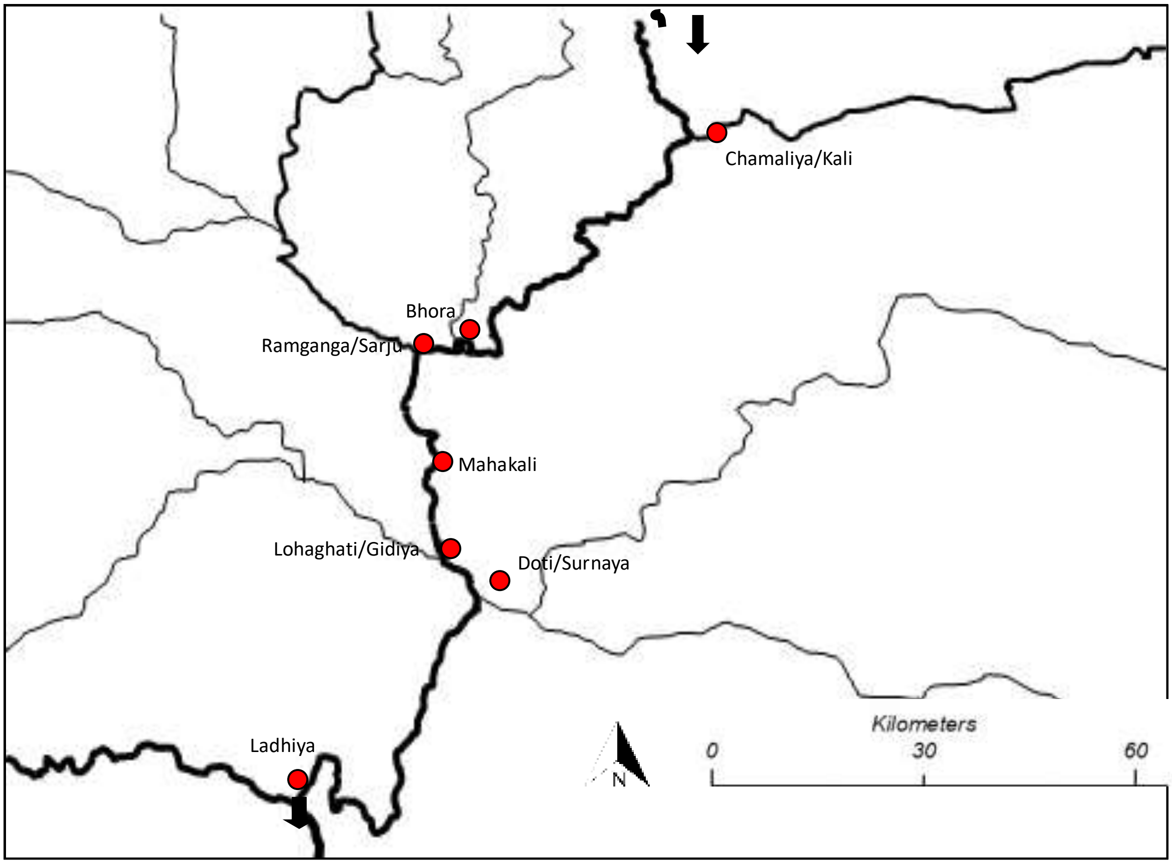


Figure 3.1: The Study Area: *Central river with flow direction arrows is the Mahakali, with seven* T. putitora *sampling sites (red dots). Peripheral rivers are breeding tributaries.*

Table 3.1: Sampling Scheme: *Fish samples collected for meristic, allometric and morphological analysis at different sites.*

Site Name	Number and Type of Fish Samples
Mahakali	13 A
Lohaghati/Gidiya	20 A, 11 B
Doti/Surnaya	23 A, 8 B
Ramganga/Sarju	4 A
Chamaliya/Kali	1 A
Bhora	2 A
Ladiya	1 A

Meristics

Physical measurements (meristics) - scale counts, fin ray counts - were recorded for each individual specimen of *T. putitora.* Meristic data were collected for 83 specimens; 64 Type A and 19 Type B, from seven different sample sites (***Fig. 3.1***) on the Mahakali River associated with ten spawning tributaries (Ramganga/Sarju site contains fish from Sarju, Jhurighar and Panar tributaries) and one sample site on the Mahakali River itself (***Table 3.1***). Due to the importance of minimising stress experienced by each individual of this endangered species, and the harsh environmental conditions (high temperatures, bright sunlight/lack of shade), a necessarily rapid, but limited method was developed for data collection: the majority of samples were caught by angling, then all samples were given a few minutes to recover on a stringer, replaced in the water between meristic and allometric measurement (approx. 5 minutes each set of measurements), again placed in the water on the stringer and left to recover for one hour before careful release with the unbound specimen held facing upstream until it departed under its own power. No bycatch was present and no fatalities occurred except in the small (~20) number of individuals obtained already dead from local fishermen. Nine parameters were measured twice for each sample by the researcher and assistant successively and in the event of discrepancies, re-measured:

Fin ray counts: number of rays in dorsal, caudal, anal, pelvic and pectoral fins.

Scale counts: number of scales along lateral line, diagonally from lateral line to dorsal fin, diagonally from lateral line to anal fin, around circumference of caudal peduncle (see ***Fig. 3.2***).

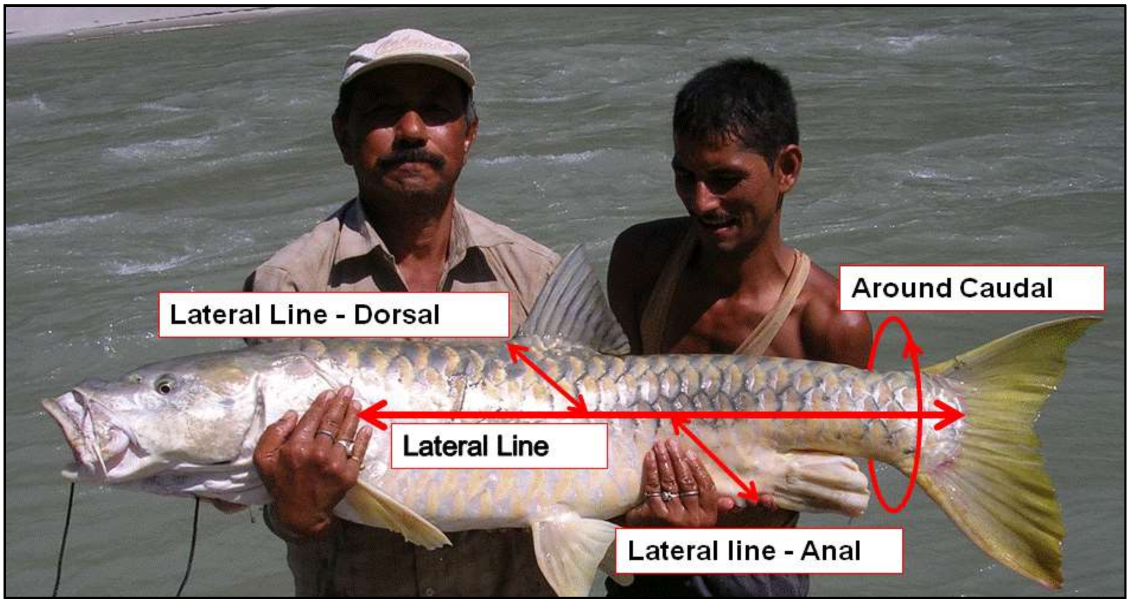


Figure 3.2: *Scale count method used for all* Tor putitora *samples.*

All data sets were tested for normality of distribution with the Anderson-Darling test. As they were all not normally distributed, the non-parametric Kruskal-Wallis test was utilised, followed by a Dunn's multiple comparison test for any significant results.

Allometry

A number of morphological parameters were recorded for each sample with a flexible tape measure to the nearest millimetre (mm). Each measurement was taken by the researcher and an assistant and in the event of discrepancies, the measurement was retaken by the researcher and assistant combined until a consensus was reached: total length (tip of snout to fork), head length (tip of snout to furthest rear point of operculum), pre-dorsal fin body circumference, pre-anal fin body circumference. Weight was measured with a Rapala RSDS-50 digital scale accurate to 7 grams (1/4 ounce). The variables in the entire sample from all tributaries of morphotype A and B, were tested for differences between them using ANCOVA in Minitab 17, with total length as the covariate for all analyses. The samples for Type A and B from each of the two tributaries containing both morphotypes (stations Lohaghati/Gidiya and Doti/Surnaya) were then tested to establish whether significant variation exists between the two morphotypes within the sympatric populations associated with these sub-catchments.

Morphometrics

Lateral and ventral view digital photographs (3.2 megapixel resolution with Nikon Coolpix E3200) of the head region of *T. putitora* specimens were taken against a background of a graph board separated into 10mm squares to allow accurate scaling of the measurements prior to the landmark-based geometric morphometric analysis (***Fig. 3.3***). Care was taken to ensure that the fish were photographed from a position perpendicular to the surface to ensure consistent measurements. After inspection for image quality, 23 Type A and 14 Type B lateral photographs, and 21 Type A and 13 Type B ventral photographs were selected for the morphometric analysis. Group membership was assumed to be known *a priori*, as in this case the distinguishing features of the Type A and Type B samples are relatively pronounced.

Fig. 3.3 shows lateral and ventral views of morphotypes A and B, respectively. Oversized versions (red dots) of the provisional landmarks were added for clarity:

Lateral view ***(Fig. 3.3a, c).***

1. upper labial contact with superior surface of head,
2. lower labial surface at widest point,
3. termination of pre-maxilla joint,
4. pre-operculum groove on a straight line with landmarks 5 and 7,
5. terminus of operculum at furthest point from landmark 3,
6. upper terminus of operculum,
7. centre of eye orbit

Ventral view ***(Fig. 3.3b, d).***

1. rear terminus of dentary joint (right side),
2. tip of dentary bone,
3. rear terminus of dentary joint (left side),
4. front terminus of dentary bone at labial groove (left side),
5. rear terminus of mental lobe/rear edge of central point of labial groove,
6. front terminus of dentary bone at labial groove (right side),

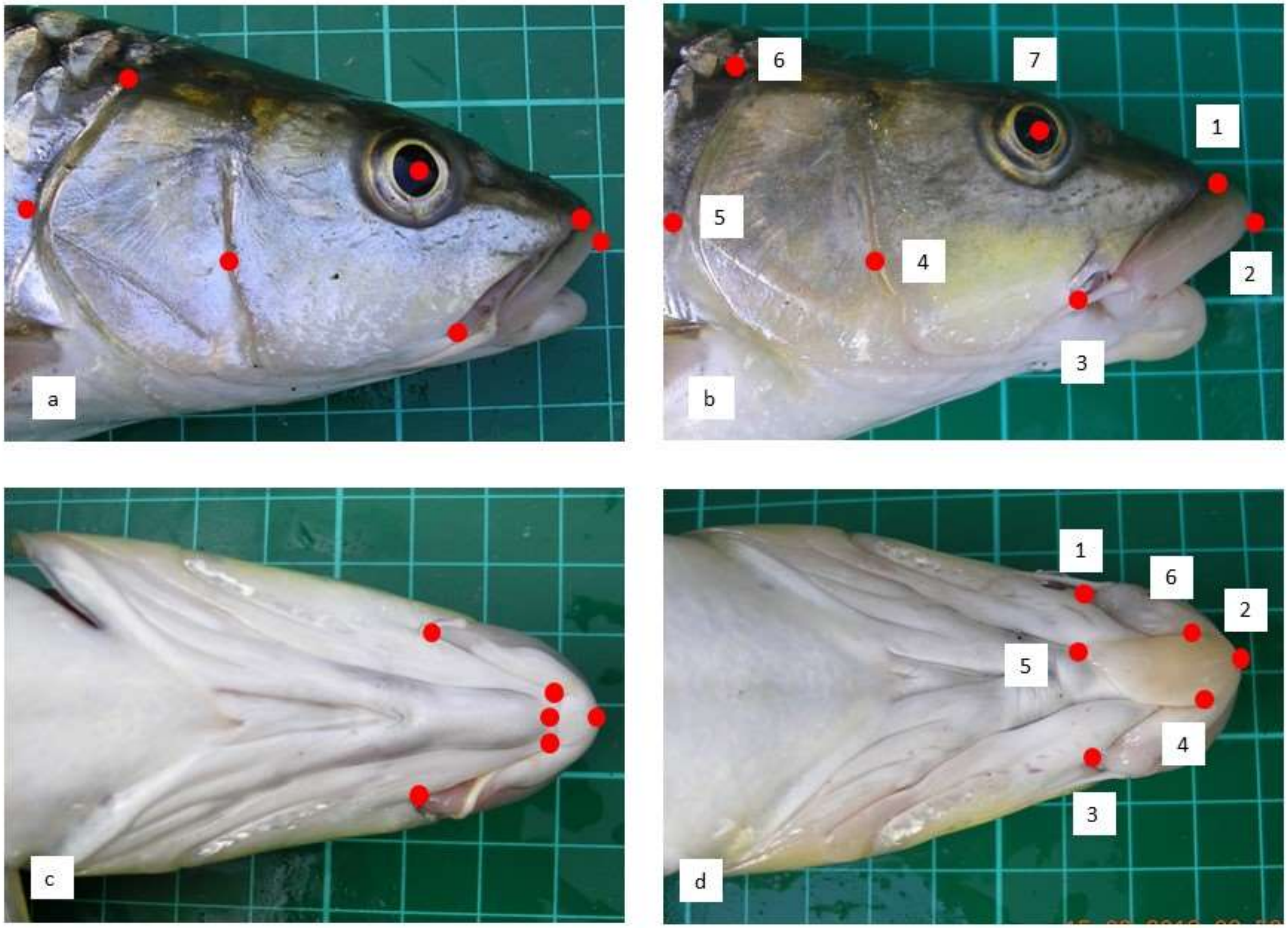


Figure 3.3: *Lateral and ventral views of* T. putitora *showing positions of landmarks (not to scale) used for morphological analysis. Type A Morphotype (a,c), Type B Morphotype (c,d).*

Analyses were undertaken with tpsDig 2 (Rohlf, 2006), which is used to define landmarks and for shape creation. Each sample was scaled to the 10mm background grid with tpsDig 2 before analysis to remove any size-dependent bias. Subsequent statistical analysis with MorphoJ 1 (Klingenburg, 2008) was based on Generalised Procrustes Analysis (GPA), a rigid shape analysis that uses isomorphic scaling, translation, and rotation to determine the most appropriate fit between two or more shapes equipped with landmarks.

Each configuration of landmarks was centred at the origin by subtracting the coordinates of its centroid (the average of X and Y coordinates of all landmarks) from the corresponding (X or Y) coordinates of each landmark. This translates each centroid to the origin and the coordinates of the landmarks now reflect their deviation from the centroid. The landmark configurations were scaled to unit centroid size by dividing each coordinate of each landmark by the centroid size of that configuration. One configuration was chosen to be the reference, then the second configuration rotated to minimize the summed squared distances between homologous landmarks (over all landmarks) between the forms. (Rohlf and Slice, 1990): The remaining configurations, representing subsequent samples, were then rotated to optimal alignment on the first, the average shape calculated, then all configurations again rotated to optimal alignment with the average, which becomes the new reference. At this point, the average shape was recalculated. If it differed from the previous reference, the rotations were recalculated using the most recent reference. When the latest reference matched the one before, the iterations stopped (Zelditch *et al.*, 2004).

Canonical Variate Analysis (CVA) was undertaken in MorphoJ to determine whether pre-defined groups (Types A and B) could be statistically distinguished based on the multivariate morphometric data derived from tpsDig2. CVA constructs new axes, which are linear combinations of the original variables, and all specimens are then ordered along these new axes. This process aims to maximise the separation of the group means relative to the variation within groups. Four covariance matrices were constructed with the Procrustes coordinates derived from the landmarks on A and B lateral and A and B ventral views. The data sets were then combined to produce Lateral and Ventral covariance matrices and analysed by Canonical Variate Analysis. CV shape change and CV score graphs plus permutation tests for Procrustes and Mahalanobis distances were produced. Finally, the canonical coefficients that relate the original shape coordinates to the CV scores were summarised.

For all three techniques, an overall comparison between all morphotype A and B specimens was undertaken, followed by consideration of variance between the forms and within groups in specific sub-catchments.

Results

A scatterplot displaying the length and weight relationship of individual samples is shown in ***Fig. 3.4.*** Type A samples numbered 64 and ranged from 15 - 130 cm total length (mean 48.5cm), Type B samples numbered 19 and ranged from 17 - 80 cm total length. Type A samples ranged in weight from 56.70g - 24947.56g. Type B from 56.70g - 5017.86g. The lowest weights recorded cannot be fully verified owing to the inaccuracy of the scales used.

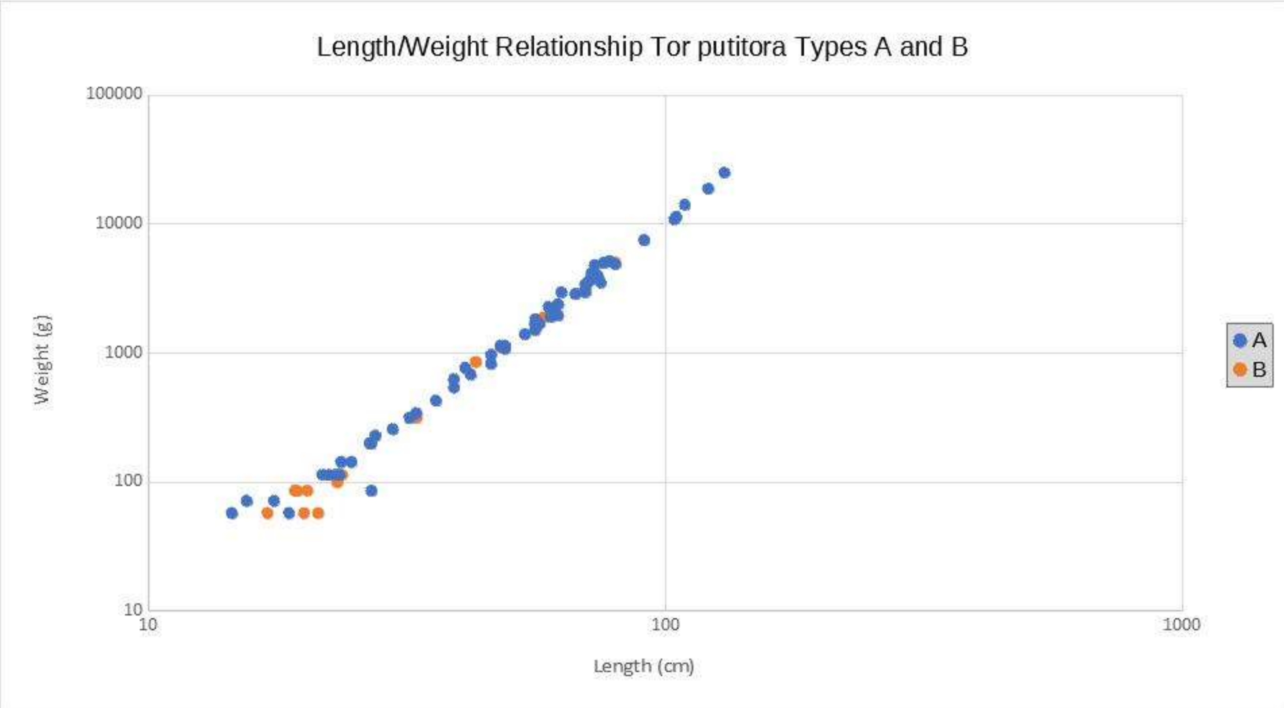


Figure 3.4: *Total length-weight scatterplot of both Type A and Type B* T. putitora *specimens obtained from all stations sampled across the Mahakali basin.*

Fin Ray Count Variation between Morphotypes A and B in overall Mahakali Catchment

Fin ray counts were recorded for Type A and Type B individuals of *T. putitora* from across the Mahakali catchment (**Fig. 3.5**).

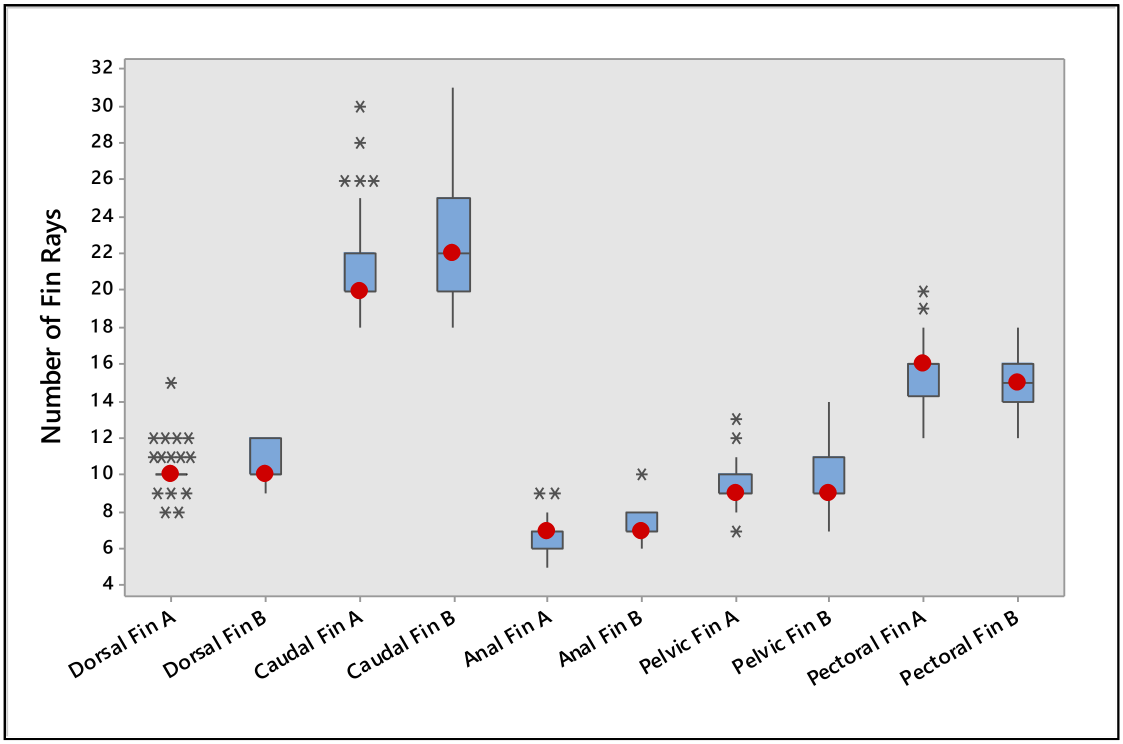


Figure 3.5: *Fin ray counts of Type A and Type B morphotypes. Combined totals of all stations. Red dots represent median values, the extent of the box indicates 25 and 65 percentiles, ends of the whiskers are maximum and minimum values and asterisks are outliers. Sample size-56 Type A, 19 Type B*

When the entire data set, consisting of all Type A and Type B samples from across the Mahakali catchment (***Fig. 3.5***), was analysed with the Mann-Whitney test for variation in counts between forms, the only significant result (p = 0.012, adjusted for ties) observed was for caudal fin rays with Type B recording a higher median (22) of rays than Type A (20) (***Table 3.2)***.

Table 3.2: *Results of Mann-Whitney tests in caudal fin ray counts - Stations Mahakali, Lohaghati/Gidiya, Doti/Surnaya, Ramganga/Sarju.*

Dataset	n	Fin	Median	DF	W	p
Total A	**56**	**Caudal**	**20**	**1**	**1926.0**	
Total B	**19**		**22**	**1**		**0.012 (adjusted for ties)**

Fin Ray Count Within Group Analyses

To examine variance among the four Type A populations with sufficient sample sizes of four or greater (stations Mahakali, Lohaghati/Gidiya, Doti/Surnaya, Ramganga/Sarju), tests for variance in fin ray counts were carried out between the stations. Type A samples from Station Mahakali showed a significantly lower median (20) for caudal fin ray count than Type A samples from Stations Lohaghati/Gidiya (21.50) and Ramganga/Sarju (22) (***Table 3.3)***. Dunn's pairwise comparisons were carried out for the six pairs of groups. There was strong evidence (p = 0.008, adjusted using the Bonferroni correction) of a difference between stations Mahakali and Lohaghati/Gidiya. There was no evidence of difference between the other pairs (***Fig. 3.6***). Station Mahakali is located on the main Mahakali River, downstream of Station Ramganga/Sarju and upstream of Stations Lohaghati/Gidiya and Doti/Surnaya, respectively. The population's origin and migratory habits are unknown.

To compare the two spatially separated Type B populations at stations Lohaghati/Gidiya and Doti/Surnaya, fin ray counts were also tested with Kruskal-Wallis. There was no significant difference observed between Type B morphotypes from either station.

Table 3.3: *Results of Kruskal-Wallis tests of Variance in fin ray counts of Type A morphotype from different sites.*

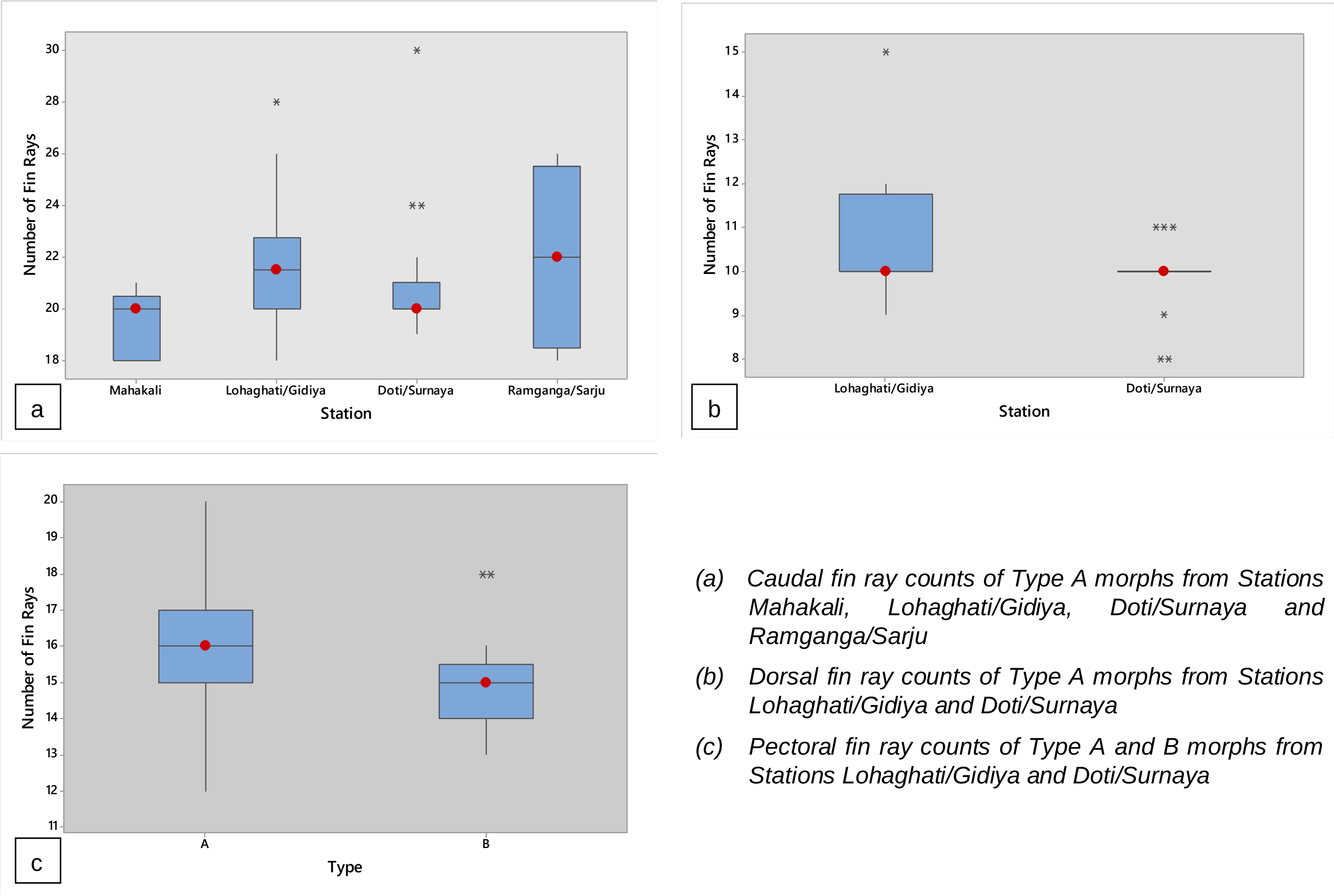
Station	n	Fin	Median	DF	H	p
1 Mahakali	**13**	**Caudal**	**20**	**3**	**10.49**	**0.015 (adjusted for ties)**
2 Lohaghati/Gidiya	**20**		**21.50**			
3 Doti/Surnaya	**23**		**20**			
4 Ramganga/Sarju	**4**		**22**			

Fin Ray Count Variation Between Morphotypes A and B in Shared Sub-catchments

To identify any variation in fin ray counts between the two morphotypes in the only two sub-catchments where they are found in sympatry, counts from fish at stations Lohaghati/Gidiya and Doti/Surnaya were tested against each other. A difference between medians was found for pectoral fin rays, Type A median = 16, Type B median = 15 (p = 0.041 adjusted for ties; ***Table 3.4***).

Table 3.4: *Results of Kruskal-Wallis tests of Variance in pectoral fin ray counts of Type A and B morphotypes from stations Lohaghati/Gidiya and Doti/Surnaya.*

Type	n	Fin	Median	DF	H	p
A	**43**	**Pectoral**	**16**	**1**	**4**	
B	**19**		**15**	**1**	**4.18**	**0.041 (adjusted for ties)**



(a) Caudal fin ray counts of Type A morphs from Stations Mahakali, Lohaghati/Gidiya, Doti/Surnaya and Ramganga/Sarju

(b) Dorsal fin ray counts of Type A morphs from Stations Lohaghati/Gidiya and Doti/Surnaya

(c) Pectoral fin ray counts of Type A and B morphs from Stations Lohaghati/Gidiya and Doti/Surnaya

Figure 3.6: *Comparison of caudal (a) and dorsal (b) fin ray counts of Type A morphs. Comparison of pectoral fin ray counts (c) of total Type A and Type B morphs from stations Lohaghati/Gidiya and Doti/Surnaya.*

Scale Counts

Scale counts were recorded for Type A and Type B individuals of *T. putitora* from across the Mahakali catchment. Mean counts were as follows: Type A lateral line, 27.92 (±2.75), lateral line to dorsal, 4.48 (±0.5), lateral line to anal, 4.17 (±0.38), around caudal, 8.58 (±1.00); Type B lateral line, 28.63 (±2.01), lateral line to dorsal, 4.32 (±0.48), lateral line to anal, 4.11 (±0.32), around caudal, 8.84 (±1.01).

Scale Count Variation Between Morphotypes A and B in Overall Mahakali Catchment

The Kruskal–Wallis test did not identify any significant difference for any of the scale counts between Type A and Type B samples derived from the entire Mahakali catchment (***Fig. 3.7***).

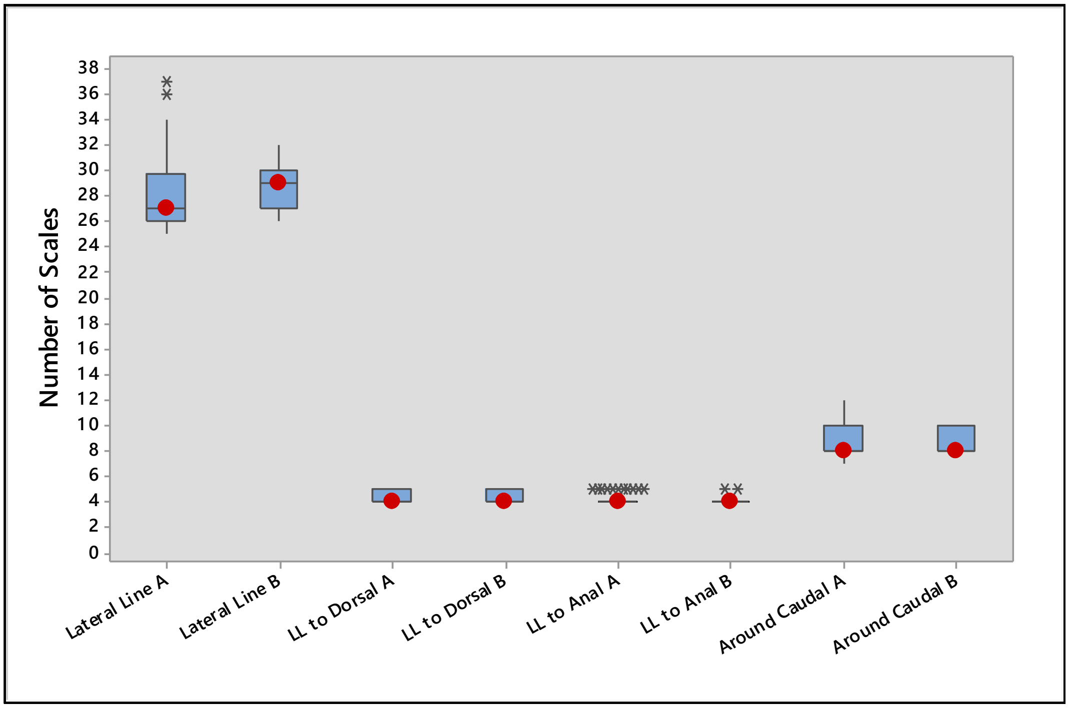


Figure 3.7: *Scale counts for Type A and B morphotypes. Combined totals of all stations. Red dots represent median values, the extent of the box indicates 25 and 65 percentiles, ends of the whiskers are maximum and minimum values and asterisks are outliers. Sample size-56 Type A, 19 Type B*

Scale Count Within Group Analyses

Type A morphotypes from Station Mahakali showed a significantly higher median (5) of lateral line to dorsal scale counts compared to Type A morphotypes from Station Lohaghati/Gidiya and Doti/Surnaya (both medians = 4), and an identical count to station Ramganga/Sarju (***Table 3.5***). Dunn's pairwise comparisons indicated a difference (p<0.001, adjusted using the Bonferroni correction) between stations Mahakali and Lohaghati/Gidiya. Stations Mahakali and Doti/Surnaya were also significantly different (p=0.014). There was no evidence of difference between the remaining pairs.

Station Mahakali Type A morphotypes also recorded a significantly lower median (27) of lateral line scales than Type A morphotypes from station Lohaghati/Gidiya (27.5), station Doti/Surnaya (28) and station Ramganga/Sarju (28.5) (see ***Table 3.5***). Dunn's pairwise comparisons were carried out for the six pairs of groups. There was strong evidence (p=0.033) of a difference between stations Mahakali and Doti/Surnaya. There was no evidence of difference between the other pairs.

Table 3.5: *Results of Kruskal-Wallis tests of scale counts of Type A morphotypes.*

Station	n	Scale Count	Median	DF	H	p
Mahakali	**13**	**Lateral Line to Dorsal**	**5**	**3**	**18.10**	**<0.001 (adjusted for ties)**
Lohaghati/Gidiya	**20**		**4**			
Doti/Surnaya	**23**		**4**			
Ramganga/Sarju	**4**		**5**			
Mahakali	**13**	**Lateral Line**	**27**	**3**	**8.82**	**0.032 (adjusted for ties)**
Lohaghati/Gidiya	**20**		**27.50**			
Doti/Surnaya	**23**		**28**			
Ramganga/Sarju	**4**		**28.50**			

Scale Count Variance Within Morphotypes A and B in Shared Sub-catchments

To further investigate potential differences between the two morphotypes, A and B in the two sub-catchments stations Lohaghati/Gidiya and Doti/Surnaya, where they are found in sympatry, scale counts were tested with the Kruskal-Wallis test. No significant difference was observed between Type A and Type B in either catchment separately, or within the two catchments combined.

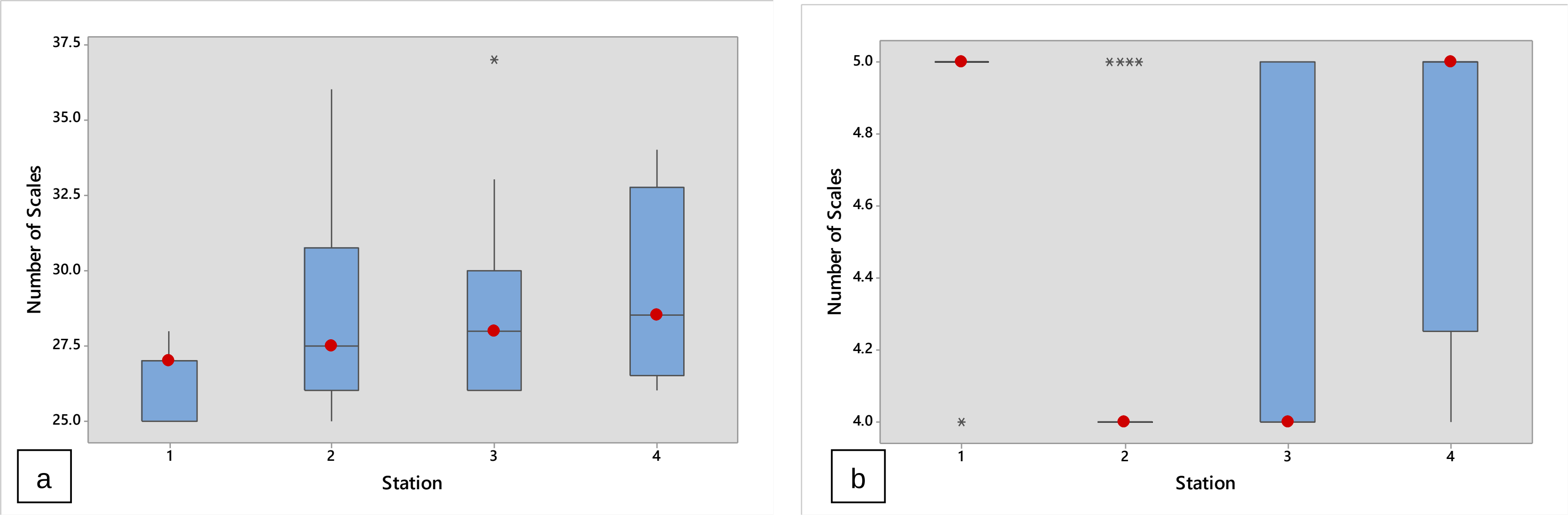


Figure 3.8: *Comparison of scale counts of Morphotype A from stations 1-Mahakali, 2-Lohaghati/Gidiya, 3-Doti/Surnaya and 4-Ramganga/Sarju*

(a) Lateral line scale counts of Morphotype A from Stations 1-4

(b) Lateral line to Dorsal fin scale counts of Morphotype A from Stations 1-4

Allometry

Allometric variation between Morphotypes A and B in overall Mahakali catchment results were as follows:

There were no significant differences between the groups in any of the measurements (total length (tip of snout to fork), head length (tip of snout to furthest rear point of operculum), pre-dorsal fin body circumference, pre-anal fin body circumference) taken (ANCOVA with total length as covariate, $p > 0.05$)

Allometric variation between Morphotypes A and B in shared sub-catchments results conformed with the overall Mahakali catchment results:

The results for Type A versus Type B samples from stations 2 and 3 also produced no significant differences between the groups in any of the measurements (total length (tip of snout to fork), head length (tip of snout to furthest rear point of operculum), pre-dorsal fin body circumference, pre-anal fin body circumference) (ANCOVA with total length as covariate, $p > 0.05$).

Morphometrics

Initial CVA analysis utilising seven landmarks for the lateral view and six for the ventral view showed a clear separation of Type A and B for both views (***Figs. 3.9*** and ***3.10***). The variation between both, A and B lateral and A and B ventral, accounted for by the canonical variates was 100%.

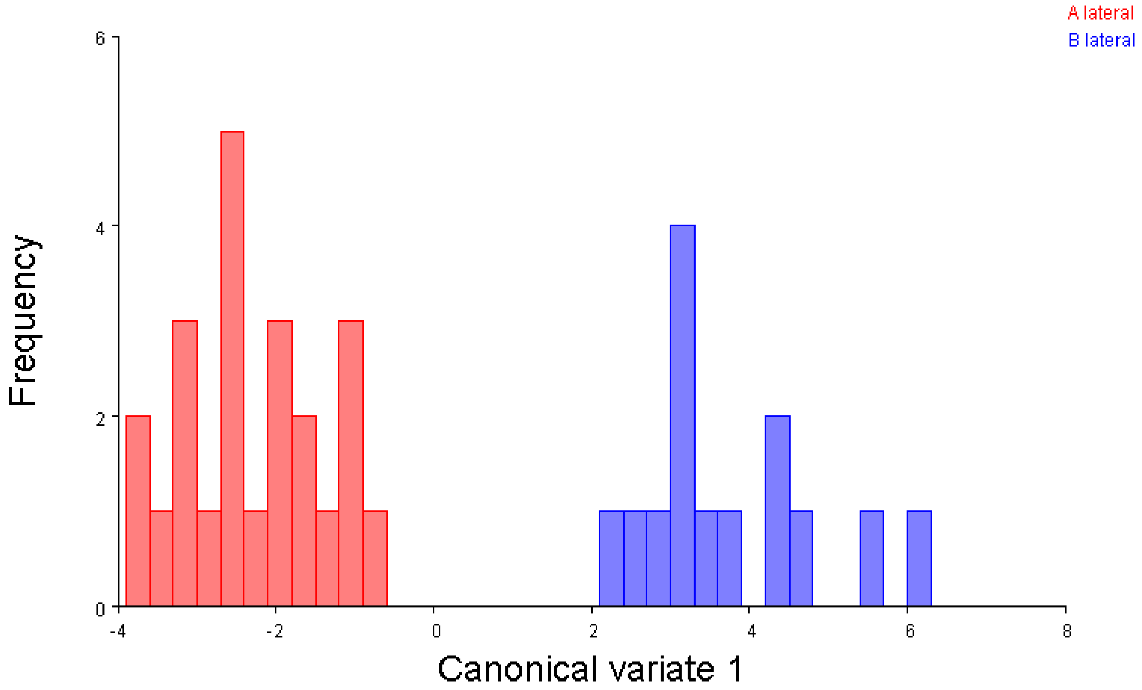


Figure 3.9: **Figure 3.9:** *Canonical Variate Analysis scores of lateral views of head shapes of 23 Type A and 14 Type B* Tor putitora *morphotypes.*

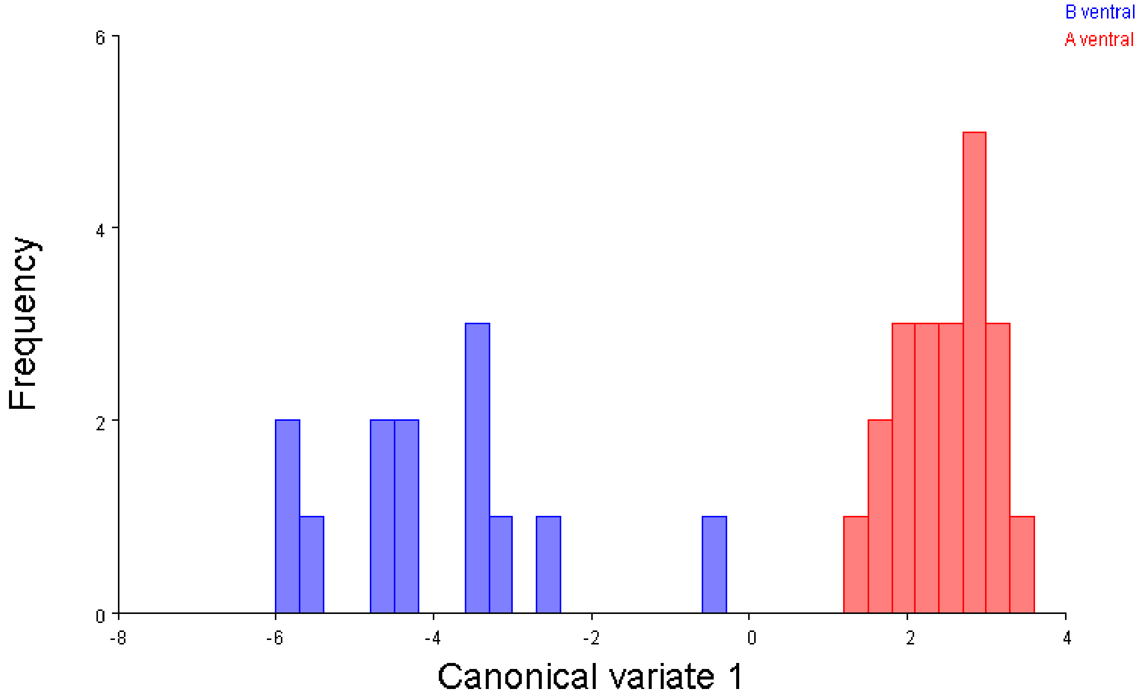


Figure 3.10: *Canonical Variate Analysis scores of ventral views of head shapes of 21 Type A and 13 Type B* Tor putitora *morphotypes.*

Table 3.6 summarises the result of the analysis, including permutation significance tests of between group differences (Eigenvalues-amount of variance, Mahalanobis distance-distance relative to the centroid and Procrustes distance-square root of the sum of squared distances in landmark position*)*. The Canonical Coefficients (a measure of the value of association between two canonical variates) are displayed in ***Table 3.7***. For both lateral and ventral views, there was a highly significant difference between the groups when considering both Mahalanobis and Procrustes distances.

Table 3.6: *Summary of Eigenvalues, Mahalanobis distance and Procrustes distance with permutation test p-values and number of observations for ventral and lateral views of T. putitora A and B morphotype landmark configurations*.

Groups	Observations	Eigenvalue	Mahalanobis distance	p-value	Procrustes distance	p-value
A Ventral	**21**	**10.32**	**6.41**	**<0.001**	**0.39**	**<0.001**
B Ventral	**13**					
A Lateral	**23**	**8.93**	**5.99**	**<0.001**	**0.07**	**<0.001**
B Lateral	**14**					

Table 3.7: *Canonical Coefficients for lateral and ventral views of T. putitora A and B morphotype landmark configurations. Lateral has seven landmarks, ventral has six.*

CV1	Lateral x	CV1	Lateral y	CV1	Ventral x	CV1	Ventral y
x1	**46.76**	**y1**	**191.93**	**x1**	**2.12**	**y1**	**-0.95**
x2	**-49.14**	**y2**	**-188.07**	**x2**	**9.29**	**y2**	**10.34**
x3	**5.90**	**y3**	**23.97**	**x3**	**-1.19**	**y3**	**-6.94**
x4	**19.11**	**y4**	**10.74**	**x4**	**-21.98**	**y4**	**8.30**
x5	**5.32**	**y5**	**4.42**	**x5**	**11.13**	**y5**	**-4.47**
x6	**-26.93**	**y6**	**-4.60**	**x6**	**0.62**	**y6**	**-6.27**
x7	**-1.01**	**y7**	**-38.38**				

The shape change results from the CVA analysis and the relative importance of each landmark in differentiating between Type A and Type B morphotypes are visualized in a lollipop graph (***Fig. 3.11***). In the lateral view landmarks 2 and 7, representing the thickness of the upper lip and the position of the eye, respectively, show the greatest variation. In the ventral view landmark 5, representing the mental lobe, has by far the largest range of any of the landmarks. The combined shape change of the other landmarks in the ventral view suggests that Type B morphotypes have a longer and narrower head than Type A morphotypes.

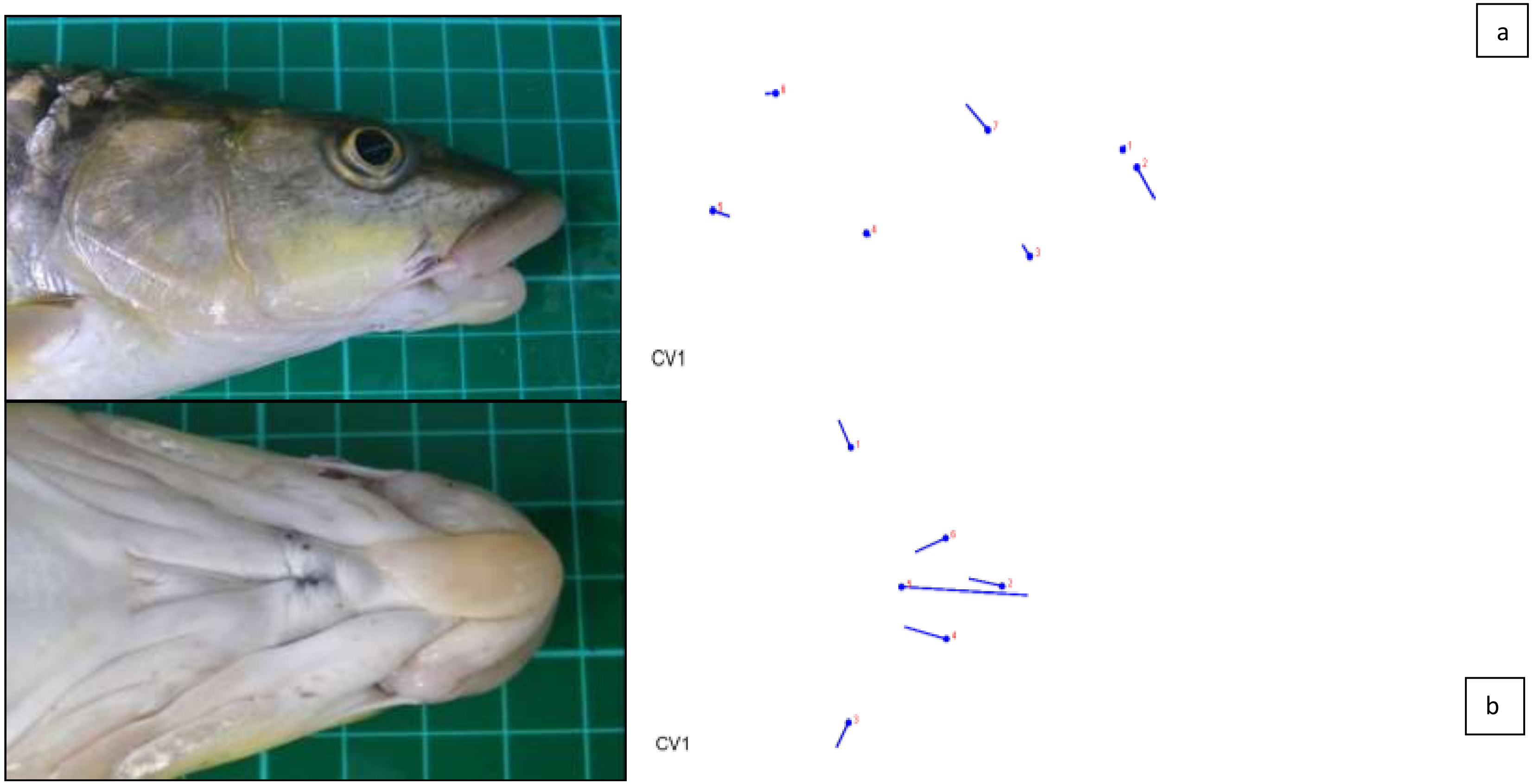


Figure 3.11: *Lollipop graphs of CV shape changes displaying relative influence of landmarks, a=lateral, b=ventral (Type B morphotype shown). The 'stem' on each lollipop in CV1a represents the change from Type B to the 'head', Type A. In CV1b the reverse is the case.*

Discussion

The use of meristic data has historically proven to be a valuable tool in taxonomy for identification of fish to species level (Dasgupta, 1991, Kurup and Radhakrishnan, 2010, Simon *et al.*, 2010), and to population, or stock, level (Bhatt *et al.*, 1998, Turan, 2004, Patiyal *et al.*, 2014). The latter two Indian studies (Patiyal *et al.,* 2014 and Bhatt *et al.,* 1998) and a Pakistani study (Pervaiz *et al*. 2012) were conducted on geographically isolated *T. putitora* populations with no connecting water bodies, and hence there was greater potential for distinction to develop owing to reproductive isolation; even then they did demonstrate that intra-specific variation commonly exists within this species. The present study is specifically concerned with populations within one interconnected river system, the Mahakali River, where the potential for spatial reproductive isolation exists but has not been demonstrated and the relative proximity (2-3 km) of the breeding sub-catchments e.g. Surnaya / Doti (station 3) and Lohaghati/Gidiya (station 2) could possibly facilitate genetic intermixing of stocks. Arora and Julka (2013) examined morphometric and meristic characteristics of two populations of *T. putitora* from the Beas River system in Himachal Pradesh, India and found few significant differences (lateral line 25.66 (±0.479), 26.5 (±0.508), lateral line to anal 2.5 (±0), 3.5 (±0) scale counts and caudal fin ray counts ±17 (±0), 19 (±0) within the data. The results obtained in the current meristic study display a similar number of significant differences (number of caudal and pectoral fin rays between Type A and Type B morphotypes, number of dorsal fin rays, lateral line and lateral line to dorsal scale counts within the Type A group between sampling sites) in the meristic data. The widespread overlap in counts presented in the boxplots (***Figs 3.5-3.8***) suggest that the meristic characteristics do not provide conclusive evidence to rely solely on these data for the identification of individual specimens to population level in the investigated portion of the Mahakali River system.

The allometric analysis in the current study produced no evidence of differences between Type A and Type B morphotypes for any of the measurements taken. However, a considerable amount of research has been conducted in India (Bhatt *et al.*, 1998, Barat *et al*., 2012, Patiyal *et al.*, 2014), Indonesia (Haryono and Tjakrawidjaja, 2006) and China (Chen and Yang, 2004) on the allometric variation within the *Tor* genus and within different populations of a single species, for the purpose of inter and intra-specific population identification. The majority of these studies have identified significant differences between populations from discrete water bodies. Bhatt *et al.* (1998) found differences in minimum body depth, pre-anal length, head depth, eye diameter and post orbital distance between samples from the River Ganges and Gobindsagar reservoir in two separate states in the north of India. In all, they measured 20 different characters in specimens obtained from local fishermen. Barat *et al.* (2012) studied populations of *T. putitora* from five rivers in four states across the breadth of north India using a truss network system of morphometric analysis and the results of their discriminant function analysis allowed them to correctly classify 100% of their samples to the respective river systems. In this case, the main differences among the populations were related to the head length and depth, body depth and thickness of caudal peduncle. A particularly relevant study, as it contains samples from one of the tributaries of the Mahakali, the Ladhiya River was conducted by Patiyal *et al.* (2014). They used multivariate analysis to identify differences in maximum and minimum body depth, orbit diameter, head length and depth, dorsal fin height and depth and managed to clearly discriminate between wild stocks of *T. putitora* from the Ladhiya River in Kumaon province, Uttarakhand and captive stocks from a fish farm in Lucknow, Uttar Pradesh. It is clear from the above studies that *T. putitora* populations from different catchments can be identified in specific cases using a detailed system of measurements. In Indonesia, Haryono *et al.* (2006) examined museum specimens of *Tor* genus utilising canonical variate analysis and identified four species from differences in inter orbital width, caudal peduncle length and depth, head width and body depth. The study by Chen and Yang (2004) in China has produced evidence for a new species, *Tor yingjiangensis*, based on differences in lateral line to anal scale counts, shorter caudal peduncle length, greater caudal peduncle depth and lesser body depth. None of these investigations were confined to populations within a single river system or lake as in this study. The previous studies have, however, all included a greater number of variables than the current investigation due to the fact that they were carried out on dead specimens, either collected in the field or obtained from museums, which allowed a more thorough, but time consuming method. This was impossible to reproduce with live specimens in the field during the current study without causing significant damage to the samples before their release.

Intra-specific morphological variation in the heads and mouthparts of various fish species are well documented and have been linked to resource partitioning (Adams and Huntingford, 2004), substrate variation (Komiya and Watanabe, 2011), and prey type (Fraser *et al.*, 1998). Adams and Huntingfords' (2004) study examined sympatric morphotypes of Arctic Charr (*Salvelinus alpinus*) from Loch Rannoch, Scotland and stated that morphological characters, which played a functional part in food acquisition were highly plastic and mainly driven by environmental factors, although a small, but decisive genetic effect was also present. Their overall conclusion suggested that resource partition-driven phenotypic plasticity plays a significant role in trophic polymorphism with longer-term implications of potential genetic divergence and subsequent speciation. Variation in substrates found in lacustrine environments has a parallel in the riverine habitats utilised by *T. putitora* (sand, boulders, gravel) where different prey species inhabit distinct areas and feed in separate parts of the water column in a manner similar to lakes. The question of whether any resource partitioning between the morphotypes of *T. putitora* can be detected is addressed in the chapter on stable isotopes below.

The landmark-based geometric morphometric analysis used in this study produced a very clear separation of the two morphotypes A and B for both lateral and ventral views as opposed to an incremental progression from one type to the other. It is therefore reasonable to deduce that there are two distinct morphological variants. The thickness of the upper lip in Type B individuals and the position of the eye, landmarks 2 and 7, respectively, show the greatest variation in the lateral view and the lip thickness could be an important visual indicator of Type B classification, whereas the eye position would be difficult to evaluate in basic in-field assessments of type identity. The length/existence of the mental lobe in the ventral view, corresponding to landmark 5 could make the presence of the mental lobe a reliable determining factor in Type B identification.

It is unknown whether morphotypes A and B, although sharing the same habitat, are reproductively isolated, spatially or temporally (Adams *et al*., 2006, Amundsen *et al.,* 2008), or whether the phenotypic variation is a result of environmental factors alone (Svanback and Bolnick, 2007, Vehanen and Huusko, 2011). It is possible for sympatric populations of different morphotypes to exist within one habitat as Adams *et al.* (2006) established in their study which showed that Arctic Charr (*Salvelinus alpinus*) populations within three connected lochs with no restrictions on movement between them displayed clear differences in head morphology and significant differences in six microsatellite loci. They concluded that high levels of breeding site fidelity and hence, spatial reproductive isolation was the greatest likely factor in divergence of phenotype. Individuals of a single species can also display morphological variation within the same habitat, while maintaining common breeding habits, as suggested by Svanback and Bolnick (2007). They manipulated population density of three-spined sticklebacks (*Gasterosteus aculeatus* (Linnaeus, 1758)) within enclosures and observed phenotypically different individuals adapt to alternative prey types correlated to their body morphologies. This would not appear to be the case in the current study as the sticklebacks displayed a continuum of morphological variation, rather than two distinct morphotypes.

Coldwater lakes and the fish species, which inhabit them, have been the focus of the majority of studies on intra-specific morphological variation of the head and mouthparts of fishes. Large post-glacial lakes contain three relatively stable environments, whose differences are dictated by light penetration and temperature, the limnetic, littoral and benthic zones. Each of these zones support distinct ecosystems whose food resources can be exploited by different morphotypes of the same fish species (Adams and Huntingford, 2002) resulting in planktivorous, benthivorous and piscivorous fish with their heads and mouthparts adapted to handle different sizes and behaviour of prey. The environmental conditions in the Mahakali River and its breeding tributaries are far more unstable in terms of temperature, light penetration, current and sediment load (see Study System above). Nevertheless, distinct zones are still identifiable, including deep pools, rapids, eddies and tributary junctions. Each zone has its specific substrate encompassing mainly large boulders (deep pools), smaller cobbles (rapids), sand (eddies) and gravel (tributary junctions), which support a range of algae, aquatic invertebrates, and prey fish. The possibility of two morphotypes of one species sharing the same habitat, but utilising different food resources in a riverine, as opposed to lacustrine, environment has been demonstrated in several studies. For example, Whiteley (2007) examined stomach and intestine contents and concluded that a long snouted morphotype of the Mountain Whitefish *Prosopium williamsoni* (Girard, 1856) in the Clark Fork River in Montana, U.S.A. exploited a greater proportion of benthic prey items than the short snouted morphotype of the same population, which mostly obtained prey from higher in the water column. Cucherousset *et al.* (2011) identified two morphotypes of the European Eel *Anguilla anguilla* (Linnaeus, 1758) in the River Frome (UK), a wide jawed morphotype, which fed at a higher trophic level in the centre of the river and whose stomach contents contained a higher percentage of fish to invertebrate prey, and a narrow jawed morphotype, which fed at a lower trophic level (more invertebrates than fish) closer to the riverbank. Nakano (1999) examined prey items found in the stomachs of a Lenok Trout *Brachymystax lenok* (Pallas, 1773) morphotype from the Artyemovka and

Arsyenevka Rivers in South Eastern Russia with sub terminal mouthparts and linked its head morphology to a diet of benthic invertebrates (Trichoptera). Its sympatric morphotype with terminal mouthparts was found to contain a higher percentage comparatively of terrestrial insect prey found drifting higher in the water column. These studies demonstrate that diet-based polymorphism does occur in rivers as well as lakes and that the morphological variation displayed in the current study should be further investigated. In order to examine whether *T. putitora* in the Mahakali River are also displaying diet-based polymorphism in a similar manner as the riverine species above by utilising different parts of the water column and the food resources which are found in each, stable isotope ratios of carbon and nitrogen, which can shed light on dietary composition by comparing uptake of the heavier isotopes, and their relationship with relative trophic position, will be discussed in Chapter 5 of the presented thesis and Chapter 4 will examine physico-chemical conditions and aquatic invertebrate populations in the breeding tributaries to determine whether the spawning and juvenile habitats display distinctive environmental conditions, which could affect prey type or availability.

Limitations on data collection as discussed above were due to the constraints imposed on taking measurements from live specimens in remote and difficult terrain (only at sites Ramganga/Sarju, Lohaghati/Gidiya and Doti/Surnaya were any samples obtained from local fishermen, the others were accessible only by raft) and in very high ambient temperatures as samples recovered by angling tended to be from some distance outside the campsites, often in precarious situations on cliff faces or unstable slopes with no shade in which to place the fish. To ensure the welfare of the fish their time out of the water was limited with the knock-on effect of severely limited time to take the required measurements, as opposed to the more relaxed conditions associated with examining dead samples transported to the laboratory or obtained from museums. Improvements in methodology could be introduced in future studies by the assembly of a larger, better equipped team of researchers with portable laboratory facilities including holding and anaesthetising tanks and their supplementary equipment to allow for a more complete set of measurements to be taken before returning the fish to the river. Substituting a higher resolution camera than the one utilised in this study could allow full body photographs with clearer views of meristic counts and a landmark based full truss analysis to be done in the laboratory, which would produce a more comprehensive and robust dataset.

In summary, there is strong evidence from the morphometric analysis of differences in the shape of the head and mouthparts of Type A and Type B morphotypes. In contrast the meristic and allometric comparisons have produced only limited evidence of differing characteristics, which are likely to be unreliable for use as defining factors in identification of the two morphological variants, due to the overlaps in counts. To investigate potential drivers of the variation in head and mouthpart morphology, subsequent chapters will explore the juvenile habitat physico-chemistry and potential food resources as represented by aquatic invertebrate communities within each breeding tributary as well as an examination of the trophic relationship of the two morphotypes through an examination of scale tissue stable isotope ratios.

Chapter Four

Physico-chemical Conditions and Aquatic Invertebrate Communities in Spawning Streams of *Tor putitora* in Western Nepal

Introduction

An understanding of the physico-chemical conditions and aquatic invertebrate communities in the habitat of *T. putitora* during its juvenile stage is important to any study interested in investigating variation within populations. Especially, when the sub-catchment is composed of two or more potential spawning/rearing streams which may differ in ecological character. Environmental conditions within a habitat and biological diversity of aquatic fauna have been shown to be closely linked (Raven *et al.,* 1998) and utilisation of the resulting different food resources has been suggested as a factor in evolutionary processes such as reproductive isolation (Amundsen, *et al.,* 2008). The Mahakali catchment contains stream ecosystems of two general types: glacier-melt/seasonal snow-melt and spring-fed. The contribution of each type to the flow of the Mahakali River varies throughout the year and is closely tied to the Indian monsoon weather pattern. During the monsoon season (June-September) around 80% of annual precipitation falls, as snow above 5000-6000m and as rain at lower altitudes (Anon., 2015). After the monsoon, only a few brief periods of precipitation, due to weather patterns known as 'western disturbances' lasting two or three days are observed with the snowline extending down to 3000m (Sharma, 1993). These periods of winter precipitation, however, are an important factor in replenishing the mass of glaciers in north western Nepal, including the Milam glacier, the main source of the Mahakali (Shrestha and Aryal, 2011). From April until the commencement of the monsoon rains, the snowline gradually recedes as snow-melt increases its input into the system until the time of highest solar insolation and temperature in July and August, when melting of the glaciers makes its maximum contribution (Thayyen and Gergan, 2010). The steep topography of the catchment has resulted in turbulent and rapidly flowing rivers of the riffle, rapid and pool type with substrates composed of gravel, cobbles, boulders and sand, which are continuously remodelled during the periods of extreme high water levels and velocities associated with the monsoon rains. The base of the food web at higher altitudes rests on benthic algae, which support communities of aquatic invertebrates in each of the habitats formed by the combinations of elements of the substrate under the influence of seasonal current velocities. Generally, taxonomic richness of aquatic invertebrate communities increases with declining altitude in Himalayan rivers (Suren, 1994). Streams which contain a high percentage of glacial meltwater are subjected to elevated sediment loads, turbidity, channel instability and low concentrations of nutrients (Uehlinger *et al.*, 2002). Milner *et al.* (2001) charted the downstream succession of macroinvertebrates in glacial rivers as: -2°C to 4°C, Chironomidae, Tipulidae, Oligochaetae; above 4°C, Perlodidae, Baetidae, Simulidae and Empididae. The streams dominated by springwater and rainwater are predominantly warmer, more abundant and diverse in macroinvertebrates, and less turbid, thermally stable and relatively nutrient rich (Malard *et al.*, 1999) with autochthonous algal growth combined with allochthonous inputs from forested catchments. The practice of terraced agriculture within stream catchments in the Himalayas has an influence on the ecology of the streams and has been demonstrated to have effects on aquatic invertebrate community composition (Brewin *et al.*, 2000). Habitats in these zones experience physical modifications (wider channels, fewer rapids, finer particle size of substrate and reduced tree cover), accompanied by increases in aquatic invertebrate abundance (Manel *et al.*, 2000). Urban sources of pollution from settlements within catchments include: untreated sewage, hydrocarbons, plastics and run off from waste disposal sites (Kirch, 2002). In order to understand potential influences on the juvenile growth phases of the two morphological variants of *T. putitora* introduced in chapters 1, 2 and 3, an examination of their habitats is essential.

The sub-catchments of most interest in determining the identities of the two morphological variants of *T. putitora* introduced in Chapter 1 are the Lohaghati/Gidiya, and the Surnaya/Doti, the only sample sites which produced both Type A and B *T. putitora* morphotypes from what is hypothesised to be sympatric populations of the same species. The comparative physico-chemical and biological profiles of the four tributaries which form the sub-catchments appeared to differ greatly during sampling trips and during previous reconnaissance visits and these conditions may be a factor in influencing reproductive and migratory behaviour patterns. A possible hypothesis is that the two morphotypes breed in the separate tributary components (*e.g.* Doti, Surnaya) of the two respective sub catchments and utilise different food resources. An analysis of physico-chemical conditions, potential prey composition and aquatic invertebrate diversity may provide insights in this area.

Aims

This chapter investigates whether differences exist in physico-chemical conditions or aquatic invertebrate community compositions between breeding tributaries of the Mahakali River and between seasons which may be linked to the occurrence of the two morphotypes of *T. putitora*.

Methods

Study system

To collect information on the physico-chemical and ecological conditions in the breeding tributaries of the Mahakali catchment, eight aquatic invertebrate and water sampling sites were chosen (***Fig. 4.1***). The selected sites were all located well below the average snowline limit with only the Chamaliya, Sarju and Panar tributaries receiving any snow-melt and/or glacial water input as a component of their flow during the pre-monsoon and monsoon melt period (***Fig. 4.2***) from their tributaries at higher altitudes (***Fig. 4.3***).

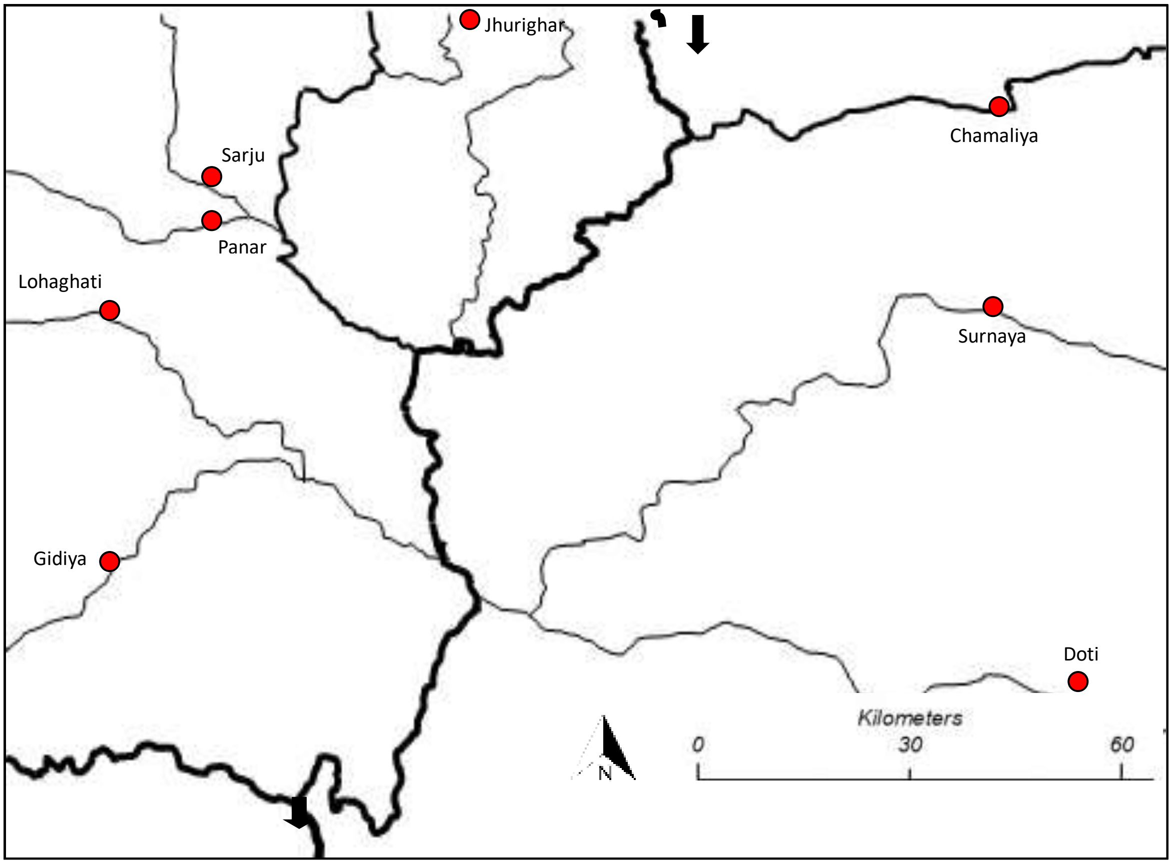


Figure 4.1. The study area: *Central river with flow direction arrows is the Mahakali. Peripheral rivers are breeding tributaries with eight aquatic invertebrate/physico-chemistry sample sites (red dots).*

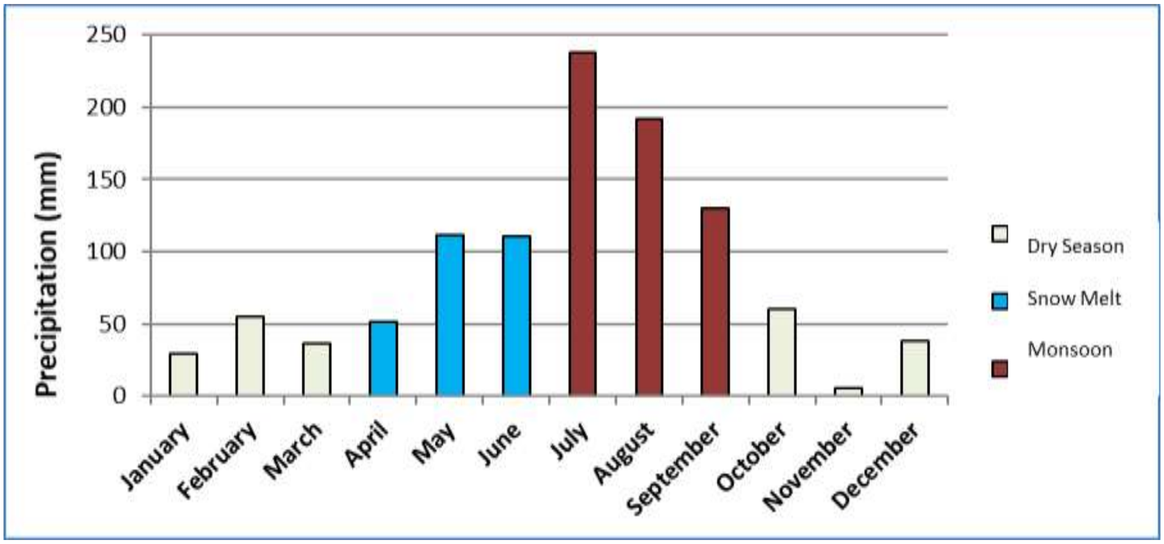


Figure 4.2: *Graph of mean monthly precipitation (mm) in Mahakali catchment including main hydrological seasons (dry, snow-melt and monsoon), data from Government of India Water Resources Study - Anon (2015).*

The tributaries Jhurighar, Lohaghati, Gidiya, Doti and Surnaya are perennial krenal, or spring, groundwater and rain fed streams, which originate in the lower hills below the snowline and record their peak volumes during the monsoon rains plus localised flood events during the period of western disturbance weather patterns.

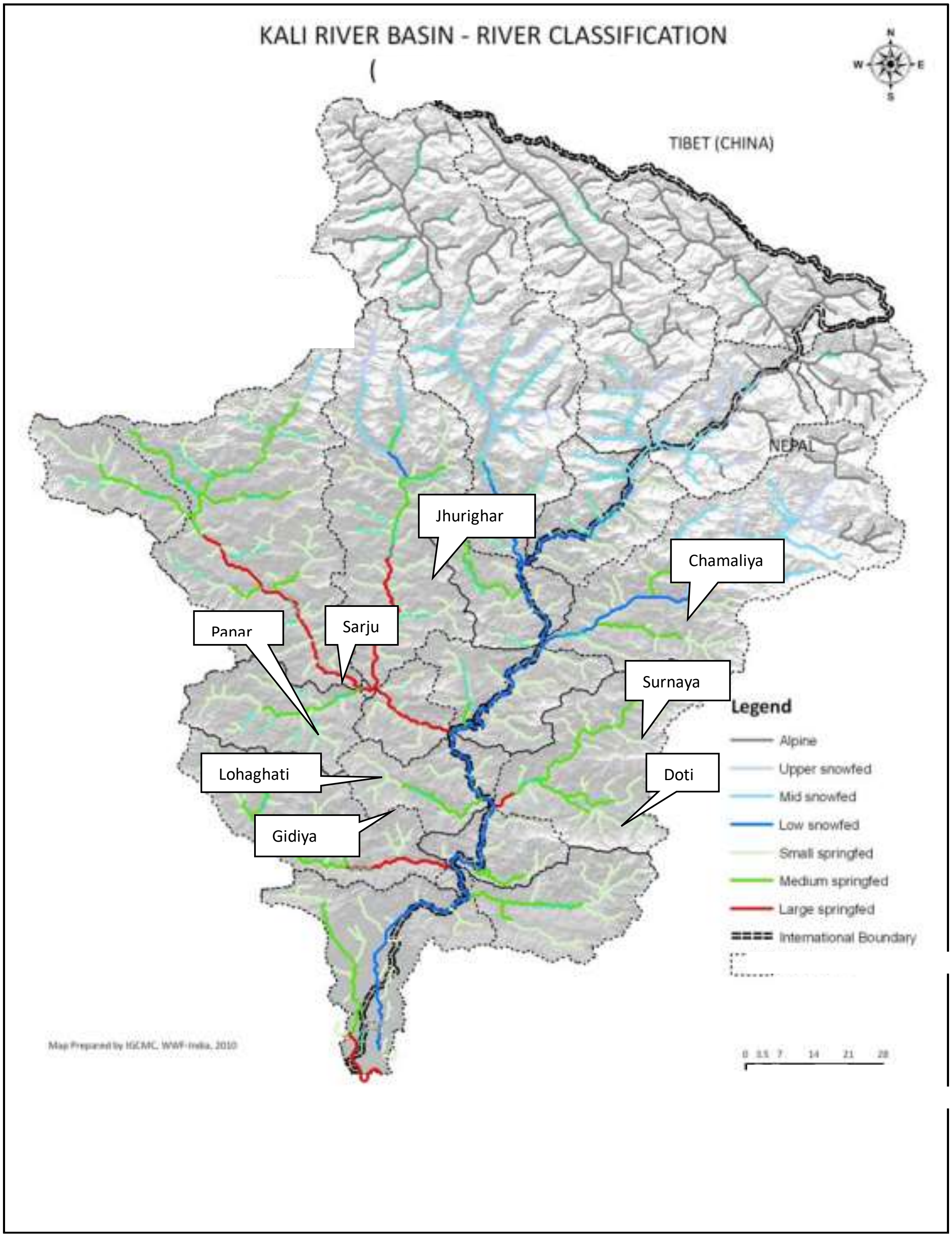


Figure 4.3: Mahakali River catchment - River classification (adapted from IGCNC WWF India 2010).

Sampling Scheme

Aquatic invertebrate and physico-chemical sampling for the post-monsoon (PM) and snow-melt (SM) season took place in Sept-Nov 2012 and Mar-Apr 2013, respectively, the two periods when both the tributary stream volume and velocities are safe to sample, and *Tor* species are active within sampling range. The Gidiya (1561m) and Lohaghati (1475m) sample sites are located at similar altitudes and the two rivers merge downstream to form one of the tributaries of the Mahakali that contains both morphotypes of *T. putitora*. The other tributary joining the Mahakali with both morphotypes is composed of the Doti and Surnaya rivers. Sample sites were located at 1503m and 1106m, respectively. The Jhurighar, Sarju and Panar rivers merge and form the largest tributary and sub-catchment of the Mahakali. These mainly rain- and groundwater fed breeding tributaries join the large snow fed East Ramganga River shortly before joining the Mahakali. The Jhurighar enters furthest upstream and the sample site is located at 981m. The Panar joins the Sarju very shortly before its junction with the East Ramganga and the two sample sites are at similar altitudes (509m, 520m), respectively. The Chamaliya River is the furthest upstream tributary of the Mahakali, which is not completely dominated by glacial water. The sample site was located at an altitude of 614m.

Aquatic Invertebrates

Aquatic invertebrate samples were collected during the snow-melt and post-monsoon seasons from eight breeding streams for quantification of invertebrate abundance and diversity within the breeding habitats of the *T. putitora* populations found in the Mahakali catchment. A series of three two-minute invertebrate kick samples were taken with a 1mm mesh net at each station in water depths of a maximum of 1m (high flow rates in these rivers, combined with slippery substrate, rendered greater depths unsafe). The three samples were combined and transferred into a two-litre white tray containing approximately 250ml of water. The sampling net was examined concurrently, and any remaining organisms transferred to the sorting tray. Organisms that escaped through the net during this stage were discounted. All invertebrates were collected with a forceps and stored in a 50% alcohol solution in individually labelled 25ml universal tubes. All universal tubes were stoppered with cotton wool before screwing on the caps to reduce damage by vibration during transportation and transferred to 70% alcohol on arrival at the laboratory at Edinburgh Napier University.

All organisms were examined under a Kyowa model SDZ-P dissection microscope (magnification x6-x40) with a Schott KL750 light source and identified to family level using primarily North American invertebrate keys (Merrit and Cummins, 1984) plus Mekong River Commission (MRC) keys (Anon., 2009) of South Asian Invertebrates, recorded in the lab book and entered into Microsoft Excel© spreadsheets.

Abundance (number of individuals), family richness (number of families) and diversity (Inverse Simpson's Index) were calculated for each tributary for both post-monsoon and snow-melt seasons. Following testing for normality with the Anderson-Darling test differences in abundance, family richness and diversity were compared between seasons across all sites with paired t-tests. Abundance data were entered into Primer 6 multivariate software package (Clarke and Gorley, 2006) and after standardisation and Log(x+1) transformation, analysed for similarity using cluster analysis, multi dimensional scaling (MDS) based on a Bray-Curtis similarity index and the significance of the seasonal differences tested using ANOSIM (analysis of similarity). The analysis was focused on establishing patterns of differences in community composition between sites and seasons (snow-melt vs post-monsoon). Tributaries were characterised in rank abundance curves with Primer 6 by grouping abundance data into taxonomic families for comparison of aquatic invertebrate community composition and evenness.

Physico-chemical Variables

Water samples were taken from approximately 1 metre depth for physico-chemical analyses of the eight tributary streams during the two main climatic seasons: post-monsoon and snow-melt. Water samples for calculating pH, nutrient and conductivity values were collected by immersing 125ml Azlon low density polyethylene sample bottles in the streams and securing the lids underwater, eliminating air from the samples. The samples were transported to the laboratory at Edinburgh Napier University and analysed in a SEAL AQ2+ Auto analyser (SEAL Analytical) for concentrations of: Total Oxidised Nitrogen (TON), Nitrite (NO_2), Soluble Reactive Phosphate (PO_4), and Ammonium (NH_4) using standard colourimetry methods (APHA, 1995). Suspended solids (SS) samples were collected in the field by pumping one litre water samples from each station through a pre-weighed Whatman GF/C 47mm filter disc with 1.2 µm pore size using a Mitivac 2 hand pump equipped with funnel and measuring bottle accessories. After filtration, each sample disc was folded in half internally to preserve any particles on the surface, dried and stored between two 100mm filter discs in pre-labelled zip lock plastic bags. In the laboratory, the discs were oven dried at 60°C for 24hrs and weighed immediately after their removal from the oven, Subtraction of the original filter weights produced a measure of the total suspended solids present. Dissolved oxygen (%) and water temperature (ºC) were recorded in the field with a YSI model 55 dissolved oxygen meter by placing the probe in flowing water and allowing the reading to settle at each sample site after calibration for altitude at each station. Readings obtained for dissolved oxygen were recorded as percentage saturation and mg/L and were logged in the field notebook. Altitude and coordinates were recorded at each site with a Garmin Dakota GPS of accuracy 5-10m latitude/longitude. Conductivity samples were filtered through Whatman cat No 1822-025 filters in the field prior to the laboratory analysis with a Hanna Instruments HI933000 conductivity meter. pH was recorded on site with a Hanna Instruments handheld pH meter.

Physico-chemical variables were tested for normality of distribution using the Anderson-Darling test and then, owing to lack of normality, Spearman rank-order correlations used to assess relationships between the different parameters recorded. All variables were separated into post-monsoon and snow-melt seasons and paired t-tests conducted for each variable to evaluate any seasonal differences. Physico-chemical data were then entered into Primer 6 multivariate software package and after standardisation and Log(x+1) transformation (Primer 6 default), analysed for similarity using cluster analysis, multi dimensional scaling (MDS) based on the Bray-Curtis similarity index and ANOSIM (analysis of similarity) analysis to test for differences between seasons.

Relationships Between physico-chemical and invertebrate Variables

To explore relationships between physico-chemical and aquatic invertebrate abundance data, BEST analysis in Primer 6 was performed on the invertebrate data and the physico-chemical variables: TON, NO_2, PO_4, NH_4 and conductivity. BEST analysis maximises a Spearman rank correlation between the two Bray-Curtis resemblance matrices produced after standardisation and square root transformation.

Results

Aquatic Invertebrates

The aquatic invertebrate sampling of the eight tributary rivers of the Mahakali identified as spawning streams of *T. putitora* revealed 40 families of aquatic invertebrates belonging to 10 taxonomic orders.

Table 4.1: *Aquatic invertebrate Orders and Families of eight tributaries of the Mahakali River.*

Order	**Families**
Ephemeroptera	Heptagenidae, Baetidae, Ephemerellidae, Leptophlebidae, Caenidae, Ephemeridae, Isonychidae.
Trichoptera	Hydropsychidae, Stenopsychidae, Philopotamidae, Brachycentridae, Goeridae, Psychomyiidae, Leptoceridae, Odontoceridae, Lepidostomatidae.
Plecoptera	Perlidae
Odonata	Gomphidae, Euphaeidae, Corduliidae
Pulmonata	Limneadae, Planorbidae
Hemiptera	Aphelocheiridae, Psephinidae, Corixidae, Gerridae, Micronectidae, Naucoridae
Coleoptera	Gyrinidae, Elmidae, Hydrophilidae, Dytiscidae. Scirtidae.
Diptera	Chironomidae, Tabanidae, Simulidae, Tipulidae. Ephydridae.
Megaloptera	Corydalinae
Arhynchobdellida	Erpobdellidae

Abundance, Family Richness and Diversity

The results presented in ***Table 4.2*** display abundance (number of individual invertebrates), family richness (total number of families in the three kick samples) and diversity as measured by the Inverse Simpson's Index.

Abundance

A wide range of abundances was found across the rivers sampled from 604 specimens in the Jhurighar River during the snow-melt season to only 37 in the Lohaghati River in the same season. Overall abundance was highest in the Jhurighar (695) and Gidiya (610) and lowest in the Chamaliya (260). The remaining tributaries' counts ranged from 302 to 493. The number of invertebrates recovered in the samples was higher in the post-monsoon season than in the snow-melt season in every individual tributary except for Jhurighar, which produced much greater numbers in the snow-melt season than the post-monsoon season (SM: 604, PM: 91). Of the others, the tributary which showed the least difference between seasons was the Panar (PM: 254, SM: 239) and the greatest difference the Lohaghati (PM: 269, SM: 37). A paired t-test comparing SM abundance vs. PM abundance for all tributaries combined found no significant difference (t=0.22, df=7, p=0.829)

Family Richness

Family richness ranged from 20 in the Gidiya River in both post-monsoon and snow-melt seasons to 7 in the River Lohaghati in the snow-melt season. The Gidiya produced the highest number of families in both seasons (PM: 20, SM: 20) and the Lohaghati both lowest scores (PM:10, SM:7). The other tributaries returned values ranging from 12 to 18. Family richness was relatively evenly spread over the seasons as four tributaries had higher values during the snow-melt season and three had lower values. A paired t-test comparing SM family richness vs PM family richness for all tributaries combined found no significant difference. (t=-0.79, df=7, p value 0.456)

Diversity

Invertebrate diversity varied greatly with the highest value of 10.39 recorded from the Surnaya River during the snow-melt season and the lowest (1.19) at the Lohaghati River during the post-monsoon season. The Surnaya scored highest for diversity during the snow-melt season (10.39) but was second lowest during the post-monsoon season (2.54). A paired t-test comparing SM Inverse Simpson's v PM Inverse Simpson's for all tributaries combined found no significant difference t=-2.20, df=7, p value=0.064.

Table 4.2: *Aquatic invertebrate abundance, family richness and diversity in T. putitora spawning tributaries of Mahakali River.*

Station/Season	Family richness	Inverse Simpson's	Abundance
Gidiya PM	**20**	**4.61**	**322**
Gidiya SM	**20**	**4.31**	**288**
Lohaghati PM	**10**	**1.19**	**269**
Lohaghati SM	**7**	**4.11**	**37**
Doti PM	**13**	**2.85**	**184**
Doti SM	**18**	**6.57**	**118**
Surnaya PM	**12**	**2.54**	**227**
Surnaya SM	**16**	**10.39**	**98**
Jhurighar PM	**15**	**3.50**	**91**
Jhurighar SM	**17**	**2.84**	**604**
Sarju PM	**17**	**3.77**	**230**
Sarju SM	**15**	**5.72**	**174**
Panar PM	**13**	**3.11**	**254**
Panar SM	**15**	**4.87**	**239**
Chamaliya PM	**15**	**3.71**	**192**
Chamaliya SM	**13**	**3.77**	**68**
Mean	**14.75**	**4.24**	**212.90**
Std Dev	**3.43**	**2.08**	**133.74**

Cluster Analysis - based on aquatic invertebrate abundance data

The cluster analysis of the invertebrate data in ***Fig. 4.4*** displays the Lohaghati standing out as an invertebrate community apart from the others in the two initial clusters representing the two climatic seasons. The communities then diverge into two groups containing either snow-melt samples or post-monsoon samples, with the exception of the Gidiya snow-melt sample. In effect, the invertebrate communities predominantly display separation not by geographical considerations but by the seasonal changes in nutrients, temperature, stream velocity, water level, sediment content and light penetration that accompany the annual climatic cycle. Comparing the Doti/Surnaya and Lohaghati/Gidiya sub-catchments, which contain both *T. putitora* morphotypes, the Doti/Surnaya ecosystem forms a separate cluster during the post-monsoon season and both tributaries appear in the predominantly snow-melt season major group suggesting that the Doti/Surnaya sub-catchment is more homogenous than the Lohaghati/Gidiya, which displays wide heterogeneity in the dendrogram. The Jhurighar, Sarju, Panar sub-catchment also displays a degree of homogeneity by forming sub clusters during both seasons, while the Chamaliya conforms generally to the post-monsoon - snow-melt separation, which applies to all the tributaries except the Lohaghati/Gidiya sub-catchment.

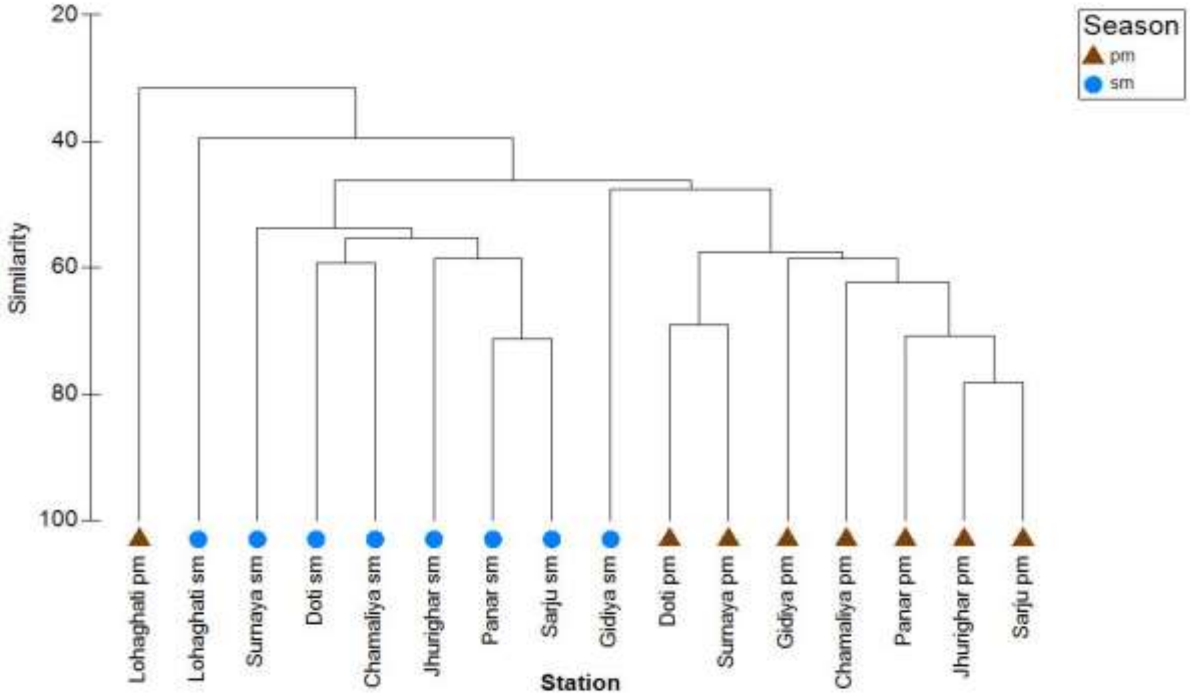


Figure 4.4: *Cluster Analysis dendrogram of Bray-Curtis similarity index from spawning tributaries of Mahakali catchment based on aquatic invertebrate abundance. Post-monsoon (PM) and snow-melt (SM) seasons are highlighted.*

Multi-Dimensional Scaling (MDS).

The MDS plot shown in ***Fig. 4.5*** displays the results of Multi-Dimensional Scaling performed in Primer 6 software. The primary division into two clusters represents samples collected during the snow-melt season and those collected during the post-monsoon season minus the Lohaghati (SM and PM) and the Gidiya (SM). The Lohaghati community appears to be showing a distinct community during both seasons from the respective same-season clusters. The sample collected from the Gidiya during the snow-melt season also falls outside the two major clusters. Of the sub-catchments that contain the two morphotypes of *T. putitora*, the Lohaghati/Gidiya ecosystem is separated from all the other tributaries whereas the Doti/Surnaya sub-catchment is contained within the respective clusters during both climatic seasons.

Figure 4.5: *MDS (Multi-Dimensional Scaling) plot of Bray-Curtis similarity based on abundance data of aquatic invertebrates from spawning tributaries of the Mahakali River. Post-monsoon (PM) and snow-melt (SM) seasons are highlighted.*

ANOSIM

In order to test the significance of the seasonal differences in community composition, ANOSIM (Analysis of Similarities) was conducted on the similarity index by assigning groups PM and SM to the data. The result of the ANOSIM analysis based on Bray-Curtis similarity log(x+1) transformed abundances produced a highly significant difference between the groups SM and PM (p=0.001). The Global R value obtained, 0.361 indicates a moderate level of separation based on 999 permutations.

Analysis of Aquatic Invertebrate Community Composition by Rank Abundance

Aquatic invertebrate families recorded from each breeding tributary were analysed in Primer 6 software and Rank abundance curves produced to allow comparisons of family richness and evenness between tributaries within each sub-catchment. Focus was placed on the Lohaghati/Gidiya and Doti/Surnaya sub-catchments, where conditions may be linked to the morphological variations of *T. putitora.*

The Lohaghati/Gidiya sub catchment.

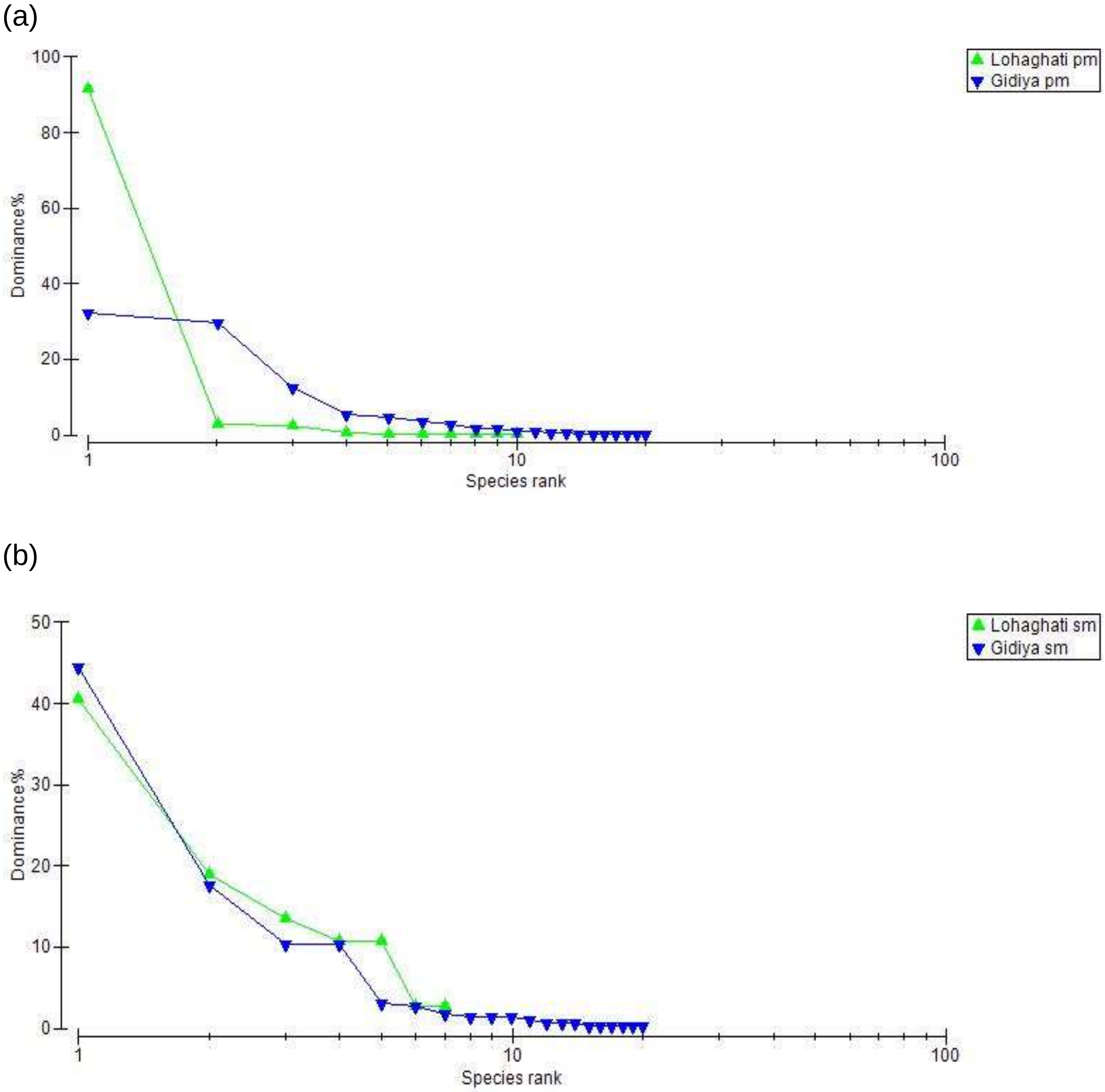


Figure 4.6: *Rank Abundance curves for Lohaghati/Gidiya sub-catchment: a=post-monsoon season (PM), b= snow-melt season (SM).*

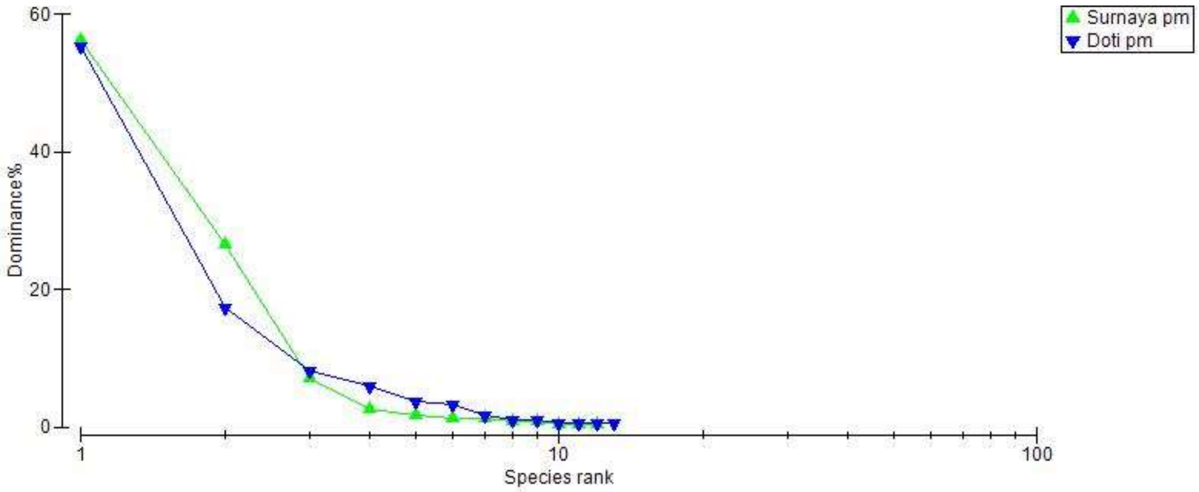
Rank abundance curves for the Lohaghati/Gidiya catchment are shown in Figure 4.6. During the post-monsoon season the Lohaghati is completely dominated by Hydropsychidae individuals (246), and only 10 families in total were identified. The Gidiya during the post-monsoon season is dominated by Baetidae (104) and

Ephemerellidae (96) but the community displays a higher number and more even spread of invertebrate families than the Lohaghati and recorded the highest value (20) for family richness of all tributaries during the post-monsoon season.

During the snow-melt season the Lohaghati sample contained only 37 invertebrates from 7 families in total and was dominated by Baetidae (15). The Gidiya produced a similar total of invertebrates during the snow-melt season to the post-monsoon season but the balance was greatly altered. Chironomidae (128) dominated the count and the spread of families approximated the Lohaghati curve for evenness. The Gidiya recorded the highest family richness (20) of all tributaries also for the snow-melt season.

The Doti and Surnaya sub catchment

(a)



(b)

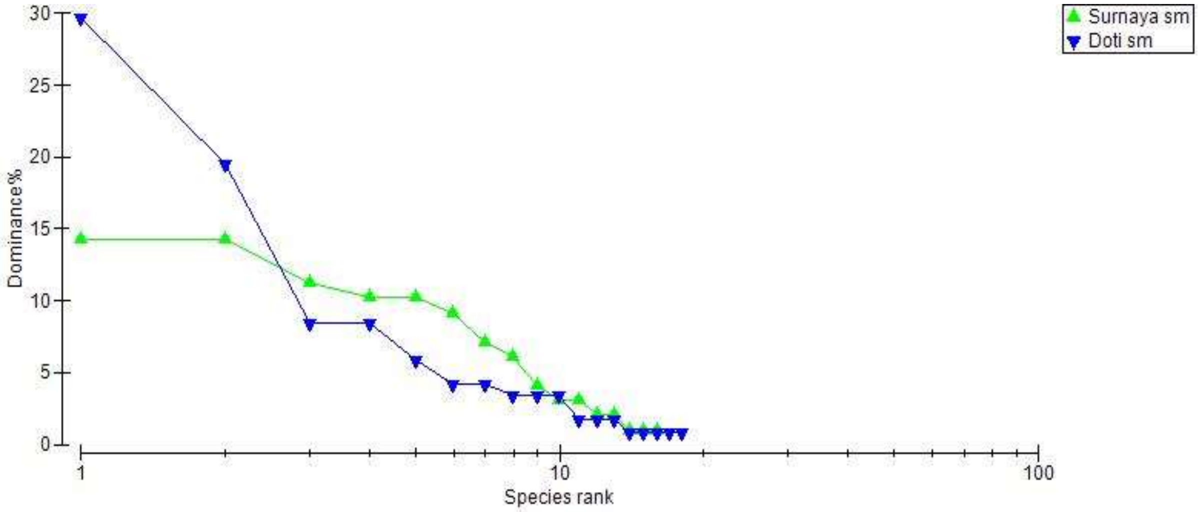


Figure 4.7: *Rank Abundance curves for Doti/Surnaya sub-catchment: a=post-monsoon season (PM), b=snow-melt season (SM).*

Overall, the balance of invertebrate families (**Fig. 4.7**) is very similar during the post-monsoon season in the Doti and Surnaya tributaries (as opposed to that found in the Lohaghati - Gidiya system) and is dominated by Baetidae (Doti 102, Surnaya 128). The similarly of rank abundance patterns was reflected in diversity values with the Doti having a value of 2.85 and the Surnaya 2.54.

During the snow-melt season the occurrence of Baetidae in the Doti was reduced to one individual and instead the dominant families were Caenidae (35) and Gomphidae (23). A similar pattern emerged in the Surnaya data as no individuals of Baetidae were recorded and Caenidae (14) and Ephemerellidae (14) produced the highest counts. The main difference between the tributaries in this season was the greater overall evenness of Families in the Surnaya.

The Jhurighar, Sarju and Panar sub catchment

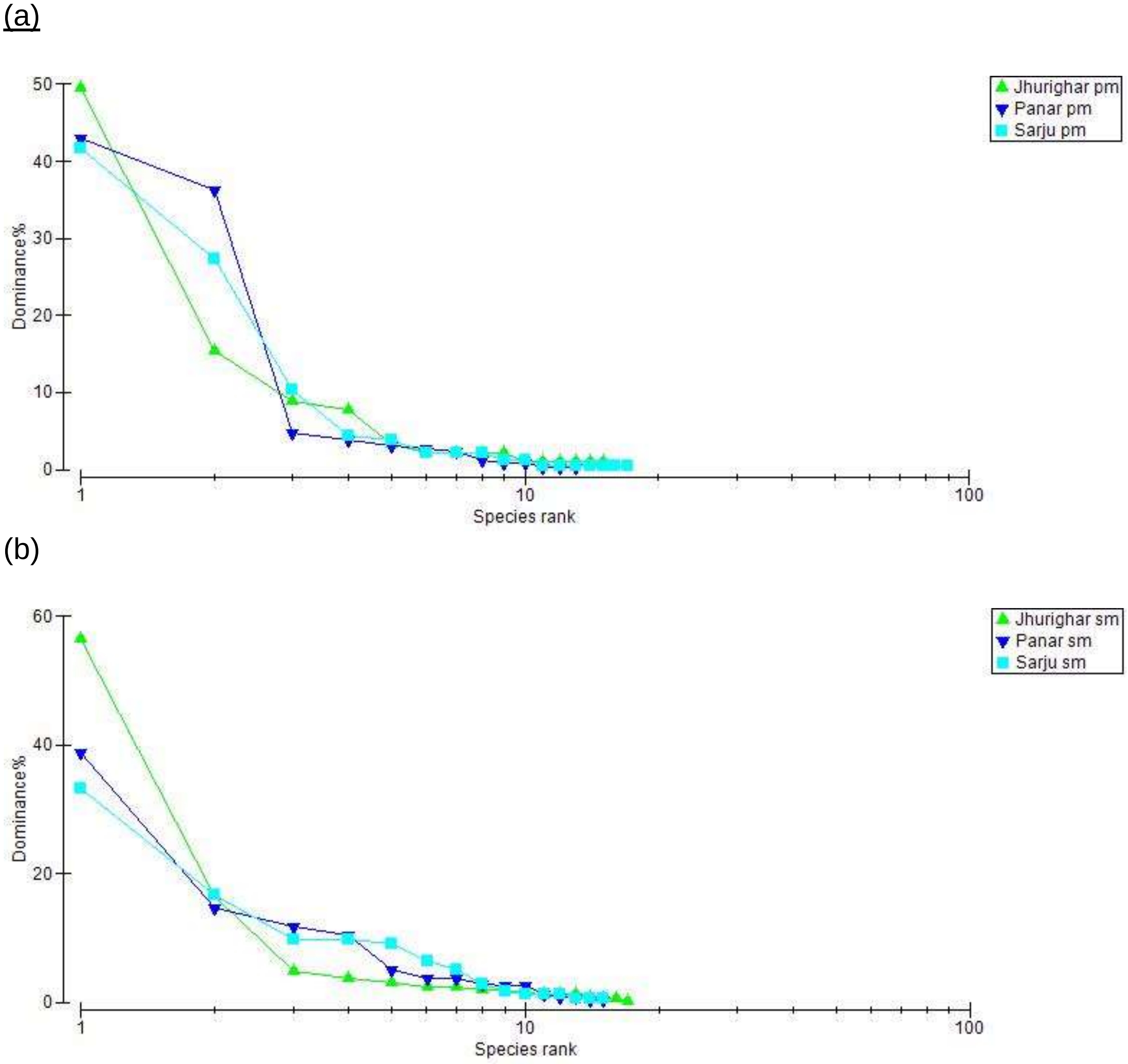


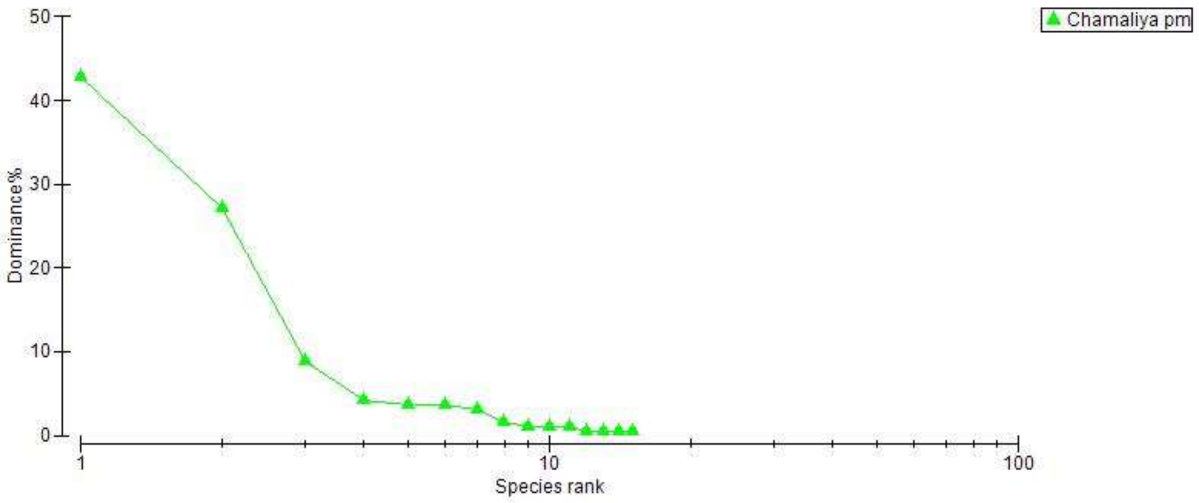
Figure 4.8: *Rank Abundance curves for Jhurighar/Sarju/Panar sub-catchment: a= post-monsoon season (PM), b= snow-melt season (SM).*

During the post-monsoon season sampling, fewer invertebrates (91) were found in the Jhurighar compared to the Panar (254) and Sarju (230). All three tributaries (**Fig. 4.8**) supported communities dominated by Baetidae, followed by Hydropsychidae and the flattening out of the curves thereafter suggests similar spreads of evenness throughout the sub-catchment during this season.

The abundance scores during the snow-melt season are quite different: Jhurighar (604), Panar (239) and Sarju (174). The dominance of the Baetidae family was restricted to the Panar (93), while Ephemerellidae were recorded in very high numbers (341) in the Jhurighar and Caenidae (58) were most prevalent in the Sarju. Family richness and evenness of spread remain relatively similar for all three tributaries. The input of snow and glacial meltwater into the Panar and Sarju is increased during this season, whereas the Jhurighar is exclusively rain and spring-fed fed.

The Chamaliya catchment

(a)



(b)

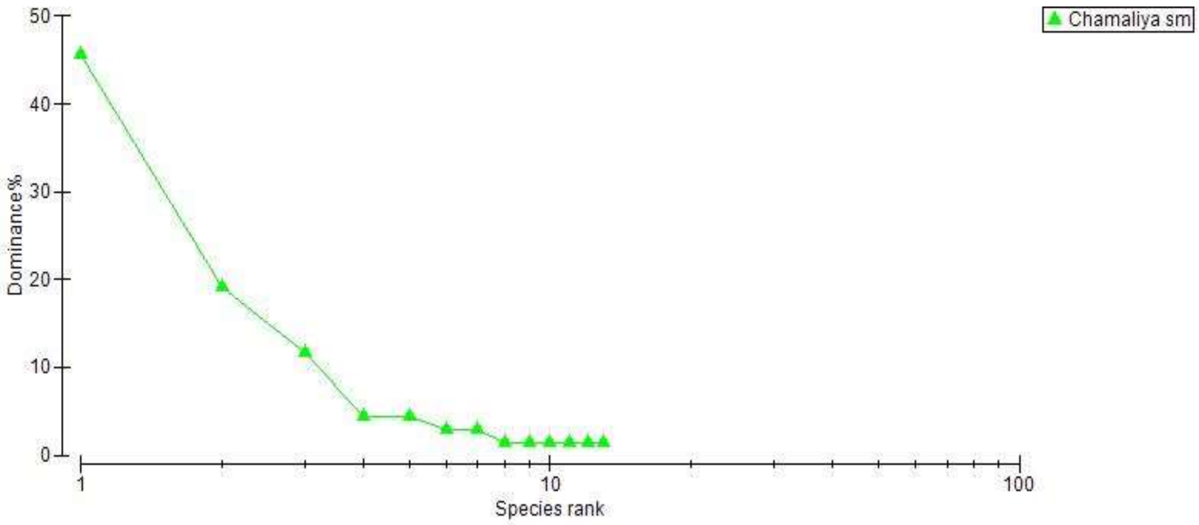


Figure 4.9: *Rank Abundance curves for Chamaliya sub-catchment: a=post-monsoon season (PM) b=snow-melt season (SM).*

In the geographically isolated and seasonally snow fed Chamaliya tributary (**Fig. 4.9**) 68 invertebrates were recorded during the snow-melt season and 192 during the post-monsoon season. Ephemerellidae (82) dominate during the post-monsoon and Caenidae (31) during the snow-melt. Family richness and evenness of abundance are broadly similar across the two seasons.

Physico-chemical Variables

The outstanding feature of the results obtained was the variation in each tributary for the majority of variables between the two seasons. The intervening period between these seasons is dominated by the monsoon itself, when river levels, water velocity and soil/debris input increase dramatically, restructuring the stream beds and altering the habitat for diatoms, macrophytes, aquatic invertebrates and fish. All results of the physico-chemical sampling are presented in ***Table 4.3****.* Post-monsoon results for pH are unavailable due to failure of the electronic meter on that sampling trip. The Gidiya River suspended solids value and temperature readings are missing due to equipment failure on the same trip.

The following graphical representations of the results have been grouped to display the tributaries next to each other within their four sub-catchments: Gidiya/Lohaghati, Doti/Surnaya, Chamaliya, Jhurighar/Sarju/Panar.

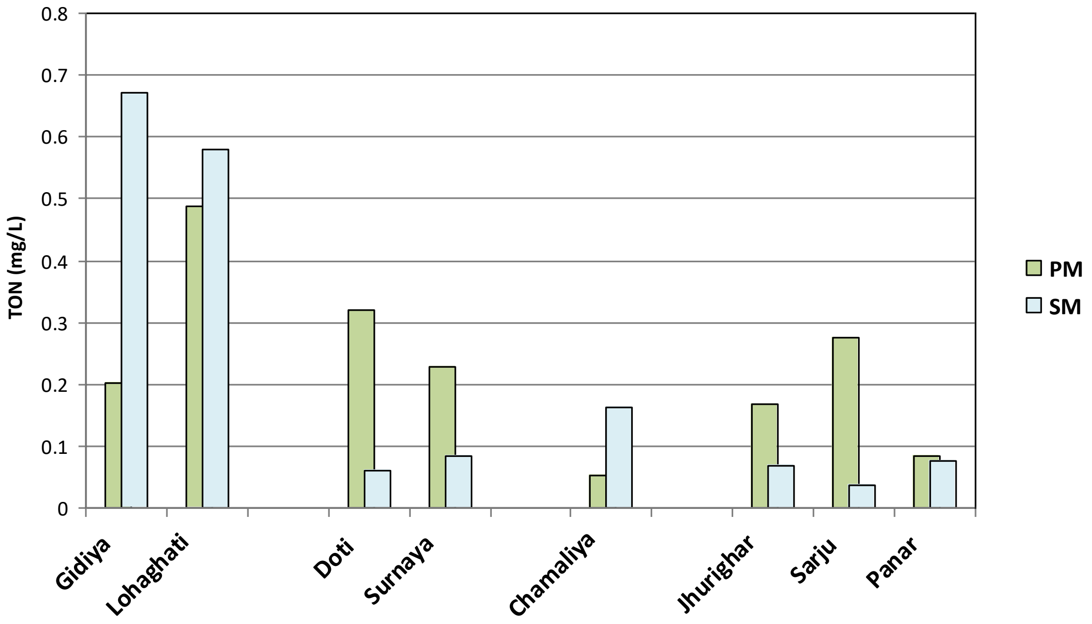


Figure 4.10: *Total Oxidised Nitrogen concentration (mg/L) in four sub-catchments of the Mahakali River.*

Total Oxidised Nitrogen concentration (***Fig. 4.10***) across the tributaries ranged from a high of 0.671 mg/L (Gidiya SM) to a low of 0.037 mg/L (Sarju SM). Both rivers in the Lohaghati/Gidiya sub catchment recorded increases in the snow-melt season, as did the Chamaliya. The three highest values were measured in the Lohaghati and Gidiya. The Doti/Surnaya and Jhurighar/Sarju/Panar sub-catchments recorded decreases in the snow-melt season.

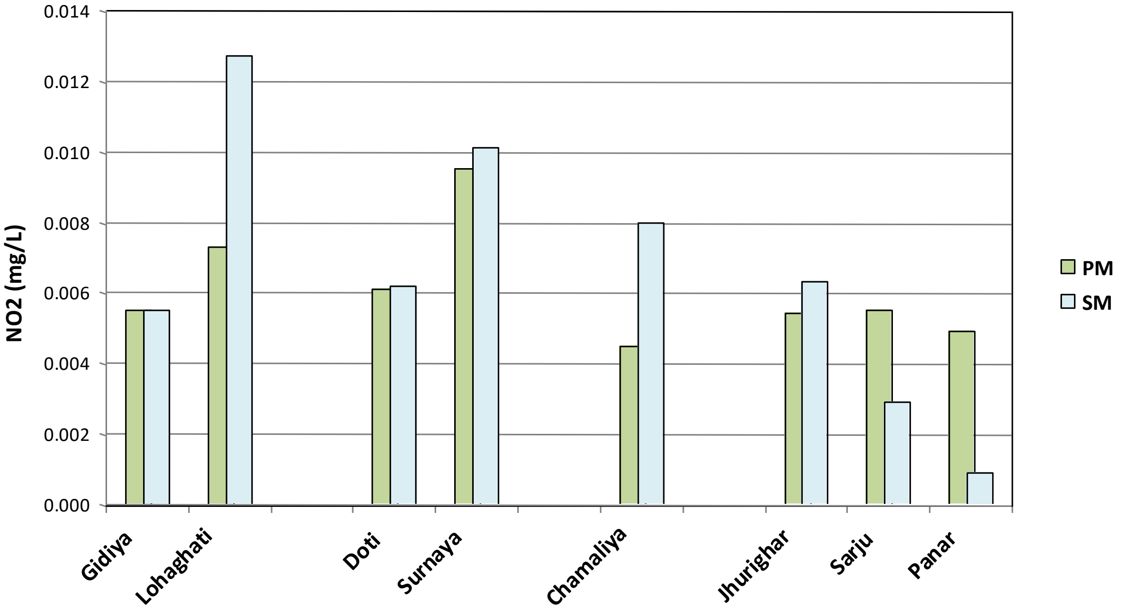


Figure 4.11: *NO_2 concentration (mg/L) in four sub-catchments of the Mahakali River.*

NO_2 concentrations (***Fig. 4.11***) varied from a maximum of 0.0127 mg/L (Lohaghati SM) to a minimum of 0.0009 mg/L (Panar SM). The Gidiya showed no change between seasons, while the Lohaghati in the same sub-catchment, recorded a higher value during the snow-melt season than the post-monsoon season. The latter pattern was also seen for both tributaries in the Doti/Surnaya sub-catchment, the Jhurighar and Chamaliya tributaries. The Sarju and Panar tributaries, however, had higher readings during the post-monsoon season.

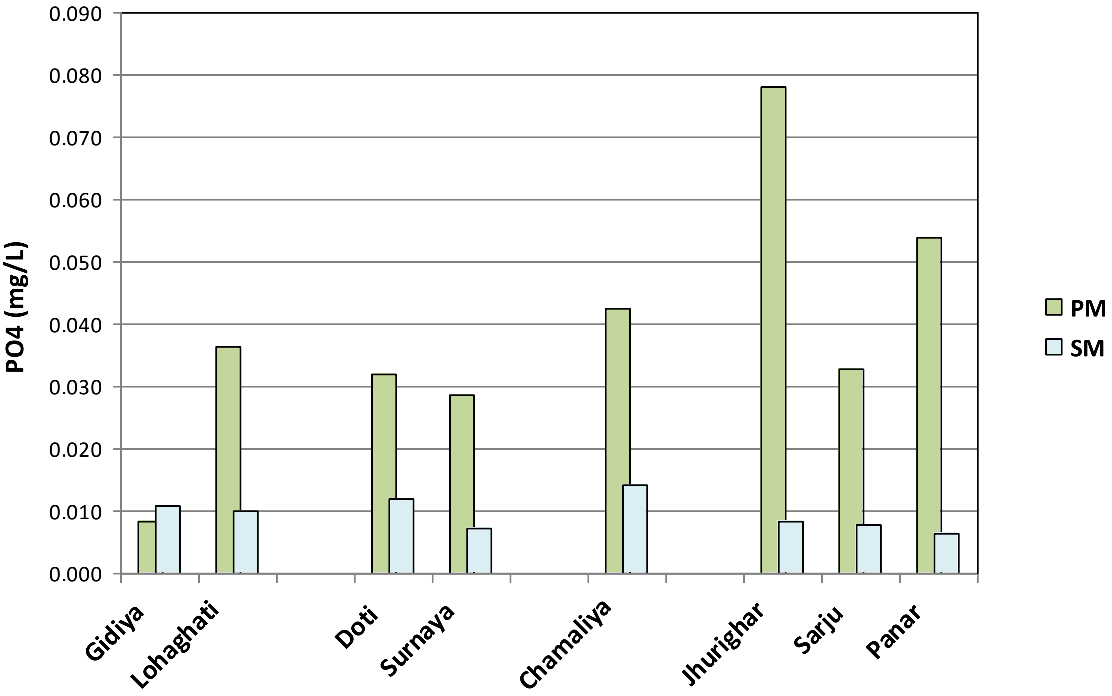


Figure 4.12: *PO_4 concentration (mg/L) in four sub-catchments of the Mahakali River.*

All tributaries with the exception of the Gidiya carried higher PO_4 loads (***Fig. 4.12***) in the post-monsoon sampling period. The Jhurighar recorded the highest value in the data set (0.08 mg/L) during the post-monsoon season and the Panar the lowest (0.006 mg/L) during the snow-melt season. Overall PO_4 concentrations for all tributaries combined were found to be significantly higher in the post-monsoon season than in the snow-melt season (paired t test, t=3.94, df=7, p = 0.006).

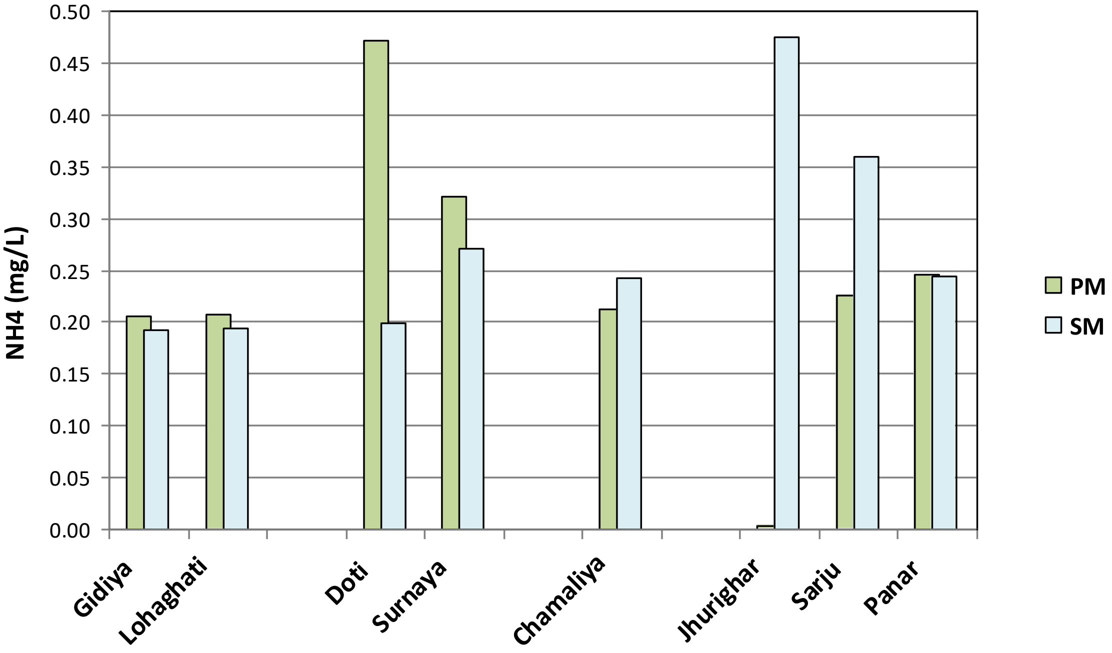


Figure 4.13: *NH_4 concentration (mg/L) in four sub-catchments of the Mahakali River.*

NH_4 concentrations (***Fig. 4.13***) ranged from a high of 0.475 mg/L to a low of 0.004mg/L. These two readings were obtained from the same tributary, the Jhurighar, in the snow-melt and post-monsoon seasons, respectively. The Sarju also recorded a decrease in the post-monsoon season while the Panar showed almost no change. The isolated Chamaliya tributary exhibited higher concentration in the snow-melt season. In the Lohaghati/Gidiya catchment, small increases of NH_4 were recorded in the post-monsoon season. In the Doti/Surnaya catchment, post-monsoon measurements exceeded snow-melt measurements by a moderate level in the Surnaya and by more than double in the Doti.

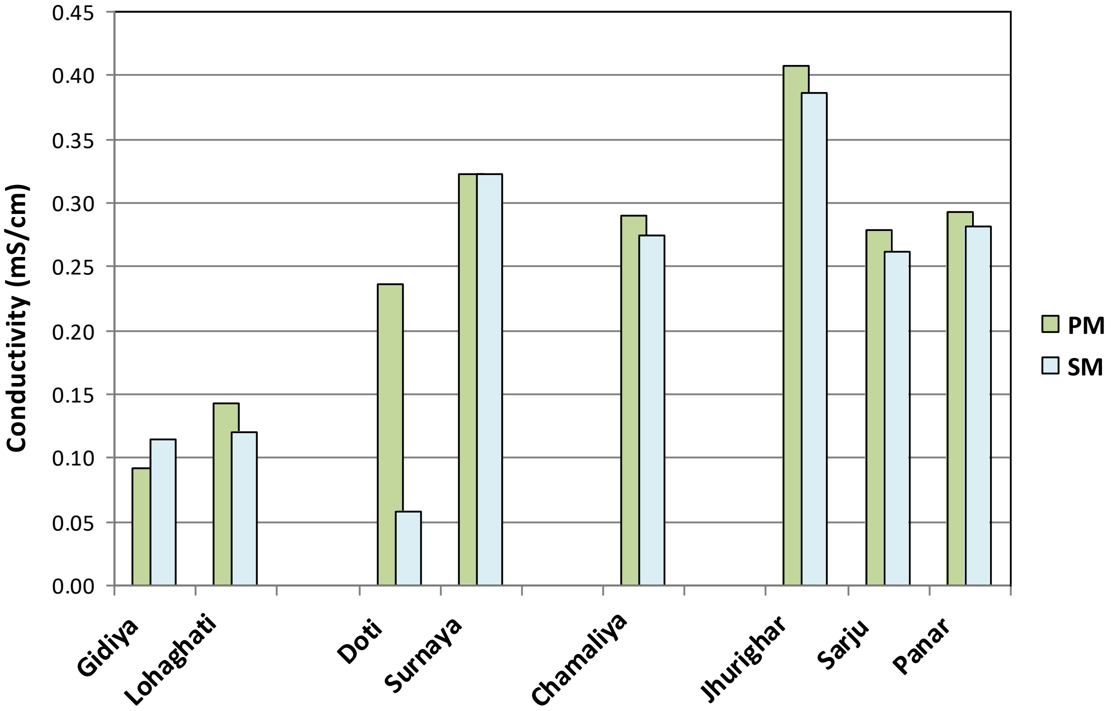


Figure 4.14: *Conductivity (mS/cm) in four sub-catchments of the Mahakali River.*

Conductivity levels (***Fig.4. 14***) showed no significant differences in all tributaries during the post-monsoon season. The highest concentration was found in the Jhurighar during the post-monsoon season (0.407 mS/cm) and the lowest in the Doti during the snow-melt season (0.058 mS/cm).

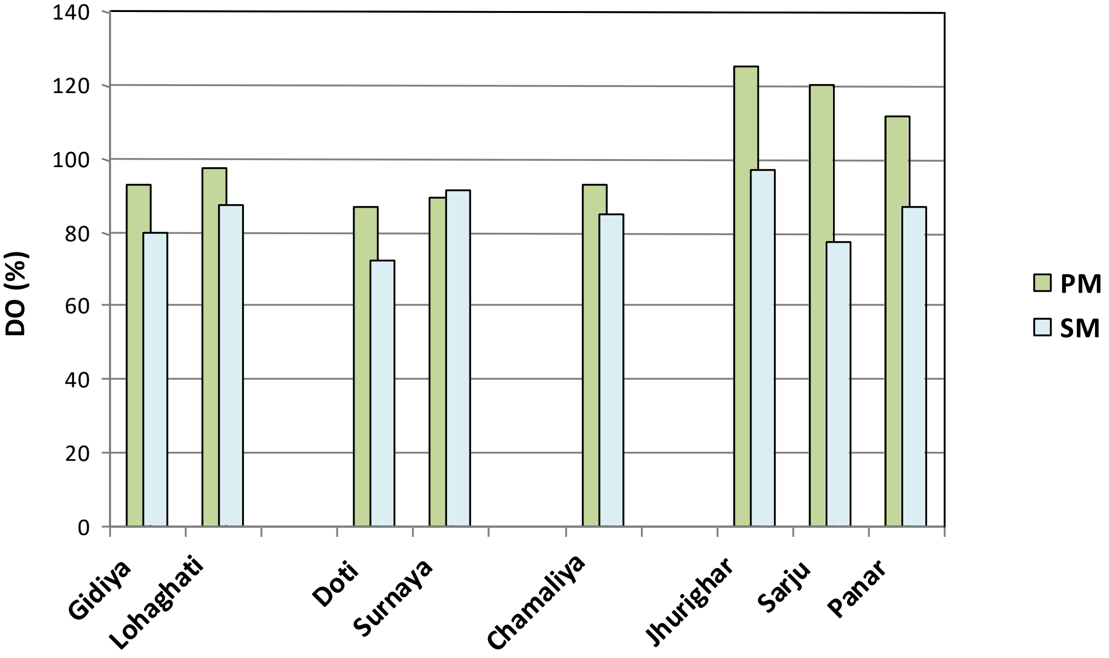


Figure 4.15: *Dissolved Oxygen (%) concentration in four sub-catchments of the Mahakali River.*

Dissolved Oxygen was observed to be higher in the post-monsoon season in every tributary except the Surnaya, which recorded a small drop from its snow-melt season D.O. value (***Fig. 4.15***). The highest concentration during the post-monsoon season was found in the Jhurighar (125.1 %), followed by the other two rivers in its sub catchment, the Sarju (119.7 %) and Panar (111.2 %). Overall Dissolved Oxygen concentrations for all tributaries combined were significantly higher during the post-monsoon season (paired t test, t=3.58, df=7, p = 0.009).

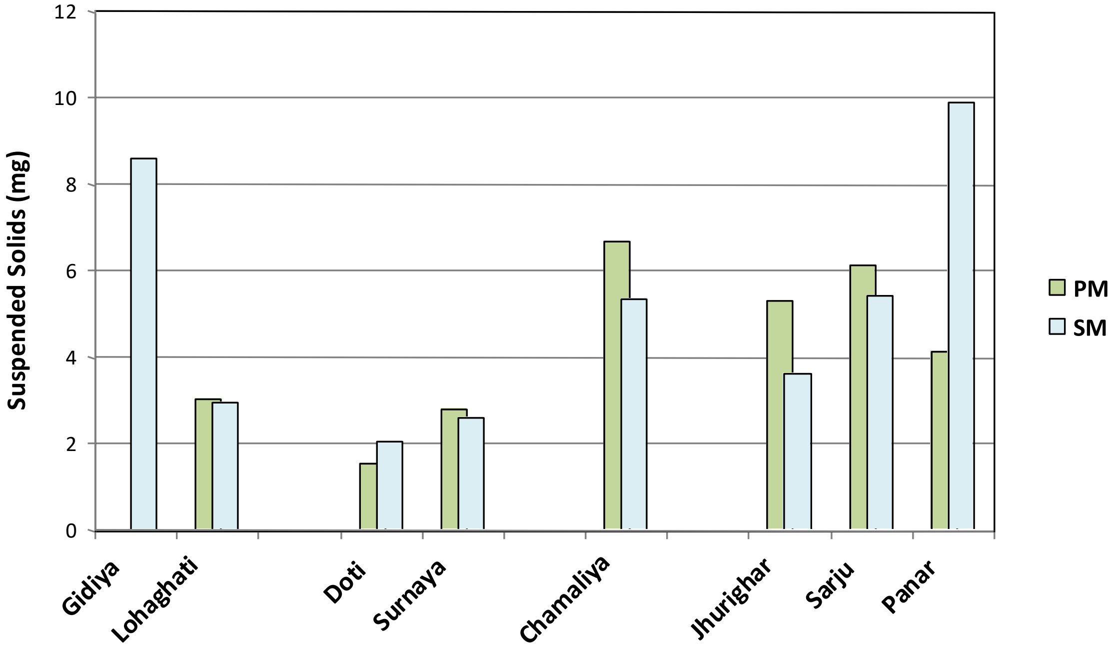


Figure 4.16: *Suspended Solids (mg) in four sub-catchments of the Mahakali River (Gidiya PM sample missing due to equipment failure).*

During the snow-melt season, the suspended solids results ranged from a high of 9.9 mg (Panar), followed by the Gidiya (8.59 mg), Sarju (5.42 mg), Chamaliya (5.32 mg), Jhurighar (3.59 mg), Lohaghati (2.95 mg), Surnaya (2.6 mg) and Doti (2.05 mg) (***Fig. 4.16***). Equipment failure resulted in no reading for the Gidiya during the post-monsoon season and the results ranged from a high of 6.67 mg (Chamaliya), followed by Sarju (6.1 mg), Jhurighar (5.3 mg), Panar (4.13 mg), Lohaghati (3.02 mg), Surnaya (2.77 mg) and Doti (1.54 mg).

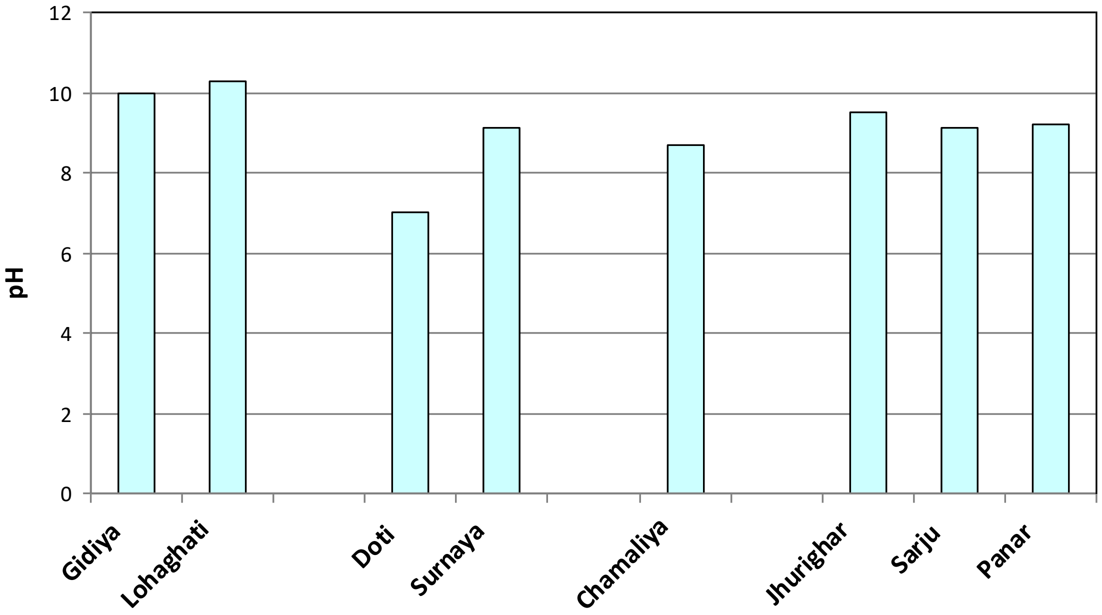


Figure 4.17: *pH values in four sub-catchments of the Mahakali River (data for PM season missing due to equipment failure).*

pH values ranged from 7 (Doti) to 10.3 (Lohaghati) across all tributaries (***Fig. 4.17***). The highest two readings were recorded in the two tributaries of the Lohaghati/Gidiya sub-catchment (Gidiya - 10.0). The Doti/Surnaya sub-catchment returned values of 7 (Doti) and 9.1 (Surnaya). Readings were more uniform in the Jhurighar/Sarju/Panar sub-catchment at 9.5, 9.1 and 9.2, respectively. The Chamaliya returned a value of 8.7.

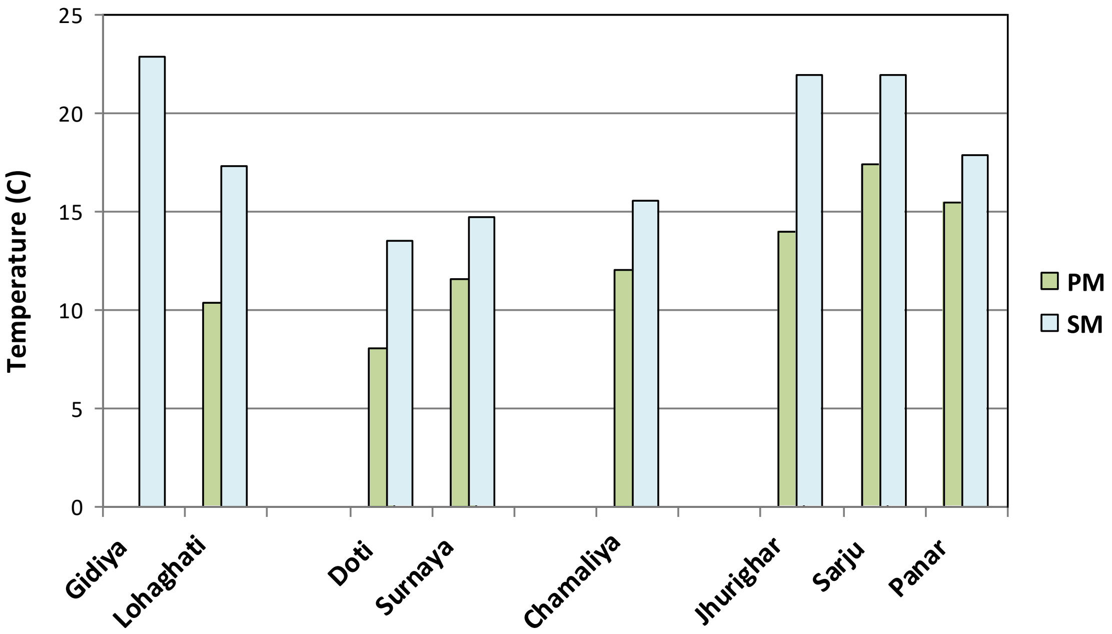


Figure 4.18: *Water Temperature (°C) in four sub-catchments of the Mahakali River.*

Water temperature (***Fig 4.18***) ranged from 22.9ºC in the Gidiya River during the snow-melt season to 13.5ºC in the Doti. The Jhurighar and Sarju both recorded a temperature of 21.9ºC, followed by the Panar (17.9ºC), Lohaghati (17.3ºC), Chamaliya (15.6ºC), Surnaya (14.7ºC) and Doti (13.5ºC). During the post-monsoon season equipment failure at the Gidiya River discounted the result. The highest post-monsoon result was recorded at the Sarju (17.4ºC), then Panar (15.5ºC), Jhurighar (14.0ºC), Chamaliya (12.0ºC), Surnaya (11.6ºC), Lohaghati (10.4ºC) and Doti (8.1ºC).

Summary of Seasonal Differences in Physico-chemical Variables

The physico-chemical data for each variable were analysed to determine whether any significant differences exist by comparing the results from the snow-melt season with those of the post-monsoon season using paired t-tests. Dissolved oxygen (%) concentration was significantly higher during the post-monsoon season (paired t test, t=3.58, df=7, p = 0.009) while water temperature (°C) was significantly lower in the post-monsoon season (paired t test, t=-6.32, df=7, p = 0.001) PO_4 concentrations were also found to be significantly higher in the post-monsoon season (paired t test, t=3.94, df=7, p = 0.006). None of the other variables mentioned in the text associated with the figures produced any significant differences.

Cluster Analysis based on Nutrient and Conductivity Data

Based on all nutrient and the conductivity data, the cluster analysis showed the Jhurighar River to be separate from three other major groups during the post-monsoon season ***(Fig. 4.19)***. Two of these groups contain either snow-melt season or post-monsoon season sample sets exclusively, while the remaining group contains three snow-melt and one post-monsoon sample set.

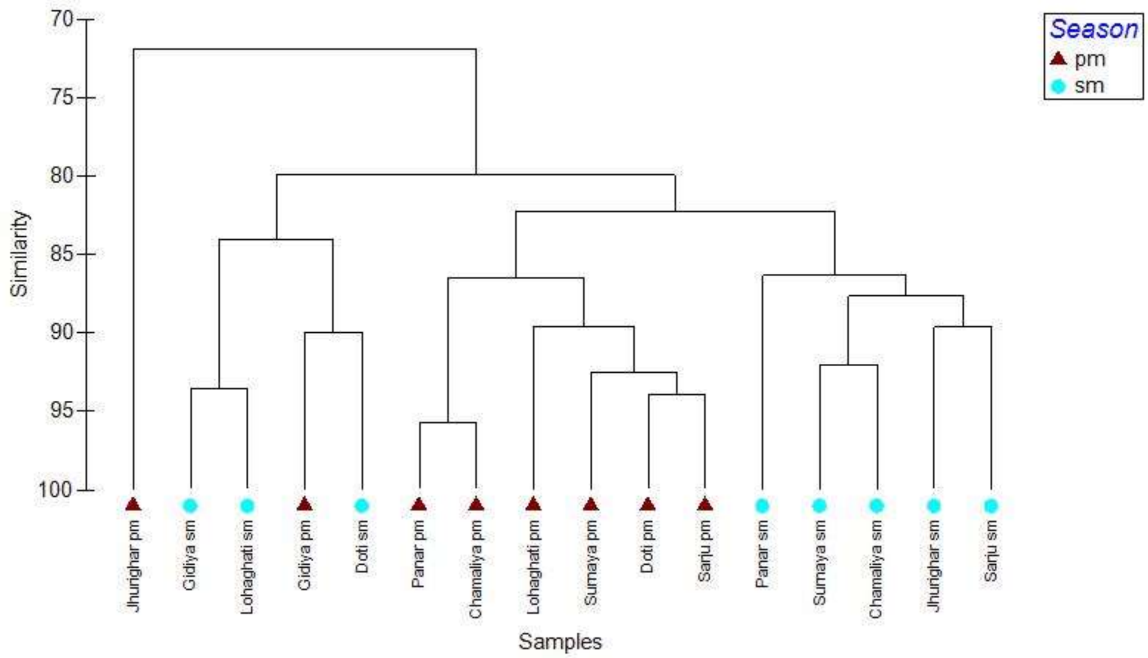


Figure 4.19: *Cluster Analysis dendrogram of Bray-Curtis similarity index from spawning tributaries of Mahakali catchment based on TON, NO_2, PO_4, NH_4, Conductivity. Post-monsoon (PM) and snow-melt (SM) seasons are highlighted.* Multi-Dimensional Scaling (MDS).

The MDS plot shows a partial separation of the left and right sides of the plot, representing samples collected during the snow-melt season and those collected during the post-monsoon season. An exception was Gidiya PM, which intruded into the snow-melt side and Jhurighar PM which is isolated from the main group ***(Fig. 4.20)***.

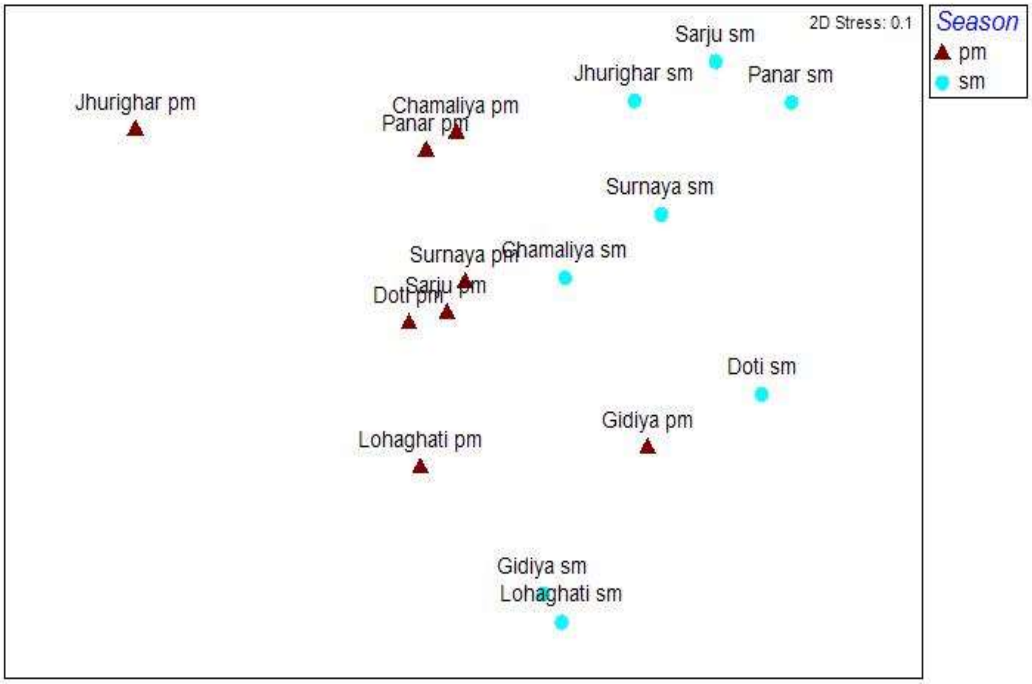


Figure 4.20: *MDS (Multi-Dimensional Scaling) plot of Bray-Curtis similarity index from spawning tributaries of Mahakali catchment based on TON, NO_2, PO_4, NH_4, Conductivity. Post-monsoon (PM) and snow-melt (SM) seasons are highlighted.*

ANOSIM

In order to test the significance of the grouping produced by the MDS plot, an ANOSIM (Analysis of Similarities) was conducted on the similarity index by assigning groups PM and SM to the data. The result of the ANOSIM analysis based on Bray-Curtis similarity log(x+1) transformed values produced a significant difference between the groups SM and PM (p=0.009). The Global R value obtained, 0.282 indicates a moderate level of separation based on 999 permutations.

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Table 4.3: *Summary of Physico-chemical data obtained from water samples collected in snow-melt (SM) and post-monsoon (PM) seasons. Ph, Total Oxidised Nitrogen (TON mg/L), Nitrite (NO_2 mg/L), Soluble Reactive Phosphate (PO_4 mg/L), Ammonium (NH_4 mg/L), Conductivity (Cond. mSv). and Suspended solids (SS mg/L), sample site Altitude in metres, Dissolved Oxygen (% saturation), Temperature (°C)*

Station	pH	TON (mg/L)	NO_2 (mg/L)	PO_4 (mg/L)	NH_4 (mg/L)	Cond. (mSv)	S.S. (mg/L)	Altitude (m)	DO (%)	Temp (°C)
Gidiya PM		0.202	0.0055	0.008	0.206	0.092		1560	93.00	
Gidiya SM	10	0.671	0.0055	0.011	0.193	0.114	8.59	1560	79.72	22.90
Lohaghati PM		0.488	0.0073	0.036	0.207	0.143	3.02	1474	97.20	10.40
Lohaghati SM	10.3	0.578	0.0127	0.01	0.194	0.12	2.95	1474	87.40	17.30
Doti PM		0.319	0.0061	0.032	0.471	0.236	1.54	1502	86.60	8.10
Doti SM	7	0.059	0.0062	0.012	0.198	0.058	2.05	1502	72.22	13.50
Surnaya PM		0.227	0.0095	0.028	0.321	0.322	2.77	1105	89.50	11.60
Surnaya SM	9.1	0.08	0.0101	0.007	0.270	0.323	2.6	1105	91.20	14.70
Jhurighar PM		0.167	0.0054	0.08	0.004	0.407	5.3	980	125.10	14.00
Jhurighar SM	9.5	0.069	0.0063	0.008	0.475	0.387	3.59	980	97.07	21.90
Sarju PM		0.276	0.0055	0.033	0.226	0.279	6.1	520	119.70	17.40
Sarju SM	9.1	0.037	0.0029	0.008	0.359	0.262	5.42	520	77.13	21.90
Panar PM		0.085	0.0049	0.054	0.246	0.293	4.13	508	111.20	15.50
Panar SM	9.2	0.075	0.0009	0.006	0.245	0.282	9.9	508	86.71	17.90
Chamaliya PM		0.051	0.0045	0.042	0.212	0.29	6.67	614	93.00	12.00
Chamaliya SM	8.7	0.162	0.008	0.014	0.242	0.274	5.32	614	84.55	15.60
Mean	9.11	0.222	0.0063	0.024	0.254	0.243	4.663	1033	93.21	15.64
Median	9.15	0.165	0.0058	0.013	0.234	0.277	4.130	1043	90.35	15.500
Std Dev	0.99	0.198	0.0028	0.021	0.113	0.106	2.418	434.4	14.53	4.343

All physico-chemical data from ***Table 4.3*** were analysed to determine if any significant correlations existed between any of the parameters. Conductivity/altitude, conductivity/D.O.%, D.O.%/PO_4 and altitude/TON produced significant results. Temperature, pH and suspended solids were omitted due to missing data. A summary of significant correlations from the physico-chemical data is displayed in ***Table 4.4***.

Table 4.4: *Summary of significant physico-chemical correlations.*

Variables	Spearman's rho (r_s)	p value
Conductivity - Altitude	**- 0.586**	**0.017**
Conductivity - D.O.%	**0.555**	**0.026**
D.O.% - PO_4	**0.528**	**0.036**
Altitude - TON	**0.515**	**0.041**

Relationships – Physico-chemical variables / Aquatic invertebrate Community Composition

To assess if the physico-chemical variables associated with invertebrate nutrient requirements could explain the pattern of invertebrate population variation, BEST analysis in Primer 6 was performed on the invertebrate data and the physico-chemical variables: TON, NO_2, PO_4, NH_4 and conductivity. The results indicated that TON and conductivity best explain the variation in the composition of the invertebrate communities as assessed by similarity index and that there was a significant negative correlation between the invertebrate similarity and the combination of these two variables: Rho, 0.347, p = 0.045, number of permutations = 999.

Discussion

The objective of the physico-chemical and aquatic invertebrate analyses was to investigate whether significantly different ecological conditions within a sub-catchment could produce differences in food resources that may be linked to the morphological variation in the heads and mouthparts of *T. putitora* morphotypes. If two rivers within one sub-catchment where both morphs are known to occur e.g. Lohaghati and Gidiya displayed significantly different environments then further investigation would be useful to determine whether breeding is spatially separated for the two morphotypes encountered in that sub-catchment in correlation to the differences seen. Alternatively, if a comparison of the breeding tributaries by season resulted in significant differences, then the possibility of temporal separation of breeding events would be the logical focus of further investigation.

Physico-Chemical Conditions

Dissolved Oxygen, Temperature and Altitude.

Overall dissolved oxygen values were high and no tributary recorded less than 72%. DO% concentrations were significantly higher during the post-monsoon season when the higher river levels and greatly increased turbulence caused by the heavy monsoon precipitation ran off compared to the more stable conditions comprising the snow-melt season, when precipitation was absent and only spring, glacier and snow-melt sources contributed to river waters. Temperatures were also significantly lower in the post-monsoon season reflecting the ability of the water to retain more dissolved oxygen at lower temperatures. The higher altitude sampling sites of Lohaghati, Gidiya, Doti and Surnaya do not appear to be any less habitable than lower altitude sampling sites regarding these parameters to aquatic fauna despite their elevation, as shown by the dissolved oxygen values which are corrected for both altitude and temperature. The rivers which are known to have input from snow-melt water in their higher altitude tributaries (Panar, Sarju and Chamaliya) appear to be minimally influenced in terms of temperature by snow-melt by the time they reached the lower altitudes where the sampling sites are located, as they were warmer during this season than during the post-monsoon season.

pH and Suspended Solids.

pH results ranged from 7 to 10.3 during the snow-melt season, all within parameters able to support diverse aquatic invertebrate communities (Berezina, 2001). Due to the failure of the pH meter during the post-monsoon sampling trip it was impossible to compare pH between the two seasons. Suspended solids varied from 9.9 to 1.54mg and may have been affected by unseasonal rainfall and agricultural activities during the sampling periods. Suspended solids in the monsoon affected rivers of the Himalayas can show wide variation but in general, it would be expected that the post-monsoon season, with its high rainfall, should produce a greater input of soil and vegetation from forested and cultivated areas into adjacent streams. This appears not to be the case (***Fig. 4.16***) in at least two of the tributaries sampled (Panar and Doti). The Panar snow-melt result can perhaps be explained by the fact that an unseasonable rainstorm occurred on the morning of the sampling trip and the river was rising rapidly as sampling took place. However, the limitations on the data are preventing any robust conclusions.

Nutrients and Ions.

Nutrients such as nitrogen, nitrites, ammonia, and phosphate, along with ions of soluble minerals derived from the bedrock - calcium, magnesium, potassium, and sodium, are required to support life within freshwater ecosystems (Bhateria and Jain, 2016). The algae which provide the base of the autochthonous food web rely on these molecules for growth and reproduction. Decomposition of allochthonous organic inputs including vegetation, invertebrates, and other detritus release nutrients as a product of bacterial digestion, fungal consumption, and invertebrate food processing activities (Vanni, 2002). The nutrient data displayed in the multi-dimensional scaling plot showed an overall separation by season, rather than by tributary or sub-catchment. ANOSIM analysis confirmed that the seasonal separation was moderate but significant. There is no evidence to suggest that the nutrient concentrations or conductivity differ significantly between the breeding tributaries Lohaghati and Gidiya or between the Doti and Surnaya so it is unlikely that this is a significant factor influencing the composition of the aquatic invertebrate communities, and hence, food preferences of *T. putitora* juveniles, which may have provided a link to trophic polymorphism.

Aquatic Invertebrate Communities

Aquatic invertebrate abundance results revealed the extent to which communities from individual tributaries were affected by the passing of the monsoon weather pattern. The Panar, Sarju and Gidiya showed little change in terms of abundance, whereas the Doti and Surnaya displayed a slight bias towards higher abundance during the post-monsoon season and the Jhurighar, Lohaghati and Chamaliya wide variation between seasons. In terms of potential prey resources in the sub-catchments where both variants of *T. putitora* are found, the Lohaghati recorded lower values for abundance, family richness and diversity than the Gidiya in both seasons and the main invertebrate resource was overwhelmingly Hydropsychidae caddis nymphs, as opposed to a mixture of mayflies, caddis, chironomids and Odonata in the Gidiya. The Doti/Surnaya sub-catchment, on the other hand, showed a more even spread of abundance plus similar family richness and diversity and contained communities similar to the Gidiya. If both of the sub-catchments had displayed similar heterogeneity, then a closer examination of the communities would have been appropriate.

The cluster analysis also separated the Lohaghati out from all the other tributaries initially, before splitting the tributaries into two seasonally defined groups with the exception of the Gidiya during the snow-melt season. The Doti/Surnaya sub catchment again displays greater similarity and both tributaries fit into the main seasonal groupings. Multi dimensional scaling confirms this representation with the Lohaghati producing the two outliers and the Gidiya SM appearing outside the snow-melt group. The ANOSIM analysis produced a highly significant result for difference between seasons which may suggest that, in general, the aquatic invertebrate communities, are separated in character temporally, rather than spatially. The BEST analysis of the relationship between nutrient concentrations and variation in aquatic invertebrate community composition indicated that TON and conductivity were the driving factors behind the variation.

The results suggest that the Lohaghati (at least in the zone sampled) has a different composition of aquatic invertebrate community from the other tributaries. This may be due to physico-chemical influences outwith the parameters of the investigation or anthropogenic factors and further examination would be required to identify these. Further consideration of the functional types and habits of the different organisms present would also be useful. The Gidiya and Lohaghati sub-catchment does show that different habitats, and therefore different prey resources for *T. putitora* can exist between two tributaries in a sub-catchment but the similarities in habitat and potential prey items between the Doti and Surnaya tributaries counteract any conclusions regarding spatial separation of *T. putitora* breeding events that may be arrived at from those differences. The significant difference between seasons revealed by the ANOSIM analysis, however, suggest that a new hypothesis may be introduced in future studies: that *T. putitora*, in the tributaries which contain both morphological variants, utilise two spawning seasons, in which the different aquatic invertebrate communities available as prey items could influence the development of morphological expression. The existence of two temporally separated spawning events has been previously suggested (Joshi *et Al.*, 2002, Sehgal, 1987, Johal and Tandon, 1981, Malik, 2011) but evidence is scarce, and this phenomenon has not been recorded within the Mahakali catchment to date.

Chapter Five

Trophic Polymorphism in the Himalayan fish *Tor putitora*, Comparison of Stable Isotope Ratios in Fish Scales

Introduction

The fish species *T. putitora* has been observed to exhibit two different morphotypes in sympatric populations in the Mahakali River in western Nepal: one with a terminally positioned, wide mouth with thin lips, and the other with a narrower, ventrally located mouth with thick hypertrophied lips and a mental lobe, as established in Chapter 3. Variations in fish head and mouthpart morphology have been reported in several species and have been linked to differences in diet. Malmquist *et al*. (1992) found Arctic charr from Lake Thingvallavatn, Iceland to exhibit two basic morphotypes, a thick-bodied type with a subterminal mouth, which foraged predominantly on slow moving littoral zoobenthos such as snails, and a slender, fusiform type with a terminal mouth aperture, which consumed mainly pelagic zooplankton and fish. Adams *et al*. (1998) extended the number of charr morphotypes to three forms in Loch Rannoch, Scotland by discriminating between planktivorous, benthivorous and piscivorous morphotypes with relative increases in jaw robustness and head size. Traditional methods of examination of fish diet such as gut content analysis (GCA) can provide information on a limited scale as it provides only a snapshot in time of dietary preferences and has the disadvantage that it is difficult to carry out without causing injury to the fish sampled. Stable isotope analysis, on the other hand, can provide an accurate picture of a fish's diet over the longer term, including discrimination between prey types if the isotopic signatures of potential food sources have been established to be distinct (Cabana and Rasmussen, 1996), or in a simpler context to evaluate potential differences in diet or trophic position in an inter-specific comparison, and sampling can be carried out in a rapid and relatively harmless manner by the simply extracting a single scale. Other tissues, which may be extracted for sampling, include fin clippings, muscle, or liver tissue (Campana and Thorrold, 2001), of which only the first can be regarded as relatively harmless in terms of fish welfare. Hayden *et al.* (2015) found that variation existed within fin tissue $\delta^{13}C$ between ray and membrane sections and also noted longitudinal variation in $\delta^{15}N$ from base to tip of fin. They suggested that data obtained from fin clippings should be calibrated against muscle or liver tissue (which displayed more homogeneity) to account for this variability or alternatively, in cases where samples must be returned unharmed, to select tissue from exactly the same areas of the fin thus standardising the sampling. Taking into account that the cross referencing with muscle or liver tissue must inevitably result in irreparable damage to the fish sampled, a single scale removed from the same area of the fish each time provides a much less harmful alternative. The architecture of fish scales and their pattern of growth can complicate stable isotope studies as layers are laid down successively over older areas, potentially biasing the values obtained. Samples taken from the scale focus would include the full isotopic life history of the fish, whereas samples taken from the most recent growth area (the outer edge) represent only the past growth season (***Fig. 5.2***). The period of the fish's growth that is important to the study determines the zone of the scale that should be analysed (Dixon *et al.,* 2015).

Therefore, stable isotope analysis has become an important tool in the study of resource use and trophic position of fishes and other animals, especially threatened or endangered species, whose populations may be detrimentally affected by the collection of large numbers of samples. Isotopes are variants of an element that differ in the number of neutrons they possess within their atoms. The stable isotopes of carbon, $\delta^{12}C$ and $\delta^{13}C$ and nitrogen, $\delta^{14}N$ and $\delta^{15}N$ are the most commonly used in studies of ecological processes. The lighter isotopes (lower superscript number) are preferentially processed compared to the heavier isotopes in biological pathways, resulting in isotopic fractionation, which leads to retention of the heavier isotopes within biomass (Evans and Bauer, 2016). Animal body tissues exhibit stepwise enrichment of ratios of $\delta^{15}N$ with increasing trophic position within food webs due to this fractionation of the isotopes during the feeding process (Minagawa and Wada, 1984), allowing the estimation of trophic positions on the basis of a 3-4‰ enrichment of a consumer in relation to its prey (stable isotope discrimination factor) (Post, 2002). More recent research by Busst and Britton (2016) has demonstrated more variation, from 2-6.9‰, dependent on the protein content of diets fed to captive samples of the omnivorous fishes European barbel (*Barbus barbus* (Linnaeus, 1758)) and chub (*Squalius cephalus* (Linnaeus, 1758)). Other studies have provided evidence suggesting that stable isotopes derived from fish scales and fin clippings provide a viable non-lethal alternative to the more established use of muscle tissue (Hanisch *et al.*, 2010, Kelly *et al.*, 2006) or otoliths (Wells *et al.*, 2003) in stable isotope studies. Ramsay *et al*. (2012) further demonstrated that classification of fishes to their sites of origin was 5% higher for δ^{15}N and δ^{13}C from scales than Sr, Mn, Ba and Mg and 4% higher than for otoliths.

Stable isotope techniques have been used to demonstrate intra-specific variation in the diets of sympatric populations of brown trout (*Salmo trutta*) in Loch Ness, Scotland (Grey, 2001). The trout samples used in the study consisted of small parr (medium sized littoral and open water inhabitants) and ferox (large piscivorous specimens). The δ^{15}N ratios increased with length of fish. These results were cross-referenced with gut content analysis, which separated the trout into the three categories mentioned above by prey type (open water parr: lotic invertebrates and zooplankton; littoral parr, littoral-benthic invertebrates, aerial insects; ferox: Arctic charr (*Salvelinus alpinus*) and brown trout). Other salmonid species such as lake dwelling Arctic charr have also been shown to exhibit variation in feeding ecology (Guiguer *et al*., 2002, Knudsen *et al.,* 2014) and differences in morphological traits linked to littoral and pelagic niche adaptations. A recent paper by Olson *et al*. (2019) found a remarkably similar case of littoral-pelagic resource polymorphism to the many Arctic charr studies, but in a distantly related species, Hovsgol grayling (*Thymallus nigrescens* (Dorogostaisky, 1923)) from Lake Hovsgol, Mongolia. They identified two feeding groups by examining C and N stable isotope ratios, stomach contents and morphology, thus demonstrating that a wide array of relatively distantly related and geographically isolated species can exhibit similar propensities toward resource polymorphism.

The bulk of this type of scientific studies have been focused on salmonids within post glacial lake environments, but importantly for this study, cyprinids have also demonstrated intra-specific morphological and dietary variation, including populations living in river systems, as is the case of *T. putitora* noted above. Levin *et al*. (2018) found six morphotypes of *Labeobarbus gananensis* (Vinciguerra, 1895) in the Genale River, Ethiopia of which five displayed divergent morphology of the mouth and headparts, corresponding to scraping (two types), lipped and generalised forms and a large mouthed phenotype. Stable isotope analysis of $\delta^{15}N$ and $\delta^{13}C$ revealed differences representing alternative foraging strategies (scraping, omnivory and piscivory). Another riverine cyprinid species, described by Roberts (1998) from the Bolovens Plateau in southern Laos, *Poropuntius bolovenensis* was observed by him to exhibit four distinct trophic morphotypes: generalised; narrow head and thick lips; short head and horny jaw sheath; large broad head and wide jaws. Unfortunately, this species has not yet been studied for any trophic relationships with stable isotope analysis to complement the gut content analyses on which the conclusions were based. Roberts and Khaironizam (2008) also noted examples of other cyprinid species displaying various morphotypes including the thick-lipped morphotype with mental lobe characteristic for the Type B morphotype of *T. putitora* examined in the current study. Their study noted the same phenomenon in African barbs, *Barbus brucii* (Boulenger, 1907), Iraqi and Iranian *Tor grypus* (Heckel, 1843), Malaysian *Neolissochilus soroides* (Duncker, 1904), and also in the more distantly related cyprinid family Schizothoracinae, widespread in Asia and present in the Mahakali catchment where the current study was undertaken. Roberts (1998) states that trophic polymorphism represents an ancient evolutionary heritage of the ‘Old World Barbels’ (including the *Tor* genus), which has played a role in the adaptive radiation, speciation and evolution of distinct genera within this group of fishes.

Aims

The occurrence of the two morphotypes of *T. putitora* in the Mahakali River is hypothesised to be a result of two differing feeding strategies: utilising resources from the mid and upper zones of the water column, and exploitation of benthic organisms on, or close to, the riverbed. The aim of this analysis is therefore to examine If a significant difference in trophic position between the two morphotypes can be established. If this is indeed the case, then it would be reasonable to assume that they utilise different food resources within the range of habitat formed by the hydrological dynamics of this seasonally variable Himalayan river. By examining both the areas of earliest scale growth (scale focus) and most recent growth (outer edge), comparisons can also be made between early and later life stages of juveniles, which may provide insights into ontogenic shifts in foraging behaviour.

Methods

Fish samples were obtained by angling, cast netting and from local fishermen (chapter 3) from two sites located on the main Mahakali River (***Fig. 5.1***) at its junctions with breeding tributaries where both morphotypes had been observed to exist in sympatry. Scales for stable isotope analysis were removed from directly in front of the anal fin of the specimens and dried in sunlight under protective netting. The dried scales were then stored in numbered ziplock bags and transported to the laboratories at Edinburgh Napier University. The number and types of samples recovered is shown in ***Table 5.1***.

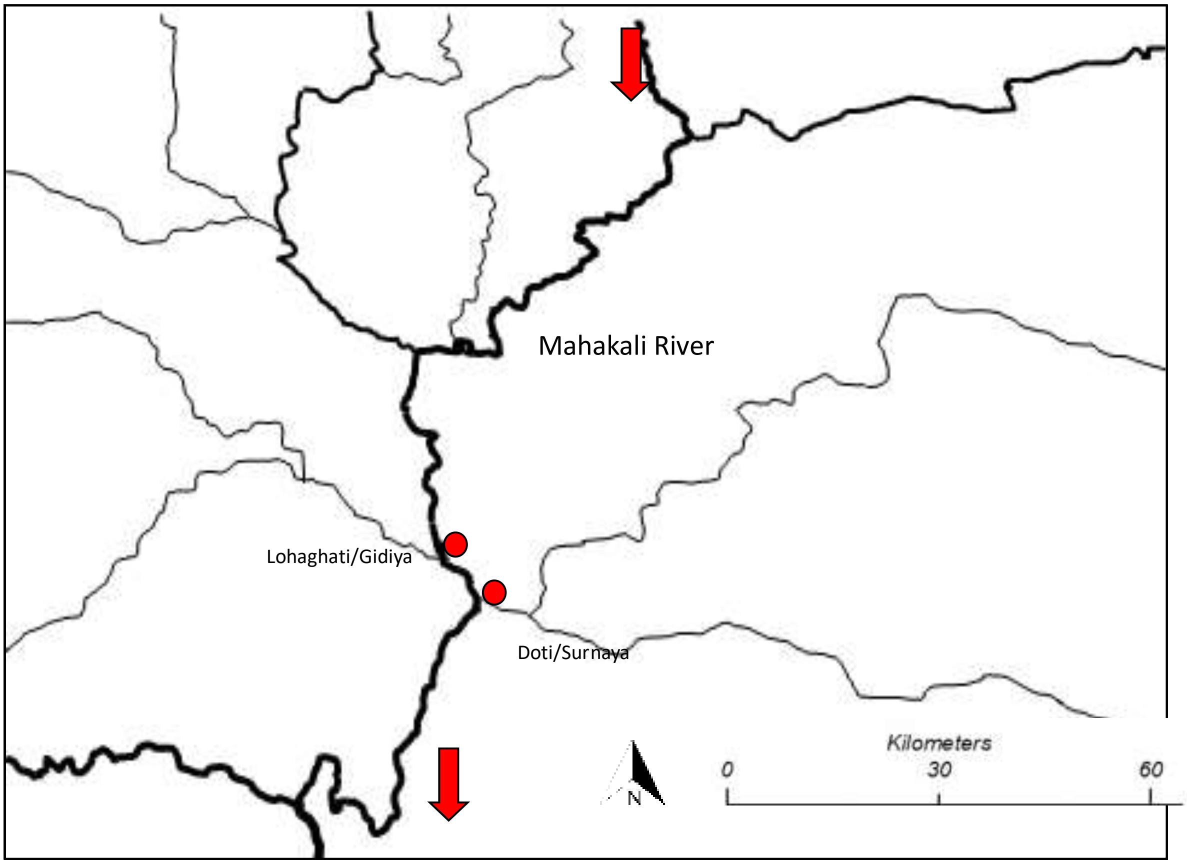


Figure 5.1: Sampling Sites: *Central river with flow direction arrows is the Mahakali, with two* T. putitora *stable isotope sampling sites (red dots).*

Table 5.1: Tor putitora *morphotype samples used in stable isotope analysis, with total length and sub-catchment sample sites.*

Sub-catchment	Type A	Total Length (cm)	Type B	Total Length (cm)
Lohaghati/Gidiya	A45	17.5	B17	23.2
Lohaghati/Gidiya	A46	15.5	B18	20.3
Lohaghati/Gidiya	A47	16.5	B19	19.2
Doti/Surnaya	A60	22.3	B2	14.5
Doti/Surnaya	A61	26.8	B14	17.0
Doti/Surnaya	A62	23.6	B15	20.0

Six samples from each morphotype (three each from both sub-catchments, ***Table 5.1***) were selected for scale analysis according to their total lengths in order to compare individuals of similar size and age class. Type A samples ranged from 15.5 to 26.8cm, Type B samples from 14.5 to 23.2cm.

The scales were individually soaked in deionized water for one hour and cleaned with toothbrushes to remove any residues of lipids or guanine. Six scales from each morphotype of *T. putitora* were then dissected under an Olympus SZ51 dissection microscope with scalpel and forceps. 0.7 mg portions measured on a Sartorius ENTRIS 124-1S scale, representing both $\delta^{13}C$ and $\delta^{15}N$ isotope samples, were taken from the focus of the scale - representing growth to date including the early developmental stage, and the outer edge of the scale - representing most recent growth (***Fig. 5.2***). They were transferred into 5mm x 3mm pressed tin capsules (Elemental Microanalysis, Okehampton) and dried in an oven at 60^{o}C overnight. The dried samples were then sent to NERC Life Sciences Mass Spectrometry Facility at East Kilbride, Scotland for mass spectrometry analysis where they were combusted in an Elemental Pyrocube elemental analyser linked by continuous flow to a mass spectrometer (Thermo Fisher Scientific-Delta Plus X). The stable isotope ratios were recorded in ‰ with delta notation δ according to the V-Pee dee belemnite and air international standards for carbon and nitrogen, respectively. The data was then separated into eight groups: A morphotype focus, B morphotype focus, A morphotype scale outer edge, B morphotype outer edge, for both $\delta^{13}C$ and $\delta^{15}N$ results. Each group was then rested for normality of distribution with the Anderson-Darling test and found to be normally distributed. To test the possibility that total length may influence the variance an Ancova analysis was performed in Minitab 17 with sub-catchment and type as factors and total length as a covariate.Morphotype A and B Means were then tested against each other with a Two Way Anova and Tukey post hoc tests applied.

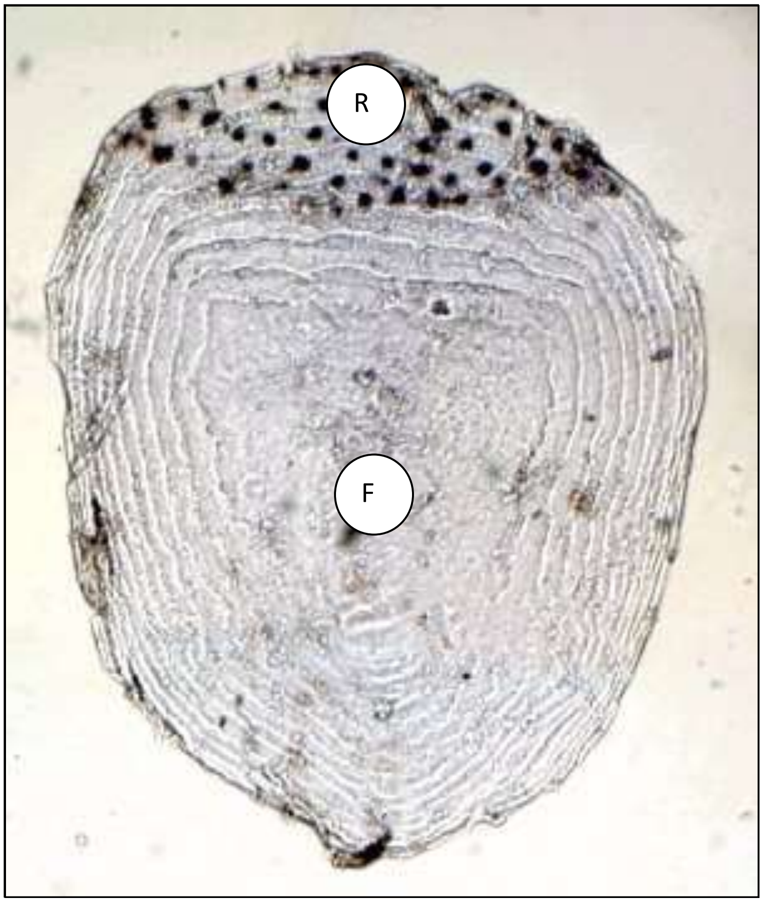


Figure 5.2: *Location of samples removed from scales of* Tor putitora *morphotypes for Mass Spectrometry analysis:* ***F****-focus (growth in early developmental stage),* ***R****-outer edge (recent growth stage. Scale shown is typical cycloid scale reference: http://www.microscopy-uk.org.uk/mag/artjan02/fishes.html*

Results

$\delta^{13}C$ ratios from focus samples ranged from -19.74 ‰ to -13.53 ‰ for Type A and -18.77 ‰ to -14.43 ‰ for Type B. $\delta^{13}C$ ratios for outer edge samples ranged from -20.18 ‰ to -13.59 ‰ for Type A and -20.36 ‰ to -15.57 ‰ for Type B. Mean $\delta^{13}C$ ratios were lower for A focus samples than B focus samples (***Table 5.2***) but lower for B outer edge samples than A outer edge samples.

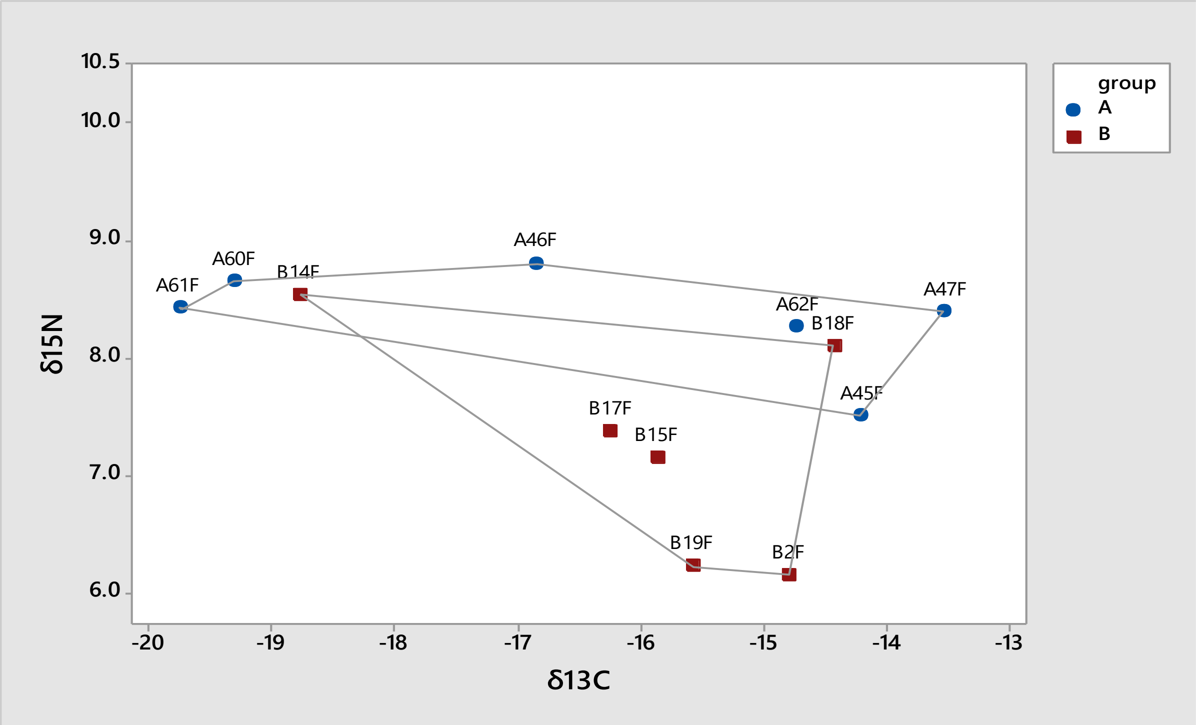
$\delta^{15}N$ ratios from focus samples for Type A morphotypes ranged from 7.51 ‰ to 8.85 ‰. $\delta^{15}N$ ratios from Type B focus samples ranged from 6.15 ‰ to 8.54 ‰.

Outer edge sample $\delta^{15}N$ ratios ranged from 8.12 ‰ to 10.17 ‰ for Type A and 6.81 ‰ to 9.48 ‰ for Type B. Mean $\delta^{15}N$ ratios were higher for A morphotypes than B morphotypes for both scale focus and outer edge samples. A summary of the means and standard deviations are shown in ***Table 5.2*, **and range of values for individual samples shown on stable isotope biplots in ***Fig***. ***5.3***.

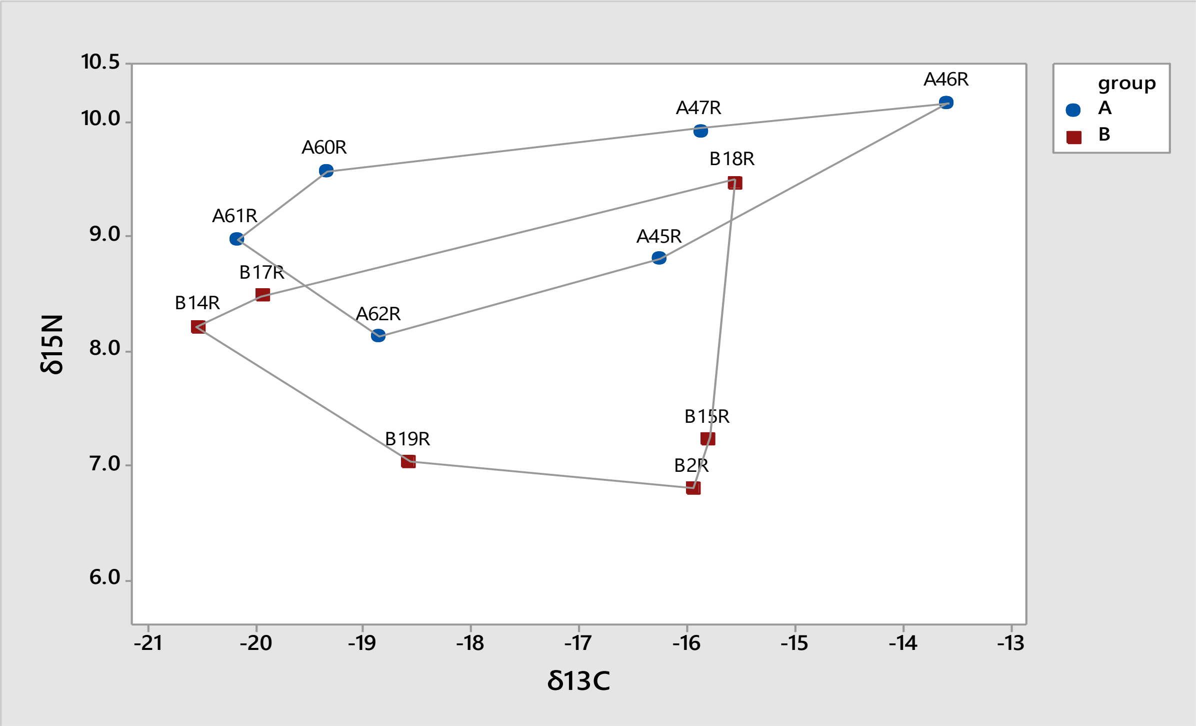
Table 5.2: *Means and Standard Deviations of stable isotopes $\delta^{13}C$ and $\delta^{15}N$ recorded from scale samples of Tor putitora. AF=Morphotype A focus sample. BF=Morphotype B focus sample. AR=Morphotype A outer edge sample. BR= Morphotype B outer edge sample.*

Sample Type	**Mean $\delta^{13}C$**	**Std. Dev.**	**Mean $\delta^{15}N$**	**Std. Dev.**
AF	-16.391	2.670	8.340	0.452
BF	-15.948	1.535	7.259	0.968
AR	-17.351	2.521	9.265	0.767
BR	-17.730	2.238	7.880	1.029

For both life stages represented by the scale focus and outer edge samples, morphotype A samples cluster towards the top half of the stable isotope biplot (***Fig. 5.3)*** demonstrating higher $\delta^{15}N$ values compared to morphotype B samples.


group
A
B
10.5
10.0
9.0
8.0
7.0
6.0
δ15N
-20
-19
-18
-17
-16
-15
-14
-13
δ13C
A61F
A60F
B14F
A46F
A47F
A62F
B18F
A45F
B17F
B15F
B19F
B2F

a


group
A
B
10.5
10.0
9.0
8.0
7.0
6.0
δ15N
-21
-20
-19
-18
-17
-16
-15
-14
-13
δ13C
A46R
A47R
A60R
B18R
A61R
A45R
B17R
B14R
A62R
B15R
B19R
B2R

b

Figure 5.3:* *(a) *Stable isotope biplot (δ13C and δ15N) for samples from scale focus of different* T putitora *morphotypes.* ***(b)*** *Stable isotope biplot (δ13C and δ15N) for samples from scale outer edge area.*

To test the possibility that total length may influence the variance an Ancova analysis was performed in Minitab 17 with sub-catchment and type as factors and total length as a covariate. Total length did not affect $\delta^{13}C$ or $\delta^{15}N$ in either scale focus or outer edge samples. There were also no interactions between sub-catchment and type for both, stable isotopes and scale sections. Morphotype A means (Two way Anova) were significantly greater than morphotype B means for $\delta^{15}N$, both for scale focus samples (F=5.57, df=1, p=0.043) and for scale outer edge samples (F=8.37, df=1, p=0.018). Tukey post hoc tests for both results confirmed the difference (scale focus difference in means -1.081, p=0.043, scale outer edge difference in means -1.385, p=0.018. No significant differences were found for $\delta^{13}C$.

Discussion

The trophic level inhabited by animals is reflected in their $\delta^{15}N$ signatures (Minagawa and Wada, 1984). Higher signatures are linked to feeding at higher trophic levels and vice versa. Any differences between two morphotypes of the same species would therefore suggest differences in diet and feeding behaviour. The results of the stable isotope analysis appear to indicate that the diets of A and B morphotypes diverge early in life and this divergence increases with age/total length, at least until the maximum age/length of the samples tested. The significant result obtained for $\delta^{15}N$ signatures confirms differences in trophic ecology both for early and recent growth stages of the *T. putitora* samples from the Lohaghati/Gidiya and Doti/Surnaya sites suggesting a relatively higher trophic position for A morphotypes. This implies a gradual separation in dietary preference during development, perhaps along the lines of ontogenetic shifts by

A morphotypes from macroinvertebrates to small fish, although this remains to be validated. Macroinvertebrate populations in the two sub catchments contain a variety of functional feeding groups: scrapers, which feed on algae and periphyton; shredders and collectors, utilising coarse and fine particulate organic matter, respectively; filterers, exploiting fine suspended organic matter; and finally, predators, which prey on the others. As carnivorous macroinvertebrates generally produce higher $\delta^{15}N$ fractionation values (Vander Zanden and Rasmussen, 2001) for a single prey-predator linkage (3.4‰ ± 0.4‰) than herbivorous macroinvertebrates (2.5 ‰ ± 2.5‰) any species of fish which consumes a mixture of the types may not display clear cut differences in $\delta^{15}N$ signatures at the 3.4‰ value necessary to be classified at a higher trophic level according to the hierarchy established by Minagawa and Wada (1984). As the fish samples examined were at a relatively early stage of development (14.5cm-26.8cm) in terms of their maximum growth potential (>2m), it is possible that the results reflect a transitional phase between insectivory and piscivory for the A morphotypes. The significant increase in $\delta^{15}N$ signature from the early growth (scale focus) samples to the recent growth (scale outer edge) samples supports this interpretation.

In order to clarify the situation, it would be appropriate to compare samples from older/longer individuals of *T. putitora*, which may be difficult to achieve due to the relative scarcity of B morphotypes of larger sizes. Ontogenetic shifts in diet can affect stable isotope values within a species as demonstrated by: Zhao *et al.* (2014) in largemouth bass (*Micropterus salmoides*), which replaced a diet of littoral macroinvertebrates with fish from the pelagic zone, concurrently increasing $\delta^{15}N$ levels; in Arctic charr (*Salvelinus alpinus*) by Guiguer *et al.* (2002), which also displayed a shift to piscivory from invertebrates; and in brown trout (*Salmo trutta*) by Grey (2001) who found that $\delta^{15}N$ signatures became progressively enriched as total length increased. As larger *T. putitora* individuals of Type A are regularly captured by using fish baits and lures which mimic them, it has been suggested that an ontogenetic shift to piscivory also takes place in this species, and that this shift may be recorded in the $\delta^{15}N$ values of larger fish. One complication, which must be faced in an investigation of older individuals is the way scale growth takes place. As a fish grows, the scales lay down successive layers of collagen with each centrifugally grown ring of new growth, resulting in the scale focus containing isotopic components from all previous growth seasons (Hutchinson and Trueman, 2006) so any further analysis of scale stable isotope signatures in larger individuals should preferably be restricted to recent growth areas to avoid unnecessary complication, as the aim of the study would only be to ascertain whether the diets of fully developed adults produce significantly different isotope signatures close to the 3.4‰ value established by Minigawa and Wada (1984). Further analysis would also benefit from inclusion of more samples as the sample sizes were necessarily restricted due to financial considerations.

Chapter Six

Conclusions

This project was conceived as a result of observations made on the Mahakali River in Western Nepal. There appeared to be two sub-catchments containing spawning tributaries where sympatric populations of *Tor putitora* expressed two different morphotypes. The aims of this study were to: 1) evaluate whether the two morphotypes could be separated by morphological, meristic and allometric traits; 2) investigate physico-chemical conditions and aquatic invertebrate community composition in breeding streams to determine if variation existed between stream ecosystems and they contained differing potential food resources that may influence morphological adaptations of fish; 3) examine if distinct trophic positions of the two morphotypes reflecting different dietary preferences could be established by means of stable isotope analysis.

The results obtained in the morphological studies indicate that the two morphotypes can be distinguished using landmark-based geometric morphometric analysis of the head and mouth areas, whereas meristic and allometric methods proved to be unreliable as identification tools. In the case of the Mahakali catchment, the widely used (e.g. Adams and Huntingford, 2004, Mattson and Belk, 2013) landmark-based geometric morphometric analysis described in Chapter 3 has provided strong evidence that the variation in the head and mouthparts is expressed as two discrete morphotypes (thick lips with mental lobe and thin lips without mental lobe) as opposed to a continuum of variation from one extreme to another. The meristic (scale and fin ray counts) and allometric (body proportions) analysis however, proved inconclusive as a means of discriminating between Type A and B variants. Overlaps in the fin ray and scale counts confounded any definitive characterisations, such as those proposed for other recently reclassified *Tor* species previously believed to be *T. putitora* by Arunkumar and Basudha (2003), *T. barakae* and ZiMing and JungXing (2004), *T. yingjiangensis*. This suggests that the polymorphism is restricted to the head and mouthparts of *T. putitora* in the individuals examined in this study. Evidence that populations of *T. putitora* containing Type B morphotypes, including those previously described as *T. macrolepis* and *T. progenius*, are found living sympatrically with Type A morphotypes in other rivers is currently scant (Laskar, 2012). Further investigation is required to examine if the existence of sympatric populations of the two morphotypes that was evident in the present study is reproduced in other river systems throughout the species geographical range. Anecdotal evidence (e.g. photographs, angling reports) from across the range of *T. putitora* during the past three centuries, provided by recreational anglers, suggests that this indeed the case.

As described in the introduction, genetic confirmation of the taxonomy of species belonging to the *Tor* genus also remains incomplete. This was recently emphasised by Raghavan (2020) at the 2nd International Mahseer Conference, held in Chiang Mai, Thailand. However, the study by Nguyen *et al.,* (2008), using regions of mitochondrial DNA (Cyt b, 16S rRNA, ATPase), plus the investigation by Khare *et al.* (2014), using another region of mitochondrial DNA (Cytochrome C Oxidase-COI), plus the work of Laskar *et al.,* (2013), also utilising COI techniques, have provided strong evidence that the *Tor* species (*T. macrolepis* and *T. progenius*), displaying morphological characteristics similar to Type B variants analysed in this study are indeed synonyms of *T. putitora*. Further investigations of polymorphism in *T. putitora* should therefore focus on the intraspecific genetic identities of populations associated with individual spawning tributaries. A very recent study by Yadav, *et al*. (2020) has combined mitochondrial DNA (Cytochrome b) and microsatellite analysis to examine relationships between populations of *T. putitora* in tributaries of the River Ganga. Their study demonstrated the utility of this technique in identifying discrete populations within one catchment although, crucially, the samples were obtained almost entirely (11 of 12 sites) from snow fed main stem rivers rather than in or at the confluences with known breeding tributaries, where populations may be linked to each breeding stream. The methodology, however, appears to be very suitable as a means of establishing a clearer picture of the extent of genetic relatedness and breeding stream fidelity of *T. putitora* in the Mahakali catchment (and elsewhere), as well as providing details of any genetic relationship between morphotypes A and B in the sympatric populations examined in this study. The results of this type of investigation could have important implications on understanding the relative influences of genetics vs environmental factors in the observed polymorphism in the sympatric populations.

The question of whether morphological variation could be linked to ecological conditions in separate tributaries of a sub-catchment, was examined in Chapter 4. The investigation of the physico-chemistry and aquatic invertebrate community composition in the breeding streams exposed differences, but these appeared to be largely temporal rather than spatial, as was originally hypothesised, leading to the formulation of a new hypothesis and potential future line of investigation. The physico-chemistry of streams has well-established effects on the composition of aquatic invertebrate communities (Brewin, *et al.* 2000, Milner, *et al.* 2001), and hence on the availability of certain food resources for local juveniles of fish species. If availability of particular prey items in spatially segregated nursery areas was a driving force behind phenotypic variation in *T. putitora* within the two sub-catchments of the Mahakali where sympatric populations of Type A and B are found (Lohaghati/Gidiya and Surnaya/Doti), then we would expect to find clear differences between the paired streams within those sub-catchments. This study found differences between the Lohaghati and Gidiya nursery areas, but not between the Surnaya and Doti nursery areas thus providing no clear evidence that the morphological variation is driven by food resource availability within spatially separated reproductive groups in the manner found by Grey (2001) in brown trout, or Guiguer *et al*. (2002) in Arctic charr. However, there was strong evidence of differences between ecological conditions of the tributary streams during the two sampling seasons, post-monsoon and snow-melt, both in physico-chemistry and aquatic invertebrate community composition. These results have prompted the formulation of a new hypothesis: *Tor putitora* morphotype A and B reproduction may be isolated temporally by two discrete breeding events, with juveniles possibly exploiting significantly different food resources during the early phases of development. Some researchers (Johal and Tandon, 1981, Sehgal, 1987, Joshi *et al.*, 2002, Malik, 2011) suggested the occurrence of two or more breeding events within a single year as noted in Chapter 4 and additional work to examine this hypothesis, along with an intraspecific genetic comparison between the two morphotypes, may provide information regarding the population identity and homogeneity or otherwise of the morphotypes. If the occurrence of more than one breeding event can be demonstrated by the capture of gravid females outside of the regular June-October breeding season, or actual spawning events witnessed at the same time, then fin clippings could be collected for the genetic analysis discussed above and compared with samples previously obtained from both morphotypes. Any specimens captured should also be analysed with the landmark-based geometric morphometric analysis to establish their classification as morphotype A or B. If no significant differences in genetic identity are discovered between the morphotypes, it will suggest that environmental factors are influencing the polymorphism, especially if both morphotypes are present and gravid at the same time. If only one morphotype is found to be in breeding condition, and significant genetic separation is established, the role of genetic identity in the polymorphism may be dominant.

Irrespective of whether the observed morphological variation stems from temporally separated spawning events as hypothesised above, the stable isotope analysis conducted in Chapter 5 has provided evidence of trophic differentiation/dietary partitioning between Type A and B morphotypes. This suggests that further analysis of later growth stages could be a fruitful area of research. The differences are small but significant and appear to increase with ontogenetic development. The samples analysed in this study were all obtained from specimens under 27cm in length, which is a relatively early stage in the development of a species for which the largest examined specimen measured 130cm. As documented in Chapter 5, a wide range of fish species experience dietary shifts from aquatic invertebrates to piscivory during their life cycles, such as the Eurasian perch studied by Hjelm, *et al.* (2000). Their study showed that perch diets shifted with ontogenetic growth from pelagic zooplankton, through benthic invertebrates and finally to almost exclusively fish. This may also be the case for *T. putitora* morphotype A individuals, whereas morphotype B specimens may be restricted to a diet of algae and aquatic invertebrates gathered from the bottom of the river. The shape of the mouthparts of the two morphotypes (forward facing in A type, downward oriented in B type) suggests that foraging takes place in different parts of the water column (Adams and Huntingford, 2004, Cucherousset, *et al.,* 2011). Further examination of larger specimens' stable isotope values would be useful in this respect. A comprehensive study cross-referencing the data with gut content analysis may be achievable without causing harm to individuals of this endangered species by the use of stomach flushing techniques (Kamler and Pope, 2001). However, personal experience and anecdotal evidence from local fishermen and recreational anglers suggests that larger *T. putitora* specimens killed for food are invariably found to have empty stomachs upon examination. This phenomenon is unexplained and the hypothesis untested but, if it is the case, then any stomach flushing exercise would prove to be futile as a means of cross-referencing stable isotope and gut content analyses.

As noted in the introduction to this study, the occurrence of cyprinid morphotypes with thick lips and a mental lobe is not restricted to *T. putitora* or even the *Tor* genus. Roberts and Khaironizam (2008) briefly examined trophic polymorphism in other barbin cyprinids from various genera across Asia, the Arabian Peninsula, and Africa. They concluded that trophic polymorphism has been an important factor in the adaptive radiation of cyprinids, their relative dominance in the habitats they occur in, and their propensity to evolve rapidly on a geological timescale. They also noted that the most extreme examples of intraspecific trophic polymorphism are observed in locations where the fish fauna is relatively depauperate, such as isolated lakes, mountain streams, or habitats containing physiographical barriers including waterfalls and extensive series of rapids. The rivers in the Mahakali catchment fit the last two descriptions exactly. The much-studied Salmonidae of the northern hemisphere share this very useful trait and have used it to their advantage to fill several ecological niches during the colonization of lakes and streams revealed by the retreat of the glaciers at the end of the last Ice Age (Knudsen *et al.,* (2006). Many studies have investigated this phenomenon in so-called First and New World countries, but it appears that much remains to be learnt from the considerably less studied cyprinids of the Old World. For instance, a lot of the interest in Salmonidae centres around their anadromous migratory behaviour in species such as Atlantic Salmon (*Salmo salar*), which undertake long and arduous journeys from their birthplaces in small upland streams downriver to the ocean as juveniles, returning as adults to breed after one or more years. Members of the *Tor* genus are also suggested to travel long distances to reach their spawning grounds (Nautiyal *et al.*, 2008), in this case entirely within freshwater systems (potamodromous migration). It remains unknown whether individuals of *T. putitora* return to their natal streams in the same manner as salmon, or if a more generalised mixing of populations takes place. In this study, the sympatric populations appear to be linked to particular sub-catchments containing breeding streams, a circumstance that casts some doubt upon Nautiyal's assumption of long trans-catchment migratory journeys in this species (Nautiyal *et al.*, 2008), at least in the Mahakali River system. Anthropogenic modification of Himalayan rivers in the form of hydroelectric dams is believed to be a leading cause of decline in populations by interrupting migration routes (Shrestha, 1998). As the construction of dams shows no sign of decreasing in this area (Anon, 2013), a comprehensive follow-up study examining migratory behaviour by tracking individual specimens, which has been proposed by the Mahseer Trust (Anon., 2019) for the highly endangered *Tor remadevii*, accompanied by a study of intraspecific genetic comparison between the populations associated with different spawning streams within the Mahakali catchment (Lohaghati/Gidiya, Surnaya/Doti, Chamaliya, Bhora, Ladhiya, Jhurigar, Panar and Sarju), would likely produce evidence to clarify the situation.

The importance of those findings cannot be understated, as conservation efforts focusing entirely on the preservation of uninterrupted major Himalayan rivers may lose sight of the importance of preserving the smaller breeding tributaries. Many of these tributaries pass through towns, farmland and villages located at higher elevations than the deep, sparsely populated and relatively inaccessible gorge of the Mahakali. Therefore, they may face greater risks in the form of pollution, sand extraction and destructive fishing methods which reduce populations of juveniles. If individual breeding tributaries support genetically distinct populations displaying limited migration within the Mahakali catchment, then surely conservation efforts should focus on the tributaries and the sections of the main river exploited by the adults of the species, an area that could hypothetically exist above, below or between any hydroelectric constructions on the Mahakali itself, such as the Pancheswar Multi-Purpose Project. The risk to such distinct populations posed by hydroelectric dam construction on the main river is not restricted to limitations imposed on migration routes, however. Changes to flow regimes can affect fish migratory stimuli (Poff, *et al.,* 1997), temperature variation can influence rates of development and survival of juvenile fish (Clarkson and Childs, 2000), sediment transport can alter the ecological status of the river (Schmutz and Sendzimir, 2018), and invasive species may be introduced to the reservoirs created (Chao et al., 2018). Irrespective of these further challenges, priority should be given to the protection of the areas which support breeding and juvenile growth stages to ensure production of future stocks to supply the entirety of the section of the main river inhabited by each population of *T. putitora*.

To summarize: evidence was presented that two morphological variants of *Tor putitora* are found in sympatric populations within the Mahakali River catchment. Further investigations of other Himalayan river systems to ascertain whether this phenomenon is widespread across the range of *T. putitora* habitat, and the implications regarding utilisation and status of those habitats, could be crucial in developing conservation strategies for the future. The newly established hypothesis considering temporal separation of breeding events within sub catchments requires testing to establish its validity - this would require a series of electrofishing surveys throughout a full year. A follow-up study of stable isotope ratio values within both morphotypes should include a greater number of samples generally, and older and larger specimens particularly, to examine if the divergence of $\delta^{15}N$ values increases continuously with growth, providing additional evidence of dietary divergence between the two morphotypes.

Genetic analysis has the potential to provide much useful information to help clarify some of the remaining questions around the distinction between the morphs that has been evidenced in this study. A variety of approaches has been used in previous work: random amplified polymorphic DNA (Mohindra, *et al*.,2007, Ghosh and Alam, 2008, Arora and Julka, 2013), ribosomal DNA (Singh, *et al.,* 2009, Kumar, *et al*., 2013), mitochondrial DNA (Nguyen, *et al*., 2008, Laskar, *et al*., 2013, Khare, *et al*., 2014) and microsatellite DNA (Esa *et al.*, 2011, Sahoo, *et al.*, 2013). Once the Mahseer Trust, an international group of scientists currently collating information on all *Tor* species, reaches final agreement on the preferred methodology for DNA analyses in cases of intraspecific variation, a study of the two morphotypes should be conducted to shed light on the question of genetic influence on the polymorphism observed in *Tor putitora* and other *Tor* species. Further investigation of the migratory habits and population genetic identity would be an important step in formulating conservation strategies within the Mahakali catchment and elsewhere in the future.

Ultimately, an overall conservation strategy needs to be established which has the ability to draw on the various lines of research applicable to migratory fish species: hydrology (dams, water and substrate abstraction), ecology and population dynamics (identity, ecology, habitat utilisation, interconnectivity of populations), other anthropogenic influences (destructive fishing practices, pollution, introduction of alien species). One tool which may prove to have the attributes necessary to encompass the above requirements is the Freshwater Protected Area (FPA). FPAs are classified “protected areas” by the IUCN as “clearly defined geographical space, recognised, dedicated, and managed, through legal or other effective means, to achieve the long-term conservation of nature with associated ecosystem services and cultural values" (IUCN 2008). A study by Bower, *et al*. (2005) examined how FPAs can be applied to migratory fish populations and recommends measures including: identification of migratory species, their ecology and their essential habitats, location of FPAs in areas likely to support the maximum number of migratory species, prioritising connectivity to support migratory behaviour, including stakeholders and community members at all stages of planning and implementation. In the case of the Mahakali, the first recommendation would take the form of a systematic effort to clarify the taxonomy of the three *Tor* species recorded in the catchment and elucidate their breeding habits, migratory range and habitat utilisation. The second and third recommendations would entail locating which tributaries of the Mahakali are used as spawning and juvenile development habitats, then prioritising those with the greatest interconnectivity. Finally, all the stakeholders in the Mahakali catchment should receive a fair and equal consideration of their views-from the riparian subsistence agriculturalists, traditional fishing communities, recreational anglers, representatives of the religious communities who carry out rituals on the riverbanks, to the forestry departments, hydropower and irrigation departments and other government agencies. It is to be hoped that this thesis may form a small part of this collective effort and assist the conservationists of the future.

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