Trait divergence in river and reservoir populations of Australian smelt (*Retropinna semoni*)

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Contents

Certificate of authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma at Charles Sturt University or any other educational institution, except where due acknowledgment is made in the thesis. Any contribution made to the research by colleagues with whom I have worked at Charles Sturt University or elsewhere during my candidature is fully acknowledged. I agree that this thesis be accessible for the purpose of study and research in accordance with the normal conditions established by the Executive Director, Library Services or nominee, for the care, loan and reproduction of theses.

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Animal ethics approval

The research presented in this thesis was approved by the Charles Sturt University Animal Care and Ethics Committee (Permit numbers 14/058, 15/002, 15/052, 15/065 and A16035).

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Abstract

Dam construction has been a major driver of ecological change in freshwater ecosystems. Fish populations have been shown to diverge in response to different flow velocity habitats. However, adaptation of fish populations to different flow velocity habitats in rivers and reservoirs have not been widely explored. Understanding how fish populations have diverged in response to ecosystem changes, such as altered flow velocity, can help predict the effects of human impacts on freshwater systems.

The aim of this thesis was to evaluate divergence of body and fin morphology, prolonged swimming speed performance and physiology in response to different flow velocities by comparing these traits among six river and five reservoir populations of Australian smelt (*Retropinna semoni*), a small-bodied fish from south-eastern Australia. Australian smelt are a suitable model species for this research because: 1) they are a short-lived species (~ 3 years) and have a short generation time (~ 6 to 12 months); 2) they are widely distributed and common throughout rivers and reservoirs in south-eastern Australia and 3) populations are highly genetically structured and therefore potentially adapted to local habitats. Reservoir habitats selected for this study have existed for at least 50 years, during which time, divergence of resident Australian smelt from river populations may have occurred over multiple generations.

Using geometric and traditional morphometric methods, I showed that mean body shape was significantly different among river and reservoir populations of Australian smelt based on Procrustes distances (D_ρ=0.007 – 0.017; F = 26.822; P < 0.001). Rivers populations had deeper bodies, larger heads, narrow caudal peduncles and terminal (horizontal) mouth position compared to reservoir conspecifics which had narrow, fusiform bodies, small heads, relatively deep caudal peduncles and superior (ventral) mouth position.

The relationship of swimming speed performance with body morphology and fin aspect ratio was assessed in Australian smelt from a subset of three river and two reservoir populations using critical swimming speed (U_{crit}) tests in a recirculating swim tunnel. River populations achieved significantly higher mean swimming speeds (U_{crit} = 46.61 ± 0.98 cm s^{-1}) than reservoir conspecifics (U_{crit}= 35.57 ± 0.83 cm s^{-1}; P < 0.05). Caudal fin and pectoral fin aspect ratios were significantly higher for river (1.71 ± 0.04 and 1.85 ± 0.03 respectively) than for reservoir populations (1.29 ± 0.02 and 1.33 ± 0.02 respectively; P < 0.05). The relationship of U_{crit} with body morphology (Partial Least Squares regression components, from geometric morphometric analyses), fin aspect ratios and standard length was evaluated using the best subset selection approach. Caudal fin and pectoral fin aspect ratios were the strongest predictors of U_{crit} (β =19.421 and 5.142; t = 9.633 and 3.036

respectively; P < 0.01). Physiological differences were evaluated from a subset of three river and two reservoir populations of Australian smelt. Citrate synthase (a metabolic enzyme) activity in lateral muscle tissue (F = 18.451; P < 0.001) and gill mass (F = 16.498; P < 0.001) were significantly higher in river populations. These fish also had higher U_{crit} values (F = 73.778; P < 0.001) than reservoir populations.

Body shape differences among river and reservoir populations of Australian smelt support previous literature that morphological divergence occurs consistently among populations of freshwater fish from river and reservoir habitats. However, the direction of body shape divergence among Australian smelt populations (i.e. deeper or shallower body in a given habitat) was inconsistent with theoretical predictions and previous studies of body shape in river and reservoir populations of *Cyprinella lutrensis*, *Lepomis gibbosus*, *Bryconops caudomaculatus* and *Biotodoma wavrini*. In contrast, body shape patterns observed in river and reservoir populations of Australian smelt, were consistent with others such studies of *Oncorhynchus nerka*, *Gasterosteus aculeatus* and *Labidesthes sicculus*. Fin aspect ratios, but not body shape, were strongly correlated with critical swimming speed. Higher citrate synthase activity and gill mass in river populations may reflect the higher swimming speed requirements of living in flowing water.

The formation of permanent lentic waterbodies following dam construction may be driving rapid morphological, swimming performance and physiological divergence among river and reservoir populations of Australian smelt. Understanding the effect of divergence in body morphology, fin shape and physiology on swimming performance in river and reservoir populations of Australian smelt has provided new insights into how freshwater fishes have adapted to different flow velocity habitats following dam construction. These results highlighted the need to consider swimming kinematics and swimming mode as central to future research into fish morphology, physiology and swimming performance. Understanding how freshwater fishes adapt and persist is important because it provides insight into how the impacts of dam construction on freshwater fishes could be managed.

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Chapter 1. General introduction

1.1. Impacts of human disturbance on freshwater fishes

1.1.1. Human driven change in freshwater ecosystems

Freshwater systems, including lakes, rivers and reservoirs are among the most impacted ecosystems on earth. A range of uses, chemical inputs, species invasions, water extraction and regulation are all contributing to significant modification from natural states (Carpenter *et al.* 2011). In addition to direct human modification, freshwater resources are under increasing pressure from global climate change that results in altered ecological processes, driving shifts in native and invasive species' distributions and abundance, declines in suitable habitat and increased thermal stress for aquatic biota, particularly fish (Poff *et al.* 2002). The most prominent human threats or causes of extinction for freshwater biota are habitat degradation, pollution, exploitation and flow modification, the latter contributing to declines or extinction among 20% of threatened or vulnerable species (Collen *et al.* 2014).

Freshwater fishes have undergone declines with as much as 40% of all species classified as vulnerable or threatened (Collen *et al.* 2014). Fishes are the most vulnerable to flow modification activities, of which dam construction has the greatest impact because it obstructs migration corridors, causes mortality due to passage through pumps and hydropower turbines, loss of genetic connectivity, and reduces spatial extent of suitable habitats (Liermann *et al.* 2012). In response to dam construction, freshwater fishes have either undergone significant population declines, shifted their range and abundance, undergone some kind of adaptation or in the worst case, extinctions (Morita & Yamamoto 2002; Todd *et al.* 2005; Cureton & Broughton 2014).

1.1.2. Population level responses to dam construction in freshwater fishes

Dam construction has been a major driver of the unprecedented global change in freshwater ecosystems (Vitousek *et al.* 1997; Liermann *et al.* 2012; Collen *et al.* 2014) and has led to increased rates of evolutionary and adaptive change, especially at the population level (Smith & Bernatchez 2007).

Ecosystem and hydrological dynamics of rivers have been dramatically modified as a result of dam construction (Poff *et al.* 2007) which have imposed ecological constraints on native biota that are adapted to the natural flow regimes that existed prior to dam construction (Poff *et al.* 1997). The impacts arising from dams in downstream reaches include cold-water pollution, defined as altered seasonal temperature profiles due to selective flow releases from reservoirs (Stanford & Ward 1979) include altered spawning cues; migration timing (Holden

1979; Sherman *et al.* 2007; Ugedal *et al.* 2008) and recruitment (Humphries *et al.* 2002) as well as lack of microhabitat due to altered flow velocities and discharge volumes (Travnichek *et al.* 1995). While the effects of altered flow regimes downstream of dams have been well described, the ecological processes and associated selective pressures arising in reservoirs have not been as well described as those for natural lakes (e.g. Jones & Hoyer 1982). Nevertheless, rivers and reservoirs are known to be fundamentally different ecosystems, with a range of physical, chemical and biological differences. Sommer & Lampert (1998) state that flow and unidirectionality are the primary factors that distinguish rivers from reservoirs. In rivers, the constant mixing and disturbance of moving water eliminates physical and chemical gradients (Sommer & Lampert 1998; Wetzel 2001), and physical limitations are imposed by high flow velocities on the organisms that inhabit river ecosystems (Poff *et al.* 1997). In contrast, reservoir systems have a low flow gradient owing to distinct hydrological zonation: they consist of riverine zones where inflowing streams enter the system, transitional zones with characteristics intermediate between rivers and natural lakes, and lacustrine zones with relatively low or no flow and greater depth (Kimmel & Groeger 1984; Soballe & Kimmel 1987).

As a result of the different biotic and abiotic characteristics, reservoirs are likely to support fish populations or communities that have habitat requirements and face selective pressures different to those from rivers (Randall *et al.* 1995; Wetzel 2001). Unfortunately, there are few syntheses that concisely contrast biotic and abiotic factors in rivers and reservoirs apart from the earliest synthesis of Baxter (1977) and Fernando and Holčik's (1991) discussion of differences in fish communities in rivers and reservoirs and how these might cause trait divergence among fish populations.

Given that populations of riverine fishes are adapted to natural flow regimes (Poff *et al.* 1997), trait divergence might be an expected response in river populations of fishes and conspecifics inhabiting reservoir systems. Travnichek *et al*. (1995) noted that the relative abundance of certain fishes could be predicted by discharge and flow velocity, indicating that flow velocity could be a strong selective pressure. In light of the contemporary ecological change arising from dam construction, further work is needed to understand divergent responses of fish populations to conditions they have not previously encountered, as modification of river systems to meet human demands for freshwater resources continues.

1.1.3. Adaptation of freshwater fishes to rapid ecological change

Rapid ecological change can have serious consequences for freshwater fishes. Responses can range from extinction due to reproductive isolation (Morita & Yamamoto 2002), significant

population declines and altered life-history patterns (Law 2000) or adaptation to selective pressures (Reznick *et al.* 1996). Recent discovery of phenotypic divergence among freshwater fish populations separated following dam construction (e.g. Haas *et al.* 2010) suggests that there are major differences in the selective pressures acting on fish populations from river and reservoir systems.

Differences in flow velocity between rivers and reservoirs are likely to cause trait divergence among fish populations, due to local adaptations necessary to enhance migratory capacity, foraging and reproductive success (Pavey *et al.* 2010). Dittman and Quinn (1996), argue that salmonids have undergone adaptive divergence in body shape and life-history due to the high variability of hydrological and foraging conditions encountered along migratory pathways. Range restriction and isolation imposed by dams has also led to concerns over the resilience of locally adapted populations (Waples *et al.* 2009). Therefore, it is reasonable to expect that the largely still-water conditions in reservoir habitats (Kimmel & Groeger 1984; Soballe & Kimmel 1987) impose different physical constraints on the riverine fishes that inhabit them following impoundment (Fernando & Holčík 1991) and lead to divergence from river populations.

Trait divergence, particularly in morphology, has been shown to arise among populations of a riverine fish species separated by impoundment (e.g. Haas *et al.* 2010; Franssen 2011). However, there is uncertainty in the magnitude and direction of this trait divergence (e.g. McGuigan *et al.* 2003; Langerhans 2008). Organisms adapted to historically high-flow velocity systems may be released from the selective pressures associated with harsh lotic conditions to which they were adapted prior to isolation in an impoundment (Poff *et al.* 1997). This means that changes in velocity in reservoirs may constitute a novel ecosystem, because they differ considerably from historical river systems. The novel ecosystem concept (Hobbs *et al.* 2006) provides a theoretical framework for studying divergence of reservoir and river fish populations based on ecosystem configurations.

Novel ecosystems have previously been defined as ecosystems with biotic and abiotic composition that have emerged as a result of deliberate or inadvertent human activity (Hobbs *et al.* 2006). However, the current definition does not capture the intense selective pressures that can be associated with the impacts of dam construction on freshwater fishes. For example, cold-water dam releases which constrain recruitment success of native fishes (Clarkson & Childs 2000; Jensen 2003; Todd *et al.* 2005) represents a strong selective pressure, because temperature and flow regimes exceed the conditions to which they were normally adapted (Lytle & Poff 2004). While the definition described previously has been adequate in capturing the ecological changes that lead to altered fish assemblages (Travnichek

et al. 1995; Moyle 2014), it does not adequately capture the selective pressures that arise and differentiate one ecosystem from another, such as rivers and reservoirs. For this thesis, the existing definition of novel ecosystems from Hobbs *et al*. (2006) is modified to describe a novel aquatic ecosystem as:

A system distinguished over time by the presence or absence of a selective pressure, which alters the ecological fitness of a species or population following human intervention.

Understanding trait divergence responses to novel ecosystems has potential to assist with quantifying and predicting the resistance and resilience of fish populations in the face of ecosystem change. Reservoir ecosystems are distinguished as novel ecosystems by the selective pressures that altered flow velocity imposes on resident fish populations (Haas *et al.* 2010) which originated in highly heterogeneous river systems (Poff *et al.* 1997). Thus, for fishes to persist, and avoid declines and extinction, their populations must adapt morphologically, functionally or physiologically, to the biotic and abiotic conditions in which they exist.

1.2. Evaluating divergence of morphological, functional and physiological traits among populations of fish

In fishes, ecological and habitat requirements have driven the evolution of swimming performance (Langerhans & Reznick 2010). This has been based on the mechanistic link between body shape and swimming performance at the inter-specific level examined by Webb (1984a; 1984b) who showed that variation in morphology at broad taxonomic scales could explain differences in swimming performance and therefore their ability to exploit a given habitat (Fig. 1.1). This is evident in the range of swimming performance functions (e.g. cruising, accelerating, manoeuvring) (Webb 1984a) and the associated phylogenetic diversity of physical (e.g. morphology) and physiological (e.g. metabolic oxygen demand) maxima in fishes (Moyes *et al.* 1992; Dickson *et al.* 1993; Dickson 1995). Swimming performance is important for survival. It has been cited as the ultimate predictor of ecological fitness in fish because selection is thought to act on swimming performance directly, which is mediated by forces in flowing water that act on morphological and physiological features (Langerhans & Reznick 2010).

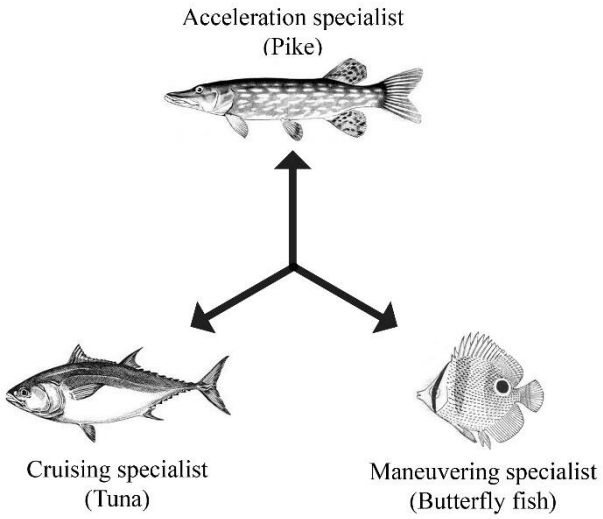

Figure 1.1. The functional morphology of fishes as described by Webb (Webb 1984a). Fishes at the corners of the triangle are specialists. Each specialist performs poorly in the other two functions. Fishes at the centre are generalists, performing each function well, but not as well as any of the specialists. Intermediate forms (between the centre of the triangle and specialists) perform a function better than the generalist, but not as well as the specialist. Modified from Webb (1984a).

Studies have shown that trait divergence in feeding structures and body shape arise among populations of the same species from river and lake habitats (Hendry *et al.* 2002; Berner *et al.* 2008). For example, predator density has altered life-history traits and burst swimming performance in geographically separate populations of guppies (*Poecilia reticulata*) (Ghalambor *et al.* 2004; Domenici *et al.* 2008; Langerhans & Makowicz 2009; Oufiero *et al.* 2011). Such changes have been observed at time-scales spanning no more than a human lifetime (Reznick *et al.* 1997; Hendry & Kinnison 1999). Divergence in body shape also appears to have been caused by the presence of dams which result in emergence of novel ecosystems in reservoirs (Langerhans *et al.* 2003; Haas *et al.* 2010; Franssen 2011; Franssen *et al.* 2013a; Cureton & Broughton 2014).

In the last decade, there has seen growing interest in intra-specific divergence in fishes following dam construction. Haas *et al*. (2010) presented the earliest evidence of body shape divergence between river and reservoir populations of *Cyprinella venusta*, a riverine fish species. They concluded that *C. venusta* populations isolated in reservoir habitats were on average, deeper bodied posteriorly with smaller heads, while river fish populations were on average, shallower bodied with larger heads. Franssen (2011) and Cureton and Broughton (2014) reported comparable patterns of morphological divergence for river and reservoir populations of *Cyprinella lutrensis* and *Pimephales vigilax.* These findings contrast with similar investigations in terms of the direction, magnitude and consistency of the morphological divergence between populations from lotic (flowing) and lentic (standing)

systems (Brinsmead & Fox 2002; McGuigan *et al.* 2003; Pavey *et al.* 2010; Franssen *et al.* 2013a). This may be due to constraints on the direction of trait divergence acting on a species (McGuigan *et al.* 2003) or species-specific swimming kinematics that determine the functional importance of body shape (Walker *et al.* 2013).

With a single exception (McGuigan *et al.* 2003), none of these studies have directly tested swimming performance and simultaneously compared body shape among river populations and their reservoir conspecifics. McGuigan *et al.* (2003) tested swimming performance among pairs of naturally occurring river and lake populations of rainbowfish (*Melanotaenia* spp.) but did not find differences in body shape or swimming performance. Therefore, despite their comprehensive analysis, no insights into trait divergence among fish populations from lotic and lentic habitats could be drawn from their study. These inconsistencies raise questions about the generality of divergence of body morphology and swimming performance among fish populations impacted by dam construction and the selective pressures emerging in reservoir ecosystems.

Much like the differences in the patterns of morphological divergence among fish populations, there have been different approaches implemented in the study of body morphology. The development in morphometric methods has greatly advanced research into the ecological importance of body shape (Strauss & Bookstein 1982; Fleming & Gross 1989; Meyer 1990; Douglas & Matthews 1992; Walker 1997; Ruber & Adams 2001). Rigorous statistical and geometric morphometric methods have been developed for evaluating shape variation under the premise that traditional morphometric methods were statistically and biologically flawed (Rohlf & Marcus 1993). The traditional method, which is based on relationships among linear measurements, might be confounded due to issues such as size covariation of measurements such as body length and depth (Parsons *et al.* 2003).

Despite the overwhelming adoption of the geometric method (Adams *et al.* 2004) some doubt remains about whether it is the most appropriate method for certain ecological problems (Parsons *et al.* 2003; Maderbacher *et al.* 2008). It has been argued that both methods may provide complementary inferential approaches. The traditional method is thought to be more appropriate for identifying patterns of covariation of body shape variables with size, while the geometric method is more appropriate for identifying fine-scale, localized shape variation (Schmieder *et al.* 2015; Ramírez-Sánchez *et al.* 2016). Thus, it is difficult to discern if the geometric method has become more popular. This may be because it provides a more powerful tool for analysis of body shape, or that there are simply disproportionately fewer studies in which other simple, linear metrics such as the fineness ratio (ratio of body length to depth; Walker *et al.* 2013) have been sufficient to address certain eco-morphological

problems. Thus, there is a need to determine if results obtained from the traditional method correspond to results obtained using the geometric method.

With the development of morphometric methods over the last 20 years, several researchers have argued that variation in body shape constrains swimming performance in different contexts including foraging (Ruber & Adams 2001), predator evasion (Langerhans *et al.* 2004) and migration (Chapman *et al.* 2015). However, with one exception (McGuigan *et al.* 2003), there have been no studies of swimming performance in fish populations from river and reservoir habitats. The assumption that body shape is a strong predictor of swimming performance and fitness among divergent populations of freshwater fish, has been based largely on inter-specific variation in body morphology and swimming performance (Webb 1984a; Domenici 2003; Blake 2004). Morphological adaptations are thought to be a mechanism by which energetic demands are minimized in fishes inhabiting different aquatic environments (e.g. trout are adapted to turbulent river habitats and tuna are adapted to cruising in the open ocean) (Vogel 1994). Specific attempts to explore the form-function relationship at the intra-specific level have yielded insightful results (Kolok 1992; Odell *et al.* 2003; Ojanguren & Braña 2003; Kilsby & Walker 2010). But these authors have tested this relationship only at an inter-individual level. Thus, these studies do not inform how swimming performance and morphological traits might change among divergent fish populations in river and reservoir habitats. Furthermore, there are very few studies that have considered the role of physiological traits in adaptation of fish populations under different selective pressures (i.e. flow velocity in river and reservoir habitats).

The relationship between body shape, swimming performance and physiological traits such as muscle composition or metabolic capacity are not always consistent and have not yet been fully explored (Langerhans 2008). Intra-specific variation of physiology has not been utilized in the context of trait divergence and rapid evolution of fish populations following dam construction. Hochachka (1961) presented one of the earliest demonstrations that heart mass significantly differed between groups of salmonids under different experimentally imposed exercise regimes. Studies of metabolic capacity following exercise training also revealed that performance of fish could be related to increases in metabolic enzyme activity (Johnston & Moon 1980; Dickson *et al.* 1993; Gibb & Dickson 2002; Norin & Malte 2012; He *et al.* 2013). Other measures of metabolic capacity such as gill structure, muscle mass and cell size, heart size and red blood cell concentration have been studied. However, results from studies of these traits have been historically equivocal or based on exercise protocols designed to test inter-individual physiological plasticity (Davison 1997). Indeed, it appears that physiological trait variation has not been used for evaluating divergence among populations at

all, except for two recent examples in which metabolic enzyme activity, heart, gill and muscle mass served as proxies for evaluating divergence among populations in predator escape response (Odell *et al.* 2003) and population-specific migrations undertaken in the early life stages of some salmonids (Patterson *et al.* 2004). These two latter studies provide insight into evolution of the physiological mechanisms that underlie differential swimming performance among populations under divergent selective pressures. However, Odell *et al*'s (2003) results were confounded because of allometric scaling of measured traits with body size.

This points to a lack of understanding of how variation in swimming speed performance among fish populations are related to physiological and morphological traits. These gaps in the evolutionary ecology of riverine fishes present an opportunity to explore the mechanistic link between swimming performance and morphological and physiological traits, and the divergence that arises among fish populations from river and reservoir habitats.

1.3. Research questions

Three important knowledge gaps exist in the field of fish eco-morphology: 1) determining how predictable divergence in morphology is in freshwater fishes; 2) understanding how swimming performance compares among river and reservoir populations of a freshwater fish and 3) quantifying the relative contribution of other morphological traits such as fins, and physiological traits, such as cardio-vascular organs, to divergence in swimming performance. To fill some of the knowledge gaps in the divergence of freshwater fishes following dam construction, the overarching question of this thesis is: *How do fish populations adapt to different flow velocity habitats?* Within this overarching question, this thesis will address three specific research questions:

1. Does body shape differ among river and reservoir fish populations?

2. Does prolonged swimming performance differ among river and reservoir fish populations and if so, how is prolonged swimming performance correlated with morphology and fin shape?

3. Do physiological traits differ among river and reservoir fish populations?

1.4. Study area

To undertake this study, selection criteria were developed to facilitate the selection of rivers and reservoirs that were appropriate to address the research questions. The reservoirs were chosen based on; a) age (time since construction of the dam), b) number of inflowing streams, c) frequency of spill-over events, and d) a suitable collection site could be found within the dam, at least 6 km from the river inflow and from the dam wall or downstream reservoir (Franssen 2011) (Table 1.1). Time since dam construction was a necessary consideration because it was assumed that the older the dams, the longer river and reservoir populations were separated, and sufficient time would have elapsed for divergence to occur (Reznick *et al.* 1997). Selecting reservoirs with fewer inflows reduced the possibility of mixing among reservoir and upstream river populations (Hendry *et al.* 2002). Frequency of spill-over events may have an effect on population mixing due to displacement of reservoir populations into downstream river habitats. Distance of sampling sites within reservoirs from inflowing rivers was maintained at least greater than 6 km to reduce the possibility of sampling individuals migrating from inflowing rivers and so that reservoir populations were less likely to experience river flow velocities. River systems were chosen based on the following criteria; a) whether the river is downstream of a dam deemed to be suitable according to criteria outlined previously, b) if there are tributaries flowing into the river downstream of the dams to be sampled, and c) sites in river habitats were at least 6 km downstream or upstream of reservoirs (Table 1.1). If a river was downstream of the sampled reservoir, the reservoir and river populations could be directly compared. Tributaries were sampled in preference to impounded rivers, as this reduced the likelihood of confounding effects of population mixing from reservoir spill-over events. Where no tributaries were available, sites within rivers were selected at least 6 km from upstream or downstream dams, to reduce the possibility of mixing with reservoir populations displaced from upstream reservoirs, or those migrated from downstream reservoirs. Free flowing rivers were preferred over impounded rivers, as there was less likelihood of mixing among displaced reservoir populations and river populations. Rivers and reservoirs were selected if they met at least two of these criteria (Table 1.1).

Table 1.1 Criteria used for selection of study systems to maximise the difference between river and reservoir populations.

Reservoir	River
● Dam constructed more than 30 years ago.	● Located downstream of reservoir to represent the ancestral river population.
● No more than 2 in-flowing rivers.	● Tributary stream on major river below sampled reservoir.
● Available sample sites in the reservoir at least 6 km from inflowing streams.	● More than 6 km between dam and river sample sites.
● No spill-over (>100% capacity) in the last 10 years.	
● Detailed information available about resident fish populations (Reservoirs and rivers)	

The rivers and reservoirs selected for this study were located in south-eastern Australia (Fig. 1.2 and 1.3; see Fig. 1.4 and 1.5 for photos). They consisted of three rivers and three reservoirs from the southern Murray-Darling Basin (Fig. 1.2) and three rivers and two reservoirs in the Hawkesbury-Nepean river catchment (Fig. 1.3). The reservoir systems were at least 50 years old (Lake Nillahcootie; Fig. 1.2) and the oldest reservoir (Waranga Basin; Fig. 1.2) was 102 years old, and all reservoir systems were in-stream (constructed in the main channel of a river) except for Waranga Basin, which was an off-stream reservoir filled by two viaducts connected to the Goulburn River (Goulburn-Murray Water, www.g-mwater.com.au) (Fig. 1.2).

Table 1.2. Study systems from the southern Murray-Darling Basin (SMDB) and Hawkesbury-Nepean catchment (HNC) regions. Water bodies are rivers or reservoirs. For reservoirs, age of dam is listed and for rivers, are they impounded (yes or no)? Distance from upstream dams or downstream reservoirs (where present) are the shortest distance as possible following the water course. Number of inflows is the number of streams or rivers entering the dam, tributary refers to whether any streams flow into the river from which fish were sampled.

Rivers	Region	Impounded?	Tributary	Distance (dam to sample site)
Broken River	SMDB	Yes	No	26 km
Kiewa River	SMDB	No	Yes	9.5 km
Ovens River	SMDB	No	No	8 km (downstream; Fig. 1.2)
Bargo River	HNC	No	Yes	Free flowing
Georges River	HNC	No	No	Free flowing
Nepean River	HNC	Yes	No	28.1 km
Reservoirs	**Region**	**Age of dam (years)**	**Number Inflows**	**Distance from sample site to nearest inflows**
Lake Hume	SMDB	81	2	>24 km
Lake Nillahcootie	SMDB	50	1	7.1 km
Waranga Basin	SMDB	102	2	>6.9 km
Lake Cordeaux	HNC	91	0	No inflows
Lake Nepean	HNC	82	1	9.3 km

Sample sites within reservoirs were located at least 7 km from the closest inflowing stream. Lake Hume has two inflowing rivers (Mitta Mitta river and Murray River; Fig. 1.2) entering the lake 24 km and 52 km from the sample sites respectively, while Lake Nillahcootie and Waranga Basin have one and two inflows respectively (Fig.1.2). The inflow into Nillahcootie was 7.1 km from the sampling sites and the Waranga Basin sampling sites were at least 6.9 km from the intermittently flowing viaducts connecting to the Goulburn River. Reservoir systems in the Hawkesbury-Nepean catchment were Lake Nepean, (dammed in 1935) which had only one inflow, the Nepean River which continues downstream below the dam (Fig. 1.3), and Lake Cordeaux (dammed by 1926), which is a headwater system with no inflows (Gehrke *et al.* 1996; NSW Office of water: www.nswwater.nsw.gov.au; Gehrke 1997; Gehrke *et al.* 2004).

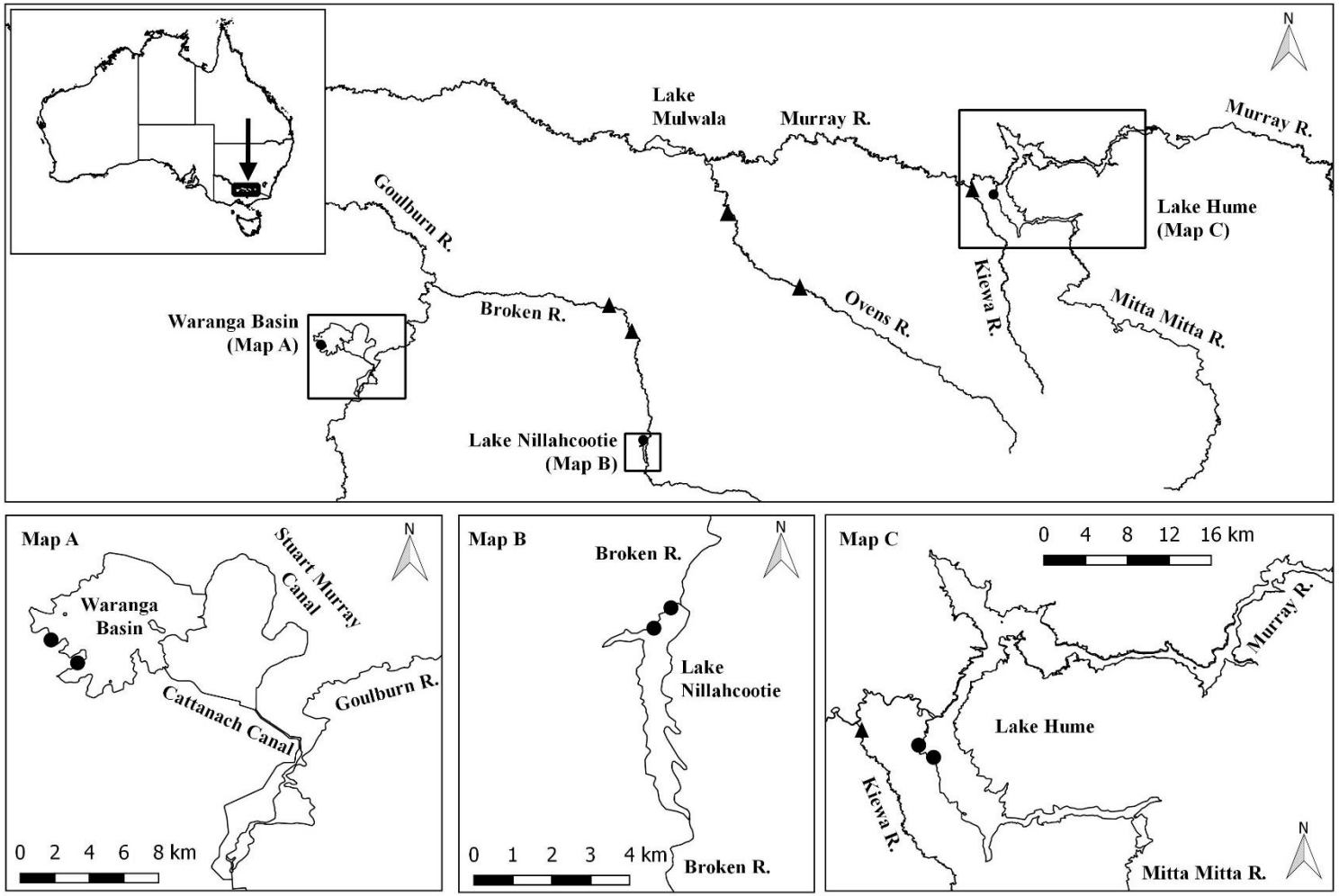

Fig. 1.2. Map of river and reservoir study sites from the southern Murray-Darling Basin. Maps A to C show enlargements of reservoirs. Triangles indicate sampling sites on rivers, circles indicate sampling sites on reservoirs. Top panel shows relative locations of rivers and reservoirs. Sampling sites within dams occurred at least 6 km from inflowing streams and sampling sites in rivers were chosen in tributaries on dammed rivers or at least 6 km from upstream or downstream reservoirs (where present; e.g. Lake Mulwala on the Ovens River). Arrow on inset map shows location of southern Murray-Darling Basin sites in Australia.

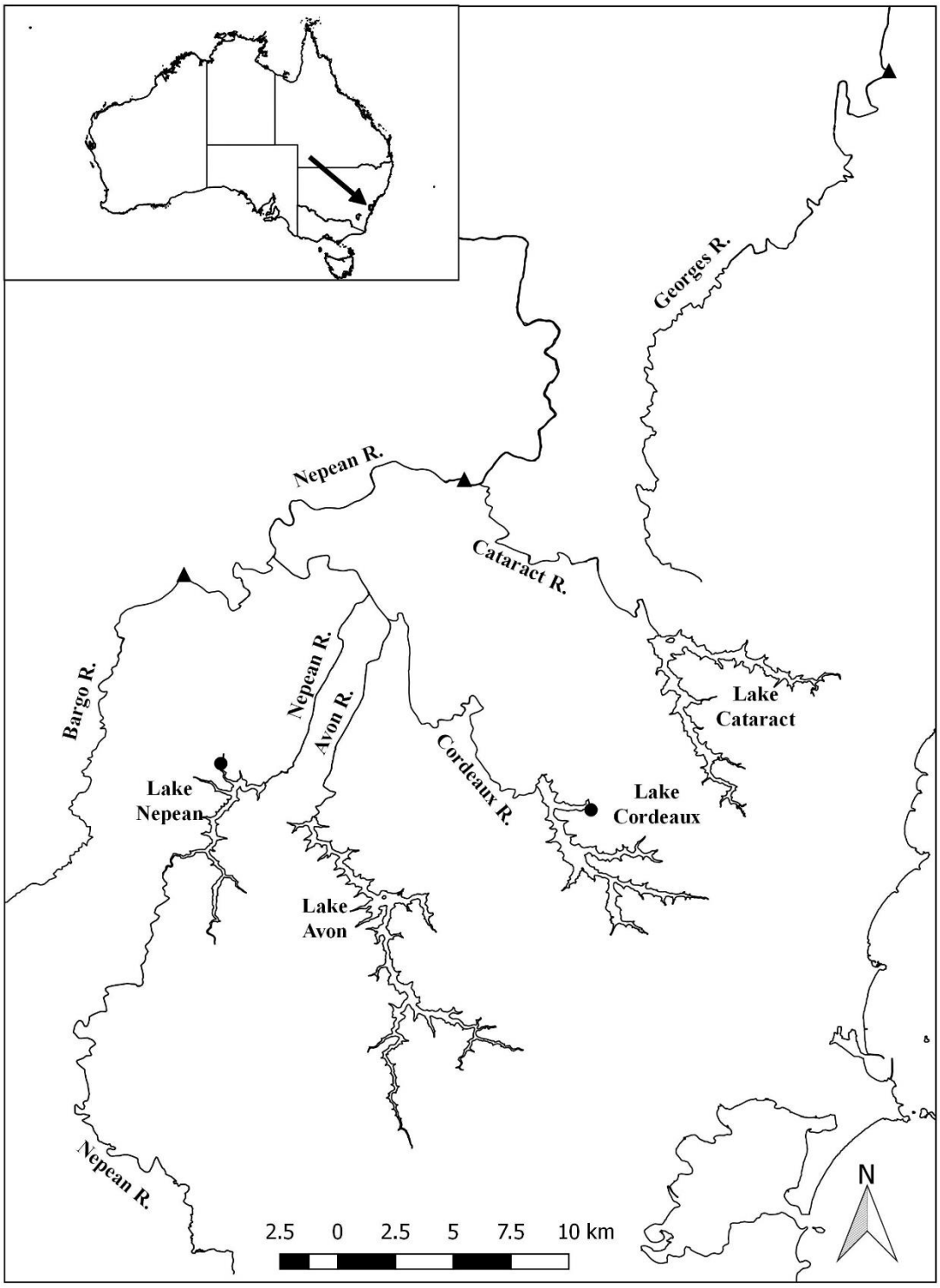

Fig 1.3. Map of river and reservoir study sites in the Hawkesbury-Nepean catchment. Triangles indicate sampling sites on rivers, circles indicate sampling sites on reservoirs. Sampling sites within dams were at least 6 km from inflowing streams and sampling sites in rivers were chosen in tributaries on dammed rivers or at least 6 km from upstream dams. Arrow on inset map indicates location of the Hawkesbury-Nepean catchment in Australia.

A third suitable reservoir could not be identified in the Hawkesbury-Nepean catchment, as there was insufficient information about resident fish populations in other reservoirs in the catchment (Gehrke 1997). Flow velocities at all sites were measured using a Marsh-McBirney Flo-Mate flow meter on each sampling trip along with other water quality parameters including dissolved oxygen, pH, turbidity, conductivity and temperature (Appendix 1). Flow velocities at the river sites were significantly higher than flow velocities at the reservoir sites (Appendix 1).

The reservoirs from the Hawkesbury-Nepean catchment were headwater systems with low productivity and limited fish populations consisting of mostly small-bodied species including Australian smelt (*Retropinna semoni*), introduced goldfish (*Carassius auratus*), flathead gudgeon (*Philypnodon grandiceps*) and eel (*Anguilla australis*) (Gehrke *et al.* 1996) while the southern Murray-Darling Basin rivers and reservoirs were large lowland systems known to consist of large and small bodied species including gudgeon (*P. grandiceps*), Australian smelt *(R. semoni*), carp gudgeon (*Hyspeleotris* spp.), silver (*Bidyanus bidyanus*) and golden perch (*Macquaria ambigua*), cod (*Maccullochella* spp.), catfish (*Tandanus* spp.) and rainbowfish (*Melanotaenia* spp.) (Humphries *et al.* 1999; Humphries & Lake 2000).

Fig. 1.4. River habitats sampled in the Hawkesbury-Nepean catchment: a) Bargo River, b) Georges River, c) Nepean River; and the southern Murray-Darling Basin: d) Kiewa River, e) Ovens River, f) Broken River.

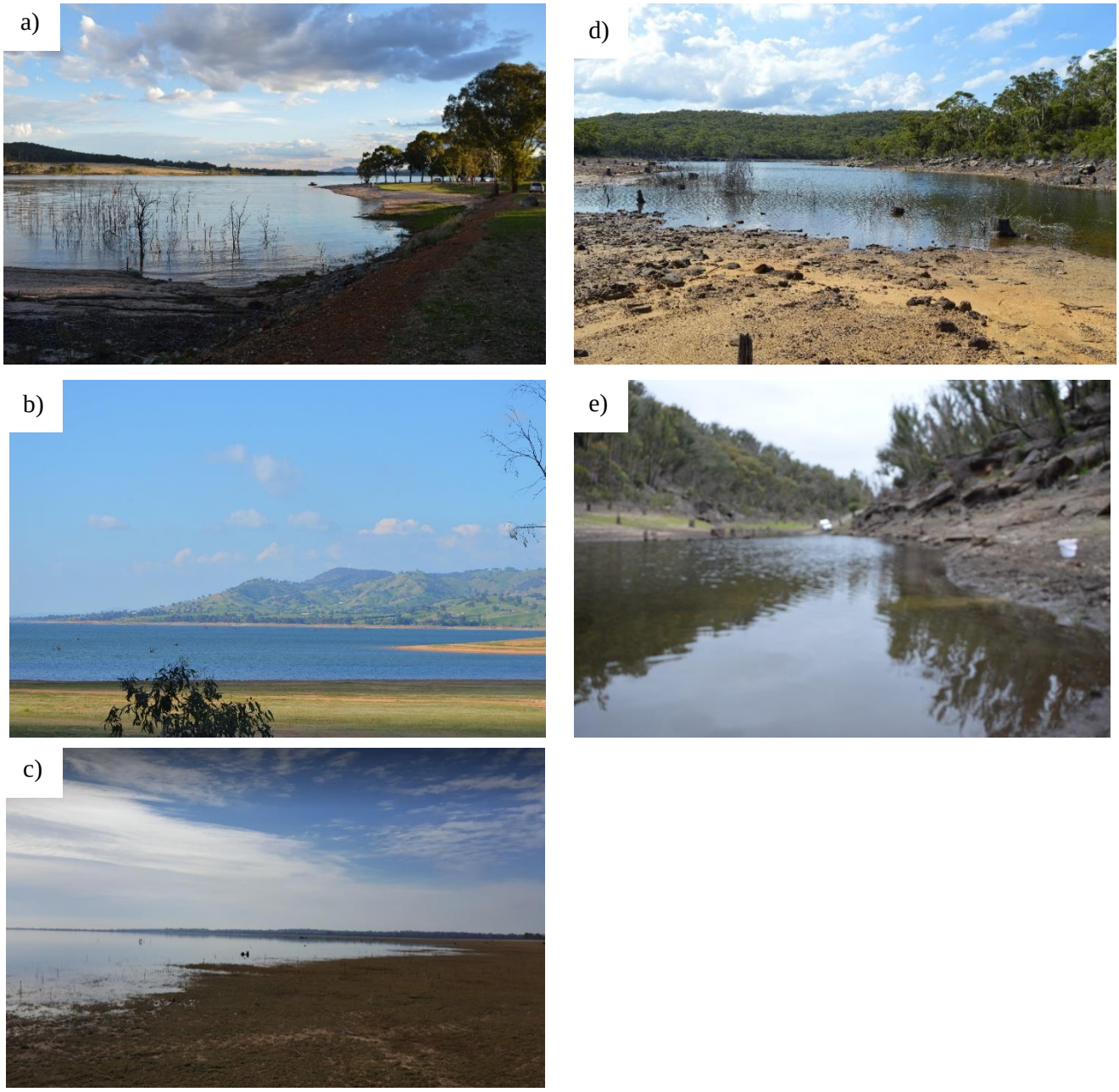

Fig. 1.5. Reservoir habitats sampled in the southern Murray-Darling basin: a) Lake Nillahcootie, b) Lake Hume, c) Waranga Basin; and the Hawkesbury-Nepean catchment: d) Lake Cordeaux, e) Lake Nepean.

1.5. Study species

A number of native fish species were considered as focus taxa for this study (Table 1.3). The study species was selected after taking into account a) conservation status (either national or state-listed), b) presence or absence in rivers and reservoirs based on published records and c) taxonomic status (i.e. known or unresolved) (Table 1.3).

Table 1.3. Candidate species considered for this thesis. Conservation status (Status) was based on state and national conservation legislation (* = NSW Fisheries Management Act 1994; † = Environmental Protection and Biodiversity Act 1994; ‡= Victorian Flora and Fauna Act 1988). Tick in taxonomy column indicates known classification.

Species	Status	Distribution	Taxonomy	Stocked
Murray cod (*Maccullochella peeli*)	Vulnerable†	Common	✓	Yes
Golden perch (*Macquaria ambigua*)	Threatened‡	Common	✓	Yes
Silver perch (*Bidyanus bidyanus*)	Critically endangered*†	Locally abundant	✓	Yes
Eel-tailed catfish (*Tandanus tandanus*)	Threatened*‡	Rare	✓	Yes
Macquarie perch (*Macquaria australasica*)	Endangered*†	Rare	✓	No
Southern pygmy perch (*Nannoperca australis*)	Endangered*	Rare	✓	No
Flathead gudgeon (*Phylipnodon grandiceps*)	Unlisted	Uncommon but locally abundant	✓	No
Mountain galaxids (*Galaxias olidus*)	Unlisted	Uncommon but locally abundant	unresolved	No
Carp gudgeon (*Hypseleotris* spp.)	Unlisted	Abundant	unresolved	No
Australian smelt (*Retropinna semoni*)	**Unlisted**	**Abundant**	✓	**No**

The species considered included Murray cod (*M. peeli*), silver perch (*B. bidyanus*), golden perch (*M. ambigua*) and catfish (*T. tandanus*). Some of these species such as *T. tandanus* were either rare or absent from many waterways (Rourke & Gilligan 2010), while others such as *M. peeli* and *B. bidyanus* were regularly stocked into both rivers and dams (Hunt *et al.* 2010) which raised concerns over the effects of domestication on local adaptation associated with hatchery rearing. Other species that were considered were Macquarie perch (*M. australasica*), southern pygmy perch (*N. australis*), flatheaded gudgeon (*P. grandiceps*) and galaxids (*Galaxias* spp.) but these four species were ruled out because the former two are

threatened species, which presented difficulties with obtaining sampling permits and animal ethics regulations and the latter two could not be reliably sampled from reservoirs. Also considered were the *Hypseleotris* spp. however, due to extensive hybridization, they present an extremely high phenotypic diversity, making accurate identification very difficult (Bertozzi *et al.* 2000), which indeed was not appropriate for this thesis.

In light of these criteria, the study species chosen for this thesis was the Australian smelt (*Retropinna semoni*) (Fig. 1.6). Australian smelt are common in reservoirs, streams, wetlands and ephemeral creeks across much of south-eastern Australia (Lintermans 2007).

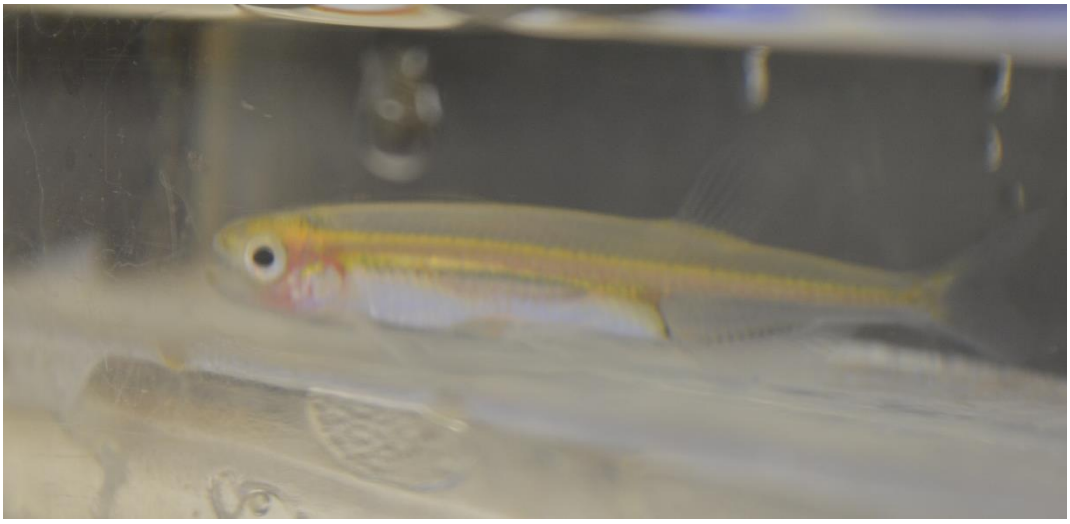

Fig. 1.6. Australian smelt acclimating to experimental conditions.

They inhabit both freshwater and brackish systems (McDowall 1979) and have a wide distribution (Woods *et al.* 2010; Hughes *et al.* 2014). They are open-water swimmers and an important forage species for larger piscivorous fish (McDowall 1979; Milton & Arthington 1985; Hammer *et al.* 2007). Australian smelt are also a short-lived species, with a life-span of up to 3 years and a generation time (time to first spawning) as short as 6 to 9 months (Milton & Arthington 1985). Other short-lived species such as Trinidadian guppies (*P. reticulata*) and three-spined sticklebacks (*G. aculeatus*) have been shown to undergo rapid phenotypic and genetic change over remarkably short time-scales (Reznick & Bryga 1987; Barrett *et al.* 2011). Similarly, Australian smelt may also be predisposed to genetic divergence and may experience a high degree of local adaptation which are desirable characteristics for testing the hypotheses proposed in this thesis. High genetic structuring among populations of Australian smelt that have been separated by physical barriers, despite the presence of fishways suggests that Australian smelt populations are indeed likely to experience a high degree of local adaptation, possibly due to site fidelity (Duncan *et al.* 2016).

Thus, potential confounding factors that were relevant for the species listed previously (Table 1.3), were thought to be of minimal concern in Australian smelt. Due to their short life-span, short term adaptation or evolutionary changes may have occurred in populations of Australian smelt in a timeframe spanning much less than a human life-time. Furthermore, they are known to inhabit most river systems and large reservoirs in south-eastern Australia in large numbers (Gehrke *et al.* 1996; Gehrke 1997; Lintermans 2007) and since they are not a recreationally important species, trait divergence in populations is not confounded by stocking from other populations. As a result, Australian smelt were the most suitable species for the study of trait divergence as a response to dam construction and the emergence of novel ecosystems.

1.6. Thesis overview

To address the three research questions, this thesis is divided into three data chapters and the general discussion. Chapter 2 presents a field study where populations of Australian smelt, were compared to test hypotheses about changes in body morphology following dam construction. Chapter 3 expands on findings in chapter 2 by testing the prolonged swimming speed performance using the critical swimming speed protocol among populations of Australian smelt from rivers and reservoirs. The body shape variation and fin aspect ratios were also quantified and tested as predictors of prolonged swimming speed performance. In chapter 4, a different sample of Australian smelt from rivers and reservoirs were used to determine the contribution of physiological traits to divergence in prolonged swimming speed performance. In this chapter, the critical swimming speed experiments from chapter 3 were repeated on these individuals and physiological traits including heart mass, gill mass and metabolic enzyme activity were quantified. Finally, chapter 5 presents a synthesis of the findings from the preceding chapters. The results are discussed in the context of divergent river and reservoir populations of Australian smelt and the contributions of this thesis for furthering our understanding of divergence among river and reservoir populations of freshwater fishes in general.

Chapter 2. Body shape variation of Australian smelt from rivers and reservoirs

2.1. Introduction

River ecosystems have been extensively modified by the construction of irrigation and water supply reservoirs affecting fundamental changes in their ecology and the selective pressures acting on the fish populations (chapter 1.1). Morphological adaptation of fishes is driven to some extent by the physical constraints imposed by movement in water and therefore often varies under different flow velocities (Vogel 1994). Changes in flow velocity, such as those associated with dam construction, have resulted in divergence among river and reservoir populations (Haas *et al.* 2010; Franssen 2011; Franssen *et al.* 2013a; Cureton & Broughton 2014). Because functional trait adaptations have enabled fishes to exploit a wide range of physically demanding habitats, there has been a great deal of interest in understanding drivers of morphological divergence among fish populations inhabiting rivers and reservoirs (Fleming & Gross 1989; Langerhans *et al.* 2003; Berner *et al.* 2008; Aguirre 2009; Pavey *et al.* 2010).

Numerous studies suggest body shape determines population-scale fitness. It is thought to directly influence the locomotion necessary to undertake life-sustaining activities such as foraging (Bodaly 1979; Webb 1984b; Pavey *et al.* 2010), predator evasion ((Langerhans *et al.* 2004; Domenici *et al.* 2008; Blob *et al.* 2010), habitat utilization (Brinsmead & Fox 2002) and migration (Chapman *et al.* 2015). However, the magnitude and direction of morphological change in fish populations following substantial ecological changes arising in novel ecosystems such as reservoirs, is not entirely clear.

Langerhans (2008) hypothesized that there is a mechanistic link between the hydrodynamic forces acting on fish populations and evolution of their body shape and fin characteristics. This hypothesis implies that patterns of phenotypic differentiation, particularly body shape, should be predictable across populations inhabiting different flow velocity habitats due to the biomechanics of fish locomotion (Langerhans & Reznick 2010) and the physical effects of drag, pressure and momentum in water (Vogel 1994). A number of studies (Brinsmead & Fox 2002; Haas *et al.* 2010; Franssen 2011; Cureton & Broughton 2014) have found that fish from reservoir populations tend to develop deeper bodies, while river fish become, on average, more streamlined, conforming to Langerhans' (2008) predictions and to Webb's (1984a) conceptual model linking form and function. In contrast, Pavey *et al.* (2010) showed that Sockeye salmon (*Oncorhyncus nerka*) are more streamlined in lake habitats and river fish have deeper and more robust bodies, while McGuigan *et al*. (2003) found no clear

indication that morphological divergence occurred among populations of rainbow fish (*Melanotaenia eachamensis* and *Melanotaenia duboulayi*) from rivers and naturally occurring lakes. In naturally occurring river and reservoir habitats, populations may have been separated for time periods at geological time scales, compared to dams which may have separated populations for as little as several decades. Thus, rainbowfish populations may have had more opportunity to diverge than populations of fish in habitats separated by dams. This indicates that contrary to Langerhans' (2008) hypothesis, the direction of divergence in body shape among populations of fish from river and reservoir habitats may be inconsistent and not easily predictable. Hence, it is unclear if morphological divergence among river and reservoir populations is a general response among species, especially when there appears to be no clear consensus between the literature and the theoretical predictions such as those of Webb (1984a) and Langerhans (2008).

Much of this information is also confined to a small number of populations of salmonid and cyprinid species across relatively small geographical ranges (< 500 km) in south-east North America and the impacts of dam construction on morphological divergence have been overlooked outside these systems. Australian rivers and reservoirs are inhabited by a number of river-adapted fishes which are abundant across the eastern states. However, little is known about morphological variation among fish populations in river and reservoir habitats outside North America. Morphological variation has been explored among paired river and natural lake populations of rainbowfish (*M. eachamensis* and *M. duboulayi*) (McGuigan *et al.* 2003) but these populations have been separated by long term geological processes, as opposed to dams.

Australian smelt (*Retropinna semoni*) are common in reservoirs, rivers, streams, wetlands and ephemeral creeks across much of south-eastern Australia (Lintermans 2007). Closely related species (e.g. *Retropinna retropinna*) also inhabit both freshwater and brackish habitats in New Zealand, where evidence from meristic, life-history and qualitative morphological studies have shown considerable population-specific morphological variation and sexual dimorphism among lacustrine and migratory estuarine populations (McDowall 1979; Ward *et al.* 1989). Australian smelt are endemic and genetically structured in many parts of the Murray-Darling Basin but are widely distributed (Woods *et al.* 2010; Hughes *et al.* 2014). They are open-water planktivores and an important forage species for larger piscivorous fish (Milton & Arthington 1985). As discussed in chapter 1, high levels of genetic structuring coupled with the fact that morphological trait divergence can have a genetic basis (Franssen 2011) suggest that it is likely that river and reservoir populations of Australian smelt have undergone trait divergence in body shape. Furthermore, Australian smelt are

known to inhabit most rivers and large reservoirs across south-eastern Australia in large numbers (McDowall 1979; Milton & Arthington 1985; Gehrke *et al.* 1996; Gehrke 1997; Gehrke *et al.* 2004; Lintermans 2007), making them a suitable model species for studying morphological adaptation. Furthermore, they are not a recreationally important species, and therefore their population structure is less likely to be influenced by stocking or translocation. To quantify the effect of flow velocity on the body shape of river and reservoir populations (research question 1), I test the following hypotheses:

> H_0: Populations from reservoir habitats will not be significantly different in body shape, head shape or caudal peduncle depth and mouth orientation compared to populations from river habitats.

> H_a: Populations from reservoir habitats will have significantly different body shape, head shape, caudal peduncle depth and mouth orientation compared to populations from river habitats (Langerhans *et al.* 2003; Langerhans 2008; Haas *et al.* 2010).

2.2. Methods

2.2.1. Sampling

Australian smelt populations were sampled from each of 6 river and 5 reservoir habitats (see chapter 1.4 for details) across the southern Murray-Darling Basin and Hawkesbury-Nepean catchment on at least two visits between October 2014 and February 2015. Between 60 and 90 individuals were collected using a 10 m (length) × 1.8 m (drop) seine net made of fine (3 mm diagonal) mesh. Sampled fish were euthanized by overdose with excess benzocaine (100 $mg\ L^{-1}$) and stored in 70% ethanol. At the site from which fish were sampled within each river and reservoir, measurements of flow velocity ($m\ s^{-1}$) were made using a Marsh-McBirney Flo Mate portable flow velocity meter at 5 to 6 random points. Measurements were taken from the middle of the water column. Water quality parameters, including dissolved oxygen (DO) concentration ($mg\ L^{-1}$), temperature (^{o}C), conductivity ($mS\ cm^{-1}$) and turbidity (NTU) were also measured at 5 random points across the same area as the flow velocity, using a Horiba U51 multi-parameter probe (Appendix 1).

2.2.2. Population demographics and dimorphism

Body shape variation can be confounded by sexual dimorphism and size at maturity (Shine 1989; Bisazza 1993; Navarro *et al.* 2009; Herler *et al.* 2010). Therefore, sexual dimorphism and size at maturity were assessed across all sampled populations. Australian smelt (and other

members of the Retropinnidae family) are thought to be sexually dimorphic for fin size, particularly the pectoral, pelvic and ventral fins, (McDowall 1979). However, this nor sexual dimorphism in body shape or size have ever been formally quantified in Australian smelt (*R. semoni*). All sampled individuals were sexed macroscopically by checking for presence of gonads and gametes. Gonads and gametes were identified following descriptions by Milton and Arthington (1985) and a decision tree was used to classify individuals as male, female, juvenile or unknown (Fig. 2.1). The juveniles and unknown individuals could not be clearly ruled out as immature unless gametes were visible and because mean standard lengths suggested that juvenile standard length was greater than adult male and female standard lengths in some cases. Individuals classed as juvenile or unknown were therefore excluded from further analyses, and only confirmed adult males and females were retained. Histograms were plotted for each river and reservoir population with standard length (SL; measured from tip of snout to posterior margin of caudal peduncle) in millimetres and centroid size (CS; computed during geometric morphometric analysis; see chapter 2.2.3 for details), against frequency of males and females to assess sexual size dimorphism. Histograms were also plotted for the aggregated data for each habitat, with SL and CS against the frequency of males and females, to assess overall sexual size dimorphism between habitats**.** These were statistically evaluated using non-parametric ANOVA using the *vegan* package (Oksanen *et al.* 2007) for R (R Core Team 2015). Individuals were rescaled to unit centroid size during the body shape analysis (see chapter 2.2.3 for details) as it is the only measure of size which is independent of shape in the absence of allometry (Zelditch *et al.* 2004).

Sexual dimorphism in body shape was assessed by including sex as a factor in statistical analyses for geometric and traditional morphometric data. The body size variation across population and habitat and sexual size dimorphism were assessed in two ways: 1) using the standard-length measurements corresponding to the distance measured between landmarks 1 and 6 in the traditional morphometric analysis and 2) the centroid size computed during the superimposition in the geometric morphometric analysis.

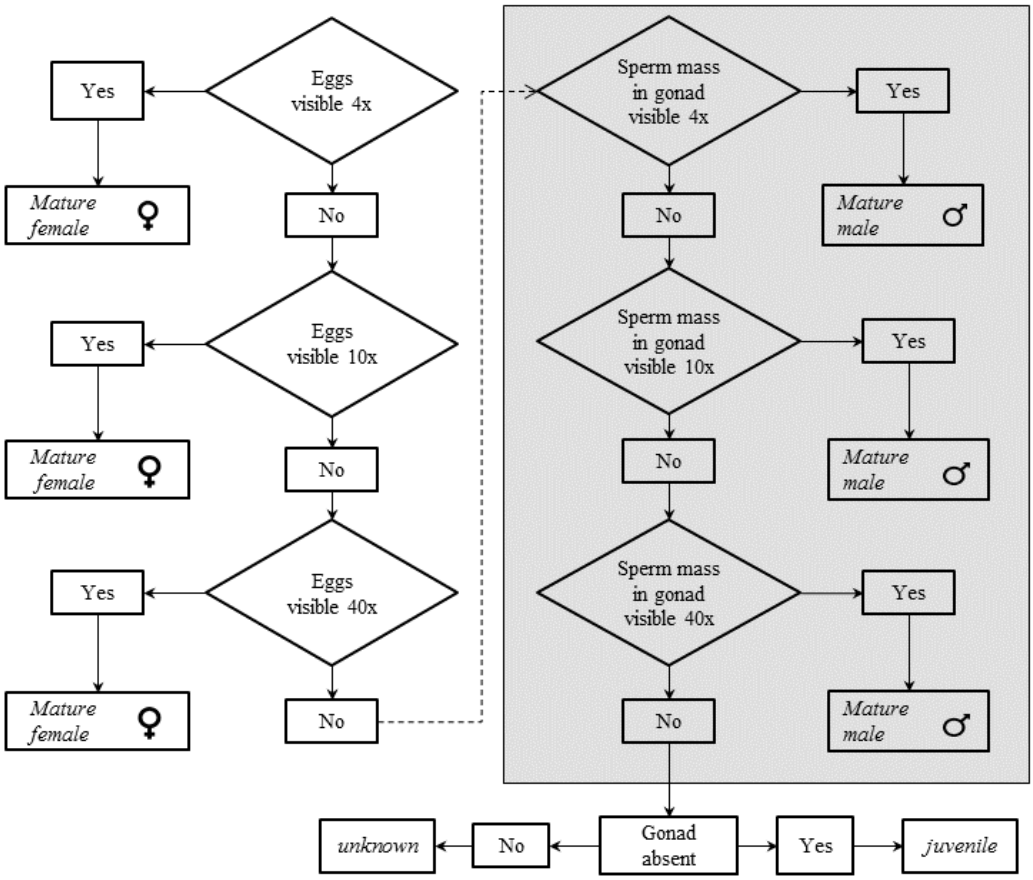

Fig. 2.1. Decision tree used to classify sampled Australian smelt as male, female, juvenile or unknown. Factors (4x, 10x, 40x) indicate magnification of objective lens used to view gonads and germ cells. Descriptions of gonads and germ cells adapted from Milton and Arthington (1985).

2.2.3 Geometric and traditional morphometric data

Morphological divergence between river and reservoir populations of Australian smelt was assessed using two methods: 1) geometric morphometrics which is based on the analysis of relative locations of landmark coordinates (Rohlf & Marcus 1993) and 2) traditional morphometrics which is based on the multivariate analysis of linear measurements, such as lengths, depths or widths, referred to as "trusses", across the form of the fish (Strauss & Bookstein 1982; Strauss & Bond 1990). A number of studies have compared results of morphological trait analyses using traditional and geometric morphometrics and while many argue that geometric morphometrics appear to provide more rigorous results (chapter 1.2), there are similarly useful results that arise from traditional morphometrics (Parsons *et al.* 2003; Maderbacher *et al.* 2008; Viscosi *et al.* 2009; Schmieder *et al.* 2015). Despite the strengths of the geometric method (Rohlf & Marcus 1993; Adams *et al.* 2004; Zelditch *et al.* 2004) there are advantages to using the traditional method. Examples of this utility include the ability to generate data that can be compared with previous studies using the traditional method (Parsons *et al.* 2003), it can provide complementary results to geometric

morphometric data to support hypotheses in exploratory studies and it can aid in identifying fewer, simpler measurements to act as proxies for the variation present in the shape of a whole organism (Ramírez-Sánchez *et al.* 2016).

In this chapter, the geometric and traditional morphometric methods were used to provide complementary tests for the proposed hypotheses, and to test if the traditional morphometric method can be used to replicate results from the geometric method. The geometric method represents a conceptually different approach to analysis of shape variation compared to traditional morphometrics; it captures the geometry of an object or organism through the two-dimensional coordinates of homologous landmarks (Rohlf & Marcus 1993). It is based on Kendall's (1977) definition of shape as "*all information that remains when the effects of location, orientation and size are removed*". The procedures used to collect and analyse landmark data is described here. All fish were photographed laterally from the left side against a rubber mat with a printed 10 mm reference grid using a Sony Alpha 7 digital SLR camera with a Canon 100 mm macro lens mounted a fixed distance from the rubber grid on a modified photo enlarger mount (Fig. 2.2). Special care was taken to ensure that each specimen was as close to the centre of the photograph as possible to minimize distortion due to lens edge effects and at the highest resolution possible (Zelditch *et al.* 2004). A blank *.tps* file used to store raw landmark coordinate data for all photographs was created using *tpsUtil* software (v1.44; Rohlf 2010). Sixteen pre-determined landmarks chosen according to the following criteria described by Zelditch *et al*. (2004) and Bookstein (1991) were digitized at homologous points on photos of each specimen (Fig. 2.3) using *tpsDig* software (v2.10; Rohlf 2006). A ten-millimetre scale bar was set on each image using the printed grid visible in the photographs to enable calibration of each image to a common scale, and subsequent rescaling (size-correction) during superimposition.

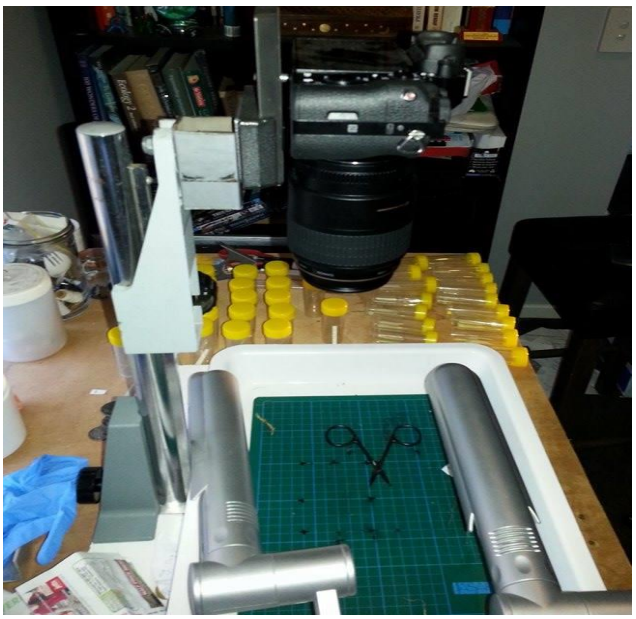

Fig. 2.2. Sony Alpha 7 digital SLR with Canon 100 mm macro lens mounted at a fixed position and distance from the focal plane, on a modified photo enlarger mount. Rubber mat with reference grid and lamps for additional lighting visible in foreground.

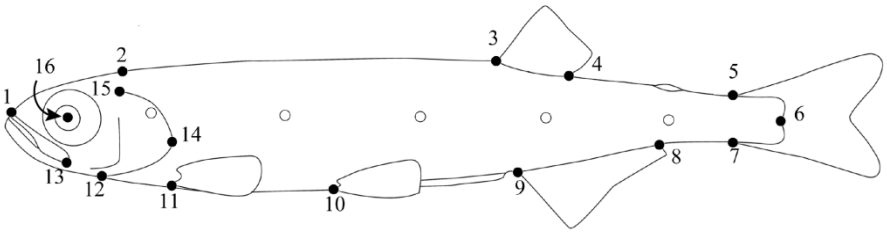

Fig. 2.3. Diagram showing 16 landmark locations digitized on each specimen for the geometric morphometrics method. Filled circles indicate the landmark locations used for analysis of shape variation. Empty circles indicate 5 additional landmarks that were placed along the lateral line for 'unbending' specimens deformed by rigor mortis or preservation. Landmark descriptions: 1 = nose, 2 = dorsal skull margin, 3 = anterior dorsal fin insertion point, 4 = posterior dorsal fin insertion point, 5 = dorsal caudal fin insertion point, 6 = notch in posterior margin of caudal peduncle, 7 = ventral caudal fin insertion point, 8 = posterior ventral fin insertion point, 9 = anterior ventral fin insertion point, 10 = pelvic fin insertion point, 11 = pectoral fin insertion point, 12 = mandible hinge, 13 = maxilla, 14 = posterior margin of operculum, 15 = dorsal terminus of operculum, 16 = eye.

All 16 landmarks were set and stored as a list of (x, y) coordinates for each specimen in the *.tps* file. Landmarks were selected using the following guidelines described by Zelditch *et al*, (2004) and Bookstein (1991):

a) selected landmarks should be homologous among all specimens,
b) the topologies of landmarks (relative positions) should not change among individuals,
c) selected landmarks should provide adequate coverage of the whole form or region of interest for all specimens and,
d) landmarks should be repeatably identifiable and their location on the specimen unambiguous.

To validate the selected landmarks against these criteria, the error due to landmark digitization was assessed by taking a random sample of 5 photographed individuals, digitizing pre-determined landmarks as described previously for the same 5 photos on 3 different dates and testing for differences in landmark positions among the 3 samples of photos using the analyses described in chapter 2.2.4 (Appendix 2). This process ensured consistency of landmark positioning and that the selected landmarks fulfilled the landmark selection criteria described previously. Once consistency of landmark digitization was checked, it was performed on the remaining specimens. The 16 selected landmarks were positioned on each specimen, and an additional 5 landmarks were positioned along the midline of each specimen (Fig. 2.3) to enable use of the 'Unbend' module in *tpsUtil* (v1.44; Rohlf 2010) to correct specimens that were deformed due to the effects of rigor mortis and preservation. Unbending was performed using the 5 additional landmarks as well as landmarks 1 and 6 on each specimen. The 'Unbend' module fits a quadratic function to a series of defined landmarks on each specimen and transforms the quadratic function into a straight line (Rohlf 2010).

To ensure that landmark coordinate data used in statistical shape analysis conformed to Kendall's (1977) definition of shape described previously, the effects of location, size and rotation were removed by superimposition of raw landmark coordinate data. Superimposition was achieved by performing a generalized Procrustes analysis (GPA) using the *geomorph* (v3.1.1; Adams & Otárola-Castillo 2013) package in the R statistical computing environment (R Core Team 2015). The *geomorph* package implements the Procrustes algorithm (Gower 1975; Goodall 1991) for GPA which performs translation, scaling (size-correction) and rotation operations in order to minimize differences among landmark configurations in the dataset that are unrelated to shape. A simplified description of the GPA based on Zelditch *et al*. (2004) and Goodall (1991) is presented in Fig. 2.4 and Table 2.1.

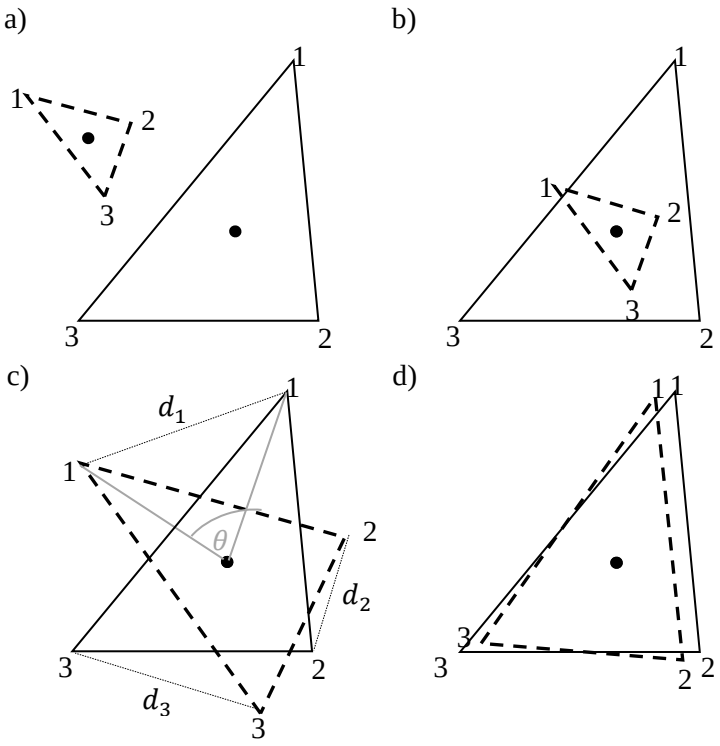

Fig. 2.4. Two hypothetical triangles that have been super-imposed using GPA by performing the following three operations; a) to b) translation; b) to c) scaling; c) to d) rotation. Numbers indicate homologous vertices; the centroid of each configuration is indicated by a filled circle; the solid and heavy broken outlines indicate the reference and target configurations, respectively; and the fine broken lines indicate the Procrustes distances between landmarks. This figure was modified from Zelditch *et al*. (2004).

Table 2.1 Operations performed by Procrustes algorithm in generalized Procrustes analysis (GPA). Adapted from Zelditch *et al*. (2004).

Operation	*Description of operation*	*Equation*
1. Compute centroid.	The centroid is the geometric centre of a landmark configuration defined as the mean of all landmark coordinates of a configuration where *K* is the number of landmarks, *X* is the *x* coordinate and *Y* is the *y* coordinate of the *j*-th landmark for X_c and Y_c, the coordinates of the centroid.	$X_c = \frac{1}{K}\sum_{j=1}^{K} X_j$ $Y_c = \frac{1}{K}\sum_{j=1}^{K} Y_j$
2. Align all landmark configurations to a common origin	All landmark configurations are aligned to a common origin (0,0) by subtracting each configurations' centroid from each landmark coordinate. where X_j and Y_j are the *j*-th landmark of a configuration.	$X_{centered} = X_j - X_c$ $Y_{centered} = Y_j - Y_c$
3. Compute centroid size and rescale all landmark configurations to unit centroid size.	The centroid size is the square root of the sum of squared distances from the centroid to each landmark of a configuration. This operation removes differences in size among the shapes being compared. It is preferred to other measures of size as it is a size metric independent of shape in the absence of allometry.	$CS = \sqrt{\sum_{i=1}^{K}\sum_{j=1}^{M}(X_{ij} - C_j)^2}$
4. Rotate each specimen to minimize the sum of squared distances (Procrustes distance, ρ) between corresponding landmarks from the reference configuration.	The Procrustes distance (square root of the sum of squared distances, ρ) between the corresponding landmarks of the target and the reference configurations is minimized by finding the angle of optimal rotation (θ). The value of θ is substituted into the equation for D^2 and solved for *D*. The reference configuration is the grand mean of all optimally rotated specimens and computed using GPA (see text for references).	$D^2 = \sum_{j=1}^{K}\left[(X_{Rj} - (X_{Tj}\cos\theta - Y_{Tj}\sin\theta))^2 + (X_{Rj} - (X_{Tj}\cos\theta - Y_{Tj}\sin\theta))^2\right]$ $\theta = arctangent\left(\frac{\sum_{j=1}^{K} Y_{R_j}X_{T_j} - X_{R_j}Y_{T_j}}{\sum_{j=1}^{K} X_{R_j}X_{T_j} + Y_{R_j}Y_{T_j}}\right)$

Superimposing raw landmark coordinate data took the landmark configurations in the data set from a multidimensional space called 'configuration space' to 'Kendall's shape space', whereby dimensions of variability in the landmark configurations were removed by translating, scaling and optimally rotating them as described previously. Configuration space is defined as all the possible configurations of *k* landmarks in *m* dimensions (*x* and *y* axes) in a $k \times m$ dimensional space (in this chapter, *k* = 16, *m* = 2) (Rohlf 1996; Zelditch *et al.* 2004). By translating and scaling each landmark configuration in the GPA, three dimensions of variability were removed (two by centering each configuration to a common origin and one by scaling each configuration to unit centroid size. This removed size differences among all specimens. After correcting for size differences among all individuals using centroid size, all configurations moved from configuration space to pre-shape space with $km - m - 1$, or more simply $2k - 3$ dimensions. Finally, optimally rotating all landmark configurations removed an additional dimension of variability, thus taking the configurations from pre-shape space to Kendall's shape space (Rohlf 1996) with $km - m - \frac{m(m-1)}{2} - 1$ dimensions or more simply, $2k - 4$ dimensions. Kendall's shape space is a geometric space with non-Euclidean geometry (Fig. 2.5), where the shape differences between reference and target configurations are measured by finding the angle of optimal rotation to minimize the Procrustes distance between corresponding landmarks of two configurations. The resulting Procrustes residuals, often referred to as warp scores (vectors describing differences between reference and target configurations; Webster & Sheets 2010) are projected to tangent space to facilitate statistical analysis of Euclidean distances, using methods such as non-parametric MANOVA. (Fig. 2.5)**.**

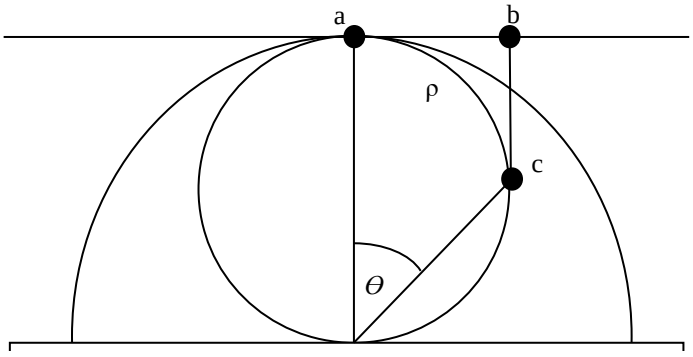

Fig. 2.5. A cross section through Kendall's shape space (inner sphere) and pre-shape space (outer hemisphere). The line tangential to the hemisphere is the tangent space to the sphere at the reference configuration (a). This example is for a sample of triangular landmark configurations. For landmark configurations with *k* > 3 landmarks, Kendall's shape space is a multi-dimensional space of 2*k*-4 dimensions and is difficult to visualize. Point a) is the reference configuration in both Kendall's shape space and tangent space, b) is the target configuration projected to tangent space and c) is the target configuration in Kendall's shape space. The angle of optimal rotation (Θ) minimizes the Procrustes distance (ρ), the arc between the reference (a) and target (c) configurations, and the equivalent Euclidean distance, a) to b), in tangent space. Figure modified from Zelditch *et al*. (2004).

The dimensions showing the most variation in landmark coordinates among the specimens, were found by performing a principal components analysis (PCA) using the *plotTangentspace* function in *geomorph*. All PCA's were performed with a covariance matrix. In geometric morphometrics, this is referred to as a relative warp analysis, the aim of which is to project the partial warp scores (herein referred to as shape variables) computed from the GPA to tangent space in order to describe major trends of variation in as few statistically orthogonal or independent dimensions as possible (Rohlf 1996; p. 124). Shape differences between river and reservoir habitat were then visualized by plotting the mean shape configurations as deformation grids (herein referred to as TPS or thin-plate spline grids). TPS's were generated by computing the deformation of each mean shape relative to the consensus configuration (Rohlf 1993; Rohlf & Marcus 1993) using the shape variables that describe the landmark displacements between them.

The traditional morphometric method was also used to assess morphological divergence among river and reservoir populations of Australian smelt and the results were compared to those obtained using geometric morphometric methods. After the specimens were unbent, as described above for the geometric morphometrics, the original *.tps* file with the raw landmark coordinate data was superimposed by GPA using the *TradMorphGen* module in *CoordGen 8* software (v8; Sheets *et al.* 2014). A series of trusses (linear measurements) were specified in the *TmorphGen8* function in *CoordGen8*, which requires a measurement protocol file to be loaded, to specify which inter-landmark distances are to be measured. The measurement protocol was plotted to an image of the consensus configuration (Fig. 2.6) showing the linear measurements (inter-landmark distances) that were measured and their descriptions (Table 2.2). These inter-landmark distances represent various lengths and depths that may be biologically important, and were chosen based on similar inter-landmark distances used by Meyer (1990). The linear measurements were saved to an Excel spreadsheet, rescaled to original measurement units (millimetres) using the scale factor for each specimen and log-transformed. A PCA was performed on the traditional morphometric data to describe major trends of variation as described for the geometric morphometric data. As reported by Bookstein (1989), PCA performed on traditional morphometric data (linear truss measurements) separates measurements that contribute to size and shape, based on covariances among the linear measurements and a designated size variable (e.g. standard length). All statistical analyses were performed using customized R scripts **(**Appendix 3**).**

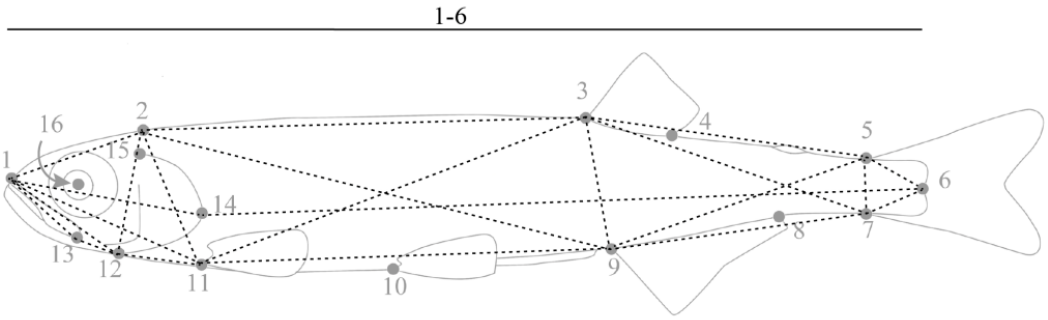

Fig. 2.6 Diagram showing 23 measurements of inter-landmark distances on each specimen for the traditional morphometrics method (broken lines). These are described in Table 2.2.

Table 2.2 Description of linear truss measurements (inter-landmark distances) made between 16 landmarks that were used in geometric morphometrics analysis of body shape of river and reservoir populations of Australian smelt.

LM's	Description
1-2	nose to skull margin (NSM);
1-6	standard length (SL);
1-11	nose to pectoral fin insertion (NPCF);
1-12	nose to mandible hinge (NMD);
1-13	nose to maxilla (NMX);
1-14	head length (HL);
2-3	skull-margin to anterior dorsal fin insertion (SMDF);
2-9	skull margin to anterior ventral fin insertion (SMVF);
2-11	body depth (BD);
2-12	skull-margin to mandible hinge (SMMD);
3-5	dorsal length of caudal peduncle (DLCP);
3-7	anterior insertion of dorsal fin to ventral insertion of caudal fin (DFVCF);
3-9	anterior insertion of dorsal fin to anterior insertion of ventral fin (DFAVF);
3-11	anterior insertion of dorsal fin to insertion of pectoral fin (DFPF);
5-6	dorsal insertion of caudal fin to notch in posterior margin of caudal peduncle (DICP);
5-7	depth of caudal peduncle (CPD);
5-9	dorsal insertion of caudal fin to anterior insertion of ventral fin (CFVF);
6-7	notch in posterior margin of caudal peduncle to ventral insertion of caudal fin (VICP);
7-11	ventral caudal fin insertion point to anterior insertion point of ventral fin (VCAV);
9-11	anterior insertion of ventral fin to insertion of pectoral fin (VFPF);
11-12	pectoral fin insertion to mandible hinge (PFMD);
12-13	mandible hinge to maxilla (MDMX)
14-6	trunk length (TL)

2.2.4. Statistical analysis

A principal components analysis was performed first to visualize the largest sources of variation in the geometric and traditional morphometric data. For both geometric and traditional morphometric data, a non-parametric MANOVA was performed to assess variation in shape attributable to habitat, population and sex. Detailed descriptions of the procedures from *geomorph* used in this chapter, are summarized in Adams and Otarolla-Castillo (2013) and Collyer *et al*. (2015) and further mathematical details are found in Anderson (2001a; 2001b), Legendre and Anderson (1999) and Goodall (1991).

Shape differences attributable to habitat, population and sex were quantified by computing the sum of squares (SS) of Procrustes distances; the magnitude of deviation between shape variables of each group mean and the reference configuration (see Table 2.1 for equation). For the traditional morphometric data, Mahalanobis distance (D_m) was used as the distance metric instead of Procrustes distance, because it scales each variable's mean by the variance-covariance matrix, placing each variable on a common scale. Variation in shape variables was evaluated by partitioning the total model SS across factors, in order to reduce residual or unexplained error in the specified model:

$$Shape \sim habitat\ (population)\ \times\ sex + log(CS)$$

where *shape* was the matrix containing the shape variables, *habitat* was a fixed factor defined as a flowing river or standing reservoir habitat, *population* was a random factor nested within *habitat, sex* was a fixed factor and log-transformed centroid size (*logCS*) was included as a covariate. Residual error was computed across populations not individuals, thereby each population was the lowest unit of replication. Log-transformed centroid size was included as a covariate in the non-parametric MANOVA analysis of geometric morphometric data, if size differences were found among the groups and log-transformed standard length was included as a covariate in the non-parametric MANOVA analysis of the traditional morphometric data. To ensure that factors were statistically treated as random or fixed, appropriate mean squares ratios for F-value calculations were determined following the expected mean squares procedures described in Anderson and terBraak (2003) (see Appendix 4).

The effect and statistical significance of each factor was assessed using a randomized residual permutation procedure (RRPP) as described by Anderson (2001b). This procedure assumed that the following null hypothesis was true; a random reallocation of observed shape variables to different levels of a factor would result in the same distribution as the original data set (Anderson 2001b). Thus, the observed shape variables among specimens were

shuffled and reallocated to different factor levels according to the randomization rules for a nested mixed effect model described in Anderson and terBraak (2003) (see Appendix 4), and *SS* and *F*-statistic were recalculated. This was repeated for 999 permutations, thereby generating the distribution of a pseudo *F*-statistic (Anderson 2001b). The *F*-statistic computed for a factor could then be used to compute a P-value as:

$$P = \frac{No.\,of\,F_{RRPP} \geq F_D}{Total\ of\ F_{RRPP}}$$

where F_{RRPP} was the total number of *F*-values computed for each permutation of the shape variables during the RRPP and F_D was the *F*-value computed for the original set of shape variables. This value provided the probability that *SS* of shape variables between habitats, among populations and between sexes, was greater than the *SS* of shape variables among specimens within each habitat, population and sex, purely by chance (α). A summary table was provided containing the *Z*-scores (standard deviations between groups and mean of the sampling distribution) indicating effect size, based on the *F*-distribution that was generated, as well as the *F*- and *P*-values for each factor.

2.3. Results

2.3.1. Population demographics and data summary

Standard lengths (SL) were not significantly different between habitats (F = 0.260; P = 0.597; Table 2.3), while they were significantly different by sex, the habitat × population interaction and the habitat × population × sex interaction (F = 21.774, 2.276 and 3.591 respectively; P < 0.05; Table 2.3). The significance of the three-way interaction indicates that there are some sex-specific differences in standard lengths for some populations, though not all. Furthermore, the *SS* for the three-way interaction constitutes less than 5% of the total variation ($SS_{system\ type\times\ population\ \times\ sex}\ /SS_{total}$ = 0.04; Table 2.3). The distribution of standard lengths (SL) generally support these observations. Of the 11 populations, most were centred around 35-40 mm SL and approximately normally distributed, except for the Broken, Ovens and Nepean rivers and Lakes Cordeaux, Nepean and Nillahcootie which had bimodal distributions (Fig. 2.7).

Table 2.3 Non-parametric ANOVA statistics for nested factorial model of standard length data from river and reservoir populations of Australian smelt as a function of habitat, sex (fixed effects) and population (random effect). F-values were calculated using a randomized residual permutation procedure (RRPP); effects were considered significant if *P*-values were less than a critical $\alpha = 0.05$.

Effect	Df	SS	MS	F	Z	P
Habitat	1	11.640	11.640	0.260	0.5	0.597
Sex	1	975.260	975.260	21.774	14.832	< 0.05
Habitat × population	9	917.640	101.960	2.276	2.685	< 0.05
Habitat × sex	1	160.860	160.860	3.591	1.854	0.062
Habitat × population × sex	9	1303.290	144.810	3.233	4.443	< 0.05
Residuals	647	28979.130	44.790			
Total	668	32347.000				

Centroid sizes were not significantly different between habitats ($F = 0.914$; $P = 0.352$; Table 2.4), though they were significantly different for the habitat × population interaction ($F = 157.556$; $P < 0.05$; Table 2.4). Even though centroid size is highly variable among populations, the overall variation in centroid size does not result in differences in centroid size between habitats (i.e. river or reservoir).

Table 2.4 Non-parametric ANOVA statistics for nested factorial model of centroid size data from river and reservoir populations of Australian smelt as a function of habitat, sex (fixed effects) and population (random effect). F-values were calculated using a randomized residual permutation procedure (RRPP); effects were considered significant if *P*-values were less than a critical $\alpha = 0.05$.

Effect	Df	SS	MS	F	Z	P
Habitat	1	2.610^{-7}	2.610^{-7}	0.914	0.060	0.352
Sex	1	1.060^{-6}	1.060^{-6}	0.370	0.446	0.549
Habitat × population	9	4.052^{-3}	4.502^{-4}	157.556	336.924	< 0.05
Habitat × sex	1	1.700^{-7}	1.700^{-7}	0.060	0.695	0.809
Habitat × population × sex	9	3.400^{-5}	3.780^{-6}	1.323	0.677	0.232
Residuals	647	1.848^{-3}	2.860^{-6}			
Total	668	5.938^{-3}				

Figures 2.7, 2.8 and 2.9 also highlight a strongly biased sex-ratio, ranging from 16:1 to 3:1 (F:M). This reflects previously reported sex ratios for Australian smelt during spawning periods in other locations, ranging from 2:1 to 4:1 (Milton & Arthington 1985). It is unclear if removing the juveniles and individuals for which sex could not be determined, affected the sex ratio. In addition, the sex ratio for the species inclusive of juvenile or immature individuals has never been reported for the species and could not be determined visually in this thesis. Milton and Arthington (1985) sampled 115 mature individuals and found that the

observed ratios were significantly different from 1:1. Given the number of individuals sampled (669) for this chapter is nearly five times greater, it is possible that the observed sex ratios closely reflect the true sex ratio of the Australian smelt river and reservoir populations in this thesis. However, this was beyond the scope of the thesis and not further analysed.

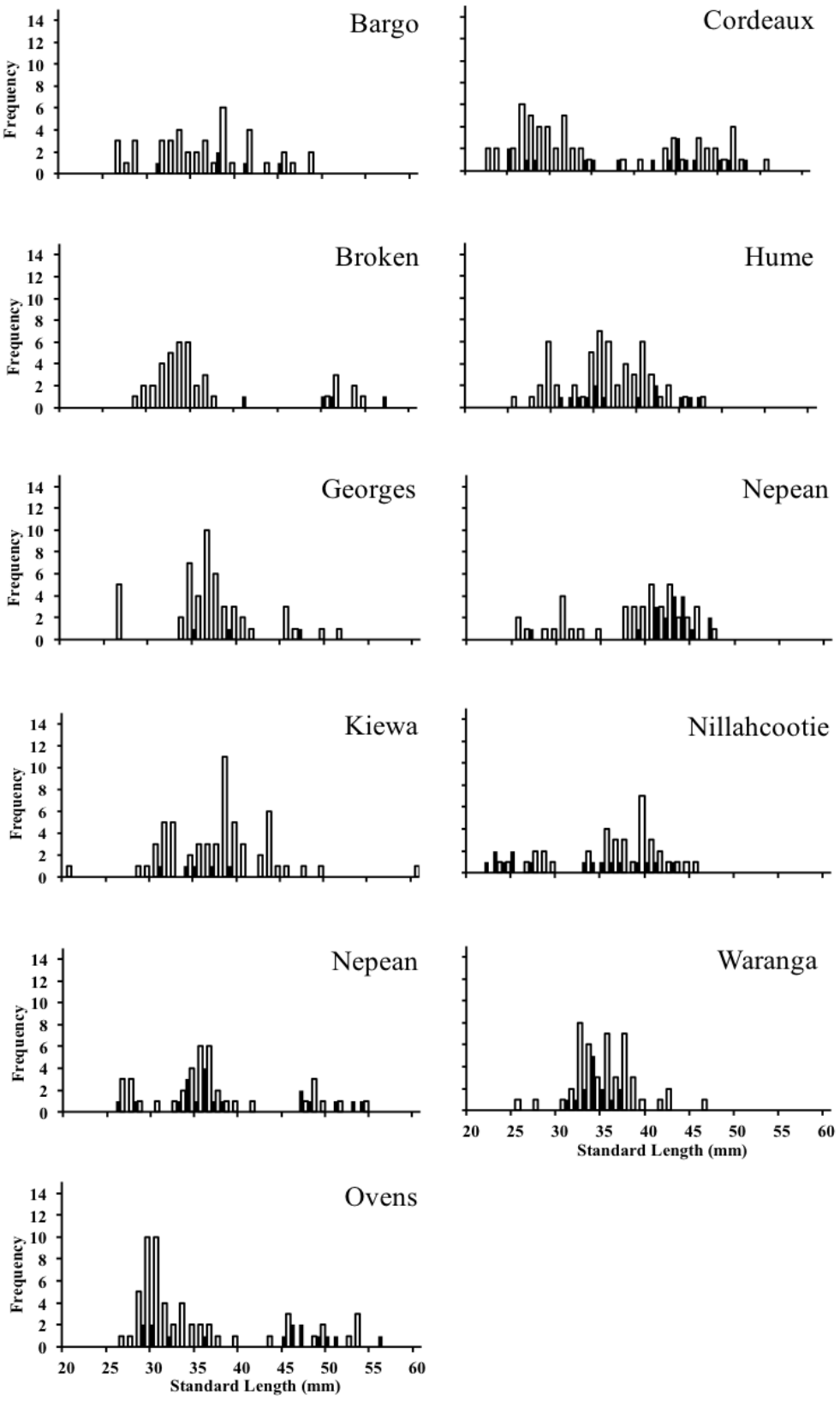

Figure 2.7. Size class (SL) distribution of 11 Australian smelt populations. Data is for clearly identified adult individuals with developed gonads. Juveniles and unsexed individuals could not be confidently identified or distinguished, thus they were excluded from further analysis. Empty bars are females, solid bars are males. Sample sizes (*n*) for Bargo, M = 5, F = 43; Broken, M = 4, F = 40; Georges, M = 3, F = 50; Kiewa, M = 5, F = 58; Nepean River, M = 20, F = 40; Ovens, M = 15, F = 57; Lake Cordeaux, M = 18, F = 60; Lake Hume, M = 14, F = 57; Lake Nepean. M = 19, F = 43; Lake Nillahcootie, M = 17, F = 38; Waranga Basin, M = 15, F = 48

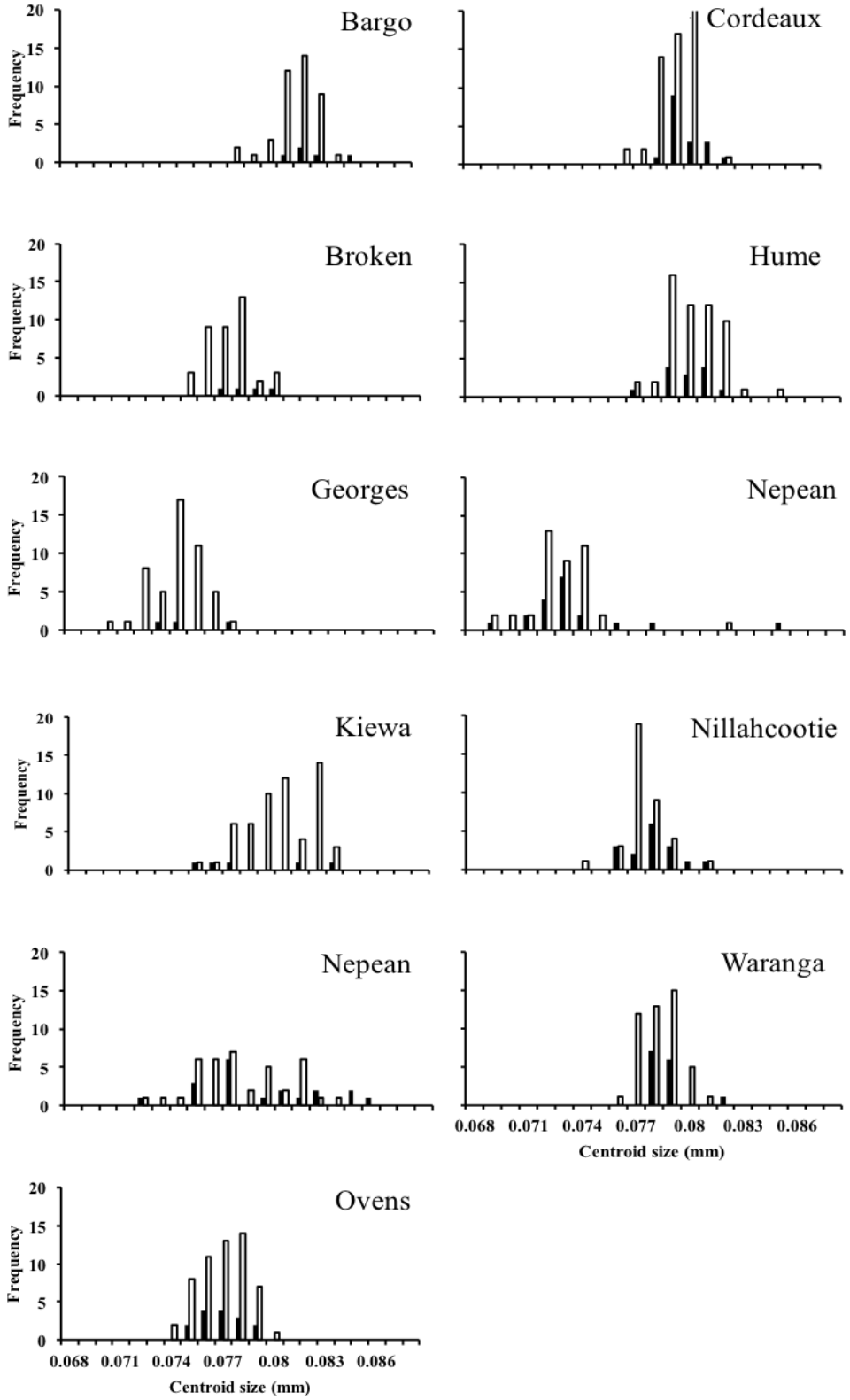

Figure 2.8. Centroid Size (CS) distribution of 11 Australian smelt populations. Data is for clearly identified adult individuals with developed gonads. Juveniles and unsexed individuals could not be confidently identified or distinguished, thus they were excluded from further analysis. Empty bars are females, solid bars are males. Sample sizes (*n*) for Bargo, M = 5, F = 43; Broken, M = 4, F = 40; Georges, M = 3, F = 50; Kiewa, M = 5, F = 58; Nepean River, M = 20, F = 40; Ovens, M = 15, F = 57; Lake Cordeaux, M = 18, F = 60; Lake Hume, M = 14, F = 57; Lake Nepean. M = 19, F = 43; Lake Nillahcootie, M = 17, F = 38; Waranga Basin, M = 15, F = 48.

Male and female size class distributions (both SL and CS) by habitat (i.e. river or reservoir) showed a positive skew (Fig. 2.9). These size distributions indicate that a large proportion of the populations may be smaller, mature fish ranging in standard length from 25 to 55 mm and centroid size of 0.068 to 0.086. (Fig. 2.9), but centroid size showed a uniform distribution, with a negative skew in both habitats (Fig. 2.9). Mean standard length (± SE) for river females was 36.51 ± 0.39 mm, river males, 40.83 ± 1.14 mm, reservoir females 36.37 ± 0.43 mm and reservoir males 38.49 ± 0.82 mm standard length. Mean CS (± SE) for river females was 7.83 $\times 10^{-2} \pm 0.02 \times 10^{-2}$, river males 7.88 $\times 10^{-2} \pm 0.04 \times 10^{-2}$, reservoir females 7.86 $\times 10^{-2} \pm 0.02 \times 10^{-2}$ and reservoir males 7.84 $\times 10^{-2} \pm 0.04 \times 10^{-2}$. Due to the presence of significant differences in size among river and reservoir populations of Australian smelt, log-transformed CS was included as a covariate in geometric morphometric analyses and log-transformed standard length was included as a covariate in traditional morphometric analyses.

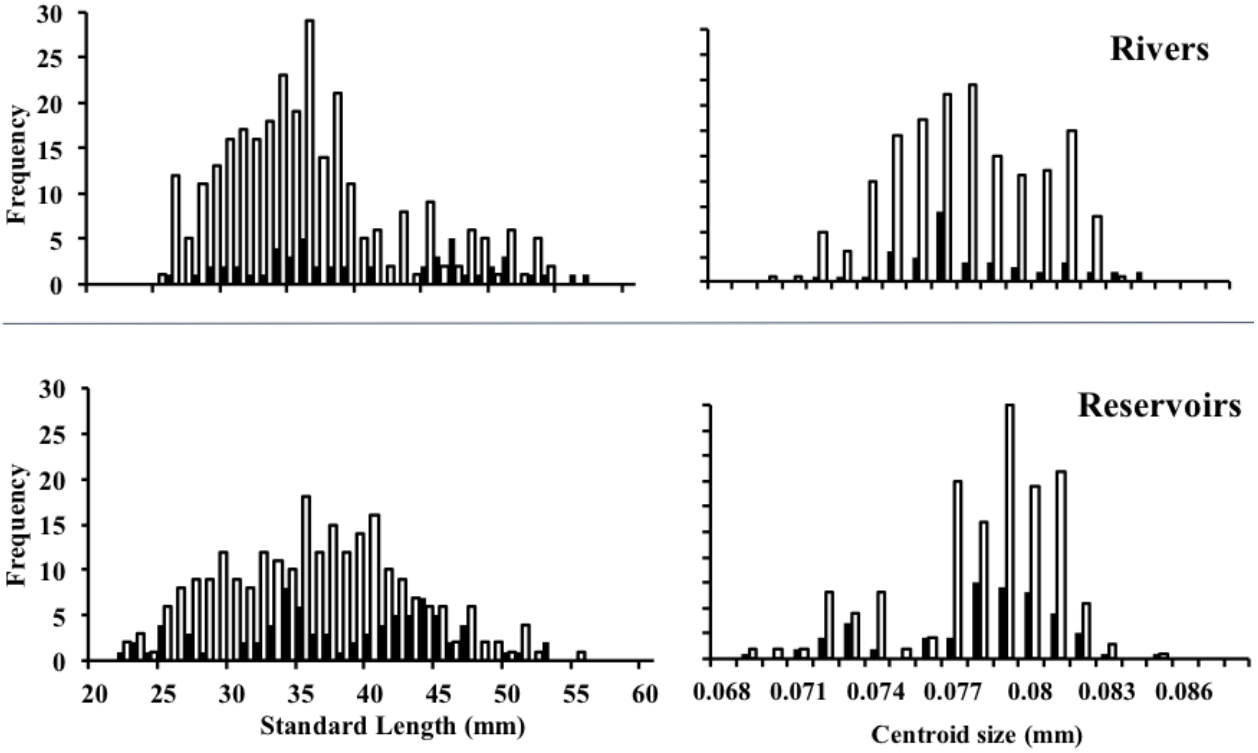

Figure 2.9. Size class distribution of Australian smelt in river and reservoir habitats based on standard length (left panels) and centroid size (right panels). Data is for clearly identified adult individuals. Juveniles and unsexed individuals could not be confidently identified or distinguished, thus they were excluded from further analysis. Solid bars are males and empty bars are females. Sample sizes (*n*) for river males = 52; river females = 288; reservoir males = 83; reservoir females = 246.

2.3.2 Geometric morphometrics

A PCA of shape variables acquired from GPA of landmark coordinate data (Fig. 2.10) showed that strong variation in mean body shape of Australian smelt between river and reservoir habitats was attributable to 4 out of the 6 river populations. The river population body shapes showed the greatest variance along PC 2 (21.1%; Fig. 2.10), whereas body shape of populations from reservoir habitats showed greater variance along PC 1 (35.6%, Fig. 2.10). The maximum variation occurring in body shape among populations from river and reservoir habitats across PC axes 1 and 2 are depicted in the TPS grids below and to the left of the PC 1 and PC 2 axes (Fig. 2.10). Reservoir population body shape means show a dorso-ventral deepening of the trunk, superior (surface) orientation of the mouth and larger head relative to the river population body shape means (Fig. 2.10). Lake Cordeaux (LCO) males and females had a unique body shape compared to the other populations with shallow bodies relative to the river populations, but also with the highest PC 1 score, indicating they had the narrowest caudal peduncle relative to body depth. The variation of reservoir population body shape means along PC 1 was greater than variation of river population body shape means. While the body shape variation along PC 1 appears similar to the variation in body shape along PC 2, the former appears to represent changes isolated mostly to the depth and antero-posterior compression of the head region and depth of the caudal peduncle (Fig. 2.10). River population body shape means were less variable and mostly characterized by a deeper head region and narrow caudal peduncle, while reservoir population body shape means were mostly characterized by uniform depth along the length of the body (Fig. 2.10). The PC loadings (see Appendix 5) reflect the concentration of shape change in landmarks in the head region for PC 1 and more uniform shape change across the whole body for PC 2.

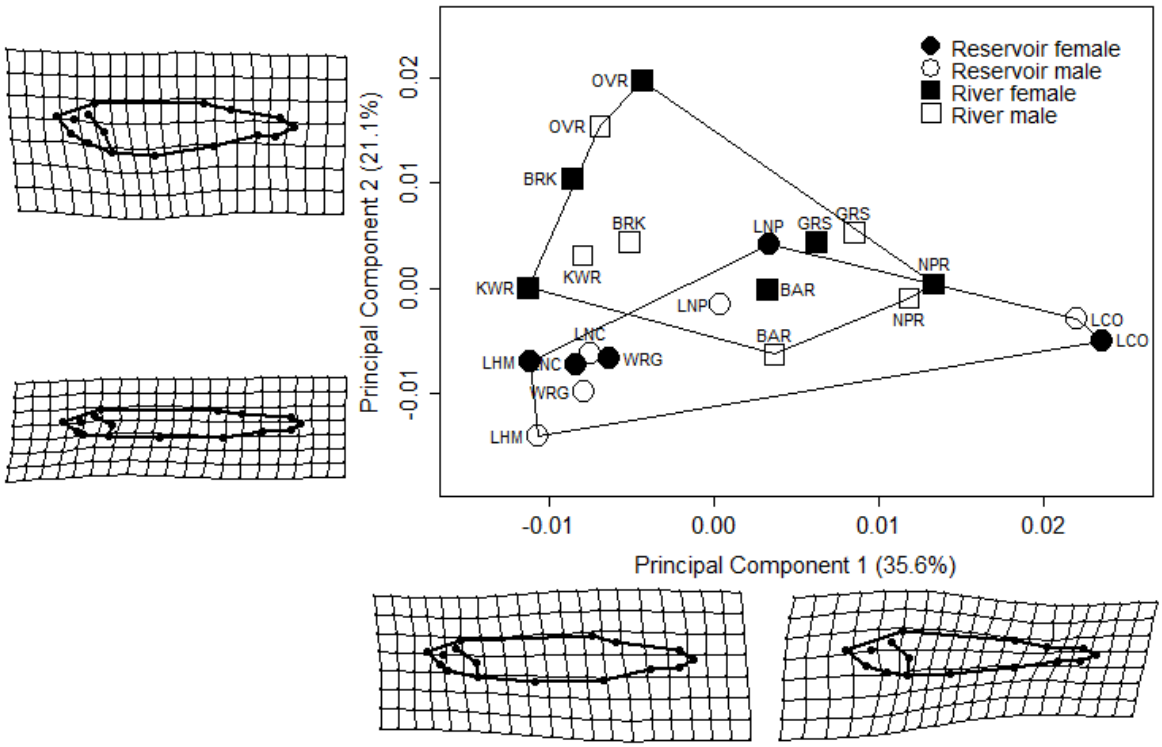

Fig. 2.10. Plot of principal component (PC) 1 and 2 for landmark coordinate (geometric morphometrics) data which represent 35.6% and 21.1% of variation in mean body shape among populations, respectively. TPS grids to the bottom and left of the PC plot depict the most 'extreme' body shape at the minimum and maximum scores along the respective axes. Shape deformations shown in TPS grids are magnified 2x for ease of visualization. Polygons indicate the variation in mean body shape among populations within each habitat. Population names are abbreviated: Bargo river, BAR; Georges river, GRS; Nepean river, NPR; Lake Nepean, LNP; Lake Cordeaux, LCO; Broken river, BRK; Kiewa river, KWR; Ovens river, OVR; Waranga Basin, WRG; Lake Hume, LHM; Lake Nillahcootie, LNC.

The non-parametric MANOVA performed on geometric morphometric data indicate that habitat, sex, habitat × population and habitat × population × sex interactions had significant effects on body shape ($F = 40.334,\ 12.607,\ 26.822$ and 1.553 ; $P < 0.05$; Table 2.5) respectively. Habitat and habitat × population effects had the largest effect sizes ($Z = 19.688$ and 16.739 respectively; Table 2.5), indicating that differences between habitats were almost as large as differences among populations. The habitat × population interaction also indicates that differences between habitats may be driven by differences between specific river and reservoir populations. While differences in body shape between river and reservoir habitats clearly exist, the differences in body shape may not consistently arise among other populations outside those considered in this study. Sex was also found to have a significant effect on body shape ($F = 12.607; P < 0.001$; Table 2.5), but the effect size of sex was less than half of the effect size of habitat ($Z = 7.999$ and 19.688 respectively; Table 2.5).

Table 2.5 Procrustes ANOVA (non-parametric MANOVA) statistics for nested factorial model of body shape based on landmark coordinate data from river and reservoir populations of Australian smelt as a function of habitat, sex (fixed effects), population (random effect) and log-transformed centroid size (LogCS; covariate). F-values were calculated using a randomized residual permutation procedure (RRPP); effects were considered significant if *P*-values were less than a critical α = 0.05

Effect	Df	SS	MS	F	Z	P
Habitat	1	0.024	0.024	40.334	19.688	< 0.001
Sex	1	0.007	0.007	12.607	7.999	< 0.001
Log(CS)	1	0.011	0.011	19.944	12.357	< 0.001
Habitat × population	9	0.142	0.017	26.822	16.739	< 0.001
Habitat × sex	1	0.001	0.001	2.155	2.009	< 0.050
Habitat × population × sex	9	0.008	0.001	1.553	1.551	< 0.001
Residuals	647	0.381	0.001			
Total	668	0.577				

The significance of the habitat × population × sex interaction ($F = 1.553,\ P < 0.001$; Table 2.5) suggests that differences in body shape between habitats are attributable to a unique pattern of sexual dimorphism in specific river and reservoir populations. The effect size of the habitat × population × sex interaction ($Z = 1.551$; Table 2.5) is comparatively smaller than the other effects in the model. The factors of interest remained significant, despite the fact that log-transformed centroid size (*log(CS)*) was a significant predictor of shape (F = 19.944; P <0.001). Thus, shape differences indicated by this analysis exist, after controlling for the correlation between body shape and size.

A post-hoc test, based on pairwise comparisons of Procrustes distances (D_ρ) among mean body shape of habitat × sex groups (Table 2.6) shows that there is sexual dimorphism within reservoirs and within rivers ($D_\rho = 0.007$ and 0.012 respectively; $P < 0.001$; Table 2.6). However, the Procrustes distance between reservoir females and river male mean body shapes is also significant ($D_\rho = 0.017$; $P < 0.001$; Table 2.6). By contrast, the distance between mean body shapes for reservoir males and river females is not significant. Therefore, differences in mean body shapes of river and reservoir habitats are also attributable partly to population-level sexual dimorphism.

Table 2.6 Pairwise Procrustes distances (D_ρ) between mean body shapes of habitat × sex groups, based on geometric morphometrics data (computed following Collyer *et al.* 2015). Procrustes distances between means (above diagonal) were computed based on the Procrustes ANOVA (non-parametric MANOVA) model in Table 2.4. Distances were considered significant if P-values (below diagonal) were less than a critical $\alpha = 0.05$.

		Reservoir		River	
		Female	Male	Female	Male
Reservoir	Female		0.007	0.012	0.017
	Male	< 0.001		0.011	0.013
River	Female	0.302	0.476		0.012
	Male	< 0.001	0.483	< 0.001	

2.3.3 Traditional morphometrics

A PCA of mean linear truss measurements (Fig. 2.11) showed that among the river and reservoir populations of Australian smelt, 79.0 % and 12.8 % of variation were described by PC 1 and PC 2 respectively.

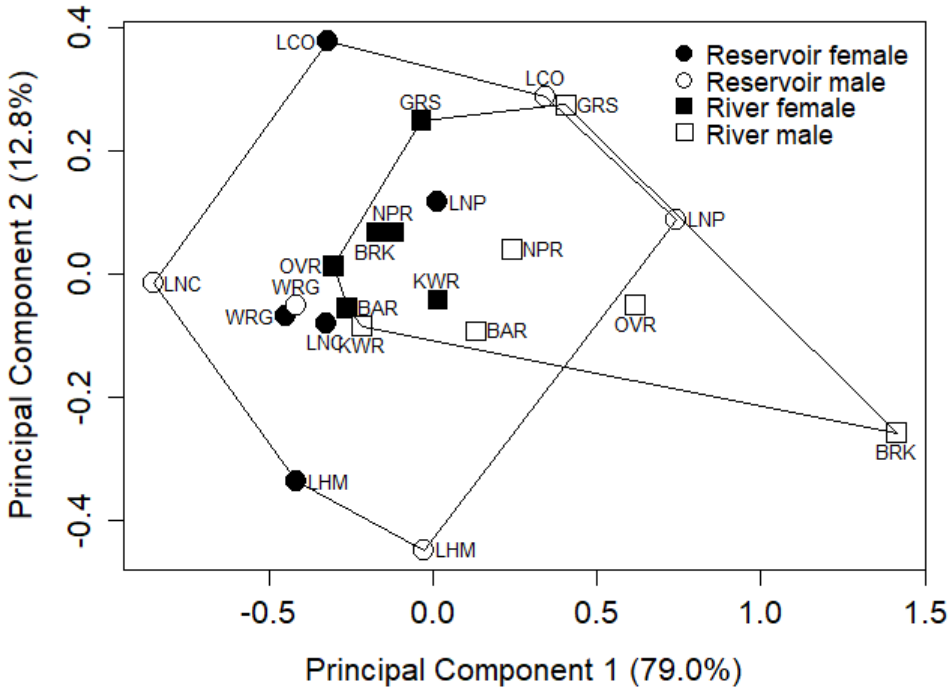

Fig. 2.11. Plot of principal components (PC) 1 and 2 for linear truss (traditional morphometric) data which represent 79.0% and 12.8% of variation in mean body shape among river and reservoir populations of Australian smelt, respectively. Polygons indicate the variation in mean body shape among populations within each habitat. Population names are abbreviated: Bargo river, BAR; Georges river, GRS; Nepean river, NPR; Lake Nepean, LNP; Lake Cordeaux, LCO; Broken river, BRK; Kiewa river, KWR; Ovens river, OVR; Waranga Basin, WRG; Lake Hume, LHM; Lake Nillahcootie, LNC.

The population variation across PC 1 (79.0 %; Fig. 2.11) was more than twice that of the same component in the PCA for the geometric morphometric data (35.6 %; Fig.2.10). The most variation appears to be among Broken River, Ovens River and Lake Nillahcootie males and Lake Hume population, with the remaining populations clustered at the centre of both PC axes (Fig. 2.11). The variation across PC 2 (12.8 %; Fig. 2.11) was approximately half that of the same component in the PCA for geometric morphometric data (21.1 %; Fig. 2.10). While the river populations appear to form a distinct cluster that mostly separates from the reservoir populations across PC 2 (Fig. 2.10), there appears to be no distinct separation between river and reservoir populations based on the PCA of the traditional morphometric data, across PC 1 or 2 (Fig. 2.11). In contrast to PC 1 of the geometric morphometric data, PC 1 of the traditional morphometric data describes more than double the variation among groups (79.0 % and 35.6 %; Fig. 2.10 and 2.11 respectively). The variable loadings for PC 1 of mean linear truss measurements on river and reservoir populations of Australian smelt were positive, ranging between 0.14 and 0.28 (Appendix 6), indicating they were all correlated with some latent variable common to the linear measurements. The loadings for PC 2 ranged from -0.26 to 0.34, with the highest being 0.66 (Appendix 6). These loadings for PC 2 demonstrate that a small proportion of the variation among groups is attributable to changes unique to each linear measurement.

The non-parametric MANOVA performed on the traditional morphometric data indicate that all fixed and random effects had significant effects on body shape (F = 14.321, 5.464, 12.346, 9.606, 2.280 and 1.295; $P < 0.001$; Table 2.7) respectively. Habitat and habitat × population had the largest effect sizes ($Z = 45.972$ and 87.843 respectively; Table 2.7), while linear measurements covaried significantly with standard length (*log(CS)*, F = 12.346; P < 0.001; Table 2.7). Differences between habitats were therefore smaller than those among populations means.

While differences in body shape between river and reservoir habitats clearly exist, the traditional morphometrics data also suggests that differences in body shape that occur between some river and reservoir populations may only be applicable among some populations from river and reservoir habitats outside those considered in this study.

Table 2.7. Non-parametric MANOVA statistics for nested factorial model of body shape based on linear truss measurements (traditional morphometrics) from river and reservoir populations of Australian smelt as a function of habitat, sex (fixed effects) and population (random effect). F-values were calculated using a randomized residual permutation procedure (RRPP); effects were considered significant if *P*-values were less than a critical α = 0.05

Effect	Df	SS	MS	F	Z	P
Habitat	1	270.300	270.300	14.321	45.972	< 0.001
Sex	1	103.000	103.148	5.464	15.303	< 0.001
Log (CS)	1	233.100	233.060	12.346	39.242	< 0.001
Habitat × population	9	1632.100	181.344	9.606	87.843	< 0.001
Habitat × sex	1	43.000	43.000	2.280	4.201	< 0.010
Habitat × population × sex	9	220.600	24.447	1.295	2.207	< 0.050
Residuals	646	12194.000	18.877			
Total	668	14696.000				

For the traditional morphometric data, sex was found to have a significant effect on body shape ($F = 5.464; P < 0.001$; Table 2.7), and the effect size ($Z = 15.303$; Table 2.7) was larger than the effect size for sex in the geometric morphometric data (Table 2.7). In contrast to the results from the geometric morphometric data, the habitat × sex interaction was significant ($F = 2.280;\ P < 0.001$; Table 2.7). As in the analysis of the geometric morphometric data, the significant habitat × population × sex effect, indicates that sexual dimorphism may not occur consistently outside the populations considered in this study based on traditional morphometric data ($F = 1.295,\ P < 0.05$; Table 2.7) and has a relatively small effect size ($Z = 2.207$; Table 2.7).

A post-hoc test was performed for traditional morphometrics data (Table 2.8). Contrary to the geometric morphometric data, the pairwise distances were significant for all group means in the traditional morphometric data (i.e. Mahalanobis distance (D_m)=2.583-9.546; P < 0.001; Table 2.8), thus mean shapes were significantly different between river and reservoir habitats and between males and females.

Table 2.8 Pairwise Mahalanobis distances (D_m) between habitat × sex combinations among mean linear measurements for each group based on traditional morphometric data (computed following Collyer *et al.* 2015). Procrustes distances among means (above diagonal) were computed based on the Procrustes ANOVA (non-parametric MANOVA) model in Table 2.11. Distances were considered significant if *P-values* (below diagonal) were less than a critical $\alpha = 0.05$.

		Reservoir		River	
		Female	Male	Female	Male
Reservoir	Female		2.583	3.466	6.418
	Male	<0.001		4.699	9.546
River	Female	<0.001	<0.001		5.262
	Male	<0.001	<0.001	<0.001	

Both the traditional and geometric morphometric data, when analysed using non-parametric MANOVA suggest that there are differences in mean shape among river and reservoir populations, and males and females. However, the geometric and traditional morphometric methods differ in the differences they detect among population means. Mean body shape was different between river and reservoir populations, with sexual dimorphism present in both habitats (Fig. 2.12), when compared against the reference (grand mean) shape. While the variance in body shape suggested by the PCA is not reflected as strongly in the mean body shapes, there are certain morphological characteristics which are clearly habitat-specific and others which are sex-specific (i.e. implying sexual dimorphism).

Australian smelt from river habitats clearly show an overall deeper, tapered body compared to reservoir conspecifics (Fig. 2.12). More specifically, river fish appear to have a deeper, more antero-posteriorly compressed head region, deep, short trunk region, narrow peduncle relative to body depth, more superior (dorsally) oriented mouth and posteriorly located pectoral fin. In comparison, the reservoir fish have a more fusiform, narrow body shape, dorso-ventrally compressed head, longer trunk region relative to standard length, deep caudal peduncle relative to body depth and terminal (horizontal) mouth orientation.

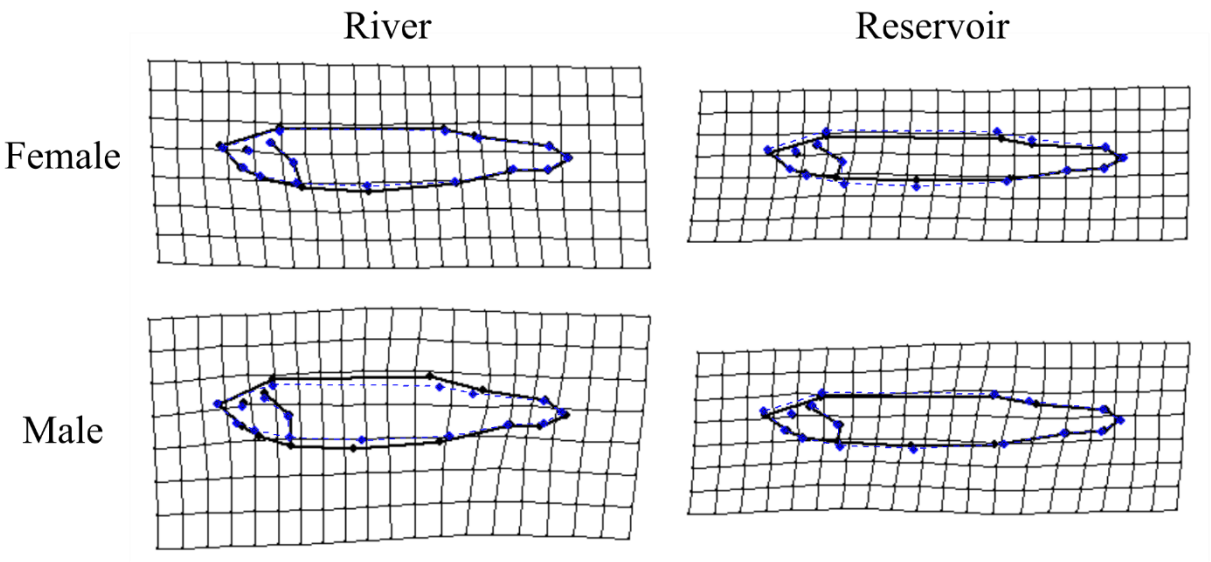

Fig. 2.12 Thin-plate spline (TPS) grids showing body shape deformations between the reference configuration (broken outline) and mean configuration of male and female Australian smelt from river and reservoir habitats (solid outline). Mean shapes were produced using geometric morphometric data analysed in this chapter. Shape deformations are magnified 5X for ease of visualization.

Sexual dimorphism is evident, particularly for the river fish, where males have a longer, flatter head with a slightly superior mouth orientation, shorter pre-dorsal length (posterior edge of skull to dorsal fin), compressed ventral region (pectoral to ventral fin), longer dorsal fin base and longer ventral fin base. In the reservoir fish, males were flatter in the head region, tapered posterio-anteriorly, have a compressed ventral and dorsal region and have a longer ventral and dorsal fin base.

2.4. Discussion

2.4.1. Flow velocity and morphological divergence

The results of this chapter support the alternative hypothesis; that populations from reservoir habitats have significantly different body shape, head shape, caudal peduncle depth and mouth orientation compared to populations from river habitats. Australian smelt from river habitats were generally deeper bodied, with narrower caudal peduncles relative to the trunk region and deeper, more antero-posteriorly compressed heads than reservoir populations and this was reflected in both the geometric and traditional morphometric analyses. Despite the fact that size (measured as log-transformed centroid size for the geometric morphometric analysis in Chapter 2.3.2) was a significant predictor of shape change, body shape was still significantly different among populations from river and reservoir habitats.

However, the direction of body shape divergence was not consistent with theoretical predictions based on flow velocity and results from previous field studies (Table 2.9) on which the hypotheses were based (Langerhans 2008; Haas *et al.* 2010; Franssen 2011).

Morphological divergence among populations of fish from river and reservoir habitats has been observed repeatedly through several empirical studies (Langerhans *et al.* 2003; Franssen *et al.* 2013a; Cureton & Broughton 2014) and meta-analyses (Langerhans 2008).

Five studies (Brinsmead & Fox 2002; Langerhans *et al.* 2003; Haas *et al.* 2010; Collin & Fumagalli 2011; Franssen 2011) have shown that fish from river habitats had narrow, fusiform bodies and fish from reservoir habitats were deeper bodied and more robust, with two studies providing evidence of opposite transformations (Pavey *et al.* 2010; Franssen *et al.* 2013a); that fish from river habitats had deeper, robust bodies, while fish from reservoir habitat were shallower bodied and more fusiform (Table 2.9).

The results of the present study conform to those of Franssen *et al*'s (2013a), Pavey *et al*'s (2010) and Hendry *et al*'s (2002) findings. Australian smelt, much like *Cyprinella venusta*, *Labidesthes sicculus*, *Lepomis macrochirus*, *Oncorhyncus nerka* and *Gasterosteus aculeatus* (Table 2.9) exhibit a similar pattern of morphological divergence, with deeper bodies, larger heads and relatively narrow caudal peduncles among populations from the lotic habitats and shallower, fusiform bodies with smaller heads among populations from lentic habitats. Franssen *et al.* (2013a), Pavey *et al.* (2010) and Hendry *et al.* (2002) argued that deeper, robust body shape in river dwelling populations allowed individuals to hold their position and enabled swimming control and manoeuvrability that may be beneficial in capturing food items passing in the current and making complex manoeuvres in turbulent and variable flow conditions. In contrast, Haas *et al*. (2010), Franssen (2011) and Langerhans *et al*. (2003) argued that the deeper bodies and caudal peduncles are evolved in reservoir fish populations due to a need for high burst swimming performance to avoid predators and increased manoeuvrability to capture food items suspended in the water column.

Exceptions to predicted and reported morphological divergence were two *Melanotaenia* spp. (McGuigan *et al.* 2003) and *Netropis atherinoides* (Franssen *et al.* 2013a), which showed no differences in body depth between natural lake (i.e. low or no flow) and river habitats (i.e. high flow velocity) and which contradict other research of divergence in body morphology (Table 2.9).

Table 2.9 Studies comparing body shape between river and reservoir populations of fish. Studies in bold are those that show the same results as those reported in this chapter. River and reservoir columns describe body shape change in populations from those habitats.

Author	River	Reservoir
Pavey *et al.* (2010) *Oncorhyncus nerka*	Deeper body and caudal peduncle, larger, longer head, posterior eye.	Narrower body and caudal peduncle, smaller compressed head, anterior eye.
Franssen *et al.* (2013a) *Cyprinella venusta* *Labidesthes sicculus* *Lepomis macrochirus* *Notropis atherinoides*	Deeper body and larger caudal peduncle, larger, longer head with terminal mouth position (no differences observed in *N. atherinoides*).	Shallower body and smaller caudal peduncle, smaller, compressed head with upturned mouth position.
Hendry *et al.* (2002) *Gasterosteus aculeatus*	Deeper bodied, shorter.	Shallower bodied, longer.
Langerhans (2008)	Shallower body	Deeper body
Haas *et al.* (2010) *Cyprinella venusta*	Shallower body, larger, longer head, posterior eye, larger more anterior dorsal fin, narrow caudal peduncle, terminal mouth position.	Deeper body, small head, anterior eye, wider anterior dorsal fin, deeper caudal peduncle, upturned mouth position.
Langerhans *et al*. (2003) *Bryconops caudomaculatus* *Biotodoma wavrini*	Shallower body and caudal peduncle, larger head, terminal mouth position	Deeper body, smaller, narrower head, deeper caudal peduncle, upturned mouth position.
Franssen (2011) *Cyprinella lutrensis*	Shallower body, larger, longer head, posterior eye, terminal mouth position	Deeper body, smaller, compressed head, anterior eye, smaller, upturned mouth position.
Collin and Fumagalli (2011) *Phoxinus phoxinus*	Shallower body, smaller caudal peduncle, shallower, longer head.	Deeper body and larger caudal peduncle, shorter, deeper head.
Brinsmead & Fox (2002) *Lepomis gibbosus* *Ambloplites rupestris*	Shallow bodied (but not consistent among all populations compared).	Deeper bodied (but not consistent among all populations compared).
McGuigan *et al*. (2003) *Melanotaenia eachamensis* *Melanotaenia duboulayi*	No significant differences in body shape between stream and lake populations.	
Cureton *et al*. (2014) *Pimephales vigilax*	Shallow, fusiform body	Deep, robust body

Interestingly, Haas *et al*'s (2010) study of morphological divergence among river and reservoir populations of *C. venusta* found differences in body shape that were the reverse of patterns observed by Franssen *et al*. (2013a) for the same species. Fish populations that originated from river habitats prior to dam construction were adapted to natural flow regimes (Lytle & Poff 2004). These results suggest that differences in flow velocity among river and emergent reservoir ecosystems are at least partly responsible for trait divergence in body shape of Australian smelt. However, the influence of other selective pressures, such as predation, cannot be ruled out as they were not evaluated in this thesis.

Predation is thought to be a strong selective pressure in some populations of fish and may also be a cause of adaptive divergence in body shape among habitats with high and low predator densities (Langerhans *et al.* 2004; Langerhans 2009). Stocking of recreational fish species is a factor leading to increased predator density in reservoir habitats (Fernando & Holčík 1991). A better understanding of the relative contribution of other selective pressures such as predator regime, may provide a better overall understanding of how morphological divergence among river and reservoir populations occurs under multiple selective pressures. The relative contribution of predation and flow velocity to body shape divergence have only been studied separately (Langerhans & Reznick 2010). Thus, morphological trade-offs and their influence on swimming performance may be better understood through an integrated study of multiple selective pressures and should be considered for future research.

The results from the geometric and traditional morphometric methods were not directly comparable. The results derived from the two methods differ in that geometric morphometrics found differences between male and female means, but no significant differences between all river and reservoir mean shapes. On the other hand, analysis of traditional morphometric data suggested that mean shapes were significantly different among all groups (habitat type and sex). Populations of Australian smelt from river habitats have deeper bodies and larger heads than reservoir conspecifics based on the geometric morphometrics. However, the results from the traditional method are more difficult to interpret. It relies on detailed knowledge of the dimensions measured and interpreting the PC loadings for main axes of variation (i.e. PC 1 and PC 2). The variation along PC 1 according to traditional morphometrics theory, should be interpreted as size related variation (i.e. allometry), while the remaining components or PC's are interpreted as purely shape variation (Bookstein 1989). This is reported as the optimal interpretation since the linear measurements on an organism are typically positively correlated with size measurements (Bookstein 1989). Thus, the PC 2 loadings (Appendix 6) for the traditional morphometric data in this chapter suggest the majority of shape variation can be described in terms of shortening or lengthening of the distance between the nose and

mandible hinge, from the dorsal to ventral fin insertion points, pectoral fin to mandible hinge and mandible hinge to the maxilla. The problem this presents, is that it is difficult to distinguish how much of this variation is attributable to sexual dimorphism, and how much is attributable to habitat differences such as flow velocity. These changes reflect some of the variation seen in Fig. 2.12, but it is difficult to ascertain which groups specifically, these changes occur in.

The results from the traditional morphometrics suggest that for the same amount of analytical and data collection effort, geometric morphometrics provide more conservative, but perhaps more thorough and interpretable evidence of shape change to that provided by traditional morphometrics, as it provides more coverage of the form and does not suffer from multi-collinearity (Zelditch *et al.* 2004). Furthermore, the results from the geometric morphometric method were easier to interpret as it provided the option to generate TPS grids which assisted with visualising the shape change that occurred among populations from river and reservoir habitats and the ability to visualize shape change attributable to sex or habitat effects.

2.4.2. Sexual dimorphism

Australian smelt populations from river and reservoir habitats were both characterized by significant sexual dimorphism. This chapter is the first attempt to quantify sexual dimorphism in Australian smelt, even though it has been described qualitatively in efforts to revise the species taxonomic status (McDowall 1979). Presently, there are a number of theories that can explain sexual dimorphism, although the literature on the topic relates to sexual size dimorphism rather than shape dimorphism (Bisazza 1993; Herler *et al.* 2010). The extent of sexual shape dimorphism in both river and reservoir populations of Australian smelt is limited to the length of the ventral and caudal fin bases.

While there are a number of theories that can explain sexual dimorphism (Herler *et al.* 2010), the niche dimorphism theory may offer a valid explanation of the sexual dimorphism observed here. Given that the median fins (dorsal and ventral) play a mostly functional role, serving as vertical stabilizers (Webb 1984a) the possibility exists that larger median fins in males may be attributable to different niche preferences between sexes in both habitats. However, Australian smelt are a schooling species (Milton & Arthington 1985; Lintermans 2007) and therefore males and females are likely to occupy similar microhabitats unless they form single-sex schools, though this is presently unknown. Comparisons could be drawn to other families such as Poecilidae in which a number of species show sexual dimorphism in fin shape and size (Endler 1984; Bisazza 1993). However, because of different life-histories and

reproductive modes between these species, this is still speculative and sex-specific habitat preference may be an area of focus for future research to disentangle the contribution of sexual selection and habitat effects to sexual dimorphism for Australian smelt and other species.

2.5. Conclusion

This chapter has presented evidence of strong morphological divergence and sexual dimorphism among populations of Australian smelt from river and reservoir habitats. The direction (increase or decrease) of change in body depth and head size were the most striking morphological shifts observed when comparing mean body shapes among populations of Australian smelt from river and reservoir habitats. In conjunction with the findings from similar studies, these results are in contrast to theoretical predictions that a more streamlined body shape will emerge among populations in lotic habitats, and deep, robust body shapes will be selected for in lentic habitat. The results of this chapter also confirm previous qualitative descriptions of sexual dimorphism in Australian smelt (McDowall 1979), which has been shown to be of similar magnitude to morphological divergence among river and reservoir populations. The comparison between traditional and geometric morphometrics methods, demonstrated that geometric morphometrics provides an approach that generates more statistically robust and intuitive results for analysis of body shape variation. The following chapter explores the relationship between body shape variation described here, fin shape and prolonged swimming speed among Australian smelt populations from river and reservoir habitats.

Chapter 3. Linking form and function: fin shape explains differences in swimming performance among river and reservoir fish populations

3.1. Introduction

The fitness that arises from swimming performance can affect a species' ability to occupy particular habitats (Vogel 1994). Consequently, the morphological traits of aquatic organisms are often shaped by flow velocity (Townsend & Hildrew 1994). Generally, fish in high flow-velocity environments, or those that undertake migrations should exhibit traits that enhance their swimming efficiency (e.g. Lytle & Poff 2004; Allan & Castillo 2007; Donaldson *et al.* 2013). For example, the distance travelled per unit of energy expenditure generally increases with body length in fish and therefore many migratory species are large-bodied, while relatively few small-bodied species undertake long distance movements (Ware 1978). Similarly, Webb (1984b; 1984a) and Langerhans (2008) suggest that optimal swimming performance and fitness in fish is attributable to morphological adaptations, including body shape and other propulsive traits such as fins which enhance their performance under certain flow velocity conditions. Some evidence has emerged which suggests that the selective pressure imposed by flow velocity on swimming traits is strong enough to generate such trait divergence in morphology in species within relatively few generations (Cureton & Broughton 2014).

The construction of dams has created novel ecological and evolutionary pressures (chapter 1), not the least of which have been changes to the mean minimum and maximum flow velocities encountered by freshwater fish populations. The formation of permanent lentic (low flow velocity or standing) waterbodies following dam construction appears to be driving morphological divergence among populations which historically inhabited flowing river ecosystems (chapter 2). Although it is clear that fish are morphologically adapted to their flow velocity environments (Vogel 1994), there are substantial questions arising over studies suggesting that functional adaptation, such as the ability to swim under given flow velocities, are driven by specific patterns of morphological change over a short period of time (Langerhans *et al.* 2003; Haas *et al.* 2010; Franssen 2011; Franssen *et al.* 2013a). Evidence has emerged which suggests that the selective pressure imposed by flow velocity is strong enough to generate changes in morphology in less than 15 generations (~20 years) (Cureton & Broughton 2014). However, the functional consequences of these morphological changes have not been fully investigated.

Haas *et al*. (2010) and Franssen (2011) found that after decades of isolation, reservoir populations of *Cyprinella venusta* and *C. lutrensis* had generally larger heads and deeper

bodies and caudal peduncles while river populations were more streamlined and fusiform in body shape. However, other intra-specific studies of paired river and reservoir populations (Taylor & McPhail 1986; Brinsmead & Fox 2002; McGuigan *et al.* 2003; Pavey *et al.* 2010) have been equivocal in their support of morphological divergence based on varying flow velocity habitats. Fish can respond in various ways to different flow velocity habitats (e.g. rivers or reservoirs), including changes in body shape alone or in conjunction with fin shapes, increases in body size or changes in muscle composition or physiology. For instance, body shape is only a reliable predictor for sustained swimming speed performance in fishes that use labriform (pectoral fin-powered) locomotion (Walker *et al.* 2013). Accordingly, caudal and pectoral fin shape are more reliable predictors of swimming speed among and within species of body-caudal fin (BCF) swimming fishes (Sambilay Jr 1990) and median-paired fin (MPF) swimming fishes, respectively (Fulton 2010; Fulton *et al.* 2013). Furthermore, populations of a single species can differ in their morphological and performance adaptations across their distribution over environmental flow velocity gradients (Fulton 2010; Fulton *et al.* 2013; Binning *et al.* 2014).

Morphological adaptations to river and reservoir habitats have been examined for a range of species including *C. venusta*, (Haas *et al.* 2010), *C. lutrensis* (Haas *et al.* 2010; Franssen *et al.* 2013a), *Lepomis macrochirus* (Franssen *et al.* 2013a), *L. gibbosus* (Brinsmead & Fox 2002), *Labidesthes sicculus* (Franssen *et al.* 2013a) and *Netropis atherinoides* (Franssen *et al.* 2013a), but swimming performance was not evaluated in any of these studies. The failure of previous studies to evaluate relationships between morphology and swimming performance provides an opportunity to explore whether or not the changes in body form, driven by the construction of dams, has altered the swimming performance of fish populations. To quantify the effect of flow velocity on the swimming performance of river and reservoir populations (research question 2), I used measurements of critical swimming speed (U_{crit}), to test the following hypotheses:

H_0: There are no differences in prolonged swimming speed performance among river and reservoir populations

H_a: River and reservoir populations have significantly different prolonged swimming speed performance.

In doing so, I also tested body morphology and fin aspect ratios as predictors of prolonged swimming speed performance.

3.2. Methods

3.2.1. Sampling

Australian smelt (*Retropinna semoni*) were collected from 3 river and 3 reservoir systems across the southern Murray-Darling Basin (see chapter 1.2 for details) between June and July 2015 using a 10 m (length) x 1.8 m (drop) fine mesh (3 mm diagonal) seine net. Individual smelt were collected from the net and placed in a 20 L plastic bucket containing water collected on site and a battery powered air-pump connected to an air stone to provide aeration. Aquarium salt (0.5 parts per thousand; ppt) was added to assist osmoregulation and minimize handling and transport stress. Once the minimum number of individuals was collected (60), water was added until the bucket was filled to the edge, and the lid was pressed on. This minimized the airspace between the water surface and the lid, thus avoiding excess agitation of the water and associated stress during transport.

3.2.2. Fish husbandry and feeding

A recirculating system consisting of fifteen 32 L aquaria, a 120 L combination sump and biological filter powered by an impeller pump with a maximum flow rate of 550 $L\ hr^{-1}$ were used for holding sampled fish during the experiments. Aquarium heaters (100 watt) for maintaining water temperatures at ~20 ^{o}C (Appendix 7) were randomly distributed throughout half of the holding aquaria, with 3 additional heaters in the sump. Exchange rate in the aquaria was maintained at 75% or 24 $L\ hr^{-1}$. The biological filter was activated for at least two weeks prior to field sampling with aged potable water. Once sampled fish were returned to the lab in 20L buckets, aquaria were prepared for receiving fish by stopping flow from the system and drawing the aquarium volume down to approximately 20% full. Source water from where the Australian smelt were sampled was used to adjust temperature and pH conditions in the aquaria until it matched water quality measurements taken from the collection site. All individuals were then transferred to the aquaria and river or reservoir water was used to refill the aquarium over a period of 6-8 hours. Fish from each population were evenly distributed across 2 aquaria to comply with densities of no more than 1 fish per litre according to animal ethics permit conditions (see Animal ethics permit numbers, pg. i). Water quality and temperature were monitored using a Horiba U51 multi-parameter probe, during transfer of fish and throughout the duration of the experiments (Appendix 7).

Fish were allowed to acclimatize to holding aquaria and routine movement by the investigator in the room for 48-72 hours, after which feeding was commenced. All critical swimming speed tests commenced within 10 days of introducing collected fish to the holding aquaria. This encompassed the number of days required to collect fish from all sampling sites

and complete acclimation to holding aquaria. If fish did not commence feeding after the initial acclimation period, feeding was attempted again at 6-hour increments until feeding behaviour was observed. All fish were fed twice daily to satiation with frozen brine shrimp. Fish were deemed to have reached satiation when they were no longer seen actively taking food items. All fish within aquaria were observed prior to each feeding for any mortality and to assess any signs of disease or stress associated with captivity. Mortalities were removed and recorded daily, and water quality measurements were taken after feeding to avoid causing stress in fish associated with movements in the room.

3.2.3. Critical swimming speed

Prolonged swimming performance defined as a spectrum of swimming modes, speeds and behaviours that can be maintained for up to 200 minutes (Beamish 1978), was investigated for Australian smelt via measurements of critical swimming speed (U_{crit}). An incremental method was used to measure U_{crit} in 15 individuals from each of three river and three reservoir population of Australian smelt. Sample sizes as low as 5 individuals (e.g. Farlinger & Beamish 1977) have been used to assess swimming performance, but typically 10 – 30 individuals are needed to allow for inter-individual variation (e.g. Taylor & McPhail 1986; Plaut 2000). A sample size of 15 fish per population was estimated to be a sufficient sample size whilst minimizing the use of animals to comply with animal care and ethics approval. Simulation experiments for nested hierarchical experimental designs suggest that a minimum of 10 individuals is needed within each group to adequately test for statistical significance of each group (Anderson, 2001). While every effort was made to collect more individuals, the combination of mortality rates of up to 75% in captivity and difficulty with capturing more than 60 individuals within the available time to undertake sampling and return sampled fish to the lab, meant that sample sizes were limited to 15 individuals per group. Nevertheless, previous studies of critical swimming speed suggested that the minimum number of replicates for sufficient statistical power in this type of experiment can range from 3 to 21 individuals per group (e.g. Kolok & Farrell 1994; Fisher *et al.* 2005; Farrell 2008). A recirculating swim tunnel was employed in U_{crit} tests, which had a cross-sectional tunnel area of 36.92 cm^2 (7.1 × 5.2 cm) and a 25 cm long swimming section (Fig. 3.1; design following Stobutzki & Bellwood 1994). Flow velocity was regulated by rotating a 40-mm ball valve against a protractor that provided a calibrated measure of flow velocity. Flow velocity calibrations were done by measuring the flow volume per unit time coming from the downstream end of the working section at randomly chosen times throughout the day and on different days for a fixed temperature of ~20^{o}C (Appendix 8). Temperature was maintained in the swim tunnel at

~20.0°C (Appendix 7) via a recirculating sump and Hailea HC-250 water chilling unit. The critical swimming speed (U_{crit}) test, a standard method for measuring prolonged swimming performance relevant to daily patterns of habitat use (Plaut 2001; Fulton 2010), involved an incremental test procedure as follows:

i) individual fish were placed into a swim tunnel at a velocity of ~ 2.5 cm s^{-1} (see over page) and acclimated for at least 30 minutes;

ii) flow velocity was increased in fixed increments at pre-determined time intervals until the fish reached fatigue, defined as the point where the fish became exhausted and was swept downstream against a mesh barrier;

iii) the experiment was terminated when the fish remained pinned against the barrier and could not resume swimming after 30 seconds.

iv) The critical swimming speed (U_{crit}) in cm s^{-1} was calculated as

$$U_{crit} = U_i + (\frac{t}{\Delta t})\Delta U_{ii}$$

where U_i was the final velocity maintained for the prescribed entire time interval prior to the interval at which fatigue occurred, *t* was the time elapsed in the time interval during which fatigue occurred, Δ*t* was the prescribed time interval at which velocity was increased, and U_{ii} was the velocity increment.

Fig. 3.1. Recirculating swim tunnel with sump and water chilling unit used to maintain temperatures during critical swimming speed experiments. Water was circulated from a 40 L sump (large blue tub at centre of photo) through the Perspex swim tunnel by an 18 hp Davey pool pump (right of sump) and through a Hailea HC-250A water chiller using a 2000 $L\ hr^{-1}$ submersible pond pump. Flow velocity in the swim tunnel was regulated by a 40 mm ball valve (right of swim tunnel).

Prior to commencing U_{crit} tests, fish were starved for 24 hours to ensure that food remaining in the gut or ongoing digestive processes did not confound performance (Garland & Arnold 1983). Given the fragility of this species to handling for measurement and transfer to the swim tunnel, individuals were anaesthetized in 30 $mg\ L^{-1}$ (0.003%) of benzocaine until the fish reached stage 3.1-3.2 anaesthesia (Zahl *et al.* 2009). Although the potential effects of anaesthesia on swimming performance were not quantified, all individuals were treated similarly to ensure that any relative differences in swimming speed performance among the populations were maintained. Each U_{crit} test was commenced with an initial acclimation velocity of approximately 2.5 $cm\ s^{-1}$ (minimum flow velocity that could be precisely set in the flume) for at least 30 minutes, and then the flow velocity was increased in 3.5 $cm\ s^{-1}$ (~ 1 standard length s^{-1} for individuals 30-40 mm standard length) increments every 5 minutes until the individual reached exhaustion. Velocity increments equivalent to approximately 1 body lengths s^{-1} and time-steps between velocity increases as low as 5 minutes have been found to provide a reliable measure of prolonged speed performance (e.g. Plaut 2000; Yan *et al.* 2012) at which optimal critical swimming speeds are achieved and measured (Farlinger & Beamish 1977). Some individuals failed to correctly orient against the direction of flow or failed to swim against the current from the beginning of the experiment, and exhibited a distinct, lethargic behaviour. These individuals were removed and euthanized using an overdose of at least 100 $mg\ L^{-1}$ (0.01%) of benzocaine. At the completion of the U_{crit} tests, all individuals were preserved in 70% ethanol. Preserved fish were photographed laterally from the left side (see chapter 2.2.3 for details).

3.2.4 Morphology, sex and maturity assessment

Body shape variation was assessed using the geometric morphometric method detailed in chapter 2.2.3 and maturity and sex were identified for all fish used in swimming performance experiments. Only clearly identified adult male and female Australian smelt with developed gonads were used in the experiments due to difficulty in identifying the sex of immature or juvenile individuals as per chapter 2.

3.2.5. Fin aspect ratios

Variation in fin aspect ratios was measured as follows; 1) the pectoral fin from the left side of each individual was detached, spread out on a 10 mm rubber grid and photographed as described in chapter 2.2.3 for body shape. Caudal fins were left attached to the body, spread out on the same grid, pinned in place and photographed at the same time as the whole individual as described in chapter 2.2.3. Measurements were made and computed as:

$$AR_p = \frac{length\ of\ leading\ edge^2}{total\ fin\ area}$$

$$AR_c = \frac{height\ of\ fin^2}{total\ fin\ area}$$

where AR_p and AR_c were pectoral and caudal fin aspect ratios. All measurements were taken to the nearest $0.01\ mm^2$ using ImageJ (v1.50i, Rasband 1997).

3.2.6. Statistical analysis

Non-parametric MANOVA was used to assess variation in body shape, caudal fin and pectoral fin aspect ratios and U_{crit} attributable to habitat (river or reservoir), population, standard length and sex, using the *vegan* package (v2.4.2, Oksanen *et al.* 2007) for R (R Core Team 2015). Shape differences attributable to habitat, population and sex were statistically analysed as per chapter 2.2.4. Variation in U_{crit} was quantified by computing the sum of squared (SS) residuals and partitioning the total model SS across factors, in order to reduce residual or unexplained error in the specified model:

$$U_{crit} \sim habitat\ (population) \times sex + standard\ length$$

where U_{crit} was critical swimming speed expressed as centimetres per second ($cm\ s^{-1}$), habitat was a fixed factor defined as a flowing river or standing reservoir, population was a random factor nested within habitat, sex was a fixed factor and standard length was included as a covariate. Residual error was computed across populations, not individuals, thereby each individual population was the lowest unit of replication. This assessment of variation was repeated for the caudal and pectoral fin aspect ratios with the same model structure as for U_{crit}. Expected mean squares were determined following the same rules as described for chapter 2.2.4.

The effect size and statistical significance of each factor on the response variables was assessed using a randomized residual permutation procedure (RRPP) as described by Anderson (2001b) and in chapter 2.2.4. This was repeated for 999 permutations to generate the distribution of the pseudo *F*-statistic (Anderson 2001b), from which *P*-values could be computed for each factor. This value provided the probability that SS among habitats, populations and sexes, is greater than the SS among specimens within each habitat,

population and sex, purely by chance (α) (i.e. among group versus within group variation, respectively). Summary tables were provided containing the *Z*-scores (standard deviations among group means and mean of the sampling distribution) indicating effect size based on the *F*-distribution that was generated, as well as the *F*- and *P*-values for each factor. The purpose of the statistical analyses in this thesis was inference about trait variation among populations. Hence group mean and standard error were plotted for univariate traits, rather than predicted values, to represent parameter values tested for significant differences using non-parametric MANOVA.

The correlation of U_{crit} with body shape variables, pectoral fin aspect ratio and caudal fin aspect ratio was assessed with a best subset modelling approach to identify the best morphological variables (body morphology and fin shape) to explain variation in swimming speed performance among river and reservoir populations of Australian smelt. Shape based on geometric morphometric data from chapter 3.2.4 was included as a predictor of U_{crit} in the best-subset selection analysis. This was achieved by using partial least squares (PLS) analysis to derive a component which consisted of the linear combination of singular shape vectors (covariation coefficients for each shape variable), which have the highest covariance with U_{crit}. Subsequently, component scores were derived to describe the position (predicted shape) of each individual Australian smelt along the shape component and these would be included as a predictor variable representing body shape in the best subset selection analysis. The PLS analysis was performed following Rohlf and Corti (2000) using the *geomorph* package (v. 3.0.5) for R.

Best subset model selection was carried out with the *leaps* package (v2.4.2; James *et al.* 2013) for R (R Core Team 2015) to determine which variables described in the preceding paragraphs, explained the greatest amount of variation in U_{crit}. Details of the best subset selection approach are provided in James *et al*. (2013) for linear models. The best subset approach using linear models in the *leaps* package compared all 2^p possible linear models, where p is the number of predictor variables, to a null model (i.e. no predictor variables), using an adjusted R^2 statistic:

$$R^2{}_{adjusted} = \frac{RSS \times (n-d-1)^{-1}}{TSS \times (n-1)^{-1}}$$

where *RSS* and *TSS* were residual sums of squares and total sums of squares for a given model, *n* was the number of observations and *d* was the number of variables. The 5 best models, one for each model size (i.e. number of predictor variables) were selected. The model

that resulted in the largest increase in adjusted R^2 value (of the 5 best models) was chosen as the best explanatory model. The adjusted R^2 statistic was used because as it penalizes models with higher numbers of predictor variables. It is a preferred statistic, over other statistics such as RSS and simple R^2 values, because these statistics may indicate that additional variables improve the fit of a model to a dataset even though they do not significantly increase the models predictive power (James *et al.* 2013). Other statistics used for model selection, such as AIC or BIC also penalize the addition of redundant variables, albeit in a different way (i.e. adjusted R^2 is based on RSS, whereas AIC or BIC are based on maximum likelihood). Thus, results for model selection based on parsimony, is the same using both metrics (James *et al.* 2013). Consequently, adjusted R^2 was used for model selection for simplicity of interpretation. Violations of linearity were checked using diagnostic plots to assess heteroscedasticity, normality and independence of errors for the chosen model (Appendix 9)**.** All statistical analyses were performed using customized R scripts (Appendix 3).

3.3. Results

3.3.1. Population demographics and data summary

Fish standard length (*SL*) was significantly different between habitats (F = 32.609; P < 0.05; Table 3.1) and habitat × population interactions (F = 11.248*;* P < 0.05; Table 3.1). There were some sex-specific differences in standard length for some populations, though not all (F = 3.878; P < 0.05; Table 3.1). The SS for the three-way interaction constitutes 8.1% of the total variation in standard length ($SS_{habitat \times population \times sex} / SS_{total}$ = 0.081; Table 3.1).

Table 3.1. Non-parametric ANOVA statistics for nested factorial model of standard length data for Australian smelt sampled from river and reservoir systems for critical swimming speed experiments, as a function of habitat, sex (fixed effects) and population (random effect). F-values were calculated using a randomized residual permutation procedure (RRPP); effects were considered significant if *P*-values were less than a critical α = 0.05.

Effect	Df	SS	MS	F	Z	P
Habitat	1	457.410	457.410	32.609	22.097	< 0.05
Sex	1	8.250	8.250	0.588	0.311	0.463
Habitat × population	3	473.350	157.780	11.248	11.030	< 0.05
Habitat × sex	1	1.020	1.020	0.073	0.631	0.808
Habitat × population × sex	3	163.190	54.400	3.878	3.207	< 0.05
Residuals	65	911.770	14.030			
Total	74	2014.990				

There were more males than females in this data set (Fig. 3.2 and 3.3), compared to the size class distributions in chapter 2.3.1. The bias in sex ratio in some populations (e.g. Broken or Kiewa River populations; Fig 3.2.) may be causing significance of the three-way interaction (F = 3.878; P < 0.05; Table 3.1). It is unknown why the sex-ratio was different to that reported in chapter 2, as the sampling methodology was the same and effectively random because sex could not be identified before running the swimming experiments. Differences in standard length distributions were confirmed when plotted by population (Fig. 3.2) and by habitat type (Fig. 3.3). Populations of Australian smelt from river habitats had greater standard length than those from reservoir habitats (Fig. 3.3). Log-transformed standard length was included as a covariate in all further analyses.

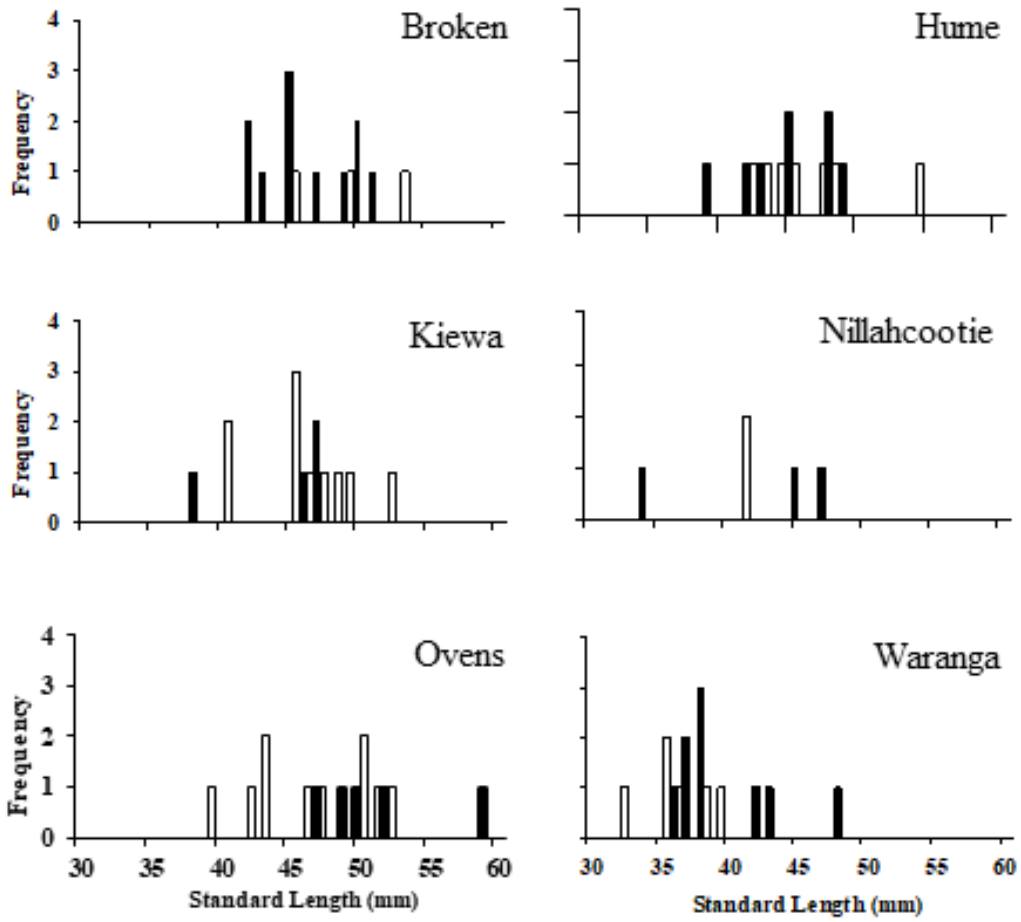

Fig. 3.2. Size class distribution based on standard length (*SL*) of 6 Australian smelt populations. Data is for clearly identified adult individuals. Due to difficulties associated with identifying sex in juveniles in chapter 2.3.1., no juvenile individuals were used in the swimming performance experiments in this chapter. Solid bars are males, empty bars are females. Sample size for Broken, M = 12, F = 3; Kiewa, M = 5, F = 10; Ovens River, M = 5, F = 10; Lake Nillahcootie, M = 3, F= 2; Waranga Basin, M = 9, F = 6; Lake Hume, M = 8, F = 7.

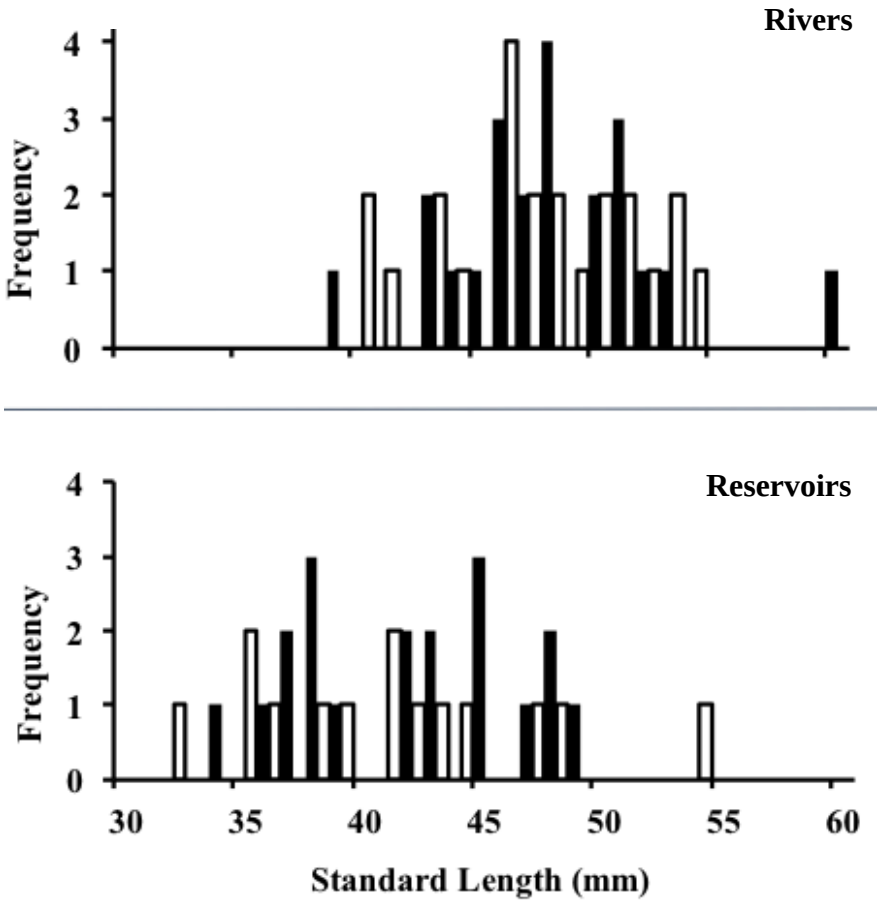

Fig. 3.3. Size class distribution based on standard length (*SL*) of Australian smelt populations from river and reservoir systems. Data is for clearly identified adult individuals. Due to difficulties associated with identifying sex in juveniles in chapter 2.3.1., no juvenile individuals were used in the swimming performance experiments in this chapter. River fish appear to have greater mean standard length than reservoir fish. Grey bars are males, empty bars are females. River males = 22; river females = 23; reservoir males = 20; reservoir females = 16.

3.3.2. Assessment of variation in swimming performance

The non-parametric ANOVA for U_{crit} data of Australian smelt from the swimming experiments indicate that habitat ($F = 67.581; P < 0.001$; Table 3.2) and standard length were significant ($F = 10.010; P < 0.001$; Table 3.2).

Table 3.2. Non-parametric ANOVA statistics for nested factorial model of critical swimming speed in river and reservoir populations of Australian smelt as a function of habitat, sex (fixed effects), population (random effect) and standard length as a covariate. *F* - values were calculated using a randomized residual permutation procedure (RRPP). Effect size (*Z* – scores) represented the standard deviations between group means for each effect. Effects were considered significant if *P*-values were less than a critical α = 0.05

Effect	Df	SS	MS	F	Z	P
Habitat	1	2133.200	2133.170	67.581	44.479	< 0.001
Sex	1	15.200	15.240	0.483	0.384	0.503
Standard length	1	316.000	315.980	10.010	5.915	< 0.001
Habitat × population	4	24.500	6.110	0.194	1.126	0.943
Population × sex	1	38.300	38.250	1.212	0.081	0.288
Habitat × population × sex	4	249.200	62.310	1.974	1.257	0.114
Residuals	67	2114.800	31.560			
Total	79	4891.200				

The standard-length effect size (Z = 5.915; Table 3.2) was smaller than habitat effect size (Z = 44.479; Table 3.2) suggesting that variation in U_{crit} was more attributable to habitat type than standard length. Mean U_{crit} was plotted by habitat and sex with error bars showing that Australian smelt populations from river systems achieved much higher U_{crit} values than those from reservoir systems (Fig. 3.4). Differences among river and reservoir populations in prolonged swimming performance, measured as critical swimming speed, were not specific to any given river or reservoir populations of Australian smelt, given that the habitat × population interaction was not significant (F = 0.194; P = 0.943; Table 3.2).

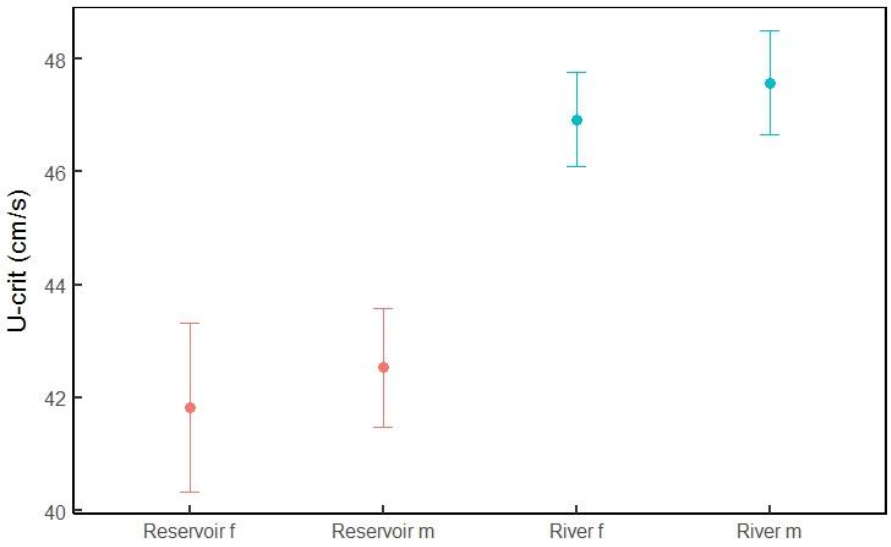

Fig. 3.4. Mean critical swimming speed ($\boldsymbol{U_{crit}}$) for male and female Australian smelt population from reservoir and river populations. Error bars represent 1 standard error around the mean. Sample size (*n*) = 3 (River male and river female populations means) and 2 (Reservoir male and reservoir female population means).

3.3.3 Assessment of variation in body shape

The body shape data in the non-parametric MANOVA did not include Lake Nillahcootie fish sampled for the swimming performance experiments due to insufficient sample size (Nillahcootie, female; *n* = 2). For unknown reasons, there were not enough smelt encountered during field sampling in this reservoir, which resulted in failure to calculate a population mean and therefore, estimate for the sex model parameter. Procrustes ANOVA (Non-parametric MANOVA) analyses showed that habitat, sex and habitat × population effects were all significant (F = 10.429, 4.312 and 5.884 respectively; $P < 0.001$; Table 3.3), while shape variation covaried significantly with standard length (log(CS) F = 1.949; P < 0.001).

Table 3.3. Procrustes ANOVA (non-parametric MANOVA) statistics for nested factorial model of body shape based on landmark coordinate data from river and reservoir populations of Australian smelt as a function of habitat, sex (fixed effects), population (random effect) and log-transformed centroid size as a covariate (log(CS)). F-values were calculated using a randomized residual permutation procedure (RRPP). Effect size (*Z* – scores) represent the standard deviations between group means for each effect. Effects were considered significant if *P*-values were less than a critical α = 0.05.

Effect	Df	SS	MS	F	Z	P
Habitat	1	0.0043	0.0043	10.429	5.336	< 0.001
Sex	1	0.0018	0.0018	4.312	3.552	< 0.001
Log (CS)	1	0.0008	0.0008	1.949	2.053	< 0.050
Habitat × population	3	0.0074	0.0024	5.884	7.113	< 0.001
Habitat × sex	1	0.0003	0.0003	0.669	0.182	0.439
Habitat × population × sex	3	0.0013	0.0004	1.038	1.982	< 0.050
Residuals	64	0.0269	0.0004			
Total	74	0.0430				

Effect size for habitat was smaller than for habitat × population but larger than sex (Z = 5.336, 7.113 and 3.552 respectively; Table 3.3) indicating that differences in body morphology between habitats may be attributable to specific river and reservoir populations.

A post-hoc test based on pairwise comparisons of Procrustes distance (D_ρ) among mean body shape of habitat × sex groups showed that sexual dimorphism was present in Australian smelt from river populations (Table 3.4). There were no other significant differences between male or female Australian smelt from river and reservoir habitats based on pairwise Procrustes distances (Table 3.4). This reflects the population-specific variation in body morphology which was greater than the variation in body shape attributable to the habitat effect ($SS_{habitat \times population} = 0.0074$; $SS_{habitat} = 0.0043$; Table 3.3).

Table 3.4 Pairwise Procrustes distances (D_ρ) between mean body shapes of males and females from river and reservoir habitats sampled for swimming experiments, based on geometric morphometric data. Procrustes distances among means (above diagonal) were computed based on the Procrustes ANOVA (non-parametric MANOVA) model in Table 3.4. Distances were considered significant if *P*-values (below diagonal) were less than a critical α = 0.05.

		Reservoir		River	
		Female	Male	Female	Male
Reservoir	Female		0.008	0.016	0.020
	Male	0.169		0.016	0.015
River	Female	0.723	0.634		0.013
	Male	0.098	0.659	<0.010	

Mean body shapes for males and females from river and reservoir habitats were plotted as TPS grids (Fig. 3.5) to visualize body shape variation. The shape transformations show that mean body shape reflected similar patterns to those observed in chapter 2.3.2, and that sexual dimorphism was present in both habitats (Fig. 3.5). Mean body shape of the river populations show a dorso-ventral deepening of the trunk, terminal (horizontal) orientation of the mouth and longer, deeper head relative to the mean body shapes of the reservoir populations (Fig. 3.5). The reservoir fish appear to have a more superior (dorsally) oriented mouth, although this is more pronounced in the reservoir females. Generally, the reservoir fish had a narrower, fusiform body shape, smaller, dorso-ventrally compressed head and longer caudal peduncle. Landmarks 11 and 12 in particular, appear to exhibit the most variation in the head region and may constitute much of variation in overall head depth (the distance between landmark 12 and 2 and possibly between landmarks 12, 13 and 1), between river and reservoir populations.

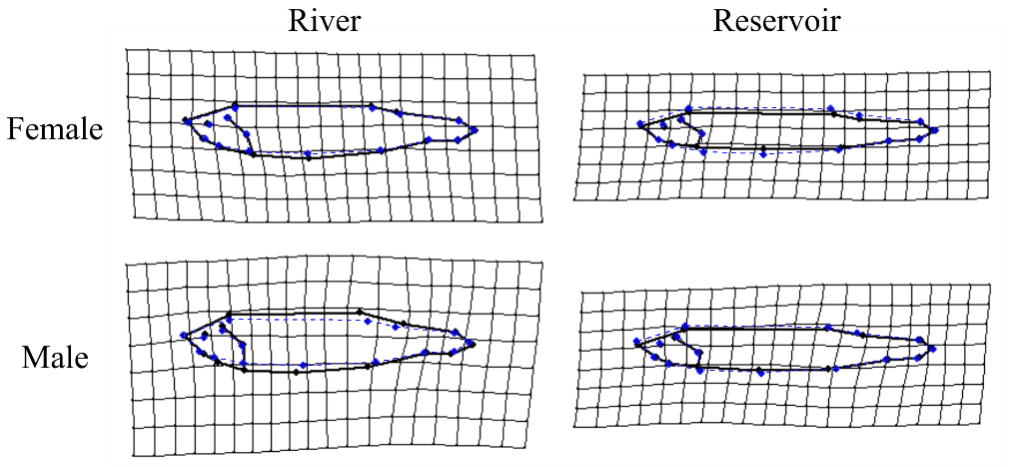

Fig. 3.5. TPS grids depicting body shape deformations between the reference configuration (broken outline) and mean configuration of male and female Australian smelt from river and reservoir habitats (solid outline). Mean shapes were produced using geometric morphometric data analysed in this chapter. Transformations have been magnified 5x for ease of visualization.

Mean body shapes of Australian smelt populations from rivers and reservoirs also showed clear indications of the sexual dimorphism observed in chapter 2.3.2. In both river and reservoir habitats, the males have a deeper trunk, shorter pre-dorsal region (posterior edge of skull to dorsal fin), compressed ventral region (pectoral to ventral fin), longer ventral fin base and narrower caudal peduncle (Fig 3.5).

Although there are clear differences in mean body shapes among male and female Australian smelt from river and reservoir habitats (Fig. 3.5), not all the variation is statistically significant, as described earlier (i.e. habitat × population interaction was significant). Australian smelt from river populations clearly show an overall deeper, tapered body when compared to Australian smelt from reservoir habitats (Fig. 3.5). In particular, river fish have a deeper head, a deeper, shorter trunk region and narrow caudal peduncle relative to body depth.

The partial least squares (PLS) analysis of geometric morphometric shape data of Australian smelt from this chapter, was used to derive a component of singular vectors describing maximum covariation between the 32 shape variables (*x* and *y* coordinates for each landmark) and observed U_{crit} values for each individual (Fig. 3.6A). The largest coefficients were for coordinates 1*x*, 3*y*, 10y, 11*x* and *y* and 15*y*. These vectors describe anterior movement of the nose, movement of the dorsal fin upwards relative to the body, posterior movement of the pelvic fin, rear- and downward movement of the pectoral fin and upward movement of the dorsal terminus of the operculum (Fig. 3.7). Component scores for every individual plotted against their respective U_{crit} (Fig. 3.6B) showed that female and male Australian smelt from river habitats (empty circles and squares) had higher component scores

and U_{crit} values, than female and male Australian smelt from reservoir habitats (solid circles and squares). The correlation coefficient indicates that there is a moderate correlation between critical swimming speed and the body shape component (*r*-PLS = 0.556; Fig. 3.6B).

A)

	Coordinate	
Landmark	*x*	*y*
1	-0.327	0.083
2	-0.044	0.185
3	-0.014	0.245
4	-0.048	0.062
5	-0.13	0.066
6	-0.156	0.163
7	-0.039	-0.123
8	0.041	-0.185
9	0.015	-0.117
10	0.182	-0.355
11	0.571	-0.224
12	-0.054	-0.066
13	0.025	-0.034
14	0.104	-0.081
15	-0.054	0.233
16	-0.071	0.146

B)

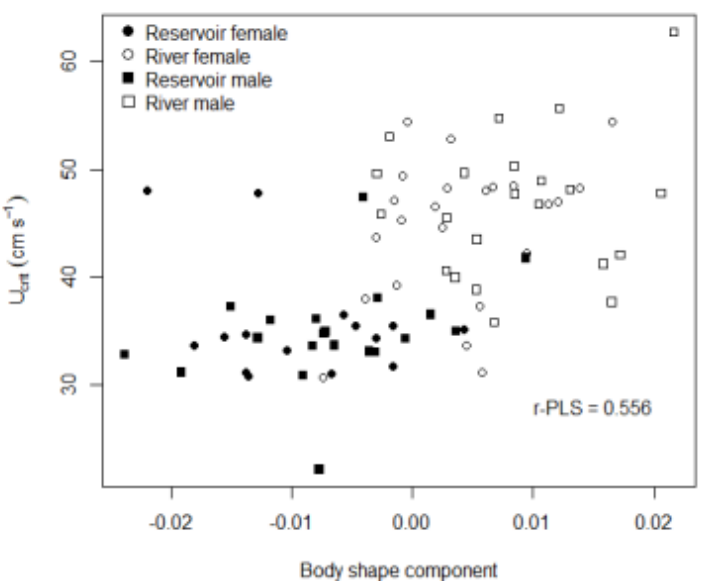

Fig. 3.6. Results for partial least squares (PLS) regression of multivariate shape (this chapter). For each landmark coordinate (*x*, *y*), a singular vector described the maximum covariation of shape with critical swimming speed or U_{crit} (Fig. 3.6A). Each individuals' component score described its projected (predicted) position along the body shape component, which was then plotted against their respective observed U_{crit} (Fig. 3.6B). The correlation coefficient was computed for the regression, showing the strength of the correlation between the two variables. The respective location of each landmark coordinate on the body of the Australian smelt is shown in Fig. 3.7.

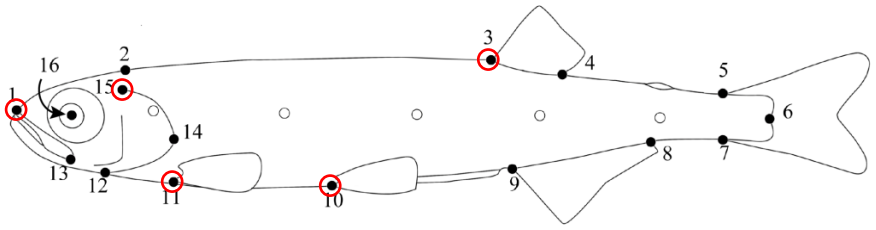

Fig. 3.7. Diagram showing 16 landmarks digitized on each specimen for the geometric morphometrics method. Filled circles indicate the landmarks used for analysis of shape variation. Empty circles indicate 5 additional landmarks that were placed along the lateral line for 'unbending' specimens deformed by rigor mortis or preservation. Landmark descriptions: 1 = nose, 2 = dorsal skull margin, 3 = anterior dorsal fin insertion point, 4 = posterior dorsal fin insertion point, 5 = dorsal caudal fin insertion point, 6 = notch in posterior margin of caudal peduncle, 7 = ventral caudal fin insertion point, 8 = posterior ventral fin insertion point, 9 = anterior ventral fin insertion point, 10 = pelvic fin insertion point, 11 = pectoral fin insertion point, 12 = mandible hinge, 13 = maxilla, 14 = posterior margin of operculum, 15 = dorsal terminus of operculum, 16 = eye. Red circles indicate landmarks at which coordinates had the largest loadings in the PLS body shape component described in this chapter.

3.3.4. Assessment of variation in fin aspect ratios

Caudal fin aspect ratio was significantly different between river and reservoir habitats ($F = 60.726; P < 0.001;$ Table 3.5) with no other significant effect. Australian smelt from river habitats had higher mean caudal fin aspect ratios ($\bar{x}$ = 1.66 – 1.75; Fig. 3.8) than those from reservoir habitats ($\bar{x}$ = 1.27 – 1.32; Fig. 3.8).

Table 3.5 Non-parametric ANOVA statistics for nested factorial model of caudal fin aspect ratio in river and reservoir populations of Australian smelt as a function of habitat, sex (fixed effects), population (random effect) and standard length as a covariate. *F* - values were calculated using a randomized residual permutation procedure (RRPP). Effect size (*Z* – scores) represented the standard deviations between group means for each effect. Effects were considered significant if *P*-values were less than a critical α = 0.05.

Effect	df	SS	MS	F	Z	P
Habitat	1	3.368	3.368	60.726	37.246	< 0.001
Sex	1	0.018	0.018	0.336	0.447	0.574
Standard length	1	0.029	0.029	0.527	0.362	0.502
Habitat × population	4	0.080	0.020	0.363	0.853	0.823
Population × sex	1	0.142	0.142	2.563	0.965	0.133
Habitat × population × sex	4	0.367	0.091	1.657	0.781	0.172
Residuals	67	3.716	0.055			
Total	79	7.723				

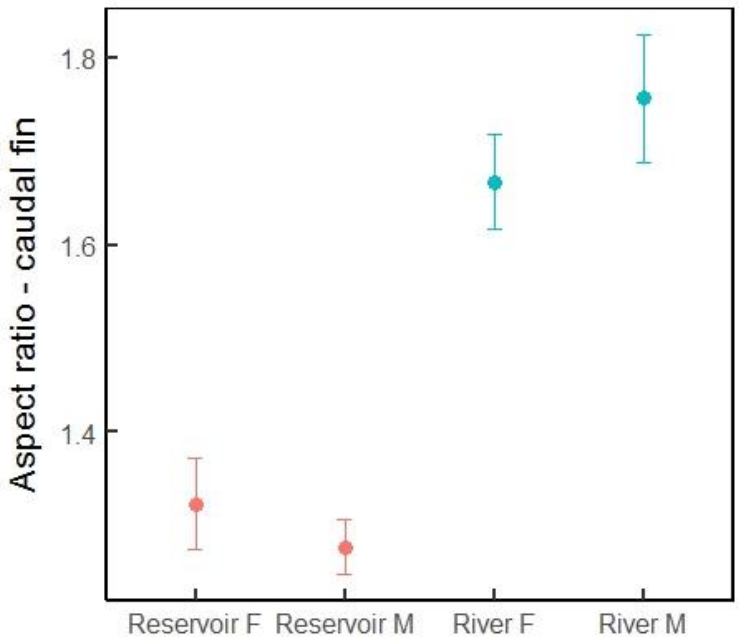

Fig. 3.8. Mean caudal fin aspect ratio for male and female Australian smelt from reservoir and river habitats. Error bars represent 1 standard error around the mean. Sample means (*n*)= 3 (River male and river female populations means) and 2 (Reservoir male and reservoir female population means).

Pectoral fin aspect ratio was also significantly different among populations of Australian smelt from river and reservoir habitats ($F = 139.490; P < 0.001$; Table 3.6) with significant habitat × population and population × sex effects ($F = 6.185; P < 0.001$ and $F = 4.829; P < 0.05$, respectively; Table 3.6). Australian smelt from river habitats had higher mean pectoral fin aspect ratios ($\bar{x}$ = 1.81 – 1.89) than those from reservoir habitats ($\bar{x}$ = 1.32 – 1.33) overall (Fig. 3.9).

Table 3.6. Non-parametric ANOVA statistics for nested factorial model of pectoral fin aspect ratio in river and reservoir populations of Australian smelt as a function of habitat, sex (fixed effects), population (random effect) and standard length as a covariate. F - values were calculated using a randomized residual permutation procedure (RRPP). Effect size (Z – scores) represented the standard deviations between group means for each effect. Effects were considered significant if P-values were less than a critical α = 0.05.

Effect	df	SS	MS	F	Z	P
Habitat	1	5.363	5.363	139.490	97.153	< 0.001
Sex	1	0.033	0.033	0.865	0.114	0.353
Standard length	1	0.004	0.004	0.126	0.648	0.736
Habitat × population	4	0.951	0.237	6.185	6.846	< 0.001
Population × sex	1	0.185	0.185	4.829	2.391	< 0.050
Habitat × population × sex	4	0.031	0.007	0.205	1.038	0.938
Residuals	67	2.576	0.038			
Total	79	9.146				

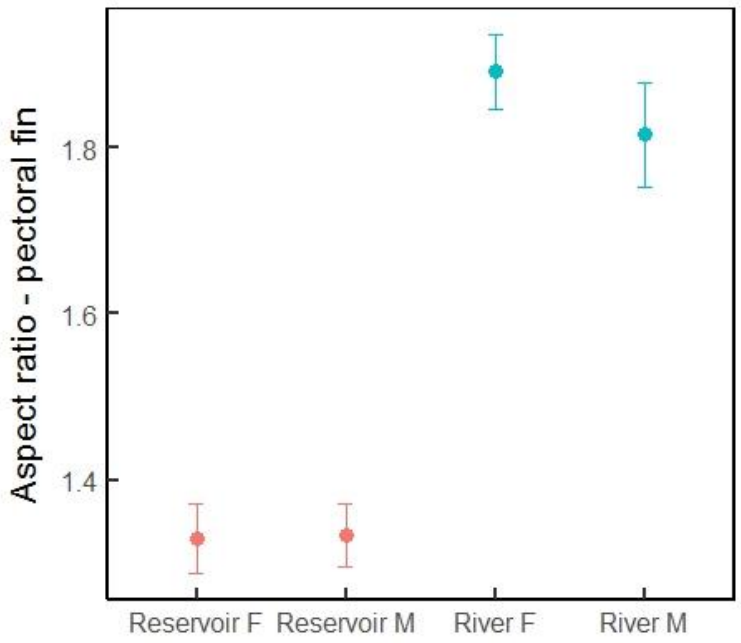

Fig. 3.9. Mean pectoral fin aspect ratio for male and female Australian smelt from reservoir and river habitats. Error bars represent 1 standard error around the mean. Sample means (*n*)= 3 (River male and river female populations means) and 2 (Reservoir male and reservoir female population means).

Compared to effect size for habitat (Z = 97.153, Table 3.6), the effect sizes for habitat × population and population × sex interactions were small (Z = 6.846 and 2.391 respectively; Table 3.6). Some variation in pectoral fin aspect ratio may be attributable to differences between specific population pairs due to the habitat × population interaction (F = 6.185; P < 0.001; Table 3.6) and sexual dimorphism in pectoral fin aspect ratios may be statistically significant only in some populations (F = 4.829; P < 0.05; Table 3.6) or this may be due to biased sex ratios in some populations (Fig. 3.2). Greater swimming speed performance of Australian smelt from river habitats, relative to those from reservoir habitats, based on experimental observations of U_{crit}, are strongly reflected by patterns of differentiation in fin aspect ratios (Fig. 3.8 – 3.9 respectively).

3.3.5. Predictors of swimming performance: correlation of critical swimming speed with morphological traits.

Four candidate models were identified as suitable linear models for U_{crit} of Australian smelt. Of these, model 2 was accepted as the most plausible linear model based on adjusted R^2 values (Table 3.7) which shows that caudal fin and pectoral fin aspect ratios are the best predictors of U_{crit} in river and reservoir populations of Australian smelt.

Table 3.7. Candidate models ranked by adjusted R^2 values from best subset selection approach used to identify the best linear models representing the relationship between 4 predictor variables and critical swimming speed of Australian smelt populations from river and reservoir habitats. Four candidate models were identified, one of each model size from 1 to all four predictor variables. Column headings are *p*; number of variables included in the model: $AR_{pectoral}$; mean pectoral fin aspect ratio variable: AR_{caudal}; mean caudal fin aspect ratio variable, SL; mean standard length, X_{PLS}; the body shape component derived from the partial least squares (PLS) analysis. Variables included in each model are denoted by a cross (×).

Model	p	$AR_{pectoral}$	AR_{caudal}	SL	X_{PLS}	${R^2}_{adj}$
1	1		×			0.946
2	2	×	×			0.973
3	3	×	×		×	0.970
4	4	×	×	×	×	0.968

Model 2 was accepted based on an adjusted R^2 value of 97.3% (Table 3.7), because it had the largest adjusted R^2 value of all the candidate models. Models 3 and 4 were not accepted because the additional variables decreased the explanatory power (${R^2}_{adj}$) by 0.3 and 0.5%, whilst model 1 was not accepted because it explained the least variation in critical swimming speed (Table 3.7). Model 2 estimates suggest that mean U_{crit} increased by 19.42

$cm\ s^{-1}$ per unit increase of caudal fin aspect ratio, while it only increased by 5.14 $cm\ s^{-1}$ per unit increase of pectoral fin aspect ratio (β = 19.42 and 5.14; *t* =9.633 and 3.04; P < 0.01; Table 3.8). These results demonstrate that caudal fin and pectoral fin aspect ratio are both strong predictors of U_{crit}. The body shape component (X_{PLS}), which summarises variation in overall body depth, does not provide a useful predictor of critical swimming speed of Australian smelt from river and reservoir habitats, even though there is moderate covariation between these two variables (Table 3.9).

Table 3.8. Model 2 summary table for linear regression of critical swimming speed of Australian smelt from river and reservoir habitats against pectoral fin and caudal fin aspect ratios, including parameter estimates, standard errors (SE), *t*-value which represents the number of standard deviations that the parameter estimate lies from 0, and *P*-values. Effects were considered significant if P-values were less than a critical α = 0.05.

Effect	Estimate	SE	*t*	P
Intercept	3.328	2.149	1.549	0.165
Pectoral fin aspect ratio	5.142	1.694	3.036	< 0.010
Caudal fin aspect ratio	19.421	2.016	9.633	< 0.001

Table 3.9. Mean U_{crit}, pectoral fin ($AR_{pectoral}$) and caudal fin (AR_{caudal}) aspect ratios, and body shape component (X_{PLS}) scores for 5 populations of Australian smelt from river and reservoir habitats. The body shape change column describes the shape change occurring along the shape change axis, from lowest to highest component scores. Habitat types are denoted as "Riv" (river) and "Res" (reservoir).

Population	Sex	U_{crit}	$\bar{x}\ AR_{pectoral}$	$\bar{x}\ AR_{caudal}$	X_{PLS}	Body shape change
Waranga (Res)	F	34.143	1.239	1.239	-0.006	*Shallow, fusiform*
Waranga (Res)	M	34.299	1.262	1.262	-0.003	
Hume (Res)	M	35.120	1.450	1.243	-0.012	
Hume (Res)	F	37.999	1.430	1.409	-0.015	
Kiewa (Riv)	F	42.756	1.757	1.608	0.005	
Ovens (Riv)	F	44.886	1.953	1.706	0.002	
Broken (Riv)	M	45.116	1.932	1.665	0.007	
Ovens (Riv)	M	46.918	1.692	1.777	0.007	
Broken (Riv)	F	49.773	2.133	1.733	0.010	
Kiewa (Riv)	M	50.072	1.660	1.954	0.011	*Deep, robust*

The regression lines and observed mean values of U_{crit} were plotted as a function of pectoral fin and caudal fin aspect ratios for model 2 (Fig. 3.10).

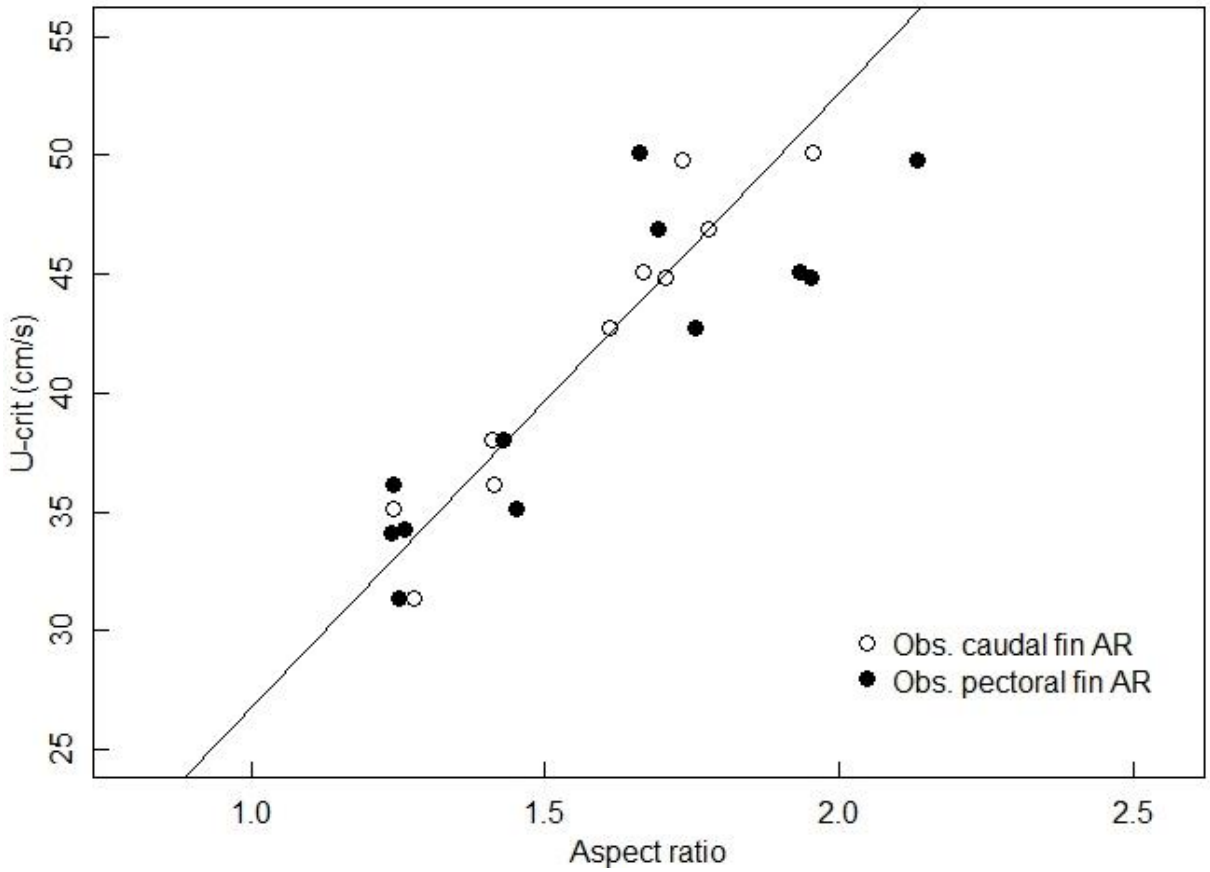

Fig. 3.10. Plot of observed pectoral fin ratios (Obs. pectoral fin AR; filled circles) and caudal fin aspect ratios (Obs. caudal fin AR; empty circles) against U-crit overlaid with fitted linear model 2.

3.4. Discussion

3.4.1. Critical swimming speed and ecological fitness

Results from this chapter support the alternative hypothesis; Australian smelt from river habitats have higher prolonged swimming speed performance than conspecifics living within reservoirs. Prolonged swimming speed was strongly correlated to fin aspect ratios, but not body shape. Standard length significantly covaried with swimming speed performance, which is expected from previous studies illustrating strong allometric relationships in a range of fishes (Beamish 1978; Odell *et al.* 2003; Fulton 2007). However, results of the best subset selection analysis, showed that standard length was in fact, not the strongest predictor of swimming speed performance. Therefore, Australian smelt in rivers may be utilising higher fin aspect ratios than reservoir conspecifics to enhance swimming efficiency. Despite the fact that standard length was not the strongest predictor of prolonged swimming speed performance based on the best subset regression analysis, significant differences in body size of Australian smelt from river and reservoir populations suggest size may contribute to some aspect of swimming performance. In other words, fin aspect ratios have a much stronger

effect on swimming speed performance differences among river and reservoir populations than standard length.

Australian smelt are a planktivorous fish, that inhabit surface waters in both river and reservoir habitats (chapter 1.5). Although foraging success was not directly evaluated for this chapter, it could be argued that the observed significant differences in U_{crit} among populations from river and reservoir habitats may underlie respective foraging strategies. Populations from river habitats may hold station in fast currents, while populations from reservoir habitats may use short bursts to capture suspended food items in open water.

Attributing differences in swimming performance among populations of Australian smelt from river and reservoir habitats to differences in flow velocity was based on the assumption that flow velocity is the primary selective pressure acting on swimming performance. The role of flow velocity in shaping various morphological traits and swimming performance has been repeatedly demonstrated in other species and systems (Langerhans *et al.* 2003; Fulton & Bellwood 2005; Donaldson *et al.* 2013). However, this chapter presents the first evidence of differences in swimming speed performance among river and reservoir populations. This result supports the hypotheses that prolonged swimming speed performance should be greater in fish from river habitats than those from reservoir habitats (e.g. Langerhans 2008; Haas *et al.* 2010; Pavey *et al.* 2010; Franssen 2011). Furthermore, this chapter presents new evidence of the mechanistic link between fin aspect ratios and swimming speed performance at the intra-specific scale.

3.4.2. Relationship between swimming performance, body morphology and fin shape

The variation in U_{crit} among populations of Australian smelt from rivers and reservoirs was strongly correlated with caudal fin and pectoral fin aspect ratios. Langerhans *et al*. (2003) proposed that fish populations from river habitats would have higher prolonged swimming performance than fish populations from reservoir habitats. Langerhans (2008) argued that these differences in swimming performance are attributable to differences in body depth, body stiffness, proportions of red and white muscle and fin aspect ratios. This chapter showed that river populations have higher prolonged swimming speed performance, supporting Langerhans' *et al.* (2003) hypothesis regarding swimming performance. However, based on results from this chapter, the observed differences in swimming speed performance are attributable to differences in caudal fin aspect ratios, but not body shape as suggested by Langerhans *et al.* (2003).

This raises uncertainty over Langerhans (2008) hypothesis that body shape is correlated with swimming performance in river and reservoir populations, and is further

complicated by observations from Franssen (2011), Franssen *et al*. (2013a), Haas *et al*. (2010) and Cureton *et al*. (2014) who presented conflicting evidence for the direction and magnitude of morphological divergence among populations from river and reservoir habitats (see chapter 2.4.1). This is because none of these studies tested swimming performance in the populations for which divergence in body morphology was evaluated. Such inconsistencies suggest that swimming performance is not readily predictable on the basis of body shape variation. Furthermore, the observed variation in body shape in this thesis, based on geometric morphometrics may suggest that the patterns of body shape variation observed among Australian smelt populations from river and reservoir habitats are not large enough to have an effect on swimming performance. Of interest however, may be the variation around the ventral side of the head region and pectoral fins (landmarks 11 and 12), which appear to contribute to the differences in size and depth of the head. While the data in this chapter do not provide any information on this, this variation in head depth may constitute variation in size of the gill arches due to differences in oxygen demand between river and reservoir populations. Studies linking gill morphology and size with head size and habitat preference (Chapman *et al.* 1999; Chapman & Hulen 2001), support this connection, because higher flow velocities may impose high oxygen demand on river populations. However, further investigation for other species such as Australian smelt, is required to confirm this.

While the literature reviewed throughout this chapter does suggest that differences in body shape among fish populations from river and reservoir systems, are highly likely due to the effects of flow velocity, it cannot be ruled out that the patterns observed here are simply not strong enough to be statistically or biologically significant and may only be starting to emerge. The reasons for this however, are beyond the scope of this thesis. More importantly, the present results have shown that fin aspect ratios were significantly different among river and reservoir populations of Australian smelt and that these traits were strongly correlated with prolonged swimming speed performance.

Fin aspect ratios were the strongest predictors of swimming speed performance. Fulton *et al.* (2005) demonstrated that pectoral fin aspect ratio was likely one of the strongest predictors of swimming performance in three families of labriform swimming fishes. Specifically, they reported that fish with tapered pectoral fins (high aspect ratio) were likely to have greater prolonged swimming speed performance, while fish with rounded fins (low aspect ratio), were more likely to have lower prolonged swimming speed performance. This translated to predictable patterns of colonization by labriform swimming fishes in high and low flow velocity habitats (Fulton & Bellwood 2005).

The mean pectoral fin aspect ratios reported in this chapter reflect the same pattern: populations from river and reservoir habitats had high and low pectoral fin aspect ratios, respectively. However, the results presented here suggest that caudal fin aspect ratio was a stronger predictor of prolonged swimming speed performance than pectoral fin aspect ratio. The swimming mode (the way in which fishes use their body and fins to generate propulsion) may provide an adequate explanation for this. Labriid fishes, which are median-paired fin (MPF) swimmers tend to use pectoral fins for propulsion (Fulton & Bellwood 2004), whereas body-caudal fin (BCF) swimmers tend to rely on undulation of their bodies and caudal fin to generate propulsion (Blake 2004). Australian smelt have not been formally described as BCF swimmers. However, their close phylogenetic relationship with the salmonids which are BCF swimmers (Webb 1984a; Blake 2004), and observations of fin movement during the critical swimming speed tests (Fig. 3.11) suggest Australian smelt are also BCF swimmers. Consequently, it is reasonable to conclude, according to the results in this chapter that the caudal fin is a primary propulsive mechanism in Australian smelt.

Figure 3.11 Image of Australian smelt taken during critical swimming speed experiments. Paired (pectoral and pelvic) fins are clearly held flat against the surface of the body.

Though there is literature reporting the importance of the caudal fin aspect ratio as a predictor of swimming speed performance (Sambilay Jr 1990; Vogel 1994; Plaut 2000; Fulton 2010), the role of the caudal fin aspect ratio in the context of morphological and functional divergence among river and reservoir fish populations has not been investigated. The biomechanical basis for higher swimming speed performance of high aspect ratio fins, is that tall, crescent shaped caudal fins, and likewise, tapered pectoral fins, incur less drag per unit of force generated (Vogel 1994; Sfakiotakis *et al.* 1999). Conversely, low fin-aspect ratios are

best suited to accelerating and burst swimming but are less suited to steady swimming as they incur more pressure drag per unit of force generated (Vogel 1994; Sfakiotakis *et al.* 1999; Fulton 2010).

Walker *et al*. (2013) also determined that body fineness (length-depth) ratio was not a strong predictor of swimming performance in fishes that utilized (BCF) swimming mode, whereas it was for those species that utilized median and pectoral fin (MPF) locomotion, This means that the importance of body shape in swimming performance may be conditional on a fishes swimming mode. Based on the results from this chapter and existing literature, the predictions regarding body shape effects on swimming performance by Langerhans (2008) do not appear to apply to divergent populations of Australian smelt. In fact, Langerhans (2008) conceded that these predictions held for only about three quarters of all case studies considered in their meta-analysis. Body shape may therefore not be relevant in understanding functional variation in Australian smelt and in order to find adequate explanations for functional variation in other species, other factors such as fin morphology and physiology, should be central to future research efforts. Finally, McGuigan *et al*. (2003) reported that although body shape may not have functional importance for swimming speed performance in river and naturally occurring lake populations of rainbow fish (*Melanotaenia* spp.), physiological traits such as muscle proportions or aerobic metabolism may be significant predictors of swimming performance. The interactions between swimming performance and metabolic capacity however, have not been explored in the context of divergent river and reservoir fish populations.

3.5. Conclusion

The results from this chapter demonstrate that fin shape, not body morphology, is the best predictor of prolonged swimming speed performance in Australian smelt. Combined with the inconsistency of observed body shape variation in the literature, the results from the present chapter place considerable doubt over the importance of body shape as a factor underlying intra-specific variation in swimming speed performance of species that utilize a BCF swimming mode. Surprisingly, the role of paired (pectoral and pelvic), median (ventral and dorsal) and caudal fins has been overlooked in studies of divergent river and reservoir populations. The evidence presented in this chapter suggests that variation in swimming speed performance must be viewed as a function of a system of locomotor mechanisms (i.e. fins and swimming mode) that facilitate optimal movement of fishes, not only body morphology.

The present chapter has provided new evidence that fish populations may have partly adapted to differences in flow velocity in river and reservoir habitats due to the demands of

living in flowing water. Physiological traits such as metabolic oxygen demand and muscle composition need to be assessed in conjunction with morphological traits to evaluate their relative contributions to swimming performance.

Chapter 4. Size-specific physiological differences among river and reservoir fish populations

4.1. Introduction

Aquatic ecosystems present a diversity of flow velocity habitats that fish must adapt to in order to survive. Because swimming is a pivotal trait in overcoming the challenges associated with flow velocity (e.g. Fulton 2010), the diverse lifestyles and habitat requirements of fish have driven the evolution of a wide range of swimming performances (Langerhans & Reznick 2010). As such, the adaptation of fishes to different habitats requires fish to achieve some balance between maximum swimming performance and the physiological cost it incurs (Claireaux & Lefrançois 2007). Therefore, populations from river and reservoir habitats can be expected to adapt physiologically, to achieve optimal swimming efficiency (Langerhans 2008).

Swimming is the most physically demanding activity for fish and is often limited by physiological traits such as oxygen consumption rate and associated organs and cellular processes (Brett & Groves 1979). Many physiological traits such as metabolic enzyme activity, haematology, muscle structure, heart size (Franklin & Davie 1992; Thorarensen *et al.* 1996) and gill morphology (e.g. Chapman *et al.* 1999) are known to facilitate oxygen supply important to prolonged swimming activity (Jones & Randall 1978). While the mechanistic link between activity levels of metabolic enzymes, heart size, gill size, red blood cells and swimming performance can provide insight into the aerobic capacity of the whole organism during swimming, this relationship has never been explored among populations separated by dams. Consequently, no evidence exists to suggest how physiological traits such as gill structure, heart size and metabolic enzyme activity influence variation in swimming performance among populations from river and reservoir habitats.

Variation in the activity levels of metabolic enzymes such as citrate synthase (CS), hexokinase (HK), β-hydroxyl CoA dehydrogenase (HOAD) and cytochrome C oxidase (CCO) in red and white skeletal muscle has been linked to intra-specific variation in swimming performance (Johnston & Moon 1980; Childress & Somero 1990; Farrell *et al.* 1990; Martinez *et al.* 2003). But these investigations considered only inter-individual variation. At the population level, Patterson *et al.* (2004) showed that intra-specific differences in CS, CCO and LDH activity in sockeye salmon (*Oncorhyncus nerka*) fry originating from different river catchments, caused the differences observed in swimming performance among their respective populations. In addition to the importance of metabolic enzymes in generating energy to power muscles involved in locomotion, the gills and heart

(Randall 1982) are important determinants of swimming performance, due to their role in oxygen uptake and distribution. Though variation in cardio-vascular organs and metabolic enzyme activity has been studied in populations from different catchments, this has not been extended to comparisons of populations from river and reservoir habitats.

Gill structure, including surface area and mass, have been important traits in the study of local adaptation of fishes and there is considerable evidence of intra-specific variation (Palzenberger & Pohla 1992). For example, several studies have shown that fish populations inhabiting hypoxic (low oxygen) environments such as swamps, have considerably higher gill surface area than fish populations from well oxygenated stream environments (Chapman & Hulen 2001; Schaack & Chapman 2003). Population-specific differences in gill surface area can be maintained by selective pressures imposed by strong dissolved oxygen gradients (Chapman *et al.* 1999; Schaack & Chapman 2003) and can be a predictor of maximum oxygen consumption rate during both burst and prolonged swimming activity (Moyes 2003; Odell *et al.* 2003). Likewise, enhanced swimming performance arising from exercise training has been attributed to greater heart mass (Hochachka 1961), although some have suggested that heart mass may be related to sex-specific swimming behaviour, rather than habitat type (Franklin & Davie 1992; Kolok 1992; Thorarensen *et al.* 1996).

Clearly, there is a mechanistic link between metabolic enzyme activity levels, heart size, gill size and swimming performance in fishes but relationships among these variables have not been compared systematically among populations separated by dams. Consequently, there is currently no empirical evidence to support hypotheses suggesting a physiological basis for observed differences in swimming capacity commonly observed among river and reservoir populations (chapter 3). Here, I investigate how fish populations adapt to different flow velocity habitats by testing the following hypothesis:

H_0: There are no differences in citrate synthase (CS) activity, heart mass and gill mass among populations of fish from river and reservoir habitats;

H_a: River populations will have significantly different citrate synthase (CS) activity, heart mass and/or gill mass from reservoir populations of fish.

4.2. Methods

4.2.1. Sampling

Australian smelt were collected from three river and two reservoir populations across the southern Murray-Darling Basin (chapter 1.2) between June and July 2016 using a 10 m (length) x 1.8 m (drop) fine mesh (3 mm diagonal) seine net. Insufficient individuals were sampled from Lake Nillahcootie (third reservoir) so it was not included in any analyses in this chapter. Sampled fish were handled and transported to holding aquaria at the laboratory, in the same manner described in chapter 3.2.

4.2.2. Fish husbandry and feeding

Fish were maintained in holding aquaria in a controlled temperature room and fed following the protocol described in chapter 3.2.2. Aquarium heaters (100 watt) used to maintain water temperatures in holding aquaria at ~20^{o}C (Appendix 7) were randomly distributed throughout half of the holding aquaria, with 3 additional heaters in the sump. Fish were introduced to holding aquaria by gradually adjusting temperature and pH conditions and river or reservoir water was used to fill the holding aquaria over a period of 6-8 hours to allow fish to acclimatize to experimental temperatures (see chapter 3.2.2).

Fish were allowed to acclimate to conditions in the holding aquaria and movement of the investigator in the room for a total of 48-72 hours, after which feeding was commenced. This also ensured that fish were able to recover from stress associated with sampling to avoid confounding experimental results. If fish did not commence feeding after the initial acclimatization period, feeding was attempted again at 6-hour increments until feeding behaviour was observed. All fish were fed twice daily to satiation with frozen brine shrimp. Mortalities were removed and recorded, and water quality measurements were taken after feeding, to avoid causing stress.

4.2.3. Sex and maturity assessment

Maturity and sex were identified for all fish used in swimming performance experiments as per chapter 2.2.2. Only clearly identified adult male and female Australian smelt with developed gonads were used in the experiments due to difficulty in identifying immature or juvenile individuals as per chapter 2.

4.2.4. Experimental design

A control sample of 8 Australian smelt from each river and reservoir population was collected to measure heart mass, gill mass and to obtain muscle tissue for CS assays prior to critical

swimming speed (U_{crit}) tests (see chapter 4.2.5). A separate sample of 15 Australian smelt from each river and reservoir population was assessed for critical swimming speed. The control sample was required to ensure that stress associated with handling prior to U_{crit} tests did not confound relative differences among river and reservoir fish.

The samples for the U_{crit} tests are referred to as 'trial' samples herein. Muscle tissue was collected from 7 of these 15 individuals and heart and gill mass were measured for the remaining 8 individuals from each river and reservoir population. Thus, in both the trial and control groups, river and reservoir habitat types were represented by at least 21 and 14 individuals, respectively, for CS activity, heart mass and gill mass. These sample sizes have been shown to be sufficient for evaluating physiological trait variation (Martinez *et al.* 2003), which minimized use of animals to comply with animal ethics approvals (see Animal ethics permit numbers, pg. i).

4.2.5 Critical swimming speed

Prolonged swimming speed performance (defined in chapter 3.2.3) was investigated for Australian smelt via measurements of critical swimming speed (U_{crit}) to evaluate the effect of CS activity, heart mass and gill mass on prolonged swimming speed performance in river and reservoir populations. An incremental method was used to measure U_{crit} in 15 individuals from each river and reservoir population of Australian smelt. A recirculating flow tank (dimensions and calibration described in chapter 3.2.3) was employed for U_{crit} tests in this chapter. Flow was regulated by rotating a 40-mm ball valve against a protractor that provided a calibrated measure of flow velocity. Temperature was maintained in the swim tunnel at ~20 ^{o}C (Appendix 7) via a recirculating sump and Hailea HC-250A water chilling unit. The U_{crit} test, a standard method for measuring prolonged swimming speed performance relevant to daily patterns of habitat use (Plaut 2001; Fulton 2010) involved an incremental test procedure (see chapter 3.2.3 for details).

Prior to commencing U_{crit} tests, fish were starved for 24 hours to ensure that food remaining in the gut or ongoing digestive processes did not confound performance (Garland & Arnold 1983). Given the fragility of this species to handling, individuals were anaesthetized as per chapter 3.2.3 for measurement and transfer to the flow tank. Recovery time for all individuals was standardized to ensure that anaesthesia did not cause any bias in the comparison of U_{crit} among river and reservoir populations. Standard length of each anaesthetized fish (nearest 0.1 mm) and mass (nearest 0.001g) were measured. Individuals were then introduced into the swim tunnel within 45 seconds and then left to recover with supplementary aeration for at least 30 minutes. While efforts were made to ensure that all fish

from river and reservoir populations collected in the field for U_{crit} tests were of a uniform size, the fish that were collected could not be measured and selectively sampled in the field, due to the fragility of the species to handling. Therefore, smelt collected from river and reservoir populations for U_{crit} tests may have differed in mean standard length due to random sampling error.

Each U_{crit} test was commenced with an initial acclimation velocity of approximately 2.5 $cm\ s^{-1}$ (minimum flow velocity that could be set in the flume) for at least 30 minutes, then the flow velocity was increased in 3.5 $cm\ s^{-1}$ (~ 1 standard length $s^{-1}$for individuals of 30-40 mm standard length) increments every 5 minutes until the test individuals reached exhaustion (indicated by resting on grid at the downstream end of the swim chamber). Velocity increments equivalent to approximately 1 body lengths s^{-1} and time-steps between velocity increases as low as 5 minutes have been found to provide a reliable measure of prolonged swimming speed performance (e.g. Plaut 2000; Yan *et al.* 2012) at which optimal U_{crit} values are achieved and measured (Farlinger & Beamish 1977). Individuals which failed to correctly orient against the direction of flow or swim against the current were removed and euthanized using an overdose of at least 100 $mg\ L^{-1}$ (0.01%) of benzocaine. Standard length was measured for fish in control and trial samples and all individuals were sexed macroscopically by checking for presence of gonads and gametes (chapter 2.2.2).

4.2.6. Tissue sampling and metabolic enzyme activity

From the individual U_{crit} tests, 7 individuals were randomly chosen to be assayed for CS. Citrate synthase is the enzyme that catalyses the first reaction (Fig. 4.1) in the citric acid cycle (Krebs' cycle) where energy is generated through the formation of adenosine triphosphate (ATP) molecules within the mitochondria of skeletal muscle cells (Wiegand & Remington 1986). Activity levels of CS have been shown to be a key indicator of aerobic performance, due to its correlation with oxygen consumption rate and mitochondrial density (Childress & Somero 1990; Somero & Childress 1990; Gibb & Dickson 2002). Consequently, CS was chosen as the indicator of differences in swimming speed performance between river and reservoir populations of Australian smelt, which are active, pelagic swimmers (Lintermans 2007) across most environments. Anaerobic enzymes were not measured because anaerobic metabolism was not in the scope of the research question, as it is associated with sprint swimming rather than prolonged swimming.

At the conclusion of each U_{crit} test, each individual fish was euthanized using an overdose of at least 100 $mg\ L^{-1}$ (0.01%) of benzocaine. The effect of anaesthesia on metabolic enzyme activity was unknown, however there have been no reported effects of

anaesthesia on metabolic enzyme activity in fish. Thus, individuals were handled similarly so that any relative differences among the control groups, trial groups and the two habitat types were maintained.

Citrate Synthase:

a. Oxaloacetate + acetyl CoA ⟶ citrate + CoA ● SH

b. CoA ● SH + DTNB ⟶ CoA + S-S-nitrobenzoate + thionitrobenzoate anion

Fig. 4.1. Formula depicting the reaction between oxaloacetate and acetyl coenzyme-A that is catalyzed by citrate synthase (a), and a subsequent reaction between coenzyme-A and dithionitrobenzoate (DTNB) (b) which results in the formation of thionitrobenzoate anions (boxed) which is measured by absorption in a spectrophotometer at wavelength of 412 nm. Figured modified from Frazier and Thorburn (2012).

Death was indicated by lack of movement in the opercula for at least 30 seconds. The entire lateral muscle tissue was then removed from both sides of the individual by running a scalpel along the backbone from the posterior margin of the opercula, to the end of the caudal peduncle. Because red, pink and white muscle types could not be distinguished visually, the entire skeletal muscle was sampled for the CS assays. Each piece of lateral muscle was immediately weighed on a micro-balance and wrapped in aluminium foil. Both pieces of muscle from the individual were inserted into a 2.5 ml cryogenic vial and plunged into dry-ice pellets to reduce the temperature of the samples to –70 ^{o}C. The entire process from removing the muscle tissue from an individual to placing the sample on dry ice was completed within 90 seconds. Samples were later transferred to a cryogenic freezer for long term storage at – 70^{o}C. An additional 7 individuals were randomly sampled from holding aquaria for each population, for collecting muscle tissue to be used as controls for the CS assays. The same protocol was followed, except that individuals were euthanized directly after removal from holding aquaria instead of preparing for U_{crit} tests.

Muscle tissue samples were sent to the mitochondrial lab at the Murdoch Children's Research Institute in Melbourne, Australia for CS assays. The CS assays were performed using kinetic spectrophotometry following the general protocol described in Frazier and Thorburn (2012). The protocol used for the assays is summarized here with details of reagent volumes and reaction times used:

1) Whole lateral muscle fillets from one side of each individual (45.5 – 234.6 mg) were homogenized in 10x volume of tissue lysis buffer (Zheng's buffer) using a chilled

glass homogenizer, then transferred to a chilled Eppendorf tube and centrifuged at 600 RCF (Relative Centrifugal Force).

2) Supernatant (liquid remaining at the surface of sample following centrifugation) was decanted, frozen and rethawed 3 times, sonicated and diluted at a ratio of 1:10 with Zheng's buffer.
3) One millilitre of CS assay buffer (50 mM KPi, pH 7.4, 0.1 mM 5,5 ¢ -dithio-bis-(2-nitrobenzoic acid) (DTNB)) was added to duplicate quartz cuvettes and equilibrated to 20°C (temperature at which U_{crit} tests were performed), followed by 2-10 µL of supernatant (depending on size of original tissue sample and volume of Zheng's buffer used for dilution). NB: KPi is potassium phosphate buffer.
4) The reaction was started by adding acetyl-CoA to a concentration of 0.1 mMol and mixing the cuvettes.
5) Linear reaction rates (linear change in absorption rate) were determined by measuring absorption of thionitrobenzoate anions at 412 nm for approximately 3 minutes (see Fig. 4.1).
6) Reaction rates (activity) of CS in nmol/min/mg were calculated as follows:

$$\text{CS (nmol/min/mg)} = ((\Delta A \times (\varepsilon \times l)^{-1}) \times V) \times c^{-1}$$

where ΔA is the change in absorbance per minute, ε is the extinction coefficient (13.6) for CS, l is the path length (width in mm) of the cuvette, V is the volume of supernatant containing the target enzyme (ml), and c is the calculated concentration of protein (tissue sample) in the supernatant (mg) for which the reaction rate was being calculated.

4.2.7. Heart and gill mass measurement

From the U_{crit} tests for each population, the remaining 8 individuals not used for CS assays were used for heart and gill mass measurements. After each individual was euthanized, the heart was dissected from behind the gill arches. Care was taken to ensure that the entire heart mass (sinus venosus, atrium, ventricle and bulbous arteriosus) was retained following Hochachka (1961). After excising the heart from the pericardial cavity, it was quickly blotted dry to remove any remaining blood or mucus and was immediately placed on pre-weighed aluminium foil and weighed to the nearest 0.001mg. Similarly, the gill structure was also removed, by excising individual gill arches (four on each side) where they connected with the surface of the buccal (mouth) cavity. Gill mass was chosen as a proxy for gill surface area,

because it has been demonstrated to be a significant predictor of variation in swimming performance (Martinez *et al.* 2003; Odell *et al.* 2003). Following Kultz and Somero (1995), gill arches were rinsed in medical saline solution, to remove as much remaining blood and mucus as possible, then quickly blotted, immediately placed on pre-weighed aluminium foil and weighed to the nearest 0.001 mg. Gills could not be perfused (flushed) with saline solution due to their small size and fragility, thus rinsing was the most appropriate method for minimizing excess fluids in the gills for accurate weighing.

4.2.8. Statistical analysis

A non-parametric ANOVA was used to assess variation in CS activity, heart mass and gill mass, standard length and raw U_{crit} values attributable to habitat, location, control or trial group, and sex, using the *vegan* package (v2.4.2; Oksanen *et al.* 2007) for R (R Core Team 2015), as per chapter 3.2.6.

CS activity, heart mass and gill mass were analysed with standard length as a covariate to determine if any differences in response variables among river and reservoir fish populations were independent of size. Size variation among groups (river, reservoir, trial and control) was evaluated using a non-parametric ANOVA following the same procedures described in chapter 3.2.6. Variation in the dependent variables (DV; U_{crit}, heart mass, gill mass, CS activity) was quantified by computing the sum of squared (SS) residuals and partitioning the total model SS across factors, to reduce residual or unexplained error in the specified model:

$$DV \sim habitat\ (population) \times sex \times control + standard\ length$$

Where DV was the dependent variable, habitat was a fixed factor (defined as a river or reservoir), sex and control (defined as control or trial group) were fixed factors, population was a random factor and standard length was a covariate. Expected mean squares were determined following the same rules as described in chapter 2.2.4.

The effect and statistical significance of each factor on the dependent variables was assessed using a randomized residual permutation procedure (RRPP) as described by Anderson (2001a) and in chapter 2.2.4. This was repeated for 999 permutations to generate the distribution of the pseudo *F*-statistic (Anderson, 2001a), from which *P*-values could be computed for each factor. This value provided the probability that *SS* between habitats, populations and sex, is greater than the SS among specimens within each habitat, population and sex, purely by chance (α). Summary tables were provided containing the *Z*-scores

(standard deviations between groups and mean of the sampling distribution) indicating effect size based on the *F*-distribution that was generated, as well as the *F*- and *P*-values for each factor.

4.3. Results

4.3.1. Population demographics and data summary

Fish standard length (*SL*) was not significantly different between habitats (F = 1.499; P = 0.220; Table 4.1) in control samples, but was significantly different among some populations from each habitat type (F = 3.324; P < 0.05; Table 4.1). Standard length for trial groups was also significantly different between habitats (F = 23.993; P < 0.05; Table 4.2). Sums of squares (SS) for the habitat effect in the trial samples constituted 25.6% of the total variation in standard length ($SS_{habitat}/SS_{total}$ = 0.256; Table 4.2). Sex ratios and size class distributions were plotted for river and reservoir habitats (Fig. 4.2).

Table 4.1 Non-parametric ANOVA statistics for nested model of standard length in control samples of river and reservoir populations of Australian smelt as a function of habitat, sex (fixed effects) and population (random effect). *F* - values were calculated using a randomized residual permutation procedure (RRPP). Effect size (*Z* – scores) represented the standard deviations between group means for each effect. Effects were considered significant if *P*-values were less than a critical α = 0.05.

Effect	Df	SS	MS	F	Z	P
Habitat	1	26.510	26.508	1.499	0.237	0.220
Sex	1	56.130	56.129	3.174	1.343	0.088
Habitat × population	3	176.310	58.771	3.324	2.243	< 0.05
Population × sex	1	7.780	7.779	0.440	-0.414	0.529
Habitat × population × sex	3	32.110	10.703	0.605	-0.528	0.644
Residuals	25	442.000	17.680			
Total	34	740.830				

Table 4.2 Non-parametric ANOVA statistics for nested model of standard length in trial samples of river and reservoir populations of Australian smelt as a function of habitat, sex (fixed effects) and population (random effect). *F* - values were calculated using a randomized residual permutation procedure (RRPP). Effect size (*Z* – scores) represented the standard deviations between group means for each effect. Effects were considered significant if *P*-values were less than a critical $\alpha = 0.05$

Effect	Df	SS	MS	F	Z	P
Habitat	1	504.390	504.390	23.993	14.916	< 0.05
Sex	1	0.140	0.140	0.006	0.721	0.941
Habitat × population	3	84.950	28.320	1.346	0.302	0.290
Population × sex	1	0.060	0.060	0.002	0.686	0.964
Habitat × population × sex	3	11.20	3.730	0.177	0.996	0.906
Residuals	65	1366.420	21.020			
Total	74	1967.150				

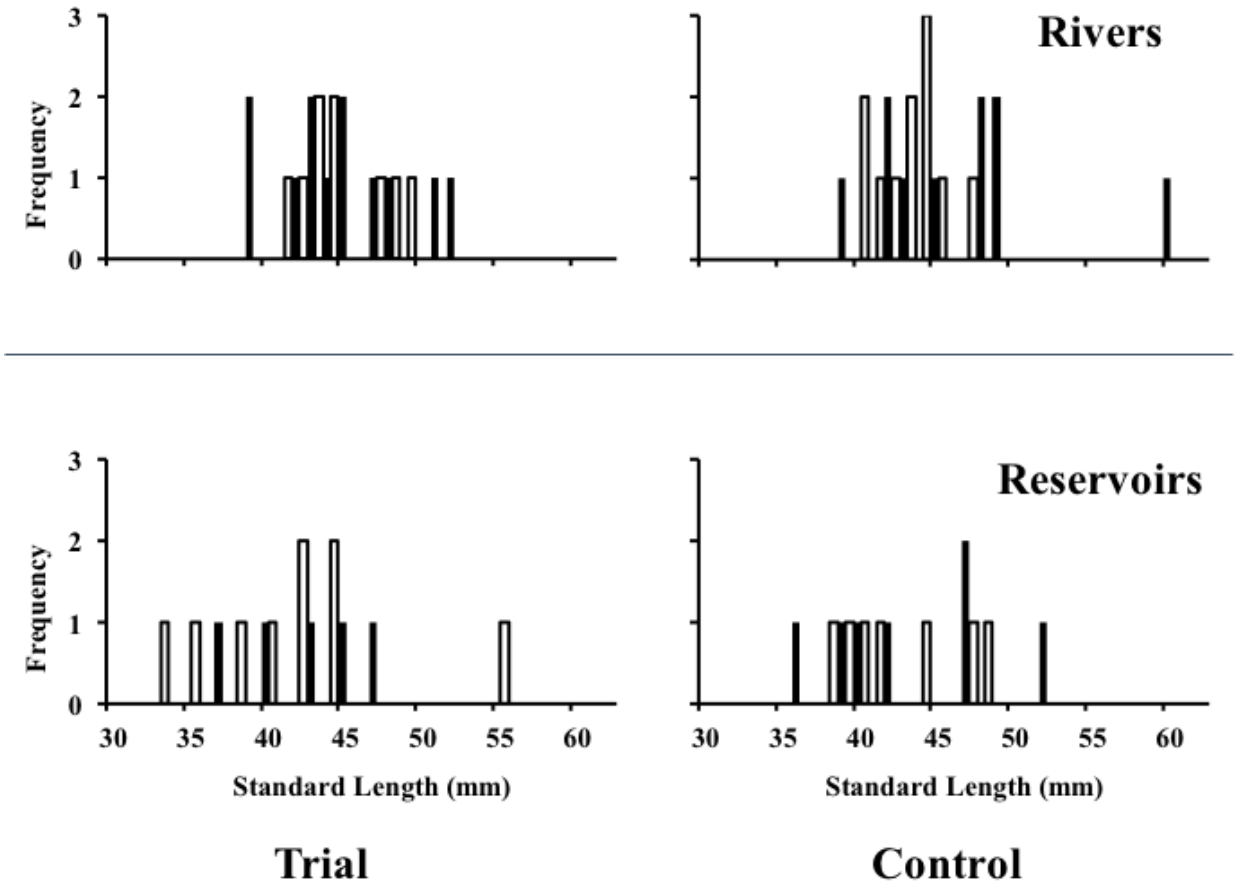

Fig. 4.2. Size class distribution based on standard length (SL) of Australian smelt control (right panels) and trial (left panels) samples from river and reservoir habitats. Data is for clearly identified adult individuals. Due to difficulties associated with identifying sex in juveniles in chapter 2.3.1., no juvenile individuals were used in the experiments in this chapter. Solid bars are males, empty bars are females. Sample size (*n*) = River males (trial), 12; River females (trial), 9; River male (control), 10; River female (control), 11; Reservoir males (trial), 6; Reservoir females (trial), 9; Reservoir males (control), 7; Reservoir females (control), 7.

4.3.2. Assessment of variation in swimming performance

The non-parametric ANOVA for U_{crit} data of the trial groups of Australian smelt, indicate that habitat and sex were significant factors ($F = 73.778; P < 0.001$ and $F = 5.116; P < 0.001$ respectively; Table 4.3). Standard length was not a significant predictor ($F = 0.232; P = 0.645$; Table 4.3). This was unexpected because swimming speed is known to scale with standard length (chapter 3.3.2). Sex was a significant factor (F = 5.116; P < 0.001; Table 4.3), however it did not contribute to differences in U_{crit} among river and reservoir populations with marginal effect size relative to habitat (Z = 2.470 and 42.605 respectively; Table 4.3). Mean U_{crit} plots with error bars (Fig. 4.3) reflect significant factors (habitat and sex) for trial samples of Australian smelt from river and reservoir habitats.

Table 4.3. Non-parametric ANOVA statistics for nested factorial model of critical swimming speed in trial samples of river and reservoir populations of Australian smelt as a function of habitat, sex (fixed effects), population (random effect) and standard length as a covariate. *F* - values were calculated using a randomized residual permutation procedure (RRPP). Effect size (*Z* – scores) represented the standard deviations between group means for each effect. Effects were considered significant if *P*-values were less than a critical α = 0.05

Effect	Df	SS	MS	F	Z	P
Habitat	1	1580.540	1580.540	73.778	42.605	< 0.001
Sex	1	109.600	109.600	5.116	2.470	< 0.001
Standard length	1	4.980	4.980	0.232	0.557	0.645
Habitat × population	3	69.600	23.180	1.082	0.001	0.375
Population × sex	1	20.300	20.290	0.947	0.079	0.350
Habitat × population × sex	3	143.400	47.820	2.232	1.339	0.107
Residuals	64	1371.100	21.420		0.415	
Total	74	3299.500				

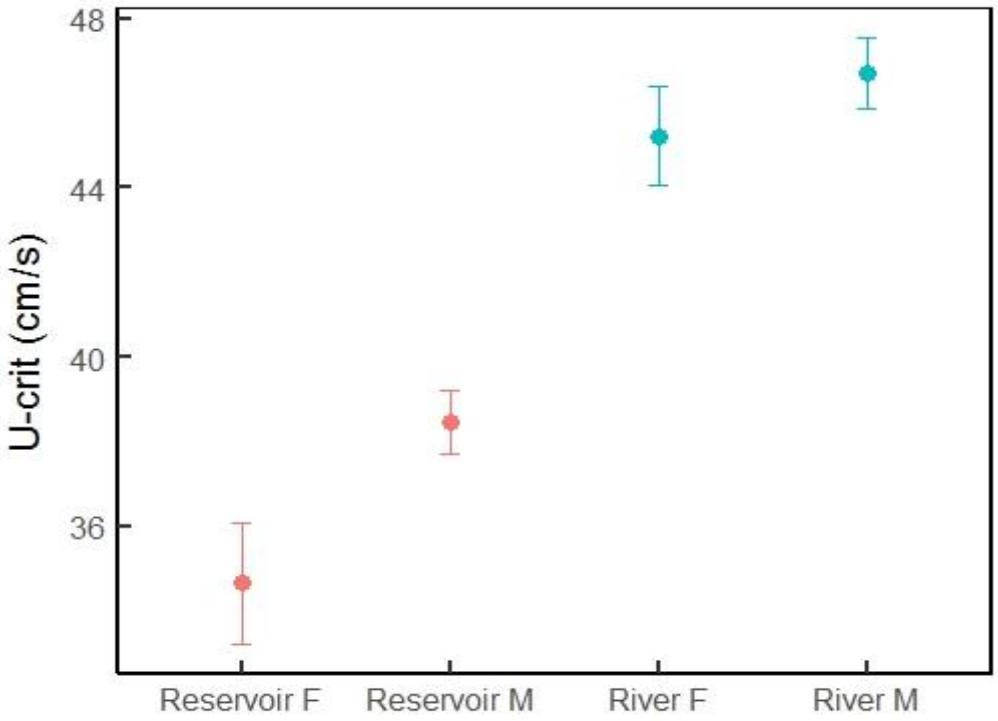

Fig. 4.3. Mean critical swimming speed (U_{crit}) for trial samples of male and female Australian smelt from reservoir (orange circles) and river populations (blue circles) used for citrate synthase (CS) assays. Sample sizes (*n*) were: River = 3; Reservoir = 2. Error bars are 1 standard error around the mean.

4.3.3. Citrate synthase assays

Citrate synthase activity was significantly different among Australian smelt populations from river and reservoir habitats (F = 57.183; P < 0.001; Table 4.4), but due to the habitat × population effect (F = 18.541; P < 0.001; Table 4.4) differences in CS activity may not be attributable to all populations. The effect size for habitat was nearly twice that of the habitat × population effect (Z = 38.620 and 20.451 respectively; Table 4.4) indicating that differences in CS activity between habitats was nearly double the differences among populations. Overall, these results indicate that CS activity is significantly different between river populations and reservoir conspecifics of Australian smelt despite size differences among them.

Table 4.4. Non-parametric ANOVA statistics for nested factorial model of citrate synthase (CS) activity in river and reservoir populations of Australian smelt as a function of habitat, sex (fixed effects), population (random effect) and control or trial sample, with standard length as a covariate. *F* -values were calculated using a randomized residual permutation procedure (RRPP). Effect size (*Z* – scores) represented the standard deviations between group means for each effect. Effects were considered significant if *P*-values were less than a critical α = 0.05.

Effect	Df	SS	MS	F	Z	P
Habitat	1	36527	36527	57.183	38.620	< 0.001
Sex	1	545	545	0.853	0.138	0.376
Control	1	11	11	0.018	0.650	0.888
Standard length	1	12857	12857	20.128	12.560	< 0.010
Habitat × population	3	35358	11786	18.451	20.451	< 0.001
Habitat × sex	1	21	21	0.032	0.684	0.859
Habitat × control	1	6	6	0.009	0.695	0.919
Sex × control	1	10	10	0.016	0.692	0.898
Habitat × population × sex	3	759	253	0.396	0.703	0.744
Habitat × population × control	3	1034	345	0.539	0.553	0.672
Habitat × sex × control	1	2609	2609	4.085	1.636	< 0.01
Habitat × population × sex × control	3	1450	483	0.757	0.299	0.532
Residuals	49	31300	639			
Total	69	122488				

There was no significant difference in CS activity between control and trial Australian smelt samples (F = 0.018; P = 0.888; Table 4.4). Thus, there was no significant effect of handling-related stress on CS activity. There were some sex and habitat-specific differences between control and trial samples of Australian smelt (F = 4.085; P < 0.01; Table 4.4) but the effect size was relatively small compared to the other effects (Z = 1.636; Table 4.4). Standard length was a significant predictor of CS activity (F = 20.128; P < 0.01; Table 4.4), but the effect size of standard length was less than a third of the habitat effect size (Z = 12.56 and 38.62, respectively; Table 4.4). Means and error bars of CS activity were plotted for Australian smelt populations and sexes to reflect significant factors with the largest effect sizes (Fig. 4.4).

Citrate synthase activity in Australian smelt from Broken river and Kiewa river populations was significantly different to that of Australian smelt from Lake Hume and Waranga Basin (Fig. 4.4). However, CS activity was not significantly different between Lake Hume (LH), Waranga basin (WB) and Ovens river (OR) populations (Fig. 4.4), accounting for the significant habitat × population interaction (Table 4.4). Overall, CS activity among most of the river populations of Australian smelt were greater than the reservoir populations.

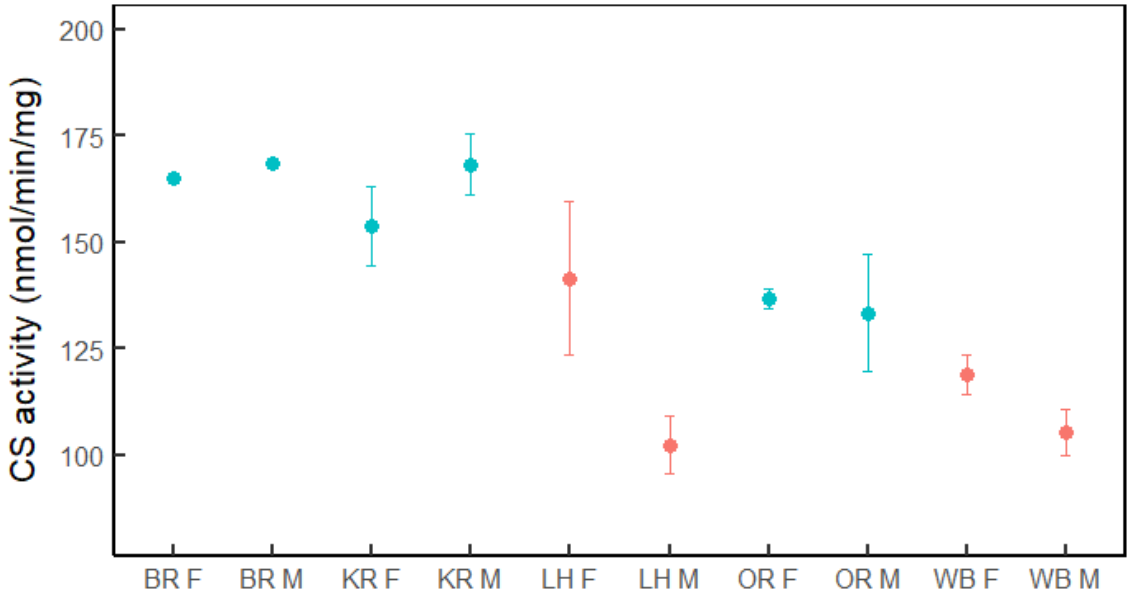

Fig. 4.4. Mean citrate synthase (CS) activity (nmol/min/mg) for trial Australian smelt from river (blue circles) and reservoir (orange circles) habitats. On the *x*-axis, 'F' indicates females, 'M' indicates males; population names indicated by abbreviations were; BR – Broken river, KR – Kiewa river, LH – Lake Hume, OR – Ovens river and WB – Waranga basin. Fish from Lake Nillahcootie were not assayed for CS activity due to insufficient sample size. Error bars indicate 1 standard error around the mean. Units of measurement for CS activity were reported as nanomoles (nmol) of citrate synthase required to precipitate formation of thionitrobenzoate anions in the first reaction of the Kreb's cycle, per minute per milligram of protein (muscle tissue).

4.3.4. Assessment of variation in heart mass

Heart mass was not significantly different for habitat, habitat × population and control effects (F = 0.021, 1.284 and 0.727 respectively; P = 0.887, 0.292 and 0.410 respectively; Table 4.5). There were however, sex-specific differences in heart mass (F = 16.458; P < 0.001; Table 4.5). Standard length was a significant predictor of variation in heart mass (F = 33.241; P < 0.001; Table 4.5). Despite the effect of standard length, heart mass was still significantly different between sexes.

Table 4.5. Non-parametric ANOVA statistics for nested factorial model of heart mass in river and reservoir populations of Australian smelt as a function of habitat, sex (fixed effects), population (random effect), control or trial sample and standard length as a covariate. *F* - values were calculated using a randomized residual permutation procedure (RRPP). Effect size (*Z* – scores) represented the standard deviations between group means for each effect. Effects were considered significant if *P*-values were less than a critical $\alpha = 0.05$.

Effect	Df	SS	MS	F	Z	P
Habitat	1	1.170^{-9}	1.170^{-9}	0.021	0.673	0.877
Sex	1	9.340^{-7}	9.340^{-7}	16.458	10.079	< 0.001
Control	1	4.130^{-8}	4.130^{-8}	0.727	0.202	0.41
Standard length	1	1.890^{-6}	1.890^{-6}	33.241	20.932	< 0.001
Habitat × population	1	2.190^{-7}	7.290^{-8}	1.284	0.313	0.292
Habitat × sex	1	2.700^{-9}	2.690^{-9}	0.047	0.645	0.821
Habitat × control	1	1.470^{-8}	1.470^{-8}	0.258	0.472	0.614
Sex × control	1	5.500^{-8}	5.510^{-8}	0.970	0.069	0.316
Habitat × population × sex	3	2.320^{-8}	7.720^{-9}	0.136	1.055	0.952
Habitat × population × control	3	2.310^{-7}	7.680^{-8}	1.354	0.250	0.269
Habitat × sex × control	1	6.700^{-9}	6.660^{-9}	0.117	0.620	0.759
Habitat × location × sex × control	3	4.280^{-8}	1.430^{-8}	0.252	0.922	0.86
Residuals	49	2.780^{-6}	5.680^{-8}			
Total	69	6.240^{-6}				

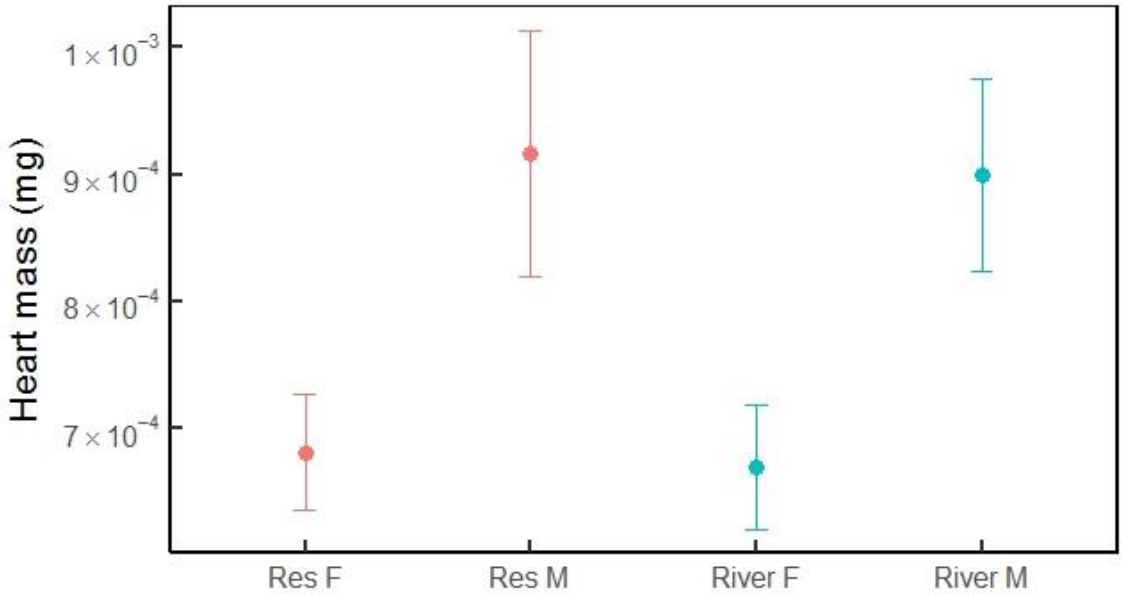

Fig. 4.5. Mean heart mass in milligrams, for male and female Australian smelt from river (blue circles) and reservoir (orange circles) habitats. Sample means (*n*) = 3 (River males and females); 2 (Reservoir males and females). Error bars indicate 1 standard error around the mean.

Mean heart mass was the same among Australian smelt of the same sex from both habitat types however, the differences between male and female heart mass, was consistent across

river and reservoir habitats (Fig. 4.5). Male Australian smelt heart mass was on about 30% higher on average, than for female Australian smelt (Fig. 4.5).

4.3.5. Assessment of variation in gill mass

Gill mass was significantly different among Australian smelt from river and reservoir habitats (F = 16.498; P < 0.001; Table 4.6). No significant differences in gill mass were observed for control and trial groups (F = 0.003; P = 0.961; Table 4.6) indicating that the effects of handling prior to U_{crit} tests had no effect on gill mass measurements. Standard length was a significant predictor of gill mass (F = 47.537; P < 0.05; Table 4.6). River populations had higher gill mass than reservoir populations (Fig. 4.6) indicating that despite size differences among Australian smelt from river and reservoir habitats, relative gill mass was still significantly different among them.

Table 4.6. Non-parametric ANOVA statistics for nested factorial model of gill mass in river and reservoir populations of Australian smelt as a function of habitat, sex (fixed effects), population (random effect), control or trial sample and standard length as a covariate. F - values were calculated using a randomized residual permutation procedure (RRPP). Effect size (Z – scores) represented the standard deviations between group means for each effect. Effects were considered significant if P-values were less than a critical α = 0.05.

Effect	Df	SS	MS	F	Z	P
Habitat	1	9.986^{-5}	9.986^{-5}	16.498	9.776	< 0.001
Sex	1	1.713^{-5}	1.713^{-5}	2.831	1.028	0.103
Control	1	2.100^{-8}	2.100^{-8}	0.003	0.750	0.961
Standard length	1	2.877^{-4}	2.877^{-4}	47.537	35.564	< 0.050
Habitat × population	3	1.497^{-5}	4.991^{-6}	0.825	0.203	0.475
Habitat × sex	1	7.997^{-6}	7.997^{-6}	1.321	0.222	0.240
Habitat × control	1	1.875^{-6}	1.875^{-6}	0.310	0.479	0.570
Sex × control	1	3.350^{-7}	3.350^{-7}	0.055	0.640	0.828
Habitat × population × sex	3	8.310^{-7}	2.770^{-7}	0.046	1.076	0.985
Habitat × population × control	3	1.104^{-5}	3.681^{-6}	0.608	0.508	0.629
Habitat × sex × control	1	2.600^{-8}	2.600^{-8}	0.004	0.716	0.954
Habitat × location × sex × control	3	1.980^{-5}	6.584^{-6}	1.088	0.043	0.403
Residuals	49	2.970^{-4}	6.053^{-6}			
Total	69	7.582^{-4}				

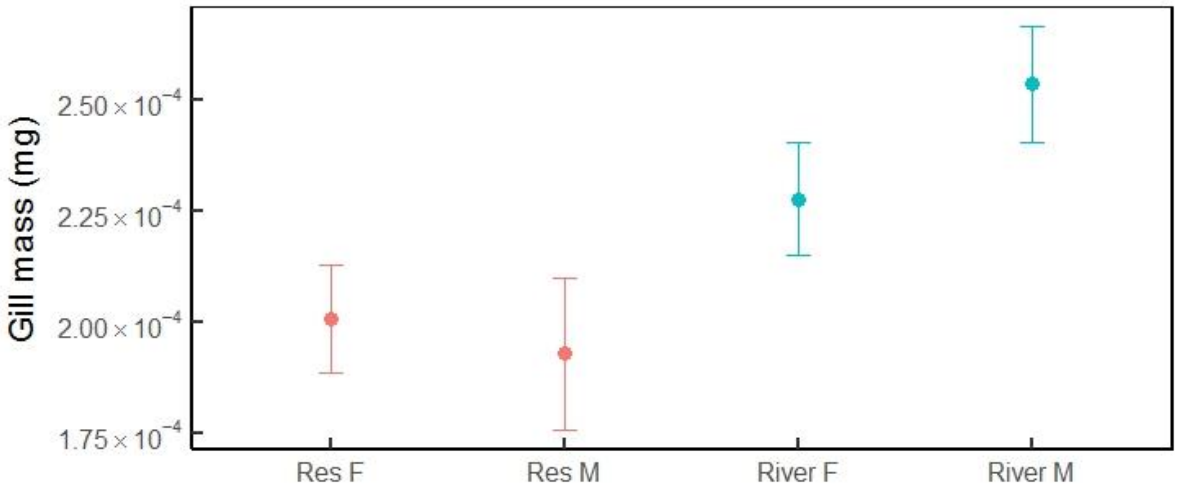

Fig. 4.6. Mean gill mass in milligrams for male and female Australian smelt from river (blue circles) and reservoir (orange circles) habitats. Sample means (*n*) = 3 (River males and females; 2 (Reservoir males and females). Error bars indicate 1 standard error around the mean.

4.4. Discussion

4.4.1. Citrate synthase activity in lateral muscle tissue of Australian smelt

The results for CS activity in lateral muscle tissue of Australian smelt supported the alternative hypothesis; that CS activity was significantly different among populations of Australian smelt from river and reservoir habitats. There is a clearly higher level of citrate synthase activity in both red and white muscle types of faster and more active fishes, than in slower, sluggish ones (Childress & Somero 1979). Yet, despite the observed differences in activity levels of citrate synthase in faster and slower swimming fishes, this has been difficult to prove, because fishes have been compared for metabolic enzyme activity by taxonomic or ecological groupings, rather than directly evaluated through tests of swimming performance (Gibb & Dickson 2002). It is possible that river and reservoir populations of Australian smelt may have had significantly different CS activity owing to different proportions of red and white muscle. White muscle should constitute the majority of CS activity, as it comprises the bulk of lateral muscle mass and thereby supports most routine activity in fishes (Childress & Somero 1979). However, this could not be evaluated because the entire lateral muscle mass of Australian smelt was used for the enzyme assays in this chapter. Nevertheless, the present chapter supports previous findings, that CS activity is strongly correlated with critical swimming speed performance, but it is unclear whether this is also associated with different red and white muscle proportions among populations from different habitat types.

The significance of the interaction between habitat and population effects indicates that the differences in CS activity in lateral muscle of Australian smelt may not have been consistent across all populations. This interaction is attributable to the similarity in CS activity

of Australian smelt from reservoir populations and the Ovens River population. Citrate synthase activity in the Ovens River population may have been lower than other river populations in the wild due to the effect of another unmeasured physiological factor. This result shows that higher swimming speed performance among Australian smelt from river habitats over those from reservoir habitats may be attributable to some extent to higher activity of metabolic enzymes, such as citrate synthase. Given the consistently smaller size of the reservoir populations, river populations may have higher swimming speed performance at least partly due to greater body size.

Citrate synthase is the primary enzyme that catalyses the first reaction in the Kreb's cycle during aerobic respiration in cellular mitochondria and is thought to be a limiting factor in prolonged swimming performance (Somero & Childress 1990; Dickson 1995; Gibb & Dickson 2002). However, metabolic enzyme activity is thought to scale only modestly, and in some cases negatively, with size (Somero & Childress 1990) thus differences in CS activity among river and reservoir populations of Australian smelt may be a combination of habitat and size effects. This may also suggest that if Childress and Somero's (1990) observation applies to Australian smelt, (that CS scales negatively with size), then size may counteract other factors that regulate CS activity in skeletal muscle cells. Further investigation is warranted to determine if CS activity also scales negatively in small-bodied fishes and across different activity levels within a species, where populations may be subject to different levels of an environmental factor such as flow velocity for multiple generations.

Though Davison (1997) suggests that exercise training, or higher activity level in general, has been shown to result in increases in aerobic enzyme activity, it is also thought to be associated with a switch to lipid metabolism in exercised fish for some species. This may be attributable to low proportion of red muscle mass and therefore low aerobic metabolic capacity with which enzymes such as citrate synthase are associated. Davison (1997) also reported that red muscle as a proportion of total lateral muscle mass was found to be associated with increased swimming speed performance. In the context of the results presented in this chapter, the reported interactions between activity levels and muscle types suggest that relatively high citrate synthase activity in lateral muscle tissue may be an indicator of higher proportions of red muscle, which in turn support higher swimming speed performance in river populations of Australian smelt.

There was no difference in the activity levels of CS among trial and control groups of Australian smelt. Thus, there was no significant effect of handling stress on relative differences in CS activity among populations from rivers and reservoirs.

4.4.2. Gill mass and swimming speed performance

The gill mass results supported the alternative hypothesis; that gill mass was significantly different among Australian smelt from river and reservoir habitats. Australian smelt from river habitats had significantly higher gill mass, after accounting for a significant standard-length covariation, than Australian smelt from reservoir habitats, suggesting that higher swimming speed performance of river populations is at least partly attributable to greater size-specific gill mass. Greater gill mass, or more frequently measured gill surface area, is an adaptation to enable higher uptake of dissolved oxygen (DO) from the fish's aquatic environment (e.g. Palzenberger & Pohla 1992). This has been demonstrated in fish populations that inhabit environments affected by chronic hypoxia, where greater gill surface area or gill size has enabled fish to undertake routine activity under low DO concentrations (Chapman *et al.* 1999; Timmerman & Chapman 2004). Australian smelt from river systems may have been expected to encounter higher DO concentrations than those from reservoir systems, due to the constant mixing and turbulence of water at high flow velocities. Mean DO concentrations of river and reservoir habitats in this thesis were not different (river habitats: 7.959 – 10.959 $mg\ L^{-1}$; reservoir habitats: 7.117 – 10.671 $mg\ L^{-1}$; Appendix 1). Therefore, different DO concentrations could not explain differences in gill mass of river and reservoir populations.

The greater size-specific gill mass in river populations of Australian smelt could be explained by higher metabolic oxygen demand associated at least partly with higher activity levels. Maximum oxygen consumption ($VO_{2_{max}}$) during prolonged swimming in guppies (*Poecilia reticulata*) has been shown to be correlated with gill mass, indicating that higher oxygen demand is a consequence of higher activity levels (Odell *et al.* 2003). Martinez *et al.* (2003) also reported a weak, but significant correlation between steady swimming performance and gill mass indicating that higher oxygen demand was associated with higher swimming speeds.

Gibb and Dickson (2002) recognized that prolonged swimming speed performance can be regulated by multiple physiological adaptations such as CS activity, heart mass and gill size. It is unclear if gill mass or CS activity was more likely responsible for the differences in prolonged swimming speed performance among river and reservoir populations because Australian smelt from river populations were on average, larger than reservoir conspecifics. The results of the CS data analysis suggest that standard length was a weaker covariate with CS activity than with gill mass (Table 4.4 and Table 4.6 respectively). Effectively, this could mean that size contributed more strongly to the differences in gill mass among river and reservoir populations, than to differences in CS activity. Future work on physiological

variation among populations should focus on measuring traits for individuals in discrete size classes, to minimize the confounding effects of size on evaluation of population divergence in other traits.

4.4.3. Sex-specific heart mass

The results for heart mass supported the null hypothesis; heart mass was not significantly different among river and reservoir populations of Australian smelt. However, there were significant sex-specific differences in both river and reservoir populations. This was contrary to observations by Hochachka (1961) where fish with higher sustained swimming speed performance had larger hearts. Davison (1997) stated that heart mass has never been recognized as a trait that is unequivocally linked with variation in swimming performance. However, Davison's (1997) review focused only on the effects of exercise training on swimming performance and physiology in controlled experiments. Heart mass may therefore be more related to life-history than habitat. This was demonstrated by Franklin and Davies (1992) who found that sex-specific variation in relative ventricle mass (corrected for body mass) represented sexual dimorphism in metabolic capacity and supported increased swimming performance in male trout, as they may engage in more demanding migratory and territorial behaviour during spawning season.

The results of this chapter suggest that heart mass is likely to be sexually dimorphic, rather than a consequence of local adaptation. There is some evidence from Odell *et al*. (2003) and Norin and Malte (2012) that heart mass does not significantly correlate with intra-specific variation in metabolic scope at all. Norin and Malte (2012) observed traits on fish reared in captivity and this may not be relevant to comparisons of wild populations. Likewise, Odell *et al*. (2003) recognized that confounding factors, particularly size differences among groups not accounted for in their methodology may have masked the effect of habitat on variation in cardiovascular organs.

4.5. Conclusion

Variation in prolonged swimming speed performance may be related to physiological variables. Evidence from phylogenetic studies that have explored variation in physiological traits in highly active species such as tunas and billfishes have demonstrated that activity level (i.e. higher swimming speed performance) does in fact reflect greater metabolic demand due to increased energy consumption (Moyes *et al.* 1992; Dickson 1995). This has also been demonstrated at the intra-specific level (see discussion). Due to minimal or negative size-scaling (Childress & Somero 1990) however, combinations of greater body size and higher

physiological trait values, differences in swimming speed performance among populations of fish from river and reservoir habitats may be attributable to differences in proportions of different muscle types. Fish are able to adapt via different physiological pathways (Davison 1997; Gibb & Dickson 2002). Thus, CS activity may influence prolonged swimming speed differences among river and reservoir populations of Australian smelt more than, or in combination with, gill mass and heart mass. The physiological adaptations of Australian smelt to river and reservoir habitats may be augmented by modifications to body size. Further work is needed to evaluate how different combinations of physiological and morphological traits can lead to intra-specific differences in swimming performance, which has not yet been sufficiently clarified (Langerhans 2009).

Chapter 5. General discussion

The aim of the research presented in this thesis was to evaluate how fish populations adapt to different flow velocity habitats. Using Australian smelt as a model species, I addressed the following research questions:

1. Does body shape vary among river and reservoir populations of fish?

2. Does prolonged swimming performance differ among river and reservoir fish populations and if so, how is prolonged swimming performance correlated with morphology and fin shape?

3. Do physiological traits differ among river and reservoir populations of Australian smelt?

River populations of Australian smelt had deeper bodies (Fig. 5.1 and chapter 2) with higher fin aspect ratios and higher prolonged swimming speeds than reservoir conspecifics (Fig. 5.1 and chapter 3). River populations were on average larger than reservoir populations in chapters 2, 3 and trial samples in chapter 4. This may have been due to the difficulties associated with reliably collecting and returning sufficient individuals to the laboratory due to unexplained mortality during transport and acclimation to experimental conditions. Several attempts were made to collect additional individuals during the experiments, to ensure that individuals from all populations sampled had homogenous size distributions. However, due to the risk of mortality associated with handling, individuals could not be measured prior to experiments, thus increasing the possibility of differences in mean size between trial and control groups. These differences in size between river and reservoir populations were observed in chapter 2, 3 and chapter 4 trial samples and were accounted for by including size as a covariate in all statistical analyses. River populations also had higher gill mass and citrate synthase activity than reservoir populations (Fig. 5.1 and chapter 4). Further analyses showed that observed differences in swimming speed performance among Australian smelt from river and reservoir populations was related to higher caudal and pectoral fin aspect ratios. Gill mass and metabolic enzyme activity were also significantly higher in river populations than reservoir conspecifics (Fig. 5.1 and chapter 4). These differences in gill mass and metabolic enzyme activity among river and reservoir populations were consistent even after accounting for covariation with size. In this chapter, I discuss these findings in the context of previous

research in eco-morphology, physiology and swimming performance, as well as possible explanations for the observed trait divergence among river and reservoir populations of Australian smelt.

5.1. Morphological divergence in river and reservoir populations of Australian smelt

Australian smelt populations from reservoirs have diverged in body morphology from those in naturally occurring river habitats (Fig. 5.1 and chapter 2). I found that Australian smelt from river habitats had deeper bodies, larger, deeper heads and relatively narrow caudal peduncles compared to their reservoir conspecifics which had shallow fusiform bodies, relatively deep caudal peduncles and smaller compressed heads.

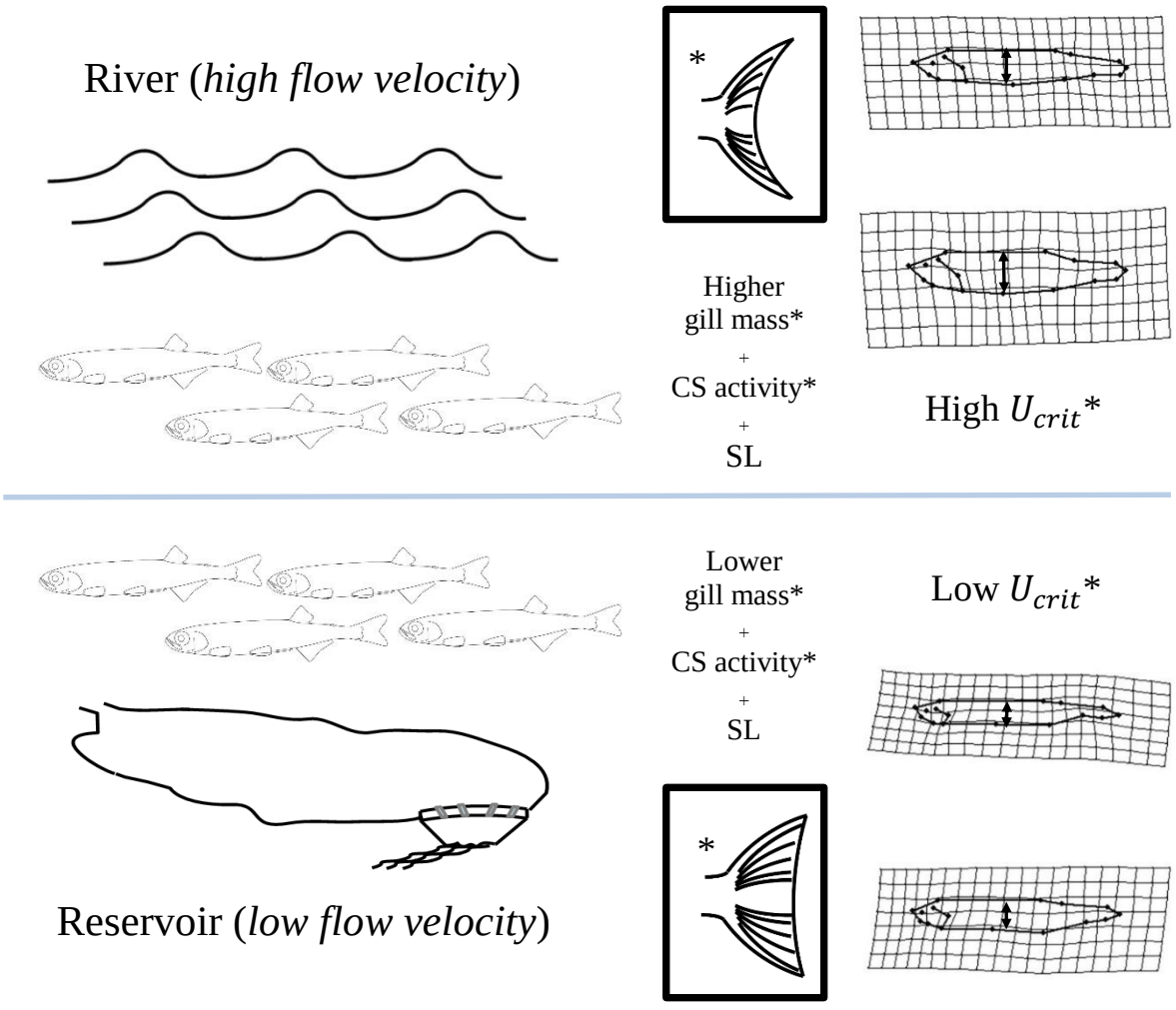

Fig. 5.1. Summary of thesis findings. River populations of Australian smelt separated by dam construction diverged into river and reservoir populations. River populations have higher fin aspect ratio and U_{crit} than reservoir conspecifics; thick continuous box indicates caudal fin aspect ratio, the best predictor of U_{crit}; River populations also had higher size-specific gill mass and citrate synthase (CS) activity than reservoir conspecifics. Asterisks (*) denote traits that have not been compared in previous studies of divergence among populations of freshwater fish.

This result was not consistent with Langerhans (2008) and several other studies which reported shallow, fusiform bodies, compressed or smaller heads and narrow caudal peduncles among river populations, and deep bodies, large heads and deep caudal peduncles in reservoir or lake populations of several species in the Cyprinidae and Centrarchidae families (Brinsmead & Fox 2002; Langerhans *et al.* 2003; Haas *et al.* 2010; Collin & Fumagalli 2011; Franssen 2011) (chapter 2). However, the body shape divergence among river and reservoir populations of Australian smelt were consistent with patterns of body shape divergence observed in several species of the Gasterosteidae, Salmonidae and Cyprinidae families (Hendry *et al.* 2002; Pavey *et al.* 2010; Franssen *et al.* 2013a). These inconsistencies in the literature suggest that even though body shape divergence among populations of fish does occur, the direction of morphological divergence (i.e. deeper or shallower bodied) is not predictable. This could suggest that ecological differences other than flow velocity play an important role in determining the morphology of fish in rivers and reservoirs. Pavey *et al*. (2010), hypothesized that deeper robust bodies conferred the ability to undertake rapid, complex manoeuvring and negotiate turbulent river currents whereas lake conspecifics were narrower and more fusiform with narrow caudal peduncles, which enhance steady, high speed-cruising. However, the swimming ability in that study (Pavey *et al.* 2010) and others studying morphological divergence among river and reservoir populations of fish (Brinsmead & Fox 2002; Langerhans *et al.* 2003; Haas *et al.* 2010; Collin & Fumagalli 2011; Franssen 2011) was never tested. Contrary to current thought, the reduction of drag (Langerhans *et al.* 2003; Haas *et al.* 2010; Franssen 2011) to increase swimming speed and efficiency (Vogel 1994), may not be the only mechanism driving morphological divergence among fish populations from different flow velocity habitats. Instead, minimization of recoil losses, the energy lost through sideways motion of the body in response to movement of the tail (Weihs 1989; Sfakiotakis *et al.* 1999), and manoeuvrability may influence body shape especially in turbulent riverine habitats.

5.2. Functional importance of body shape and the caudal fin in Australian smelt

Body shape did not account for the differences in prolonged swimming speed performance among river and reservoir populations of Australian smelt (chapter 3) which was inconsistent with the hypothesis that steady swimming performance is enhanced by reducing pressure drag through streamlining (achieved with a narrow, fusiform body) (Webb 1975; Vogel 1994; Langerhans 2008; Langerhans & Reznick 2010). Pressure drag arises from the movement of water around an object which results in turbulence in the form of a downstream wake, where velocity is lower behind than it is in front of, or at the sides, of the body (Fig. 5.2a) and

streamlining (increasing fineness or length-to-depth ratio) minimizes this problem (Fig. 5.2b, c).

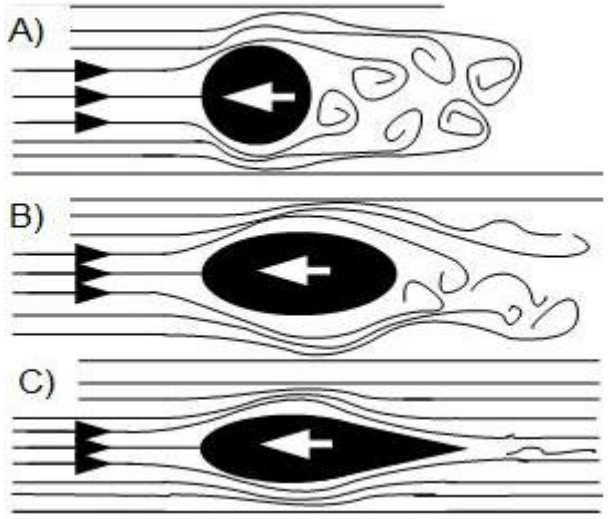

Fig 5.2. Pressure drag, visualized as a turbulent wake downstream of A) a circular and B) an ovoid longitudinal section. A streamlined shape C), minimizes the turbulence associated with a wake, by delaying the 'separation point' of flow (where the fluid becomes turbulent) until a point at the posterior margin of the object or organism. Black arrows indicate direction of flow; white arrows indicate direction of movement of the object or organism relative to the flow. Modified from Webb (1975) and Vogel (1994).

Despite the inconsistency in the direction of morphological divergence among river and reservoir populations, the hypothesis that a shallow fusiform body shape (due to the effect of drag) is necessary for increased swimming speed performance, is pervasive in the literature (Webb 1984a; Langerhans 2008; Haas *et al.* 2010; Franssen 2011). Species and families of fishes with narrower fusiform bodies have been shown to have higher prolonged swimming speed performance than those with deeper, robust bodies (e.g. Weihs 1989; Fulton & Bellwood 2005). Yet prior to this thesis, this relationship between body shape and swimming speed performance has never been empirically tested among river and reservoir populations of fish.

Despite significant differences in body morphology among river and reservoir populations, the caudal fin aspect ratio, not body shape, was the strongest predictor of prolonged swimming speed performance in Australian smelt (chapter 3). High swimming speed performance is enabled by high aspect ratio fins, as they generate propulsion more efficiently than low aspect ratio fins (Sfakiotakis *et al.* 1999). Therefore, differences in body shape in river and reservoir populations of Australian smelt may not be related to the maximum propulsion that they can generate.

Many fishes (e.g. tuna) that are body-caudal fin (BCF) swimmers generate thrust with a caudal fin that has a high aspect ratio and tend to have a streamlined, but relatively deep, robust body towards the shoulder or mid-section to minimize recoil losses (energy lost through sideways motion of the body in response to movement of the tail) (Weihs 1989; Sfakiotakis *et al.* 1999). A streamlined body may be generally advantageous to a BCF swimmer, such as Australian smelt, for reducing drag. However, this may not be an advantage for river populations, where they not only need to maintain position in strong currents but may also need to use bursts of acceleration and rapid manoeuvring to move across turbulent, complex currents. A deeper body in river populations could assist with minimizing recoil losses during acceleration, high speed sustained swimming and sharp turning manoeuvres. Conversely, a narrow, fusiform body coupled with low-aspect ratio caudal fin could provide optimal burst swimming performance and low-speed cruising for reservoir populations of Australian smelt.

5.3. Functional importance of the pectoral fin in Australian smelt

The pectoral fin aspect ratio was significantly higher in river populations of Australian smelt than in reservoir conspecifics and strongly correlated with prolonged swimming speed performance (chapter 3). This suggests that the pectoral fin may have an important role in swimming speed performance of this species. In other species, the pectoral fin has been shown to be functionally relevant for foraging, low speed cruising and contributing strongly to steering, braking and manoeuvring (Bellwood & Wainwright 2001; Blake 2004; Fulton 2007) and may have similar functions during prolonged swimming performance in BCF swimmers (Drucker & Lauder 2003; Blake 2004). In salmoniform fishes such as trout, the pectoral fins perform a mostly stabilizing role under turbulent flows in riverine habitats with complex rocky and woody substrates (Liao 2007). Australian smelt are closely related to the Salmonidae family (McDowall 1979), thus the pectoral fins may serve a similar function. For example, when trout are exposed to high velocity flows, they will use BCF motion (body undulations) as their main propulsion mechanism, but when flows become turbulent, they switch from BCF to MPF swimming and use only their pectoral fins to steer into low velocity spaces between currents to minimize energy expenditure (Liao *et al.* 2003). It is possible that Australian smelt from river populations may switch from BCF swimming to MPF swimming in turbulent flow to minimize energy expenditure in a similar way. Reservoir populations, which encounter still, or non-turbulent conditions would not benefit from this.

Changing swimming mode however, is a behavioural adaptation (Blake 2004; Liao 2007) that may allow fish to minimize energy consumption under different hydrological

conditions and activities (i.e. cruising versus manoeuvring) (Tytell 2007; Tytell *et al.* 2010). The role of behavioural swimming adaptations in divergence of prolonged swimming speed performance may present an opportunity for future research, because it is a form of adaptation that can precede morphological and physiological adaptation.

5.4. Physiological and size divergence between river and reservoir populations

The significant differences in citrate synthase activity and gill mass observed among river and reservoir populations of Australian smelt (chapter 4) is likely to reflect greater frequency of movement and energy expenditure of the river populations, which achieved greater maximum critical swimming speeds than reservoir populations. Higher oxygen demand results in an increase over time in the size of gill structures (Chapman *et al.* 1999; Moyes 2003; Martínez *et al.* 2009; Paterson *et al.* 2010). Similarly, higher metabolic enzyme activity levels tend to be associated with higher swimming speed or energy expenditure levels (Farrell *et al.* 1991; Moyes *et al.* 1992; Garenc *et al.* 1999; Gibb & Dickson 2002; Martinez *et al.* 2003). Indeed, modification of physiological traits that are responsible for delivering oxygen and metabolic fuels to the muscle cells most efficiently (Brett & Groves 1979; Gibb & Dickson 2002; Evans *et al.* 2004), is one way to enhance swimming performance. Yet, until only recently, have these physiological traits been thought of as limiting factors that underlie divergence in steady swimming performance (Odell *et al.* 2003).

In the case of Australian smelt, increased gill mass and higher metabolic enzyme activity in riverine populations may reflect higher dependence on prolonged swimming when holding station in flowing water and negotiating complex hydrological conditions, including high velocity currents. However, this conclusion must be made with caution, because physiological traits such as metabolic enzymes can scale with size in some species (Childress & Somero 1990; Somero & Childress 1990). Indeed, gill mass and citrate synthase activity did co-vary with standard length, most likely because river populations of Australian smelt were on average, larger than reservoir populations (chapter 4). Future work comparing variation in traits such as gill mass and citrate synthase activity should ensure that comparisons are made within discrete size classes from river and reservoir populations of fish to minimize confounding effects of size-scaling. This thesis presents new evidence that divergence in swimming speed performance may be regulated by variation in physiological traits such as gill mass or citrate synthase activity.

5.5. Mechanisms of population divergence

Australian smelt populations from river and reservoir habitats showed significant differences in fin shape, swimming speed performance and physiological traits. There are several mechanisms by which these changes may have arisen including gene flow, genetic drift and divergent selection (Futuyma 2006).

Trait variation in river and reservoir populations of freshwater fish are most likely to be regulated through divergent natural selection and gene flow (Hendry *et al.* 2002; Langerhans 2008). Divergent natural selection is a predicted cause of trait variation among river and reservoir populations because differences in flow velocity may impose selective pressures on populations not previously adapted to constant low-flow velocity or standing conditions (Baxter 1977; Fernando & Holčík 1991; Paller 1997). Gene flow, the result of breeding among individuals from contiguous populations (Carvalho 1993), is a mechanism that constrains selection particularly in reservoir habitats where resident populations may come into contact with river populations of fish in upstream river habitats (Hendry *et al.* 2002). However, provided that populations in reservoir habitats are separated from populations of fish in upstream river habitats, gene flow is unlikely to significantly constrain divergent selection among fish populations (Langerhans *et al.* 2003; Franssen 2011). Franssen *et al*. (2013b) argued that natural selection was the cause of morphological divergence among river and reservoir populations of *C. venusta*, because population sizes of the species in reservoir habitats likely limited the effects of gene flow and genetic drift on morphological traits.

Genetic drift, defined as differences arising in a population due to chance in an isolated or finite sub-population which then increases over time (Slatkin 1987), may be responsible for population divergence in some cases. However, random genetic drift is unlikely to be the mechanism by which the observed differences in swimming speed performance and physiological traits arose consistently among river and reservoir populations of Australian smelt. Repeated trait divergence such as this, would not be expected under the genetic drift hypothesis, because genetic drift is a gradual process occurring in all populations all the time (Futuyma 2006). Furthermore, genetic drift acts more strongly on small populations than on large ones. Australian smelt are a wide-spread, highly abundant species in the systems studied, so we would expect the trait divergence to be comparable among all populations studied here. Thus, genetic drift is unlikely to have caused the repeated trait variation observed in these systems.

Australian smelt are a highly abundant species in river and reservoir habitats (Gehrke *et al.* 1996; Gehrke 1997; Gehrke & Harris 2001; Lintermans 2007). High population structure in Australian smelt has suggested that there is minimal gene flow among populations even

within catchments (Woods *et al.* 2010). For example, genetic divergence in Australian smelt can occur between populations separated by a low-level weir (Duncan *et al.* 2016), let alone the large dams and long distances separating populations studied in this thesis. It is therefore possible that in the presence of divergent selective pressures such as flow velocity or other ecological factors, the trait differences observed among river and reservoir populations of Australian smelt are likely due to divergent natural selection. It remains unknown whether the morphological and physiological trait differences are genetically based, although these traits have been shown to be heritable (Patterson *et al.* 2004; Franssen 2011). If the differences observed between river and reservoir populations of Australian smelt are an example of trait divergence, then this has occurred in as little as 50 years based on dates of dam construction (chapter 1), but is feasible because Australian smelt have a life-span of only about 3 years (Lintermans 2007). Trait divergence has been shown to arise in other short-lived species such as *P. reticulata* (Reznick *et al.* 1997) and *Pimephales vigilax* in as few as 15 generations or 10 to 20 years (Cureton & Broughton 2014).

5.6. Opportunities for future research: alternative selective pressures and biomechanics of fish swimming

Based on the results of this thesis, the following recommendations are made for future research efforts. In efforts to evaluate differences in swimming performance among groups (populations or species), alternative selective pressures (such as predation and flow velocity) should be identified and their relative contributions to swimming performance should be evaluated through adequately designed experiments or field studies. Because suitable replicated field sites where different levels or intensities of the selective pressures of interest exist, might be difficult to find, controlled split-plot designs in which samples of a population are placed under different combinations of selective pressures, might be more appropriate than field studies. For example, swimming performance, oxygen consumption and enzyme activity might be compared for river and reservoir populations exposed to several combinations of high and low predation and high and low flow velocity to evaluate how the sampled populations might respond to combinations of these conditions in the wild. In addition to providing new insights into the morphological, functional and physiological differences among fish populations from river and reservoir habitats, this thesis revealed opportunities for research into alternative selective pressures that might affect divergence in freshwater fishes.

Alongside flow velocity, predation is one of the most widely cited selective pressures as a cause of divergence among fish populations (Langerhans *et al.* 2004; Langerhans 2009;

Oufiero *et al.* 2011). Selection pressure from higher predatory fish densities can arise through fish stocking programs (Fernando & Holčík 1991), owing to the effect that increased predator densities can have on smaller non-piscivorous fish assemblages (Eby *et al.* 2006; Potthoff *et al.* 2008; Friederichs *et al.* 2011; Roseman *et al.* 2014). Indeed, stocking of large, predatory sportfish has been common across rivers and reservoirs in the Murray Darling Basin for at least 30 years (Cadwallader & Gooley 1985; Lintermans 2000; Hutchinson *et al.* 2006). Thus, there is an opportunity to explore the role of stocking as a cause of altered predator density in river and reservoir habitats and the effects on divergence among populations of forage fishes such as Australian smelt. Studies of swimming performance and body shape divergence typically exploit existing or experimentally manipulated predator densities; however, these rarely coincide with different flow environments (e.g. Belk 1998; Reimchen & Nosil 2004; Domenici *et al.* 2008; Reznick *et al.* 2008; Ingley *et al.* 2014). Therefore, future research could consider whether alternative selective pressures such as flow velocity and predation interact in river and reservoir fish populations as suggested by theoretical predictions (Langerhans 2008; Langerhans & Reznick 2010).

If predator density was higher in reservoir habitats, resident Australian smelt may have adapted enhanced escape responses through increased burst swimming performance, and dependence on anaerobic, instead of aerobic, metabolism in reservoir populations (Langerhans 2009). This would have resulted in higher activity levels of anaerobic metabolic enzymes such as lactate dehydrogenase, rather than citrate synthase (Garenc *et al.* 1999; Kieffer 2000) and reduction in gill size due to reduced oxygen demand in response to reduced activity levels, whilst being sufficient to support minimum oxygen consumption rates required to repay oxygen debts following predator escape responses. Future studies of physiological and performance adaptations to different habitats should incorporate measures of both aerobic and anaerobic metabolic enzymes alongside measures of oxygen consumption.

The importance of alternative selective pressures, their interactions and their effect on physiological, morphological and functional divergence are still unclear, possibly because their effects on trait divergence have only been studied in isolation (Reznick & Ghalambor 2001; Nelson *et al.* 2003; Schaack & Chapman 2003; Franssen 2011; Oufiero *et al.* 2011). Therefore, integrative research across multiple organismal systems (i.e. physiology, morphology and performance) such as the research presented in this thesis, is needed to improve our understanding of trait divergence in response to rapid ecological change.

Further understanding of the relationship of body shape with swimming performance may be achieved through identifying and formally describing the swimming mode (BCF or MPF) and kinematics (amplitude and wavelength of fin and body undulations) for fishes used

in studies of trait divergence. The assumption that underlies many studies of trait divergence in body shape among fish populations from different flow velocity habitats (Hendry *et al.* 2002; Haas *et al.* 2010; Pavey *et al.* 2010; Franssen 2011; Franssen *et al.* 2013a; Cureton & Broughton 2014) is that swimming performance is predictable as a direct response to variation in body shape (Langerhans 2008; Fig. 5.3A). However, this may be an oversimplification. Subsequently, Langerhans (2009) suggested that variation in swimming performance may not always be directly linked with variation in functional traits (i.e. body shape, fins, etc) and that the same levels of performance in one group might be achieved with different trait combinations and kinematics in another group. Several recent attempts using computational modelling have demonstrated that there is considerable interaction among swimming speed, kinematics, energetics and body shape and that this complicates predictions of swimming performance based purely on body shape (Tytell 2007; Borazjani & Sotiropoulos 2009; Tytell *et al.* 2010; Tokić & Yue 2012). Subsequently, Walker *et al.* (2013) proposed that intra-specific body shape variation may arise across divergent selective pressures such as flow velocity or wave disturbance gradients only in species that swim with rigid bodies (MPF swimmers), but not in those that use body undulations and the caudal fin to generate propulsion (BCF swimmers) (Fig. 5.3B). The findings of this thesis support Walker *et al*'s (2013) findings: Australian smelt are a BCF swimmer and therefore, a species in which significant variation in body shape does not underlie significant variation in swimming speed performance (unshaded regions; Fig. 5.3B).

Consequently, it is recommended that for future studies of divergence in swimming speed performance and morphology of fishes, evaluating the swimming mode and kinematics (wave-length and amplitude of body undulation) for a range of swimming functions (i.e. cruising, accelerating and manoeuvring) should be central to formulating hypotheses and experimental design. The swimming mode and kinematics of a species should be established for groups through preliminary kinematic studies where results can be incorporated into subsequent performance trials (e.g. Domenici & Blake 1997; Liao 2007).

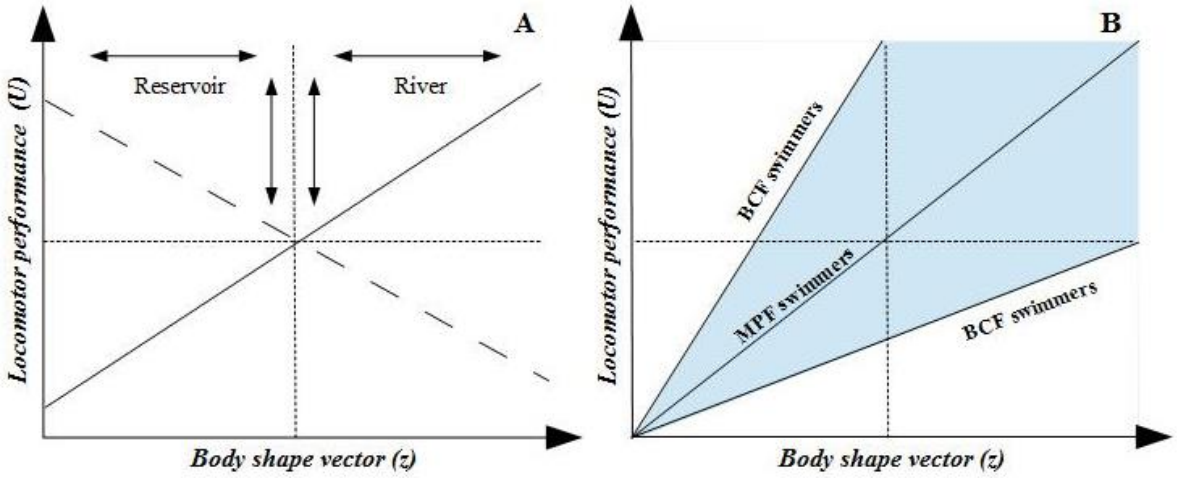

Fig. 5.3. Generalized models of the relationship between body shape and swimming performance. Panel A): Langerhans' (2009) conceptual model: steady swimming performance is positively correlated with a hypothetical body shape vector (solid line) and unsteady (burst or fast-start) swimming performance is negatively correlated with the body shape vector (broken line). Upper left and right quadrants represent predicted optimal body shape and swimming performance values for reservoir and river populations of fish, respectively. Panel B): a qualitative model based on description from Walker *et al*. (2013) which shows that the correlation between body shape and swimming speed performance is mediated by swimming mode (BCF vs MPF). Shaded region (MPF swimmers) represents significant functional relationship (strong correlation) between body shape and swimming performance; unshaded region (BCF swimmers) represents non-significant functional relationship (weak or no correlation) between body shape and swimming performance.

Alternatively, kinematics can be directly evaluated through swimming performance trials (e.g. Langerhans 2009), particle image velocimetry (e.g. Tytell 2007; Tytell *et al.* 2010) or field observations (e.g. Fulton & Bellwood 2004; Fulton 2007). Once the form-function relationship is better understood, the trait divergence responses to different habitats and fitness consequences of selective pressures such as dam construction, may be more easily evaluated or predicted.

Lastly, future studies of trait divergence should endeavour to quantify the rate of evolutionary change via complementary studies of genetic structuring or gene expression among populations that are morphologically, functionally or physiologically different. This may also present opportunities to investigate trait variation and adaptive potential for species that are regularly stocked in river and reservoir systems across south-east Australia, such Murray cod (*Maccullochella peeli*), Australian bass (*Macquaria novamaculeata*) or golden perch (*Macquaria ambigua*). This may be particularly insightful among populations that are subject to intense recreational angling activity, which can be a strong selective pressure on game fish species (e.g. Redpath *et al.* 2010).

5.7. Conclusion

Quantifying divergence among river populations of Australian smelt and reservoir conspecifics, has shed light on morphological, performance and physiological differences by which the species may have adapted and persisted in the face of rapid, ecological change.

The effect of drag on energy expenditure and consequently, swimming performance of fishes has given rise to a presumption that body shape is directly linked with swimming performance in river and reservoir populations of fish (Vogel 1994; Vogel 2003; Lytle & Poff 2004). In this thesis, fin morphology, not body shape, was the strongest morphological predictor of swimming speed, despite the biomechanical underpinnings of theoretical predictions which suggest that body shape should be strongly correlated with swimming speed performance (Webb 1984a; Langerhans 2008). In doing so, I provide new evidence that patterns of trait divergence inconsistent with those predicted by theory (Langerhans 2008;2009) may achieve the same swimming performance outcomes in river and reservoir populations of freshwater fishes as predicted by those generalized models (Langerhans 2008). Furthermore, this thesis presented new evidence that divergence among river and reservoir fish populations is partly underpinned by physiological trait differences.

The main strength of this thesis is that swimming speed performance, body morphology, heart mass, gill mass and metabolic enzyme activity were evaluated for the same individuals from river and reservoir populations of Australian smelt. Most studies focus on single trait comparisons among populations or compare multiple traits at the among-species or inter-individual level (e.g. Reidy *et al.* 2000; Nelson *et al.* 2003; Norin & Malte 2012; He *et al.* 2013). A comprehensive evaluation of the traits that are thought to underlie variation in swimming speed performance has not previously been attempted in the study of trait divergence of river and reservoir populations of freshwater fishes.

Size was acknowledged as a confounding factor in the prolonged swimming speed results of chapter 3 and the physiological trait differences among river and reservoir populations in chapter 4. The effect of size relative to the effect of flow environment (river or reservoir) on physiological trait differences among populations can be statistically accounted for in the results. It is known that in many cases physiological traits such as those studied for this thesis, do scale with size (Somero & Childress 1990). Thus, I conclude that populations of Australian smelt have adapted to river and reservoir habitats via a combination of size, physiological, fin shape and swimming speed performance modifications.

Variation in swimming performance among fish populations from different flow velocity habitats may be a response to unprecedented ecosystem change. Understanding patterns of swimming performance, morphological and physiological trait divergence

following unprecedented ecosystem change will be essential for drawing accurate predictions of evolutionary responses among fishes as societal demands on water resources intensify.

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7. Appendices

Appendix 1 – Water quality parameters (Chapter 2 -4)

Water quality parameters measured were temperature (ºC), pH, turbidity (NTU), conductivity ($mS\ cm^{-1}$), dissolved oxygen (DO; $mg\ L^{-1}$) and flow velocity ($m\ s^{-1}$) (Tables 7.1 and 7.2). Five point measurements were made for each parameter, and mean (μ) was calculated for each sampling trip (*st*):

$$\mu_{st} = \Sigma(m_1 \dots m_5)/5$$

Where *m* is a point measurement taken on a sampling trip to a river or reservoir.
The sampling trip (*st*) means for each variable were calculated monthly for periods when sampling was undertaken (October 2014 to February 2015, June 2015 and May 2016).

Table 7.1. Water quality parameters for reservoir systems from which Australian smelt were sampled. Parameters were temperature ($T\ ^oC$), pH, conductivity ($mS\ cm^{-1}$), turbidity (*NTU*), dissolved oxygen concentration, DO ($mg\ L^{-1}$) and flow velocity, U_{flow} ($m\ s^{-1}$) with standard errors (*s.e.*). All means and standard errors are calculated from 5 point measurements at the site where sampling was undertaken in that system.

Date	System	System type	$T\ ^oC$ ± s.e.		*pH* ± s.e.		$mS\ cm^{-1}$ ± s.e.		*NTU* ± s.e.		*DO* ($mg\ L^{-1}$) ± s.e.		U_{flow} ± s.e.	
30/10/2014	L. Hume	reservoir	18.06	± 0.042	6.982	± 0.022	0.036	± 0.001	0.648	± 0.103	9.734	±0.074	0.005	±0.003
30/10/2014	L. Nillahcootie	reservoir	18.362	± 0.109	7.672	± 0.014	0.119	± 0.001	3.040	± 0.233	10.014	±0.427	0.005	±0.003
11/11/2014	L. Hume	reservoir	19.192	± 0.040	6.336	± 0.006	0.048	± 0.001	1.940	± 0.323	11.680	±0.032	0.005	±0.002
19/11/2014	L. Nillahcootie	reservoir	19.476	± 0.029	6.492	± 0.006	0.130	± 0.000	2.460	± 0.091	12.382	±0.096	0.005	±0.002
27/11/2014	L. Cordeaux	reservoir	22.354	± 0.011	6.712	± 0.013	0.085	± 0.000	9.400	± 0.871	11.636	±0.074	0.040	±0.001
28/11/2014	L. Nepean	reservoir	20.078	± 0.053	6.618	± 0.031	0.072	± 0.001	9.800	± 0.954	11.766	±0.161	0.042	±0.002
17/12/2014	L. Nepean	reservoir	22.172	± 0.055	6.766	± 0.020	0.054	± 0.003	23.720	± 2.197	11.148	±0.031	0.005	±0.000
18/12/2014	L. Cordeaux	reservoir	22.956	± 0.014	6.612	± 0.020	0.116	± 0.006	13.800	± 3.001	7.350	±0.072	0.005	±0.002
21/01/2015	L. Nillahcootie	reservoir	24.754	± 0.149	7.678	± 0.011	0.138	± 0.001	14.020	± 1.570	7.010	±0.101	0.038	±0.002
22/01/2015	Waranga Basin	reservoir	32.14	± 0.029	8.724	± 0.078	0.091	± 0.001	91.580	±21.703	5.778	±0.233	0.040	±0.002
31/01/2015	Waranga Basin	reservoir	20.752	± 0.056	7.926	± 0.069	0.081	± 0.001	113.120	±12.827	10.674	±0.045	0.030	±0.002
11/02/2015	L. Cordeaux	reservoir	24.77	± 0.097	6.838	± 0.032	0.132	± 0.015	30.760	± 0.884	3.526	±0.233	0.036	±0.002
18/06/2015	L. Nillahcootie	reservoir	10.011	± 0.023	7.21	± 0.066	0.051	± 0.000	15.991	± 1.998	11.343	±0.093	0.015	±0.003
19/06/2015	L. Hume	reservoir	9.726	± 0.015	7.204	± 0.020	0.042	± 0.000	29.400	± 4.479	10.598	±0.077	0.004	±0.002
23/06/2015	Waranga Basin	reservoir	10.514	± 0.043	7.234	± 0.028	0.061	± 0.000	198.800	± 16.131	12.014	±0.080	0.010	±0.006
18/05/2016	L. Hume	reservoir	9.682	± 0.002	7.48	± 0.001	0.148	± 0.001	29.020	± 3.396	9.882	±0.043	0.004	±0.002
23/05/2016	L. Nillahcootie	reservoir	13.94	± 0.009	7.058	± 0.072	0.037	± 0.001	56.540	± 3.612	11.822	±0.146	0.011	±0.002
25/05/2016	Waranga Basin	reservoir	10.724	± 0.010	6.948	± 0.025	0.351	± 0.005	94.260	± 3.565	7.464	±0.264	0.008	±0.003
		Mean	*18.315*	*±0.355*	*7.132*	*± 0.032*	*0.100*	*± 0.004*	*41.017*	*± 2.908*	*9.768*	*±0.139*	*0.016*	*±0.001*

Table 7.2. Water quality parameters for river systems from which Australian smelt were sampled. Parameters were temperature (T^oC), pH, conductivity ($mS\ cm^{-1}$), turbidity (NTU), dissolved oxygen concentration, DO ($mg\ L^{-1}$) and flow velocity, U_{flow} ($m\ s^{-1}$) with standard errors (*s.e.*). All means and standard errors are calculated from 5 point measurements at the site where sampling was undertaken in that system

Date	System	System type	$T\ ^oC$ ± *s.e.*		pH ± *s.e.*		$mS\ cm^{-1}$ ± *s.e.*		NTU ± *s.e.*		$DO\ (mg\ L^{-1})$ ± *s.e.*		U_{flow} ± *s.e.*	
01/12/2014	Georges River	river	22.08	±0.136	6.392	±0.019	0.176	±0.002	51.740	±4.625	11.668	±0.052	0.13	±0.004
30/10/2014	Kiewa River	river	17.032	±0.008	6.574	±0.025	0.008	±0.001	21.260	±2.188	9.438	±0.089	0.26	±0.032
11/11/2014	Kiewa River	river	16.376	±0.024	6.428	±0.048	0.03	±0.001	2.920	±0.784	14.44	±0.246	0.222	±0.009
14/11/2014	Broken River	river	21.89	±0.009	6.506	±0.014	0.125	±0.003	23.180	±2.881	11.95	±0.092	0.148	±0.003
18/11/2014	Kiewa River	river	21.122	±0.007	6.544	±0.034	0.041	±0.000	27.020	±1.071	13.378	±0.182	0.204	±0.027
19/11/2014	Broken River	river	20.154	±0.007	6.508	±0.005	0.13	±0.000	25.680	±0.345	12.796	±0.11	0.232	±0.016
27/11/2014	Nepean River	river	23.728	±0.028	7.154	±0.039	0.222	±0.002	0.440	±0.018	12.4	±0.093	0.202	±0.008
28/11/2014	Bargo River	river	23.77	±0.048	6.574	±0.008	0.217	±0.000	5.160	±0.978	11.146	±0.095	0.098	±0.002
17/12/2014	Bargo River	river	24.202	±0.040	7.09	±0.020	0.185	±0.010	80.340	±14.104	7.126	±0.054	0.174	±0.020
18/12/2014	Nepean River	river	24.032	±0.024	7.23	±0.026	0.414	±0.062	0.582	±0.004	13.214	±0.113	0.154	±0.003
18/01/2015	Ovens River	river	24.78	±0.015	6.956	±0.026	0.065	±0.000	39.90	±5.383	6.036	±0.133	0.236	±0.016
18/01/2015	Ovens River	river	23.586	±0.005	6.95	±0.031	0.067	±0.001	33.520	±1.401	6.16	±0.044	0.204	±0.013
21/01/2015	Ovens River	river	22.586	±0.067	7.194	±0.008	0.045	±0.000	8.220	±0.551	8.69	±0.075	0.216	±0.022
23/01/2015	Broken River	river	24.77	±0.097	6.838	±0.032	0.141	±0.005	30.760	±0.884	3.526	±0.233	0.206	±0.009
31/01/2015	Broken River	river	23.896	±0.053	7.36	±0.031	0.149	±0.001	32.060	±2.958	7.438	±0.150	0.242	±0.017
11/02/2015	Bargo River	river	27.214	±0.005	7.312	±0.008	0.168	±0.000	23.660	±2.318	4.892	±0.144	0.13	±0.006
11/02/2015	Georges River	river	26.516	±0.029	7.188	±0.007	0.146	±0.004	36.420	±5.233	4.024	±0.080	0.144	±0.006
27/02/2015	Kiewa River	river	23.646	±0.013	6.542	±0.057	0.023	±0.001	6.320	±1.426	8.268	±0.037	0.186	±0.007
18/06/2015	Ovens River	river	10.206	±0.005	6.818	±0.074	0.037	±0.000	3.080	±0.079	10.948	±0.104	0.28	±0.04
23/06/2015	Broken River	river	7.384	±0.011	7.272	±0.046	0.139	±0.000	13.920	±0.184	12.052	±0.129	0.174	±0.019
26/06/2015	Kiewa River	river	10.694	±0.106	7.544	±0.031	0.072	±0.001	11.792	±0.304	8.943	±0.146	0.226	±0.010
15/05/2016	Ovens River	river	13.16	±0.066	7.59	±0.039	0.076	±0.001	2.442	±0.089	2.442	±0.076	0.272	±0.016
20/05/2016	Kiewa River	river	13.233	±0.039	7.168	±0.003	0.182	±0.002	46.120	±1.456	12.502	±0.121	0.266	±0.015
22/05/2016	Broken River	river	13.556	±0.117	7.132	±0.018	0.055	±0.002	44.000	±4.971	14.782	±0.136	0.21	±0.011
		Mean	*19.984*	*0.239*	*6.952*	*0.015*	*0.121*	*0.004*	*23.772*	*0.828*	*9.511*	*3.634*	*0.201*	*±0.049*

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Appendix 2 – Validation of landmark identification

A common source of error in morphology studies is landmark digitization error. This is the systematic error and bias associated with mis-identifying and incorrectly digitizing landmarks on sampled specimens (Zelditch *et al.* 2004). To evaluate the consistency and accuracy of the landmark digitization procedure and possibility of bias due to misidentification of landmark locations, a trial was carried out on a sub-sample of the Australian smelt sampled for chapter 2, using the landmark digitization and statistical analysis procedures described in that chapter. Ten individuals were randomly chosen from the original 668 and landmarked in a single day. This process was repeated on 2 other randomly allocated days with the same 10 individuals. Each day, the order of the 10 individuals to be landmarked, were randomized to avoid bias arising from familiarity with the sample. The 3 sub-samples of 10 individuals were then statistically compared following the methods described in chapter 2.

There was no significant difference among the 3 sub-samples (F = 0.332; P = 0.995; Table 7.3). Landmarking digitization among the 3 samples constituted only 2.4% of variation and less than half a standard deviation separating the 3 means ($SS_{sub-sample}/SS_{total}$ = 0.0240; Z = 0.3176; Table 7.3).

Table 7.3. Procrustes ANOVA (non-parametric MANOVA) statistics for model of landmark digitization error for 16 landmarks, among 3 sub-samples of Australian smelt sampled for chapter 2. *F* - values were calculated using a randomized residual permutation procedure (RRPP). Effect size (*Z* – scores) represent the standard deviations between group means for each effect. Effects were considered significant if *P*-values were less than a critical α = 0.05

Effect	Df	SS	MS	F	Z	P
Sub-sample	2	4.656^{-4}	2.328^{-4}	0.332	0.3176	0.995
Residuals	27	1.893^{-2}	7.012^{-4}			
Total	29	1.939^{-2}				

Thus, sampling error was considered minimal and not likely to confound true variation in the total sample. The repeatability and consistency of landmark digitization was also visualized by plotting the landmarks for individuals from the 3 sub-samples and the mean (consensus) configuration calculated in the GPA based on those sub-samples (Fig. 7.1).

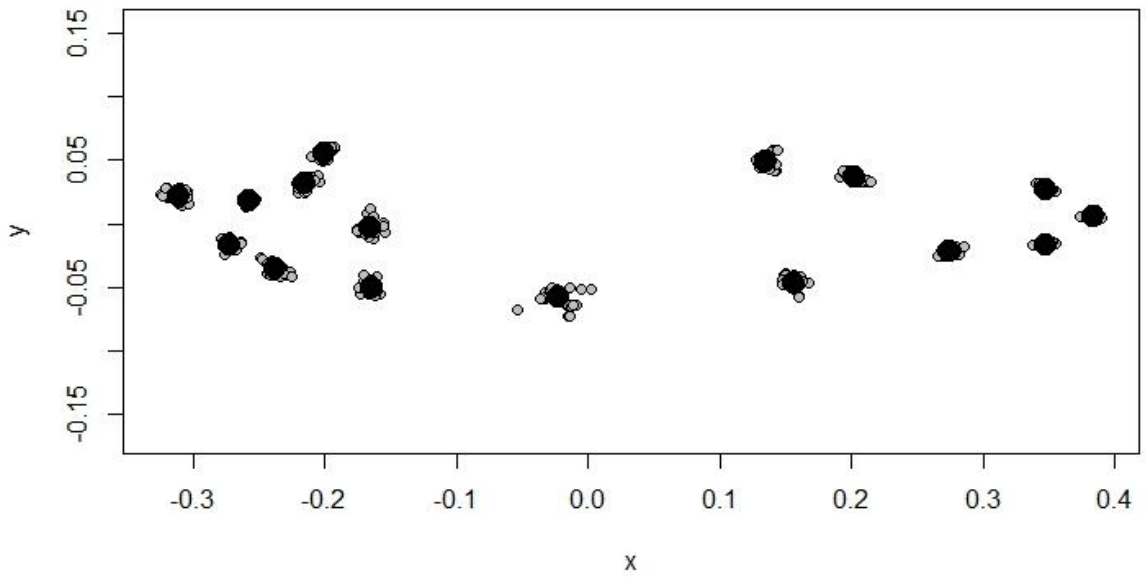

Fig. 7.1. Optimally aligned landmark configurations for all 30 individuals (small grey circles) superimposed on mean (consensus) configuration computed using GPA (large black circles). Circular scatter indicates random errors, rather than errors associated with bias or misidentification during landmark digitization (Zelditch *et al*. 2004).

Assuming that the same landmark digitization procedure was followed, and individuals from different groups were landmarked in a non-systematic way (i.e. randomize individuals from rivers and reservoir systems before landmarking), then the procedure can be considered repeatable and unbiased.

Appendix 3 - R code for statistical analyses

Scripts containing the code used to generate plots and run statistical analyses seen throughout the thesis. An example has been provided of functions that were repeatedly or frequently used. All other functions used have been described in full. All functions are preceded by comments (delimited by # symbols) used in the R script file, that describe their purpose or contents. Additional descriptions have been included in the comments to denote an example of a repeated or frequently used function. Any text not delimited by # symbols is active code.

```
###### SIZE AT MATURITY PLOTS – CHAPTERS 2, 3 & 4 #######
##### REPEAT FOR SYSTEM TYPE AND FOR CENTROID SIZE (CS) #####

# SET THE WORKING DIRECTORY WHERE DATA FILES AND SCRIPT FILE ARE STORED #
SETWD("D:/WORKING DIRECTORY")

# READ IN THE .CSV FILE CONTAINING THE STANDARD LENGTH AND CENTROID SIZE DATA #
MYDATA<-READ.CSV(AS.MATRIX("D:/WORKING DIRECTORY/SIZEATMATURITY0117.CSV", HEADER=T))

# USING BASE GRAPHICS; LOADED AT START OF SESSION BY DEFAULT #
# SUBSET DATA FOR MALES AND FEMALES FOR EACH POPULATION. REPEAT FOR ALL POPULATIONS #
# BARGO RIVER (EXAMPLE) #
MALES.BAR<-SUBSET(MYDATA, MYDATA$LOCATION=="BARGO" & MYDATA$SEX=="M", SELECT="SL")
```

```
MALES.BAR.SL<-AS.NUMERIC(MALES.BAR$SL)
FEMALES.BAR<-SUBSET(MYDATA, MYDATA$LOCATION=="BARGO" & MYDATA$SEX=="F", SELECT="SL")
FEMALES.BAR.SL<-AS.NUMERIC(FEMALES.BAR$SL)

#### SET UP FIGURE PLOT AREA ####

# SET FIGURE PLOT MATRIX #
PAR(MFROW=C(2,6))

# BARGO RIVER (EXAMPLE); PLOT REPEATED FOR OTHER POPULATIONS #
PAR(MAR=C(0.5,2.25,1.5,.25), MGP=C(1.25,.75,0))
HIST(MALES.BAR.SL, AXES=F, COL=RGB(0, 0, 0, 1), XLIM=C(18, 60), YLIM=C(0,16), BREAKS=SEQ(18,60,BY=1),
MAIN=NULL, XLAB=NULL, YLAB="FREQUENCY")
HIST(FEMALES.BAR.SL, AXES=F, COL=RGB(0, 0, 0, 0), BREAKS=SEQ(18, 60,BY=1), ADD=T)
TITLE("BARGO RIVER", LINE=-2)
BOX()
AXIS(1, AT=SEQ(20,60,BY=5), labels=F, tck=.02, padj = -1)
axis(2, at=seq(0,15,by=3), labels=T, tck=.02, padj=1)

# Make a legend if required #
par(xpd=NA)
legend(80, 5, c("female", "male"), fill=c(rgb(0, 0, 0, 1),rgb(0, 0, 0, 0)))

############################################################
####### GEOMETRIC MORPHOMETRIC ANALYSIS OF SHAPE #######
################### for chapter 2 and 3 ####################
######## All analyses here, except for GPA and TPS grids, #########
###### are performed with traditional morphometrics data #######
############################################################

# Set the working directory and read in raw data #
library("geomorph") # open geomorph package
setwd("C:/Users/Daniel Svozil/Dropbox/CSU/Geomorph data 2015/morphoanalysis2016")

# read in data and write to .csv file
mydata<-read.csv("geomorphfinal0117fixb.csv", header=T, stringsAsFactors=F)

# Check column names so variables can be identified
colnames(mydata)

# Create each variable as an object
pop<-as.matrix(mydata[,3])
system.type<-as.matrix(mydata[,2])
Y.gm<-as.matrix(mydata[,5:36])
sex<-as.matrix(mydata[,4])

# convert to 3D array #
land<-arrayspecs(Y.gm, p=16, k=2)

# plot all individuals to inspect scatter of landmarks #
plotAllSpecimens(land)



### Procrustes superimposition ###

# Generalized Procrustes analysis on all specimens
smeltshp.gpa<-gpagen(land)
```

```
# Plot reference shape
plot(smeltshp.gpa$coords)

# inspect landmark configurations for outliers
plotOutliers(smeltshp.gpa$coords)

# inspect individual outliers
plotRefToTarget(mshape(smeltshp.gpa$coords), smeltshp.gpa$coords[,,390], method="vector")

# create an object containing mean shape for all individuals or reference #
ref<-mshape(smeltshp.gpa$coords)

# read in links file. Contains landmark pairs for drawing wireframe to aid visualization
link.file<-read.csv("links.csv")
links<-matrix(link.file$links, ncol=2, byrow=T)

# calculate group means
shpmean.riv.m<-mshape(smeltshp.gpa$coords[,,which(system.type=="river" & sex=="m")])
shpmean.riv.f<-mshape(smeltshp.gpa$coords[,,which(system.type=="river" & sex=="f")])
shpmean.res.m<-mshape(smeltshp.gpa$coords[,,which(system.type=="reservoir" & sex=="m")])
shpmean.res.f<-mshape(smeltshp.gpa$coords[,,which(system.type=="reservoir" & sex=="f")])

# create figure plot area
mat <- matrix(c(1,2,3,4), 2)

# set the size of the rows and columns
layout(mat, widths=c(1,1), heights=c(1,1))

# plot group means magnified 5 times to aid visualization
riv.f<-plotRefToTarget(ref,shpmean.riv.f,mag=5, method="TPS", links=links)
riv.m<-plotRefToTarget(ref,shpmean.riv.m,mag=5, method="TPS", links=links)
res.f<-plotRefToTarget(ref,shpmean.res.f,mag=5, method="TPS", links=links)
res.m<-plotRefToTarget(ref,shpmean.res.m,mag=5, method="TPS", links=links)

### Principal components analysis ###

# Run the PCA
smeltshp.pca<-plotTangentSpace(smeltshp.gpa$coords)

# Get the PC axis importance. i.e. variance explained
smeltshp.pca$pc.summary$importance

# Plot aggregated body shape data
# aggregate the data
group <- factor(paste(pop, sex)) # make a 4 level factor
levels(group) # see the levels


# function requires a 2D array format,
# also returns the group names in the first column so we omit that
means <- (aggregate(two.d.array(smeltshp.gpa$coords) ~ group, FUN=mean))[,-1]
rownames(means) <- levels(group)

# make a 3D array again
means <- arrayspecs(means, 16, 2)
means

# Repeat the PCA with aggregated data
smeltshp.pca<-plotTangentSpace(means)
```

```
class(smeltshp.pca$coords)

# Get the PC axis importance
smeltshp.pca$pc.summary$importance

# Get the PC scores for axes 1 and 2
smeltshp.pca$pc.scores[,1]
smeltshp.pca$pc.scores[,2]

# Get the loadings (rotations)
smeltshp.pca$rotation[,1]
smeltshp.pca$rotation[,2]

# Create object containing loadings
df.load<-data.frame(smeltshp.pca$rotation[,1], smeltshp.pca$rotation[,2])
df.load

# generate a new dataframe with PC scores, system type and population factors
pc1<-smeltshp.pca$pc.scores[,1]
pc2<-smeltshp.pca$pc.scores[,2]
st<-as.factor(c("riv", "riv", "riv", "riv", "lake", "lake", "riv", "riv", "lake", "lake", "riv", "riv", "lake", "lake", "riv",
                "riv", "lake", "lake", "riv", "riv", "lake", "lake"))
pop<-as.factor(c("bargo", "bargo", "broken", "broken", "cordeaux", "cordeaux", "georges", "georges", "hume",
                "hume", "kiewa", "kiewa", "lake nep", "lake nep", "nep riv", "nep riv", "nilla", "nilla", "ovens",
                "ovens", "waranga", "waranga"))
sex<-as.factor(c("m", "f","m", "f","m", "f","m", "f","m", "f","m", "f","m", "f","m", "f","m", "f","m", "f","m", "f"))
label<-as.factor(c("BAR", "BAR", "BRK", "BRK", "LCO", "LCO", "GRS", "GRS", "LHM", "LHM", "KWR", "KWR",
                "LNP","LNP", "NPR", "NPR", "LNC", "LNC", "OVR", "OVR", "WRG", "WRG"))
df.shp<-data.frame(pc1, pc2, st, pop, sex)

# Create the vectors that define the outline of the convex hulls (boundaries showing scatter of the population
means
# For river populations
df.shp[st=="riv",]
coord.x.rivpoly<-c(-0.011271502, 0.003645462,  0.011813140, 0.013341412, -0.004361134, -0.006908106)
coord.y.rivpoly<-c(7.964063e-05, -6.241528e-03,-9.210664e-04, 4.141259e-04,  1.972153e-02, 1.541818e-02)
# For reservoir populations
df.shp[st=="lake",]
coord.x.respoly<-c(-0.0106336090, 0.0235796936,0.0219850114, 0.0033473946,  -0.0111529097 )
coord.y.respoly<-c(-0.014057368,-0.004910238,-0.002910420, 0.004131358 , -0.006977869 )
# Objects for the axes labels
xlab <- paste("Principal Component 1 (35.6%)")
ylab <- paste("Principal Component 2 (21.1%)")

# Define the wireframe links for the deformation grids
ref=mshape(smeltshp.gpa$coords)
wf<-read.csv("links.csv") #create wireframe links

### Set up the matrix for the multi-part figure
par(mar=c(4, 4, 1, 1)) # sets the margins
mat <- matrix(c(4,5,0,1,1,2,1,1,3), 3)
layout(mat, widths=c(.9,1,1), heights=c(1,1,0.6))# set the size of the rows and columns
plot(df.shp$pc1,df.shp$pc2,
    pch=c(21,22)[as.factor(df.shp$st)],
    bg=c("black", "white")[as.factor(df.shp$sex)],
    cex=3, xlab=xlab, ylab=ylab, asp=F, xlim=c(-.015,0.025), ylim=c(-0.018,0.025), cex.lab=1.4, cex.axis=1.4)
identify(df.shp$pc1, df.shp$pc2, labels = label, plot=TRUE, offset=1, cex=.7)
legend(0.012, 0.025,
     legend= c("Reservoir female", "Reservoir male", "River female", "River male"), pt.cex=2.5,
```

```
    bg=c("black", "white", "black", "white"),
    y.intersp=1,
    pch=c(16, 1, 15, 0), cex=1.3, bty="n")

### To plot convex hulls or polygons around the group means, use the Polygon function,
polygon(coord.x.rivpoly, coord.y.rivpoly)
polygon(coord.x.respoly, coord.y.respoly)

# sets the margins
par(mar = c(0,0,0,0))

# Plot shape deformations corresponding to upper and lower ends of PC axes
plotRefToTarget(ref,smeltshp.pca$pc.shapes$PC1min,mag=3, method="TPS", links=wf)
plotRefToTarget(ref,smeltshp.pca$pc.shapes$PC1max,mag=3, method="TPS", links=wf)
plotRefToTarget(ref,smeltshp.pca$pc.shapes$PC2max,mag=3, method="TPS", links=wf)
plotRefToTarget(ref,smeltshp.pca$pc.shapes$PC2min,mag=3, method="TPS", links=wf)

#### Linear model of body shape as function of system type (habitat), population (location) and sex ###
smelt.shp0<-procD.lm(smeltshp.gpa$coords~(habitat/location)*sex, iter=999)
# Get ANOVA summary table
print(smelt.shp0)
# Get random sums of squares
smelt.shp0$random.SS

# find expected means squares for the model terms
terms(~(habitat/location)*sex)

# find degrees of freedom
smelt.shp0$df

# calculate F values for system type (habitat) fixed factor
random.MS.hf<-smelt.shp0$random.SS[1,]/smelt.shp0$df[1] # random MS for habitat
random.MS.ranef<-smelt.shp0$random.SS[5,]/smelt.shp0$df[5] # random MS for 3 way interaction
random.MS.hf[1]
random.MS.ranef[1]
random.Fs.hf<-random.MS.hf/random.MS.ranef # random F for habitat
random.Fs.hf[1] # observed F value for habitat

# calculate F values for sex fixed factor
random.MS.sf<-smelt.shp0$random.SS[2,]/smelt.shp0$df[2] # random MS for sex
random.Fs.sf<-random.MS.sf/random.MS.ranef # random Fs for sex
random.MS.sf[1] # observed MS value for sex
random.Fs.sf[1] # observed F value for sex

# F values for hab:loc interaction random effect
MSE<-0.0005931 # model error
random.MS.hlr<-smelt.shp0$random.SS[3,]/smelt.shp0$df[3] # random MS for hab.loc interaction
random.Fs.hlr<-random.MS.hlr/MSE # random F-value printed for habitat
random.MS.hlr[1] #observed F value for random effect
random.Fs.hlr[1] #observed F value for random effect

# F values for hab:sex interaction fixed effect
random.MS.hsf<-smelt.shp0$random.SS[4,]/smelt.shp0$df[4]
random.Fs.hsf<-random.MS.hsf/random.MS.ranef
random.Fs.hsf[1]

# F value for hab:loc:sex interaction random effect
random.Fs.ranef<-random.MS.ranef/MSE
random.Fs.ranef[1]
```

```
### new Z scores and P values ###
# habitat z and p
geomorph:::pval(random.Fs.hf) # P-value
geomorph:::effect.size(random.Fs.hf) # Z score

# sex z and p
geomorph:::pval(random.Fs.sf) # P-value
geomorph:::effect.size(random.Fs.sf) # Z score

# habitat*location z and p
geomorph:::pval(random.Fs.hlr) # P-value
geomorph:::effect.size(random.Fs.hlr) # Z score

# habitat*sex z and p
geomorph:::pval(random.Fs.hsf) # P-value
geomorph:::effect.size(random.Fs.hsf) # Z score

# habitat*loc*sex
geomorph:::pval(random.Fs.ranef) # P-value
geomorph:::effect.size(random.Fs.ranef) # Z score

### Test if sex needs to be included as a factor
# Make geomorph data frame
gdf<-geomorph.data.frame(shape=smeltshp.gpa$coords, habitat=mydata$habitat, location=mydata$location,
sex=mydata$sex) # make geomorph data frame

### Run advanced procrustes ANOVA for post hoc analysis
# Specify two models (one with sex and one without) to test if sex needs to be included
smelt.shp1<-advanced.procD.lm(shape~(habitat/location)*sex, ~habitat/location, groups=~habitat*sex,
iter=999, data=gdf)
summary(smelt.shp1)

# Get the post-hoc analysis (procrustes distances among least squares means and P-values)
smelt.shp1$Terms
smelt.shp1$LS.means.dist
?advanced.procD.lm
head(gdf)
str(gdf)
aov.smelt.shp1

############################################################
######### PERMANCOVA FOR Chapters 3 and 4 ################
############################################################

# Open packages
library("vegan")
library("ggplot2")
library("geomorph")
library("Hmisc")

# set working directory
setwd("C:/Users/Daniel Svozil/Dropbox/CSU/PhD/U-crit/U-crit data")

# Read in the data
mydata<-read.csv("Ucrit body shape 2015 070317.csv", header = T, stringsAsFactors = F)
mydata2<-read.csv("Ucrit body shape 2015 070317 minNIL.csv", header = T, stringsAsFactors = T)

# Run PERMANCOVA for critical swimming speed with standard length as covariate
```

```
# Same code used for chapter 3 (fin aspect ratio, fineness ratio) and chapter 4 (heart mass, metabolic # enzyme
activity and gill mass
ucrit.aov<-adonis(ucrit~(habitat/location)*sex+sl, method="euclidean", data=mydata, nperm=999)
ucrit.aov

# Get z values
perm.test<-permustats(ucrit.aov, nperm=999, pairwise=TRUE)
summary(perm.test)

#plot means and standard errors
ucritplot <- ggplot(mydata,aes(x=paste(habitat, sex), y=sl, colour=habitat))
ucritplot +
  stat_summary(fun.data = "mean_se", geom = "errorbar", width=0.1, mapping=aes(group="habitat")) +
 stat_summary(fun.y = "mean", geom = "point", size=3, mapping=aes(group="habitat"))+
 labs(y="U-crit (cm/s)", x=NULL)+
 theme(
   legend.position = "none",
   plot.title = element_text(hjust=0.5),
   axis.line = element_line(size=1),
   panel.border = element_rect(fill=NA, size=1),
   panel.background = element_blank(),
   axis.text.y=element_text(size=12, margin=margin(r=10)),
   axis.text.x=element_text(size=12, margin=margin(t=10)),
   axis.title.y=element_text(size=16, margin=margin(r=10)),
   axis.title.x=element_text(size=16, vjust=1, margin=margin(t=15, b=15)),
   axis.ticks.x = element_line(size=.75),
   axis.ticks.y = element_line(size=.75),
   axis.ticks.length=unit(-.2,"cm"),
   plot.margin = margin(10,10,10,10))

# Geometric morphometric analysis for body shape data using code described previously for chapter # 2

############################################################
###### BEST SUBSET SELECTION AND REGRESSION ANALYSIS ######
############################################################

# Open packages
library(“leaps”)

# set working directory
setwd("C:/Users/Daniel Svozil/Dropbox/CSU/PhD/U-crit/U-crit data")

# Open the data file containing mean ucrit, fin aspect ratios, and fineness ratios for sex*system type # groups
subset.data<-read.csv("subsetselection080316.csv", header=T)

# Create data frame of predictor variables and response variable
predictors2<-subset.data[,6:10]
ucrit<-subset.data$mean.ucrit
df<-data.frame(ucrit, predictors2)

# run subset selection for 2 best models of each model size (i.e. no. of predictor variables)
best.pred3<-regsubsets(ucrit~., data=predictors2, nbest=2, nvmax=5, method="forward")
summ3<-summary(best.pred3)
summ3$outmat # matrix of variables in each model
summ3$adjr2 # adjust R-squared values

# regression for linear model 3
lm.3<-lm(mean.ucrit~mean.pfar+mean.cfar, data=subset.data)
summary(lm.3)
```

```
# regression for linear model 5
lm.5<-lm(mean.ucrit~mean.pfar+mean.cfar+pc.score.2, data=subset.data)
summary(lm.5)

# plot the linear models from lm.3 and lm.5
# set figure parameters
par(tck=0.02)
par(mar=c(2.5,2.5,.25,.25), mgp=c(1.5,.4,0), tck=0.02)
plot(subset.data$mean.cfar, subset.data$mean.ucrit, ylim=c(15,60), xlim=c(0.9,2.6), pch=21, cex=1.3,
      col="black", bg="white", xlab="Aspect ratio", ylab="U-crit (cm/s)")
points(subset.data$mean.pfar, subset.data$mean.ucrit, pch=21, cex=1.3, bg="black", col="black")

# lm.3
abline(0.8346, 25.9216, lty=2)
# lm.5
abline(-1.781, 27.785)

# add a legend
legend(2.1, 34, c("Model 3", "Model 5"), bty="n", lty=c(2, 1), cex=1)
legend(2.1, 31, c("Obs. caudal fin AR", "Obs. pectoral fin AR"), bty="n", pch=c(1,16,17), bg=c("black", "white"),
cex=1, pt.cex=1.3)

# Test assumptions of linearity
plot (lm.3)
```

Appendix 4 – Expected mean squares (EMS) table for non-parametric ANOVA/MANOVA

In three-way nested factorial experiments, *F*-ratios are calculated using the following expected mean squares which state the expected variation attributable to a model term (either residual error or fixed effects). Table 7.4 shows the model terms, the mean squares for each term and the expected mean squares and *F*-ratios used in the calculation of significance (P-values) for each model term.

Table 7.4. Factors, mean squares (MS), sources of variation attributable to each model term (expected mean squares; EMS) and the F-ratio for calculating significance of each model term.

Factor	MS	EMS	F-ratio
Habitat SS_A	$SS_A/(a-1)$	$\sigma^2 + n\sigma^2{}_{AC} + bnK_A$	$\frac{SS_A}{SS_{AB}}$
Sex SS_B	$SS_b/(b-1)$	$\sigma^2 + nK_{AB} + abnK_B$	$\frac{SS_B}{SS_{AB}}$
CS SS_{cov}	$SS_{cov}/1$	σ^2	$\frac{SS_B}{SS_R}$
Habitat × sex SS_{AB}	$SS_T - SS_{A+B+C+AC} - SS_R$	$\sigma^2 + nbc\sigma^2{}_{ABC} + nK_{AB}$	$\frac{SS_{AB}}{SS_{ABC}}$
Habitat × location SS_{AC}	$SS_T - SS_{A+B+C+AB} - SS_R$	$\sigma^2 + n\sigma^2{}_{AC}$	$\frac{SS_{AC}}{SS_{ABC}}$
Habitat × location × sex SS_{ABC}	$SS_T - SS_{A+B+C+AC+AB} - SS_R$	$\sigma^2 + n\sigma^2{}_{ABC}$	$\frac{SS_{ABC}}{SS_R}$
Residual SS_R	$SS_R = \frac{1}{n}\sum_{i=1}^{N-1}\sum_{j=i+1}^{N} d_{ij}^2 \epsilon_{ij}^C$		
Total	$SS_T = \frac{1}{N}\sum_{i=1}^{N-1}\sum_{j=i+1}^{N} d_{ij}^2$		

Appendix 5 – Variable loadings for PC 1 and PC 2 from PCA on geometric morphometric data (Chapter 2.3.2).

The table of loadings from a PCA is not conventionally used to interpret shape variation. It can however, provide insight into the magnitude of the changes in landmark locations relative the two main axes of variation (PC 1 and PC 2) in a principal components analysis.

Table 7.5 Variable loadings for PC 1 and 2 from PCA of body shape variation among populations of Australian smelt from river and reservoir habitat based on geometric morphometric analysis of 16 landmarks. Most variable loadings lie between 0.10 and 0.25, with those exceeding 0.25 in bold-type. Columns *x* and *y* are the *x* and *y* coordinates for 16 landmarks.

	PC1		PC2	
Landmark	*x*	*y*	*x*	*y*
1	**-0.417**	0.012	0.001	**0.287**
2	**0.326**	0.208	0.031	0.128
3	0.132	-0.051	-0.140	**0.304**
4	**0.508**	-0.110	-0.202	**0.264**
5	-0.144	0.014	-0.051	0.083
6	-0.084	0.026	0.000	-0.040
7	-0.214	0.035	-0.049	-0.095
8	0.004	0.082	0.079	-0.021
9	-0.154	0.102	-0.036	-0.157
10	-0.227	-0.050	0.217	**-0.481**
11	0.115	-0.160	**0.296**	**-0.399**
12	0.175	-0.201	0.021	-0.098
13	-0.163	-0.116	-0.075	0.091
14	0.133	0.004	-0.055	-0.154
15	0.121	0.124	0.006	0.104
16	-0.113	0.079	-0.042	0.185

Appendix 6 – Variable loadings for PC 1 and PC 2 from PCA on traditional morphometric data (Chapter 2.3.3).

The table of loadings from a PCA is not conventionally used to interpret shape variation. It can however, provide insight into the magnitude of the changes in linear truss measurements along the two main axes of variation (PC 1 and PC 2) in a principal components analysis.

Table 7.6 Variable loadings for PC 1 and 2 from PCA of body shape variation among populations of Australian smelt from river and reservoir habitat based on traditional morphometric analysis of 23 linear measurements. Most variable loadings lie between 0.03 and 0.25, with those exceeding 0.25 in bold-type. Acronyms for linear truss descriptions are defined in Table 2.2 (p. 34).

Linear truss	PC1	PC2
nsm	0.199	0.212
sl	0.185	-0.064
npecf	0.201	0.096
nmdb	0.238	**0.341**
nmx	0.230	0.161
hl	0.190	0.079
smdf	0.172	-0.086
smvcf	0.172	-0.133
bd	0.244	0.056
smmdb	0.222	0.084
dcp	0.197	-0.151
dcpv	0.185	-0.173
dfaf	0.241	**-0.264**
dfpecf	0.183	-0.070
dcfcp	0.213	-0.206
cpd	0.213	-0.157
vcfcp	0.270	-0.131
vcfaf	0.187	-0.088
afpf	0.169	-0.124
pfmdb	0.146	**-0.254**
mdbmx	**0.280**	**0.661**
tl	0.184	-0.103

Appendix 7– Aquarium and swim flume temperatures measured during experiments for chapter 3 and 4

Temperatures measured in aquaria in which fish were held prior to critical swimming speed tests for chapter 3 and 4 using Hobo U22-001 temperature loggers (Table 7.7). Temperatures in holding aquaria were logged at 10 minute intervals for the entire experimental period (July 4 to June 30, 2015 and June 5 to June 22, 2016). Temperatures were also monitored throughout the critical swimming speed tests, using a Hobo U22-001 temperature logger in the sump supplying water to the swim tunnel (Table 7.8).

Table 7.7. Temperatures for holding aquaria measured during the experimental period in 2015 and 2016. S.E. is 1 standard error.

	Chapter 3 (2015)			Chapter 4 (2016)	
Aquarium	^oC	± S.E.	Aquarium	^oC	± S.E.
T1	20.767	0.0010	T1	20.685	0.0001
T4	20.638	0.0001	T4	20.670	0.0001
T7	20.779	0.0001	T7	20.838	0.0001
T10	20.549	0.0001	T10	20.676	0.0001
T13	20.631	0.0001	T13)	20.589	0.0001
T15	20.779	0.0001	T15	20.692	0.0001

Table 7.8. Temperatures measured in swim flume at 10 minute intervals for all critical swimming speed tests in chapter 3 (2015) and chapter 4 (2016).

Chapter 3 (2015)	Chapter 4 (2016)
20.613 ± 0.001	20.318 ± 0.002

Appendix 8 – Swim flume calibration

The swim flume used for the critical swimming speed experiments in chapters 3 and 4 was calibrated by measuring the average flow rate per unit of time ($L\ s^{-1}$). The ball valve at the entry to the swim flume was set at a particular angle. A stopwatch was used to measure the time taken to fill a 10L bucket at 21°C (proposed experimental temperature) and the depth of the water inside the working section of the swim flume chamber (cm) was measured with a ruler. This was repeated 3 times, each on a different day and used to calculate a mean flow rate for 19 different ball valve positions (angles). The mean flow rates, in (cm^3) were then converted to flow velocities (U_{flow} in $cm\ s^{-1}$) as:

$$U_{flow} = \frac{flow\ rate}{w\ \times\ h}$$

Where *w* is the width of the swim flume chamber and *h* is the water depth (*cm*) inside the swim flume chamber. The mean flow velocities were then plotted as a linear regression against the corresponding ball valve angle (Fig. 7.2 and 7.3) to derive a linear relationship between ball valve angle and flow velocity. The linear equation was then used to determine the required ball valve angle to achieve a desired flow velocity inside the swim flume chamber.

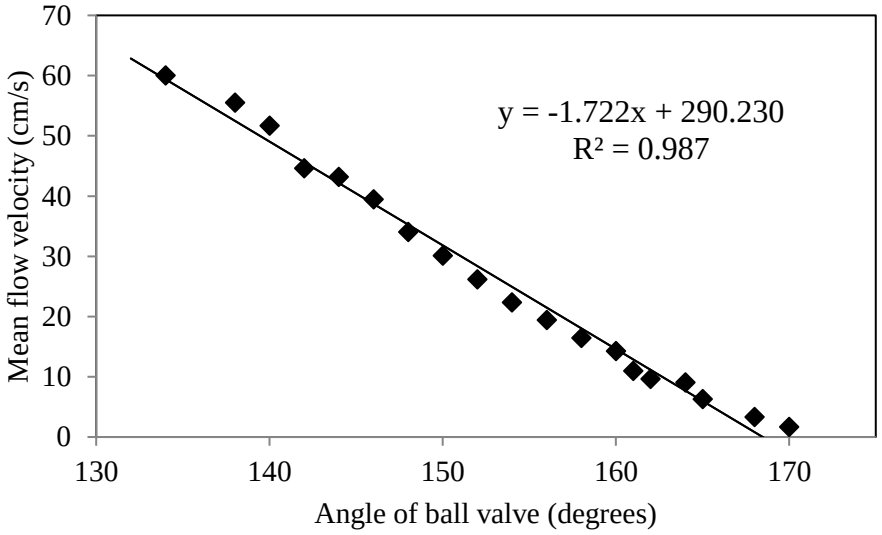

Fig. 7.2. Linear model of mean flow velocity as a function of ball valve angle. These results are from the critical swimming speed experiments for chapter 3 carried out in 2015.

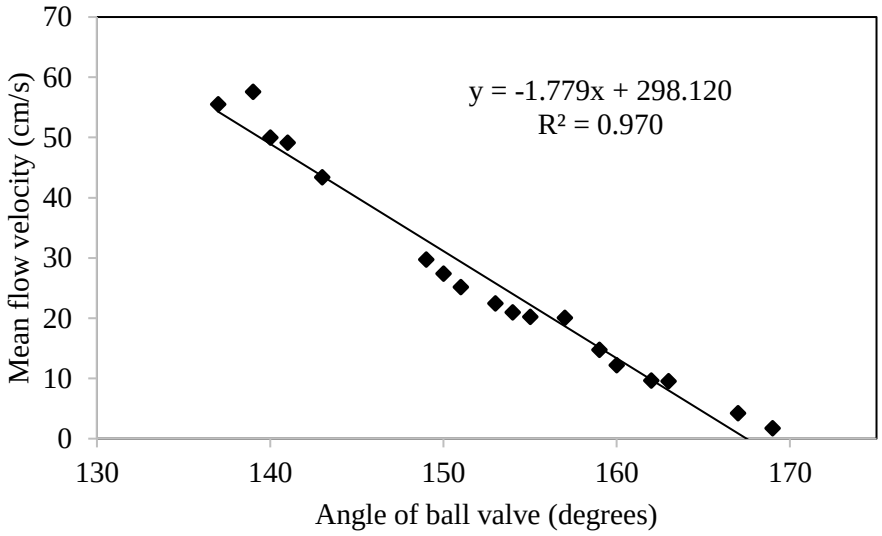

Fig. 7.3. Linear model of mean flow velocity as a function of ball valve angle. These results are from the critical swimming speed experiments for chapter 4 carried out in 2016.

Appendix 9 – Testing assumptions of linearity: linear regression chapter 3

The residuals for critical swimming speed (U_{crit}) from linear regression analyses in chapter 3 were tested for linearity using Cook's distance (Fig. 7.4), scale-location plot (Fig. 7.5) and quantile-quantile plots (Fig. 7.6).

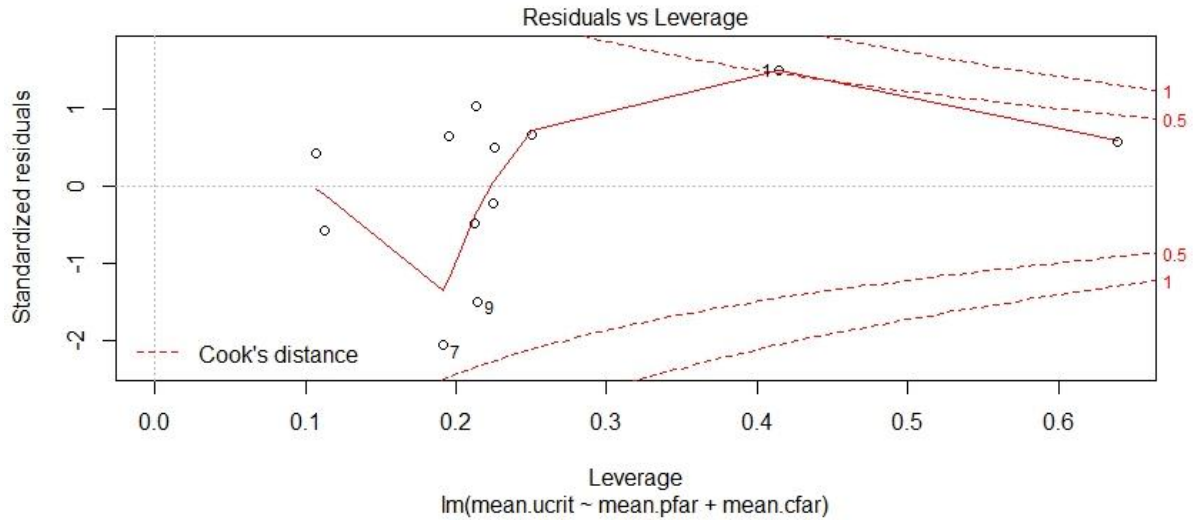

Fig. 7.4. Cook's distance plot for linear model 2 from chapter 3. Cook's distance is defined as the difference between parameter estimates (β) and its value if the *i*th observation was deleted (β_{-i}), otherwise referred to as leverage (McDonald 2002).

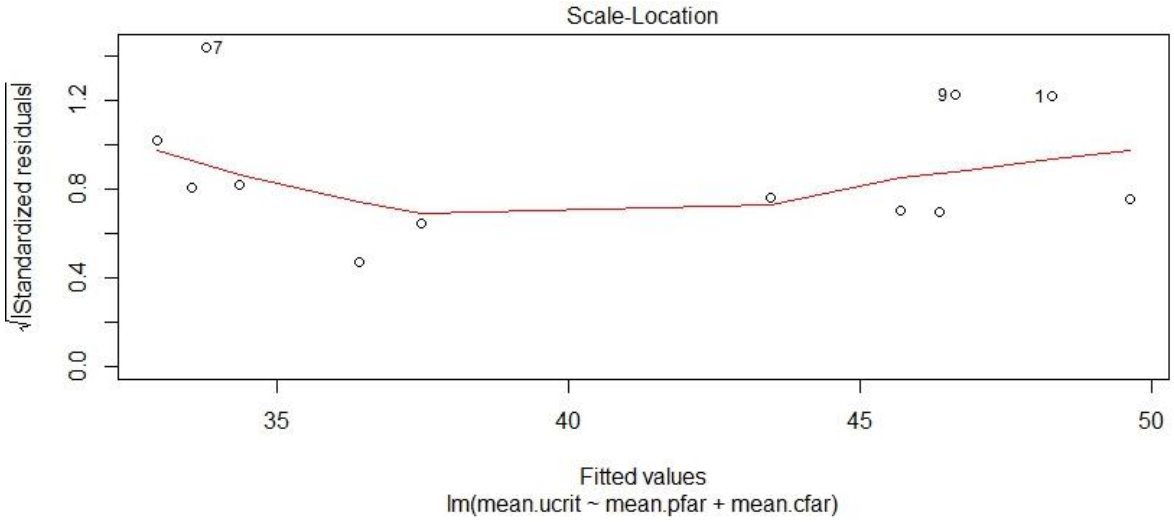

Fig. 7.5. Quantile-quantile (Q-Q) plot for linear model 2 in chapter 3, showing plot of residuals against predicted critical swimming speed values (theoretical quantiles).

Two observations (top right corner, Fig. 7.4) appears to have relatively high leverage to the other observations, however these observations are not influential on the regression parameters as they do not exceed the lowest cited Cook's distance value of $D_i = 0.5$ (inner broken line labelled on right margin) (McDonald, 2002). Residuals are approximately normally distributed, as there no large deviations of residuals from predicted values (Fig. 7.5;

broken line) and have a good degree of homoscedasticity (consistent variation) of standardized residuals across the range of predicted critical swimming speeds (Fig. 7.6; fitted values). Generally, residuals from linear models of critical swimming speed from chapter 3 do not violate assumptions of normality and all data points were retained.

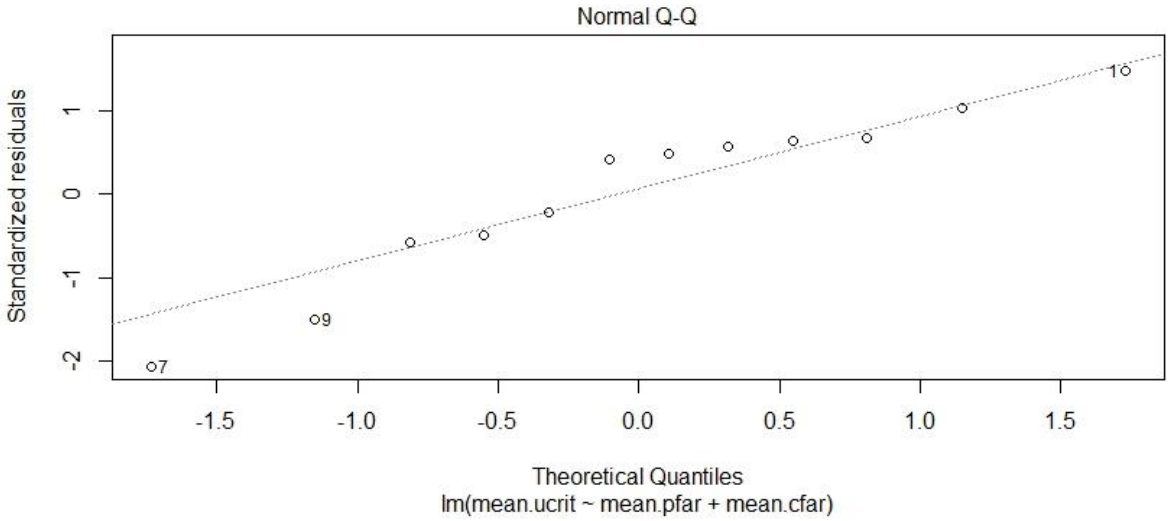

Fig. 7.6. Scale-location (heteroscedsticity) plot showing spread (relative scale) of residuals for predicted values of critical swimming speed for model 2 in chapter 3.