QUANTITATIVE ANALYSIS OF THE FINE STRUCTURE OF THE FISH GILL: ENVIRONMENTAL RESPONSE AND RELATION TO WELFARE.

Fish Feeding by Hawaa Jenjan

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Thesis submitted for degress of Doctor of Philosophy

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September, 2011

Abstract

- Methods were developed to quantify variation in gill size and microstructure and applied to three fish species: brown trout, Arctic charr and common carp. Measurements of arch length, number and length of gill rakers, number and length of gill filaments and number, length and spacing of the lamellae were taken for each gill arch and combined by principal component analyses to give length-independent scores of gill size. Levels of fluctuating asymmetry in gill arch length were also examined. Buccal and gill cavity volumes were measured from silicon moulds. Standard histological methods were used to examine gill microstructure

- Benthic-feeding charr from a sample collected in Loch Awe, Scotland had relatively larger heads and buccal cavities than did sympatric pelagic-feeding fish Allowing for body size, they also had a more extensive respiratory surface, perhaps reflecting exposure to poorly oxygenated water while feeding on the loch bottom and/or a more active life style. Levels of asymmetry in gill arch length were higher in the pelagic-feeding form, which grow faster than the benthic-feeding form (Chapter 2).

- Gill size and structure were compared in carp (Chapter 3) and trout (Chapter 4) classified by a standard test as having proactive, reactive or intermediate stress copping styles. Proactive carp and trout had more extensive respiratory surfaces and lower levels of hyperplasia than did reactive fish, intermediate fish lying in between. The opposite was the case for density of mucous cells, which was highest in reactive fish and lowest in proactive ones. These data suggest that maintaining a large respiratory surface may represent an unrecognised cost of a proactive coping style.

- Common carp were held in mixed groups of proactive and reactive fish in one of 6 combinations of temperature (20^{o}C and 25^{o}C) and dissolved oxygen (3-4, 5-6 and 7-8 mg O_2 L-^{1}) for 10 weeks. At the higher temperature fish had relatively larger heads and longer secondary lamellae, but had fewer mucous cells and a lower percentage of hyperplasia. At the lowest oxygen levels fish had relatively larger heads and a higher degree of hyperplasia than those held in normoxic and hyperoxic conditions. These results suggest that, over weeks, carp are able to "remodel" their respiratory structures in response to their current oxygen requirements. Few clear differences in response were found between proactive and reactive fish (Chapter 5).

- In semi-extensively farmed carp sampled over their final production year. Short-term, acute husbandry stressors (grading and crowding) produced striking changes in

several potential welfare indicators, including reduced body condition, increased in plasma glucose, lactate and cortisol levels and higher level of body damage. Percentage hyperplasia and secondary lamella number and length also increased. Long-term acute stress (pre-harvest crowding in concrete tanks) was associated with increased levels of skin and fin damage and in hyperplasia and mucus cell number, reflecting high stress levels and/or poor water quality. Glucose, lactate and cortisol levels fell, suggesting either habituation to current conditions or differential mortality by physiological stress status (Chapter 6).

- The results of Chapters 2-6 are synthesised in a general discussion (Chapter 7) and considered in the context of the existing literature on trophic polymorphism, on stress coping strategies, on the effects of environmental conditions of the welfare of cultured fish and on how gill structure and microstructure relate to other indicators of welfare.

Dedications

To the soul of my late father and to my beloved mother

To you my wife Asma

To my daughters Hawaa and Azad.

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Acknowledgements

My sincere solutions to Allah the almighty for his blessings throughout my work.

I am especially grateful to my first supervisor Professor Felicity Huntingford for her continuous encouragement, guidance and support, not only giving me academic advice, but also for the tremendous amount of moral support when things (e.g., my English language when I started my PhD or the bad situation in my home country Libya at these moments) not going too well. Her high commitment to my work even after her retirement was impressive and greatly appreciated. Also I wish to express my deep gratitude and thanks to my second supervisor Professor Colin Adams for suggesting the research problem, guidance, fruitful advice and enlightening comments throughout the course of study.

I would like also to express thanks to Lorna and Florence for all your smile, all help and the kindness.

Thanks also to Dr. Maciej Pilarczyk in Poland for tank and pond common carp data that you collected and for all your help.

I am grateful to John Laurie, Patricia McLaughlin, Graham Law, Graham Adams, Alistair, George and SCENE people for all help in all things technical.

I wish also to acknowledge the help of Dr Ian Montgomery, Andrew Lozkhart, David Russele and Catheriwe Boyd for assistance in fish gill histology and Margaret Mullin and Dr. Lawrence Tetley for help in electron microscope.

A special thanks to my dear friends and my office mates; Flavia, Fabio, Helen, Maria, Fathi, Aiad, Emma and Lyndsey.

I am grateful to the Libyan embassy-UK for financial support. Also I would like to extend my thanks to University of Benghazi (Garyounis).

Finally, a warm thanks to my family in Libya, My wife and my daughters for all support.

Author's Declaration

I declare that the work recorded in this thesis is my own, and no part of the work here has been submitted for any other degree. Supervisions by F.A. Huntingford and C. Adams have helped develop ideas through this work.

Hussein B.B. Jenjan

CHAPTER 1

GENERAL INTRODUCTION

1 General introduction

1.1 Introduction

Together, the five pieces of work described in this thesis were designed to quantify the extent of natural within-species variation in the fine structure of gills in three species of teleost fish, namely brown trout (*Salmo trutta*), Arctic charr (*Salvelinus alpinus*) and common carp (*Cyprinus carpio*), relate such variability to differences in life style and environmental conditions and explore the relationships between gill status and other commonly used indicators of welfare in cultured fish. To put this work in context, this chapter starts with an account of the structure and function of fish gills. It then moves on to consider what welfare means, whether the term applies to fish, how welfare is usually measured (including stress responses in fish) and how it may be compromised in aquaculture. The chapter then described the biology of the three study species and aspects of intra-specific variation that might be reflectes in differences in gill structure, before summarising the aims and structure of the thesis as a whole.

1.2 Gill structure and function

Gills provide a very large surface allowing contact between the circulatory system of the fish and the water in which it lives. Their primary function is to allow the uptake of oxygen and excretion of carbon dioxide, but they also contains several cell types that play an important role in ionic regulation. For example, when euryhaline fish are transferred from fresh water to sea water, chloride celles are increased in number and size, along with an increase in Na^+ / K^+ _ ATPase activity (Jenjan 2002). In freshwater teleost gills some pavement cells can play an active role in ion uptake and acid-base transport by the fish gills. In general, fish gills are allow the movement of other substances into and out of the fish and so are particularly sensitive to any changes in the environment (Burggren *et al.,* 1992; Chapman, 2007; Saadatfar and Shahsavani, 2011). Gills are highly complex structures, so describing and measuring them is much more difficult than is the case for more simple organs, such as the skin, intestine, bladder or operculum (Wilson and Laurent, 2002). Gills should be regarded as a whole organ, including several types of epithelia and many different kinds of cell, linked to a compound system of vessels and under complex neural control (Dunel-Erb *et al.,* 1994).

1.2.1 Gill structure

Overall, the structure of respiratory surfaces is relatively constant across the different groups of fish, especially in the teleosts (all the common bony fishes) and holosteans (bony fish such as the bowfin, *Amia calva* that show primitive characteristics). Gill structure is more variable in the chondrichtyes (cartilaginous fishes such as sharks, rays and skates. Daborn *et al.,* 2001; Wilson and Laurent, 2002). In the bony fish, the respiratory surface is supported by gill arches, but in the chondrichtyes the gill filaments are supported by an inter-branchial septum that runs from the gill arch to the outer body wall, while in the cyclostomes (jawless fishes including lampreys and hagfish) the gills are strengthened by divisions of the pharyngeal pouch.

In teleosts, each gill arch (Figure 1.1) consists of large number of gill filaments and a number of gill rakers. The individual gill arches are supported by vertical elements of the gill skeleton, which run through the medial portion of each arch. The respiratory surface itself is positionning on the gill lamellae, associated with abductor muscles that are essential for locating of the gill filament in the water flow (Hughes, 1984). The gill filaments are dorso-ventrally flattened and filament surface area is wholly made up of folds or secondary lamellae. The felaments support the secondary lamellae, which comprise the main respiratory surface and its supporting system of capillary blood vessels, providing an exchange region within the gill filament. A system of muscles controls the precise orientation of the gas exchange surface (Evans *et al.,* 2005).

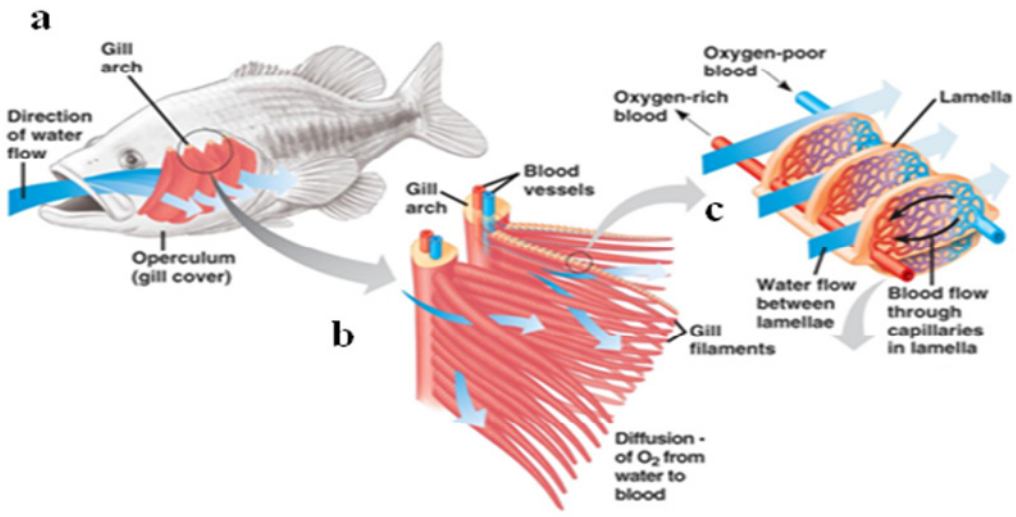


Figure 1.1. Schematic diagram of the teleost fish gill. a) location of gills without gill cover (operculum), revealing four gill arches on each side, b) segments of gill arches shows gill arches, number of gill filament, blood vessels and direction of water flow and c) dissection of gill filament showing the gill secondary lamellae, blood capillaries and the direction of blood and water flow. Figure from website: http://quizlet.com/2213010/ch-42-circulation-gas-exchange-ap-bio-flash-cards/

The gill rakers on the gill arches (Figure 1.2) act as a food filtration mechanism (Eggold and Motta, 1992; Sanderson et al., 1996; Sanderson et al., 2001; Amundsen *et al.,* 2004; Østbye *et al.,* 2005), being used by fish for straining food and other material from the water (Munshi *et al.,* 1984; Schluter, 1994). The feeding habits of fish have usually been found to relate to the number and length of gill rakers. Generally fish with high gill raker numbers are planktivoures, whereas those with lowest gill raker numbers are usually benthivorous (Doherty and McCarthy, 2004; Amundsen *et al.,* 2004).

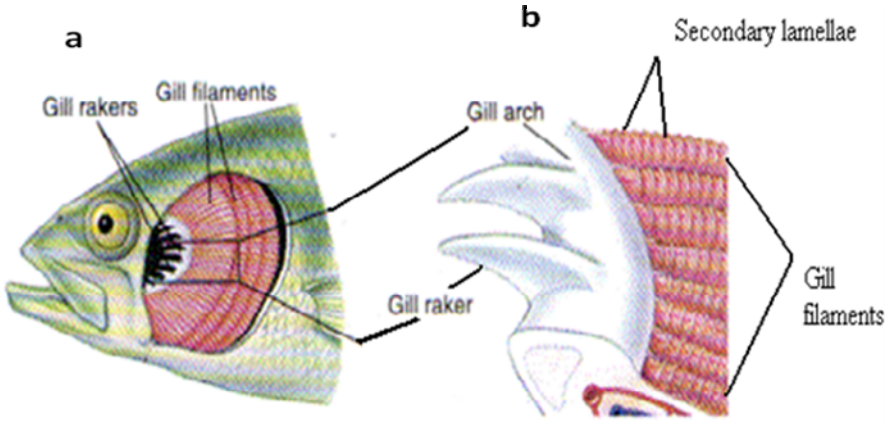


Figure 1.2. Schematic diagram of the teleost fish gill. a) location of gills without gill cover (operculum), revealing four gill arches on each side, gill filaments and gill rakers, b) segments of gill arch show gill arch, gill rakers, number of gill filament and number of secondary lamellae. Figure from website: http://www.cabrillo.edu/~jcarothers/lab/notes/fishapods/index.html

The teleosts are characterized by the presence of a bony gill cover (operculum), which develops from a fold in the hyoid arch and is a hard structure composed of dermal bone, with a crescent-shaped caudal opening (Romer and Parsons, 1986).

1.2.2 Gill microstructure

The secondary lamellae are shaped by cross-folding of the upper and lower sides of the gill filament (Figures 1.1 and 1.2) and are in regularly spaced rows along the length of the gill filament, though in the gill arches they are inclined at a slight angle to the long axis of the gill filament. The filaments and lamellae are bounded on both sides by a thin epithelium (Laurent and Dunel, 1980; Khoshnood *et al.,* 2011. See Figures 1.3 and 1.4) which is thinner in the lamellae than on the filament (Olson, 2002). The lamellae from adjacent gill filaments come into close proximity to each other, forming a sieve-like arrangement through which water currents pass, before travelling out at the back of the rows of

secondary lamellae. The respiratory currents run counter to the lamellar blood flow, improving the efficiency of gas exchange.

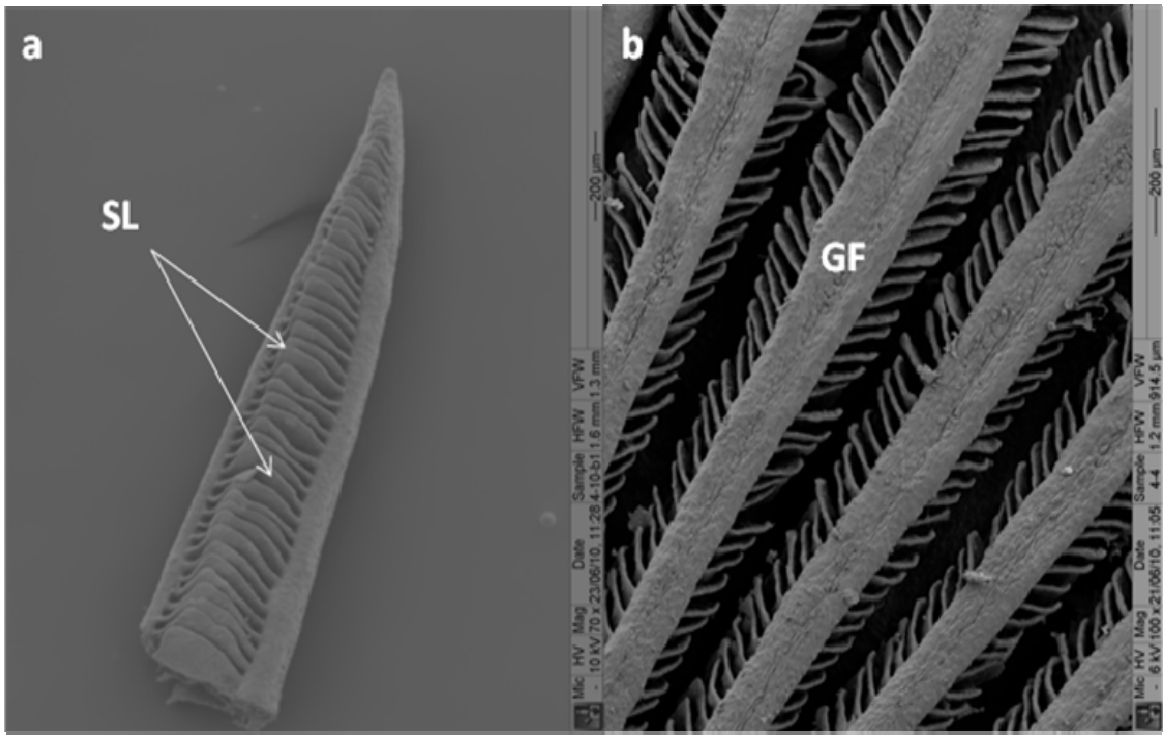


Figure 1.3. Scanning electron micrograph of six gill filaments of the brown trout (*Salmo trutta*). One gill filament is lying flat (a) while another five (b) have leading edge facing up. From these two micrographs the gill secondary lamellae (SL), which are present on two faces of the gill filaments (GF).

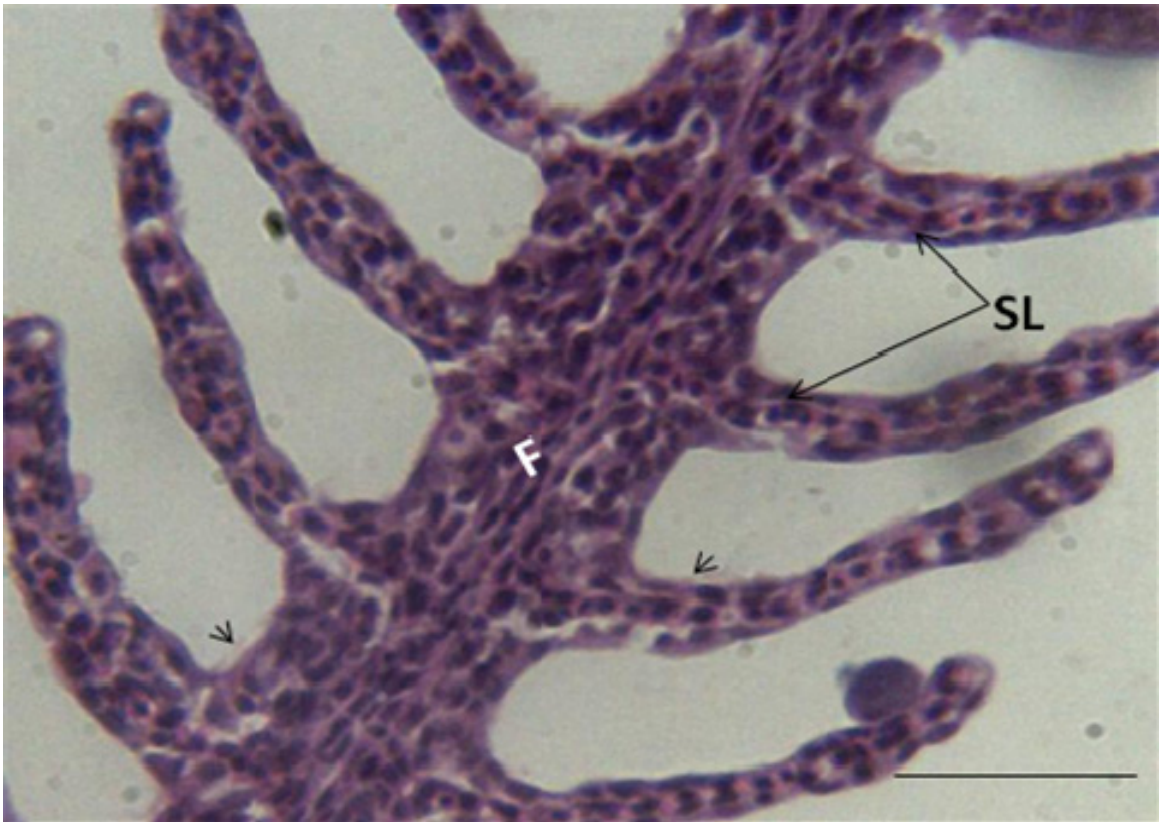


Figure 1.4. Histological section of gill of brown trout (*Salmo trutta*) shows gill filament (F), gill secondary lamellae (SL) and thin epithelium cover (arrowhead), scale bar = 0.50 mm (H, E and PAS stain and 400X magnification).

In terms of cellular structure, the surface of the gill epithelium consists mainly of cuboidal and squamous pavement cells, which make up about 90% of the gill surface area (Wilson and Laurent, 2002). The epithelium contains other kinds of cells. These include chloride cells, mitochondium-rich cells that are involved in ion transport and serve to regulate acid-ion balance in relation to the water salinity (Jenjan, 2002; Marshall, 2002; Wilson and Laurent, 2002). The epithelium also contains pillar cells, which have a cylindrical body linking the two parallel sheets of the gill epithelium (Olson, 2002; Wilson and Laurent, 2002) and can change the diameter of the vascular channels (Kudo *et al.,* 2007).

Also found in the gill epithelium are mucous cells, which are large ovoid cells, composed mainly of apical mucus secretory granules (Dezfuli *et al.,* 2010); the nucleus and cytoplasm are usually being compressed in a basal location. Within the cytoplasm is the cellular machinery for producing mucin; this is a heavily glycosylated protein that coats the gill membranes when fish are exposed to environmental stressors including parasites and pollutants (Peuranen *et al.,* 1994; Wilson and Laurent, 2002; Andrews *et al.,* 2010; Dezfuli *et al.,* 2010; Flores and Fand Tomaz, 2011). The mucous cells are commonly

found in the filament epithelium and in the space at the base of the lamellae in both healthy and infected fish (Reimschuessel et al., 1991; Dezfuli *et al.,* 2010). Mucous cells are involved in a wide range of functions, including respiration, osmoregulation, feeding and disease resistance (Yan *et al.,* 2007; Schroers *et al.,* 2009; Dezfuli *et al.,* 2010). Their density is variable between species and relates to habitat, being relatively low in marine compared to freshwater fish (Laurent and Hebibi, 1989). Density of mucous cells also varies within a given species, for example being higher in infected than in uninfected fish (Andrews *et al.,* 2010).

The fish gills are the primary place of sodium and chloride transport, actively taking up salts in fresh water and secreting them in seawater. Even though pavement cells wrap the majority of the gill respiratory surface area, they are mainly considered to play an inactive role in the gill physiology of most fishes. Pavement cells are supposed to be important for gas exchange because they are thin cells with a wide apical surface area and are generally the main cell type that covers the sites of gills that use for gas exchange (Carmona *et al.,* 2004).

In freshwater teleost gills some pavement cells can play an active role in ion uptake and acid-base transport by the fish gills (Wilson *et al.,* 2000) Furthermore, pavement cells present structural type indicative of a high metabolic activity. In contrast to pavement cells, chloride cells reside in a much smaller part of the gill epithelial surface area, however they are measured to be the primary sites of active physiological procedures in the gills. While pavement cells are located in all area of gill filaments, chloride cells are generally more common on the afferent edge of gill filaments, as well as the distances between gill lamellae. Furthermore, chloride cells are generally not found on the epithelium tissue that covering the gill lamellae (Perry 1997; Jenjan 2002).

1.2.3 Habits, habitats and gill structure in fish

Numerous studies have explored the relationship between gill morphometrics (for example, the number and length of the gill filaments and the number, length and spacing of the secondary lamellae) and the habits, life style and habitat of fish concerned (Hughes, 1984; Matey *et al.,* 2008; Turko *et al.,* 2011). Development of the respiratory surface is often estimated geometrically, based on the product of the mean estimated area of the individual gill secondary lamellae (from their mean length and height) and their estimated

total number in the whole gill system. A comparison of fast-swimming pelagic fish such as the tuna (*Thunnus albacores*) and sluggish benthic fish such as the toadfish (*Opsanus tua*) shows striking differences in number and size of the gill filaments and secondary lamellae, all of which are markedly higher in tuna, matching the respiratory requirements of the fish. In general, fast-swimming fishes have a larger gill surface area and a thinner epithelium than do sluggish fish, and hence the blood to water distance across the lamella is smaller in the fast-swimming fish than sluggish fish (Hughes, 1984). Such relationships between gill structure and life style and habitat are discussed further in later chapters of this thesis.

In addition to fixed adaptations in gill structure at the species level, reversible changes also occur in relation to environmental conditions (Roberts and Rowell, 1988; Ghalambor *et al.,* 2010). For example, in freshwater carp (*Carassius carassius*), gill features such as the thickness of the filament epithelial and hence the water-blood diffusion distance can change as a response to differences in water temperature and oxygen levels (Matey *et al.,* 2008; Mitrovic and Perry, 2009; Turko *et al.,* 2011). So-called "gill remodelling" has been reported in relation to changes in oxygen levels and salinity (Ong *et al.*, 2007; LeBlanc *et al.,* 2010). This involves, among other responses, changes in the extent to which the respiratory membrane is occluded by epithelial cells sloughed into the space between the secondary lamellae (or hyperplasia) (i.e. increased mitotic activity in underlying cells. Concurrently, a number of the newly shaped epithelial cells differentiate into mucous and chloride cells, (Temmink *et al.,* 1983)), which reduces functional gill surface area (Graham, 1997; Turko *et al.,* 2011). Increases in hyperplasia between secondary lamellae may serve to reduce dehydration and prevent the secondary lamellae from collapsing when fish are exposed to air, for example, and may reduce ion loss when fish are exposed to freshwater (LeBlanc *et al.* 2010; Turko *et al.,* 2011).

Changes in water quality, for example low pH or rapid changes in water temperature and in salinity, as well as handling or crowding, can result in changes in gill structure and function. Most of such changes are nonspecific in nature, representing a combination of direct damage and compensatory response (Mallatt, 1985). All these stressors can cause water uptake and passive ion loss in freshwater fish and water loss and ion fluxes in seawater fish (Wendelaar Bonga, 1997; Jenjan, 2002). Moreover, acidic water and toxic water pollutants can lead to damage of gills and can impair uptake of oxygen (Evans, 1987; Wendelaar and Bonga, 1997). Compensatory responses to stress at the level of the gills include hyperplasia and increased mucous cell numbers, with hypersecretion of mucous in both freshwater and seawater fishes (Wendelaar and Bonga, 1997; Schroer et al., 2009).

Such relationships between gill structure and environmental conditions are discussed further in later chapters of this thesis and also have implications for how fish are affected by adverse conditions, and hence to the topic of fish welfare.

1.3 Fish stress and welfare

1.3.1 What is animal welfare?

Animal welfare is a complex and controversial subject, partly because the term has been defined in different ways in the literature. In general, definitions of welfare fall into three main types: feelings-based, nature-based and function-based. Feelings-based definitions are concerned with the subjective experience of the animal and require for good welfare that the animal concerned be free from negative experiences such as pain and have access to positive experiences such as companionship in social species (Chandroo *et al.,* 2004; Huntingford *et al.,* 2006; Segner *et al.,* 2011). Nature-based definitions centre on the ability of an animal to live a natural life, requiring for good welfare that a captive animal lives and behaves in a manner comparable to that of its wild counterparts. Function-based definitions focus on the capability of the animal to survive and adapt to its current environment without being affected beyond its physical ability (Huntingford *et al.,* 2006). Thus, animal welfare refers to an animal's "state as regards its attempts to cope with its environment" (Fraser and Broom, 1990). According to such definitions, good welfare requires the ability to maintain homeostasis and regular biological functions, reflected in good health and freedom from disease as well as growth and reproduction (Huntingford *et al.,* 2006; Turnbull and Kadri, 2007; Segner *et al.,* 2011). These different kinds of definitions are not right or wrong, but reflect different facets of a very complex topic.

1.3.2 Can the term welfare be applied to fishes?

Controversy about how animal welfare is best defined is particularly vigorous in the case of fishes, which are very different kinds of animal from farmed terrestrial species such as mammals and birds. In particular, there is much discussion about whether feelings-based definitions of welfare can be applied to fish. The argument against this is based on the assumption that fish behaviour is largely the result of simple stimulus-response reflexes and that fish show little capacity for long time memory and so are little affected by adverse

events (e.g. Rose, 2007). This view has been contested by number of authors, who point out that fish are capable of highly complex behaviour (for example, forming mental maps and learning by observation how good conspecifics rivals are at fighting) and readily form memories, for example of an adverse event such as attack by a predator, and retain these for long periods (e.g. Braithwaite 2010). There is currently not enough information to distinguish clearly between these views. However, in this thesis welfare is assessed by a variety of indicators of a fishs ability to cope with its environment, which relate to the less controversial function-based definition of welfare.

1.3.3 Measures of welfare

The measurement of fish welfare has been discussed at length in several different reviews, for example, Fraser and Broom, (1990); Dautzer, (1991); Dawkins, (2006); Huntingford *et al.,* (2006). Most measures of welfare relate to physical condition, life history events or physiological and behavioural responses, and so concern how well the body is functioning (Huntingford and Kadri, 2008). Use of such measures is based on the argument that if the biological fitness of the fish is reduced by the environmental conditions in which it is held, then its welfare will be poor. In terms of life history variables, poor welfare might be reflected in slow growth, delayed maturation, lengthened periods between successive breeding episodes and early death. Direct measures of physical condition such as the incidence of injury, damage ordisease are clearly related to welfare, defined in terms of effective functioning (Huntingford and Kadri, 2009), since diseased and injured fish are highly likely to function less well than healthy fish (Segner *et al.,* 2011). Effective immune function is also an important welfare indicator, since if fish or other animals are kept in such a way that their immune systems are less effective in combating disease, there is clearly some inadequacy in the management of the housing system (Broom, 1988; Fraser and Broom, 1990).

Physiological stress responses can also provide measures of good or poor welfare. These are discussed further in the following section, but interpretation is not simple. For example, production of the adrenal hormones adrenaline and cortisol represents an early component of the stress response (see below). These hormones activate several responses that help the animal concerned to cope with whatever challenge has initiated the response, and so generally in the short term they indicate that the animal can and is coping, so arguably its welfare is good. However, if such responses fail to remove or counteract the stressor, levels of these same hormones may be chronically elevated, which may indicate

failure to adapt and hence poor welfare (Moberg, 1985; Buller and Morris, 2003). Behaviour is changed as a response to numerous environmental problems (Huntingford *et al.,* 2006; Huntingford and Kadri, 2008) and such changes are critical in how animals cope with emergencies. Therefore behaviour and the performance of abnormal behaviour can be used as welfare indicators (Broom and Johnson, 1993; Ashley, 2007). Such measures can potential be used for both farmed and wild fish (Segner *et al.,* 2011; Lupatsch *et al.,* 2010), although the welfare of wild fish has received relatively little attention.

1.3.4 The stress response

1.3.4.1 Overview of the stress response: Studies of animal welfare draw heavily on the natural mechanisms that allow animals, including fish, to deal with environmental challenges, in other words on the stress response. Stress can be defined as a condition in which a hazard to the biological functions of an organism is detected by that organism and a suite of physiological and behavioral responses are mounted to offset this challenge (Reid *et al.,* 1998; Gilmour *et al.,* 2005; Kittilsen *et al.,* 2009; Johasen *et al.,* 2011). The stress response in fish shows similarities to that of other vertebrates and involves the principal messengers of the brain-sympathetic-adrenal medulla axis (resulting in the production and release of adrenaline) and the brain-pituitary-adrenal axis (resulting in the production and release of cortisol; Peter, 2011). These two hormones trigger a number of physiological responses that are reflected at all levels of biological organization (Dini *et al.,* 2006; Iwama *et al.,* 2006; Peter, 2011), making adaptations that eventually help the animal to acclimate to the environment (Lock and Wendelaar Bonga, 2008; Peter, 2010). The stress response can consequently be considered to be part of a coping strategy (Vijayan and Moon, 1994). The various components of the stress response, in fish and in other animals, have been characterised as primary, secondary and tertiary responses (Bowers *et al.,* 2000; Peter, 2011).

*1.3.4.2 Primary stress responses***:** Primary stress responses, which involve rapid and abrupt neuroendocrine responses, involve the release of catecholamines, primarily adrenaline, from chromaffin tissue (Gingerich and Drottar, 1989; Reid et al., 1998; Barton, 2002) and stimulation of the hypothalamic-pituitary-interrenal axis, culminating in the discharge of corticosteroid hormones to circulation (Okawara *et al.,* 1992; Fevolder and Roed, 1993; Weld *et al.,* 1987; Wendelaar Bonga, 1997; Mommsen *et al*., 1999 ; Barton, 2002. Sumptor, 1997). The pattern of cortisol release, and hence plasma levels of this hormone,

depend on the size and duration of the stressor. Response to an acute, short-term challenge usually takes the form of a sharp increase in plasma cortisol levels lasting for periods of the order of hours (Barton and Iwawa, 1991; Waring *et al.* 1996; Cutts *et al.,* 1998). However, exposure to continuous, chronic stress can result in elevated cortisol levels lasting for much longer periods, of the order of days and weeks (Pottinger *et al.,* 1994; Barton, 2002). Under some conditions, fish will adapt to a repeated stressor and stop to show a stress response, even while originally responding with high cortisol levels (Pickering and Pottinger, 1985). Other hormones are involved, though to a lesser extent, in the stress response; these include thyroxine (Barton, 2002), prolactin (Pottinger *et al.,* 1992) and somatolactin (Kakizawa *et al.,* 1995).

In general, the majority of fish species show their highest plasma cortisol increase within about 0.5–1 hour after a brief stressful event (Barton, 2002). There are some exceptions to this; for example, Vijayan and Moon (1994) found that plasma cortisol in the sea raven (*Hemitripterus americanus)* took about 4 hours to reach its peak level of cortisol (about 260 ng/ml) after a strong stressor (1 minute of air exposure folowed by 1 minute pursuit). The authors suggest that the slow rate of response to the stressor could help save energy in a generally inactive fish species that has a low metabolic rate. Other examples of adaptive between-species differences in cortisol release have been reported (Barton, 2002). For example, in scaphirhynchid sturgeons (*Scaphirhynchus* spp) peak levels in cortisol following an acute handling stressor were low (Barton *et al.,* 1998).

1.3.4.3 Secondary stress responses: The neuro-endocrine changes that make up the primary stress response initiate a number of changes, characterised as secondary stress responses, that activate a number of metabolic pathways, modifying blood biochemistry and respiration and preparing the animal for action (Vijayan *et al.,* 1994; Iwama *et al.,* 2004; Gabriel and Akinrotima, 2011). Thus exposure to a stressor results in a number of changes in plasma, tissue ion, metabolic rate and respiration, haematological characters, respiration, acid-base status, immune function and hydromineral balance (Iwama *et al.,* 1997; Mommsen *et al.,* 1999; Barton, *et al.,* 1998). Raised plasma glucose concentrations are an important component of the secondary stress response, resulting from glucose production from glycogen, particularly in the liver, and its release into the circulation (Iwama *et al.,* 2006). The glucose produced in response to stress provides a source of additional energy to tissues such as the brain, the muscles and (in fish) the gills, to meet the increased energy demmand. Levels of cortisol and adrenalin are correlated to glucose

concentrations in fish (Vijayan *et al*., 1994; Gabriel and Akinrotima, 2011), so plasma glucose is regularly used as an indicator of stressed state in fish (Gabriel and Akinrotima, 2011).

1.3.4.4 Tertiary stress responses: The tertiary responses involve long term adaptations to stressors and the physiological responses that they produce, and can be seen at the level of the whole animal and at the population level. Many tertiary stress responses arise because stress-induced increases in metabolic rate divert energy from essential life procedures, resulting in reduced growth and body condition, poor resistance to disease, decreased reproductive ability (since stress reduces production of reproductive hormones (Pankhurst and Dedual, 1994; Haddy and Pankhurst, 1999; Barton, 2002)) and poor survival (Wedemeyer *et al.,* 1990; Iwama *et al.,* 2004).

1.3.4.5 Behavioural responses to challenge: The stress responses in fish, behavioural as well as physiological, are reasonably well known and are similar to those of other vertebrates (Galhardo *et al.,* 2011). In some respects, behavioural responses are an animal's first line of defence against unfavourable environmental change, regularly being activated by the same stimuli that start the primary stress response (Martinez-Porchas *et al.,* 2009). When behavioural defence mechanisms, such as avoidance and escape responses, are not enough to remove the animal from the cause of risk, more striking changes of behaviour may occur, especially in response to chronic stress, including shelter seeking and suppression of activity and feeding (Schreck *et al.,* 1997; Galhardo and Oliveria, 2009).

1.4. Aquaculture and fish welfare

1.4.1 What aquaculture is

The primary, secondary and tertiary stress responses, including behavioural responses, can have harmful consequences for cultured fish and have been much discussed in this context (Gabriel and Akinrotima, 2011). Aquaculture is the culture of plants and animals in fresh, brackish and marine waters and is considered to be a part of agriculture. Some forms of aquaculture, for example the culture of carp, have taken place for thousands of years (Balon, 2004), but these have mostly been extensive or semi-intensive, with fish held in relatively large water bodies at relatively low densities with various forms of

supplementary feeding. Intensive aquaculture developed relatively recently and has quickly expanded, so that it is now a major component of the food production industry (Pèrez-Casanova *et al.,* 2009). As for other parts of agriculture, aquaculture practices are now being examined to determine their impact both on the environment and on the welfare of the cultured fish (Conte, 2004).

1.4.2 Aspects of aquaculture that may compromise fish welfare

1.4.2.1 The five freedoms: In agriculture generally, the Farm Animal Welfare Commission (FAWC)'s concept of the "five freedoms" has proved a valuable tool for considering and protecting animal welfare. According to this view, to guarantee welfare, there are five freedoms that captive animals must have (FAWC, 1979). These are depicted in Figure 1.3 and, between them aspects of modern aquaculture have the potential to infringe all of these 5 freedoms.

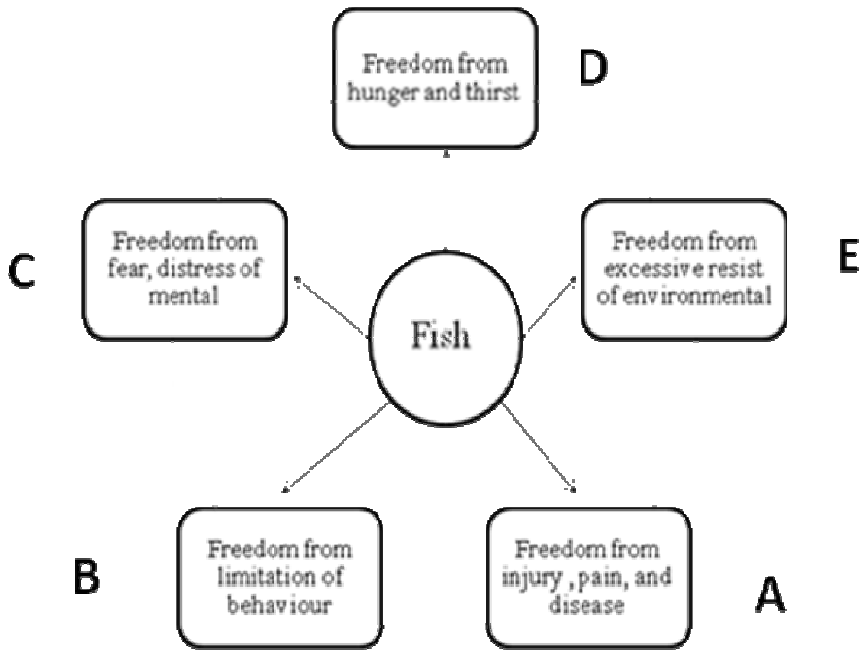


Figure 1.5. Schematic representation of the five freedoms that guarantee good animal welfare.

In aquatic farming, health and welfare are often understood as the absence of obvious, overt disease and injury, reflecting freedom from injury, pain and disease (A in Figure 1.5). Much emphsis has been given to prevention of disease and reduction of mortality in aquaculture. Good health in these terms is essential for good welfare (Duncan 2005; Ashley 2007), but the term health should be extended beyond the simple absence of disease to cover the absence of pathology at the tissue and cell level (Broom, 2007, Conte, 2004). From this perspective, health means the capability of an animal for normal

physiological functioning and maintenance of homeostasis. Farmed fish may be exposed to a variety of stressors that may compromise their welfare according to this broad definition (Huntingford *et al.,* 2006). These include crowding and high densities, poor water quality, aggressive interactions, handling and removal from water, low oxygen concentrations especially during transport, high incidence of disease and pain and stress at slaughter.

1.4.2.2 Crowding: Crowding is often loosely described as high fish loading density and can arise in a diversity of ways, depending on the aquaculture system in which the fish are controlled (Robb, 2008). Crowding problems are usually related to intensive fish culture, especially when the water supply fails and/or aeration is decreased (Lembo *et al.,* 2008). Fish become physiologically stressed from psychological stressors crowding, compromising freedom C in Figure 1.5. In addition crowding often relates to the behavioural requirements of the fish for physical space, relating to freedom B in Figure 1.5. Failure to provide cultured fish with sufficient space can reduce growth and overall fitness (Wedemeyer, 1996), though the limits at which this occurs will be species specific (Ellis *et al.,* 2002). For example, Arctic charr (*Salvelinus alpines)* exhibit stress-related behaviours at low density, showing fewer behavioural indicators of stress as density is increased. In general, when fish are crowded at high densities, there is smaller amount oxygen available (Lembo, 2008) and the concentration of waste products will increase as fish density in increases, so freedom E in Figure 1.5 is also compromised by crowding (Ellis *et al.,* 2002; Robb, 2008). The chronic stress of low oxygen in both crowded and uncrowded tanks can cause increased plasma cortisol levels in fish (Pickering *et al.,* 1991; Robb, 2008). The longer the fish are crowded, the poorer their condition will be, the extent depending on the species concerned. For example, levels of cortisol and glucose increased after 7, 9 and 22 hours at high loading density in satellite sturgeon (*Acipenser stellatus*) and not until 10 days in Russian sturgeon (*Acipensergueldenstaedtii*) (Bayunova *et al.,* 2002).

1.4.2.3 Poor water quality: Regardless of stocking density, water quality is critical for the welfare of farmed fish, since poor water quality places excessive physiological demands on the fish (compromising freedom E and eventually A in Figure 3). The term "water quality" encompasses the total of physical, biological and chemical factors such as low or high water temperature, low or high dissolved oxygen, high concentration of nitrogenous compounds such as ammonia and nitrate as well as other toxic substances (Caldwell and Hinshaw, 1994; Mmochi *et al.,* 2002). Water quality is consequently an important parameter to be measured when planning for high aquaculture production. Also it can

affect the growth, body functions health and welfare of cultured fishes (Pickering, 1993; Mmochi *et al.,* 2002). Of these variables, dissolved oxygen concentrations, together with water temperature, are perhaps the most important (Timmons *et al*. 2001; Caldwell and Hinshaw, 1994). Both surplus and shortage of dissolved oxygen can lead to mass mortality among cultured fish (Krom *et al.,* 1985). The interacting effects of these two water quality parameters are discussed in detail in Chapter 5.

Oxygen is essential in respiration and metabolism, and in fish the metabolic rate is highly affected by the oxygen concentration in the culture environment. When levels of dissolved oxygen decrease, respiration initially increases by way of compensation. Feeding activities of fish often decrease with falling oxygen levels (Tom, 1998); for example, Thetmeyer *et al*. (1999) found that, rainbow trout (*Oncorhynchus mykiss*) reduced feed intake when oxygen saturation fell below approximately 60%. Similar results have been obtained from European sea bass (*Dicentrarchus labrax, L*), blue tilapia (*Oreochromis aureus*) *(*Papoutsoglou and Tziha, 1996), channel catfish (*Ictalurus punctatus*) Buentello *et al*., 2000), juvenile turbot (*Scophthalmus maximus*) (Pichavant *et al.* 2000), showing reduced growth when exposed to low oxygen levels. As a consequence, growth rates are reduced in many species of fish as oxygen levels fall and, at the same time, the probability of contracting a disease is increased. In general welfare, health and physiological conditions are best when the dissolved oxygen is maintained close to saturation. When the levels of oxygen fall, the growth rate of the fish can be highly affected by increase in stress, decrease activity and also decreased immunity to diseases (Wedemeyer, 1996).

As ectotherms, fish take on the temperature of their environment and are intolerant of quick fluctuations of temperature. This makes water a perfect living environment for them, because water has high heat capacity and thus allows a large amount of heat energy to be absorbed with little equivalent change of temperature. Fish can be classified in terms of their temperature tolerances into cold water, warm water and tropical species. Up to a point, rising water temperature can increase both feed intake and growth of fish (Baras *et al.,* 2001; Handeland *et al.,* 2008; Xie *et al.,* 2011; Soto-Zarazúa *et al.,* 2010). However, very high temperatures lead to decreased growth and feed utilisation efficiency, with decreased feed intake and increased energy usage during respiration (Maricondi- Massari *et al.,* 1998; Baras *et al.,* 2001; Handeland *et al,.* 2008). For example, high water temperatures reduce the energy retention in juvenile cobia (*Rachycentron canadum.* Sun *et al.* 2006). There is a minimum and maximum tolerance limit for each species of fish, as well as a most favourable temperature range for growth (Soto-Zarazúa *et al.,* 2010),

sometimes known as the standard environmental temperature. Water temperatures outside this range causes stress and slow growth, which in fish culture adversely affects the quality and quantity of fish produced as well as time to harvest (Soto-Zarazúa *et al.,* 2010). Fish normally experience stress and disease spread when the water temperature remains close to their maximum tolerance for prolonged periods, or when it fluctuates abruptly.

Such effects of temperature on the energy budget in fish interact with those of other factors such as tank shape or volume (Xie *et al.,* 2011) and also the pH of the water. Satisfactory pH levels for fish culture usually lie between pH 6.5 and pH 9.0 (Parra and Baldisserotto, 2007). When water is extremely alkaline (i.e. above pH 9), ammonium ions in water are changed to toxic ammonia, which can kill fish. Conversely, acidic water (less than pH 5) removes metals from rocks and sediments into solution, many of which may have adverse effects on metabolic rates and gill function and can be lethal. Garcia *et al.* (2011) found high mortality rates in fingerlings of silver catfish, (*Rhamdia quelen*) exposed to pH 5 and 9. Alkaline carbonate and bicarbonate ions are important determinants of water quality, since they serve to counteract water acidity and to balance pH (Adhikari *et al.,* 2007). In semi-intensive aquaculture systems where the water is fertilised, the correct pH is important for successful aquaculture. Fertilisers, which may contain nitrogen, phosphorous and potassium are added to encourage the growth of phytoplankton. Phytoplankton breaks down waste into harmless ammonia, and is also the food of zooplankton animals which in turn feed fish. The hardness of the water in which fish live or are housed (i.e. levels of dissolved calcium ions) is another important determinant of water quality. Water hardness can impair fertilization of the eggs of aquatic animals (Spade and Bristow, 1999) and has a major effect on survival and development in fish eggs, larvae and adults (Ketola *et al.,* 1988; Spade and Bristow 1999). Perschbacher and Wurts, (1999) found that high calcium levels (i.e. hard water) significantly affected survival of juvenile channel catfish (*Ictalurus punctatus*) exposed to a toxic concentration of copper sulphate in low alkalinity water.

1.5 The study species

As mentioned above, the work described in this thesis considers three different species of fish, all of which are farmed to a greater or lesser extent; taking these in turn, they are the Arctic charr, the brown trout and the common carp. A brief account is given here of the

biology and aquaculture of these species; more details are provided later in the relevant chapters.

1.5.1 Arctic charr and trophic polymorphism

The Arctic char (*Salvelinus alpinus*) is a salmonid fish closely related to salmon and trout and sharing many characteristics with these two species. The Arctic charr is a cold water fish, being found in deep, cold lakes at maximum temperature of 10^{o}C (Frost, 2001). Colouration is changeable, depending on size and habitat, but in general these fish are olive green or brown with red spots along the body sides. Length at maturity ranges from 10 to 150cms, and the weight at maturity ranges between 2 to 5 kg. fish can weigh 9 kg or more.

The Arctic charr has a number of features that are advantageous for efficient culture, with feed conversion ratios (ratio of biomass of flesh produced to feed provided) of about 1:1. They are a relatively large species that thrives at high densities, tolerating culture densities of up to 120kg/m^3 (Jorgensen *et al.,* 1993) and can also survive short-term exposure to low dissolved oxygen levels (Delabbio, 1995; Magnan *et al.,* 2002). They are a cold-water species, exhibiting maximum growth at temperatures of 10–15^{o}C (Larsson and Berglund, 1998; Svenning *et al.,* 2007), so are suitable for culture at high latitudes. Female Arctic charr spawn about between 2000 to 4000 eggs every two to three years, making provision of stock relatively easy. Arctic char has good quality, attractive flesh (Kim, 1993; Aarset, 1999) and has a fillet yield that is approximately 7–8% higher than a fillet of rainbow trout yield, due to its relatively large body and small head (Glandfield, 1993). At present the cultured Arctic charr is a high value product, attracting good wholesale prices due to currently limited aquaculture production (Delabbio, 1995).

The main interest in Arctic charr in the context of the present thesis lies in the fact that, in the wild this species commonly shows trophic polymorphism, that is the coexistence within a single population of individuals that are specialised (morphologically and behaviourally) for different diets. The co-existence of sympatric variants is common in Arctic charr, numerous lakes having coexisting populations that feed in different ways and sometimes breed at different times of year and in different habitats. Sympatric morphs of Arctic charr vary in habitat, morphology (including colour) as well as behaviour. Such difference within populations is habitually linked with differences in foraging habits and coexisting benthic, pelagic and sometimes piscivorous morphs of charr have been reported at several sites

(Adams *et al.,* 1998). Possibly the best studied system is Lake Thingvallvatn, in Iceland, where four sympatric forms exist differing in shape and body size and trophic biology (Smith and Skulason, 1996). Polymorphic feeding morphology and behaviour have been described for a number of other populations, for instance, Arctic charr in Loch Rannoch, Scotland (Adams and Huntingford, 2002). Sympatric forms normally vary in the number of their gill rakers (Amundsen, 1988) and are associated with variation in foraging habitat and food types (Adams *et al*., 2007; Skulason and Smith, 1995). Commonly, the morph that mostly lives on small pelagic prey has more, longer gill rakers than does that feeding on larger, benthic prey (Foote *et al.,* 1999). Since gill arches serve the combined function of supporting the respiratory tissue and of collecting food and since different foraging specialisations may result in fish inhabiting water with different dissolved oxygen levels, it may be that trophic polymorphism is associated with every difference in gill structure and function, as has previously been shown at the species level.

1.5.2 Brown trout and within species variability in behaviour and physiology

Brown trout are salmonid fishes that are local to Europe, Iceland and the Northwest coast of Europe, along the Mediterranean also western Asia. However, brown trout have been introduced at least 24 countries within the last 90 years (Ozvarol *et al.,* 2010). Given suitable spawning substrata, good temperature conditions and water of suitable quality, brown trout can inhabit water bodies ranging in size from very small streams to the largest rivers (Behnke 1986; Klemetsen *et al.,* 2003).The reasons for the large geographical distribution of brown trout lies in its ecological changeability and excellent ability to inhabit new water bodies (Klemetsen *et al.,* 2003). Brown trout differ in size, growth rate, feeding habits and habitat use within and between water courses (Pakkasmaa and Piironen 2001).

The body of the brown trout is olive brown or dark green and yellowish white on the belly. The sides of the fish have red spots, most bounded by a pale halo. The environment of brown trout is defined by physical and biotic factors. Rivers and streams are highly changeable habitats consequently wild brown trout are physically exposed to a diversity of environmental systems, with reference for example to flow rate, temperature of water and

levels of dissolved oxygen. Trout are reasonable tolerant of environmental change, for instance surviving within a temperature range of 0.5 – 22 °C. (Bell, 2006). Among the physical factors that affect the ecology of the species, water current is an important factor in the habitat of brown trout (Bagliniere and Maisse, 1999), trout being morphologically adapted to life in flowing fresh water (Brown, 1975). Macrophytes as well play a function in the habitat structure by restricting visual limits and also influencing water oxygenation and pH. Brown trout are negatively phototactic until resorption of yolk sac is complete, but this changes through ontogeny, as that fry are positively phototactic and adult stages are more and stronger negative phototactic (Ottaway and Clarke, 1981).

Brown trout mostly eat aquatic insects and crustaceans and, when larger, other fish. The main prey of juvenile brown trout however is aquatic invertebrates (Ruginis, 2008; Bridcut, 2000). The food utilised by a brown trout is dependent upon availability and by the behaviour of the trout (Montori *et al.,* 2006). Prey availability differs with time (Fochetti *et al.,* 2003); for instance, through October to February, the brown trout are more likely to feed on bottom fauna (Johanson, 1978) and through May and September they are more likely to feed on insects taken at the water surface (Björnsson, 2001). Small brown trout are sit and wait predators that choose an area for feeding that they defend as a territory and prey upon organisms that drift downstream in the current. The diet of large brown trout is more varied than that of younger brown trout, consisting of large aquatic insects such as *Hexagania*, mayflies and larger species of caddisflies, as well as crustaceans, snails, salamanders, amphibians. Adult trout feed mostly at night, especially through the summer (Bell, 2006). Brown trout commonly show defensive behaviour, and during the reproductive period, females are less aggressive and show a lower level of territoriality than males (Montori *et al.,* 2006).

As a result of the wide tolerance range, the growth rate of brown trout is greatly changeable in different localities, perhaps connected to water temperature, nutrition or to genetic factors. Thus fish might grow to to 200g from eyed eggs within 10 and 20 months (Bromage *et al.,* 1990). Adult brown trout are usually 33-40 cm in length, though old individuals can reach a much larger size. The world record weight for angler-caught trout is in excess of 20kg. In Western Australian the trout fishery is based upon young, fast growing fish, mainly 1+ year old fish which reach 1.0kg in weight (Kheyrandish *et al.,* 2010).

Males of brown trout normally mature for the first time at age 1or 2. The mean size of mature males at age 1+ is larger than of those that are immature (Maisse *et al.,* 1987). For females, the great mature majority for first time at 2 or 3 years old however some females can mature at age 1+, but this is very unusual. Maturing 1+ females are commonly larger than immature females and males of the same age (Bagliniere and Maisse, 1999). Brown trout spawn in the autumn (from October to December) when the temperature of the water drops to 5 – 10°C (Pender and Kwak, 2002). Eggs are laid on stone and stony bottoms, usually in running waters, although lake spawning populations happen uncommonly (Schneider *et al.,* 2000; Brabrand *et al.,* 2002). Reproduction occurs earlier at higher latitudes and altitudes as a result of lower water temperature and longer egg incubation time. Large females can produce 400 to 1200 eggs. Trout mate every year, and they are not likely to have the same mate year after year. The eggs hatch the following spring (Bell, 2006).

The main interest in brown trout in the present thesis, besides providing another salmonid species in which to quantify individual variability in gill development, lies in the fact that a number of studies have documented consistent individual differences in behavioural and physiological responses to stress. In a number of salmonids, risk-taking, aggressive life style is linked with high resting metabolic rate (Yamamoto *et al.,* 1998). In a study by Lahtl *et al.* (2002) on juvenile brown trout, both metabolic rate and the amount of aggressive behaviour were found to vary between four populations. High metabolic rate and high aggressiveness can give a selective advantage to individual trout living in environments with constant, predictable food (Lahti *et al.,* 2002). Such individual differences in metabolic rate and associated differences in aggressiveness may be linked to within-species differences in gill development and fine structure, as reported above at the species level.

1.5.3 Common carp and variable coping styles

The common carp has a very long history of domestication, probably being the oldest cultured and most domesticated fish in the world (Wohlfarth, 1984; Michaels, 1988; Hulata, 1995), with several locally adapted cultured stocks (Bauer and Schlett, 2004; Sun and Liang, 2004). It is the most important culture fish in eastern European and Asian countries (Kucharczyk, 2008) and shows a number of advantages for fish farming. For example, it is one of the larger fresh water species and tolerates and grows well in a wide

variety of environments, allowing high productivity. As adult, carp are benthic-feeding omnivores (Nathanael and Edirisnghe, 2001), taking a varied and variable diet together with water plants, insects, crustaceans and annelids (Goldstein and Simon, 1999). The species can therefore be cultured using natural feeds or low protein prepared diets. Common carp breed readily in captivity, making them suitable for selective breeding programmes.

The main interest in common carp in the context of the present thesis lies in the fact that, while generally these are healthy fish and relatively resistant to husbandry stressors such as transportation, consistent, stable individual differences have been described in behavioural and physiological response to stress. In other words, distinct stress coping styles exist in common carp, as they do in many other species of animal (Huntingford *et al.,* 1994; Koolhaas *et al.*, 1999). Even for fish of a similar size consistent differences in risk taking, in capacity for scramble competition and in cortisol responsiveness have been reported (Tanck *et al.,* 2002; Huntingford *et al.,* 2010). These are associated with differences in metabolic rate, once environmental conditions such as temperature and feeding history, which are known to affect both metabolic rate and stress responsiveness (Ruane *et al.*, 2002), have been standardised. Using the terminology often applied to different stress coping styles, proactive, aggressive, risk-taking carp have a higher resting metabolic rate than do reactive, non-aggressive, risk avoiding fish, which also show stronger responses to handling stress. As in the case of brown trout, such individual differences in behavioural and physiological response to stress may be associated with differences in gill development and fine structure.

1.6 Aims and structure of this thesis

The broad aims of the work described in this thesis were as follows:

- To develop multivariate methods for quantifying within-species variability in the development and status of the respiratory surfaces and to compare these to existing methods used for this purpose. Development of these multivariate methods is described in detail in Chapter 2, using brown trout and Arctic charr, and they are then used in Chapters 3-6.

- Using light and electron microscopy to explore and quantify variation in the fine structure of the fish gills, using brown trout and common carp. The methodologies

are described in Chapter 3 (on common carp) and various aspects of them used in Chapter 4 (on brown trout).

- To relate variation in gill development and fine structure to within- species differences in life style that place different energetic demands on individual fish. These include trophic polymorphisms in Arctic charr (Chapter 2) and different stress coping styles in common carp (Chapter 3) and brown trout (Chapter 4).

- To explore the relationship between variability in gill development and fine structure and other commonly used measures of welfare in common carp held at high densities and exposed experimentally to high temperature and low oxygen levels (Chapter 5) and in extensively-farmed carp exposed to various husbandry pressures during one year of production (Chapter 6).

CHAPTER 2

A STUDY OF GILL DEVELOPMENT IN SALMONID FISH IN RELATION TO LIFE STYLE.

The work described in this chapter was on preserved samples of wild brown trout and Arctic charr collected by Dr. Monica Garduno-Paz and Prof. Colin Adams. All the morphometric data reported here new data collected by Hussein Jenjan.

2.1 Introduction

2.1.1 Trophic polymorphism in fishes

Trophic polymorphism refers to the coexistence within a population of the same species of individuals specialised for acquiring food in different ways (Noakes 2008). There is an increasing awareness of the importance of such polymorphism in both an ecological and evolutionary context (Bolnick *et al*., 2003; Swanson *et al.,* 2003). Trophic polymorphisms are associated with differences in both morphology and behaviour, such that with the evolution of trophic specialisation, phenotypically uniform populations become diversified (Jonsson and Jonsson, 2001; Maerz *et al*., 2006; Whit *et al*., 2007).

Sympatric trophic polymorphism has been described in many species of fish, including cichlids (East African cichlids, Meyer, 1990), rainbow smelt (*Osmerus mordax,* Taylor and Bentzen, 1993), whitefish (*Goregonus sp*) (Bernatchez and Dodson, 1991) and sticklebacks (*Gasterosteus aculeatus*; Cresko and Baker, 1996; reviewed by Ruzzante *et al.,* 1998). Generally, diversification into co-existing trophic morphs has occurred in species-poor fish communities, so trophic polymorphism is particularly common in fish inhabiting freshwater bodies in northern latitudes (Proulx and Magnan, 2004). Many such water bodies present two separate and distinct functional habitats, the littoral shore line and the pelagic, mid-water zone. Trophic polymorphisms very often take the form of co-existing benthic-feeding and pelagic-feeding types (Skulason and Smith, 1995; Proulx and Mognan, 2004), with morphology and behaviour adapted to feeding in these different habitats (Adams and Huntingford, 2002).

Table 2.1 summarises some case studies of trophic polymorphism in fish. For example, sticklebacks are distributed in the northern hemisphere, being found in seawater, brackish water and freshwater and frequntly exhibit different forms depending on local environmental factors (McPhail, 1994). In a few small lakes in New Western America , pairs of benthic and limntic (pelagic) forms cohabit, showing clear ecological separation. The benthic form is large, with a few short gill rakers, while the pelagic form is smaller, with longer and more numerous gill rakers (McPhail, 1984); the two forms are at least partially isolated reproductively (Schluter and McPhail, 1992). Liem and Kaufman (1984) describe two morphs of cichlid species *Cichlasoma minckleyi* in Mexico, one herbivorous

and the other carnivorous. The herbivorous morph has a small head, long intestine and small papilliform pharyngeal jaw teeth, while the carnivorous form has a big head, small intestine and large molariform pharyngeal teeth.

Table 2.1. Trophic polymorphisms in some fish species and the nature of the separation between forms.

Fish species	**Nature of separate and distinct ecological differences**	**Phenotypic difference**	**Reference**
Salmo trutta	Benthivorous, herbivorous	Behavioural, morphological and life history	Ferguson, 1989; Ferguson and Taggart, 1991
Gasterosteus aculeatus	Herbivorous and benthivorous	Behavioural and morphological	McPhail, 1984 and 1994
Cichlasoma mincklyi	Herbivorous and carnivorous	Behavioural and morphological	Kornfield *et al.,* 1982

The evolutionary significance of trophic polymorphism is the subject of some discussion (Smith and Bush, 1996; Ruzzante *et al*., 1998; Hulsey, 2006; Ruehl and DeWitt, 2005). Smith and Bush, (1996) suggest that where there is a degree of reproductive isolation between morphs, trophic polymorphism may be a step in the process of speciation. As well as the immediate specialisation for feeding described above, fish with different feeding specialisations often show associated differences in other aspects of their biology. For example, as a result of different profitabilities of different food types, they may differ in growth rates, life history patterns and reproductive biology (Fraser, 2009). The brown trout (*Salmo trutta* L.) population referred to in Table 2.1 shows variability in many features of its morphology, behaviour and ecology, including different time and place of spawning (Ferguson, 1989). The variation between morphs of trout is clearest with respect to colour pattern, but colour variation is indictive of a wide range of other characteristics. There is evidence for reproductive isolation between the two morphs; in a few cases morphological variation in brown trout is interpretable in genetic terms.

Most relevant in the context of the work described here, specialisation on different diets (benthic and pelagic for example) may take the fish into different habitats that may themselves exert divergent selection pressure on other aspects of the fishes' biology. For example, feeding on benthic rather than pelagic prey might potentially expose fish to deep water with low levels of dissolved oxygen (Wetzel and Likens, 1991; Horne and Goldman, 1994), which may in turn influence gill structure and function. Such correlated effects of trophic polymorphisms have not been extensively studied. The aim of the work described in

this chapter is, having developed a protocol for examining gill microstructure in the brown trout (*Salmo trutta* L), to use this to compare gill microstructure in two sympatric forms of Arctic charr (*Salvelinus alpinus* L) that specialise on benthic and pelagic prey.

2.1.2 The biology of Arctic charr

The Arctic charr is a salmonid fish that has a wide distribution at high latitudes in the northern hemisphere and is found further north than any other freshwater fish (McCarthy, 2007; Berrill and McCarthy, 2008). It is predominantly a freshwater species, occurring in lakes in many countries including Scotland, northern North America, Iceland, Greenland, Scandinavia and Russia, but anadromous forms that migrate to sea are common at high latitudes. The Arctic charr is a cold water species, being found only in deep, cold lakes with a summer/autumn maximum temperature of 10 ^{o}C (Frost, 1976; Svenning *et al*., 2007). Like its relatives the Atlantic salmon (*Salmo salar* L) and the brown trout (*Salmo trutta* L), the charr has variable coloration, depending on size and habitat. Generally, these fish are olive green or brown with pink or red spots along the sides of the body. The charr is a carnivorous fish, feeding on a range of invertebrates and vertebrates, including zooplankton, insects and small fish. Average length and weight at maturity in Arctic charr are highly variable, both between and within populations. Thus, length at maturity ranges from 10 to 150 cms, with an average of between 70 and 80 cms; average weight at maturity ranges from 2 to 5 kg, with an average of 3 – 4 kg. The mean age of sexual maturity is also variable within and between populations, ranging from three to five years. Arctic charr may spawn either in spring or in autumn, but spawning is most common in the autumn (October and November). Spawning sites are usually in shallow water substrates in lakes and rivers. Females spawn between 2000 and 4000 eggs every two to three years. The eggs are buried under the gravel and hatch in the spring, but the young remain in the small rocks for several months (Frost, 2001).

2.1.3 Trophic polymorphism in arctic charr

The co-existence of sympatric variants is common in Arctic charr. For example, many lakes contain coexisting populations that spawn at different times. Sympatric morphs of Arctic charr may also differ in habitat, morphology (including colour) and behaviour. Such variation within populations is often associated with differences in diet; thus coexisting

benthic, pelagic and sometimes piscivorous forms of charr have been reported at many sites (Adams *et al.,* 1998; See Table 2.2). Perhaps the best studied system is Lake Thingvallvatn, in Iceland, where four sympatric morphs exist, differing in shape and adult body size as well as trophic biology (Smith and Skulason, 1996). Polymorphic feeding morphology and behaviour have been described for a number of other populations of Arctic charr, for example, charr in Loch Rannoch, Scotland (Adams and Huntingford, 2002). Sympatric morphs commonly differ in the number of their gill rakers (Amundsen, 1988) and are associated with differences in choice of foraging habitat and/or prey types (McCarthy *et al.,* 2004; Adams *et al*., 2007; Skulason and Smith, 1995). Generally, fish that mainly live on small pelagic prey have more, longer gill rakers than do those feeding on larger, benthic prey (Foote *et al.,* 1999). Since gill arches serve the combined function of supporting the respiratory tissue and of collecting food, it is appropriate to examine the structure of the gill arches in polymorphic forms of Arctic charr, to determine whether trophic polymorphism is associated with any differences in respiratory function.

Table 2.2. Examples of trophic polymorphism in arctic charr *Salvelinus alpinus* and the nature of the separation between morphs.

Location	Number of morphs	Ecological differences	Phenotypic differences	Reference
Lake Thingvallanat	4	Benthivorous, plankivorous and piscivorous	Morphological characteristics, head structure, gill raker length, gill raker number and spawning time. There are two benthic forms have blunt snouts and sub-terminal mouths and the piscivorous and plankivorous forms have pointed snouts and terminal mouths. The benthic forms have fewer gill rakers. Also, the small benthic forms spawn between September and November and the large benthic form spawn between July and August.	Snorrason *et al.,* 1994
Loch Rannoch	3	Benthivorous and piscivorous	Morphological characteristics, head structure, body size and feeding habitat. The pelagic morph can be very succeful and greatly respected form the other by its coloration and body shape. Head structures (head length, head depth, jaw length, jaw width and snout curvature) differ between morphs. Feeding ecology, differences between morphs have been found. The Benthivorous form had acanthocephalan parasites but not found in piscivorous form.	Adams *et al.,* 1998; Adams and Huntingford, 2002
Loch Ericht	2	Zooplankton, macro-invertebrates and piscivorous	Morphological, sympatric morphs, a pale form and a coloured form, that differ related with head size and shape. Feeding ecology, the pale morph feed on benthic macro-invertebrates and the coloured morph feed on zooplankton.	Fraser *et al.,* 1998
Loch Awe	2	Bottom feeders and midwater feeders	Spring charr spawning about March each year and autumn charr around October that they come from different gene pools. They differ in feeding ecology, that the autumn morph feeding in the pelagic zone and the spring morph feeding in the littoral zone.	Garduno-Paz and Adams, (unpublished)

2.1.4 Gill structure and function in fish

As a group, fish are characterised by the use of gills as the primary means of extracting oxygen from water (Moyle and Chech, 1996). During vertebrate evolution, gills developed gradually as the primary gas exchange system and essentially consist of a number of capillaries surrounded by an epithelium that forms a thin barrier between the fishes' blood and the surrounding water. The gill system of teleost fish is made up of a series of arches (Figure 2.1) that serve as anchor points for filaments that bear the respiratory membranes of gill. These comprise numerous, small secondary lamella that protrude from the sides of each filament and form the primary gas exchange sites (Bond, 1979; Moyle and Chech, 1996; Palaniappan *et al.,* 2008).

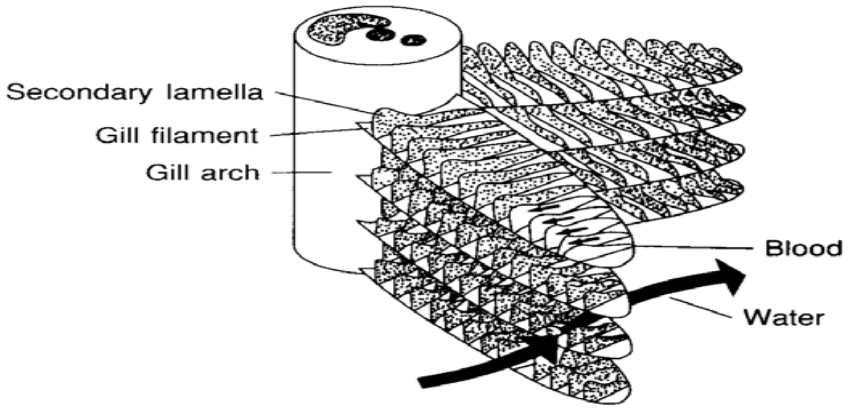


Figure 2.1. Schematic representation of the structure of gill structure in a teleost fish (from http://www.fishdoc.co.uk/koi/koigills.htm).

Studies of gill structure and function have been performed for a large number of fish species (Hughes and Morgan, 1973; Khangarot and Tripathi, 1990; Perry, 1997; Kharakatsouli *et al*., 2006; Nilsson, 2007). Details of gill structure depend on taxonomic position and stage of development (Wells and Pinder, 1996), but are also adapted to local environmental condition (Hughes and Morgan, 1973), especially water quality (Severi *et al.,* 1997). Through their involvement in the physiological process of gas transfer, gills are considered one of the first organs to be affected by poor water quality (Fischer-Scherl and Hoffmann, 1988) and are very sensitive to physical and chemical changes of the aquatic environment (Crespo *et al.*, 1988). Not surprisingly, many studies have examined the relationship between gill structure and both the metabolic demands of fishes and the oxygen content of the water in which they live (Palzenberger and Pohla, 1992).

Development of methods for quantifying the structure and organisation of gill filaments have facilitated studies of the relationship between gill structure and function and the habitat and habits of fishes (Hughes, 1984; Hartl *et al*., 2000; Timmerman and Chapman, 2004; Karakatsouli *et al*., 2006). Most researchers follow Hughes (1984) in estimating total gill area by the formula:

$$A\ (mm) = L.\ n.\ bl$$

where A is total gill surface area in mm^2, L is the total length of all the filaments (mm), n is the number of the secondary lamellae on both sides of the filaments (mm) and bl is the average bilateral area of the secondary lamellae (mm^2).

The gill area of fish increases with fish size and is correlated with activity, speed of swimming and water conditions (Saroglia *et al*., 2002; Chapman and Hulen, 2001). In general, more active fishes with high oxygen requirements have longer gill filaments and

more secondary lamellae than do sluggish fish (Bhagwant and Elahee, 2002). Fish species known to live in, and tolerate, low oxygen conditions have a relatively large gill surface area, through increases in the number and length of both gill filaments and secondary lamellae (Chapman *et al*., 2000; Timmerman and Chapman, 2004). Morphological changes in gills in response to environmental quality have been observed in several teleost species, so that in general, gill morphology is a good indicator of the condition of wild and farmed fish (Peters *et al.,* 1984).

In this study, the quantitative methods to define gill function developed by Hughes, (1984) and others, to salmonids have been aplied, initially using brown trout and then Arctic charr from two sympatric morphs that may well forage under different oxygen regimes (spring spawning, bottom feeders and autumn spawning, midwater feeders).

2.1.5 Fluctuating asymmetry in gill structure

The differential development of a bilateral trait between the left and right side of the body of an individual animal generates asymmetry (Sheffer *et al.,* 1998; Jawad, 2003). Deviations from symmetry can be directional (as in the case of handedness in humans, where most individuals are right handed) or may represent random departures from complete bilateral symmetry, in which case this is referred to fluctuating asymmetry. One cause of fluctuating asymmetry is environmental stress, which interferes with the homeostatic regulatory mechanism that usually equalise development on the two sides of the body (Estes *et al*., 2006). Fluctuating asymmetry can therefore be used as a measure of early exposure to adverse environmental conditions, including pollutants (Jawad, 2003, Ayoade *et al.,* 2004; Almeida *et al*., 2008). According to Jawad, (2003) and Almeida *et al.,* (2008), there is a direct correlation between environmental stress due to pollution in the aquatic environment and asymmetry in fishes. Asymmetries may appear at levels of pollution lower than those that cause extensive morbidity, so might potentially be used to provide early warning of environmental disturbance (Bengtsson and Hindberg, 1985; Ayoade *et al*., 2004).

A relationship between environmental stress and the levels of asymmetry in various bilateral structures has been reported in several species of fish. For example, Almeida *et al*. (2008) found an increase in asymmetry for length of pectoral and ventral fins of goldfish and carp with falling water quality (dissolved oxygen, total suspended solids, total nitrogen and total

phosphorous). The level of fluctuating asymmetry reflects exposure to isopropyl methylphosphonic acid in channel catfish (Estes *et al.,* 2006) and to high concentrations of mercury and low pH in several fish species (Jagoe and Haines, 1985). Figure 2.3 shows higher levels of asymmetry in pond-reared as opposed to wild carp in several structures (Almeida *et al.,* 2008). Ayoada *et al.,* (2004) suggest that fish gills are a particularly sensitive indicator of stress in fish and Oxnevad *et al*., (2002) found high levels of asymmetry for gill raker number in perch (*Perca fluviatilis* L) exposed to lake acidification. The present study therefore included an examination of levels of asymmetry in various gill structures in two sympatric morphs of Arctic charr that might naturally experience differences in water quality.

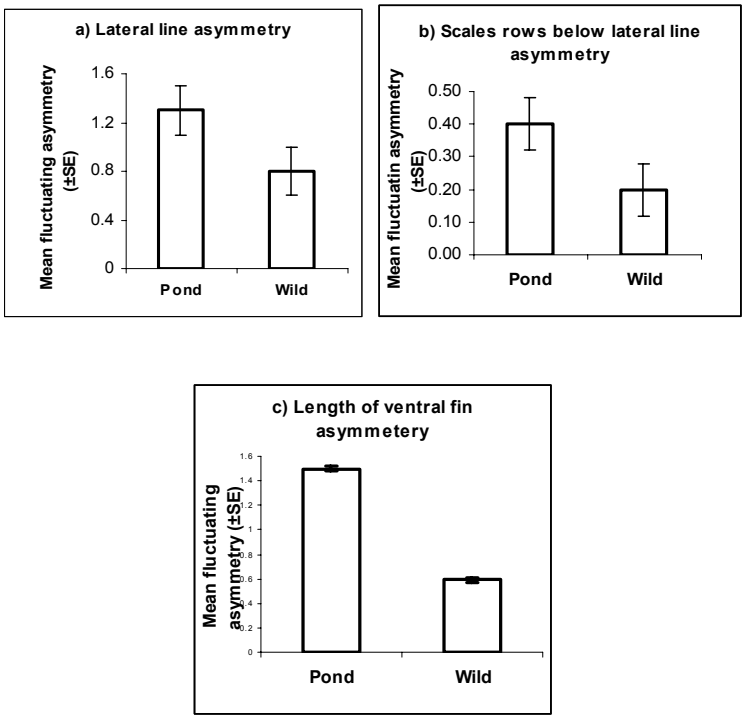


Figure 2.3. Variation in fluctuating mean asymmetry (± SE) calculated as (right – left) for several bilateral characters: wild vs. pond reared carp for a) lateral line, b) scales below lateral line and c) length of ventral fin. Replotted from Almeida *et al.*, (2008).

2.1.6 Aims of the present study

With this background, the broad aim of the study described in this chapter was to make a quantitative comparison of the structure of the respiratory structures in two sympatric morphs of Arctic charr with different feeding ecology and, possibly, different exposure to different oxygen regimes. The main specific objectives were:

- To develop methods for quantifying the structure of the respiratory apparatus and levels of asymmetry in gill structure in brown trout.
- To apply these methods to Arctic charr
- To compare gill structure in two sympatric morphs of Arctic charr occupying habitats likely to differ in levels of dissolved oxygen.
- Supplementary aims were to examine levels of asymmetry in gill structure in both species and also carry out an overall comparison of respiratory structure in charr and trout.

2.2 Material and Methods

2.2.1 Subjects

Twenty four Arctic charr from two sympatric morphs of Arctic charr and twenty nine brown trout were used to study respiratory function. Brown trout (average length 14.4 cm) were collected from Loch Lomond by gill-net during autumn. Arctic charr (average length 18.9 cm) were collected from Loch Awe, Scotland by gill-net, ten during the autumn spawning seasons and fourteen during the spring spawning season. The spring morph feeds on benthos on the loch bottom and the autumn morph feeds in mid water. Fishes were killed by a schedule 1 method (benzocaine, overdose, followed by a blow to the head), frozen and transported directly to the laboratory (Glasgow University's Scottish Centre for Ecology and the Natural Environment, Rowardennan, Loch Lomondside).

2.2.2 Morphometrics

2.2.2.1. *General morphometrics*: Total length (to the nearest 1 mm) and head length (to the nearest 1 mm) of all fish were measured. The opercular bones on the two sides of each fish were removed from their ligaments, cut from the head and soaked for a few minutes in potassium hydroxide. They were lightly brushed with a stiff tooth brush to remove attached tissue. After these procedures, opercular height and width were measured (Figure 2.4).

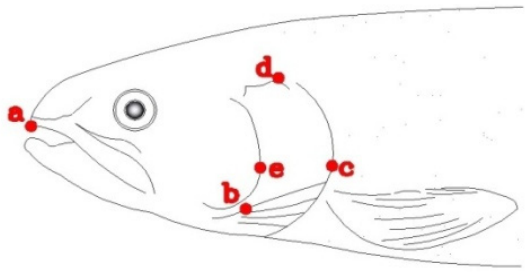


Figure 2.4. Variables of head morphometry measured in Arctic charr and brown trout: head length (a-c); operculum height (b-d) and operculum width (c-e).

2.2.2.2 *Buccal and gill cavity volume*: Buccal cavity volume (cm^3) was measured using a mould method modified after Okuda *et al*. (2002). For both study species, a mould of the buccal and gill cavity was obtained by injecting silicon (and for the first few trout to be examined, putty) into the mouth. "Care was taken to inject at a constant pressure, until the entire buccal cavity was full", leaving the mouth slightly opened, and the operculum held closed. The moulds were left to dry for 20 min with the fish on ice. The buccal moulds were removed and their volume then determined by water displacement, duplicate measurements being taken for each silicon mould and the mean calculated.

2.2.2.3 *Gill morphometrics*: All gill arches from both sides of each fish were dissected from fish and placed in 0.9 % normal saline. The gills from two sides of the fishes were excised, keeping the rakers and filaments intact. The gill arches were separated, mucous was removed by gentle scraping, using a scalpel at an angle of 45° and the measurements indicated in Table 2.3 taken for the left and the right sides of each arch using a binocular microscope at a magnification of 3X with an eyepiece micrometer (after Hughes, 1984).To quantify individual respiratory status with respect to those variables, the mean value for the right and left sides were used.

Table 2.3. Summary of the measurements made to characterise gill structure on brown trout and Arctic charr.

Structure	Arctic charr	Brown trout
Arch	Length of each gill arch	Length of each gill arch
Raker number	Total number of gill raker for all gill arches	Total gill raker number for all arches
Raker length	Length of each gill raker	
Filament number	Total number of gill filament for all gill arches	Total gill filament number for all arches
Filament length	Length of every tenth filament for all gill arches	
Lamellar number	Number of secondary lamellae per mm on every tenth gill filament of all gill arches	
Lamellar length	Length of secondary lamellae on top, middle and base of every tenth gill filament of all gill arches	
Lamellar distance	Distance between secondary lamellae on top, middle and base of every tenth gill filament of all gill arches	

2.2.3 Measuring fluctuating asymmetry

Fluctuating asymmetry (FA) in paired structures was calculated after Evans and Hatchwell, (1993) as follows:

$$FA = (XR - XL) / Max (XR, XL)$$

where XR is right variable side and XL is left variable side.

For both brown trout and Arctic charr this formula was applied to the length of each gill arche.

Measurement error is a problem when small levels of asymmetry are involved. Therefore analysis of FA was only carried out for structures when asymmetry measures were repeated clearly. This was the case for gill arches (Table 2.4), so only three structures were used.

Table 2. 4. **Regression analyses of duplicate measurements for length of gill arches 1st and 2nd for Arctic charr.**

Variable	Regression equation	F	P	R^2
Asymmetry 1st arch length (cm)	Y= -0.056 + 0.095 X	10.17	*<0.001*	41.4
Asymmetry 2nd arch length (cm)	Y= -0.0115 + 1.02 X	21.15	*<0.001*	42.0

2.2.4 Measuring body condition

The relationship between weight and length for fish (brown trout) was estimated using the condition factor (K):

$$K = W / L^3$$

where W and L are weight (g) and length (mm) of the same fish.

2.2.5 Otolith size and age data

The otholith surface was ground, polished and examined according to Fraser *et al.,* (1998). The age of each Arctic char was estimated by counting annular rings in the otolith, taking nine counts per otolith. Length at age was estimated using a calibration comprising a regression of otolith size on fish body length over all fish (both morphs) ($F_{1, 40} = 4.92$ and $p = 0.012$). There was no effect of morph on the relationship between body size and otolith size ($F_{1, 40} = 1.61$ and $p = 0.211$).

2.2.6 Statistical analysis

The following statistical procedures were carried out, using the MINITAB statistical package. Firstly, the data were checked for normality and transformations performed where necessary. Initial scrutiny of the data was carried out using means and standard errors (SE). Regression analysis was then used to explore the relationship between all variables and body length. Residuals from the regression on length were used to generate length-independent variables where appropriate (as described by Reist, 1985; Adams and Huntingford, 2004). Relations among measured variables were studied by correlation analysis. Principal Components Analyses (PCA) was used to examine covariance among related variables and to generate economical compound scores where appropriate. For simplicity, separate PCAs were carried out for gill arch length, gill raker means and gill filament means. The main derived scores from those analyses were combined by a further PCA and ANCOVA used to examine relationships between the main measure of respiratory development and body length and head length in the two categories of fish. T-tests were used to compare to the morphology of spring and autumn morphs.

2.3 Results

2.3.1 Gill structure and respiratory function in brown trout:

The trout data were collected by way of method testing, but are included here for completeness and for comparative purposes.

2.3.1.1 General morphometrics: Table 2.5 shows the mean values (± SE) of all the measured variables averaged over the left and right sides of the head for brown trout, together with the results of ANOVA by gill arch number.

Table 2.5. Mean and standard error of the mean ± SE for arch length, filament number and gill raker number of 29 brown trout, together with the results of ANOVA and *post hoc* comparison using the Tukey test.

Gill arch	Arch (cm)	Filament	Raker
	Mean ± SE	**Mean ± SE**	**Mean ± SE**
1st	1.9 ± 0.03	88.2 ± 1.29	20.4 ± 0.36
2nd	1.7 ± 0.03	77.7 ± 1.24	17.5 ± 0.44
3rd	1.5 ± 0.02	66.5 ± 1.37	14.1 ± 0.39
4th	1.2 ± 0.03	51.6 ± 1.47	10.8 ± 0.42
F3,88	90.7	90.5	62.8
P	*<0.001*	*<0.001*	*<0.001*
Tukey test	1≠2,3,4; 2≠3,4 and 3≠4	1≠2,3,4; 2≠3,4 and 3≠4	1≠2,3,4; 2≠3,4 and 3≠4

Gill arch length deceased significantly from front to back, with *post hoc* tests confirming that each successive arch was shorter than the one in front. Both number of filaments and number of raker also decreased significantly from the front to the back of the head, with decreasing arch length. Again *post hoc* tests confirmed that these two measures decreased for each successive gill arch from filament number for second gill arch, also filament number for fourth arch was different from filament number for third gill arch.

2.3.1.2 Relationship between measured variables and body length in brown trout: Table 2.6 shows the results of regression analyses of the relationship of all measured variables with body length. A significant positive relationship with body length was found for the following variables: head length, length of arches 1, 2, 3, filament number for all arches and height and width of the operculum. For further analysis, these variables were therefore expressed as residuals from the regression with length and are referred to as "length corrected" in the following text.

Table 2.6. Regression analyses of total body length (cm) against head length (cm), condition factor, the arch length (cm), the filament number (mm), raker number, opercular height and width (cm) and buccal cavity volume (ml) for 29 brown trout. Italics indicate significant relationships.

Variable	Arch	Regression	$F_{1,28}$	P-value	R^2
Total body weight (cm)		Y= - 41.3 + 4.92 X	119.30	*<0.001*	80.29
Head length (cm)		Y= 0.6 + 0.157 X	23.81	*<0.001*	44.9
Condition factor		Y= - 0.28 +0.067 X	41.02	*<0.001*	58.8
Arch length (cm)	1	Y= 1.0 + 0.133 X	7.68	*0.01*	19.8
	2	Y= 0.9 + 0.116 X	5.40	*0.03*	14.0
	3	Y= 0.7 + 0.098 X	4.77	*0.04*	12.3
	4	Y= 0.9 +0.051 X	1.74	0.19	2.4
Filament number	1	Y= 44.2 + 6.09 X	10.62	*0.00*	27.0
	2	Y= 51.1 + 4.48 X	6.56	*0.02*	17.6
	3	Y= 30.4+ 4.74 X	5.15	*0.03*	13.8
	4	Y= 13.3 + 4.41 X	4.10	*0.05*	10.6
Raker number	1	Y= 15.1 + 0.401 X	1.05	0.32	0.2
	2	Y= 15.2 + 0.150 X	0.14	0.72	0.0
	3	Y= 12.2 + 0.122 X	0.09	0.77	0.0
	4	Y= 15.8 + 0.367	0.70	0.41	0.0
Opercular height (cm)		Y= 0.50 + 0.03 X	8.78	*0.01*	23.0
Opercular width (cm)		Y= 0.31 + 0.03 X	5.64	*0.03*	15.1
Buccal cavity volume (ml)		Y= 3.03 - 0.04 X	0.04	0.98	0.00

2.3.1.3 Correlations among measured variables: Table 2.7 shows the matrix of correlations for arch length across all arches (Table 2.7a), for gill raker number across all arches (Table 2.7b), for gill filament number across all arches (Table 2.7c) and for opercular width and height and buccal cavity volume, all length-corrected as appropriate (Table 2.7d).

Table 2.7. The matrix of correlations for arch length across all arches (a), gill raker number across all arches (b), gill filament number across all arches (c) and opercular width and height and buccal cavity volume (d) of brown trout. In each cell, the top figure is the correlation coefficient and the bottom figure is the P-value.

a) Arch length

	1st arch	2nd arch	3rd arch
2nd arch	0.927 0.000		
3rd arch	0.805 0.000	0.832 0.000	
4th arch	0.607 0.001	0.588 0.001	0.853 0.000

b) Raker number

	1st arch	2nd arch	3rd arch
2nd arch	0.847 0.000		
3rd arch	0.559 0.002	0.617 0.001	
4th arch	0.556 0.003	0.563 0.002	0.737 0.000

c) Filament number

	1st arch	2nd arch	3rd arch
2nd arch	0.883 0.000		
3rd arch	0.564 0.002	0.631 0.000	
4th arch	0.627 0.000	0.615 0.001	0.602 0.001

d) Opercular size and buccal cavity

	Opercular height	Opercular width
Opercular width	0.553 0.003	
Buccal cavity	-0.157 0.003	-0.178 0.395

Many of the variables were highly correlated with each other even though body length was allowed for. In order to explore the relationships among these variables and, if possible, to condense the data, three principal component analyses (PCA) were carried out, taking the data for gill arch length, gill filaments and gill rakers separately. PCA on gill arch length (Table 2.8a) shows that the first 3 PCAs explained 99% of variation in gill arch length. PC1 accounted for 79% of the total variation, with positive loading for all arches. This therefore represents variation in overall gill arch size, over and above variation in body length. PC2 accounted 16% of the total variance and had negative loading for length of the first and second arches and positive loading for the third and fourth arches. This represents differential development of gill arches at the front and back of the gill cavity. PC3 (accounting for just 4% of variance) opposed length of the second and fourth gill arches against length of arches 1 and 4.

Table 2.8. Principal Component coefficients for the first three for a) gill arch length, b) gill filament number and c) gill raker number for all arches of brown trout.

Gill arch	a) Arch length			b) filament number			c) raker number		
	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3
1st	*0.50*	*- 0.51*	- 0.58	*0.51*	*-0.34*	*0.64*	*0.49*	*0.56*	*0.47*
2nd	*0.52*	*- 0.41*	0.34	*0.53*	*-0.34*	-0.05	*0.52*	*0.43*	*-0.47*
3rd	*0.53*	0.27	0.60	*0.52*	-0.03	*-0.74*	*0.50*	*-0.46*	*-0.50*
4th	*0.45*	*0.71*	- 0.45	*0.43*	*0.88*	0.20	*0.49*	*-0.54*	*0.55*

PCA for gill filament counts (Table 2.8b) shows that the first 3 principal components explained 87% of variation in this data set. PC1, which accounted for 77% of the total variance, has positive loadings for both variables and so represents variability in gill filament number, independent of body size. PC2 accounted for 14% of the total variance and represents differential variation in anterior arch filament number opposed to the posterior arch. These components have negative loadings for filament number for arches 1, 2 and 3 but positive loading for the fourth arch. PC3 (accounting for just 8% of variance) opposes filament number for the second and third arches.

For gill raker numbers (Table 2.8c), the first three components explained 95% of the total variance. PC1 (explaining 68% of the variance) has positive loadings for all scores and so again reflects variation in overall raker number, independent of overall body length. PC2 (18% of variance) opposes gill raker number for the third and fourth arches to the first and second arch represents differential developmental in front and back of head. PC3 (9% of variance) had positive loading for raker number for the first and fourth arches.

2.3.1.4 Asymmetry in gill arch length: Table 2.9a shows mean ±SE asymmetry scores for the length of each arch for brown trout. Table 2.9b shows the correlations among the 4 asymmetry measures and Table 2.9c shows the results of a Principal Components on these values. The correlation matrix showed positive relationships between asymmetry scores for all gill arches. PC1 accounted for 79 % of the total variance and had positive loadings for asymmetry in all arches; the score on this component is therefore used as an index of overall asymmetry in gill arch length. PC2 (14%) has negative loadings for the front arches and positive loadings for the back of the head and thus defuse differences in asymmetry in front and back of head.

Table 2.9. a) Mean (SE) asymmetry scores for length of each gill arch in b) Correlations between mean asymmetry scores for the 4 arches; italics indicate significant relationship, (In each cell, the top figure is the correlation coefficient and the bottom figure is the P- value) . c) Principal Component Analysis for asymmetry in gill arch length for 29 brown trout.

a)

Gill arch	Asymmetry arch length
	Mean ± (SE)
1st	0.003±**0.017**
2nd	0.005±**0.022**
3rd	0.009±**0.022**
4th	0.027±**0.028**

b)

Gill arch	**Asymmetry PCA**	
	PCA1	**PCA2**
1st	0.50	- 0.44
2nd	0.53	- 0.29
3rd	*0.53*	0.02

c)

	1st arch	**2nd arch**	**3rd arch**
2nd arch l	*0.853* *0.000*		
3rd arch	*0.773* *0.000*	*0.880* *0.000*	
4th d arch	*0.494* *0.008*	*0.572* *0.001*	*0.687* *0.000*

There were no significant relationships between overall asymmetry in gill arch length, as measured by PC1, and either length (r^2 = 0.09, N 24, P= 0.63), weight (r^2 = 0.03, N= 24, P= 0.80) and condition factor (r^2 = 0.02, N= 24, P= 0.91).

2.3.2 Gill structure and respiratory function in Arctic charr:

2.3.2.1 General morphometrics: Table 2.10 shows the mean values (± SE) for all measured variables in Arctic charr averaged across the two sides of the head for each gill arch, together with the results of ANOVA by arch number. *Post hoc* testing confirmed that the first arch was the longest (Table 2.10a) and that mean arch length decreased successively between the 2nd, 3rd and 4th arches. Gill raker length and number differed significantly between gill arches (Table 2.10b and c). For gill raker length, *post hoc* tests showed no difference between the first two arches, but significantly shorter gill rakers for the third and fourth arches. For both variables, *post hoc* tests revealed no significant difference between

the first and second gill arches, but values for the third and fourth gill arches were smaller than those for the first arch.

Mean length and number of the gill filaments (Table 2.10d and e), as well as the mean length of the secondary lamellae (Table 2.10f), also decreased significantly towards the back of the head, with the longer first and second arches bearing many, long gill filaments and relatively long secondary lamellae. For filament length, post hoc tests showed significant differences between scores for all arches, whereas for filament number the fourth arches had significantly different scores from the second and third arches. For secondary lamella length, post hoc testing showed that scores for the fourth gill arch were just different from those for the first arch. The numbers of lamellae per mm of filament (Table 2.10g) did not differ significantly between gill arches (Table 2.10h).

Table 2.10. Mean ±SE for a) gill arches length and b) gill raker length and c) gill raker number and d) filament length and e) filament number and f) secondary lamellae length and g) secondary lamellae number and h) distance between secondary lamellae, for 24 Arctic charr, from Loch Awe. Results of

Gill arch	a) Arch length (cm)	b) Raker length (mm)	c) Raker number	d)Filament length (mm)	e) Filament number	f) lamellar length (mm)	g) lamellar number (per mm)	h) lamellar distance (mm)
	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
1st	2.7 ± 0.10	1.20 ± 0.05	19.4 ± 0.55	3.9 ± 0.16	81.9 ± 1.75	0.10 ± 0.01	60.2± 4.4	0.05 ± 0.002
2nd	2.4± 0.09	1.10 ± 0.06	18.6 ± 0.46	3.8 ± 0.17	73.8 ± 1.31	0.09 ± 0.01	58.3± 5.2	0.05 ± 0.003
3rd	2.0± 0.09	0.93 ± 0.05	16.8 ± 0.43	3.6 ± 0.14	65.0 ± 1.42	0.08± 0.01	57.4± 5.5	0.04 ± 0.003
4th	1.6± 0.09	0.70 ± 0.04	13.3 ± 0.52	3.0 ± 0.15	51.5 ± 1.53	0.08 ± 0.01	55.6± 5.9	0.04 ± 0.003
F3,92	28.5	16.7	30.2	7.1	73.8	4.1	0.5	0.8
P	0.00	0.00	0.00	0.00	0.00	0.01	0.68	0.48
Tukey test	1≠2,3,4; 2≠3,4 and 3≠4	1≠3,4; 2≠3,4 and 3≠4	1≠3,4; 2≠4 and 3≠4	1≠3,4; 2≠3,4 and 3≠4	2≠4 and 3≠4	1≠4		

ANOVA and *post hoc* test are also shown.

Figure 2.5 shows the distribution of estimated total filament lengths (mm) for all charr. The overall mean ±SE value is 1976.6 (± 94.3) mm, with considerable variation about this mean, from a minimum of 1100 to a maximum of 2600.

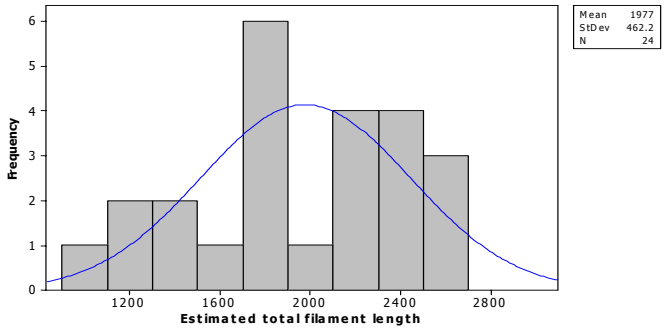


Figure 2.5 Frequency distribution of estimated total filament lengths (mm) for all Arctic charr.

2.3.2.2 Relationship between measured variables and body length: Table 2.11 shows the results of regression analyses of the relationship of all variables with body length. Significant positive relationships were found for the following variables: head length, length of all arches, and filament length for all arches, filament number for arches 1 and 4, raker length for arch 1, opercular height and width and volume of gill cavity and buccal cavity. For further analysis, these variables were therefore expressed as residuals from the regression with length and are referred to as "length-corrected" in the following text.

Table 2.11. Regression analyses of total body length (cm) and head length (mm), average arch length (cm), average filament length (mm), average filament number, average secondary lamellae length (mm), average secondary lamellae number and average distance between secondary lamellae (mm), average gill raker number, average gill raker length (mm), average height and width of the operculum (cm), average buccal cavity volume (ml) and average gill cavity volume (ml) for 24 Arctic charr *Savelinus alpinus*. Italics indicate significant relationships.

Variable	Arch	Regression equation	F	P	R^2
Arch length	1	Y= 1.72 + 0.12 X	15.25	*0.00*	*38.8*
	2	Y= 1.80 + 0.09 X	7.83	*0.01*	*26.0*
	3	Y= 1.04 + 0.10 X	10.32	*0.00*	*31.0*
	4	Y= 0.49 + 0.09 X	9.11	*0.01*	*27.7*
Filament length	1	Y= 2.47 + 0.18 X	9.55	*0.01*	*26.6*
	2	Y= 2.59 + 0.15 X	6.63	*0.02*	*18.1*
	3	Y= 3.22 + 0.13 X	6.51	*0.02*	*21.6*
	4	Y= 1.07 + 0.10 X	6.93	*0.02*	*17.0*
Filament number	1	Y= 95.90 + 1.44 X	4.35	*0.05*	*9.7*
	2	Y= 92.80 + 0.96 X	3.94	0.06	7.0
	3	Y= 79.80 + 0.94 X	2.72	0.11	19.9
	4	Y= 50.80 + 1.41 X	5.56	*0.03*	*0.0*
Lamellar length	1	Y= 0.16 + 0.00 X	0.02	0.89	0.6
	2	Y= 0.17 – 0.00 X	0.26	0.61	0.0
	3	Y= 0.19 – 0.00 X	1.71	0.21	1.8
	4	Y= 0.16 – 0.00 X	0.73	0.40	0.0
Lamellar number	1	Y= 51.10 + 0.48 X	1.59	0.45	0.0
	2	Y= 60.60 + 0.12 X	0.03	0.87	0.0
	3	Y= 35.4 + 1.16 X	2.32	0.14	5.4
	4	Y= 27.8 + 1.47 X	3.34	0.08	9.2
Lamellar distance	1	Y= 0.08 – 0.00 X	0.10	0.75	0.0
	2	Y= 0.09 – 0.00 X	1.38	0.25	1.9
	3	Y= 0.09 – 0.00 X	1.49	0.24	2.4
	4	Y= 0.09 – 0.00 X	1.64	0.21	2.8
Raker number	1	Y= 23.40 + 0.31X	1.82	0.19	4.4
	2	Y= 0.59 + 0.05 X	3.91	0.06	9.6
	3	Y= 19.70 + 0.29X	2.90	0.10	9.1
	4	Y= 18.00 + 0.11X	0.23	0.63	0.0
Raker length	1	Y= 0.78 + 0.05 X	5.72	*0.03*	*14.6*
	2	Y= 24.50 + 0.19 X	0.94	0.34	11.3
	3	Y= 0.74 + 0.04 X	2.67	0.12	7.0
	4	Y= 0.34 + 0.03 X	2.78	0.11	7.6
Head length		Y= 2.365 + 0.07X	5.19	*0.03*	*15.4*
Height opercular		Y= 0.460 + 0.03X	15.22	*0.00*	*38.0*
Width opercular		Y= 0.445 + 0.03 X	15.09	*0.00*	*38.0*
Buccal cavity volume		Y= - 0.02 + 0.09X	6.06	*0.02*	*18.0*
Gill cavity volume		Y= -1.12 + 0.14 X	16.62	*0.00*	

2.3.2.3 Correlations among measured variables: Table 2.12 shows the matrix of correlations for arch length for all arches, for gill raker number and length across all arches (Table 2.12a), for gill filament number and length for all arches (Table 2.12d), for secondary lamella number, length and spacing for all arches and for opercular width and height and buccal cavity volume (Table 2.12e), body size-corrected as appropriate.

Table 2.12a. Correlation matrix for body size corrected gill arches length, all fish combined. In each cell, the top figure is the correlation coefficient and the bottom figure is the P- value.

	1st arch	2nd arch	3rd arch
2nd arch	0.959 0.000		
3rd arch	0.914 0.000	0.942 0.000	
4th arch	0.905 0.000	0.952 0.000	0.955 0.000

Table 2.12b. Correlation matrix for length corrected gill raker length and gill raker number for all gill arches, all fish combined. In each cell, the top figure is the correlation coefficient and the bottom figure is the P- value.

	1st raker length	2ndraker length	3rdraker length	4th raker length	1st raker number	2ndraker number	3rd raker number
2ndraker length	0.777 0.000						
3rdraker length	0.502 0.012	0.804 0.000					
4th raker length	0.450 0.028	0.430 0.036	0.669 0.000				
1st raker number	0.167 0.435	0.174 0.415	0.176 0.412	-0.029 0.892			
2ndraker number	0.381 0.066	0.553 0.005	0.376 0.070	0.239 0.261	0.281 0.183		
3rd raker number	0.056 0.793	0.210 0.325	0.484 0.017	0.356 0.088	0.307 0.145	0.453 0.026	
4th raker number	-0.100 0.641	0.132 0.539	0.145 0.498	-0.117 0.585	0.171 0.424	0.356 0.088	0.546 0.006

Table 2.12c. Correlation matrix for length corrected gill filament length and gill filament number for all gill arches, all fish combined. In each cell, the top figure is the correlation coefficient and the bottom figure is the P- value.

	1st filament length	2ndfilament length	3rdfilament length	4thfilament length	1st filament number	2ndfilament number	3rdfilament number
2ndfilament length	0.477 0.019						
3rdfilament length	0.684 0.000	0.727 0.000					
4thfilament length	0.473 0.020	0.766 0.036	0.519 0.009				
1st filament number	0.286 0.176	0.557 0.005	0.331 0.114	0.401 0.052			
2ndfilament number	0.281 0.184	0.464 0.022	0.264 0.213	0.353 0.091	0.879 0.000		
3rd filament number	0.309 0.142	0..552 0.005	0.333 0.112	0.406 0.049	0.828 0.000	0.891 0.000	
4thfilament number	0.491 0.015	0.587 0.003	0.282 0.181	0.483 0.017	0.540 0.006	0.634 0.001	0.687 0.000

Table 2.12d. Correlation matrix for length corrected gill secondary lamellae length and gill secondary lamellae number and spacing between secondary lamellae for all gill arches, all fish combined. In each cell, the top figure is the correlation coefficient and the bottom figure is the P- value.

	1st lamellar length	2nd lamellar length	3rd lamellar length	4th lamellar length	1st lamellar number	2nd lamellar number	3rd Lamellar number	4th lamellar number	1st lamellar distance	2nd lamellar distance	3rd lamellar distance
2nd lamellar length	0.217 0.308										
3rdlamellar length	0.292 0.167	0.727 0.000									
4thlamellar length	0.244 0.250	0.771 0.000	0.869 0.000								
1stlamelar number	-0.015 0.945	-0.316 0.133	-0.008 0.969	-0.108 0.614							
2ndlamellar number	-0.135 0.529	-0.434 0.034	-0.294 0.164	-0.376 0.070	0.759 0.000						
3rdlamellar number	-0.065 0.763	-0.217 0.309	0.068 0.753	0.049 0.821	0.802 0.000	0.528 0.008					
4thlamellar number	-0.069 0.749	-0.166 0.438	0.030 0.889	0.011 0.958	0.829 0.000	0.601 0.002	0.896 0.000				
1stlamellar distance	0.314 0.135	-0.152 0.478	-0.254 0.232	-0.232 0.275	-0.469 0.021	-0.424 0.039	-0.562 0.004	-0.563 0.004			
2ndlamellar distance	-0.098 0.650	0.275 0.194	0.173 0.419	0.173 0.419	-0.138 0.520	-0.074 0.732	-0.273 0.197	-0.260 0.219	0.221 0.300		
3rd lamellar distance	-0.114 0.596	0.343 0.101	0.102 0.635	-0.045 0.834	-0.275 0.193	-0.034 0.874	-0.476 0.019	-0.381 0.066	0.243 0.253	0.522 0.009	
4th lamellae distance	-0.081 0.708	0.413 0.045	0.141 0.512	-0.004 0.986	-0.322 0.126	-0.087 0.685	-0.496 0.014	-0.400 0.052	0.216 0.310	0.482 0.017	0.988 0.000

Table 2.12e. Correlation matrix for length corrected buccal cavity volume, gill cavity volume, opercular height and opercular width, all fish combined. In each cell, the top figure is the correlation coefficient and the bottom figure is the P- value.

	Buccal volume	Gill volume	Opercular height
Gill volume	0.900 0.000		
Opercular height	0.740 0.000	0.741 0.000	
Opercular width	0.671 0.000	0.703 0.000	0.961 0.000

In order to explore these relationships, principal component analyses (PCA) were carried out on the data for gill arch length, gill rakers, gill filaments and secondary lamellae separately (Tables 2.13 and 2.14).

Table 2.13a. The principal component coefficients for the first three component of a PCA for a) gill arch length and b) gill raker variables for all arches of Arctic charr (n= 24).

Arch	a) Arch			b) Rakers			
	PC1	PC2	PC3		PC1	PC2	PC3
1st	0.49	0.71	-0.14	Length	0.30	0.46	0.38
				Number	0.18	-0.29	0.51
2nd	0.51	0.23	0.24	Length	0.48	0.22	0.21
				Number	0.39	-0.19	0.30
3rd	0.49	-0.45	-0.73	Length	0.48	0.10	-0.25
				Number	0.33	-0.47	-0.33
4th	0.50	-0.47	0.62	Length	0.36	0.24	-0.55
				Number	0.17	-0.58	0.02
% variation explained	93	4	2		41	*22*	13

The principal components analysis for the gill arch length (Table 2.13a) showed that the first three components explained 99% of the variation in the data set. PC1 accounted for 93% of total variance and had positive loading for all variables, representing overall gill arch size, regardless of body size. PC2 accounted for 4% of the total variance and opposed length of the third and fourth gill arch lengths to those of first and second gill arches, representing differential development of the anterior and posterior gill arches. PC3 (accounting for just 2% of total variance) opposes length for the first and third arches to those for the second and third arches. For the gill raker variables (Table 2.13b), the first 3 components explained 76% of the total variance. PC1 (41%) has positive loadings for all scores and so reflects variation in overall raker development, independent of overall body size; fish with many, long rakers get a high score on this component and fish with few and short raker get low scores. PC2 (22%) opposed gill raker number to gill raker lengths; fish with many short rakers get a high score on this component, and the converse. Loadings for PC3 (13%) opposed raker scores for the third and fourth arches to those for the first and second arches, representing relative development of rakers on the front and back arches.

Table 2.14 shows the results of PCA on respiratory structures. The first three components resulting from PCA for gill filament measurements (Table 2.14a) explained 82% of variation in this data set. PC1, which accounted for 48% of the total variance, had positive loadings for all variables and so represents variability in filament size and number (representing an index of overall filament development) independent of body size. PC2 accounted for 23% of the total variance and had positive loadings for filament length on all arches and negative loadings for filament number; fish with few, long filaments gain high

scores in this axis. PC3 (11%) opposed filament scores for the first and second arches to those for the third and fourth arches, reflecting differential development of filaments at the front and back of the gill cavity.

Table 2.14. Principal components coefficiant from a PCA for a) filament number and filament length b) secondary lamella number, length and distance for Arctic charr. Italics indicate significant relationships.

Arch		a) Filament				b) Secondary lamellae		
		PC1	PC2	PC3		PC1	PC2	PC3
1st	Length	0.21	0.43	-0.66	Length	0.06	-0.22	0.01
	Number	0.41	-0.31	-0.04	Number	0.39	0.10	0.39
					Distance	0.07	0.23	-0.31
2nd	Length	0.25	0.52	-0.23	Length	0.25	-0.42	0.00
	Number	0.41	-0.38	-0.12	Number	0.33	0.08	0.44
					Distance	0.21	-0.02	-0.52
3rd	Length	0.39	0.32	0.36	Length	0.10	-0.57	-0.13
	Number	0.44	-0.29	-0.08	Number	0.41	0.12	0.15
					Distance	0.35	0.14	-0.37
4th	Length	0.29	0.34	0.59	Length	0.12	-0.57	0.03
	Number	0.35	-0.09	-0.04	Number	0.41	0.13	0.11
					Distance	0.37	0.11	-0.33
% variation explained		48	23	11		40	23	16

For the secondary lamellae (Table 2.14b), the first three components explained 79% of the variation in the data set. The first component accounts for 40% and has positive loadings for most variables, but particularly for secondary lamellae number. It therefore represents an index of overall development of the secondary respiratory surfaces. PC2 explains 23% of total variance, with high negative loadings for secondary lamellae length, particularly for gill arches 2-4. PC3 (16%) mainly relates to between secondary lamellae on first arch.

2.3.2.4 Combining PCA derived scores: Table 2.15a shows correlations between the PCA scores for gill arch, rakers, filaments and lamellae. Positive relationships were found among arch PC1, filament PC1 and lamellae PC1 and the scores on these derived axes were combined using a for the PCA (Table 2.15b).

Table 2.15a. Correlation matrix between the PCA scores for gill arch, gill rakers, gill filaments and secondary lamellae. In each cell, the top figure is the correlation coefficient and the bottom figure is the P- value.

	Arch PC1	Arch PC2	Arch PC3	Raker PC1	Raker PC2	Raker PC3	Filament PC1	Filament PC2	Filament PC3	lamellar PC1	lamellar PC2
Arch PC2	0.000 1.000										
Arch PC3	0.000 1.000	-0.000 1.000									
Raker PC1	1.000 *	0.000 1.000	0.000 1.000								
Raker PC2	0.000 1.000	1.000 *	-0.000 1.000	0.000 1.000							
Raker PC3	0.000 1.000	-0.000 1.000	1.000 *	0.000 1.000	-0.000 1.000						
Filament PC1	0.556 0.005	0.201 0.347	0.093 0.316	0.556 0.005	0.201 0.347	0.093 0.666					
Filament PC2	0.006 0.978	0.225 0.291	0.214 0.316	0.006 0.978	0.225 0.291	0.214 0.316	0.000 1.000				
Filament PC3	0.264 0.212	0.081 0.706	-0.590 0.002	0.264 0.212	0.081 0.706	-0.590 0.002	0.000 1.000	0.000 1.000			
Lamellar PC1	0.465 0.022	0.157 0.546	-0.443 0.030	0.480 0.017	0.118 0.582	-0.443 0.030	0.202 0.345	-0.324 0.123	0.366 0.079		
Lamellar PC2	0.130 0.544	0.429 0.037	0.286 0.175	0.277 0.190	0.429 0.037	0.286 0.175	0.293 0.165	0.366 0.078	0.092 0.668	0.000 1.000	
lamellar PC3	0.025 0.909	-0.154 0.473	0.136 0.527	0.134 0.531	-0.154 0.473	0.136 0.527	-0.158 0.462	0.200 0.348	-0.011 0.959	-0.000 1.000	-0.000 1.000

Table 2.15b. Principal component coefficients for the first three principal components of PC analysis of PC scores of gill arch length (PC1,Table 2.13a), filament length and number (PC1,Table 2.14) and secondary lamellae number and distance between secondary lamellae (PC1,Table 2.14).

Variable	PC1	PC2	PC3
Arch PC1	0.598	-0.441	0.669
Fil1ament size PC1	0.614	-0.284	-0.736
Lamellae number and distance PC1	0.590	0.291	0.753
% variation explained	72	20	8

The first component, which accounted for 72% of the total variance, is an overall measure of development of respiratory structures, independent of length. This variable is significantly related to estimated gill area (regression F= 15.38, P= 0.001; Figure 2.6). The second component (accounted for 20 %) of the variance and separates fish with gill having relatively small arches and small primary filaments but a large number of well spaced secondary lamellae from the converse differentiates the front and back of the gill structures.

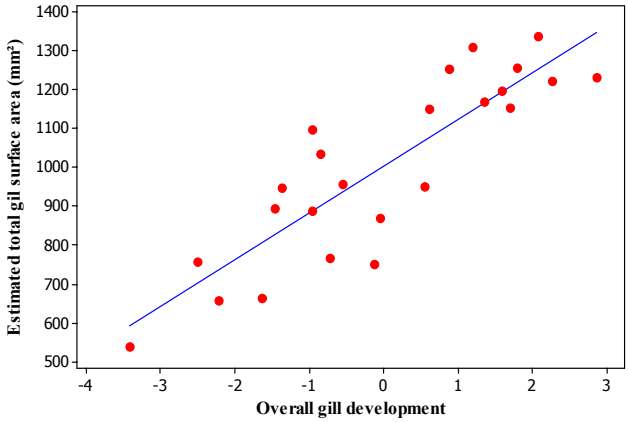


Figure 2.6. Relationship between the PCA derived measure of length-independent overall gill development and estimated gill area (mm²).

2.3.2.5 Asymmetry in gill arch length: Table 2.16a shows mean values ± SE for asymmetry scores for length of each of the 4 gill arches. Table 16b shows correlations among these values and Table 16c shows the results of a PCA of asymmetry scores for the 4 arches. PC1 (accounting for 61% of the total variance) has positive loadings for all arches, providing an index of general asymmetry. PC2 accounted for 22% of the total variance and opposed asymmetry in the first and second arches to that in the third and fourth arch length.

Table 2.16. a) Mean (±SE) values for asymmetry scores for arch length, b) correlation between asymmetry scores in the four gill arches (In each cell, the top figure is the correlation coefficient and the bottom figure is the P- value) and c) results of PCA gill arches asymmetry scores in 24 Arch charr.

2.16a)

Arch	Asymmetry
1st	0.02 ± 0.02
2nd	-0.01± 0.02
3rd	0.02± 0.03
4th	0.02± 0.03

2.16b)

	1st arch	2nd arch	3rd arch
2nd arch	0.536 0.007		
3rd arch	0.327 0.119	0.603 0.002	
4th arch	0.207 0.331	0.665 0.000	0.692 0.000

2.16c)

	PC1	PC2
1st	0.38	0.82
2nd	0.56	0.15
3rd	0.50	- 0.36
4th	0.53	-0.41
Variation %	61	22

2.3.2.6 Comparison between autumn and spring Arctic charr morphs: Table 2.17a shows mean ± SE values for all univariate variables in the spring and autumn charr morphs and Table 2.17b shows mean ± SE of the PCA derived summary scores, both with the results of T-tests. The two morphs did not differ in body length, but fish of the spring morph had significantly larger heads than those of the autumn morph, reflected in head length, opercular dimensions and length of the gill arches. In terms of trophic apparatus, the spring

morph had larger buccal cavity volume and shorter gill rakers. In terms of respiratory structure, compared with the autumn morph, fish of the spring morph had a larger gill cavity volume, more, longer gill filaments, more and more closely spaced secondary lamellae and, consequently, a strikingly larger (*c* 30%) estimated respiratory area.

To determine where the differences in estimated gill area and overall gill development were simply the result of a relatively large head in spring morph, PC scores for gill were regressed on head length. These regressions were significant for estimated gill area (0.017) and for overall gill development (0.001). Residuals were used to give head length corrected scores. T test showed significant between morphs for both scores, with spring morph larger than autumn morph (Estimated gill area mean = 49241, - 68938; T= - 2.28; P= 0.036) and (Overall gill development = 0.642, - 0.899; T= - 5.19; P= 0.000).

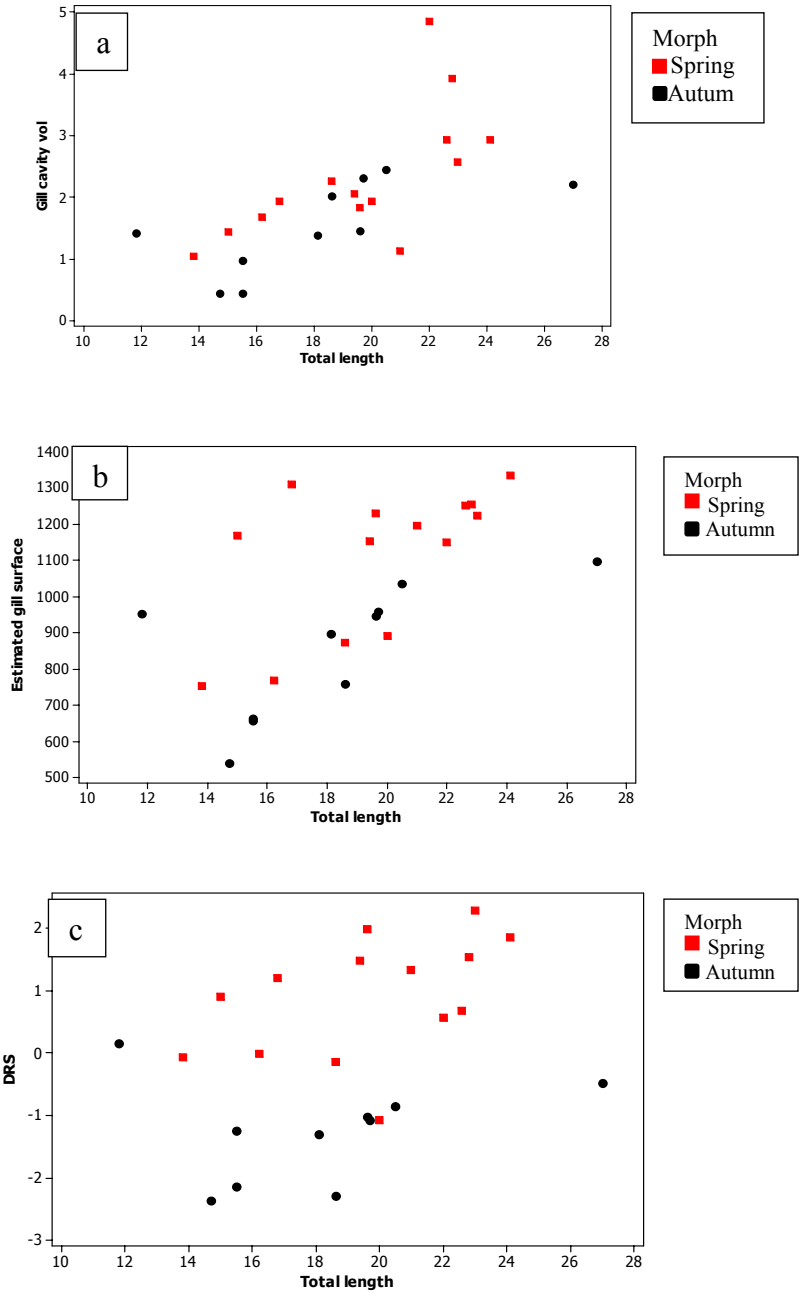
Table 2.17. A comparison of the mean ± SE of a) a range of simple morphometrics measure and b) PC scores, for 24 Arctic charr, of the 14 spring morph and 10 autumn morph.

a)	**Mean ± SE (X)**		**T**	**P**
	Spring morph	**Autumn morph**		
Body length (cm)	19.6 ± 0.86	18.1± 1.3	1.02	0.33
Head dimensions				
Head length (cm)	3.9 ± 0.18	3.4 ± 0.09	2.73	*0.01*
Arch length (cm)	2.4 ± 0.11	1.8 ± 0.08	-4.11	*<0.001*
Operculum height (cm)	1.1 ± 0.05	0.9 ± 0.04	- 2.72	*0.01*
Operculum width (cm)	1.0 ± 0.05	0.8 ± 0.04	-2.83	*0.01*
Raker length (mm)	0.9 ± 0.05	1.1 ± 0.08	-2.28	*0.04*
Raker number	17.0 ± 0.52	17.2 ± 0.45	0.25	0.81
Respiratory structures				
Gill cavity volume (ml)	1.8 ± 0.21	1.1 ± 0.18	-2.22	*0.04*
Filament length (mm)	3.9 ± 0.14	3.2 ± 0.19	-2.62	*0.02*
Filament number	70.8 ± 1.50	64.2 ± 1.90	-2.73	*0.01*
Lamellar length (mm)	0.60 ± 0.03	0.5 ± 0.02	-.99	0.33
Lamellar number/ mm	67.19± 0.73	44.90 ± 1.0	-17.47	*<0.001*
lamellar distance (mm)	0.04 ± 0.003	0.05 ±0.002	3.05	*0.01*

b)	Mean ± SE (X)		P	T
	Spring morph	Autumn morph		
Arch PC1	1.01± 1.75	-1.42 ± 1.13	4.13	*<0.001*
Raker PC1	0.50 ± 0.51	-0.69 ± 0.48	-1.72	0.10
Raker PC1	0.32 ± 0.30	-0.45 ± 0.48	-1.35	0.19
Raker PC1	-0.221± 0.27	0.31 ± 0.32	1.28	0.21
Filament	0.05± 0.37	-0.08± 0.44	- 0.23	0.82
Filament PC1	- 0.23 ± 0.29	0.320 ± 0.17	1.63	0.11
Lamellar PC1	1.52 ± 0.34	-2.13 ± 0.21	-9.22	*<0.001*
Lamellar PC1	0.29 ± 0.19	-0.41± 0.19	-0.88	0.40
Arch length asymmetry PC1	0.80 ± 0.40	1.13 ± 0.58	2.80	*0.01*
Estimated total filament length	1112 ± 201	851 ± 184	3.29	*0.004*
Head length PC1 *Gill area	0.60 ± 0.33	-0.84 ± 0.16	3.94	<0.001
Head length PC1 * Overall PCA	0.76±0.28	-1.07±0.21	5.26	<0.001

2.3.2.7 Relationship between overall gill development, gill cavity volume and estimated gill surface and head length in brown trout: Figure 2.7 shows the relationship between head length and the 3 scores of gill development. For gill cavity volume and estimated gill surface, there was a significant positive relationship (ANCOVA: gill cavity volume $F_{1,21}$ = 6.81. P = 0.016; estimated gill surface $F_{1,21}$ =19.05, P <0.001; development of respiratory surfaces $F_{1,21}$). Once this was allowed for, EGS (ANCOVA: $F_{1,21}$= 3.48. P = 0.08) but not gill cavity volume (ANCOVA: $F_{1,21}$ = 0.77. P = 0.39) was pitched marginally higher in benthic feeding fish. In contrast development of respiratory surfaces was not significantly related to head length (ANCOVA: $F_{1,21}$= 2.79. P = 0.11), but was significantly higher in benthic-feeding fish (ANCOVA: $F_{1,21}$= 20.8. P < 0.001).

Figure 2.7. Relationship between body length and three measures of gill development in benthic-and pelagic-feeding morphs of Arctic charr from Loch Awe. a) Gill cavity volume measured by silicon injection; b) estimated gill surface (EGS) calculated from a modified version of Hughes' formula. And c) overall development of the respiratory surfaces (DRS) derived from successive principal components analyses of gill morphometrics).



2.3.2.8 Age and growth: Figure 2.8 shown the relationship between fish length and otolith diameter at capture in charr of the two morphs which overall was significant positive ($F_{1,40}$ = 5.98, P= 0.02 and R^2 = 0.17). There was difference in this relationship between morphs ($F_{1,40}$ = 2.59, p= 0.12).

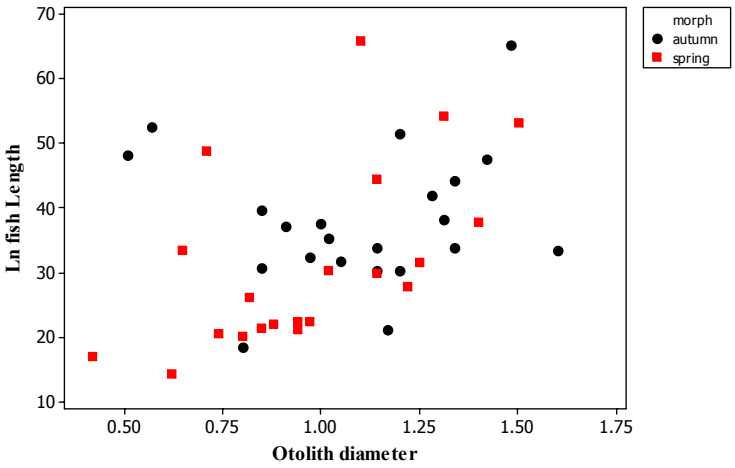


Figure 2.8. The relationship between fish length and otolith diameter at capture in two morphs of charr.

Analysis of covariance showed a highly significance increase in estimated fish length with age (T=5.49 and p= <0.001) and a marginally significant morph effect (T= 1.99 and p= 0.05) (Figure 2.9). The autumn charr was slightly larger than spring charr at any given age (inter capture = 1.142 for spring charr and 1.154 for autumn charr).

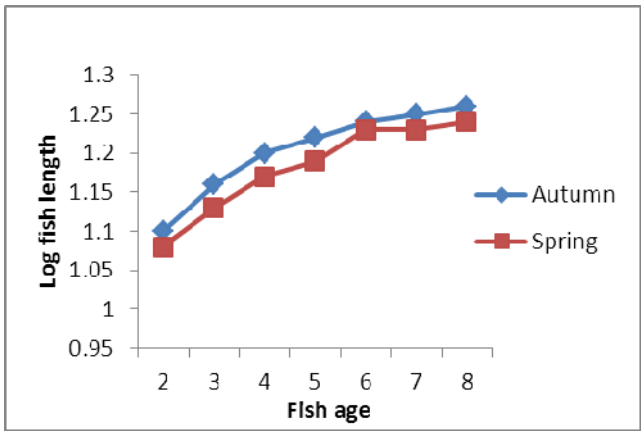


Figure 2.9. The relationship between fish length and age in two morphs of charr.

2.3.2.9 Comparison between Brown trout and Arctic charr morphs: To compare gill morphometrics in trout and charr, data for body length, gill arch length, gill raker number and gill filament number for two species were combined. Regression analyses of these variables on length was carried out for the data set and, where significant relationships were found, residuals were used to produce gill variables independent of fish are show loading. As for the separate species, PCA analyses were carried out for gill arch length, gill raker number and gill filament number. In each case, the first PCA reflected overall of gill structure variation test. Table 2.18 shows mean (± SE) of the PCA derived summary

scores, both with the results of T tests comparing species. Brown trout had significantly larger gill arch and better development raker and filaments compard to Arctic charr.

Table 2.18. Means ± SE PCA1 scores for gill arch length, gill raker number and gill filament number results of T-tests comparing for the brown trout and arctic charr.

	Mean ± SE (X)		T	P
	Brown trout	Arctic charr		
PCA1 gill arch length	0..51± 0.21	-0.59 ± 0.50	-2.02	*0.05*
PCA1 gill raker	0..39± 0.15	-0.44 ± 0.23	-3.05	*0.00*
PCA1 gill filament	1.21± 0.23	-1.36 ± 0.35	-6.20	*0.00*

2.4 Discussion

2.4.1 The aims of this study

The main aim of the present study was to investigate whether the known differences in foraging mode and microhabitat use between two sympatric forms of Arctic charr, spring and autumn, are reflected in their respiratory apparatus. In order to achieve this aim firstly methods for quantifying the fine structure of the respiratory apparatus in salmonid fishes, using brown trout were explored, and then applied these to Arctic charr of the two morphs. Because levels of asymmetry can be informative about exposure to environmental stress, were also explored asymmetry in gill arch length in brown trout and in the two charr morphs. Since detailed morphometric studies have not been previously carried out in Arctic charr, a broad comparison of gill structure in Arctic charr and brown trout was also made.

2.4.2 Gill structure in charr and trout

The present study indicates that the broad structure of the gills of brown trout and Arctic charr is similar to other teleost fish, freshwater and marine. There are four gill arches on each side of the gill chamber consisting of a curved bony bar bearing many fine filaments as well as a number of gill rakers (Weitzman, 1962; Hughes and Mittal, 1980; Severi *et al*., 2000). Within this general structure, there was considerable variability in detailed gill morphometrics and in buccal and gill cavity volume in both species. Such differences within fish species have been reported in other studies (Fernandes and Rantin, 1986; Severi

et al., 2000). Variation in size of the respiratory structures can partly be explained by gill position, arch length decreasing from front to back in both species, but is partly due to differences in body size. A positive relationship between gill morphometrics and body size has been commonly reported for teleost fish, for example *Hetropneustes fossilis* (Hughes, 1974). *Piaractus mesoptamicus* (Severi *et al.,* 1997). This relationship is thought to depend on increasing oxygen requirements with increasing body size. Variability about the general relationships can be explained in part by the activity of the fish, its mode of breathing and the environmental conditions in which it lives (Severi *et al.,* 1997). In general, relatively large gills suggests an adaptation for an active mode of life (Severi *et al.,* 1997; Rosenberger and Chapman, 2000) and measurements of gill structure and function have permitted conclusions about habitat and habit of fish. Specifically, more active fish species with high requirements for oxygen have large respiratory exchange gill areas (Mazon *et al.,* 1998. In the snail-eating *A. alluaudia,* head volume is 31% larger than in fish from an insect-eating group; this is associated with internal reallocation of respiratory apparatus through a change in the shape of the gills (Smith *et al.,* 1996).

2.4.3 Variability in gill raker morphometircs in trout and charr

The current study has demonstrated variable numbers of gill rakers in both species and variable raker length in charr. This is partly dependent on body length, but is also related to feeding habit. Numbers and lengths of gill raker reported in this study for trout and charr are comparable to those in other studies on charr (Heese, 1993) and various teleost (Ruzzante *et al.,* 1998 and 2003). The gill raker number in brown trout (11-20) and Arctic charr (14-25) reported in the present study was in accordance with the studies by Langeland and Nost (1995) on Brown trout (*Salmo trutta*) (11.8-20.4) and Arctic charr (*Salvelinus alpinus*) (13.3-19.4). However, the gill raker lengths for Arctic charr reported here are quite low (0.7-1.20 mm) in comparison to the charr (1.42 – 2.65 mm) and trout (1.17- 4.07 mm) studied by Langeland and Nost, (1995) and quite high in comparison to *Lepomis megaletis* (0.2-1.0 mm) Shoup and Hill, (1997).

Variation in raker number and length is functionally connected to food type in a diversity species (Robinson and Wilson, 1994; Ruzzante *et al.,* 1998). Gill rakers are used by fish for straining food and other materials from the water and hence raker number and structure vary with the food and feeding habit of the fish (Munshi *et al.,* 1984). An increase in the number of gill rakers is assumed to decrease the pore size through which the water is filtered, allowing the fish to retain small particles. Conversely, fish that feed on large food

items tend to have fewer, shorter gill rakers. Gill raker length is correlated with the size of prey and substrate, as well as with head size; relatively long gill rakers may increase zooplankton retention in charr (Doherty and McCarthy, 2004). Ruzzante *et al.,* (1998) found that differences in gill raker morphology between two benthic groups of *Percichthys trucha* were correlated with food and habitat; both forms were benthic feeders, but one (found at depths greater than 10 m) had shorter gill rakers than the other, which was found in the littoral area between 10 m to 20 m depths. Both forms feed on benthic invertebrates, but the deep benthic form fed less on larval Anisoptera than did the littoral form, that are likely to be mainly abundant on the littoral zone than did those of the deep benthic form (Ruzzante *et al.,*1998).

2.4.4 Variability in respiratory structures

As for gill arch length, a positive relationship was found in the present study between body length and both gill filament number and length, for both trout and charr. This reflects the need for a greater gill respiratory area to service the metabolic needs of larger fish (Mazon *et al.,* 1998).The total number of gill filaments in the two species (88.2 and 272.2) in trout and char was relatively low compared to various other species of fishes. For example, mean gill filament number was 661.5 in *Botia lohachtta* (Sharma *et al.,* 1982), 1210.9 in *Garra lamta* (Ojha *et al.,* 1989) and 368 in *Xenentodon cancila* (Ham.) (Dahal, 2003).

The present study showed that the length of filaments on the different arches was variable, being shorter for the more posterior arches; other studies have also reported differences in length between the anterior and posterior hemibranches (Severi *et al.,* 2000). It has been suggested that both morphological and physiological constraints arising from the shape of the head determine this pattern of variability (Galis and Barel, 1980; Palzenberger and Pohla, 1992). In both trout and charr, the numbers of gill filament (and gill filament length in charr) contribute to, but account for only a part of, the overall gill area.

Filament number (and length in arctic charr) of each gill arch increase with fish length, but even when this was allowed for, there was individual variation in the extent of length-dependent development of the filaments. Above a certain size, the only way in which overall filament development can be increased is through an increase in length rather than number. In both trout and charr, the number and length of the gill filaments decreased from the front to the back gill arches; this is evident both from the univariate measures and from the principal component analysis, PC2 from the multivariate analysis of gill filaments

having a strong front to back polarisation. Similar results have been found for *Dicentrarchus labrax* (Silan and Maillard, 1987) and *Liza ramada* (Severi *et al.,* 2000). The length of the gill filaments in trout and charr is almost equal to that of sea bass (*Dicentrarchus labrax*) (Saroglia *et al.,* 2002), and larger than that of *Colisa fasciatus* (Prasad, 1988).

In the present study, length, number and spacing of the secondary lamellae were unrelated to total body length, suggesting that these aspects of gill structure may instead be tuned to the life style of the fish concerned and/or ambient levels of dissolved oxygen. Similar findings and a similar explanation have been given by Rosenberger and Chapman, (2000) and Saliu and Olonire, (2007). The average number of secondary lamellae per mm of gill filament fell from the front to the back arches. Again, this was reflected both in the univariate statistics and in the results of the principal component analysis, with PC2 from the multivariate analysis of lamellae dimensions also having a strong front to back gradient.

The number of secondary lamellae per mm (*ca* 60) in Arctic charr is not high when compared to other fish species, such as *ca* 66/mm in *Gaira lamta* (Ojha *et al.,* 1982) and *ca* 64/mm in *Glyptothorax pectinopterus* (Sinha and Agarwal, 1991). The number of secondary lamellae/mm on a gill filament is not less than 60 in active fish species such as mackerel (*Scomberomrus sp*) and menhaden (*Brevoortia sp*), whilst it is 22-24 in sluggish species such as toadfish (*Opsanus tau)* and about 44 in intermediate species such as sea robin (*Prionotus sp)* Hughes, 1966). Not surprisingly, differences in number of lamellae are reflected, inversely, in differences in the spacing between them. The average of distance between secondary lamellae in Arctic charr is low (0.04-0.05mm) in comparison for example to 0.07 in *L.piscatorius.* (Hughes, 1966). The differences in distance between secondary lamellae in different fish species (freshwater and marine) can be related to the ecological niches (Prasad, 1988). Arctic charr have closely spaced lamellae (0.04-0.05mm), suggesting Hughes, (1966) an active way of life, and comparable to values for active swimmers such as *Thunnus sp* (0.06mm. Muir and Hughes, 1969) and herring (0.058mm, De Silva, (1974) and in contrast to slow-moving speices such as the plaice (*Pleuronectes sp*) which have secondary lamellae spacing *ca* 0.077mm. De Silva, 1974). According to all these gill criteria, the Arctic charr in the present study would be considered as relatively active fish. The length of the secondary lamellae is an important determinant of gill surface area, tends to be species specific and correlates fairly well with

the life-style of the fish concerned (Hughes and Morgan, 1973), though physiological as well morphological constraints limit the achievable range for this (Galis and Barel, 1980; Palzenberger and Pohla, 1992). Decreasing secondary lamellae length would lower resistance to water flow across the gills, but would also decrease total gill area. Overall, gill area is determined by a combination of length of the gill arches, number and length of the gill filaments and number and length of the secondary lamellae. This explains why in the present study there was a positive relationship between the PCA-derived score of the extensiveness of the respiratory structures and total gill areas as estimated area using Hughes' equation, (1984).

2.4.5 Asymmetry

Measurement of asymmetry in the length of the gill arches was repeatable when the same fish was measured on 2 separate occasions but were variable across individual fish for both trout and charr. For both species, principal components analysis identified a dimension of general asymmetry reflected in the length of all four arches and in PC2, a measure of differential asymmetry in the front and back of the head. Fluctuating asymmetry reflects the inability of bilateral organisms to fully control development and has therefore been proposed as a measure of developmental stability (Felley, 1980; Almeida *et al*., 2008). Two possible origins for fluctuating asymmetry have been suggested, namely genetic effects such as levels of inbreeding and environmental (exogenous) effects such as exposure to pollutants during development (Jawad, 2003; Ayoade *et al*., 2004; Eriksen *et al.*, 2008).

2.4.6 Differences between the two charr morphs

The two morphs of Arctic charr, while not differing in overall body size, showed marked differences in several length-corrected head measurements. Overall, the benthic-feeding spring morph had a relatively larger head than the autumn, pelagic feeding form, including larger buccal and gill cavity volumes and slightly fewer gill rakers. This can be related to their diet with benthic invertebrate prey being significantly larger in size than pelagic prey fed upon by charr (Fraser *et al.,* 1998). Similar trophic adaptations have been described for benthic feeding forms of Arctic charr from other water bodies (Snorrason *et al.,* 1994). The present study showed that autumn morph grows faster than spring morph at age, despite no different in body size. The growth at ages difference between the two morphs of Arctic

charr may be connected with habit and habitat of fish morphs (Kopf *et al.,* 2011). The seasonal growth cycles could be connected to feeding system, cycle of reproductive as well as to physiological changes during the season for example changes in temperature and oxygen levels (Pajuelo and Lorenzo, 2003). In addition, differences in growth at age between the two morphs used in the present study may be related to genetic variances. The autumn morph showed slightly higher levels of fluctuating asymmetry in gill arch length, which may reflect the fact that, as indicated by otoliths analysis, fish of this morph had grown faster in their early years and the fact that fast growth is costly (Garduno-Poz and Adams in preparation).

A striking difference found in the present study was the markedly more extensive development of the respiratory apparatus in the spring-breeding, benthic feeding form reflected both in the PCA derived measure and in Hughes' estimate, even when corrected for head size. The difference in overall development of the respiratory surface is accompanied by, and results from, a number of morphological differences between the two forms of charr. Thus, the spring, benthic-feeding morph has a larger buccal cavity volume than the autumn, planktonic feeding form, which brings with it longer gill arches and correlated differences in filament number and length and secondary lamellae height.

There are at least two possible explanations, which are not mutually exclusive, for this difference in respiratory structures, which has not previously been described for sympatric trophic morphs. In the first place, it may reflect exposure to different levels of oxygen in the benthic and pelagic feeding zones that the two forms exploit and metabolic need (Mathias *et al.,* 1998). Alternatively, differences in respiratory function between morphs may reflect differences in activity patterns between morphs. Plankton feeding charr living in the limnetic zone tend to be continuously mobile (Snorrason *et al.,* 1994). Thus the rate of water flow over the gills may be higher in the autumn morph as a result, allowing reduced investments in respiratory tissue. Another feature that may affect activity is that, because, unlike zooplankton, benthic prey are clumped, predictable and defensible, benthic feeding charr have been shown to be more aggressive than pelagic fish (Mikheev *et al.,* 1996). Aggression is energetically costly and in salmonids generally, aggressive fish often have higher resting metabolic rates (Huntingford *et al.,* 2010). It may therefore be that the benthic charr in the present study had greater oxygen requirements resulting from higher levels of aggressive behaviour than their pelagic counterparts and so require a larger respiratory surface.

2.5 Conclusions

The method used here to quantify development of the respiratory surface was based on successive PCAs of measurements of the various gill components. This produces a comparable variable (overall gill development) that was broadly equivalent to gill surface area based on Hughes' equation, but also showed up additional dimensions of variation. Another method, gill cavity volume estimated from silicone moulds was related to, but less sensitive than estimate gill area, but was markedly more rapid to derive. Apart from these technical points, the main finding of the work described here is that benthic-feeding spring morph of Arctic Charr from Loch Awe has a markedly larger respiratory surface than the pelagic, autumn form. This may reflect exposure to water with lower dissolved oxygen levels and/or a more active lifestyle in the benthic form. Developing and maintaining a relatively large respiratory surface could be seen as a hidden cost of a benthic feeding habit. On the other hand higher levels of asymmetry in the autumn morph may be a cost of the faster growth seen in this form.

CHAPTER 3

COPING STRATEGY AND RESPIRATORY DEVELOPMENT IN COMMON CARP

The carp used in the work described here was screened for coping strategy by Dr. Flavia Mesquita in discussion with Hussein Jenjan. The morphometric and histological results presented here are new and this work performed by Hussein Jenjan.

3. 1 Introduction

3. 1.1 Behavioural syndromes and coping strategies

Numerous studies on behaviour in vertebrates, including rats (Koolhass *et al.,* 1999), chickens (van Hierdn *et al.,* 2002) and fish (Moretz *et al.,* 2007, Wilson and Godin, 2009) have shown that individuals of a given species, population, age category and gender may differ consistently in their behavioural properties. This is sufficiently striking to be compared to personality differences among humans (Wilson, 1989 and 1994; Gosling 2001; Schjolden *et al.,* 2005*;* Bergmüller and Taborsky, 2007). The term "behavioural syndrome" is sometimes used to describe situations in which behaviour shown by individuals in different contexts is correlated (Sih *et al.,* 2004; Bell, 2007). For example, individuals often show variable and correlated levels of risk-taking in a variety of different situations; in such cases, there is said to be a shy-bold continuum, or occasionally two peaks in a bimodal distribution (representing that may be called shy or bold) (Wilson *et a*l., 1993; Sinn and Moltschaniwskyj, 2005). Risk-taking individuals, at the bold end of the shy-bold continuum, tend to make active responses to changes in their environment. Thus they readily investigate unfamiliar things, behave aggressively when challenged by a rival and attempt to escape when challenged by other negative events (Schjolden *et al.,* 2005). Additionally, risk-taking individuals tend to build up routines and to show little behavioural plasticity when exposed to change. In contrast, risk-avoiding individuals from the shy end of the shy-bold continuum show low levels of aggression, approach new objects with marked caution, do not develop routines to the same extent and are generally flexible in their behavioural responses to change. Van Oers *et al.,* (2005) concluded that reliable character differences in one context can therefore predict how animals will behave in other environments, provided the response norms and exact ecological conditions have been determined.

Such behavioural variants are often associated with differences in physiological responsiveness to stress, in which case, they are sometimes referred to as stress coping styles, as described in rodents and in humans (Schjolden *et al*., 2005). In this framework, there exists what is sometimes called the proactive-reactive axis (Reale and Festa-Bianchet, 2003). Proactive individuals are aggressive, risk taking and inflexible, respond actively to challenge and in physiological terms give a predominantly sympathetic, adrenaline-based response. By contrast, reactive individuals are risk-avoiding, non-

aggressive and flexible, respond to challenge by freezing or hiding and show a predominantly para-sympathtic, cortisol-based response (Koolhaas *et al.,* 1999. Korte *et al.,* 2005). For example, in the European rabbit (*Oryctolagus cuniclus*) there is a correlation between physiological and behavioural responses; individuals that react to the odour of a potential predator (a fox) with a low rate of scanning showing increased plasma corticosterone levels in response to challenge (Rödel *et al.,* 2006). However, the behavioural and physiological aspects of responding to challenge are not always linked in this way.

3.1.2 The shy-bold continuum and coping strategies in fish

Much of the work on coping strategies in non-human animals has been carried out on mammals, but a number of studies that have demonstrated the existence of elements of a behavioural syndrome and coping strategies in fish (Clement *et al.,* 2005; Huntingford and Adams, 2005; Schjolden et *al*., 2005; Øverli *et al.,* 2007). For example, in cichlid fish (*Nannacara anomala)*, individual differences in boldness towards a model predator correlate with fighting performance (Brick and Jakobsson, 2002). Huntingford (1976) established that aggressive behaviour shown by breeding male three-spined sticklebacks (*Gasterosteus aculeatus)* toward conspecific rivals is related to boldness toward a predator outside the breeding season. There are several possible explanations for such an association, one being that these two dimensions of behaviour in sticklebacks share common physiological mechanisms. Coleman and Wilson, (1998) found that sunfish (*Lepomis gibbosus)* classified as bold on the basis of response to a non threatening motivation (a novel food source) and a potentially threatening stimulus (a red-tipped metre stick extended towards the individual) acclimated quickly to the laboratory, fed more on prey, were more exposed and difficult to capture, and engaged in more predator inspection than did shy sunfish. In addition, a study on brown trout showed that individuals classified as bold on the basis of their readiness to inspect a novel object had an advantage in pairwise fights with co-specifics classified as shy (Sundstrom *et al.,* 2004).

In addition, some studies on rainbow trout (*Orcoihynchus mykiss*) propose the presence of differential coping styles similar to those described in mammals. Rainbow trout strains selectively bred for high and low levels of plasma cortisol in response to a standard stressor, show other differences in physiology and behaviour that may reflect different stress coping strategies. Thus individuals of the low reactive strain take more risks (for example, feeding sooner after being placed in an unfamiliar tank) than do those from high reactive strain, and

tend to dominate fish from the high responsiveness strain in pair-wise fights (Fevolden and Røed, 1993; Øverli *et al.,* 2004, Schjolden *et al.,* 2005). Correlated behavioural and physiological variation typical of stress coping strategies have also been described in unselected strains of both brown trout (*Salmo troutta*) (Brelin *et al.,* 2008) and rainbow trout (Schjolden *et al.,* 2005).

Stress coping strategies also have implications for respiratory physiology in fishes. For example, common carp (*Cyprinus carpio*) classified as proactive on the basis of risk-taking in a novel environment and low cortisol reactivity have higher resting metabolic rates than do those classified as reactive (Huntingford *et al*., 2010). Rainbow trout exposed experimentally to hypoxia show different behavioural strategies, some individuals displaying exhausting escaping behaviour and others remaining motionless (Van Raaij *et al.*, 1996). The active individuals (which had a much lower chance of surviving) showed a rise in plasma catecholamines that was four- to five times greater than that observed in the calm individuals, which in turn had a larger increase in plasma cortisol during hypoxic. These differences in plasma chemistry between trout are similar to stress coping strategies shown in higher vertebrates and in fact determine survival through hypoxic stress. (Van Raaij *et al.*, 1996)

3.1.3 Consequences of coping strategies for fitness in nature

In nature, there is often no single optimum state with respect to behaviour, physiology or morphology that maximises fitness; instead, depending on environmental circumstances, different combinations of traits (or different coping styles, in this case) may promote survival, growth and/or reproductive success (Schjolden *et al.,* 2005). For instance, bold individuals may put themselves at risk in environments in which predators are common, but in the absence of predators may have a competitive advantage, since they can forage unchallenged (Huntingford *et al.,* 1990). In general, aggressive, risk-taking, pro-active individuals tend to do better at high population densities when resources are abundant and predictable; conversely reactive animals do better at low densities, when resources are dispersed and unpredictable and a degree of flexibility is needed to locate them. This is the case for over-winter survival in great tits (*Parus major*) (Dingemanse *et al.,* 2002) and, possibly, for reproductive success in house mice (*Mus musculus*), reported in Korte *et al*., (2005). Brown trout inhabit a range of environments, in which population densities and productivity may vary unpredictably. If the fitness of individuals with different coping style in brown trout depends on the social environment and on resource distribution, the

distribution of coping styles is likely to differ between populations exposed to different environments (Brelin *et al.,* 2008).

3.1.4 Consequences for growth and survival in captivity

The environment in which fish are farmed differs strikingly and in many ways from that encountered by wild fish. These include the fact that fish are held at high densities, that food is abundant and often predictable and that the fish are to a large extent protected from predators. These are all features of the environment that are critical for the relative success of individuals at different points on the proactive-reactive continuum. The environment in which fish are cultured may influence the behaviour and physiology of cultured fish in a number of ways. Firstly, during a single generation the very different experiences of cultured and wild fish may modify the way behaviour develops; for example, in the absence of experience of direct predatory attacks they may fail to develop adequate anti-predator behaviour (Brown and Laland, 2001, Sundstrom *et al.,* 2004). Secondly, again during a single generation, differential mortality among fish with different coping strategies may alter the proportion of these phenotypes in cultured populations. Thirdly, over generations, such differential mortality may result in the establishment of permanent, inherited differences between wild and farmed fish, through the process of domestication. A number of studies support the theory that domestication imposes alterations in the behaviour of fish (reviewed in Huntingford, 2004; Huntingford and Adams, 2005). Fascinatingly, many of the differences reported between wild and hatchery fish resemble characteristics that differentiate proactive and reactive copers (Sih and Bell, 2008).

The study reported in this chapter links these different strands of research by examining a proxy for respiratory function in proactive and reactive individuals in a species that has been cultured for millennia, mainly in extensive or semi-intensive systems, namely the common carp (*Cyprinus carpio*).

3.1.5 Gill structure and function

The gills are the main site of gas exchanges and ion regulation in fish (Chang *et al.,* 2002; Ogundiran *et al.,* 2009) and their functional morphology has been extensively studied (Chapter 2 and see Kantham and Richards, 1995; Perry, 1997). The fine structure of the gills can change in the short term, in response to environmental conditions. For example, Ong *et al.,* (2007) showed that significant increases occur in the height of the interlamellar

cells of Killifish (*Kryptolebias marmoratus*) after one week of air exposure and a significant decrease in interlamellar cells after recovery in water. Gill morphology can therefore be used as an indicator of the health status of fish and, indirectly, of water quality (Ogundiran *et al.,* 2009). Gill structure of fish also varies on a longer, evolutionary time scale, in relation to level of activity and habitat of the fish concerned. Thus, active fishes with high metabolic needs or those inhabiting hypoxic environments usually have gill specializations facilitating gas transport (Mandic *et al.,* 2009). These specialisations include overall size of the gill arches, as well as the length of gill filaments and the number and area of the secondary lamellae, all of which respond to selection for increased oxygen uptake from the water.

Study of ventilatory mechanics and gill morphology suggests that the gills of teleost fish represent a balance between the need for gas exchange to meet metabolic demands and the energetic costs of the biphasic buccal pump system used to aerate the gills, costs that are magnified by branchial resistance (Hughes and Morgan, 1973). Hughes (1966) suggested that gill area could be most effectively increased by developing long gill filaments with large lamellae and this has subsequently been seen in many groups of fishes, including African swamp teleosts living in hypoxic waters (Chapman, 2007) and marine species living within the oxygen minimum layer (Graham, 2007). Large, fast swimming fish such as tuna (*Gymnosarda unicolor*) and swordfish (*Xiphias gladius*) have greater metabolic demands than do smaller, less active fish (Korsmeyer and Dewar, 2001). These metabolic demands are met by employing ram ventilation, in which the forward momentum of continuous swimming drives water over the gills (Roberts and Rowell, 1988). The morphometric characters contributing to the large gill surface areas of these high energy demand fish include more, and longer gill filaments, more secondary lamellae per mm of filament and longer secondary lamellae (Wegner *et al.,* 2010a).

Such changes in gill structure provide a larger area for gas exchange, but they also provide a larger area for the passive movement of ions between the fish and the surrounding water and so potentially compromise osmotic regulation (Hartl *et al.,* 2000; Nilsson, 2007). One way in which fish adapt is to adjust the number of epithelial cells between the secondary lamellae and hence the area of gill that is actually exposed to the water (Sollid *et al.,* 2003). Regression of the interlamellar cells during hypoxia results in greater exposure of the lamellae, thereby greatly increasing the respiratory gill surface area (Nilsson, 2007). Where fish are exposed to adverse conditions, such adaptive development of the epithelia cells, or hyperplasia, becomes a sign of poor environmental condition (Khoshnood *et al.,* 2011).

Hyperplasia can be induced by many factors, including chronic inflammation, hormone dysfunction and many sorts irritants, and serves to block harmful substances from the gills or to support rapid repair following injury or irritation (Lawler, 2005).

3.1.6 Asymmetry

Differential development of a bilateral nature on the two sides of an animal gereates asymmetry (Leary and Allendorf, 1989; Jawad, 2003). The term fluctuating asymmetry refers to the case in which a characteristic present on both body sides does not go through identical growth or development, with deviations being random with respect to laterality. Fluctuating asymmetry provides a measure of developmental sensitivity to environmental stress (Møller and Pomiankowski, 1993, Almeida *et al.,* 2008). Fluctuating asymmetry usually increases under environmental stress due to the failure of the homeostatic regulatory mechanism. In fish, levels of fluctuating asymmetry can be positively related to a wide range of stressors, for example loss of genetic variatiation (Mazzi *et al.,* 2002) and biotic stressors such as the toxic chemicals in the water (Estes *et al.,* 2006).

3.1.7 The biology of common carp (Cyprinus carpio)

3.1.7.1 General biology and ecology: The common carp (*Cyprinus carpio* L., 1758, Figure 3.1) is one of the larger fresh water species and also is one of the most important for fresh water aquaculture (Kucharczyk, 2002; Stuar and Jones, 2002). Carp can reach a maximum weight of about 60 kg and a maximum length of more than 1.20 m (Koehn *et al.,* 2000). Adult carp are benthic-feeding omnivores (Nathanael and Edirisnghe, 2001), taking a mixed and variable diet including water plants, insects, crustaceans and annelids (Goldstein and Simon, 1999). The mouth is terminal in young fish, which feed on zooplankton, becoming sub-terminal with age, with a barble on both sides. The eyes are positioned high on the head. The carp usually has a yellow body with orange fins, though there are many lighter variants. Carp live in the middle and lower reaches of lakes, often in shallow waters.

Figure 3. 1. The common carp (*Cyprinus carpio* L., 1758). Photograph by Jenjan (2008), bar=1.14 cm.

This is a highly adaptable species found in still and running water (Nathanael and Edirisinche, 2001) and able to survive in temperature ranges of 4-35 ^{o}C (Cole and Johanston, 2001), although they grow best in the range of 23-30 ^{o}C. Carp can tolerate salinities of 3.4 to 8.3 ppt (Stouthar et al., 1998; Whiterod, 2001). The optimal pH is 6-9 and they can survive oxygen concentrations as low as 0.3-0.5 mg/l.

3.1.7.2.Distribution: The common carp has a global distribution, originating in Europe in fresh water around the Black Sea and the Aegean basin (Tekin-özan *et al.,* 2008), but with subsequent introductions in many parts of the world (Figure 3.2). This wide geographical range in combination with a long history of culture has led to a diversification of types of carp, differentiated on the basis of morphological characteristics as well as geography. The main recognised forms are the common carp *Cyprinus carpio carpio* (found predominantly in Europe and Central Asia), the Amur carp *Cyprinus carpio haematopterus* (found in East Asia) and the wild carp *Cyprinus carpio viridiviolaceus* (found in Southeast Asia).

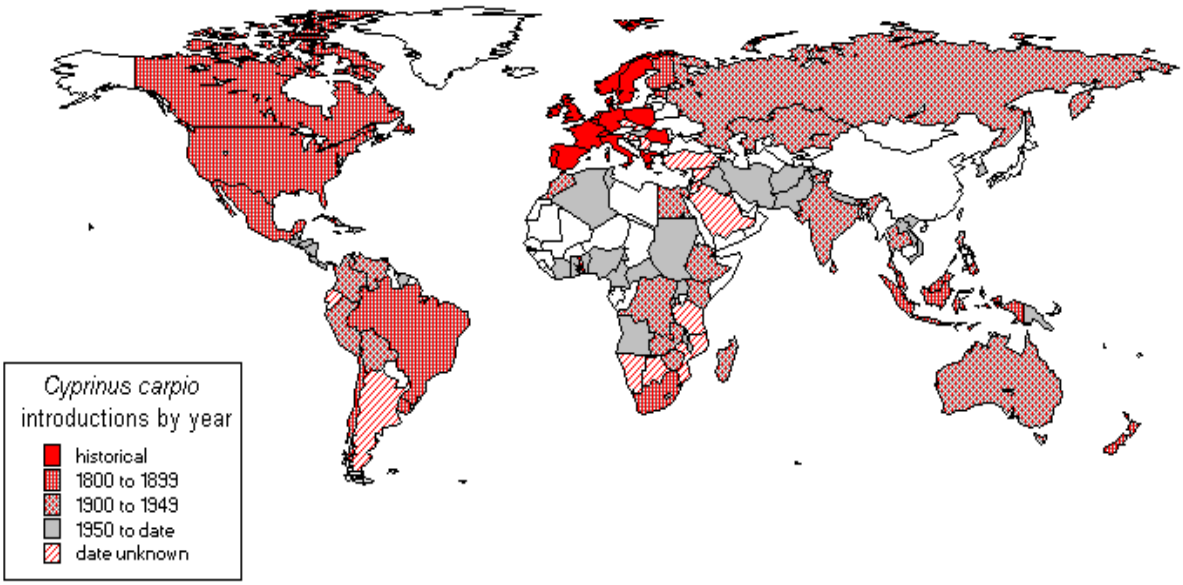


Figure 3.2. Global distribution of common carp, *Cyprinus carpio* (from Sorensen, 2006)

3.1.7.3. Growth and life history traits: Growth rates for carp in moderate latitudes are extremely variable and in some cases this may be related to genetic variability (Vilizzi and Walker, 1999; Tempero *et al.,* 2006). Age at maturity is also variable and is strongly dependent on both temperature and latitude. Reproduction occurs in males at 2-4 years of age (at a length of about 300mm) and in females at 3-4 years (at 300-500mm length; Tempero *et al.,* 2006). Breeding females produce up to approximately 30 million eggs, released with white glue that sticks the eggs to plants. The minimum temperature required to initiate spawning varies from 10 to 25^{o}C (Stuart and Jones, 2002). Spawning tends to occur during periods of high water when meadows are flooded to a depth of 20-50 centimetres (Rodrigues-Ruiz and Granado-Lorencio, 1992).

3.1.8 Coping strategies of common carp

Variable stress coping strategies have been reported in common carp (Huntingford et al. 2010). At the behavioural level, some (proactive) fish tend to take risks (emerging more rapidly from shelter to explore a novel, potentially dangerous environment) and are better able to compete for limited, spatially restricted food than are reactive fish, which avoid risk (tending to remain in shelter rather than emerging into a novel environment). In terms of physiological differences, reactive carp show greater cortisol responsiveness than do proactive fish, reflected in higher levels of expression of cortisol receptors in both head kidney and brain. They also show *ca.* 17% lower resting metabolic rates (Huntingford *et al.,* 2010). Given the association between gill structure and metabolic demands described at the between-species level (section 3.1.5 above), it is of interest to establish whether

proactive and reactive carp differ in the area of their respiratory surface. If so, potential loss of ions through such an extended gill area could represent an additional, indirect cost of the proactive strategy in fish.

3.1.9 Aims

With this background, the aims of the study described in this chapter are:

1. To screen individual common carp for coping strategy, using time to emerge from shelter into a novel environment as an easily-measured indicator.

2. To examine the relationship between various aspects of gill morphology and derive composite measures of the extent of development of the respiratory surfaces.

3. To examine gill microstructure in these same categories of fish, looking specifically at the extent to which the surface of the gill lamellae is covered by epithelial cells and the abundance of mucous cells.

4. To measure the degree of fluctuating asymmetry in the first gill arch, as possible indicator of past exposure to stress.

5. To compare gill morphometrics and microstructure in fish with different coping strategies.

3.2 Material and Methods

3.2.1 Subjects

Sixty seven common carp were obtained from Barony College, Dumfries, UK, and transported directly to the experimental aquaria in Glasgow University's Division of Ecology and Evolutionary Biology. They were screened for coping strategy and (after being used in a behavioural study not reported here, conducted by Flavia Mesquita, PhD student in the Division) were killed by a schedule 1 method (benzocaine, overdose, followed by a blow to the head) and their gill structure examined.

3.2.2 Screening for coping strategy

The fish were weighed (mean initial weight = 10.11g) and measured (mean initial total length = 8.41cm) and housed in two groups (of 27 and 33 fish) in 2 glass tanks (100 x 38 x 31.5 cm), each tank with a re-circulating filter and airstones. The temperature of the tanks was 18^{o}C. Carp were individually-marked using alcian blue dye (HO Licence number 60/3679) and photographed for future identification on the basis of a combination of dye mark and scale pattern. Risk-taking was assessed by the time taken for the fish to emerge from a darkened compartment with no food into a well lit, novel and potentially dangerous area in which they could see and smell food. This is known to be a consistent behavioural trait and predictive of coping strategy in carp (Huntingford *et al.,* 2010). Since carp are highly stressed by social isolation, they were tested in groups. The screening tank was 100 cm by 38 cm by 31.5 cm, with a water depth of 30.5 cm. Temperature was matched to that of the holding tanks (18^{o}C). At one end of the screening tank there was an enclosed, darkened settling chamber (30 cm in length), from which a plastic tunnel formed an exit into the main section of the tank. The opening to the tunnel was fitted with a removable plastic cover. Food (frozen chironomid larvae, or bloodworm) was placed on a clear container (6 cm in diameter and 30 cm in height) located in the centre of the main section, visible through the tunnel once the cover had been removed. The fish did not have access to the food in the container.

Before testing, defrosted bloodworm were placed in the clear container with an airstone to create movement. Prior to each trial, fish were deprived of food for at least 12h and then placed in the covered compartment in groups of 8 and allowed to settle down for 20 minutes. Olfactory cues were introduced to the open section of the tank by adding a small volume of water in which bloodworm had been macerated. The time (in seconds) taken by each fish to leave the settling chamber was recorded, up to a maximum of 20 minutes; fish that failed to emerge were given a notional, high score of 2000 seconds. This screening procedure was repeated 3 times for each fish, with a gap of at least 24 hours between trials. The fish were selected randomly for each trial from one of the holding tanks, to reduce the possibility that social interactions within established groups might influence behaviour during screening.

Figure 3.3 shows mean ($\pm$SE) emergence time for all the fish in each tank. A clear distinction can be made between those fish that emerged quickly in all 3 tests (fish number

1-5 in tank 1 and 1-10 in tank 2) and those that consistently failed to emerge (fish number 20-27 in tank 1 and 28-36 in tank 2). The fast emerging fish were designated as risk-taking or proactive and the slow emergers as risk-avoiding or reactive. The remaining fish were designated as intermediate, although since these fish sometimes took a long time to emerge and sometimes emerged quickly, it might be more accurate to describe them as flexible.

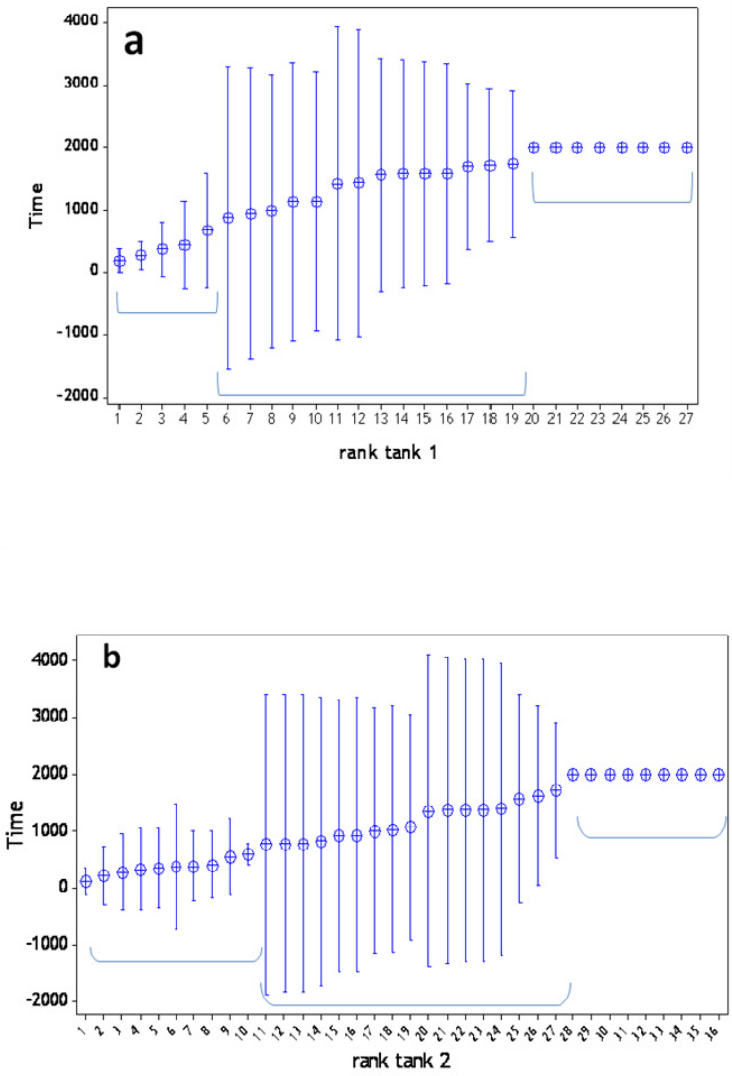


Figure 3.3. Interval plot of mean (SEM) emergence times for all carp in the novel environment test in each tank. a) tank 1 and b) tank 2. Brackets sign the division between the risk-taking phenotypes (reproduced with permission from Mesquita, 2010).

3.2.3 Morphometrics

3.2.3.1 *General morphometrics*: The body weight (g), total length (to the nearest mm), and head length (to the nearest mm) of all fish were measured. At the time of sacrifice, mean weight was 20.54 (g) and mean total length 10.37 (cm). Body condition was estimated using the condition factor (K):

$$K = W / L^3$$

where W and L are weight (g) and length (mm) of the same fish

3.2.3.2 *Buccal and gill cavity volume*: Buccal cavity volume (ml^3) was measured using a mould method modified from Okuda *et al*. (2002), in which a mould of the buccal and gill cavity was obtained by injecting silicon into the mouth, as described in Chapter 2. Care was taken to inject at a constant pressure until the entire buccal cavity was full, leaving the mouth slightly opened and the operculum closed. The moulds were left to dry for 20 min with the fish on ice. The buccal moulds were removed and their volume then determined by water displacement, duplicate measurements being taken for each silicon mould and the mean calculated.

3.2.3.3 *Operculum bone size:* The opercular bones on the two sides of each fish were removed from their ligaments, cut from the head and soaked for a few minutes in potassium hydroxide. They were lightly brushed with a stiff tooth brush to remove attached tissue. After these procedures, opercular length and width were measured as shown in Figure 2.4 (referring to chapter 2).

3.2.3.4 Gill morphometrics: All four gill arches from both sides of each fish were dissected out and placed in 10% normal saline. The gills from the two sides of each fish were excised, keeping the rakers and filaments intact. The gill arches were separated and the measurements, indicated in Table 3.1, taken for the left and the right sides of each arch using a binocular microscope at a magnification of 3X with an eyepiece micrometer (after Hughes, 1984). To quantify individual status with respect to those variables, the mean value for the right and left sides were used.

Table 3.1. Summary of the measurements made on all four arches to characterise structure in common carp

Structure	Common carp
Gill arch	Length of gill arch
Gill raker number	Total number of gill rakers
Gill raker length	Length of each gill raker
Gill filament number	Total number of gill filament
Gill filament length	Length of every tenth filament
Secondary lamellae number	Number of secondary lamellae per mm on every tenth gill filament
Secondary lamellae length	Length of secondary lamellae at 3 points of every tenth gill filament
Distances between secondary lamellae	Distances between secondary lamellae at 3 points of every tenth gill filament

3.2.3.5 Measuring fluctuating asymmetry: Fluctuating asymmetry (FA) for gill arch length was calculated after Evans and Hatchwell, (1993) as follows:

$$FA = (XR - XL) / Max (XR, XL)$$

where XR is variable measured on the right side and XL is variable measured on the left side. To assess the accuracy of asymmetry scores, duplicate measurements were made for the first two gill arches of all groups. The repeated scores were significantly related for both arches (Table 3.2) so the asymmetry measures were assumed to represent real biological variability and not measurement error.

Table 3.2. Regression analyses of duplicate measurements asymmetry for length of gill arches 1st and 2nd for common carp.

Variable	Regression equation	F	P	R^2
Asymmetry 1st arch length (cm)	Y= -0.005 + 0.352 X	11.92	*0.001*	41.4
Asymmetry 2nd arch length (cm)	Y= -0.017 + 0.534 X	35.08	*<0.001*	60.0

3.2.4 Light microscopy

The second gill arch from the left side was preserved for light microscopy. Tissues for histological analysis were placed into buffered formalin and embedded in wax following standard procedures. Wax embedded tissues were sectioned (thickness of section = 5 μm) and stained with haematoxylin (H) and eosin (E), according to the method outlined in Clark (1981). Figure 3.4 shows a typical section. The number of mucous cells (indicated in Figure 3.4) per millimetre of lamellar surface and the height of interlamellar cells as a

percentage of the height of the adjacent lamellae (indicated in Figure 3.4) were quantified on different filaments. The number of mucous cells, the height of the interlammellar epithelial cells and the height of the adjacent lamellae were measured for 9 lamellae per filament and mean values were calculated.

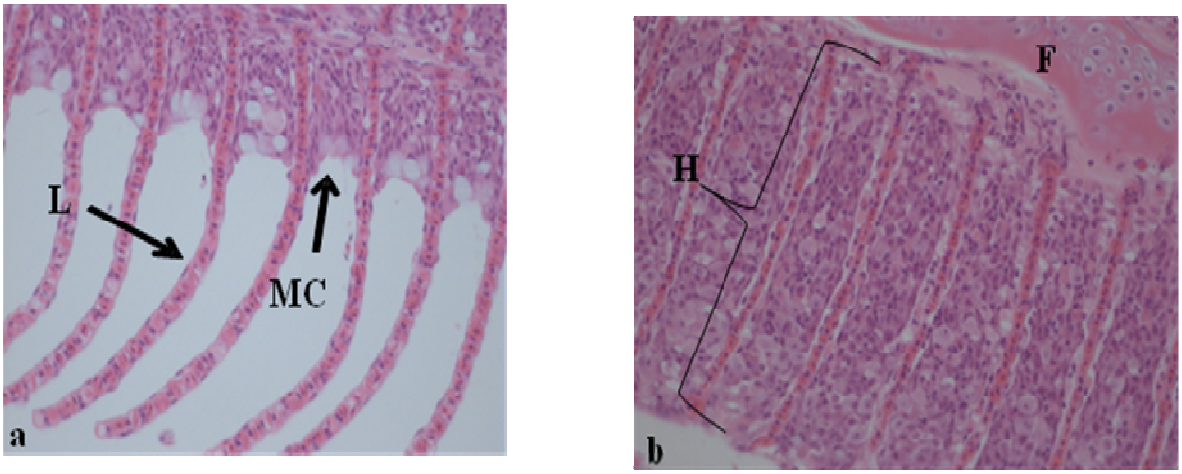


Figure 3.4.a and b. typical section through the gill filament (F) of common carp, with secondary lamellae (L) mucus cells (MC) and hyperplasia between gill lamellae (H) indicated. (H and E stain and 400X magnification).

3.2.5 Statistical analysis

The following statistical procedures were carried out, using the MINITAB statistical package. Firstly, the data were checked for normality and transformations performed where necessary. Initial scrutiny of the data was carried out using means and standard errors. Regression analysis was then used to explore the relationship between all variables and body length. Residuals from the regression on length were used to generate length-independent variables where appropriate (as described by Reist, 1985 and Adams and Huntingford, 2004). Relations among measured variables were studied by correlation analysis, followed by Principal Components Analyses (PCA), which was also used generate compound scores where appropriate. For simplicity, separate PCAs were carried out for gill arch length, gill raker means and gill filament means. Derived scores from this PC analysis were combined further by multi-variate analysis of the main PC scores. Finally, ANOVAs were used to compare to the morphology of risk-taking, intermediate and risk-avoiding carp.

3.3 Results

3.3.1 Overall morphometrics

The basic structure of common carp gills is similar to that found in other teleostean fish. Four pairs of gill arches are present in gill cavities (i.e. four gill arches in one side of the fish head), each gill arch carrying hemibranchs posterior and anterior on each filament of gill. The filaments of each hemibranchs recline parallel to each other and perpendicular to the surface of the arch. Each gill filament carrying a number of flattened secondary lamellae that are arranged in two rows from a side of the filament.

Scrutiny of the stained gill sections showed that mucous cells, which tended to cluster at thetip and thae base of the secondary lamellae, were characterized by numerous secretion (vresicles with apical openings (Figure 3.5.)

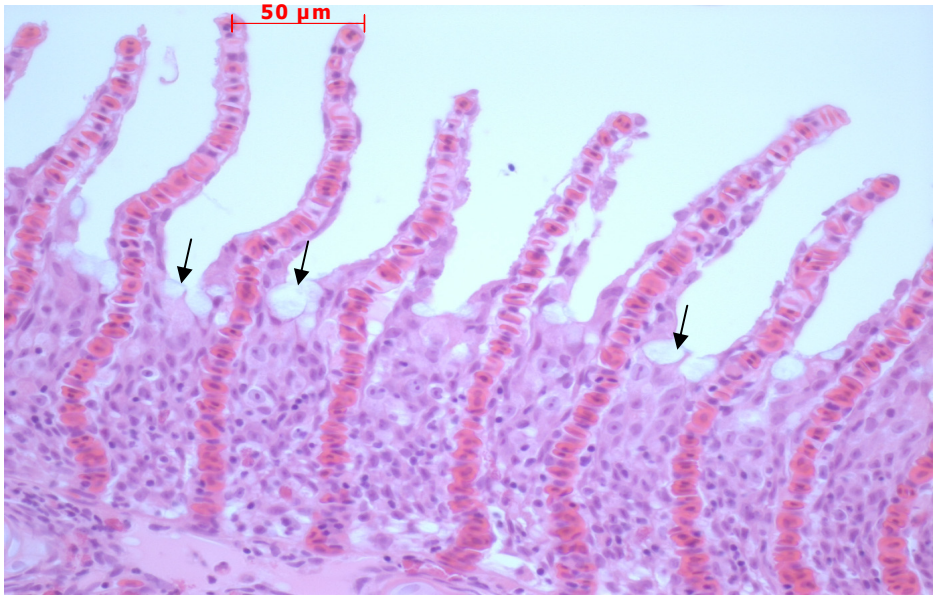


Figure 3.5. Light micrograph of gill secondary lamellae of common carp showing mucous cells (arrows), bar= 50 m µ. (H and E stain and 400X magnification).

Table 3.3 shows the mean values (± SE) for all measured variables averaged across the two sides of the head for each gill arch in the common carp, together with the results of ANOVA by arch number. For gill arch length *post hoc* testing confirmed that the first arch was the longest and that mean arch length decreased successively between the 2nd, 3rd and 4th arches. Gill raker length and number differed significantly between gill arches. For

raker length, *post hoc* tests showed the value for the first gill arch to be significantly larger than those for the second, third and fourth arches, but no differences along the remaining arches. For gill raker number, *post hoc* tests revealed significant differences between the raker numbers of all gill arches, with raker number decking with successive gill arches.

Table 3.3. Mean and standard error for gill arches length, gill raker length, gill raker number, filament length, filament number, secondary lamellae length, secondary lamellae number and distance between secondary lamellae, for 67 common carp, are also shown results of ANOVA and *post hoc* test.

Gill arch	Arch length	Raker length	Raker number	Filament length	Filament number	Lamellar length	Lamellar number	Lamellar distance
	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
1st	1.61 ± 0.06	0.62 ± 0.05	19.4 ± 0.55	3.0 ± 0.17	62.4 ± 1.58	0.16 ± 0.01	37.1 ± 0.90	0.04 ± 0.01
2nd	1.39 ± 0.05	0.49 ± 0.04	17.9 ± 0.27	2.8 ± 0.16	55.5 ± 1.86	0.15± 0.01	35.7 ± 0.93	0.04 ± 0.01
3rd	1.18 ± 0.05	0.40 ± 0.03	15.5 ± 0.35	2.6 ± 0.15	49.0 ± 2.14	0.14± 0.01	34.4 ± 0.90	0.04 ± 0.01
4th	0.99 ± 0.05	0.32 ± 0.03	13.0 ± 0.54	2.3 ± 0.14	41.9 ± 2.36	0.14± 0.01	33.2 ± 0.83	0.04 ± 0.01
$F_{3,267}$	26.37	12.61	114.25	3.53	19.29	0.77	3.64	0.21
P	0.00	0.00	0.00	0.02	0.00	0.51	0.01	0.89
Tukey test	1≠2,3,4; 2≠3,4;3≠4	1≠2,3,4; 2=3≠4;3=4	1≠2,3,4; 2≠3,4;3≠4	1=2,3; 1≠4; 2=3,4;3=4	1≠2,3,4; 2=3,2≠4;3≠4	1=2,3,4;2=3,4;3=4	1=2,3; 1≠4; 2=3,4;3=4	1=2,3,4;2=3,4;3=4

Mean length and number of the gill filaments as well as the mean number of the secondary lamellae also decreased towards the back of the head, with the longer first and second arches bearing many, long gill filaments and a relatively large number of secondary lamellae. For filament length, *post hoc* tests showed significant differences between scores for first and fourth arches, whereas for filament number, the fourth arches had significantly different scores from the second and third arches. The length of the secondary lamellae did not differ significantly between gill arches, but the length of secondary lamellae on the fourth gill arch was marginally less than for the first and second arches.

3.3.2 Estimated total filament length

Figure 3.6 shows the distribution of residual estimated total filament lengths (mm) regressed on fish body size for all common carp, to indicate normality of data . The mean (±SD) value is 2.8246 (±289.5) mm.

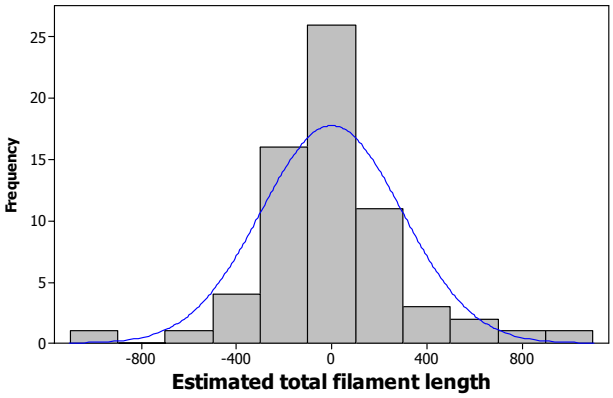


Figure 3.6. Frequency distribution of residual estimated total filament lengths (mm) for all carp.

3.3.3 Relationships between measured variables and body length

Table 3.4 show the results of regression analyses of the relationship of all variables with body length. Significant positive relationships were found for the following variables: length of all arches, filament length, filament number for all arches, secondary lamellae length and number for all arches, raker length for all arches, body weight, body length, opercular height, opercular width and estimated total filament length. For further analysis, these variables were therefore expressed as residuals from the regression with length and are referred to as “length-corrected” in the following text.

Table 3.4. Regression analyses of total body length (cm) and, average arch length (cm), average filament length (mm), average filament number, average secondary lamellae length (mm), average secondary lamellae number and average distance between secondary lamellae (mm), average gill raker number, average gill raker length (mm), average head length (cm), average body weight (gm), average height and width of the operculum (cm), average buccal cavity volume (ml), average gill cavity volume (ml) and estimated gill area (mm²) for 67 carp. Italics indicate significant relationships.

Variable	Arch	Regression equation	$F_{1,66}$	P	R^2
Arch length	1	Y= 0.069+0.148 X	180.19	*0.00*	73.1
	2	Y= - 0.072+0.141 X	205.80	*0.00*	75.6
	3	Y= - 0.227+0.135 X	202.35	*0.00*	75.3
	4	Y= - 0.253+0.120 X	170.15	*0.00*	71.9
Filament length	1	Y= - 1.080+0.394 X	108.31	*0.00*	61.9
	2	Y= - 1.130+0.377 X	105.43	*0.00*	61.6
	3	Y= - 1.100+0.351 X	98.38	*0.00*	60.0
	4	Y= - 17.300+5.690 X	117.38	*0.00*	57.1
Filament number	1	Y= 28.300+3.280 X	59.09	*0.00*	47.2
	2	Y= 12.20 0+4.160 X	79.71	*0.00*	54.8
	3	Y= - 2.550+4.950 X	93.93	*0.00*	58.8
	4	Y= -17.300+5.690 X	117.38	*0.00*	64.2
Secondary lamellae length	1	Y= - 0.162 + 0.031X	183.70	*0.00*	70.2
	2	Y= - 0.091 + 0.023X	154.17	*0.00*	73.8
	3	Y= - 0.094 + 0.023X	149.60	*0.00*	69.6
	4	Y= - 0.108 + 0.024X	168.16	*0.00*	72.0
Secondary lamellae number	1	Y= 23.000+ 1.360 X	21.41	*0.00*	23.6
	2	Y= 23.200 + 1.200X	14.79	*0.00*	17.3
	3	Y= 23.300 + 1.070 X	12.71	*0.00*	15.1
	4	Y= 25.900 + 0.700 X	5.49	*0.02*	6.4
Distance between secondary lamellae	1	Y= 0.010 + 0.000 X	3.25	0.07	3.3
	2	Y= 0.020 + 0.000 X	0.88	0.06	3.1
	3	Y= 0.00 0+ 0.000 X	3.43	0.07	3.6
	4	Y= 0.010 + 0.000 X	3.16	0.08	3.3
Raker number	1	Y= 20.900 - 0.026 X	0.07	0.79	0.0
	2	Y= 18.600 - 0.059 X	0.36	0.55	0.0
	3	Y= 14.4 00– 0.134 X	0.63	0.43	0.0
	4	Y= 18.000 + 0.110 X	1.11	0.29	0.2
Raker length	1	Y= - 0.491 + 0.107 X	70.35	*0.00*	51.2
	2	Y= - 0.354 + 0.082 X	64.25	*0.00*	48.9
	3	Y= - 0.238 + 0.062 X	69.68	*0.00*	51.0
	4	Y= - 0.196 + 0.049 X	48.41	*0.00*	41.8
Body weight		Y= -31.600 + 5.060X	369.04	*0.00*	90.9
Head length		Y= - 0.321 +0.279 X	806.18	*0.00*	97.6
Height opercular		Y= 1.100 + 0.024X	7.09	*0.01*	9.2
Width opercular		Y= 0.841 + 0.021X	4.68	*0.03*	6.0
Buccal cavity volume		Y= 1.140 + 0.001X	0.00	0.98	0.0
Gill cavity volume		Y= 0.245 + 0.010 X	1.19	0.28	0.0
Total estimated gill area		Y= - 817.0 + 111.0 X	54.14	*0.00*	36.4

3.3.4 Relationships among length-independent variables

Because so many variables were measured, successive principle component analyses were used to examine the relationships among them and to condense these into a smaller number

of variables where appropriate. Table 3.5a shows the matrix of correlations for arch length for all arches, together with the results of principal components analysis for these variables. Lengths of the different gill arches are highly correlated. The principal components analysis for the gill arch length (Table 3.5b) showed that the first two components explained 99% of the variation in the data set. PC1 accounted for 92% of total variance and had positive coefficient loading for all variables, representing overall gill arch size, regardless of body size. PC2 accounted for 7% of the total variance and opposed length of the third and fourth gill arch lengths, to those of first and second gill arches, representing differential development of the anterior and posterior gill arches.

Table 3.5. a) Correlation matrix for length of all gill arches. b) Principal Components coefficient for the first two PCAs in a PCA on gill arch length.

a)	Length	Length 2	Length 3
Length 2	0.980 0.000		
Length 3	0.965 0.000	0.993 0.000	
Length 4	0.943 0.000	0.973 0.000	0.985 0.000

b)		
Arch	**PC1**	**PC2**
1st arch	0.48	0.75
2nd arch	0.51	0.12
3rd arc	0.51	-0.24
4th arch	0.49	-0.61

Tables 3.6a and b show the correlations between gill raker number and length across all arches, corrected for fish body length where appropriate, together with the results of PCA on these variables. Raker lengths for the different arches were correlated, as were raker numbers, but in general, length and number were uncorrelated. For these variables, the first 2 components explained 66% of the total variance. PC1 (41%) opposes gill raker length to gill raker number. PC2 (22%) positive loadings for all scorers with exception for raker number for 3rd gill arch and so reflects variation in overall raker development, independent of overall body size (Table 3.6b).

Table 3.6a. Correlation matrix for gill raker length and gill raker number for all gill arches, b) Principal Components coefficients for the first two PCAs in a PCA on gill raker length and number.

a)

	Length 1	Length 2	Length 3	Length 4	Number 1	Number 2	Number 3
Length 2	0.968 0.000						
Length 3	0.903 0.000	0.897 0.000					
Length 4	0.862 0.000	0.851 0.000	0.956 0.000				
Number 1	-0.044 0.721	-0.023 0.855	-0.026 0.838	-0.033 0.792			
Number 2	-0.064 0.610	-0.044 0.727	-0.074 0.549	-0.048 0.702	0.914 0.000		
Number 3	-0.063 0.614	-0.042 0.0.736	-0.047 0.708	-0.074 0.551	0.802 0.000	0.838 0.000	
Number 4	-0.114 0.358	-0.110 0.0.375	-0.116 0.350	-0.097 0.437	0.765 0.000	0.861 0.000	0.844 0.000

b)

Arch	**Raker**	**PC1**	**PC2**
1st	Length	-0.21	*0.46*
	Number	*0.45*	0.20
2nd	Length	-0.20	*0.46*
	Number	*0.47*	-0.22
3rd	Length	-0.21	*0.46*
	Number	*0.45*	0.22
4th	Length	-0.20	*0.45*
	Number	*0.46*	0.19

Tables 3.7a and b show the matrix of correlations between gill filament number and length for all arches, together with the results of principal components analysis for these variables. Filament lengths and numbers for the 4 arches are all strongly correlated. The first two principal components (Table 3.7b) explain 95% of variation in this data set. PC1, which accounts for 84% of the total variance, has positive loadings for all variables and so represents variability in filament size and number, (providing an index of overall filament development), independent of body size. PC2 accounts for 11% of the total variance and has negative loadings for filament length on all arches and positive loadings for filament number; fish with few, long filaments gain high scores in this axis.

Table 3.7a. Correlation matrix for gill filament length and gill filament number for all gill arches. In each cell, the top figure is the correlation coefficient and the bottom figure is the P value, b) Principal Components coefficients for the first two PCAs in a PCA on gill filament length and number.

	Length 1	Length 2	Length 3	Length 4	Number 1	Number 2	Number 3
Length 2	0.998 0.000						
Length	0.991 0.000	0.996 0.000					
Length	0.983 0.000	0.990 0.000	0.997 0.000				
Number 1	0.835 0.000	0.839 0.000	0.840 0.000	0.839 0.000			
Number 2	0.888 0.000	0.889 0.000	0.884 0.000	0.879 0.000	0.924 0.000		
Number 3	0.905 0.000	0..905 0.000	0.897 0.000	0.890 0.000	0.905 0.000	0.983 0.000	
Number 4	0.933 0.000	0.932 0.000	0.924 0.000	0.918 0.000	0.869 0.000	0.948 0.000	0.973 0.000

b)

Arch	**Gill filament**		
		PC1	**PC2**
1st	Length	0.36	-0.32
	Number	0.32	*0.43*
2nd	Length	0.37	-0.33
	Number	0.37	*0.43*
3rd	Length	0.37	-0.35
	Number	0.35	0.39
4th	Length	0.36	-0.34
	Number	0.36	0.19

Tables 3.8a and b show the correlation matrix for secondary lamellae measures for all arches, together with the results of Principal Components Analysis on these variables. Number and length of secondary lamellae are correlated across arches and with each other. Distances between lamellae are correlated across arches, but do not show any consistent pattern of correlation with the other scores. For the secondary lamellae (Table 3.8b), the first two components explained 73% of the variation in the data set. The first component accounts for 43% and has positive coefficients loadings for most variables, but negative for distance between secondary lamellae. It therefore represents an index overall development of the secondary respiratory surfaces. PC2 explains 30% of total variance, with high negative loadings for secondary lamellae length and distance between secondary lamellae, but positive for number of secondary lamellae.

Table 3.8a. Correlation matrix for gill secondary lamellae length and gill secondary lamellae number and spacing between secondary lamellae for all gill arches. b) Principal Components coefficients for the first two PCAs in a PCA on gill secondary lamellae length and number and distance between secondary lamellae.

3.8a

	Length 1	**Length 2**	**Length 3**	**Length 4^t**	**Number 1**	**Number 2**	**Number 3**	**Number 4**	**Space 1**	**Space 2**	**Space 3**
Length 2	0.991 0.000										
Length 3	0.991 0.000	0.988 0.000									
Length 4	0.991 0.000	0.989 0.000	0.994 0.000								
Number 1	0.551 0.000	0.565 0.000	0.519 0.000	0.525 0.000							
Number 2	0.493 0.000	0.504 0.000	0.461 0.000	0.464 0.000	0.968 0.000						
Number 3	0.467 0.000	0.485 0.000	0.440 0.000	0.443 0.000	0.953 0.000	0.0.962 0.000					
Number 4	0.352 0.004	0.375 0.002	0.327 0.007	0.331 0.007	0.913 0.000	0.933 0.000	0.978 0.000				
Space 1	0.174 0.161	0.171 0.169	0.161 0.197	0.154 0.218	-0.068 0.586	-0.070 0.578	-0.092 0.461	-0.145 0.246			
Space 2	0.218 0.079	0.204 0.100	0.215 0.083	0.213 0.086	-0.154 0.217	-0.171 0.171	-0.192 0.123	-0.24 0.04	0.911 0.000		
Space 3	0.162 0.195	0.154 0.218	0.154 0.216	0.156 0.211	-0.094 0.451	-0.104 0.404	-0.121 0.333	-0.164 0.189	0.880 0.000	0.841 0.000	
Space 4^t	0.184 0.139	0.174 0.162	0.181 0.146	0.176 0.158	-0.150 0.228	-0.163 0.192	-0.176 0.157	-0.224 0.070	0.918 0.000	0.812 0.000	0.961 0.000

3.8b)

Arch	**b) Secondary lamellae**		
		PC1	**PC2**
1st	Length	0.30	-0.31
	Number	0.36	0.04
	Distance	-0.20	-0.39
2nd	Length	0.31	-0.31
	Number	0.35	0.04
	Distance	-0.13	-0.37
3rd	Length	0.29	*-0.31*
	Number	0.35	0.05
	Distance	-0.22	-0.39
4th	Length	0.29	*-0.31*
	Number	0.35	0.06
	Distance	-0.22	-0.41

Table 3.9 shows the matrix of correlations between the measures of volume for buccal and gill cavity and for height and width of the opercular bones. Buccal and gill volume are positively correlated. Gill volume is positively related to both opercular height and opercular width (which are themselves positively correlated), but buccal volume is unrelated to either measure.

Table 3.9. Correlation matrix for buccal cavity volume, buccal and gill cavity volume, gill cavity volume, opercular height and opercular width.

	Buccal volume	Gill volume	Opercular height
Gill volume	0.403 0.001		
Opercular height	0.176 0.151	0.306 0.011	
Opercular width	0.170 0.165	0.309 0.010	0.914 0.000

Finally, to compare, and if appropriate combine, across the different components of the gills, correlations were calculated for the main PCA scores for gill arch, gill rakers, gill filaments and secondary lamellae (Table 3.10a). Positive relationships were found among scores on the first component for arch length (arch PC1), filament number and length (filament PC1) and lamellae number and length (lamellae PC1). So finally these were combined using a PCA on these 3 variables only (Table 3.10b). The first component, which accounts for 67% of the total variance, is an overall measure of the development of respiratory structures, independent of fish length.

Table 3.10a. Correlation matrix between the PCA scores for gill arch, gill rakers, gill filaments and secondary lamellae. b) PCA coefficients of PCA on arch PC1, filament PC1 and lamellae PC1.

a)

	Arch PC1	Arch PC2	Raker PC1	Raker PC2	Filament PC1	Filament PC2
Raker PC1	0.643 0.000	0.131 0.291				
Raker PC2	-0.477 0.000	0.153 0.216				
Filament PC1	0.798 0.000	0.278 0.024	0.722 0.0.00	-0.343 0.005		
Filament PC2	-0.226 0.068	0.231 0.062	-0.041 0.746	0.126 0.312		
Secondary Llamellae PC1	0.113 0.336	0.392 0.001	0.331 0.007	-0.087 0.490	0.543 0.000	0.587 0.000
Secondary lamellae PC2	-0.314 0.010	0.036 0.771	-0.272 0.027	0.223 0.071	-0.317 0.010	0.791 0.000

b)

Variable	PC1	PC2
Arch 1	0.59	-0.56
Fillament 1	0.69	-0.40
Lamellae 1	0.34	0.83

Figure 3.7 shows the relationship between the PC derived measure of overall development of the respiratory structures and estimated gill area. These two variables are significantly related (Y= -1.17 + 0.003X, $F_{1.66}$= 171.02, P= 0.00; R^2 value = 75.5% Figure 6). Although some fish with very low estimated gill area have high PCA carried scores.

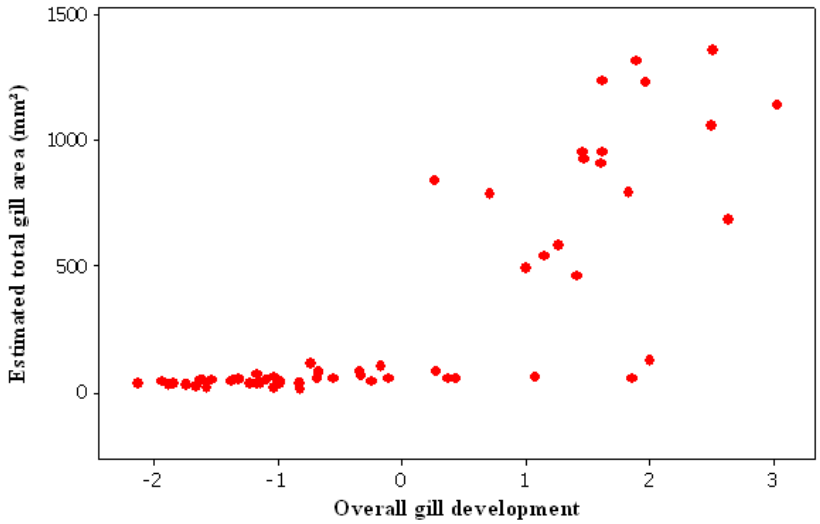


Figure 3.7. Relationship between the PCA derived measure of overall gill development and estimated gill area (mm²).

3.3.4 Asymmetry in gill arch length

Table 3.11a shows mean (± SE) values for asymmetry scores for length of each of the 4 gill arches. Table 3.11b shows correlations among these values and Table 3.11c shows the results of a PCA of asymmetry scores for the 4 arches. Levels of asymmetry in the first 3 arches were mutually positively correlated, but none were related to the scores for arch 4. PC1 (accounting for 45% of the total variance) has positive loadings for first, second and third arches and negative for fourth gill arch, providing an index of general asymmetry of the front of the gill basket. PC2 accounted for 21% of the total variance and opposes asymmetry in the first arch to that in the second, third and fourth arch length. There was a marginally significant positive relationship between overall asymmetry (PCAs) and body length (regression Y= 1.09 + 0.105 X. $F_{1, 66}$ = 3.34, P = 0.07).

Table 3.11a) Mean ±SE values for asymmetry scores for arch length, b) correlation between asymmetry scores in the four gill arches and c) PC coefficients of PCA gill arches from asymmetry scores common carp.

a)

3.11a	Arch	Asymmetry scores
	1st	-0.01±0.01
	2nd	-0.02±0.01
	3rd	0.02± 0.01
	4th	0.48±0.04

b)

	1st arch	2nd arch	3rd arch
2nd arch	0.255 0.036		
3rd arch	0.342 0.004	0.336 0.005	
4th arch	0.124 0.313	-0.111 0.370	-0.111 0.368

c)

3.11c	Arch	PC1	PC2
	1	*0.52*	-0.01
	2	*0.49*	0.51
	3	*0.56*	0.20
	4	*-0.42*	0.84

3.3.5 Gill microstructure

Great variability in hyperplasia of epithelial cells between gill lamellae was observed in gills, some having multiple layers of epithelial cells on all the filaments that were in contact with the secondary lamellae (Figure 3.8a.b). Others had no hyperplasia and the secondary lamellae was separate (Figure 3.8a.a). Others were observed to have some hyperplasia hyperplasia of epithelial cells between lamellae (Figure 3.8a. c).

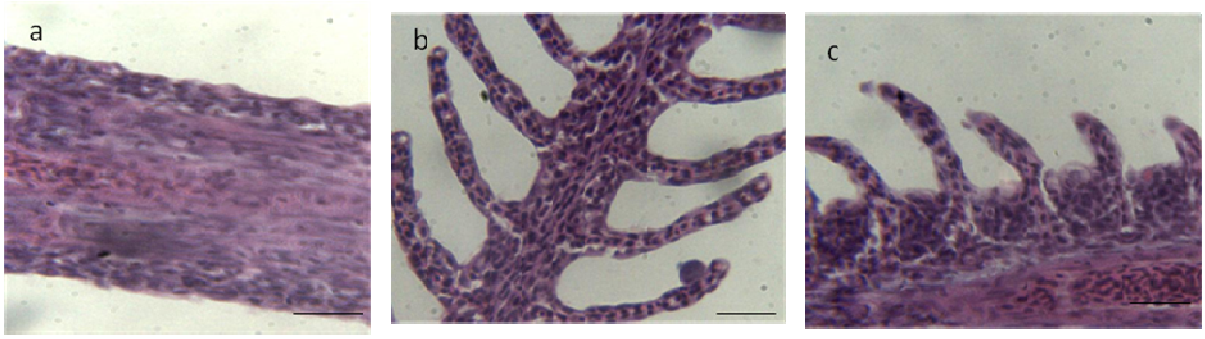


Figure 3.8a. Light micrograph of gill secondary lamellae of brown trout showing variation in hyperplasia hyperplasia of epithelial cells between gill lamellae **, a) almost complete hyperplasia, b) no hyperplasia and c) some hyperplasia between gill lamellae, (bar = 60** m µ**). H, E and** PAS **stain and magnification 400 X.**

Figure 3.8b shows the distribution of relative hyperplasia hyperplasia of epithelial cells between gill lamellae scores. A considerable degree of hyperplasia was observed (mean = .(.(34.5%

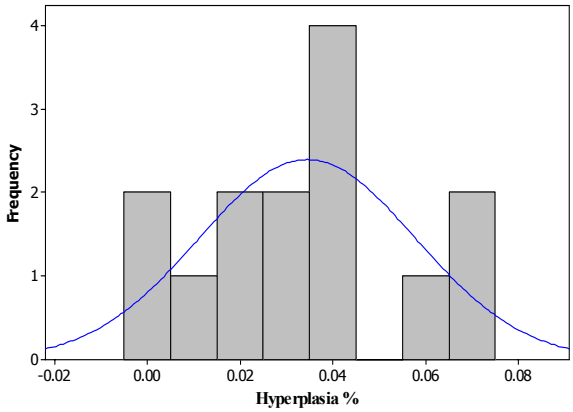


Figure 3.8b. Distribution of relative hyperplasia scores

3.3.6. Gill structure in carp with different coping strategies.

For reference, Table 3.12 shows mean (± SD) for all univariate measures for risk-taking, risk-avoiding and intermediate carp. The only variables for which the groups differed was the number of secondary lamellae, which was significantly greater (by about 33%) in the risk taking fish, than in the other two categories. *Post hoc* testing on secondary lamellae number showed significantly higher values for risk-taking fish compared with both intermediate and risk-avoiding fish, but no significant difference between the intermediate and risk-avoiding categories.

Table 3.12. Mean (± SD) univariate scores of body size and respiratory variablesin carp with the 3 risk taking phenotypes, together with the results of ANOVA for the three risks taking in carp.

Variable	Mean (±SD)			F2,65	P
	Risk-taking	Intermediate	Risk avoiding		
Body weight	19.8 ±14.4	19.70±15.54	22.17±16.61	0.18	0.84
Body length	10.48 ± 2.82	10.10 ±2.69	10.59± 2.72	0.21	0.81
Head dimensions					
Head length (cm)	2.62 ± 0.86	2.47 ±0.76	2.61 ± 0.876	0.24	0.79
Arch length (cm)	1.36 ± 0.43	1.25 ±0.45	1.27 ± 0.39	0.37	0.69
Buccal cavity volume	1.19±0.87	1.18±0.58	1.05±0.57	0.32	0.73
Operculum height (cm)	1.37 ± 0.23	1.31±0.17	1.35 ± 0.22	0.51	0.60
Operculum width (cm)	1.06 ± 0.23	1.04±0.19	1.05 ± 0.24	0.06	0.94
Raker length (mm)	0.53 ± 0.37	0.44 ±0.26	0.41 ± 0.18	1.14	0.33
Raker number	16.53±1.93	16.65±2.54	17.12±2.44	0.37	0.69
Respiratory structures					
Gill cavity volume (ml)	0.31 ± 0.21	0.35±0.19	0.351 ± 0.16	0.44	0.64
Filament length (mm)	2.89 ± 1.32	2.69 ±1.28	2.46 ± 1.18	0.59	0.56
Filament number	53.61 ± 16.73	50.88±16.30	52.49 ± 1.90	0.16	0.85
Lamellar length (mm)	0.16±0.09	0.15±0.07	0.13±0.07	0.54	0.59
Lamellar number/ mm	40.27 ± 6.28	33.19±6.89	32.82 ± 5.82	8.80	*0.00*
Lamellar distance (mm)	0.03 ± 0.02	0.04±0.01	0.05 ±0.06	0.85	0.43

Table 3.13 compares the main derived scores for the gills in carp with different coping styles. Risk taking fish had significantly higher scores than the risk avoiding fish, with intermediate fish occupying an intermediate position, for filament PC1, lamellar PC1 and overall PCA. This is not reflected in any differences estimated gill filament length or estimated gill area. For lamellar PC1 the *post hoc* test showed risk-taking fish to be significantly different from intermediate and risk-avoiding fish, with risk-avoiders just different from intermediate fish. For overall PCAs *post hoc* test showed risk-takers to have significantly higher scores than intermediate and risk-avoiding fish, but no significant difference between the two latter categories.

Table 3.13. Mean (± SD) of the PCA derived summary scores and estimated total filament length and gill surface area, with results of ANOVA test.

Variable	Mean ± SD (X)			F2,65	P
	Risk taking	Intermediate	Risk avoiding		
Arch PC1	0.32± 2.02	-0.18±2.13	-0.07 ± 1.13	1.81	0.69
Raker PC1	0.55 ± 2.47	-0.04±1.95	- 0.44 ± 1.95	1.30	0.28
Raker PC2	-0.12 ± 1.75	0.11±1.97	- 0.03 ± 1.80	0.09	0.91
Filament PC1	1.19 ± 2.40	-0.36±2.36	- 0.62 ± 2.17	3.58	*0.03*
Filament PC2	0.78 ± 0.71	-0.13±0.73	-0.52 ± 3.17	2.43	0.09
Lamellar PC1	1.54 ± 1.81	-0.418±1.73	-0.76 ± 1.64	1..71	*0.00*
Lamellar PC2	-0.06 ± 1.41	-0.06 ± 0.37	0.11 ± 0.29	2.35	0.10
Overall PCAs	0.77 ± 1.36	-0.27 ± 1.47	-0.36 ± 1.23	4.31	*0.02*
Arch length asymmetry PC1	-0.25 ± 1.34	0.07±1.32	0.14 ± 1.26	0.51	0.60
Estimated total filament length	702.0±504.3	631.8±483.2	584.4±461.8	0.30	0.74
Estimated gill surface area	517.7±482.2	438.4±518.3	298.7±498.7	0.93	0.39

Table 3.14 shows mean (± SD) mucus cell density and percentage epithelial cells hyperplasia in carp from the three risk taking categories, together with the results of a one way ANOVA. Risk avoiding fish have significantly more mucus cells per mm of lamellae and also a greater percentage of hyperplasia of epithelial cells between secondary lamellae than risk taking and intermediate fish. *Post hoc* test for mucus cell number and hyperplasia percentage revealed that risk-taking differ from intermediate and risk-avoiding also intermediate group differ from risk-avoiding group.

Table 3.14. Mean (± SD) number of mucus cells and hyperplasia % in carp with the 3 risk taking phenotypes, together with the results of ANOVA for the three groups.

	Mean ±(SD)			F2,65	P
	Risk taking	Intermediate	Risk avoiding		
Number of mucus cells	2.00±0.47	1.65±0.60	2.58±0.22	5.13	*0.03*
Hyperplasia%	20±10.9	30±0.02	70±0.02	10.9	*0.002*

3.4 Discussion

3.4.1 The aims of this study

The aims of the present study were 1) to screen individual common carp for coping strategy, exposed to a novel environment, 2) to examine the relationship between various aspects of gill morphology and derive composite measures of the extent of development of the respiratory surfaces. 3) to examine gill microstructure in these same categories of fish, looking specifically at the extent to which the surface of the gill lamellae is covered by epithelial cells and the abundance of mucous cells. 4) To measure the degree of fluctuating asymmetry in the first gill arch, as an indicator of past exposure to stress. 5) to compare gill morphometrics and microstructure in fish with different coping strategies.

3.4.2 Screening for coping strategy

Even though time to emerge from the settling section into the novel environment fell with temperature, as previously found for common carp (Huntingford *et al.,* 2010), the carp in the present study showed significant individual difference in risk-taking. Thus some carp (classified as risk-takers) emerged and explored the novel environment quickly in all tests, and others (classified as risk-avoiders) failing to emerge in all tests. Other fish showed variable emergence times, tending to emerge more quickly in successive tests (Mesquita 2010); these fish are classified here as 'intermediate', but might be better described as 'flexible'. Risk-taking fish that emerge from shelter quickly are likely to gain preferential access to any food in the novel area that they enter, but will also be more vulnerable to any predators it may contain. Individual differences in speed of exploration of a novel environment predicts coping strategy (including cortisol responsiveness and resting metabolic rate) in common carp (Huntingford *et al.,* 2010). The screening used here has therefore achieved the aim of classifying fish by coping strategy, making it appropriate to compare gill structure and function in the three identified categories of fish. Consistent individual differences in response to novelty have been described for a number of species of fish, including sunfish (*Lepomis gibbosus*; Wilson *et al.,* 1993) and wrasse (*Symphylus ocellatus*) (Budaev, 1997). Other studies show more flexible responses; for example perch (*Perica fluviatilis;* Magnhagen and Staffan, 2005) and rainbow trout (*Oncorhynchus mykiss;* Ruiz-Gomez *et al., 2008*)

3.4.3 Overall gill structure in common carp

The results presented here also show that in common carp, gill structure is similar to other bony fish. There are four gill arches on right and left sides made up of numerous filaments and as well a number of gill rakers (Rantin and Fernandes, 1986; Severi *et al*., 2000; Jenjan in preparation, chapter 2). In this structure, there was considerable changeability in gill morphometrics in common carp. Similar differences between fish species have found in other studies (Severi *et al.,* 2000; Jenjan *et al* in preparation, (chapter 2). This can be partially explained by gill location, gill arch length increasing from back to front, however also partly by differences in body size. A connection between gill morphometrics and body size has been reported for other teleost fish, for example *Sparus aurata* (Karakatsouli *et al.,* 2006), Arctic charr and brown trout (Jenjan *et al* in preparation, chapter 2) This relationship is probably dependent on increasing oxygen needs with increasing body size and changeability of it can be give explaned in part by fish activity, its type of respiration and the ecological conditions in which it lives (Severi *et al.,* 1997).

3.4.4 *Variability in gill rakers in common carp*

The numbers and lengths of gill raker reported in this study are within a range reported for other teleost fish (Ruzzante *et al.,* 1998; Foot *et al.,* 1999). In addition the gill raker numbers found for common carp in the present study (13.0-19.4) agree with those of Ross (2001).

The results of this study also demonstrated changeable numbers and length of gill rakers in common carp, some related to overall body size. The mean length of the gill rakers in common carp is low (0.32-0.62) in comparison to the Arctic charr, for example, (0.7-1.20. Jenjan in preparation, chapter 2) and in sockey salmon (*Oncorhynchus nerka*) (5.43) studied by Foot *et al.,* (1999). Gill raker length was highly correlated with body size, in both *Mugil cephalus* (Eggold and Motta, 1992) and *Alosa pseudoharengus* (MacNeill and Brandt, 1990). Gill raker length also is hypothesized to be related with pharynx size. Thus pharynx size many constrain raker length, with rakers simply growing to fit the available space. Differences in raker length and number are functionally related to food nature in a variety fish species (Amundsen *et al.,* 2004). For example, in stickleback (*Gasterosteus aculeatus*) the gill raker number and length have been shown to affect feeding performance in a wide diversity of fish species (Lavin and McPhail, 1986). The feeding behaviour in

fish has usually been found to connect with gill raker number and gill raker length (Amundsen *et al.,* 2004).

An increase in number of gill rakers is thought to decrease the pore size through which water is filtered, allowing the fish to keep smaller unit. Gill raker number and length are therefore related to substrate and food size. On the other hand, a negative relationship exists between gill raker number and length, and food size (Budy *et al.* 2005); fish feeding on large food have fewer, shorter gill rakers. Therefore, a large number and long gill rakers is common in planktivorous morphs, but benthic morphs regularly show a lower number and shorter gill rakers (Schluter and McPhail 1992; Robinson and Parsons 2002; Kahilainen *et al.,* 2011).

For example, the feeding behaviour of whitefish (*Coregonus lavaretus*) morphs have generally been found to relate with the number of gill rakers. The group with the lowest raker number are generally benthivorous and the group with high raker numbers are plankivorous (Østbye *et al.,* 2005). In the study presented here, the gill raker number and length decreased in size posteriorly through the gill baskets of common carp. Gill raker number and length could be related to gill cavity and buccal cavity shape (Jenjan *et al.,* in preparation).

3.4.5 Variability in respiratory structures

The data presented here on gill filaments showed a strong highly positive relationship between body length and both gill filament number and length, in common carp. The growth of specialized respiratory structures often relates to body size and the ecological conditions to which the fish species is exposed, and with its phylogeny (Severi *et al.,* 1997). For example, the gill area of *P. mesopotamicus* increased during growth. In other teleost fish, gill size is also thought to be correlated with fish activity, mode of fish respiration and environmental conditions (Severi *et al.,* 1997). Gill filament number could prove helpful in species identifications, where other morphological traits are inconclusive. Information about the structure of fish gills is helpful for understanding the anatomical limits to respiration in fish (Hughes and Morgan, 1973). The total number of gill filaments in the common carp (208.8) is not large, compared to a variety of other species of fishes for example, Arctic charr (272.2) (Jenjan *et al.,* in preparation, chapter 2) and *Lipidocephalichthy guntea,* (Singh *et al.,* 1991).

The present study showed that the length and number of filaments on the different arches decreased from the front to the back hemibranchs. The difference found in gill filament length and gill filament number along the different gill arches, may be connected to the head anatomy and with oxygen absorption capacity. Morphological and physiological restriction determines the pattern of variability in filament number and length (Palzenberger and Pohla, 1992). Whilst increased filament number and length result in a larger gill surface area, many, long filaments take up more space in the fish head, which may be incompatible with a streamlined body shape or with spatial constraints imposed by other systems (Palzenberger and Pohla, 1992). Gill filament number and its length are strongly related to the size of ventral pharyngeal bone (ceratobranchial). This has also been recognized in other species, including Arctic charr (Jenjan *et al.,* in preparation) and brown trout (Crespo *et al.,* 1988).

In the present study, filament number and length of each gill arch increased with fish length in common carp, however even when this was allowed for, there was character difference in the degree of growth of the filaments. The filament length in common carp is approximately equal to that of sea bass (*Dicentrarchus labrax*) (Saroglia *et al.,* 2002), but larger than that of *Glyptothorax pectinopterus* (Singh and Agarwal, 1991). In general, filament number may be correlated to the body size of the species concerned rather than to its ecology or physiology.

This study did not identify any significant relationship between secondary lamellae length and spacing and total body length, suggesting that this aspect of gill structure may be tuned to the life style of the fish concerned and/or ambient levels of dissolved oxygen. Similar results and a similar explanation have been given by Saliu and Olonire (2007) and Jenjan *et al.* (in preparation). The average number of secondary lamellae per mm of gill filament decreased from the front to the back arches. This is revealed together in the univariate statistics and in the results of the principal component analysis (PC2 has a strong front to back gradient). The number of secondary lamellae in common carp (37.1) is low in comparison with other fish species, for example, *Glyptothorax pectinopterus* (64.1) (Sinha and Agarwal, 1991), ruffe (*Gymnocephalus cernuus*) (40) (Satora *et al.,* 2010) and Arctic charr (60.1) (Jenjan *et al.,* in prepation, chapter 2). The average of distance between secondary lamellae in common carp is low (0.04 mm) in comparison for example to *Gaira lamta* (Ojha *et al.,* 1989). The differences in distance between secondary lamellae in different fish species could be correlated to the environmental positions (Prasad, 1988).

Common carp have closely spaced lamellae (0.04mm), suggesting an active mode of living (Hughes, 1966) ; in general, active species have closely spaced lamellae like (*Thunnus sp*) (0.06 mm; active; Muir and Hughes, 1969) and Arctic charr (0.04-0.05 mm; active; Jenjan *et al*., in prepataration, chapter 2), while sluggish species like plaice have more widely spaced lamellae (0.077 mm, slow-moving; De Silva, 1974). Using such criteria, common carp possible clarify as a quite active fish.

The length of the secondary lamellae is an essential determinant of gill surface area, and it is likely that it connects with the fish habit and habitat (Hughes and Morgan, 1973; Satora and Romek, 2010). However, physiological and morphological limitations the possible range for this variable (Palzenberger and Pohla, 1992). Increasing secondary lamellae length would definitely increase gill area, but would also increase resistance to water flow across the gill filaments. In general, gill area is determined by the number and length of the gill filaments and the number and length of the gill secondary lamellae. This describes why there is a positive correlation between the PCA-derived score of the extensiveness of the respiratory structures (which has positive loadings for secondary lamellae) and total gill areas.

3.4.6 Asymmetry in gill morphometrics

Levels of asymmetry in the length of the gill arches was repeatable when the same fish were measured on separate occasions. Principal components analysis identified a measurement of general asymmetry reflected in the length of three arches and in PC2, a measure of differential asymmetry in the front and back of the head. Individual scores in both these measures of asymmetry were variable, but there was no relationship with any body dimensions. The weak positive relationship between asymmetry, body length and head length could be reflecting a cost of fast growth. Several studies have found a relationship between the asymmetry and length of fish, for example, in cat fish, (*Heteropneustes fossilis*), (Al- Hassan *et al.,* 1990) and in *Decapterus russelli* (Jawad *et al.,* 2010) where there was a trend for an increasing in the asymmetry value with the increase length of fish. Fluctuating asymmetry reflects the incapability of bilateral animals completely to control development and has consequently proposed as a measure of developmental stability (Almeida *et al*., 2008). Two possible mechanisms or proximate causes for fluctuating asymmetry have been suggested, genetic effects and ecological effects (Eriksen *et al.*, 2008). On the other hand, low asymmetry may be vulnerable to

environmental stress or genetic ones, responsible for the asymmetry in some fish (Jawad *et al.,* 2010).

3.4.7 Microscopy

In fish, because of their respiratory and excretory functions gills are essential organs. In this study, hyperplasia of epithelial cells between secondary lamellae and mucous cells in the secondary lamellae were found in the gills in some common carp. Karan *et al.,* (2000) showed that from the increase of the hyperplasia between the lamellae in the gills of *Lepistes reticulatus* exposed to stress.

The observed hyperplasia of the gill epithelium is thus likely a direct response to the action of stress. The gill responses recorded in this study include hyperplasia and an increase in mucous cells number. Gill hyperplasia might serve as a defensive mechanism leading to a decrease in the gill surface area or may helpe to prevent water loss across the gills. This defence response will however take place at the expense of the efficiency of the respiratory function of the gills. The hyperplasia between secondary lamellae may serve to separate the secondary lamellae allowing them to function as respiratory structures (Ong *et al.,* 2007)

3.4.8 Comparison of gill structure in carp with different coping strategies

In this study, there were no significantly differences between carp with different coping strategies except in secondary lamellae number, which was highly significantly different between risk taking categorises. The present study found that common carp classified behaviourally as risk-takers have a larger gill area than do risk-avoiders, having more, longer filaments, as well as more secondary lamellae number. Fish classified as intermediate fell in between these two extremes. Gill surface area is related to fish activity and life style. Selection acts on many aspects of gill structure to increase or decrease gill surface area in relation to need (Wagner *et al.,* 2010a). According to Gray, (1954) and De Jager *et al.,* (1977), more active fish have a larger gill surface area than do sluggish fish. Generally, as fish activity increases, so does gill filament number, and length also increases; in addition, active fish tend to have more, closely spaced and larger, secondary lamellae than do generally inactive fish (De Jager and Dekkers, 1975; Satora and Romek,

2010). The large number of secondary lamellae (40.27/mm of gill filament) observed in risk-takers, may be considered typical of active fish, such as *Micropterus dolomieui* (40/mm of gill filament. Hughes, 1972). Overall therefore, the common carp in the present study that were classified as risk-takers have a larger respiratory surface that do risk-avoiders, suggesting a more active way of life and relating also to the fact that these fish have a higher resting metabolic rate (Huntingford *et al.,* 2010). Thus they need a larger respiratory surface to service a greater oxygen requirement.

In addition to these changes in relative development of the respiratory surface, the present study also found differences between risk-taking categories at the microscopic level. Thus risk-avoiding carp had more mucous cells as well a higher percentage of interlamellae cells than did risk-takers, with intermediate fish again being in between. Moron *et al.,* (2009) suggest that mucous cells in gill secondary lamellae increase the water-blood barrier for diffusion of respiratory gases, and subsequently reduce oxygen uptake and carbon dioxide excretion (Fernandes *et al.,* 1998; Sakuragui *et al.,* 2003). Interlamallar epithelial cells also represent a barrier to diffusion and can be adjusted over a short time scale in response to local environmental conditions (Sollid *et al.,* 2003). Thus both these cellular differences in carp with different coping strategies mean that in risk-avoiding fish the respiratory surface, as well as being smaller, is also less exposed to the surrounding water.

The higher mucous cell number in risk-avoiders may help this group to reduce loss of water in hypotonic solution. Furthermore, the risk-avoiders group (low filament length, low filament number, low secondary lamellae length, low secondary lamellae number as well as small gill surface area) may expending additional metabolic energy to maintain the respiratory function in water thus reducing scape for general activity, i.e. less muscular work (slow emergenc) in the risk-avoiding group may mean reduced need for oxygen. This, in turn, reduces the energy ratio required for activities.

Gill morphometrics and fine structure in fish represent a compromise between the need to acquire oxygen to sustain metabolism and general activity and the need to avoid excessive loss of ions across the exposed respiratory surface, It seems that in risk-taking, proactive carp, the balance is in favour of efficient oxygen uptake at the expense of ionic movements across the gills. In contrast, in reactive, risk-avoiding fish, the balance is shifted in favour of a lower rate of oxygen uptake and reduced ionic movements. The need to maintain a large, exposed respiratory surface, with an associated increased osmoregulatory pressure is therefore a hidden cost of an aggressive, proactive life style. This may help to explain how

selection maintains variable coping strategies within carp populations even though proactive fish gain preferential access to valuable resources.

3.5 Conclusions

Successive principal component analyses of gill morphometrics in common carp generated an index of overall development of the respiratory surfaces that is comparable to estimated surface area base in Hughes equation (1984), but that also identified different dimensions of variability. These gill morphometrics, together with studies at the microscopic level, show that risk-taking proactive carp have a larger exposed respiratory surface than do reactive, risk avoiding carp, with intermediate risk-takers falling in between. The opposite was the case for mucous cell number, possibly confirming the original allocation of the fish to coping style. The larger surface area probably serves the greater metabolic needs of proactive fish. Both maintaining a large respiratory surface and continuing an exchange across it may well represent a collateral cost of a proactive lifestyle.

CHAPTER 4

COPING STRATEGY AND RESPIRATORY DEVELOPMENT IN BROWN TROUT

The study described in this chapter was designed and excuted by Hussein Jenjan.

4.1 Introduction

4.1.1 Coping strategies in fish

When an animal encounters a challenging situation, a number of physiological and behavioural responses occur that are often described as a stress response. These are adaptive responses to challenge, preparing the body for effective action; however, stress responses may be chronically activated, resulting in adverse effects on growth, disease resistance and other processes, which can be seen as evidence for poor welfare (Huntingford *et al.,* 2006). In some respects, stress responses are typical of the species concerned, but consistent differences in how individual animals of a particular species respond, behaviourally and physiologically, to a diversity of challenges have been reported for number of animal groups, including fish (Wilson and Godin, 2009). These are sometimes called coping strategies and have been defined by Koolhass *et al.,* (1999) as a set of behavioural and physiological stress responses that are consistent over time and characteristic of a certain individual or group of individuals within a species. The so-called proactive strategy involves sympathetic, adrenaline-based fight-flight responses and, in behavioural terms, an active, aggressive, risk-taking life style. In contrast, the reactive strategy is characterised by parasympathetic, cortisol-based passive freeze-hide response and, behaviourally, a passive, risk avoiding style. Individuals exhibiting these two suites of responses are sometimes categorised as bold or risk taking and shy or risk avoiding, correspondingly. One component of coping strategy is how individual animals respond to risk, which may range from strong aversion to risk to extreme recklessness. This shy-bold continuum is a basic axis of behavioural variation in humans and other animals, existing regardless of the recognizable categories of age, sex and size (Wilson *et al.,* 1993).

Distinct coping strategies have been described for a number of mammalian species (Koolhass *et al.,* 1999) as well as for birds. For example, in great tits (*Parus major*) individuals from the same population show extreme phenotypic variation, with some being fast explorers in a novel environment (or bold) and others being slow explorers (or shy. Verbeek *et al.,* 1994; Van Hierdn *et al.,* 2002). Consistent individual differences in behaviour and physiology have also been described in fish (Huntingford and Adams, 2005; Wilson and Godin, 2009), which show similar physiological stress responses to those seen in mammals (Schreck, 2000; Barton, 2002; Conte, 2004). For example, consistent individual differences in risk-taking in the presence of a predator in threespined sticklebacks (*Gasterosteus aculeatus*) reported by Huntingford, (1976) are connected with

differences in stress physiology and brain biochemistry (Bell *et al.,* 2007). Bell *et al.,* also reported that individual differences in the concentration of the brain mono-amines were related to the differences among individuals in aggressiveness. For example more aggressive individuals had lower hypothalamic concentrations of serotonin.

In common carp (*Cyprinus carpio*), differences in risk-taking in a novel environment are alined to differences in competitive ability and in the expression of cortisol receptors in the head, kidney and brain (Huntingford *et al.,* 2010). Perhaps the best documented example of coping strategies in fish is provided by rainbow trout (*Onchorhynchus mykiss*), which show differences in risk-taking; bold fish are more active and learn faster in a conditioning task compared to their shy counterparts (Sneddon *et al.,* 2003). Studies on two strains of rainbow trout, high reactive and low reactive, found that the low reactive strain takes more risks than the high reactive strain and tend to dominate high reactive fish in pairwise fights (Schjolden *et al.,* 2005). Related behavioural and physiological variations due to stress have also been described between unselected strains of brown trout, *Salmo trutta* (Brelin *et al.,* 2008).

A number of studies of salmonid fish have demonstrated an association between an aggressive way of life and high resting metabolic rate (Yamamoto *et al.,* 1998). Similarity in common carp, risk-taking, proactive fish have a higher resting metabolic rate than do risk-avoiding, reactive individuals (Huntingford *et al.,* 2010). It seems that, compared with reactive fish, proactive fish have a high-energy life style. From this it may be predicted that their gill respiratory surface areas would be well-developed compared with those of reactive fish, to meet their greater oxygen needs. This is the case in common carp, in which risk-taking, proactive fish have comparatively better developed and more exposed gill surface areas than risk-avoiding, reactive fish (Chapter 3. Jenjan *et al.,* in preparation). The aim of the study described in this chapter is to examine whether the same is true for another species of freshwater fish, the brown trout (*Salmo trutta*).

4.1.2 Biology of brown trout

4.1.2.1 Distribution: Brown trout are native to Europe, Iceland and the Northwest coast of Europe, along the Mediterranean as well as Western Asia, but have been introduced into at least 24 countries within 90 years for example, (Elliott, 1994; Ozvarol, 2010).

4.1.2.2 General biology and ecology: Brown trout are salmonid fishes whose bodies are olive brown or green shading to a yellowish white on the belly. The sides of the fish have red spots surrounded by a pale halo (Figure 4.1).

Figure 4.1. Brown trout *Salmo trutta* Linnaeus 1758 (703 x 289px) by Chad Thomas, Texas State University-San Marcos.

The habitat of brown trout is defined by physical and biotic factors. Rivers and streams are variable highly habitats so wild brown trout are naturally exposed to a variety of environmental regimes, with respect for example to flow rate, water temperature and dissolved oxygen levels. Trout are reasonably tolerant of environmental change. For example, they can tolerate a temperature range of 0.5 – 22 °C. (Coutant, 1977). The physical descriptors affect the ecology of the species. For example, current is an important factor in the habitat of brown trout (Bagliniere and Maisse, 1999), trout being morphologically adapted to life in flowing fresh water (Brown, 1975). Macrophytes also play a function in the habitat structure by constraining visual limits (Haury and Bagloniere, 1990) and influence water oxygenation and pH (Begliniere and Maisse, 1999). The phototactism of brown trout shows ontogenetic changes, for example, the early stages have been shown negatively phototactic until resorption of yolk sac is complete, whereas the fry of brown trout are positively phototactic but the adult stages of brown trout are more and stronger negative phototactic (Ottaway and Clarke, 1981; Haury and Bagliniere, 1990).

Brown trout eat mainly other fish, aquatic insects and crustaceans. The major prey of juvenile brown trout however is aquatic invertebrates (Ruginis, 2008; Bridcut, 2000). The prey utilised by a brown trout is dependent upon availability and upon trout behaviour (Montoria *et al.,* 2006). Prey availability varies with the time of year (Fochetti *et al.,* 2003). For example, during October to February, the brown trout are more likely to feed on bottom fauna (Johansen, 1978) and during May to September are more likely to feed on insects taken at the water surface (Björnsson, 2001). Small brown trout are sitting and waiting predators that select an area for feeding that they defend as a territory and prey upon organisms that drift downstream in the current. The diet of large brown trout is more diverse than that of younger brown trout, consisting of large aquatic insects such as Hexagania, mayflies and larger species of caddisflies, as well as crustaceans, snails, salamanders and other amphibians. Adult trout feed mainly at night, especially during the summer (Bell, 2006). Brown trout regularly show aggressive behaviour, but during the reproductive period, females are less aggressive and show a lower level of territoriality than males (Montoria *et al.,* 2006).

4.1.2.3 Growth and life history traits: As a consequence of the wide tolerance range, the growth rate of brown trout is highly variable in dissimilar localities (Arslan *et al.,* 2007). This may be related to water temperature, nutrition or to genetic factors (Arslan *et al.,* 2007). Thus fish may grow to 200 grams from eyed eggs, taking between 10 and 20 months (Bromage *et al.,* 1990). Adult brown trout are generally 33-40 cm in length, although old individuals can reach a much larger size. The world record weight for angler-caught trout is in excess of 20 kilograms. In Western Australian the trout fishery is based upon young, fast growing fish, mainly 1+ year old fish which reach 1.0 kg in weight (Kheyrandish *et al.,* 2010).

Brown trout males commonly mature for the first time at age 1 or 2. The mean size of mature males at age 1+ is larger than that of those which are immature (Maisse *et al.,* 1987). For females, the great mature majority for first time at 2 or 3 years old but some females can mature at age 1+. Generally females maturing at 1+ are rarely larger than immature females and males of the same age (Bagliniere and Maisse, 1999). Brown trout spawn in the autumn (from October to December) when the temperature of the water drops to 5 – 10 °C (Pender and Kwak, 2002).. Trout mate every year, and they are not likely to have the same mate year after year. Large females produce 400 to 1200 eggs. the eggs hatch the following spring (Bell, 2006).

4.1.3 Gill structure and function

Brown trout are an active species, inhabiting flowing water and with high energetic demands, and their gill structure reflects this life style. The gill of fish is a multifunctional organ and the main site of gas exchange and osmoregulation. Details of the structure of the fish respiratory system are given in Chapter 2 and have been reviewed by Hughes (1984). Briefly, the gills are supported by four branchial arches. Each gill arch supports gill rakers and two columns of filaments with rows of gill secondary lamellae. The gill secondary lamellae are the primary site of gas exchange (Evans, 1987). The gill rakers are used to retain and filter food arriving in the fishes' mouth.

The size and organisation of the gill arches, the gill filament and the secondary lamellae allow some conclusions about habit and habitat of fish to be drawn (Nilsson, 2007). The gill surface area is correlated with fish activity (Satora and Romek, 2010). Fish with high metabolic needs usually have gill specializations that facilitate gas exchange (Wegner *et al.,* 2010), with gill dimensions increasing to enlarge the gill surface area and to increase the rate of uptake of oxygen from the water. Hughes and Morgan, (1973) suggested that gill morphometrics in bony fish represent an optimal balance between, on the one hand, gas exchange to meet metabolic demands and, on the other hand, the energetic cost associated with the buccal-gill pump system. The morphometric characters that contribute to a large gill surface area include more, longer gill filaments, more secondary lamellae per mm of filament and longer secondary lamellae, as found in active fish such as scombrids and billfishes (Wegner *et al.,* 2010a).

With this background, comparison between species shows that, in general, less active fish with low oxygen requirements have a smaller gill surface (Mazon *et al.,* 1998). Variability in gill area is also found within species and this reflects the life style of the fish concerned. For example, in Arctic charr, gill area is greater in the benthic-feeding (spring morph) morph (Chapter 2; Jenjan *et al* in preparation). Several aspects of gill structure were found to be variable among common carp and this was related stress coping strategy, with proactive carp having a larger respiratory surface than do reactive fish of the same size category (Chapter 3; Jenjan *et al* in preparation).

A large gill surface area facilitates gas exchange, but also presents a larger surface area for the passive movement of ions between the fish and the surrounding water and so potentially compromises osmotic regulation (Nilsson, 2007). One way in which fish

control such ionic movements is to regulate the number of epithelial cells between the secondary lamellae and therefore the area of gill that is actually exposed to the water (Sollid *et al.,* 2003). Regression of the interlamellar cells through hypoxia results in greater exposure of the lamellae, thus greatly increasing the respiratory surface area (Nilsson, 2007). Hyperplasia increases when fish are exposed to adverse conditions, possibly to protect the gill surface or to avoid taking in toxins, and so the degree of hyperplasia can be used as an indicator of exposed to poor environmental conditions (Daoust *et al.,* 1983; Andrews *et al.,* 2010). Conversely hyperplasia can be induced by many factors, including chronic inflammation, hormone dysfunction and many irritants, and serves to prevent dangerous substances from reaching the gills or for fast repair subsequent to irritation (Lawler, 2005).

4.1.4 Aims of the present chapter.

The overall aim of this study was to determine if gill surface area and gill fine structure varies in relation to risk-taking phenotype in brown trout. This was achieved through a series of sub-aims, as follows:

- To develop and deploy a method for assessing risk-taking in juvenile brown trout.
- To quantify overall development of the respiratory surface area in brown trout.
- To use scanning electron microscopy to examine the fine structure of gills in brown trout.
- To quantify the percentage of gill hyperplasia and mucus cells number by examining the structure of the gill in histological sections in brown trout.
- To compare gill morphometrics and gill structure in brown trout classified as risk-taking, risk-avoiding and intermediate.

4.2 Material and Methods

4.2.1 Subjects

Sixty brown trout (mean length 18.11cm and mean weight 57.63g) were obtained from Stirling University‘s trout hatchery at Howietoun in December 2009 and transported to the research laboratory at Glasgow University‘s Scottish Centre for Ecology and Natural

Environment, Rowardennan. They were maintained in recirculating flumes, fed daily to excess with a mixture of commercial fish pellets and frozen chironomid larvae (bloodworm). In April 2010, they were transported to the experimental aquaria in the Division of Ecology and Evolutionary Biology, Glasgow University. The fish were maintained in holding tanks of $1m^3$ supplied with recirculating water at a temperature 12.8 ± 0.2 ºC and allowed two weeks of acclimatisation, being fed daily to excess with fish pellets and frozen chironomid larvae. Fish were screened for coping strategy and killed by a Schedule 1 method (benzocaine, overdose, followed by a blow to the head) and their gill structure examined.

4.2.2 Screening for coping strategy

The risk-taking phenotype, a proxy for coping strategy of individual fish, was determined based on the sequence in which fish held in groups emerged from a small shelter to explore a novel, potentially-dangerous environment. The protocol was adapted to minimise the need for handling the trout, which are easily disturbed. The novel environment test is a well-established method for assessing risk-taking phenotype and coping strategy in fish (Huntingford and Coyle, 2007). Three or four days prior to screening, the fish were anaesthetised and marked with Alcian blue dye using Panjet marks on the fins and tails, (Hart and Pitcher, 1969), under HO Project License number 60/2930. Markes were placed at different points on all the fins and the tail together giving 20 unique combinations. The fish were weighed (mean initial weight = 72.13g) and measured (mean initial total length = 16.55 cm) and housed in the behavioural screening tank. The screening tank (103cm×103cm×45cm), consisted of two compartments: a covered compartment (51.5cm×51.5cm×45cm) and an open compartment (51.5cm×51.5cm×45cm), separated by a plastic screen containing two round apertures (dimeter ca 9 cm) fitted with removable doors.

Groups of 20 individually-marked fish were confined without food in the covered compartment over night. The following day, the scent of food (in the form of filtered water in which bloodworm had been macerated) was added to the open section of the tank and the door was then opened. Ten fish were allowed to emerge, at which point the door was closed and the identities of the emerging fish were checked. The fish were then returned to the covered compartment, left over night and the test was repeated on two successive days, after which they were then killed. Fish that emerged consistently in all 3 tests were designated as risk-taking or proactive; those that consistently failed to emerge were

categorised as risk-avoiding or reactive. The remaining fish were designated as intermediate, although since these fish sometimes took a long time to emerge but sometimes emerged quickly, it might be more accurate to describe them as "flexible". The majority of fish (53) were frozen directly for further studies; for the remainder, the 2nd left gill arch was removed and preserved for histological studies and the third left gill arch was removed and preserved for scanning electron microscopy before the rest of the body was frozen. Usually behavioural phenotype was created after 3 trials, but in some case a 4th trial was requred to clarify by classification.

4.2.3 Morphometrics

4.2.3.1 General morphometrics: The body weight (g), total length (to the nearest mm), and head length (to the nearest mm) of all fish were measured. At the time of sacrifice, mean weight was 57.63 (g) and mean total length 18.11 (cm). The relationship between weight and length for fish was estimated using the condition factor (K):

$$K = W / L^3$$

where W and L are weight (g) and length (mm) of the same fish. The opercular bones on the right side of each fish were removed from their ligaments, cut from the head and soaked for a few minutes in potassium hydroxide. They were lightly brushed with a stiff tooth brush to remove attached tissue. After these procedures, opercular length and width were measured using electronic callipers.

4.2.3.2. *Gill morphometrics*: All four gill arches from the right side of each fish were dissected out with the rakers and filaments intact and placed in 0.9 % normal saline. The gill arches were separated and the measurements indicated in Table 4.1 taken for each arch using a binocular microscope at a magnification of 3X and a compound light microscope at 40X and 100X with an eyepiece micrometer (after Hughes, 1984). To quantify individual status with respect to all these variables, the mean values for each arch on the right side were used.

Table 4.1. Summary of the measurements made on all four arches on the right side of the head for characterisation of gill structure in brown trout

Structure	Brown trout
Gill arch	Gill arches length
Gill raker number	Total number of gill rakers
Gill raker length	Gill raker length
Gill filament number	Total number of gill filaments
Gill filament length	Length of every fifth gill filament
Secondary lamellae number	Number of secondary lamellae per mm on every fifth gill filament
Secondary lamellae length	Length of secondary lamellae at 3 points of every fifth gill filament
Distance between secondary lamellae	Distances between secondary lamellae at 3 points of every fifth gill filament

4.2.3.3. Estimated total filament length and gill surface area: Total filament length was estimated by the equation:

$$(Fn_1 * Fl_1) + (Fn_2 * Fl_2) + (Fn_3 * Fl_3) + (Fn_4 * Fl_4)$$

Where Fn_1, Fn_2, Fn_3 and Fn_4 are the filament number on first gill arch, the filament number on second gill arch, the filament number on third gill arch and the filament number on fourth gill arches and Fl_1, Fl_2, Fl_3 and Fl_4 are the filament length on the first, second, third and fourth gill arches. Gill surface areas were estimated using methods established by Hughes (1984) and calculated by the equation:

$$A = l.\ n.\ bl$$

Where A is the total gill surface area, l is the total length of the gill filament, n is the mean number of secondary lamellae per mm length on two sides of a filament and bl is the mean bilateral secondary lamellae surface area.

4.2.4 Light microscopy

The second gill arch from the left side was preserved for light microscopy. Tissues for histological analysis were placed into 10% buffered formalin, passed through increasing concentrations of alcohol to dehydration and embedded in Paraplast (wax) following standard procedures. Wax embedded tissues were sectioned (thickness sections 8 µm) were stained with haematoxylin eosin and periodic acidic-Schiff (PAS) and haematoxylin reaction on a glass microscopy slides, according to the method outlined in Clark (1981). Sections were examined under light microscopy at a magnification of 100X and 400X. Figure 4.2 shows a typical section. The number of mucous cells (indicated in Figure 4.2a)

per millimetre of lamellar surface and the height of interlamellar cells as a percentage of lamella height (indicated in Figure 4.2b) were quantified on *ca* 14 different filaments. The number of mucous cells, the height of the interlamellar epithelial cells and the height of the adjacent lamellae were measured for nine lamellae at the tip, middle and base of every fifth gill filament and mean values were calculated to give a score for the abundance of mucous cells and percentage of hyperplasia.

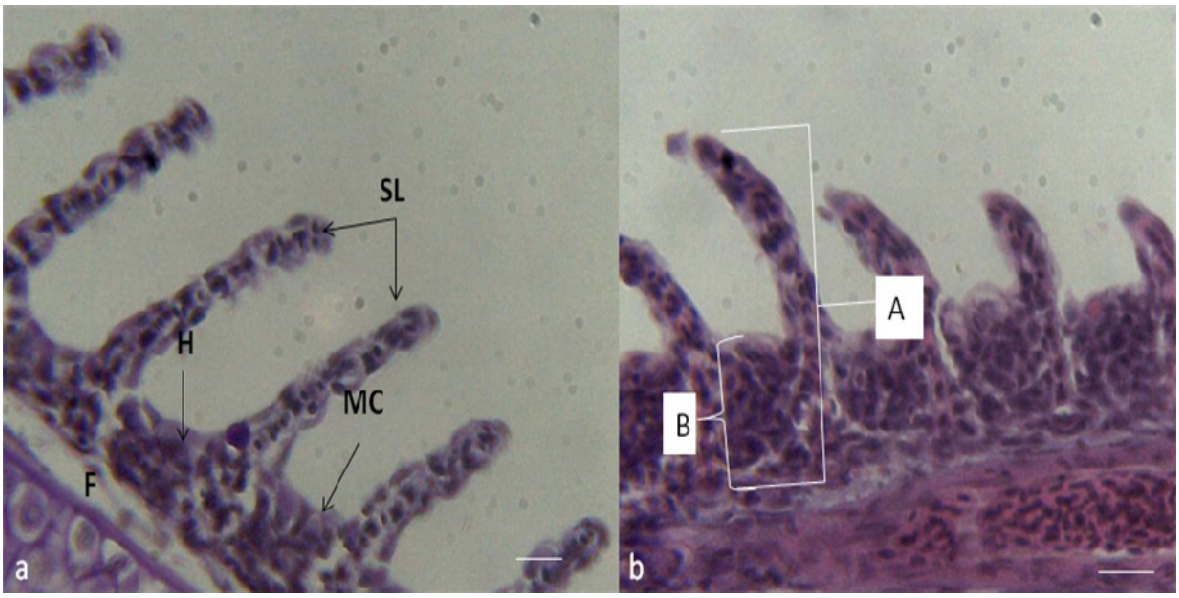


Figure 4.2a) Typical section through the gill filament (F) of brown trout, with secondary lamellae (SL) mucous cells (MC) and hyperplasia (H) indicated and b) (A) represents the height of one secondary lamellae and (B) represents the height of the interlamellar hyperplasia, bar = 60 m µ. H, E and PAS stain and magnification 400 X.

4.2.5 Scanning Electron Microscopy

For scanning electron microscope studies, the third left gill arch from each of 9 fish (5 proactive and 4 reactive) was fixed in 2.5% glutarldehyde at 4 °C for 24 hours followed by 2.5% glutarldehyde in 0.1M sodium cacodylate buffer at 4 °C for 2 hours. The fixed gill arches were then washed in 3 changes of 30 min each of 0.1M sodium cacodylate buffer and then transferred to 1% osmium tetroxide with 0.05 % sodium cacodylate for 1 hour and then washed in 3 changes distilled water for 30 mins each. The gill arches were dehydrated in a graded alcohol series and then transferred to 1:1 propylene oxide:Epon resin, left overnight and then placed in to pure Epon 218 (Epon is an epoxide resin that is used as a plastic embedding material and helps in preventing the collapse of the specimen) for few hours. The tissues were then transferred to Epon 218 Resin containing an acceleraror, left overnight and then stored to fresh Embed at 60 °C for overnight. The gills were exposed to critical point drying using CO_2. Dried gills were mounted on brass stubs

and placed within a gold coating unit. Examination of tissues was by a Jeol 6400 scanning electron microscope.

4.2.6. Statistical analysis

Statistical analyses were carried out, using the MINITAB statistical package, series 15. First of all, the data were checked for normality and transformations performed where necessary. Initial scrutiny of the data was carried out using means and standard errors. Regression analysis was then used to explore the relationship between all variables and body length. Residuals from the regression on length were used to generate length-independent variables where appropriate (Reist, 1989; Adams and Huntingford, 2004). Relations among measured variables were studied by correlation analysis, followed by Principal Components Analyses (PCA), which was also used to generate compound scores where appropriate. For simplicity, separate PCAs were carried out for mean per fish values for gill arch length, number and length of gill rakerand filaments and number, length and distance between secondary lamellae. Derived scores from these PC analysis were combined further by multi-variate analysis of the main PC scores. Finally, ANOVA was used to compare to the morphology of the three risk-taking phenotypes.

4.3 Results

4.3.1 Behaviour in the novel environment test

Table 4.2 shows emergence/non-emergence of each of the 53 brown trout tested in each trial, together with their classification with respect to risk-taking phenotype. Clearly the fish were variable in their behaviour during the trials. Some fish (for examples numbers 6 and 7) emerged on every occasion and were classified as risk takers (RT), while other fish (for examples numbers 3 and 9) did not emerge in any of the trials and were classified as risk-avoiders (RA). Yet other fish (for examples numbers 4 and 5) were variable, emerging in some trials but not others; these fish were classified as intermediate (I), although “flexible” might be a better term

Table 4.2. Summary the response of all fish in the three or four novel environment test. X represent staying in shelter and √ represent emerging into the novel environment. RA = risk-avoiding, I= intermediate and RT= risk-taker.

Fish number	Trial 1	Trial 2	Trial 3	Trial 4	Classification	Fish number	Trial 1	Trial 2	Trial 3	Trial 4	Classification
3	×	×	×	-	RA	35	√	√	×	×	I
9	×	×	×	-	RA	37	√	√	×	×	I
15	×	×	×	-	RA	42	√	×	√	×	I
16	×	×	×	-	RA	43	×	×	√	×	I
17	×	×	×	-	RA	46	√	×	×	√	I
23	×	×	×	-	RA	47	×	√	×	√	I
24	×	×	×	-	RA	49	√	×	√	×	I
25	×	×	×	-	RA	53	×	√	√	×	I
28	×	×	×	-	RA	6	√	√	√	-	RT
31	×	×	×	-	RA	7	√	√	√	-	RT
34	×	×	×	×	RA	10	√	√	√	-	RT
36	×	×	×	×	RA	13	√	√	√	-	RT
39	×	×	×	×	RA	14	√	√	√	-	RT
40	×	×	×	×	RA	20	√	√	√	-	RT
41	×	×	×	×	RA	21	√	√	√	-	RT
45	×	×	×	×	RA	22	√	√	√	-	RT
50	×	×	×	×	RA	26	√	√	√	-	RT
1	×	×	√	-	I	29	√	√	√	-	RT
2	√	×	×	-	I	30	√	√	√	-	RT
4	×	×	√	-	1	32	√	√	√	√	RT
5	×	√	×	-	1	38	×	√	√	√	RT
8	√	×	×	-	1	44	√	√	√	×	RT
11	×	×	√	-	I	48	×	√	√	√	RT
12	√	×	×	-	I	51	√	√	√	√	RT
18	√	×	×	-	I	52	√	√	×	√	RT
19	√	×	√	-	I						
27	×	×	√	-	I						
33	√	×	√	×	I						

4.3.2 Overall morphometrics

Table 4.3 shows the mean values (± SE) for all measured variables for the 4 right hand gill arches, together with the results of ANOVA testing between arch differences in each variable. For gill arch length *post hoc* testing confirmed that the first arch was the longest and that mean arch length decreased successively from 1st to 4th arch. Gill raker length and number differed significantly between gill arches. For raker length, *post hoc* tests showed the value for the first gill arch to be significantly larger than those for the second, third and fourth arches, but no differences along the remaining arches except between second and fourth arches. For gill raker number, *post hoc* test revealed significant difference between the raker numbers of all gill arches.

Table 4.3. Mean and standard error of the mean (± SE) for a) gill arches length, gill raker length, gill raker number, filament length, filament number, lamellar length, lamellar number and lamellar distance, for 53 brown trout, are also shown results of ANOVA and *post hoc* test.

Gill arch	Arch length	Raker length	Raker number	Filament length	Filament number	Lamellar length	Lamellar number	Lamellar distance
	Mean	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**
1st	2.63 ± 0.05	1.11 ± 0.06	14.6 ± 0.41	4.3 ± 0.07	76.3 ± 1.51	0.18 ± 0.06	43.0 ± 0.95	0.03 ± 0.002
2nd	2.39 ± 0.05	0.88 ± 0.03	12.7 ± 0.34	3.9 ± 0.07	69.5 ± 1.46	0.17± 0.06	40.2 ± 2.13	0.03 ± 0.006
3rd	2.15 ± 0.05	0.73 ± 0.03	10.7 ± 0.33	3.7 ± 0.07	62.3 ± 1.64	0.17± 0.07	39.4 ± 2.00	0.03 ± 0.004
4th	1.81 ± 0.06	0.57 ± 0.03	8.74 ± 0.37	3.4 ± 0.08	54.2 ± 2.09	0.16± 0.07	38.5 ± 2.15	0.03 ± 0.004
$F_{3,267}$	45.35	21.67	47.94	24.56	31.34	1.34	1.09	0.31
P	<0.001	<0.001	<0.001	<0.001	<0.001	0.26	0.35	0.82
Tukey test	1≠2,3,4; 2≠3,4;3≠4	1≠2,3,4; 2=3;2≠4;3=4	1≠2,3,4; 2≠3,4;3≠4	1≠2,3,4; 2≠3,4;3≠4	1≠2,3,4; 2≠3, 4;3≠4	1=2,3,4;2=3,4; 3=4	1=2,3,4;2=3,4 ;3=4	1=2,3,4;2=3,4; 3=4

Mean length and number of the gill filaments also decreased significantly towards the back of the head, with the longer first and second arches bearing many, long gill filaments. For filament length and filament number, *post hoc* tests showed significant differences between all gill arches. Secondary lamellae length, number and distance did not differ significantly between gill arches.

4.3.3 Relationship between measured variables and body length

Table 4.4 shows the results of regression analyses of the relationship of all variables with body length. Significant positive relationships were found for the following variables: length of all arches, filament length for all gill arches, secondary lamellae length for all arches, raker length for all arches, raker number for 1st, 2nd and 3rd gill arches, body weight, head length, estimated total filament length and total gill surface area. For further analysis, these variables were therefore expressed as residuals from the regression with body length and are referred to as "length-corrected" in the following text.

Table 4.4. Regression analyses of all measured variables against total body length (cm) for 53 brown trout (17 Bold, 19 Intermediate and 17 Shy). Italics indicate significant relationships.

Variable	Arch	Regression equation	F 1,52	P	R^2
Arch length (cm)	1	*Y=0.267+0.132X*	*49.56*	*<0.001*	55.5
	2	*Y=0.041+0.131X*	*52.09*	*<0.001*	56.7
	3	*Y=-0.261+0.135X*	*54.22*	*<0.001*	57.7
	4	*Y=0.795+0.146X*	*47.40*	*<0.001*	54.3
Filament length (mm)	1	*Y=2.53+0.0974X*	*5.78*	*0.021*	10.7
	2	*Y=2.35+0.0912X*	*7.16*	*0.011*	13.3
	3	*Y=2.05+0.0932X*	*8.32*	*0.006*	15.5
	4	*Y=1.92+0.0837X*	*5.28*	*0.027*	9.7
Filament number	1	Y=67.10+0.502X	0.42	0.523	0.0
	2	Y=60.70+0.473X	0.40	0.531	0.0
	3	Y=44.40+0.968X	1.25	0.270	0.6
	4	Y=40.20+0.740X	0.43	0.513	0.0
Secondary lamellae length (mm)	1	*Y=0.026+0.008X*	*6.90*	*0.012*	12.9
	2	*Y=0.024+0.008X*	*6.79*	*0.013*	12.6
	3	*Y=0.002+0.009X*	*8.50*	*0.006*	15.8
	4	*Y=-0.062+0.012X*	*13.61*	*0.001*	24.0
Secondary lamellae number	1	Y=44.80-0.0960X	0.03	0.857	0.0
	2	Y=16.50+1.320X	1.29	0.263	0.7
	3	Y=25.00+0.800X	0.53	0.470	0.0
	4	Y=14.00+1.350X	1.33	0.256	0.8
Distance between secondary lamellae (mm)	1	Y=0.05-0.00149X	3.19	0.082	5.2
	2	Y=0.082+0.003X	0.59	0.448	0.0
	3	Y=25.00+0.800X	0.53	0.470	0.0
	4	Y=0.059+0.001X	0.39	0.54	0.0
Raker number	1	*Y=5.49+0.494X*	*6.22*	*0.02*	11.5
	2	*Y=3.80+0.481X*	*8.70*	*0.005*	16.1
	3	*Y=3.49+0.391X*	*6.16*	*0.017*	11.4
	4	Y=4.02+0.247X	1.81	0.186	2.1
Raker length (mm)	1	Y= - 0.50+0.89 X	8.91	0.005	16.5
	2	Y= - 0.57+0.081X	12.0	0.001	21.6
	3	Y= - 0.13+0.048 X	5.35	0.03	9.8
	4	Y= - 0.37+0.053 X	6.98	0.027	14.8
Body weight (gm)		Y=-57.10+6.39X	74.57	<0.001	63.7
Head length (cm)		Y=-0.428+0.244X	26.62	<0.001	39.0
Height opercular (cm)		Y= 0.85 + 0.03 X	1.68	0.20	2.0
Width opercular (cm)		Y= 0.52+ 0.03X	0.66	0.42	0.0
Estimated total filament length (mm)		Y= 413+ 33.70 X	4.17	0.05	7.3
Total estimated gill area mm²		Y= - 1..4 + 103 X	6.90	0.01	12.9

4.3.4 Relationship among length-independent variables

4.3.4.1 Gill morphometrics: Because so many variables were measured, successive principle component analyses were used to examine the relationships among them and to condense these into a smaller number of variables where appropriate. Table 4.5a shows the matrix of correlations for arch length for all right hand arches, together with the results of principal components analysis for these variables. Lengths of the different gill arches are highly correlated. The principal components analysis for the gill arch length (Table 4.5b) showed that the first two components explains 96% of the variation in the data set. PC1 accounts for 84% of total variance and has positive loading for all variables, representing overall gill arch size, regardless of body size. PC2 accounts for 12% of the total variance and opposes length of the first, second and third gill arch lengths (negative and falling) to the fourth gill arch (positive), representing differential development of the anterior and posterior gill arches.

Table 4.5a). Correlation matrix for right hand side gill arch length. In each cell, the top figure is the correlation coefficient and the bottom figure is the P- value, b) PCA results.

Table 4.5a)	**1st**	**2nd**	**3rd**
2nd	0.962 <0.001		
3rd	0.937 <0.001	0.974 <0.001	
4th	0.806 <0.001	0.973 <0.001	0.985 <0.001

Table 4.5b)	**PC1**	**PC2**
1st	0.51	-0.31
2nd	0.52	-0.32
3rd	0.53	-0.11
4th	0.43	0.89
Variation %	84	12

Table 4.6a and b shows the correlations between gill raker number and length across all arches, length corrected where appropriate, together with the results of PCA on these variables. Raker lengths for the different arches were correlated, as were raker numbers, but in general length and number were uncorrelated. For these variables, the first 2 components explain 86% of the total variance. PC1 (61%) opposes gill raker length (negatively) to gill raker number. PC2 (25%) has positive loadings for all scores and so reflects variation in overall raker development, independent of overall body size.

Table 4.6a). Correlation matrix for right hand side gill raker length and gill raker number for all gill arches. In each cell, the top figure is the correlation coefficient and the bottom figure is the P- value, b) PCA results.

a)

	Length 1	Length 2	Length 3	Length 4	Number 1	Number 2	Number 3
Length 2	0.899 <0.001						
Length 3	0.742 <0.001	0.726 <0.001					
Length 4	0.664 <0.001	0.666 <0.001	0.935 <0.001				
Number 1	-0.159 0.301	-0.158 0.305	-0.295 0.051	-0.209 0.185			
Number 2	0.021 0.891	0.022 0.885	-0.174 0.260	-0.108 0.497	0.872 <0.001		
Number 3	-0.066 0.671	-0.088 0.571	-0.295 0.051	-0.220 0.162	0.873 <0.001	0.914 <0.001	
Number 4	-0.194 0.219	-0.158 0.241	-0.374 0.015	-0.298 0.055	0.864 <0.001	0.808 <0.001	0.883 <0.001

b)

Arch	Gill arch	PC1	PC2
1st	Length	-0.31	0.41
	Number	0.38	0.28
2nd	Length	-0.33	0.38
	Number	0.34	0.41
3rd	Length	-0.37	0.33
	Number	0.37	0.36
4th	Length	-0.33	0.34
	Number	0.38	0.29
Variation%		61	25

Table 4.7a and b shows the matrix of correlations between gill filament number and length for all arches, together with the results of principal components analysis for these variables. Filament lengths for the gill arches are all strongly correlated, as are filament numbers, but length and number of gill filaments were uncorrelated. The first two components (Table 4.7b) explain 88% of variation in this data set. PC1, which accounts for 50% of the total variance, has positive loadings for all variables and so represents variability in filament size and number (representing an index of overall filament development) independent of body size. PC2 accounts for 38% of the total variance and has negative loadings for filament length on all arches and positive loadings for filament number; fish with few, short filaments gain high scores in this axis.

Table 4.7a). Correlation matrix for right hand side gill filament length and number for all gill arches. In each cell, the top figure is the correlation coefficient and the bottom figure is the P value, b) PCA results.

	Length 1	Length 2	Length 3	Length 4	Number 1	Number 2	Number 3
Length 2	0.936 <0.001						
Length	0.863 <0.001	0.969 <0.001					
Length	0.718 <0.001	0.863 <0.001	0.916 <0.001				
Number 1	0.213 0.166	0.158 0.304	0.079 0.609	0.036 0.814			
Number 2	0.140 0.364	0.076 0.622	0.018 0.909	0.009 0.956	0.899 <0.001		
Number 3	0.121 0.434	0.060 0.697	0.022 0.889	0.008 0.960	0.747 <0.001	0.903 <0.001	
Number 4	0.163 0.291	0.079 0.612	0.024 0.877	0.056 0.720	0.661 <0.001	0.833 <0.001	0.895 <0.001

b)

Arch	**Gill filament**		
		PC1	**PC2**
1st	Length	0.38	-0.30
	Number	0.33	0.33
2nd	Length	0.39	-0.34
	Number	0.35	0.39
3rd	Length	0.37	0.36
	Number	0.32	0.40
4th	Length	0.35	-0.32
	Number	0.33	0.36
Variation%		50	38

Table 4.8a and b shows the correlation matrix for lamellar measures for all arches, together with the results of Principal Components Analysis on these variables. Number and length of secondary lamellae are correlated across arches and with each other. Distances between lamellae are correlated across arches, but do not show any consistent pattern of correlation with the other scores. For the lamellar length (Table 4.8b), the first two components explain 96% of the variation in the data set. The first component accounts for 90% and has positive loadings for all variables. PC2 accounts for 6% and has negative loadings for first and second arches but positive for third and fourth arches. For secondary lamellae number (Table 4.8b), the first two components also explain 96% of the variation in the data set. The first component accounts for 75% and has positive loadings for all variables. PC2 accounts for 21% and has negative loadings for first arch but positive for second third and fourth arches. For distance between secondary lamellae (Table 4.8b) the first two components explain 57% of the variation in the data set. PC1 accounts for 31% and has

positive loadings for all variables except distance between secondary lamellae on third gill arch. PC2 accounts for 26% and has negative loadings for first and fourth arches but positive for second and third arches.

Table 4.8a). Correlation matrix for gill secondary lamellae length and number and spacing between secondary lamellae for all gill arches. In each cell, the top figure is the correlation coefficient and the bottom figure is the P value, b) Results of PCA.

	Length 1	**Length 2**	**Length 3**	**Length 4**	**Number 1**	**Number 2**	**Number 3**	**Number 4**	**Space 1**	**Space 2**	**Space 3**
Length 2	0.951 <0.001										
Length 3	0.896 <0.001	0.899 <0.001									
Length 4	0.844 <0.001	0.830 <0.001	0.897 <0.001								
Number 1	0.112 0.470	0.157 0.308	0.051 0.741	0.157 0.309							
Number 2	0.123 0.427	0.239 0.118	0.044 0.778	0.007 0.962	0.324 0.032						
Number 3	0.144 0.351	0.272 0.074	0.069 0.657	0.034 0.826	0.392 0.009	0.963 <0.001					
Number 4	0.178 0.246	0.283 0.063	0.119 0.442	0.095 0.538	0.294 0.053	0.871 <0.001	0.897 <0.001				
Space 1	-0.191 0.213	-0.176 0.254	-0.254 0.096	-0.350 0.020	0.396 0.008	0.201 0.190	0.210 0.171	0.203 0.187			
Space 2	-0.190 0.217	-0.213 0.165	-0.192 0.213	-0.188 0.221	-0.042 0.785	-0.166 0.280	-0.191 0.215	-0.188 0.223	0.242 0.114		
Space 3	0.006 0.971	-0.018 0.907	-0.004 0.981	-0.003 0.983	-0.120 0.438	0.112 0.470	0.099 0.521	0.143 0.355	-0.030 0.848	-0.009 0.955	
Space 4	-0.002 0.991	0.053 0.735	0.002 0.991	0.013 0.933	-0.025 0.873	0.172 0.265	0.156 0.313	0.203 0.187	0.072 0.644	-0.051 0.741	-0.038 0.809

b)

Arch	**Length**		**Number**		**Distance**	
	PC1	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**
1st	*0.51*	-0.42	0.28	-0.96	*0.71*	-0.08
2nd	*0.50*	-0.49	*0.56*	0.18	*0.69*	0.28
3rd	*0.51*	0.20	*0.57*	0.10	-0.12	*0.55*
4th	*0.48*	*0.74*	*0.54*	0.21	0.08	-0.79
Variation %	90	6	75	21	31	26

Finally, to compare and if appropriate combine across the different components of the gills, correlations were calculated for the first PCA scores for gill arches and the respiratory components of the gill structures, namely gill filaments and secondary lamellae (Table 4.9a). Weakly significant negative relationships were found between arch length PC1 and filament PC1 and arch length PC1 and secondary lamella number PC1. No other significant relationships were identified. Principal component analysis of thee variables did not produce any easily identified compound variables (Table 4.9b).

Table 4.9a). Correlation matrix between the PCA scores for gill arch, gill filaments and secondary lamellae. In each cell, the top figure is the correlation coefficient and the bottom figure is the P value. Table 9b. Results of a Principal Component Analysis between PC1 filaments, PC1 arches, PC1 lamellae length, PC1 lamellae number and PC1 distance between secondary lamellae.

	PC1 filaments	PC1 arches	PC1 lamellae length	PC1 lamellae number
PC1 arches	-0.277 0.084			
PC1 lamellae length	0.113 0.481	-0.301 0.059		
PC1 lamellae number	0.181 0.258	0.018 0.911	0.101 0.531	
PC1 distance between secondary lamellae	0.151 0.346	0.100 0.541	- 0.221 0.166	0.057 0.712

a)

b

Variable	PC1	PC2	PC3
PC1 Filaments	0.436	-0.550	-0.228
PC1 arches	-0.620	-0.041	0.432
PC1 lamellae length	0.571	0.299	0.226
PC1 lamellae number	0.234	-0.407	0.810
PC1 distance between secondary lamellae	-0.209	-0.664	-0.233
Variation %	30	26	19

Figure 4.3 shows the distribution of estimated total filament lengths (mm) for all brown trout. The mean (±SD) value is 1014 (±212.3) mm. The coefficient of variation for this measure is 21%, indicated considerable variability within the sample.

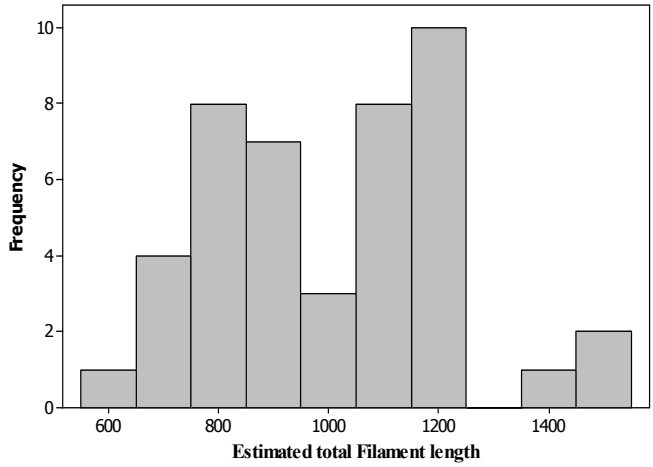


Figure 4.3. Frequency distribution of estimated gill total filament lengths (mm) for all brown trout.

4.3.4.2 Relationship between gill and head morphometrics: The height and width of the opercular bones were positively related (R = 0.608 and P <0.001). Table 4.10 shows the relationship between the various derived scores of gill development (which have been set

up to be independent of total body length) as well as estimated total gill area and head length. None of these are significant.

Table 4.10. Regression analyses of all PC1 filament, PC1 arches, PC1 secondary lamellae length, PC1 secondary lamellae number and PC1 distance between secondary lamellae against head length for 53 brown trout (17 Bold, 19 Intermediate and 17 Shy).

Variable	Regression equation	F	P	R^2 %
PC1 filament	Y= 1.06-0.252X	0.30	0.585	0.0
PC1 arches	Y= -2.21+0.563X	1.82	0.186	١٤٢٠/١ ٠/٢٦
PC1 secondary lamellae length	Y= 1.75-0.425X	1.00	0.323	0.0
PC1 secondary lamellae number	Y= -2.00+0.510X	1.58	0.216	١٤٣٢/٠ ١٤/٢٨
PC1 distance between secondary lamellae	Y= 0.85-0.214X	0.65	0.423	0.0
Estimated total filament length	Y= 943+19.30X	0.15	0.704	0.0

Figure 4.4a shows the relationship between overall gill development and residual head length.

These variables are significantly related (P=0.027 and R^2 = 10.0 %).

Figure 4.4b shows the relationship between the residual head length and residual total filament length. These variables are not significantly related (P=0.061 and R^2 = 6.5 %).

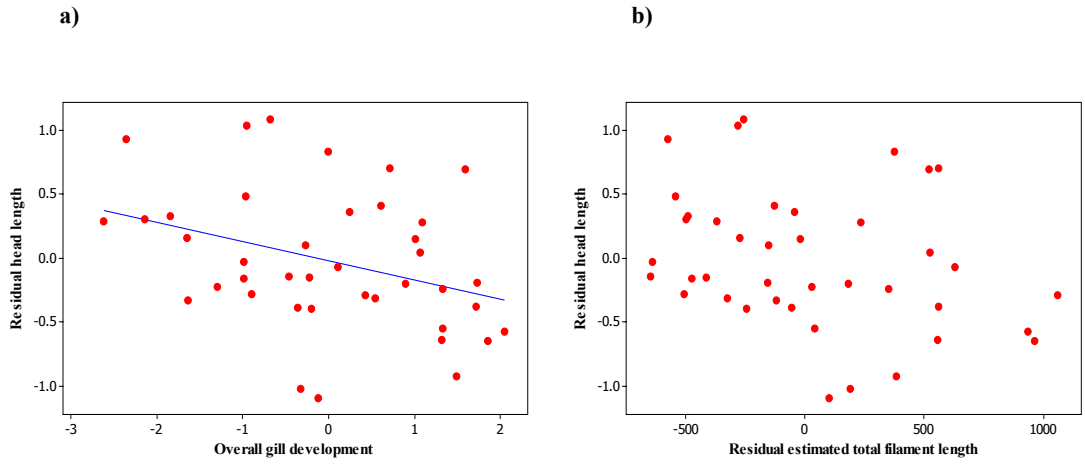


Figure 4.4a) Relationship between overall gill development and residual head length and 4.4b) Relationship between residual estimated total filament length and residual head length.

4.3.5 Gill structure

The basic structure of brown trout gills is similar to that found in other teleostean fish. Four pairs of gill arches are present in gill cavities, each gill arch carry a number of filament of gill. Each gill filament carries a number of flattened secondary lamellae that are arranged in two rows from the filament sides (Figure 4.5a). Scrutiny of stained (Figure 4.5b) and scanning electron microscope (Figure 4.5c) gill sections showed that mucous cells, which tended to cluster at the base of the secondary lamellae, werecharacterized by numerous secretory vesicles and opening..

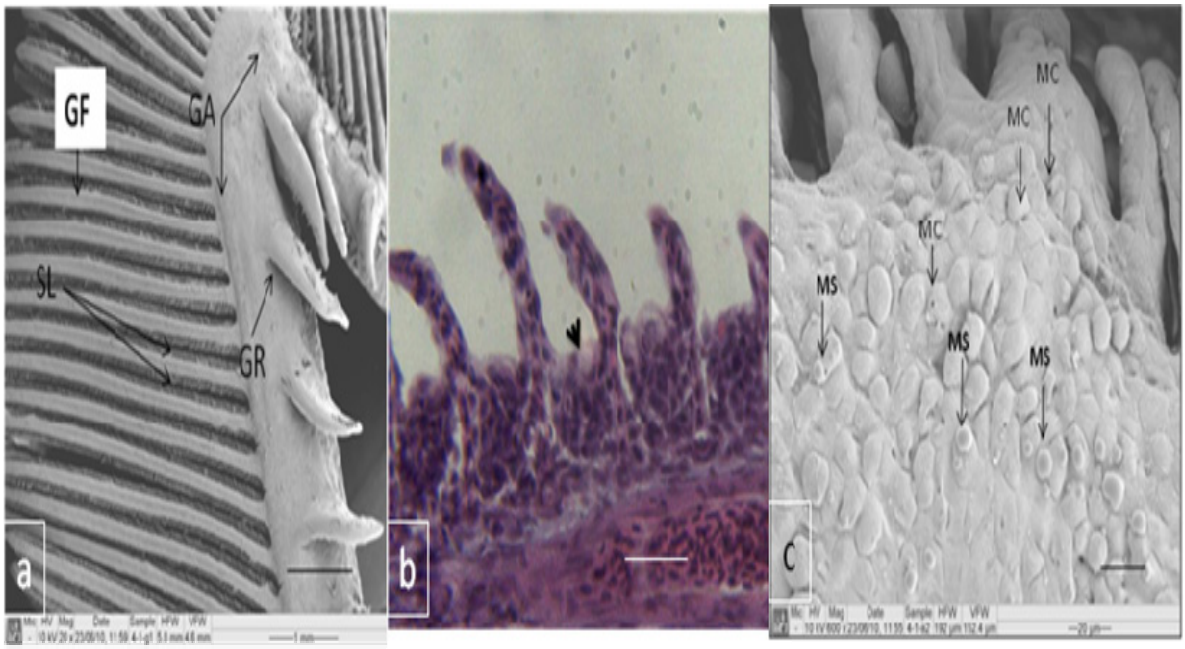


Figure 4.5. a) scanning electron micrograph of basic structure of brown trout gill showing SL= secondary lamellae, GF= gill filament, GA= gill arch and GR= gill raker, bar= 1000 µ m, figure b) light micrograph of gill secondary lamellae of brown trout showing mucous cell (MC with arrows), bar= 60 µ m (H, E and PAS stain and magnification 400 X) and figure c) scanning electron micrograph of gill secondary lamellae of brown trout showing MC= mucus cells and MS= mucus secretion, bar= 20 µ m.

Great variability in hyperplasia of epithelial cells between lamellae was observed in gills, some having multiple layers of epithelial cells on all the filaments that were in contact with the secondary lamellae (Figure 4.6A). Others had no hyperplasia and the secondary lamellae was separate (Figure 4.6B). Others were observed to have hyperplasia on some filaments only (Figure 4.6C).

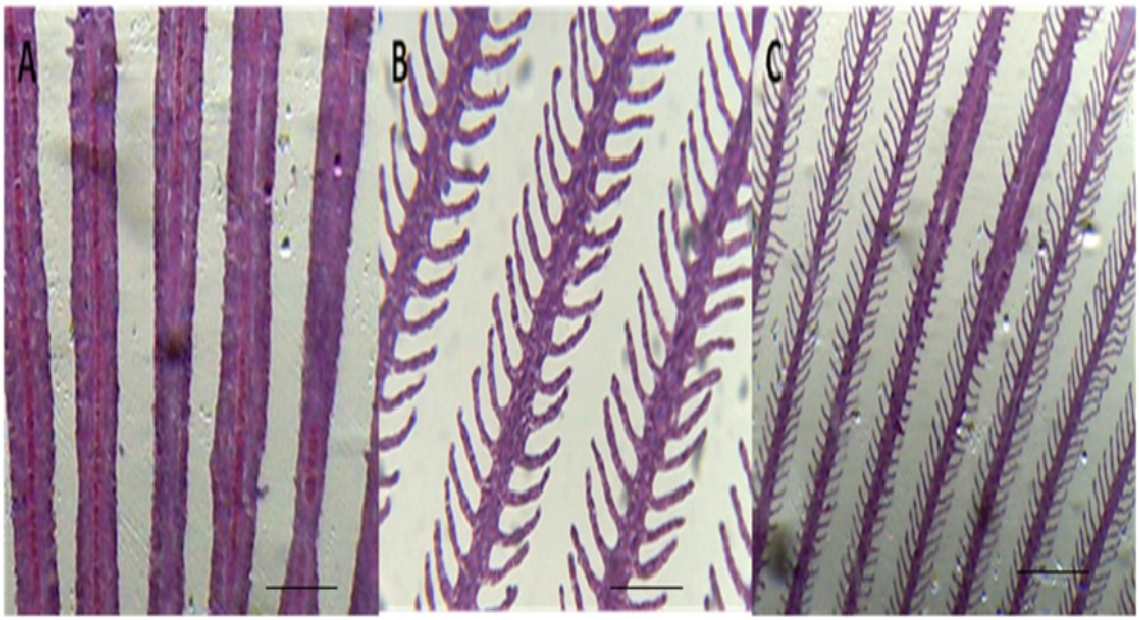


Figure 4.6. Light micrograph of gill secondary lamellae of brown trout showing variation in hyperplasia, A) almost complete hyperplasia of epithelial cells between lamellae, (bar = 0.17mm), B) no hyperplasia, (bar = 0.17mm) and C) hyperplasia on some filament, (bar = 0.34 mm) (E and S stain and 100 X magnification .

Figure 4.7 shows the distribution of relative hyperplasia scores. A considerable rangeof hyperplasia was observed (mean = 38.3 %) with a minimum of 4.4 % and a maximum 57.9 %.

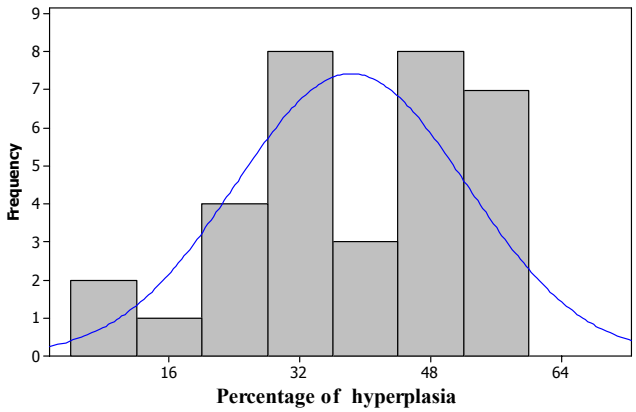


Figure 4.7. The distribution of percentage hyperplasia scores across the whole sample of brown trout

4.3.6 Comparison of trout with different coping strategies

Table 4.11 shows the means (± SE) for all univariate measures for risk-taking, risk-avoiding and intermediate brown trout. The only significant differences between stress coping strategies were in the number of gill filament and the length and number of secondary lamellae, all of which were larger in risk-taking trout and smaller in reactive fish. Thus the number of gill filament was greater by ca 10%, secondary lamellae length by ca 2.0% and the number of secondary lamellae by ca 30%. A p*ost hoc* test on filament number showed significantly higher values for the risk-taking group compared with both intermediate and risk-avoiding groups, but no significant difference between the intermediate and risk-avoiding groups. For secondary lamellae length *post hoc* tests showed significantly differences between risk-taking fish compared to risk-avoiding fish, but no significant difference between risk-taking and intermediate fish or between intermediate and risk-avoiding fish. For secondary lamellae number *post hoc* test showed a significant difference between the risk-taking and risk-avoiding group, but intermediate fish did not differ from either of the other two categories.

Table 4.11. Mean (± SD) univariate scores in brown trout with the 3 risk taking phenotypes, together with the results of ANOVA for the three risks taking in brown trout. Mean values sharing a superscript letter in common are not significantly different.

Variable	**Mean (±SD)**			**F2,39**	**P**
	Risk-taking	**Intermediate**	**Risk avoiding**		
Body weight	59.55 ±10.11	56.86±21.67	56.61±13.88	0.18	0.87
Body length	18.56± 1.18	17.81 ±2.42	18.81± 1.95	0.56	0.57
Body condition	1.07± 0.16	1.08±0.22	1.15± 0.15	0.15	0.86
Head dimensions					
Head length (cm)	4.05 ± 0.89	3.76 ±0.61	4.11 ± 0.64	1.07	0.35
Operculum height (cm)	1.35 ± 0.22	1.39±032	1.41 ± 0.18	0.61	0.85
Operculum width (cm)	1.00 ± 0.23	1.06±0.31	1.21 ± 0.77	0.59	0.56
Arch length (cm)	2.30 ± 0.22	2.22 ±0.40	2.22 ± 0.31	0.28	0.76
Feeding structures					
Raker length (mm)	0.70 ± 0.24	0.84 ±0.35	0.89 ± 0.23	1.25	0.23
Raker number	11.75±1.96	11.72±2.09	11.68±2.92	0.00	0.99
Respiratory structures					
Filament length (mm)	3.81 ± 0.47	3.81 ±0.53	3.84 ± 0.45	0.02	0.98
Filament number	72.48^{a} ± 11.17	62.66^{b} ±8.41	62.65^{b}± 9.07	4.91	*0.01*
Lamellar length (mm)	0.19^{a} ±0.03	0.17^{ab}±0.05	0.15^{b}±0.02	3.78	*0.03*
Lamellar number/ mm	36.19^{a}± 9.21	38.93^{ab}±10.04	45.66 ± 11.35	3.11	*0.05*
Lamellar distance (mm)	0.030 ± 0.02	0.028±0.01	0.035 ±0.01	0.82	0.45

Table 4.12 compares the main derived scores for the gills in brown trout with different coping styles. Risk-taking fish had significantly higher scores than the risk-avoiding fish, with intermediate fish occupying an intermediate position, for secondary lamellae length PC1. This is not reflected in any differences estimated gill filament length or estimated gill area. For secondary lamellae length PC1 *post hoc* test showed risk-taking fish to be significantly different from risk-avoiding fish, but neither risk-taking nor risk-avoiding fish are different from intermediate fish. There was a marginally significant difference in the PCA derived score for secondary lamellar number, and in this case risk avoiding fish had higher scores than risk-taking fish, with intermediate fish lying in between.

Table 4.12. Mean (± SD) of the PCA derived summary scores and estimated total filament length and gill surface area, with results of ANOVA test for the three risks taking in brown trout.

Variable	Mean ± SD			F2,37	P
	Risk taking	Intermediate	Risk avoiding		
Arch PC1	-0.31± 0.98	0.18±2.31	0.07 ± 1.87	25	0.78
Arch PC2	-0.15 ± 0.59	0.21± 0.64	-0.13± 0.83	1.19	0.32
Raker PC1	0.61 ± 1.92	-0.25±2.34	- 0.23± 2.33	0.57	0.57
Raker PC2	-0.77 ± 1.43	0.29±0.94	0.31 ± 1.66	2.47	0.99
Filament PC1	0.70 ± 2.19	-0.19±1.57	- 0.42 ± 2.26	1.09	0.35
Filament PC2	-0.95 ± 1.13	0.40±2.17	0.38 ± 1.37	2.72	0.08
Lamellae length PC1	0.85^{a} ± 1.32	0.14^{b}±2.29	-0.95^{b} ± 1.44	3.22	*0.05*
Lamellae length PC2	0.03 ± 0.39	0.05 ± 0.45	-0.09 ± 0.67	0.30	0.75
Lamellar number PC1	-0.66 ± 1.49	-0.21± 1.61	0.87± 1.83	3.09	0.06
Lamellar number PC2	0.02± 0.55	-0.08± 1.01	0.08± 1.09	0.13	0.88
Lamellar distance PC1	-0.51± 1.62	0.04± 0.92	0.43± 0.45	2.56	0.09
Lamellar distance PC2	0.09± 1.91	-0.01± 0.22	-0.07± 0.22	0.08	0.92
Estimated total filament length	1118.9±239.5	963.9±168.9	977.3±212.7	2.42	0.10
Estimated gill surface area	941.1±437.3	867.9±610.1	736.9±456.6	0.55	0.58

Table 4.13 shows mean (±SD) mucus cell density and percentage hyperplasia in brown trout from the three risk taking categories, together with the results on a one way ANOVA. Risk-avoiding fish have significantly more mucus cells per mm of lamellae than do risk-taking and intermediate fish.

Table 4.13. Mean (± SD) number of mucus cells and hyperplasia % in brown trout with the 3 risk taking phenotypes, together with the results of ANOVA test for the three risks taking in brown trout. Mean values sharing a superscript letter in common are not significantly different.

Variable	Coping style			F2,28	P
	Risk taking	Intermediate	Risk avoiding		
Number of mucous cells	1.12^{a}±0.40	1.48^{ab}±0.52	1.91^{b}±0.51	7.28	*0.003*
Hyperplasia %	32.2±13.90	36.97±13.91	45.49±12.60	0.083	0.271

Post hoc test for mucous cell number revealed that risk-taking fish did not differ from the intermediate, and intermediate group did not differ from risk-avoiding group however, risk-taking fish were shown to be significantly different from risk-avoiding group.

4.4 Discussion

4.4.1 The aims of this study

The aims of the present study were 1) to develop and deploy a method for assessing risk-taking in juvenile brown trout, 2) to quantify the overall development of the respiratory surface area in brown trout, 3) to use scanning electron microscopy to examine the fine structure of gills in brown trout, 4) to measure the extent of development of hyperplasia and mucous cells in histological sections from the gills of brown trout and 5) to compare gill morphometrics and gill fine structure in brown trout with different behavioural strategies. In this section, each of these aims is taken in turn and the extent to which they have been achieved is discussed and related to current understanding.

4.4.2 Screening trout for risk taking phenotype

In the present study, the sequence in which fish left a shelter to explore a novel, potentially dangerous environment was used to examine risk taking, an approach that has been commonly used in fish and other animal groups (Huntingford and Coyle, 2007). Overall, the individual brown trout used in the present study differed markedly in their tendency to emerge over successive tests, with some leaving cover in all three tests and others never doing so. In addition however, a group of fish was identified that were more flexible in their behaviour and did not fit neatly into either a risk avoiding or a risk taking phenotype. Overall, the novel environment test worked well in differentiating between extreme risk taking phenotypes in brown trout. The results of this study suggest that, as in many other species of fish as well as animals from other vertebrate groups, brown trout show a range of risk taking, with fish that ignore risk to exploit a profitable but potentially dangerous environment at one extreme and those that opt for safety at the other. In this respect, it agrees with a study by Sundstrom *et al.,* (2004), who found marked and reliable differences between wild juvenile brown trout in their tendency to examine a novel, potentially dangerous object. It also agrees with Brelin *et al.,* (2008), who found that four populations of brown trout with different derivations but reared under the same conditions varied in their endocrine stress response and behaviour when exposed to hypoxia and aggression. Moreover, if individuals are classified as high and low responsive based on post stress blood plasma noradrenalin levels, the frequency distribution shows that

populations of hatchery origin are biased towards having higher frequencies of high responsive individuals.

The existence of a category of fish that is flexible in response to a novel environment, as found in the present study, has been reported for other species, (for example, common carp, Mesquita, 2010); such fish are probably better classified as "flexible" and would be worth further study. It is general practice to remove individuals with intermediate emergence times, in order to focus on the clear differences between the risk-avoiding and risk-taking animals (for example, Huntingford *et al.,* 2010 on common carp), but this tactic is likely to miss an important aspect of behavioural variation.

4.4.3 Risk-taking and body status

In the present study, no differences between risk-taking phenotypes were found in length, weight or condition factor. There are two different perspectives on the probability of such a relationship, which, while not mutually-exclusive, make opposite predictions. On the one hand, according to the growth-mortality trade off view (Stamps, 2007), certain individuals adopt a fast growth and life history strategy, which among other traits involves consistent risk-taking in a variety of contexts. Other individuals adopt the reverse growth strategy and, again among other traits, show risk avoidance. On this basis, risk taking individuals are expected to be larger and (possibly) in better condition. According to an alternative view (based on the concept of "asset protection" Laland and Reader, 1999) fish that are small and of poor nutritional status will be more highly motivated to forage under risk than those that are in good condition, suggesting that individuals classified as risk avoiding will be those with good nutrient reserves, and the converse. Support for both predictions can be found in the literature on fish, since previous studies on the relationship between risk-taking phenotype and body status in fish have produced variable results. For instance, Brown *et al.,* (2007) found that *Branchyraphis episcopi* (Steindachner) that emerged faster from shelter were heavier at a given standard length than were those that emerged more slowly. Also, risk-taking three spine sticklebacks were found to be larger than risk-avoiding fish (Ward *et al.,* 2004). These results could be said to support Stamp's concept of a growth-mortality trade off (Stamps, 2007). Nevertheless, Brown and Braithwaite, (2007) reported that smaller *B. episco* emerged from shelter before larger fish. This indicates that fish with good nutrient funds do indeed protect these by staying in cover,

while fish with poor reserves emerge early because of high immediate nutritional needs. Obviously various different effects are involved and more study is needed to clarify the position, which may be different in different species and contexts.

4.4.4 Variability in gill structure in brown trout

As reported in Chapter 2, the results presented here indicate that overall gill structure in brown trout is similar to that seen in other teleosts. Each gill was found to consist of a gill arch, gill filaments and gill rakers (Mir and Channa, 2009). However, within this broad structure, considerable variability in detailed morphometrics was found. Similar variability has been found in other studies between fish species (Severi *et al.,* 2000; Chapters 2 and 3 of the present thesis; Jenjan *et al* in preparation). This can be explained partially by the position of the gill concerned, with arch length increasing from back to front. It can also be partially explained by differences in body size, since a positive relationship between gill morphometrics and body size was found for many gill structures. Such a relationship has previously been reported for other teleost fish, for instance *Sparus aurata* (Karakatsouli *et al.,* 2006) and common carp (Chapter 3; Jenjan *et al.,* in preparation). This connection is thought to be dependent on increasing oxygen requirements with increasing body size. Variability around the general relationship or gill development with body size can be explained in part by activity levels in the fish concerned, their mode of respiration and the environmental conditions where they live (Severi *et al.,* 1997).

In the current study, gill raker number and length decreased in size posteriorly through the gill baskets of brown trout. Jenjan *et al.,* (in preparation. Chapter 2) noted a similar for raker number and length in the Arctic charr (*Salvelinus alpinus*) and wild brown trout (*Salmo trutta*) as well as in the common carp (*Cyprinus carpio*) gill raker size might be linked to gill cavity and buccal cavity volume and shape and also connected to the ecophenotypism of fish character.

The numbers and lengths of gill rakers found for brown trout in this study fall within the range seen in other teleost fish (Ruzzante *et al.,* 2003). However, the observed values for gill raker number (8.74 -14.60) are quite low in comparison to other studies of this species; for example values of 11.8 – 20.4 were found by Langeland and Nost (1995). The mean length of the gill rakers in the present sample of brown trout is quite low (0.57- 1.11)

in comparison to the trout of other studies (1.17- 4.07. Langeland and Nost, 1995). Table 4.14 compares gill raker measures in the present study with these presented for brown trout by Langeland and Nost, (1995). The lengths and numbers of gill rakers reported by Langeland and Nost were slightly larger than those found in present study. Within the sample of brown trout examined in the present study, variability was observed in numbers and length of gill rakers, some of which was related to overall body size. Length-corrected measures were therefore campared (Table 4.14). Differences in length-corrected gill raker number are much less marked, but length-corrected raker lengths are still smaller in trout than in the present study. A difference in this component of the trophic apparatus may arise because the fish in the present study were hatchery-reared, whereas those used by Langeland and Nost were captured in the wild. Farmed trout are fed from very early age on formulated feed that is designed to be easy to process. Since trophic morphology varies flexibly with diet in fish (Fugi *et al.,* 2001), the shorter gill rake observed in the present study may be the result of such developmental flexibility in trophic morphology.

Table 4.14. Body length, mean gill raker number and length and length-corrected raker number and length in brown trout in the present study with these studied by Langeland and Nost, (1995).

	Body length	**Raker number**	**Raker length**	**Raker number/Raker length**	**Raker length/Body length**
Present study	18.1	11.7	0.98	0.65	0.05
Langeland and Nost, 1995	23.5	16.1	2.60	0.69	0.11

The results indicated a relationship between gill raker length and body size. This has been shown previously for in *Salmo trutta* (Langeland and Nost, 1995) and *Cyprinus carpio* (Jenjan *et al.,* preparation, in chapter 2). Gill raker length is hypothesized to be related with pharynx size (Hulsey *et al.,* 2006). Fish with large pharynxes would have larger gill arches and longer gill rakers. Differences in raker length and number are functionally connected to food type in a variety fish species (Amundsen *et al.,* 2004). For instance, in stickleback (*Gasterosteus aculeatus*) the gill raker number and length have been shown to affect feeding performance (Lavin and McPhail, 1986). Gill rakers could play a key role in food particle retention and the feeding habit in fish has generally been found to connect with gill raker number and (Amundsen *et al.,* 2004). Gill raker number and length are consequently connected to food size and a character with a geographic variation (Loy *et al.,* 1999; Matsumoto and Kohda, 2000). For instance, the feeding activities of whitefish (*Coregonus lavaretus*) forms have usually been found to connect with the number of gill rakers (Amundsen *et al.,* 2004).

4.4.5 Variability in respiratory structures

The data presented here on gill filaments showed a positive relationship between body length and gill filament length in brown trout. The growth of specialized respiratory structures frequently relates to body size, to the ecological situations to which the fish species is exposed, and to its phylogeny (Severi *et al.,* 1997). For instance, the gill surface area of ruffe (*Gymnocephalus cernuus*) increased with body weight (Satora and Romek, 2010) and also gill area was usually increased with fish activity, mode of fish respiration and ecological conditions (Severi *et al.,* 1997). The total number of gill filaments in the brown trout (268.3) recorded here is comparable to that found in an earlier study of the same species (*Salmo trutta*) (Chapter 2, Jenjan *et al.,* in preparation. Mean number = 284.0) and is also similar to those seen in other salmonid species, for example, Arctic charr (272.2; Jenjan *et al.,* in preparation).

The current study showed that the length and number of filaments on the different arches decreased from the front to the back gill arches. The variation found in gill filament length and gill filament number along the different gill arches could be related to the head structure and with oxygen levels. Structural and functional limits resolve the model of variability in filament number and length (Palzenberger and Pohla, 1992). Although increased filament number and length result in a larger gill surface area, many long filaments require more space in the fish head, which may be unsuited to a streamlined body shape (Palzenberger and Pohla, 1992). The number of gill filaments and their length are related to the size of the ventral head bone. A similar description of the relationship between gill filament and pharyngeal bone length has been recognized in other fish, including common carp (Jenjan *et al.,* in preparation, chapter 3) and *Salmo trutta* (Crespo *et al.,* 1988).

In the present study, in general, mean filament length for each gill arch increased with fish length, but there were individual differences in the level of filament development. The mean number of filaments on each gill arch found here for brown trout (65.25) is close to the mean filament numbers of the brown trout (69.90) and Arctic charr (66.70; Chapter 2, (Jenjan *et al.,* in preparation). Filament number may be related to the body size and taxonomy rather than to species ecology (Emery and Szczepanski, 1986), (i.e. filament number of gill might be helpful in definite species identifications).

The present study found no significant relationship between secondary lamellae number and spacing and total body length, which are tuned to the life style of the fish concerned and ambient levels of dissolved oxygen (Jenjan *et al* .in preparation). The average number of secondary lamellae per millimetre on both sides of the gill filament increased from the back to the front gill arches. This is revealed both in the univariate statistics and in the results of the principal component analysis, PC2 had a strong front to back gradient. The mean numbers of secondary lamellae per millimetre of gill filament in the brown trout in the present study (40.3) is high in comparison to *Tetrapturus audax (ca* 23; Wegner *et al.,* 2010b) but is low in comparison to other fish species, *Garra lamta* (66.6; Ojha *et al.,*1989), *Synodontis membranaceaus* (64.56; Saliu and Olonire, 2007) and Arctic charr (60.0; Chapter 2, Jenjan *et al.,* in preparation). The average distance between secondary lamellae reported here for brown trout is low (0.03) in comparison for example to *Thunnus sp* (0.06; Muir and Hughes, 1969). The differences in distance between secondary lamellae in different fish species may be related to the mode of fish life (Prasad, 1988). Brown trout have closely spaced secondary lamellae (0.03mm), suggesting an active way of living. In general, active species have many, closely spaced lamellae, while slow moving species such as plaice have fewer more widely spaced lamellae (Hughes, 1966).

The length of the secondary lamellae a key determinant of gill surface area, tends to be species specific and links with the fish mode life (Hughes and Morgan, 1973). Nevertheless, physiological and morphological constraints place limits on variability in this trait (Palzenberger and Pohla, 1992). Increasing secondary lamellae length would undoubtedly increase gill surface area, however it may also have influence on respiratory efficiency of water flow across the gill filaments. Generally, gill surface area is determined by the number and length of the gill filaments and the number and surface area of the secondary lamellae. This again explains why there is a positive relationship between the PCA-derived score of the extensiveness of the respiratory structures (which has positive loadings for secondary lamellae) and total gill areas as estimated using Hughes equation (1984).

4.4.6 Microscopy

In this study, histological analysis of the gills shows that the brown trout has a classic gill structure. Hyperplasia of epithelial cells between secondary lamellae and the presence of mucous cells in the secondary lamellae were found to vary extensively in the gills in individual brown trout. Both hyperplasia and mucus cells can be seen as a response to

stress, principally induced by low water quality. Gill hyperplasia might serve a protective function, leading to a decrease in exposed gill surface area, which would help to prevent transfer of valuable ions out of the body across the gills (in freshwater species) and transfer of harmful substances into the body. Such protection would be gained at the expense of the efficiency of the respiratory function of the gills. The hyperplasia between secondary lamellae may act to separte the gill secondary lamellae allowing them to function as a respiratory structure when fish out of water (Ong *et al.,* 2007). Also this alteration serves as a defence mechanism, leading to decreased respiratory surface and increased blood diffusion distance (Reimscheussel *et al.,* 1991). Therefore, these defensive responses impair the normal physiology of the fish, stopping the normal diffusion of oxygen (Ballesteros *et al.,* 2007).

In general, mucous cell numbers differ among fish species and in relation to environmental condition (Zuchelkowski *et al.,* 1981; Moron *et al.,* 2009). The mucous cells in the secondary lamellae may be related to the mode of respiration of fish. Mucous cells in secondary lamellae increase the water blood barrier for respiratory gas diffusion (Fernandes and Perna-Martins, 2002), and subsequently reduce oxygen uptake and carbon dioxide excretion (Moron *et al.,* 2009). Also mucous on the gills and gill rakers may help in filter food in teleost fish (Shephard, 2009).

4.4.7 Comparison of gill structure in brown trout with different coping strategies

In the current study, significant differences were found in gill morphometrics between brown trout groups with different coping strategies especially in filament length, lamellar length and lamellar number. Therefore brown trout classified as risk-takers had more gill filaments and longer secondary lamellae and consequently tended to have a larger gill area than did risk-avoiders group. Fish classified as intermediate were in between these two extremes. In general, fish gill surface area is related to fish activity and life style, with variability in several feature of gill structure increasing or decreasing gill surface area in relative to need (Wagner *et al.,* 2010a). According to Gray, (1954) and De Jager *et al.* (1977), more active fish have a larger respiratory surface area than do slow moving fish. Generally, as fish activity increases, subsequently gill filament number and length increases as well. Additionally, active fish often have more, closely spaced and larger

secondary lamellae than more sluggish fish (Satora and Romek, 2010). In general, the brown trout in the present study that were classified as risk-takers have a larger respiratory surface than did risk-avoiders, suggesting a more active mode of life that may linked to resting metabolic rate (Huntingford *et al.,* 2010). This agrees with the results of carp reported in chapter 3, but is less clear cut

The present study also found differences between risk-taking groups at the microscopic level. Consequently the risk-avoiding group of brown trout had more mucous cells than did risk-takers group, with intermediate fish again being intermediate this may be seen as validate of the initial behaviour-based classification. Moron *et al.,* (2009) suggest that mucous cells in gill secondary lamellae increase the water blood barrier for flow of respiratory gases, and conseguantly decreases oxygen uptake and carbon dioxide excretion (Sakuragui *et al.,* 2003). This would fit with a lower-energy life style in risk-avoiding fish, but could also reflect greater stress responsiveness in this group.

Gill morphometrics and gill structure in fish represents a compromise between the requirement to get oxygen to support metabolism and other activities, and the require to avoid excessive transfer of ions across the exposed gill surface. It appears that in risk-taking, proactive brown trout the balance is in favour of competent oxygen uptake at the expense of ionic movements across the gills. In contrast, in reactive, risk-avoiding fish, the balance is shifted in favour of a lower rate of oxygen uptake and decreased ionic movements. The need to maintain a large, exposed respiratory surface, with a related increased osmoregulatory pressure, is therefore a hidden cost of an aggressive, proactive life style. This could help to explan how variety maintains variable coping strategies are maintained within brown trout populations even when proactive fish gain special access to valuable resources.

4.5 Conclusions

As is Chapters 2 and 3, counting gill morphometrics by sequenced PCAs produced compound scores that summarise key aspects of variation in respiratory structures in trout, dependent on differences in body size. Trout classified as risk-taking tended to have larger respiratory structures than those classified as risk-avoiders, with the intermediate group lying in between. This is similar but somewhat less clear than is seen in common carp

(Chapter 3). This may be because the initial screening for coping style was less effective; though the greater number of mucous cells in risk-avoiding will support the initial classification. It might also be that in domesticated trout reared at high densities, there is less variability in coping style, most fish tending toward the proactive state.

CHAPTER 5

AN EXPIRiMENTAL STUDY OF THE EFFECTS OF DISSOLVED OXYGEN LEVELS AND TEMPERATURE ON WELFARE INDICATORS IN COMMON CARP HELD AT HIGH DENSiTIES.

This experiment was contucted at Polish Academy of Sciences Institute of Ichthyobiology and Aquacultures, Zaborze, Poland, by Dr. Maciej Pilarczyk who collected the data on body size and condition. Analysis of histological specimens and measurements of secondary lamellae are new and was carried out by Hussein Jenjan in discussion with Dr. Maciej Pilarczyk.

5.1 Introduction

5.1.1 Importance of dissolved oxygen levels.

5.1.1.1 Effects of dissolved oxygen on fish: Dissolved oxygen is possibly the most essential aspect of water quality, being necessary to the metabolism of aquatic organisms (Leclercq *et al.,* 1999). Dissolved oxygen is usually expressed as a percentage of the total amount of oxygen that could be held in solution in the volume water, given current conditions. The term anoxic is used when water is effectively devoid of oxygen, and hypoxic refers to concentrations of oxygen in water below 30%. Generally fish cannot live below 30% oxygen saturation and good aquatic environments should rarely experience dissolved oxygen of less than 80%. On the other hand, unduly high levels of dissolved oxygen, which can occur in nature, may also have adverse effects (Lefèvre *et al.,* 2009). However, for most fish, and particularly those in intensive culture, low oxygen levels are a more common threat. Exposure to low levels of oxygen triggers reversible adaptive responses and excessive or chronic exposure can cause pathological effects. Both of these kinds of effect are considered here.

5.1.1.2 Reversible adaptive responses: The immediate response of a fish to the experience of low oxygen levels may be behavioral, in that fish may avoid these conditions by moving away or escaping. If forced to remain in water with less than optimal oxygen levels, fish show adaptive responses on different time scales. At the behavioral level, they may cease activities such as movement or feeding that have high energy use and hence increase their need for oxygen. For example, both feeding and reproductive activity in fish are reduced at low oxygen levels (Van den Thillart and Van Waarde, 1985; Kramer, 1987). Spawning behavior is reduced and replaced by defensive responses in black crappie (*Pomoxis nigromaculatus*) exposed to dissolved oxygen levels of 2.5 mg/L dissolved oxygen (Carlson and Herman, 1978). When oxygen levels fall to 5-10 mg/L, juvenile paddlefish stop swimming (Burggren and Bemis, 1992). Different individuals of the same species and population may show different behavioral responses to low oxygen. For instance, Van Raaij *et al.,* (1996) found that some rainbow trout responded to low oxygen with active avoidance, while others became inactive. The fish that attempted to escape showed higher levels of plasma catecholamine and were more likely to die than were fish responding to the oxygen stressor by inactivity. Brown trout from different populations differ in their

behavioural and endocrinological responses to hypoxia, possibly as an adaptation to the oxygen regimes at their site of capture (Brelin *et al.,* 2008).

Conductance of oxygen into the blood can be increased by increasing the water flow over the gills (Jensen *et al.,* 1993) and many species of fish are known to response to reduced oxygen by increasing ventilation rate (Kramer, 1987; Robb and Abrahams, 2003), changing the regularity and amplitude of opercular movements with decreasing levels of dissolved oxygen (Doudoroff and Shumway, 1970). For example, Arctic char larvae (*Salvelinus alpinus*) studied by McDonald and McMahon, (1977) exposed to hypoxic conditions (33 mmHg) for 47 days showed higher and more harmonised buccal and opercular movements than did larvae exposed to normoxic conditions (121 mmHg). On a longer timescale, fish can adapt to reduced oxygen levels by developing additional secondary lamellae on their gills thus increasing the functional respiratory area (Johansen, 1982). McDonald and McMahon, (1977) found that Arctic char larvae held in hypoxia for 47 days had fewer gill filaments and secondary lamellae than the normoxic larvae. There are, nevertheless, limits to the increased respiratory surface area attainable. The space between the secondary lamellae is narrow in common carp *(Cyprinus carpio*), the subject of the study described in the present chapter, (about 0.04 mm, Jenjan *et al.,* in preparation) and water will tend to flow past the tips of the gill filaments rather than between them when the respiratory flow rate is high, thus by-passing the gill surfaces. In addition, increased ventilation rate is itself energetically costly and increasing the ventilation rate results in an increased the exposure to toxic materials in the water (Boyd and Tucker, 1998).

Another adaptation to low oxygen levels involves increasing the oxygen carrying capacity of the blood, for example by increasing plasma concentrations of haemoglobin or in the affinity of hemoglobin for dissolved oxygen (Jensen *et al.,* 1993; Robb and Abrahams, 2003). Lebelo *et al.,* 2001) demonstrated that in striped bass fish (*Morone saxatili*) exposure to hypoxic conditions may cause a release of red blood cells from the spleen or an increase production of red blood cells (Lebelo *et al.,* 2001) and consequently higher plasma haemoglobin concentrations (Gallugher and Farrell, 1998). In addition to increases in the capacity for oxygen transport based on high concentration of hemoglobin and increased affinity of hemoglobin for dissolved oxygen, compensation for reduced oxygen tension can take the form of increased cardiac output (Jensen *et al.,* 1993).

Given the various stress-induced changes in blood composition, haematological analyses can give valuable information on the health and condition the wild and cultured fish with hematological indices such as haemoglobin and heamatocrit (the number of red blood cells per unit volume) changing depending on the water condition (Adeyemo *et al.,* 2003). Haematology also helps to detect physiological changes following different stress conditions such as low oxygen levels and diseases (Adeyemo *et al.,* 2003; Dimarco *et al.,* 2011). Thus haematocrit and haemoglobin concentration may vary in response to oxygen levels in fish. For example in fathead minnows (*Pimephales promelas*) and yellow perch (*Perch flavescens*), haemoglobin concentration increased in hypoxic conditions (Robb and Abrahames, 2003). Haemoglobin concentration and haematocrit do not always show similar responses. For example, Marinsky *et al.,* (1990) found that rainbow trout hemoglobin levels were similar at two levels of oxygen (>80% and 40% saturation), but difference in the haematocrit were observed. There are physiological limits to the ability of a fish to respond to low oxygen by increasing total haemoglobin or red blood cells imposed by the speed to production for both of these responses (Wells and Weber, 1991).

5.1.1.3 Longer term pathological responses: Overall, fish health is best when the dissolved oxygen is kept close to saturation, whereas prolonged low levels of dissolved oxygen result in disturbed ecosystems and fish mortality (Thomann and Mueller, 1987). At lower concentrations of dissolved oxygen, respiration and feeding decrease. In addition, when oxygen is low fish are less efficient at assimilating consumed food (Tom, 1998). For example, juvenile catfish (*Ictalurus punctatus*) experienced a decrease in body condition at about 64% dissolved oxygen saturation over a 20-day exposure period (Peterson and Brown-Petrson, 1992). The severity of the effects of exposure to low oxygen levels depends on fish size and age (Robb and Abrahams, 2003), in some species at least (Smale and Rabeni, 1995). For example, adult grayling (*Thymallus thymallus*) are more sensitive to low dissolved oxygen than are fry at the same water temperature (Feldmeth and Eriksen, 1978). According to Tom (1998), requirements of oxygen per unit weight of fish decline significantly with increasing individual weight. Some species are markedly more tolerant of hypoxia than others, leading to differential survival through extended times of hypoxia (Poon *et al.,* 2002).

5.1.2 Effects of water temperature on fish

Environmental temperature is known to affect the structural and functional body systems in ectothermic animals such as fishes, not least because changes in temperature modify the

properties of their enzymes (Klyachko and Ozernyuk, 1998; Le Francxois *et al.,* 2002; Matsumoto and Kohda, 2000; Kausar and Salim, 2006). However, fish are flexible and are often able to display normal activity across a range of temperatures, as cellular processes may be preserved following a period of thermal adaptation (Gerlach *et al.,* 1991). All the same, changes in water temperature affect many processes in fish, with implications for their distribution, growth, reproduction and survival, among other functions (Aune *et al.,* 1997; Adeyemo *et al.,* 2003). Consequently, water temperature is another critical ecological variable for fish and each species has a defined range of temperature within which it can survive (Gadowaski and Caddell, 1991) and a smaller optimal range within which it can flourish (Beschta *et al*., 1987). In nature and in fish culture, changes in water temperature occur regularly, for example with the night-day cycle and as the seasons change, but irregular short term changes, for example due to immediate weather conditions, are superimposed on such regular cycles. The normal range of temperature to which tropical fish in general fish are adapted is between 22-35 °C (Howerton, 2001) and for common carp is at 28-32 °C (Wilson and Taylor, 1993). Through winter, temperature falls, therefore influencing biological fish body functions. For example, *Labeo rohita* is an important freshwater fish cultured in Asia, therefore, growth rate of this fish decreased through the low temperature of water period (Das *et al.,* 2005).

When fish encounter unfavourable temperatures, they may avoid this by moving into more favourable areas (Adeyemo *et al.,* 2003). High water temperatures increase the metabolic rates of fish, resulting in increased demand of food. Physiological responses to long term changes in temperature may compensate, completely or partially, for the effects of temperature on body function (Aho and Vornanen, 1999; Tiitu and Vornanen, 2002). Donaldson *et al.* (2008) found that rapid increases in water temperature could result in behavioural and sub-lethal physiological responses. In warmer water, fish such as silver strip (*Spratelloides gracilis*) grow faster but have a shorter life span than those in cool water (Durieux *et al.,* 2009).

Stress induced by low temperature can result in higher plasma cortisol levels (Donaldson *et al.,* 2008). For example, Tanck *et al.,* (2000) found that cortisol levels were increased with temperature decreases in common carp. Cortisol itself induces several stress-related changes that can affect fish growth rate and biological and chemical body function (Adeyemo *et al.,* 2003). For example, growth rate of *Hippoglossues hipoglossus* depends on water temperature. Lermen *et al.,* (2004) found that fatty acid levels in silver catfish (*Ramdia quelen*) decreased at high temperature (31 ^{o}C), but lactate and glucose levels

increased at high temperature. High levels of blood glucose could be indicative of metabolic activity, as well as an index of sub-lethal stress. A small increase in cortisol can have positive effects on fish; for example, by promoting glucogenesis cortisol secretion generates a rapid increase in available energy, which may promote survival (Rottmann *et al.,* 1992), maintaining homeostasis in the fish´s body (Stein-Behrens and Sapolisky, 1992) and allowing body function to return to normal following a stressful event (Mommsen *et al.,* 1999).

5.1.3 Effects of oxygen and temperature in common carp

The common carp, an important commercial species with a global distribution (Zhou *et al.,* 2000), is exposed to a wide variety of ambient oxygen levels as a result of their geographic and environmental distribution. This species is often found in the wild living in shallow hypoxic waters (Scott and Crossman, 1973, Zhou *et al.,* 2000), particularly in summer when aquatic plant respiration at night can be high (Beamish, 1964). Therefore common carp might be exposed in the wild to hypoxic environments on both a daily and a seasonal basis, over a wide variety of temperatures.

The common carp is a robust, tolerant species that can survive hypoxia and even anoxia for periods ranging from hours to days (van den Thillart and van Waarde, 1985). At low temperature carp begin feeding at about 8-10°C and the best temperatures for the common carp growth are between 20-25°C (Horvath *et al.,* 1992). Carp can tolerate temperatures of about 4^{o}C by reducing their metabolism to its minimum level (Horvath *et al.,* 1992) and can stay alive in cold water and at low oxygen levels (0.3-0.5 mg.l $^{-}$1) (Flajšhans and Hulata, 1992). Van Raaij *et al*., (1996) have shown that individual carp vary in their ability to survive at low oxygen conditions.

Physiological changes in common carp exposed to low temperatures have been studied. For example, blood cortisol and glucose levels showed significant increases in common carp after exposure to 4^{o}C (Sun and Liang, 2004). However, lactate levels were significantly decreased in common carp after exposure to 4°C-8^{o}C (Sun and Laing, 2004). These fish have a number of biochemical mechanisms that allow them to survive at low oxygen levels (Hochachka and Somero, 1984). Carp tissues can hold great reserves of glycogen and a few additional metabolic adaptations are in addition well developed, depression of metabolic rate at low oxygen levels being one of the most important (Storey and Storey, 2004). For example, Zhou *et al.,* (2002) found that, the common carp is able to

preserve constant level of muscle glycogen, muscle ATP and liver citrate synthase during a 168-hour experimental period. Changes in the activities of liver lactate dehydrogenase and muscle cytochrome c oxidase were only observed at 168 hours, indicating that these fish can change metabolic pathways in response to long-term hypoxia exposure. They also found that oxygen consumption was decreased under hypoxia, but returned to normoxic levels within 2 hours upon return to normoxic situation.

In general, these results indicate that carp adopt different strategies in a challenge to deal with short term and long term hypoxia in the natural environment.

5.1.4 Coping strategy and respiratory physiology in common carp

As discussion in Chapters 3 and 4, individuals of many species show consistent differences in their physiological and behavioural responses to stress; in other words, they show different stress coping styles (Korte *et al*., 2005). Several aspects of behaviour vary with coping style, including risk-taking and aggression (Silva**.** *et al.,* 2010). Although coping styles have been described best in mammals, they have been reported in several species of fish (Huntingford *et al.,* 2011). For example, in common carp variation in risk-taking in a potentially dangerous environment is likely to differences in competitive ability and to the expression of cortisol receptors in the head kidney and brain (Huntingford *et al.,* 2010). Additionally, in common carp higher resting metabolic rates are found in fish with a proactive coping strategy compared to those with a reactive strategy (Huntingford *et al.,* 2010). In teleost fish, gill function and structure are correlated with activity levels (Severi *et al.,* 1997). In addition, the study described in Chapter 3 of this thesis showed that proactive carp have a larger area of exposed gill (see also Satora and Romek, 2010). These various differences suggest that proactive and reactive carp might respond differently to stress in the form of low oxygen levels associated with high temperatures. For this reason, stress coping style was assessed in the fish used in the present study before they were exposed to the experimental regimes.

5.1.5 Aims of the present study

With this background, the aims of the study described in this chapter were:

- To collect information on a range of potential welfare indicators, including gill status, in common carp held under experimental conditions with varying levels of oxygen and temperature.
- To examine the relationships among these variables.
- To compare the range of welfare indicators in carp with different coping strategies held in mixed groups at two temperatures and three oxygen levels.

5.2 Materials and methods

5.2.1 Subjects

Approximately 700 one year old common carp (mean weight 24gm) were collected from winter ponds at the Polish Academy of Sciences' Institute of Ichthyobiology and Aquaculture, Zaborze, Poland in April 2006. The fish were treated for infection using 0.05% $KMnO_4$ and oxytetracyline and stored in groups of about 70 in ten 35 litre tanks with filtered, recirculating water (10% water replacement per day). After settling for one week, fish were deprived of food for a minimum of 12 hours and tested for coping strategy.

5.2.2 Screening for coping strategy

Fish were screened for coping strategy using the rate at which they explored an unfamiliar-potentially-dangerous environment (Huntingford and Coyle, 2007). This has been identified to be a reliable feature in carp that is predictive of behaviour in other contexts and also of metabolic and stress physiology (Huntingford *et al*. 2010). As carp are strongly schooling fish and become very stressed in isolation, they were screened in small groups.
Ten fish (randomly-selected) were removed from their holding tank in covered containers and moved quietly into a settling area at one corner of a well-lit tank (1.5 x 1m). The settling area consisted of a covered circular thick black compartment (diameter 50 cm) fitted at the bottom with a closeable exit tube (diameter 7.5 cm). A sheltered area at the opposite end of the main tank was connected by a closable entrance to a fish collection area. The fish were allowed to settle for 10 minutes, during which a small quantity of an extract made by soaking food pellets in water was tipped into the test section in front of the outlet tube. The cover of the outlet tube was removed and a two phase observation period started. After the first three fish had come out from the settling area, or after a time of 10 minutes if less than three fish emerged during this time, the outlet tube was closed and the fish that had emerged were gently confined in the fish collection area. These fish were

classified as the fast-emerging, proactive group. A second small amount of feed extract was added in front of the outlet tube, which was then opened, and a second recording time started until an additional four fish had emerged (or 15 minutes had elapsed. and the outlet tube was closed once more. These fish were classified as showing an intermediate coping strategy. The three fish remaining in the starting shelter were confined in the shelter by replacing the cover and classified as reactive. Following screening, reactive and proactive fish were weighed (gm) measured (mm) and were given a batch mark on the side of the tail using a panjet marker with alcian blue dye under anesthesia using 0.2% water solution of Propiscin, IRS, Zabieniec, Poland, agent based on etomidate. The intermediate fish were unused.

5.2.3 Experimental regimes

Fish from the proactive and reactive groups were used in a two way design, with three different concentrations of oxygen and two different water temperatures. Concentrations of oxygen matched to levels in rearing ponds at times of low, normal and high oxygen content (3-4, 5-6 and 7-8 mg O_2 L^{1-} respectively). Water temperatures conditions were matched to normal (20°C) and high (25°C) temperatures in the summer period. Each treatment was triplicated and 10 proactive and 10 reactive fish (size matched as far as possible) were held together in each tank. Carp were fed 2mm diameter fish feed pellets (Aller Aqua, Denmark) at a rate of roughly 2% of initial fish biomass per day.

5.2.4 Data collection

After 69 days, all carp from each tank were anesthetized, weighed (g) and the body and head length (mm) were measured. Immediately after the fish were killed, blood samples were collected from the caudal vein. For 5 proactive fish from each tank, total haemoglobin concentration was measured according to a modified method described by Tentori and Salvati, (1981). Drabkin's reagent (1 ml) was mixed with 0.5 ml of saponin (0.05 %) and 20 ml of haemolysate (product of haemolysis). For haematocrit, blood samples were collected into heparanized haematocrit tubes and centrifuged immediately and haematocrit % was measured.

The second gill arch from the right side was dissected out and preserved in 4% buffered (pH 7.0) formaldehyde for light microscopy. Tissues for histological analysis were

embedded in wax following standard procedures. Wax embedded tissues were sectioned (thickness of section = 5 μm) and stained with haematoxylin and eosin, according to method outlined in Clark (1981). Figure 5.1 shows a representative section.

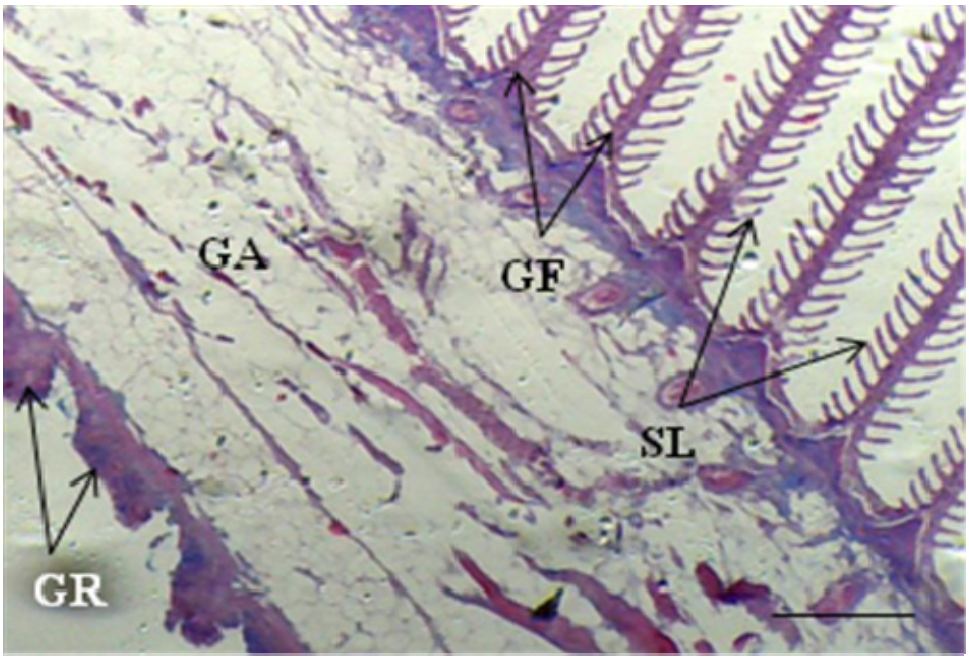


Figure 5.1. Light micrograph of one gill arch show gill arch (GA), gill filament (GF), gill secondary lamellae (SL) and gill raker (GR), bar = 0.14mm (H,E and PAS stain and 100X magnification) .

For every fifth gill filament at the tip, middle and base, nine secondary lamellae were measured to give the number of secondary lamellae per millimetre of filament, the height of secondary lamellae and the distance between secondary lamellae. The number of mucous cells per millimetre of lamellar surface, the height of interlamellae cells (as a percentage of lamellae height) and the presence of clavate lamellae (defined as lamellae with an accumulation of epithelial cells at its tip) were also recorded on about 20 different gill filaments. Mucous cell number, height of interlamellae cells and number of clavate lamellae were measured for nine gill lamellae at the tip, middle and base of every fifth gill filament and mean value were calculated.

5.2.5 Statistical analysis

The data were assessed for normality and parametric or non-parametric testing carried out as appropriate. The condition factor of each fish (K) was estimated from the relationship between weight and length as follows:

$$K = W / L^3$$

where W and L are weight (g) and length (mm). The relationship between morphometric variables and body length were examined by regression analysis (Table 5.1). Only head length was significantly related to body length and for further analysis of relative head length, the residuals of this relationship were used, providing a measurement of head length independent of body length. The relationships among the various welfare indicators were examined using correlation analysis.

Table 5.1. Regression analysis of morphometric variables on total body length (cm). Bold indicate significant relationships

Variable	Regression equation	F	P	R^2 %
Head length (cm0	Y = 0.92 + 0.21 X	68.77	**<0.001**	78.2
Lamellar height (mm)	Y = 0.02 + 0.01 X	1.67	0.206	1.1
Lamellar number per mm	Y = 52.5 + 0.15 X	0.00	0.963	0.0
Lamellar distance (mm)	Y = 0.08 - 0.00 X	0.56	0.462	0.0

To avoid pseudo-replication, the effects of coping strategy were examined using the mean values for the proactive and reactive fish in each tank. Welfare indicators for proactive and reactive fish were compared using t-tests paired within tanks and interactions between environmental conditions and coping strategy were analysed using 2 way analysis of variance on the difference between the mean per-tank scores for proactive and reactive fish. An effect of temperature was found for two variables and these were scrutinised with a two ways anova by temperature and coping strategy. Since few statistically significant effects were found, coping strategy was ignored in subsequent investigation of the interacting effects of oxygen and temperature, for which a two-way analysis of variance on tank means was used. All analyses were carried out using MINITAB 16.

5.3 Results

5.3.1 Relationship among welfare indicators

Table 5.2 shows the means (± SE mean) for all measured variables for this sample of common carp, together with their coefficients of variation. Percentage hyperplasia, the number of mucus cells and clavate lamellae and the distance between the secondary lamellae were particularly variable.

Table 5.2. Mean and standard error of the mean (± SE) and coefficient of variable for variables measured at the end of the study.

Variable	Mean ± SE Mean	Coefficient of variation (%)
Body weight (g)	71.97 ± 1.670	14
Body length (cm)	15.14 ± 0.107	4
Head length (cm)	3.51 ± 0.026	4
Condition factor	2.01 ± 0.019	5
Haematocrit (%)	42.34 ± 0.455	6
Haemaglobin (g/dl)	9.88 ± 0.150	9
Hyperplasia %	6.62 ± 2.420	200
Mucus cell number/mm	0.13 ± 0.043	192
Clavate lamella number/mm	0.24 ± 0.080	183
Lamellar height (mm)	0.09 ± 0.003	22
Lamellar number	54.29 ± 1.840	19
Lamellar distance	0.03 ± 0.003	170

Table 5.3 shows the matrix of correlations between the various welfare indicators, corrected for fish body length as appropriate. For most variables, there were no significant correlations, indicating that these measures were varying independently. However, haemoglobin and haematocrit levels were positive related. Condition factor was positively related to haematocrit but negatively related to secondary lamellae length.

Table 5.3. Matrix of product-moment correlations between the various potential welfare indicators across fish. Top number in each cell = correlation coefficient. Bottom number = significance level.

	Hyperplasia (%)	Mucus cell number	Cleavage lamella number	2ndry lamella length	2ndry lamellae number	2ndry lamella distance	Haematocrit (%)	Hb
Mucus cell number	-0.103 0.588							
Clavate lamellar umber	0.085 0.657	-0.207 0.232						
Lamellar length	0.063 0.740	-0.079 0.668	-0.187 0.305					
Lamellar number	0.143 0.458	-0.193 0.291	0.081 0.660	-0.257 0.179				
Lamella distance	-0.049 0.808	-0.070 0.702	0.201 0.269	0.183 0.342	-0.156 0.420			
Haematocrit (%)	-0.270 0.149	-0.014 0.936	0.093 0.593	-0.058 0.753	0.050 0.740	-0.058 0.749		
Hb	-0.271 0.148	0.193 0.267	-0.151 0.388	0.061 0.741	-0.069 0.705	0.127 0.480	***0.404*** ***0.015***	
Condition factor	-0.148 0.435	0.053 0.762	0.089 0.611	-0.447 0.010	0.169 0.354	-0.045 0.802	***0.464*** ***0.004***	0.274 0.106

5.3.2 Effect of coping strategy

Table 5.4a shows mean values (±SE) for all the measured variables for proactive and reactive fish, together with the results of paired t tests. Proactive fish were significantly larger than reactive fish, but there were no significant differences proactive and reactive

fish for any of the welfare variables. Table 5.4b shows the results of two-way analysis of variance on the differences between proactive and reactive fish in relation to temperature and oxygen levels. For haematocrit and secondary lamella number only, temperature had a significant effect on the relative scores of reactive and proactive fish. In the case of haematocrit, scores were higher in reactive fish at the higher temperature (mean difference score $\pm$ SE = 2.5 $\pm$ 1.0), but this was reversed at the lower temperature (mean difference score $\pm$ SE = -1.3 $\pm$ 0.93). At the higher temperature reactive fish had strikingly more secondary lamellae than did proactive fish (mean difference score $\pm$ SE = -12.03 $\pm$ 9.3), but the converse was the case at the lower temperature (mean difference score $\pm$ SE = 26.31 $\pm$ 9.3).

Table 5.4a. Mean ($\pm$ SE) welfare indicators for proactive and reactive carp, with the results of pairwise T tests at the tank level. b. Results of two-way analysis of variance of the difference between reactive and proactive carp in relation to temperature and oxygen levels.

a) Variable	**Proactive**	**Reactive**	T	**P**
Body weight	75.79 ±2.9	68.85 ±1.6	2.12	***0.05***
Body length	15.34 ± 0.18	14.9 ± 0.10	2.25	***0.04***
Head length	3.55±0.04	3.46± 0.03	1.64	0.12
Condition factor	2.01±0.03	2.00±0.03	0.12	0.99
Haemoglobin	10.2 ± 0.23	9.6 ± 0.18	1.78	0.09
Haematocrit (%)	42.0 ± 0.63	42.4 ± 0.81	0.5	0.62
Hyperplasia %	6.17±3.67	2.73 ± 1.93	0.81	0.44
Mucus cell number	0.18± 0.07	0.09± 0.05	1.00	0.33
Clavate lamella number	0.21 ± 0.08	0.29 ± 0.14	0.72	0.48
Lamellar height	0.08 ± 0.005	0.09 ±0.005	1.06	0.31
Lamellar number	52.78 ± 2.4	55.60± 2.8	-0.78	0.44
Lamellar distance	0.03 ± 0.005	0.03 ±0.004	0.53	0.60

b) Variable	**Temperature**		**Oxygen**		**Temperature * Oxygen**	
	$F_{1,31}$	P	**$F_{1,31}$**	P	**$F_{1,31}$**	P
Body length	0.82	0.39	0.29	0.77	0.42	0.67
Head length	0.69	0.43	0.18	0.84	0.41	0.67
Condition factor	0.07	0.80	0.03	0.97	0.21	0.81
Haemoglobin	0.00	0.95	0.52	0.61	0.85	0.45
Haematocrit (%)	7.48	**0.02**	3.17	*0.08*	2.21	0.16
Hyperplasia %	0.44	0.53	1.38	0,32	1.39	0.32
Mucus cell number	2.63	0.13	0.02	0.98	0.50	0.62
Clavate lamella number	0.54	0.48	0.26	0.14	1.19	0.34
lamellar height	0.09	0.77	0.10	0.91	0.11	0.90
Lamellar number	8.47	**0.02**	1.34	0.31	0.24	0.79
Lamellar distance	0.16	0.70	0.04	0.96	0.41	0.68

5.3.3 Effects of body size

Table 5.5 shows the relationship between various welfare indicators and body size. A significant positive relationship was found for haemoglobin and a significant negative relationship for mucus cell number. Thus carp that were large at the end of the experiment had relatively higher plasma haemoglobin concentrations and fewer mucus cells/mm than did smaller fish.

Table 5.5. Regression analysis of measured welfare variables against body length in common carp. Bold indicate significant relationships.

Variable	Regression equation	F	P	R^2 %
Haemoglobin	Y = 2.44 + 0.60 X	6.35	**0.017**	15.7
Haematocrit (%)	Y = 29.7 + 1.03 X	4.9	0.193	4.9
Hyperplasia %	Y = 110 - 6.85 X	3.34	0.078	8.7
Mucus cell number	Y = 2.17 - 0.14 X	4.27	**0.047**	11.5
Clavate lamella number	Y = 1.87 - 0.11 X	0.79	0.379	1.7
Lamellar height	Y = 0.02 + 0.01 X	1.67	0.206	1.1
Lamellar number	Y = 52.5 + 0.15 X	0.00	0.963	0.00
Lamellar distance	Y = 0.08 - 0.00 X	0.01	0.931	0.00

5.3.4 Effects of environmental conditions

Since there were so few effects of coping strategy, this factor was ignored in subsequent analyses. Table 5.6 summaries the results of two way analysis of variance by temperature and oxygen regardless of coping strategy. It also shows that the results of a two way analysis by temperature and coping strategy for the two variables where there was an effect of temperature on the relative scores of proactive and reactive fish (haematocrit and lamellar number, see above).

Table 5.6a. Results of two way analysis of variance of various potential welfare indicators by temperature and oxygen. b. Results of two-way analysis of variance of haematocrit and lamellar number in relation to temperature and coping strategy. Bold indicate significant relationships Bold indicate significant relationships.

a) Variable	Temperature		Oxygen		Temperature * Oxygen	
	$F_{1,31}$	P	$F_{1,31}$	P	$F_{1,31}$	P
Body weight	0.50	0.48	**3.83**	**0.03**	0.13	0.88
Body length	6.46	**0.02**	5.28	**0.01**	0.33	0.72
Relative head length	5.25	**0.03**	3.96	**0.03**	0.37	0.70
Condition factor	15.35	**<0.001**	1.87	0.17	7.63	**0.002**
Haemoglobin	0.02	0.90	0.03	0.97	1.86	0.17
Haematocrit (%)	0.61	0.70	0.92	0.41	1.24	0.31
Hyperplasia %	8.09	**0.01**	2.87	0.08	1.04	0.37
Mucus cell number	5.57	**0.03**	1.57	0.23	0.45	0.64
Clavate lamella number	3.08	0.09	0.72	0.50	2.10	0.14
2^{ndy} lamella height	4.04	0.06	0.04	0.96	0.08	0.93
2^{ndy} lamella number	1.55	***0.22***	0.16	0.86	0.73	0.49
2^{ndy} lamella distance	0.21	0.65	0.30	0.75	0.47	0.63

b) Variable	Temperature		Coping strategy		Temperature * Coping strategy	
	$F_{1,31}$	P	$F_{1,31}$	P	$F_{1,31}$	P
Haematocrit (%)	2.74	0.11	0.47	0.50	4.35	0.05
2^{ndy} lamella number	1.48	0.23	0.58	0.45	5.42	0.03

There were significant temperature effects for body length, condition factor, relative head length, percentage hyperplasia, mucous cell number and, marginally, for secondary lamellae height. Figure 5.2 shows the effects of temperature for those significant variables for which there was no interaction between oxygen and temperature. Fish held at the higher temperature were longer and had relatively larger heads and longer secondary lamellae, but fewer mucous cells and a lower percentage of hyperplasia, than those held at the lower temperature.

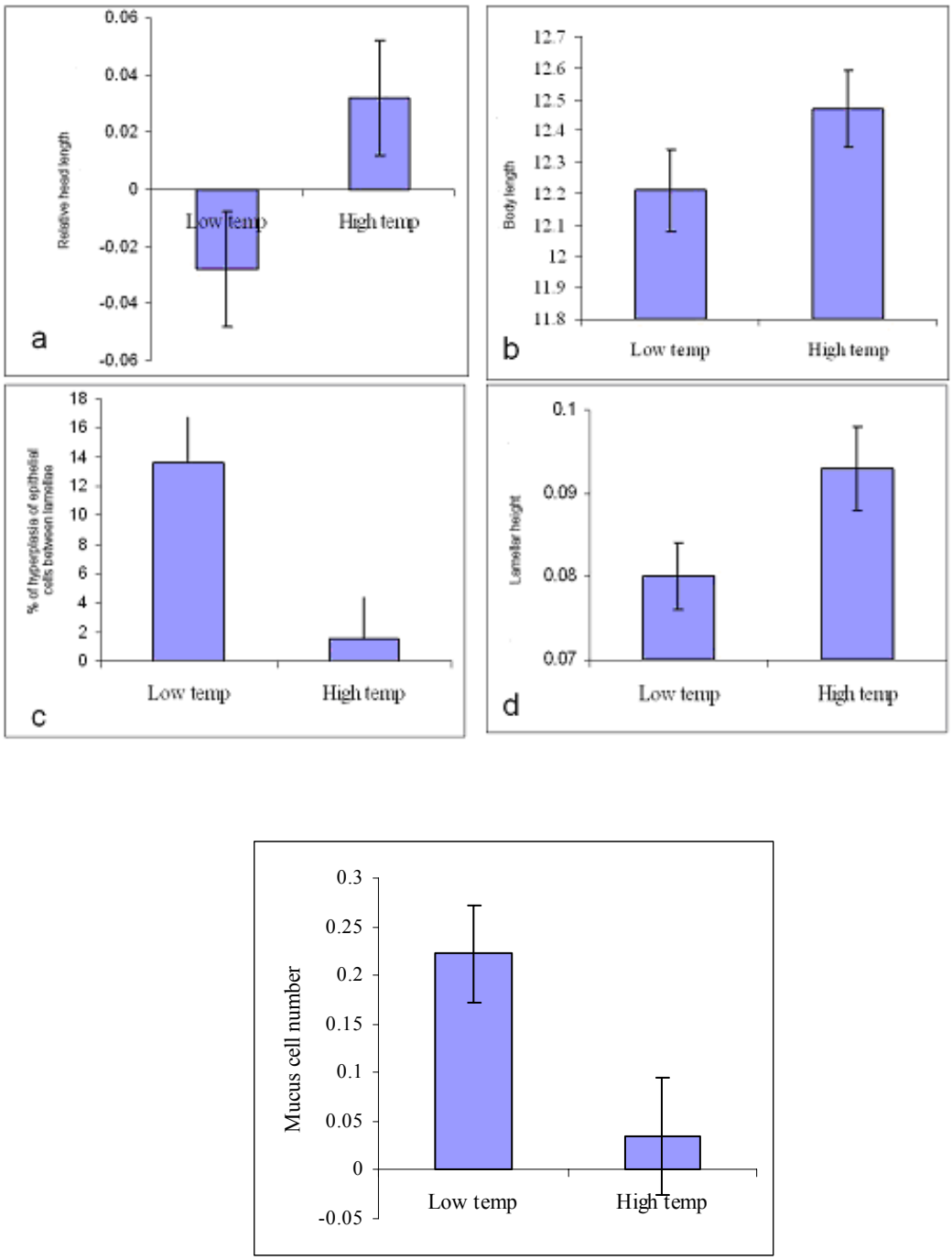


Figure 5.2. Mean (±SE) for those variables for which there was a significant temperature effect and no interaction. a) Body length (cm). b) relative head length c) percentage hyperplasia, d) mean lamella height and e) mucous cell number.

There were significant effects of oxygen concentration on body weight and length and relative head length. Figure 5.3 shows the effects of oxygen temperature for these significant variables. Fish held at the lowest oxygen levels were smaller and lighter than those held in normoxic and hyperoxic conditions and also had relatively larger heads and a higher degree of hyperplasia.

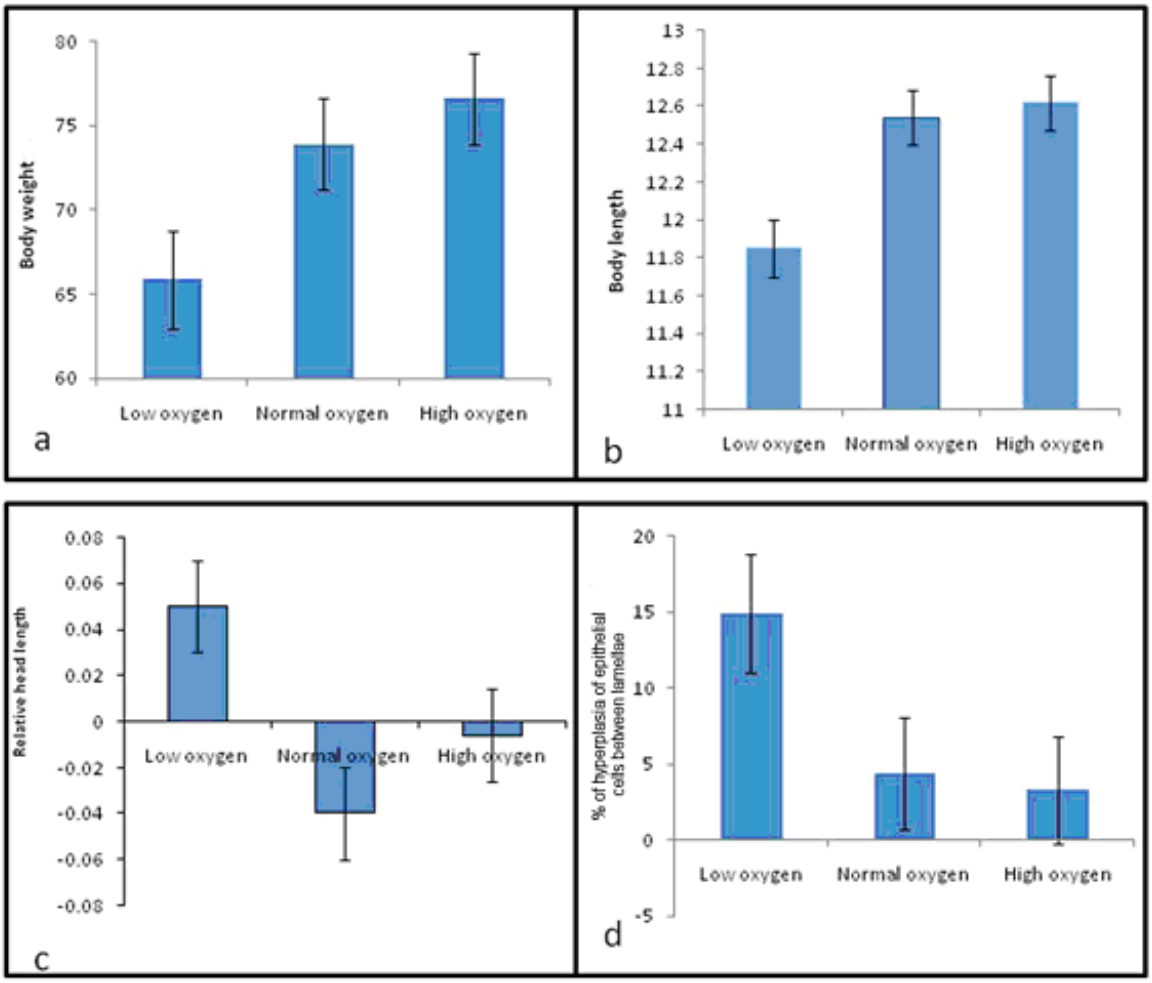


Figure 5.3. Mean (±SE) for those variables for which there was a significant oxygen effect and no interaction. a) body weight, b) body length, c) relative head length and d) percentage hyperplasia.

For condition factor there was a significant temperature effect and also a significant interaction between the effects of temperature and oxygen (Figure 5.4). At the lower temperature, condition factor increased with increasing oxygen levels, but at the higher temperature, condition factor was lowest in fish held at the high oxygen level.

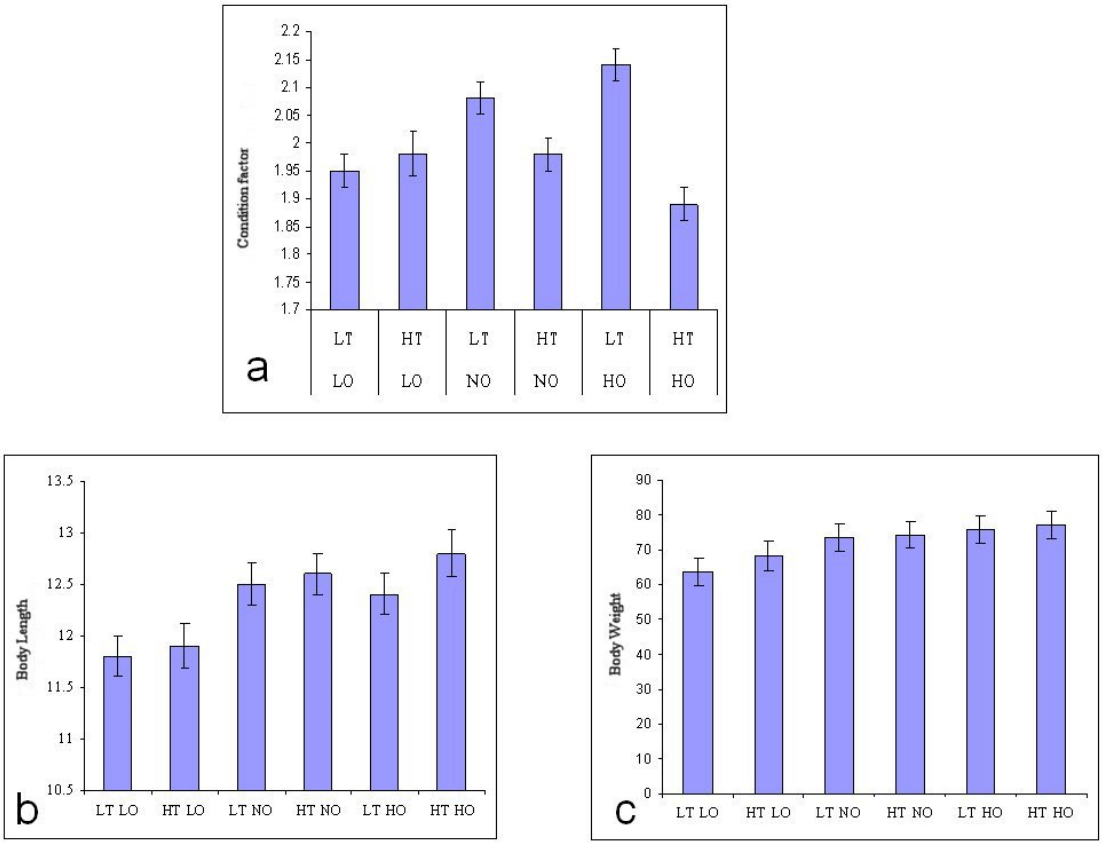


Figure 5.4. Mean (±SE) a) condition factor, b) body length and c) body weight in all six temperature and oxygen conditions.

Two ways ANOVA by temperature and coping strategy (Table 5.6b) showed no significant main effects, but a significant interaction between temperature and coping strategy for both variables. Haematocrit scores in proactive fish were higher at 20°C than at 25°C (Figure 5.5a), whereas reactive fish had intermediate scores at both temperatures. Reactive fish had markedly more secondary lamellae at 20°C (Figure 5.4b), while proactive fish had slightly more lamellae at 25°C.

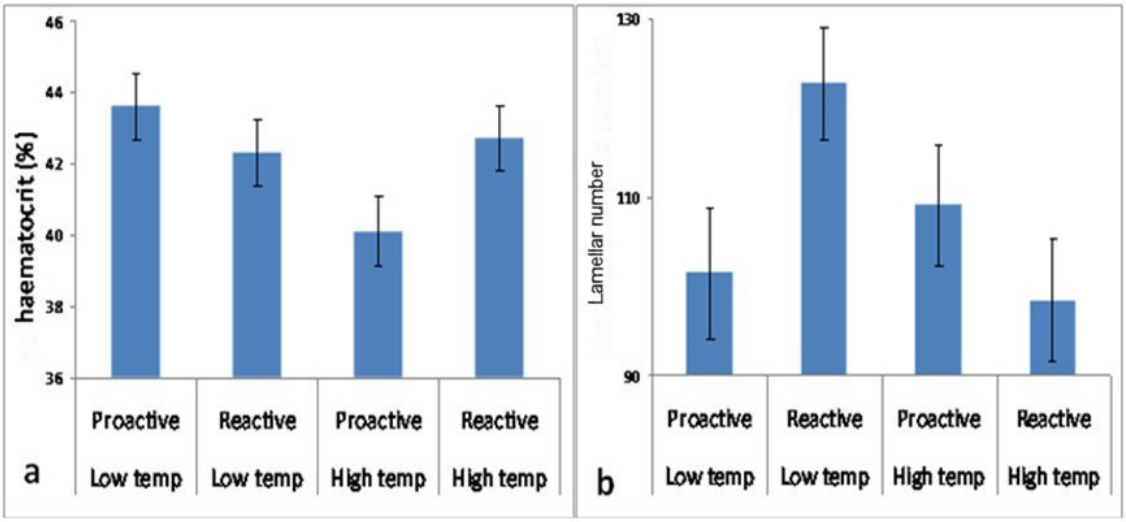


Figure 5.5. Mean (±SE) scores in reactive and proactive carp held at the higher and lower temperature for a) haematocrit and b) lamellar number.

5.4 Discussion

5.4.1 Welfare indicators

The objectives of this study were to collect information on a range of potential welfare indicators, together with gill condition, in common carp exposed to experimental oxygen and temperature levels, to observe the relationships between these variables and to compare the variety of welfare indicators in common carp with different coping strategies exposed to two temperatures and three oxygen levels.

Considering the various potential welfare indicators, there was moderate level of variability (coefficient of variation in the range of 4 to 22%), however four of the gill measurements (percentage hyperplasia, the number of mucus cells and clavate lamellae and the distance between the secondary lamellae) were particularly variable (coefficient of variation more than 100%), suggesting that these are particularly sensitive to the environmental challenges imposed on the subjects of the present study. Looking at the relationships between the various indicators, haemoglobin and haematocrit levels were positive related, suggesting that, over the range of conditions studied, total haemoglobin concentration changes are the result of changes in red blood cells number. Overall, however, there were few significant correlations between the indicators, which to a large extent varied independently in the present data set.

5.4.2 Effects of temperature and oxygen regime on growth and condition

In general and across all conditions there were no differences between carp classified as proactive and reactive on the basis of the novel environment test used in the present study. The major effects reported in this study therefore involved temperature, oxygen levels and, for one variable, an interaction between these two effects. Thus, fish held at the higher temperature were longer and heavier than those at the lower temperature. Since fish in the different experimental groups were the same size at the start of the study, the carp had been growing faster at 25^{o}C. These same two variables were also influenced by oxygen levels, with fish held at the lowest oxygen level being smaller and lighter than those from the other two conditions. Weight and length were not affected equally by the experimental manipulations, with the result that the condition factor increased with increasing oxygen

levels at 20 ^{O}C, but at 25 ^{O}C was lowest in fish held at the highest oxygen level. Fish at 20 ^{O}C had higher condition scores than did fish at 25 ^{O}C. These data suggest that the ratio between weight and length in common carp is influenced by water temperature. Fish at high temperature might stop feeding for some days, leading to weight loss until usual feeding has started again (Elliott and Hurley, 1999). Effects of temperature and oxygen on rates of growth have been reported for many other species of fish (Robb and Abrahams, 2003; Kausar and Salim, 2006). These results are agreement with those for salmon (*Salmo salar*; Seymour *et al.,* 1992), Pollack (*Pollachius pollachius*; Rug *et al.,* 2006) and bull trout (*Salvelinus confluentus*) (Selong *et al.,* 2001). For example, the growth rate of bull trout (*Salvelinus confluentus*) decreased sharply at temperature of more than 15.4^{O}C and the lowest growth rate was observed at 20^{o}C (Selong *et al.,* 2001).

5.4.3 Effects of oxygen and temperature on gills and related structures

In addition to effects on overall growth, both temperature and oxygen levels had an effect on relative head length, with relatively larger heads being observed in fish held at the higher temperature and in those held in hypoxic conditions. Theoretically, there are several possible reasons why head length might be larger in fish held at low oxygen levels. For example, they may be related to adaptive increased gill surface area, giving the gills more free space inside the head, increasing gill cavity volume and/or increasing buccal cavity volume (Smits, 1996).

Thus the relatively larger heads seen in fish held under low oxygen could reflect the increased space required for a large gill apparatus. This fits with the fact that in the present study carp held the higher temperature had somewhat longer secondary lamellae than those held at the lower temperature, indicating an increase in the capacity for uptake of oxygen. At the species level, fish that live in well oxygenated water have larger gill apparatus than the same size and species living in hypoxic water (Schaack and Chapman, 2003). Similar observations have been reported in African cichlid fish collected from hypoxic water (Chapman *et al.,* 2008), larger head length being recorded in groups from hypoxic water. Herbing *et al.,* (2000) found that larval Atlantic cod (*Gadus morhua*) at 10^{o}C had longer and more numerous gill filaments and secondary lamellae compared to fish held at 5^{o}C. In addition, in the same species, growth of the operculum bone (gill cover) correlated with the gill development (Herbing *et al.,* 2001). Again, such increases in head size in response to

low oxygen levels and high temperature may serve to increase in buccal cavity volume (Nilsson, 2007).

In the present study, rather few effects of oxygen and temperature on secondary lamellae structure were found, suggesting that these features of the respiratory structures represent permanent adaptations to the life style of the fish concerned. The number of secondary lamellae in common carp used in the present study (approximately 54/mm) is high in comparison to those reported in other studies, including Chapter 3 of this thesis in which mean lamellae number was approximately 37/mm (Jenjan *et al.,* in preparation). This may be because the carp used in the study were larger (mean weight = 24g) than those used in the study described in Chapter 3 (mean weight = 10.11g). However, secondary lamellae number is low in comparison to other fish species, yellowtail *Seriola quinqueradiata* (mean number = approximately 72/mm; Kobayshi *et al.,* 1988) and Arctic charr (mean number = approx 60/mm, (Jenjan *et al.,* in preparation, in chapter 2). Differences in the number of lamellae are reflected, inversely in differences in the spacing between them. The average distance between secondary lamellae in common carp is low (0.03mm) in comparison for example to *Mugil cephalus sp* (*ca.* 0.05; Bhagwant and Elahee, 2000) and *Clarias anguillaris* (0.04; Saliu and Olonine, 2007) and high in comparison for instance to *Chrysichthys longifilis* (0.02; Saliu and Olonine, 2007). The differences in distance between secondary lamellae in different fish species could be related to environmental niche (Prasad, 1988). The closely spaced lamellae of common carp suggest an active mode of living (Hughes, 1966); in general, active species such as Arctic charr have closely spaced lamellae (0.04-0.05 mm; Chapter 2. Jenjan *et al*., in prep), while slow moving species such as plaice (*Pleuronectes platessa*) have more widely spaced lamellae (0.077mm; De Silva, 1974). The height of the secondary lamellae also tends to be related to the mode of life concerned (Hughes and Morgan, 1973). However, physiological and morphological constraints limit the possible range for this variable (Palzenberger and Pohla, 1992). Larger secondary lamellae would definitely mean an increase in gill surface area per mm^2, but would also increase resistance to water flow across the gill filaments. Generally, gill surface area is determined by the number and length of the gill filaments and the number and surface area of the secondary lamellae.

In the present study, carp held at the higher temperature had a lower percentage hyperplasia and fewer mucous cells than those held at the lower temperature, while those held at the lowest oxygen levels had a higher degree of hyperplasia. Reduced hyperplasia at the higher temperature could be a response to higher oxygen requirements at 25 ^{o}C

compared with 20 ^{o}C. The increase in hyperplasia explain at the lowest oxygen levels is harder to explain, but might represent a stress response. Generally, the number of mucous cells in the secondary lamellae varies both with species and with environmental conditions (Moron *et al.,* 2009). More mucous cell in the gill lamellae results in a thicker layer of mucous over the gills and hence a greater barrier between water and blood for the diffusion of respiratory gases (Fernandes *et al.,* 1998). More mucous cells in the gill lamellae of carp exposed to lower temperature may be a response to ionic loss. If respiratory capacity exceeds need at low temperature, one potential value in having a thicker mucous layer on the gills would be to reduce loss of ions to the hypotonic surrounding fresh water.

In general, the present results have shown that common carp have the capacity to remodel their gills in response to oxygen and temperature regimes, by changing the percentage of the epithelial cells between the secondary lamellae and by producing more mucus cells, resulting in changes in functional respiratory surface area. Such observations are comparable to other studies conducted on different fish for example, crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*) which remodel their gills in response to oxygen levels and water temperature (Sollid *et al.,* 2005). The gill surface area was reduced by development of interlamellae cells

5.4.4 Effects of oxygen and temperature on blood respiratory variables

The present study found no effects of experimental temperature or oxygen regimes on haematocrit and haemaglobin levels. This is in contrast to previous studies that have reported a decrease in these variables in common carp. For example, the haemoglobin and haematocrit in catfish (*Clarias gariepinus*) studied by Adeyemo *et al.,* (2003) were decreased when fish were transferred from low temperature (29 ^{O}C) to high temperature (41 ^{O}C). It is recognized that a decreased numbers of red blood cells and lower haemoglobin level result in lower oxygen carrying capacity. As well as the transport of oxygen, erythrocytes have other functional responsibilities in the body, a deficient quantity and quality of red cells would consequently have some extra effects on metabolism beyond the simple oxygen provide for metabolism of tissue (Adeyemo *et al.,* 2003).

These studies and others have led some to suggest that changes in haematocrit and haemoglobin are helpful indicators of respiratory stress in fish (Langston *et al.,* 2002).

5.4.5 Comparison of welfare indicators in carp with different coping strategies

This study found no differences in any of the various potential welfare indicators between carp with different coping strategies. However, there were some significant interactions between coping strategy and the effects of environmental conditions. Thus in proactive fish, condition factor was highest at the lower temperature and at higher oxygen levels, whereas reactive fish condition was highest at the low temperature and high oxygen. Patterns of growth in weight and length clearly respond differently to environmental condition in proactive and reactive fish.

Haematocrit scores in proactive carp were higher at 20^{o}C (at which temperature their scores were broadly comparable to those of reactive fish) than at 25^{o}C (at which temperature their scores were lower than those of reactive fish), suggesting that proactive fish need more oxygen carrying capacity at the lower temperature. Proactive fish had slightly more secondary lamellae at 25^{o}C, at which temperature their mean number were higher than those of reactive fish).

In contrast, reactive fish had markedly more secondary lamellae at 20^{o}C, their scores being higher than those of proactive fish at this temperature. These results should not be over-interpreted, but, since proactive carp have a higher resting metabolic rate than do reactive carp (Huntingford *et al.,* 2010), their oxygen requirements might also be higher, especially at high temperatures, hence the need for a larger respiratory surface in this condition.

5.5 Conclusions

Of the welfare indicators studied, four gill measures (degree of hyperplasia, number of mucus cells and clavate lamellae and the distance between secondary lamellae) were particularly variable. They may therefore provide sensitive indicators of the impact of temperature and oxygen in fish. Perhaps not surprising fish held at the higher temperature and normal high oxygen levels were larger than those held in other conditions. Relative head size was highest in carp held at high temperature and low oxygen, possibly reflecting an adaptation to greater need to extract oxygen from the water. This interpretation is supported by lower levels of hyperplasia at high temperatures. There were few significant

clear differences between fish with different stress coping strategies for welfare indicators measured in this study.

CHAPTER 6

POTENIAL WELFARE INDICATORS IN SEMI-INTENSIVELY CULTURED COMMON CARP OVER ONE PRODUCTION YEAR.

This experiment was contucted at Polish Academy of Sciences Institute of Ichthyobiology and Aquacultures, Zaborze, Poland, by Dr. Maciej Pilarczyk who collected the data on body size and condition. Analysis of histological specimens and measurements of secondary lamellae are new and was carried out by Hussein Jenjan in discussion with Dr. Maciej Pilarczyk.

6.1 Introduction

6.1.1 General introduction to aquaculture

Aquaculture is the farming of water plants and animals in fresh, brackish and marine waters (Le Francois *et al.*, 2010). The form that aquaculture takes, and in particular the level of human intervention, is variable and depends on the species being cultured. For example, culture of bivalve molluscs usually involves little more than provision of sites in which the bivalves can grow, occasional thinning of the stock and harvesting. In contrast, farming finfish may involve human input throughout the life of the fish concerned, in the form of feeding, disease treatment and protection from predators. Aquaculture systems are often classified on a scale from extensive systems, with low animal density in relative to volume of water, to intensive systems, in which higher animal density is used, with strong control of water quality and feed availability, perhaps with recirculation of water (Figure 6.1).

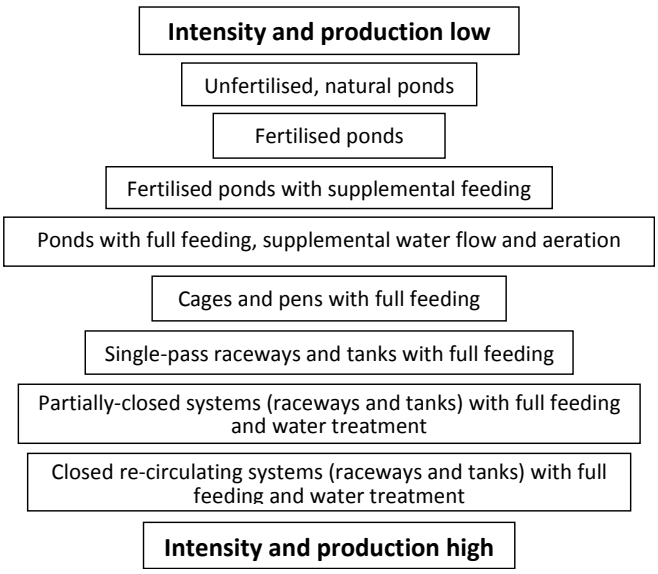


Figure 6.1. Schematic representation of fish culture systems, classified in terms of intensity. Le Francois *et al.*, 2010.

Extensive culture systems often take the form of natural earthern ponds, with the ponds being unfertilised and worked at low fish density with few external resources and water being added principally to compensate for evaporation and aeration used through periods of summer, or after levels of oxygen approach emergency conditions. Ponds may also be used for semi-intensive or intensive fish culture, intensification being achieved successively by fertilisation, by supplemental feeding using cheap ingredients and, finally,

by full feeding with aeration and rapid water turnover (Le Francois *et al.* 2010). The important water quality parameters for culturing cold water and warm water fish species are well recognized for ponds (Boyd, 1990). Poor water quality can lead to direct harm to fish by disrupting physiological functions such as water-ionic regulation, gill structure and function, or by obliterating the fish's mucous covering, which is an important defence against parasitic attack (Post, 1987; Klontz, 1993). Effective pond culture depends on reconditioning of the pond water, using sediment microbial activity to remove ammonia and carbon dioxide, with oxygen being replenished by photosynthesis of water plants, and by exchange of atmospheric gas (Boyd, 1990).

6.1.2 Welfare bottlenecks in common carp aquaculture

Fertilized ponds have traditionally been used for farming carp, including common carp (*Cyprinus carpio*) and grass carp (*Ctenopharyngodon idella*). The common carp is farmed in many countries and generates some 144,000 a tonnes of fish per annum. The best temperature for carp rearing lies between 20 and 28^{o}C (Horva´th *et al.,* 1992; Bauera and Schlott, 2004).

6.1.2.1 Effects of low winter temperatures: The common carp is a freshwater fish of the northern hemisphere that shows limited activity in winter at temperate latitudes (Bauer and Schlott, 2004), when both energy requirements and the ability for food processing are low (Bauera and Schlott, 2004). The winter is known to be a vital time in the common carp production, with the risk of a heavy loss of valuable fish. Therefore, in order to optimise wintering (Bauer and Schlott, 2004), wintering ponds must be carefully prepared before stocking with carp. Common carp stop feeding and moving at the low water temperature found in winter (Bauera and Schlott, 2004). The carp crowd together in large groups, often shaping a depression in the bottom of the pond where they stay through the winter with no movement or feeding. Young common carp at the end of their first summer show little activity at water temperatures of less than 1^{o}C, but older carp may show some activity in such conditions (Bauera and Schlott, 2004).

6.1.2.2 Effects of capture and grading: Capture and handling fish are important for enumerating and examining populations of fish. Well conducted capture exercises should have minimum effects on the communities of fish being studied. Researchers regularly presume that fish handled through capture survive and behave normally after they are released. However non-lethal capture methods such as hooking, may cause injuries and

mortality (Huntingford *et al.,* 2006; Coggins *et al.,* 2007) which might occur hours or days after release because fish are unable to improve from physical injury or capture-related stress (Barton 2002).

Regular capture and handling during procedures such as weighing, measuring, and classification are essential tools the fish farmer and researcher to provide, for example, estimates of population size, studies of fish growth and movement and studies of fish behaviour (Gorman and Stone, 1999). Nevertheless, fish physiological responses to capture and handling differ with type of mechanism, capture methods and water temperature (Kelsch and Shields, 1996). Numerous studies have observed the stress response in fish as it pertains to capture and handling (Aboul Hosn *et al.,* 2000; Barton, 2002). All features of capture and handling can expose fish to air, increased heart rate and can cause reduced growth (Barton *et al.,* 1987; Huntingford *et al.,* 2006). It can also reduce the fitness of fish and disease resitance (Aboul Hosn *et al.,* 2000; Paukert *et al,.* 2001). For example, bonytail chub (*Gila elegans*), recaptured more than once showed a lower weight and length gain than bonytail chub fish that were not recaptured (Paukert *et al.,* 2001) Sampling timing might influence the level of response potential of a fish (Pope and Willis, 1996: Stares et al., 2007) because of seasonal variation and water temperature linked with condition of spawning and the physiological responses (Knapp, 1996; Gooday, 2002). Capturing fish through the spawning period can detrimentally affect the ability of captured fish to reproduce (Pankhurst and Dedual, 1992, Haddy and Pankhurst, 1999). For example, capture and handling can suppress reproductive function in brown trout (Huntingford *et al.,* 2006).

6.1.2.3 Effects of summer conditions: During the summer season increased temperatures can have important consequences for aquatic organisms (Hirayama *et al.,* 2003). Fish are ectothermic animals and are affected by the temperature of the environment, which controls body temperature, heart rate, growth rates, reproductive and other body functions (Britz *et al*., 1997; Chapovetsky and Katz, 2003). High temperatures (e.g. in summer) generally result in higher rates for important metabolic processes, affecting both the production of the wild and farmed fish and the activity of microbial communities which lead to fish disease. Consequently, water temperature is a driving force in fish physiology since its effects are more than any other biological factor. Body functions such as growth and spawning, as well as survival in fish are highest within a distinct temperature range that differs between species (Gadowaski and Caddell, 1991). For example, Dorts *et al.* (2011) showed that, exposure to elevated temperatures within due to climate warming

might affect the reproductive success of European bullhead (*Cottus gobio*). Geographical distribution of brook trout, *Salvelinus fontinalis* is strongly constrained by highest summer temperature (Xu *et al.,* 2010). Also Sotripoulos *et al.* (2006) and Carlson *et al.* (2007) showed that a warm summer temperature is stressful on brook trout, as indicated by a low rate of food consumption and low growth rate. While short-term changes, for example due to temporary climate conditions, might influence fish for a one or two days, longer term, seasonal changes also have effects on fish. For example, fish move into more favorable areas of water to regulate their temperatures of body. In summer (warmer environments) fish have a period of faster growth ; however a faster growth rate may lead to reduced life span compared with slow growth in cool water (winter). High water temperatures during the summer lead to an increase in the fish metabolic rates (Toledo *et al.,* 2008), and a consequential increased food requirement.

The effect of seasonal changes in water temperature on gill structure can interact with those of other variables, such as fluctuating ion concentrations in the lake. For instance during summer, the epithelia of the gills of Arctic charr (*Salvelinus alpinus*) showed many chloride cells and a thick blood-to-water barrier (Hofer *et al.,* 2000). Thus, low ion concentrations during summer could affect the osmoregulatory function of fish. Through summer some fish such as Arctic charr (Hofer *et al.,* 2000) can show signs of stress, including an abnormally increased in immature blood cells and reduced the total number of red blood cells. Moreover, the capacity to activate additional blood cells from storage organs is reduced during summer, as it is also for liver glycogen reserves.

6.1.2.4 Effects of crowding and harvesting: Crowding occurs when, for husbandry purposes, fish are held at unusually high stocking densities in terms of fish weight per water volume unit. (Wedemeyer, 1996). It is known to be stressful to the fish and can lead to damage to scales and skin ulceration, possibly as a result of increased aggression (Ellis *et al.* 2002). There are species-specific differences in the response to high stocking density; for example, Arctic charr (*Salvelinus alpinus*) show stress-related behavioural responses at low density that is reduced as higher densities, at least up to a threshold density (Jorgensen and Jobling, 1991; Alanärä and Brännäs, 1996).

Thus during the production cycle for pond-reared common carp, the main critical points for welfare are during the winter when the fish are challenged by low temperature, the end of winter when the fish are captured, graded and stocked into growing ponds, during the summer, when the fish often experience a combination of high temperature and low

oxygen levels, and in the autumn when they are captured, graded and moved to concrete ponds, where they remain for several weeks at high densities prior to further crowding and harvest. The aim of the study decribed in this chapter was to explore the effects of these challenges on carp welfare, using a range of potential welfare indicators.

6.1.3 Indicators of fish welfare

6.1.3.1 Assessing fish welfare: Animal welfare has recently moved focus towards commercial and recreational activities that exploit fish populations (Huntingford *et al.,* 2006; Arlinghaus *et al.,* 2007). It is not easy to define animal welfare because the word welfare can be used in different contexts and with different meanings (Huntingford *et al.,* 2006; Branson, 2008). As explained in chapter 1, the term welfare as applied to farm animals can be defined in different ways:

- Function-based, reflecting the ability of the animal to perform in the farm environment, but this doesn't take into account the feelings of the animal.
- Feelings-based, reflecting whether the animal has what it wants and how it feels. The environment should be free from negative experiences and provide access to positive experiences.
- Nature-based, reflecting whether the animal can express its natural behaviour. This definition raises some questions for example, is all natural behaviour good welfare. For example, salmon fish migrating, is this a need to migrate or do they migrate because they need food, therefore in the farming environment when food is not an issue is the fact that they cannot migrate a welfare problem.

The welfare of an animal has been described as "its state as regards its attempts to cope with its environment" (Fraser and Broom, 1990). Animals under reduced welfare regularly show irregular behaviour patterns and can have more disease. In fish, disease or poor health are common indicators of poor welfare (Lymbery, 2002). To ensure fish welfare, there are five freedoms of fish welfare it can uses as indicators of welfare (FAWC, 1979; Table 6.1).

Table 6.1.The five freedoms of animal welfare (FAWC, 1979) and the possible easily indicators used to measure welfare of fish (Huntingford *et al.,* 2006; Lembo and Zupa, 2008).

Five Freedoms of Animal Welfare	Indicators
1. Freedom from hunger and thirst	Reduced food intake, body condition loss and low growth rate
2. Freedom from excessive negative environmental conditions	Water quality observing (for examples, low or high dissolved oxygen levels and changes in pH), reduced food availability, disturbance through tourism and chronic and acute exposure to pollutants.
3. Freedom from injury , pain and disease	Fin damage, scratches and gill diseases.
4. Freedom from limitation of behavior	Changed swimming speed, abnormal behaviour and behaviour response to unfavourable situation.
5. Freedom from fear, distress of mental	Changes in blood biochemistry (plasma cortisol, glucose, lactate), changes in body colour and rate of respiration

6.1.3.2 Production welfare indicators: Many of the stress effects described above cause reduced intake of food and increased utilisation of energy, consequently prolonged activation of the hypothalamic-pituitary-interrenal axis is probable, thus indirectly affecting growth (Ellis *et al.*, 2002; Huntingford *et al.*, 2006). Additionally, the secretion of growth hormones in teleost fish is suppressed through periods of stress (Farbridge and Leatherland, 1992), consequently stress has in addition a direct control on the mechanisms of fish growth (Huntingford *et al.,* 2006). While these variations can be interpreted as natural responses to difficult conditions and so part of normal functioning, generally changes in growth rates have been used as an indicator of negative fish welfare (Ellis *et al.*, 2002). Crowding is not the only cause of changing fish growth rates; environmental stressors such as changes in salinity (Jenjan, 2002), water pH, reduced dissolved oxygen can lead to changes in growth rate (Huntingford *et al.*, 2006).

Physical condition can also be used as a welfare indicator in wild and farmed fish. This includes disease, ectoparasites, endparasites, injuries and fin damage (Huntingford and Kadri, 2008). Infectious diseases and parasite problems generally result from exposure to other harmful organisms, many of which are everywhere in most natural surface waters (Conte, 2004). When fish are stressed, their capability to resist physical attack is reduced, and stress may be a primary contributing factor that leads to damage of health in farmed fish and may be a sign of poor welfare (Huntingford *et al.,* 2006, Iwama *et al.,* 1997). With higher stocking densities comes the issue of increased crowding stress, and injuries such as fin damage gained by these adverse conditions have been used as indicators of poor welfare (Etscheidt and Manz, 1992). The causes of increased fin damage at higher stocking densities are not fully understood (North *et al.*, 2006), but suggestions include aggressive and accidental damage through feeding and abrasion attacks by conspecifics (Huntingford *et al.,* 2006).

Intensive aquaculture studies on the incidence and severity of fin damage and relation to density of stocking have focused on salmonid fish (Turnbull *et al.*, 1998; North *et al.*, 2006). In species where aggression is common, stress of crowding can lead to increased fights between individuals and consequently to increased fin damage. For example, North *et al.* (2006) found increased levels of fin damage in rainbow trout (*Oncorhynchus mykiss*) at densities of 40 and 80 kg m^{-3} compared to 10 kg m^{-3} treatment, and suggested that the possiblities for aggressive or unintentional damage increased because of higher numbers of fish. This trend has also been shown for Arctic charr (*Salvelinus alpinus*) (Damsgard *et al.*, 1997).

6.1.3.3 Physiological variables based on the stress response: Greater understanding of how fish respond to difficult environments comes from the wide literature on the stress biology (Huntingford *et al.*, 2006). In general, as with other vertebrates, fish display a range of behavioural and physiological mechanisms that help them to cope with challenge; these are known as the general stress response (Figure 6.2. Iwama, 2007), and is regularly used as an indicator of fish welfare. The stress response reflects an adaptive physiological strategy to cope with a perceived hazard to homeostasis (Sutanto and de Kloet, 1994), but signs of physiological stress feature widely in welfare research as indicators of poor welfare (Huntingford *et al.*, 2006; Ashley, 2007).

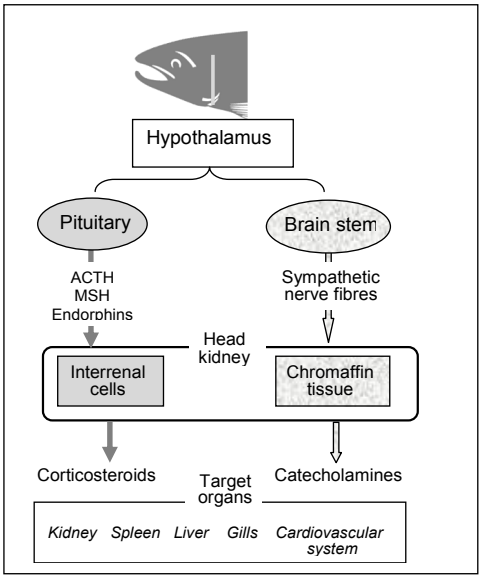


Figure 6.2. Schematic representation of the mechanisms involved in the physiological stress response in fish.

The physiological stress response has been categorized into primary, secondary and tertiary responses (FSBI, 2002). The primary response consists of a neuroendocrine response, which involves the release of catecholamines and the activation of the hypothalamic-pituitary-interrenal axis. Corticotropin stimulating factor from the hypothalamus operates on the pituitary to synthesise and relaese adrenocorticotropic hormone that subsequently stimulates the synthesis of cortisol from the interrenal tissue which is in the kidney (Figure 6.2. Huntingford *et al.*, 2006; Ashley, 2007).

The size and timing of stress-derived elevations in plasma cortisol levels are generally proportional to the time and severity of the stressor with recovery from a short term, sharp stress taking up to a few hours (Pickering and Pottinger, 1987). Raised cortisol levels may persist during continuous stress, of which stocking density could be a source (Pottinger, 1993). Plasma cortisol is commonly measured as an indicator of stress in fish, (Barton and Iwama, 1991; Fevolden *et al.,* 2002). Individual differences in the scale of the corticosteroid stress response have been recognized, and consistently high and low stress responders may be identified within strains of fish species (Pottinger *et al.,* 1994; Weil *et al.,* 2001). A decreased level of the cortisol stress response by selective breeding has been achieved in rainbow trout (*Oncorhynchus mykiss*) (Jentoft *et al.,* 2005, Pottinger and Carrick, 1999) and common carp (*Cyprinus carpio*) (Tanck *et al.,* 2002). Fevolden *et al.* (2002) report that rainbow trout selected for low post-stress cortisol levels showed better growth. The return rate of the cortisol response to basal levels usually takes place within hours after exposure of fish to a brief stressor (Jentoft *et al.,* 2005). However, the release of cortisol in fish can precede a secondary stress response as energy stores are mobilized (Pickering, 1981), by inducing liver gluconeogenesis and aminotransferases (Van der Boon et al., 1991; Mommsen *et al.,* 1999; Barton, 2002).

6.1.3.4 Behavioural variables and stress coping strategies: In conjunction with the physiological response, stress may lead to strong changes in behavior, which have been classified and interpreted in a variety of ways (Wingfield, 2003; Kortan and Adamek, 2011). In fish, behavioral responses are the first line of defence to an unfavorable change of ecological conditions, regularly being activated by the same stimuli that initiate the primary stress response. The correct behavioral response depends on the stressor disturbed. A distinction is regularly made between proactive and reactive responses (Henry, 1993; Koolhaas et al., 1999). For example, a proactive stress coping strategy is characterized by active responses to the stressful motivation, including escape and aggression (Sluyter et al.,

1996). A reactive coping strategy, in contrast, involves passive responses to challenge and low levels of aggression. Behavioural reactions to aversive motivation experiences have been observed in zebra fish in response to fear-extracting stimuli (Gerlai, 2010; Maximino *et al.,* 2010) and the novelty of the environment increased the frequency of tail-beating behavior in zebra fish (Maximino, 2011).

6.1.3.5 Gill structure and function as potential welfare indicators: Fish share with other vertebrates many general developmental pathways, body systems and physiological functions. The challenge of life in water results in a leading role for structures such as gills, not just in terms of respiration, but through osmoregulation, through which the gill plays an essential role in the physiological responses to ecological and internal changes. The large surface area of the gill is a main route through which numerous biotic or abiotic compounds enter the fish body (Evans *et al.,* 2005). Because gills perform a number of essential functions and have a large surface area in contact with the outside environment, they are very sensitive to environmental changes, both chemical and physical, in the aquatic environment and are the main target organ in fish for any environmental changes (Mallatt, 1985). For example, the effects of low oxygen levels on fish gill morphology have been studied in many species (Jenjan *et al.,* in preparation; see Chapter 5). The magnitude of the gill changes observed depends on the species' sensitivity and to the extent of the stressor. Effects of the environment on the ultrastructure of the gill, include changes in pavement cells (the most abundant cell type, which covers much of the lamellar and filament surfaces of the gill), mucous cell, chloride cells and gill epithelia (Pelgrom *et al*., 1995; Jenjan*,* 2002; Lock and Wendelaar Bonga, 2008). Chloride cells are or mitochondrion-rich cells which are sensibly certain to be responsible for salt secretion (Evans *et al.,* 2005; Jenjan 2002). Morever, filamental chloride cells activated in sea water are likely to play a role in ion secretion in sea water, and lamellar chloride cells that disappear during sea water adaptation seem to be involved in ion uptake in fresh water (Jenjan 2002). Pavement cells in general have low mitochondrial densities and uncomplicated basolateral membranes. While these cells, particularly on the gill lamellae, are supposed to be the site of trans-epithelial gas transfer, and may also play a role in ion and acid-base regulation (osmotic regulation) in freshwater fish (Evans *et al.,* 1999).

In the fish gill, the ambient water is separated from the blood by an epithelial barrier comprised principally these three cell types (Perry and Laurent, 1993). They form an extensive thin surface through which exchange of respiratory gases (CO_2 and O_2) occurs. Mucous cells secrete a mucus layer over the gill epithelium. The physiological function of

mucous is unclear, even though it is usually supposed to impede diffusive ion loss and assist active ion uptake (Perry and Laurent, 1993). The chloride cells are found distributed over the gill filament and between the secondary lamellae.

6.1.4 Aims and predicted results

The aims of the work reported in this chapter were to collect information on a variety of different welfare indicators in common carp held in extensive production over a period of 9 months in the run up to harvest and to examine how these indicators change in response to relatively long term exposure to poor environmental conditions and to a number of short-lived husbandry interventions. These variables include one aspect of the primary stress response (specifically cortisol release), as well as indicators of the secondary stress response (specifically plasma glucose and lactate levels and indirect measures of the production of red blood cells) and of tertiary responses (including body size and condition, and potentially adaptive changes to the respiratory surfaces). In addition, the incidence of damage to the fins and body surface as well as the gill filaments was recorded. Table 6.2 summarizes an attempt to predict which of these various indicators are likely to respond to short-lived, acute husbandry interventions (grading of the fish in spring and autumn and crowding prior to harvest) and to longer-lasting, chronic challenge (exposure to high temperatures and low oxygen levels during summer and crowding in rough concrete tanks after autumn grading and prior to harvest).

Table 6.2. Predicted changes to indicators of stress in response to explosive to acute and chronic stressors on fish farm.

INDICATOR	ACUTE CHALLENGE		CHRONIC CHALLENGE	
	Spring and autumn grading (1 day)	**Final crowding and harvest (1 day)**	**High summer temperature and low oxygen levels (several weeks)**	**Rough concrete tanks at high densities (several weeks)**
Body weight	May fall due to stress and reduced feeding opportunities	May fall due to stress and reduced feeding opportunities	Growth likely to be slow or negative	Growth may be slow or negative
Body length	No change	No change	Growth may be slow	Growth may be slow
Condition factor	May fall	May fall	Likely to fall	Likely to fall
Glucose/lactate	Sharp rise expected	Sharp rise expected	May stabilise at low levels if fish can adapt or may be chronically elevated if not	May stabilise at low levels if fish can adapt or may be chronically elevated if not
Cortisol	Sharp rise expected	Sharp rise expected	May stabilise at low levels if fish can adapt or may be chronically elevated if not	May stabilise at low levels if fish can adapt or may be chronically elevated if not
Haematocrit Haemaglobin	Some adaptive changes possible	Some adaptive changes possible	Likely to be chronically elevated to meet challenge	Likely to be chronically elevated to meet challenge
Gill filament development	Adaptive changes unlikely but damage possible	Adaptive changes unlikely but damage possible	May be extended to improve gas exchange	May be reduced to protect against poor water quality
Hyperplasia	Adaptive changes unlikely	Adaptive changes unlikely	May be reduced to improve gas exchange	May be increased to protect against poor water quality
Mucous cells	Adaptive changes unlikely	Adaptive changes unlikely	May be reduced to improve gas exchange	May be increased to protect against poor water quality
Clavate lamellae	Some damage possible	Some damage possible	Uncertain	Extensive damage possible
Body and fin condition	Some damage possible	Extensive damage possible	Poor recovery from damage expected	Extensive damage possible

6.2 Material and methods

6.2.1 Sampling schedule

Third production season common carp *Cyprinus carpio* were collected from winter ponds at the Polish Academy of Sciences Institute of Ichthyobiology and Aquacultures, Zaborze, Poland (Figure 6.3) using a seine net from standard production ponds, and were kept under standard management. After stocking into on-growing ponds (Figure 6.3), fish were sampled at eight times between early April and late December (Table 6.3).

All sampled fish were anaesthetized, weighed (g) and body and head length (mm) were measured. The condition factor of each fish (K) was estimated from the relationship between weight and length as follows:

$$K = W/L^3$$

where W and L are weight (g) and length (mm).).

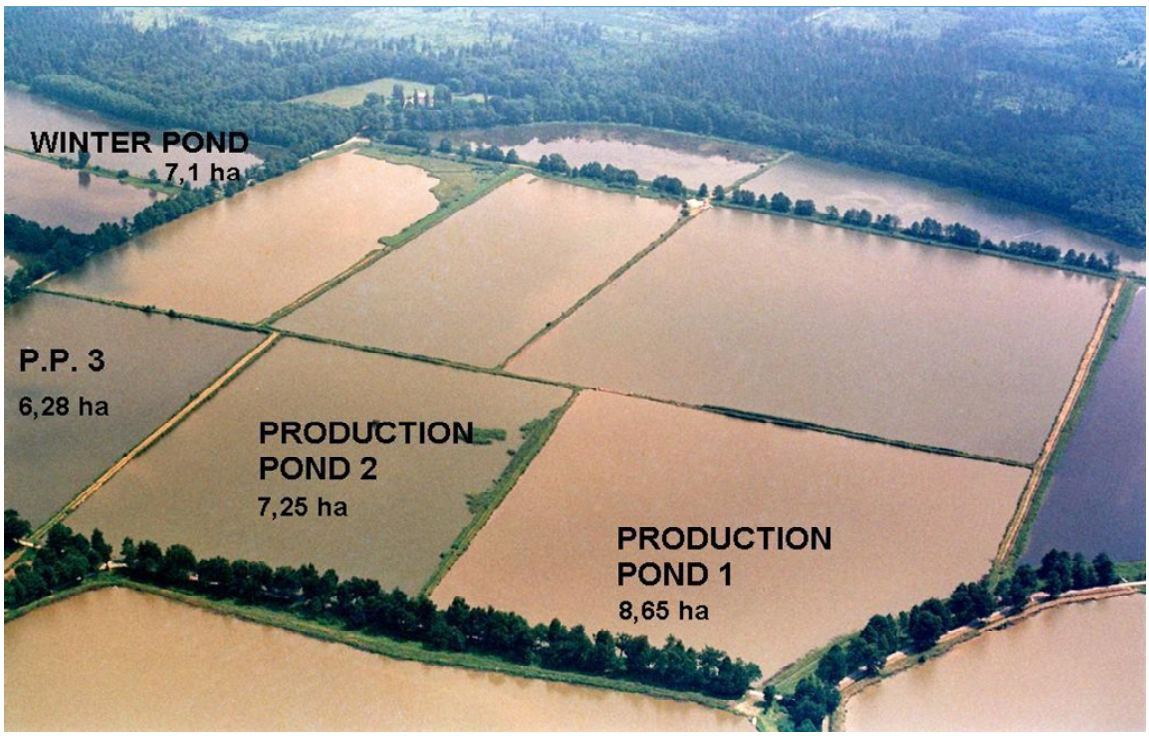


Figure 6.3. The winter ponds and production ponds from which carp were sampled in the present study.

Table 6.3. Details of sampling procedures carried out at eight points during the final year of production in semi-intensively farmed common carp.

Point	Date (2006)	Husbandry event or circumstances	Treatment	Body size
A	06 April	Before spring grading	20 carp sacrificed immediately after being collected from one winter pond	Weight 543.0 ± 23.0g Length 28.5± 0.46 cm
B	07April	After spring grading	After capture, grading & transport, 14 carp sacrificed from groups to be placed in each of 3 earthern growing ponds	Weight 456.0±24.0 g Length 27.8 ± 0.45 cm
C	20.June	Start of summer	Prior to summer, 14 carp collected from each of the 3 ponds & sacrificed.	Weight 1248.0 ±66.0g Length 36.7 ± 0.58cm
D	06 Sept	End of summer	At the end of summer, 14 carp collected from each of the 3 ponds & sacrificed	Weight 2086.0 ±92.0g Length 43.1 ± 0.66cm
E	16 Oct	Before autumn grading	Prior autumn grading, 14 carp collected from each of the 3 ponds & sacrificed	Weight 2132.0 ±124.0g Length 45.3 ± 0.94cm
F	17 Oct	After autumn grading	After autumn grading, 14 carp from each of the 3 ponds sacrificed. Remaining fish from all ponds placed together in a single concrete storage tank, already stocked with other carp.	Weight 2104 ± 69.0g Length 45.0 ± 0.51cm
G	07 Dec	Start of pre-harvest crowding	20 carp collected from the concrete storage pond & sacrificed. Remainder crowded prior to harvest	Weight 1750.0 ±92.0g Length 41.9 ±0.62cm
H	20 Dec	End of pre-harvest crowding	At final harvest, 20 carp collected from concrete single pond collected & sacrificed	Weight 1444.0 ±71.0g Length 40.1 ± 0.66cm

6.2.2 Blood analysis

Immediately after the fish were killed from each sample, blood samples were collected from the caudal artery and vein.

6.2.2.1 Blood biochemistry (glucose, lactate and cortisol: Blood samples were centrifuged and plasma was collected. The glucose concentration was measured using on enzyme based colorimetric method using glucose oxidase, lactate was measured using enzyme based colorimetric methods using lactate Dry-Fast and cortisol was measured using Microwell ELISA method. Glucose and lactate scores were combined into a single measure of blood biochemistry by a principal component anlaysis; the first component accounted for 62% of the total variation in the data set and had ositive loadings for all 3 variables.

6.2.2.1 Blood respiratory status (red blood cells count, haematocrit and haemoglobin): Red blood cells were counted using a Burker chamber after dilution in Hayme liquid. Total haemoglobin concentration was measured according to a modified method described by Tentori and Salvati (1981). Drabkin's reagent (1 ml) was mixed with 0.5 ml of saponin

(0.05 %) and 20 ml of haemolysate (a product of haemolysis). For haematocrit, blood samples were collected into heparanized haematocrit tubes and centrifuged immediately and haematocrit % was measured. These variables were combined into a single measure of blood respiratory status by a principal component anlaysis; the first component accounted for 61% of the total variation in the data set and had positive loadings for all 3 variables.

6.2.3 Condition of the external body surface

For each sampled fish, the external surface was examined thoroughly for *Argulus spp,* which were removed carefully from the fish using forceps and counted. The presnece or absence of whitespot (*Ichthyophthirius multifiliis*) was also recorded. The incidence of splits in all fins and the number of broken fin rays were noted. Principal components analysis was used to generate an overall score of damage to all fins (except the pelvic fins, which showed very little damage), the first component accounting for 34% of the total variance and having positive loadings for splits and broken rays across all fins.The number of scratches per cm² of fish skin was also recorded.

6.2.4 Gill condition

The second gill arch from the right side was removed by dissection and preserved in 4% buffered (pH 7.0) formaline for light microscopy. Tissues for histological analysis were embedded in wax following standard procedures. Wax embedded tissues were sectioned (thickness of section = 5 μm) and stained with haematoxylin and eosin, according to method outlined in Clark (1980). At the tip, middle and base of every fifth gill filaments, nine secondary lamellae were measured for the number of secondary lamellae per millimeter of gill filament, the height of secondary lamellae and the distance between secondary lamellae. The number of mucous cells per millimeter of lamellar surface, the height of interlamellae cells (expressed as a percentage of the height of the adjacent lamellae, to give a measure of percentage hyperplasia) and the presence of clavate lamellae (defined as lamellae with an accumulation of epithelial cells accumulation at the tip of secondary lamellae which increase the cell layer number) were also recorded on about 20 different gill filaments. Mucous cells number, height of interlamellae cells and clavate lamellae were averaged for each fish to give a score for the overall abundance of mucous

cells and clavate lamellae and percentage hyperplasia. Principal components analysis was used to derive a compound score for the extent of development of the secondary lamellae, the first component accounting for 66% of the total variation and having positive loadings for both number and length of the lamellae.

6.2.5 Statistical analysis

Initial scrutiny of the data was based on comparisons of means and standard errors. The relationships among the measured variables were determied using correlation analyses. Differences between male and female carp were examined by unpaired T tests and differences among the 3 holding ponds by oneway analysis of variance. Relationships between measured variables and body length were investigated by regression analysis. Comparisons of measured variables between relevant pairs of sample points were made using unpaired T tests. The number of *Argulus* spp per fish at the different sampling points was examined using one way anova and the relationship between *Argulus spp* infestation and the various welfare indicators by regression analysis. The incidence of fish with whitespot at the various sample points was compared by Chi^2 analysis and the status of the various welfare indicators in fish with and without whitespot was compared by unpaired T tests. All analyses were carried out using the MINITAB statistical package.

6.3 Results

6.3.1 Overall range of measures variables

Table 6.4 shows the mean values (±SE) and the coefficient of variation for all the univariate measures used to assess performance and welfare in the common carp used in this study, averaged over the whole study period. As expected, all measures varied considerably, with plasma cortisol levels and the number of mucous cells and clavate lamellae having particularly high coefficients of variation.

Table 6.4. Mean and standard error of the mean (±SE) and coefficient of variation for all univariate measures used to characterise production and welfare in common carp

Variable	Mean (±SE mean)	Coefficient of variation (%)
Body weight	1525.30 ± 49.50	53
Body length	39.01 ± 0.47	20
Condition factor	2.29 ± 0.02	17
Glucose	78.62 ± 2.25	47
Lactate	61.17 ± 1.39	37
Cortisol	182.81 ± 8.04	72
Haemoglobin (g/dI)	8.36 ± 0.11	21
Hematocrite (%)	33.07 ± 0.30	15
Red blood cell	1123.30 ±22.10	32
Secondary lamellae height	0.16 ± 0.00	37
Secondary lamellae number (mm)	36.34 ± 0.31	14
Distance between secondary lamellae	0.02 ± 0.00	35
Hyperplasia %	39.70 ± 2.79	98
Mucus cell number (mm)	0.87 ± 0.06	118
Clavate lamellae number (mm)	0.89 ± 0.11	200
Total skin lesion	1.18 ± 0.02	159
Total skin scratches	3.07 ± 0.25	133
Total broken rays	0.33 ± 0.03	142
Total fin split (-PVF)	215.60 ± 12.30	94

6.3.2 Relationships among variables

Table 6.5 summarises the significant correlations between all measured variables, with univariate measures replaced by PCA-derived measures as appropriate, for the whole study period and for specific sampling points. Blood biochemistry, blood respiratory status and cortisol were mutually positively correlated, representing a general axis of physiological stress and these variables were all negatively related to body condition. Percentage hyperplasia and number of mucous cells were positively correlated, indicating common responses of the gill surfaces to stress, and both were negatively related to blood biochemistry. Damage and number of scratches were also positively related, while damage was positively related to respiratory status and lamellar development and negatively related to blood biochemistry. The number of clavate lamellae was positively associated with condition.

There were relatively few significant correlations at the 4 neutral sampling points (A, C, E and G), possibly due to small sample sizes. In period A, a negative correlation between condition and cortisol was found, as seen in the correlation matrix for the whole data set. In period E, positive correlations were reported for cortisol and blood biochemistry and respiratory status and in period G there was a positive relationship between overall damage and scratches, in both cases as seen in the correlation matrix for the whole data set. The

other correlations for the separate neutral sample points are particular to the relevant period.

Table 6.5. Correlations between potential welfare indicators. a) over the whole study period. b) at sampling points A, C, E and G. -, -- and - - - represent negative correlations at P< 0.05, 0.01 and 0.001 respectively. +, ++ and +++ represent positive correlations at P< 0.05, 0.01 and 0.001 respectively.

a	Condition	Bio-chemistry	Cortisol	Respiratory status	Lamellar development	Hyper-plasia	Mucous cells	Clavate lamellae	Damage	Scratches
Biochemistry	-									
Cortisol	- - -	+++								
Respiratory status	- - -	++	+++							
Lamellar development										
Hyperplasia		- - -								
Mucous cells		- - -				++				
Clavate lamellae	+++					-				
Damage		- -		++	+++					+++
Scratches										

b	Condition	Biochemistry	Cortisol	Respiratory status	Lamellar development	Hyperplasia %	Mucous cells	Clavate lamellae	Overall damage	Scratches
Blood biochemistry			G^{+++}							
Cortisol	A^{-}									
Respiratory status			E^{+}							
Lamellar development				E^{+}						
Hyperplasia%	E^{+}									
Mucous cells					C^{++}					
Clavate lamellae						A^{+}	A^{-} C^{-}			
Damage						G^{+}	C^{-}			G^{+}
Scratches					A^{-}					

6.3.3 Effects of gender

Table 6.6 shows mean ± SE values for all measured variables in male and female carp separately, combined by PCA-derived scores where appropriate, with the results of T tests. Overall, there were few differences between the sexes, male fish had significantly higher scores for blood respiratory status and more whitespot colonies than did females. Gender differences were therefore ignored in subsequent analyses.

Table 6.6. A comparison of the mean ± SE of an all measured variables for male and female of 1269 common carp. Bold font indicates significant relationships.

Variable	Male	Female	T	P
Body weight	1533.00 ± 70.00	1519.00 ± 70.00	-0.13	0.89
Body length	39.38 ± 0.70	38.70 ± 0.60	-0.72	0.47
Condition factor	2.27 ± 0.00	2.26 ± 0.00	1.25	0.21
Blood biochemistry	0.01 ± 0.10	-0.01 ± 0.10	-0.14	0.89
Cortisol	2.20 ± 0.02	2.15 ± 0.03	-1.60	0.11
Blood respiratory status	0.26 ± 0.12	-0.21 ± 0.11	-2.86	**0.01**
Lamellar development	0.01 ± 0.10	- 0.01± 0.10	-0.10	0.92
Hyperplasia %	39.20 ± 4.30	40.10 ± 3.60	0.16	0.88
Mucous cell number/mm	0.86 ± 0.10	0.88 ± 0.10	0.22	0.83
Clavate lamellae number/ mm	0.98 ± 0.20	0.83 ± 0.10	-0.69	0.49
Damage	-0.12 ± 0.10	0.10 ± 0.09	1.60	0.11
Scratches	2.81± 0.34	3.27± 0.34	0.93	0.35
Argulus	3.67 ± 0.40	4.01 ± 0.40	0.66	0.51
% fish with whitespot	15%	6%	Chi^2=6	**0.01**

6.3.4 Effects of holding pond

Table 6.7 summarises the results of one way ANOVA of all measured variables by pond. There were a few differences between fish from the different holding ponds; for example, fish from pond 3 were lighter in weight, (marginally) shorter in length and had (marginally) fewer clavate lamellae. Fish in pond 2 had significantly higher levels of infestation with *Argulus* than did those from the other ponds and this pond effect was allowed for in subsequent analyses. For body weight a *post hoc* test showed fish in pond 1 to be significantly different from fish in pond 3, but fish in pond 2 did not different from fish in pond 1 and in pond 3. For body length, a *post hoc* test showed a marginally significant difference between pond 1 and pond 3, as well as between pond 2 and pond 3, but no significantly difference between pond 1 and pond 2. For clavate lamellae, *a post hoc* test showed significantly differences between fish in pond 1 and fish in pond 3, but no significantly difference between fish in ponds 1 and 2 as well as between fish in pond 2 and fish in pond 3. A *post hoc* test for *Argulus* spp showed that, fish in pond 1 significantly different from fish in pond 2, but fish did not different from fish in pond 3, and fish in pond 2 significantly different from fish in pond 3. Since there were relatively

few consistent effects of on-growing pond on the measured variables, fish from all three ponds were combined in subsequent analyses.

Table 6.7. Mean (±SD) for all measured variables for common carp held in the three on-growing ponds, with the results of a one way ANOVA. Mean values sharing a superscript letter in common are not significantly different.

Variable	Pond 1	Pond 2	Pond 3	F	P
Body weight	1789 (± 988)[a]	1661 (± 833)[ab]	1365 (± 645)[b]	4.75	0.01
Body length	40.7 (± 8.7)[a]	40.3 (± 8.2)[a]	37.9 (± 6.1)[b]	2.79	0.06
Condition factor	2.34 (± 0.47)	2.24 (± 0.40)	2.31 (± 0.39)	0.97	0.38
Blood biochemistry	0.18 (±1.02)	-0.08 (± 1.38)	0.32 (± 0.88)	2.42	0.09
Cortisol	171.9 (± 100.1)	165.1 (± 96.7)	148.4 (± 60.2)	0.30	0.74
Blood respiratory status	0.35 (± 1.21)	-0.06 (± 1.50)	-0.09 (± 1.27)	2.40	0.09
Lamellar development	-0.17 (± 1.2)	0.01 (± 1.2)	-0.18 (± 1.2)	0.43	0.65
Hyperplasia %	25.9 (± 38.0)	33.9 (± 35.7)	26.7 (± 33.0)	0.71	0.49
Mucous cell number/mm	0.61 (± 0.87)	0.83 (± 1.02)	0.75 (± 0.93)	0.93	0.40
Clavate lamellae number/ mm	1.43 (± 2.67)[a]	1.03 (± 2.06)[ab]	0.61 (± 0.80)[b]	2.89	0.06
Damage	-0.02 (± 1.20)	-0.04 (± 0.88)	-0.30 (± 0.94)	1.57	0.21
Scratches	2.90 (± 3.99)	3.69 (± 5.00)	2.53 (± 4.09)	1.27	0.28
Argulus	3.30 (± 3.00)[b]	5.76 (± 4.73)[a]	4.00 (± 5.40)[b]	5.57	0.004
% with whitespot	7.2%	2.9%	1.4%	3.4	0.19

6.3.5 Measured variables and body length

Table 6.8 shows the results of regression analyses of the relationship of all variables with body length across the whole study period, with univariate scores replaced by PCA-derived scores where appropriate. Significant positive relationships were found for condition, clavate lamella number and (marginally) overall damage. Significant negative relationships were found with blood biochemistry, cortisol, blood respiratory status, percentage hyperplasia and number of scratches.

Table 6.8. Regression analyses of potential welfare indicators against total body length (cm), with univariate measures replaced by PCA-derived scores where appropriate. Bold type indicate significant relationships.

Variable	Regression equation	F 1,268	P
Condition	Y=2.05 + 0.007X	4.50	**0.04**
Blood biochemistry	Y= 1.68 - 0.040X	21.51	**<0.001**
Cortisol	Y= 2.47 - 0.010X	12.39	**0.001**
Blood respiratory status	Y= 0.80 - 0.020X	3.69	**0.005**
Lamellar development	Y= 0.03 - 0.001X	0.01	0.930
Hyperplasia %	Y= 84.90 -1.180X	11.19	**0.001**
Mucous cell number/mm	Y= 1.14 - 0.010X	0.72	0.398
Clavate lamellae number/mm	Y= -1.25 + 0.05X	14.92	**<0.001**
Damage	Y= -0.64 + 0.02X	3.12	0.079
Scratches	Y= 7.01 - 0.100X	9.90	**0.002**

To examine possible relationships between potential welfare indicators and body size while allowing for any seasonal effects, regression analysis was also carried out on carp collected at the first of each pair of sample points (points A, C, E and G, Table 6.9). These represent points at which the fish had been in their previous condition for some time and had not been subjected to any acute husbandry practice. Given the number of comparisons made, there are effectively no significant relationships between length and any of the possible welfare indicators, although blood status and (marginally) lamellar development are positively related to body length in period G.

Table 6.9. Regression analysis of potential welfare indicators on body length in common carp sampled at 4 points. Point A = before the spring grading. C = before the summer. E = before autumn grading. G = before final harvest. Bold type indicate significant relationships.

Point	Variable	Regression coefficient	F	P	R^2 %
A	Condition	-0.0385	2.95	0.103	9.3
A	Blood biochemistry	+0.006	0.02	0.89	0.0
A	Cortisol	+0.018	1.27	0.28	1.4
A	Blood respiratory status	-0.111	1.49	0.238	2.5
A	Lamellar development	+0.053	0.32	0.58	0.0
A	Hyperplasia %	+0.92	0.14	0.71	0.0
A	Mucous cell number/mm	+0.007	0.00	0.954	0.0
A	Clavate lamellae number/mm	+0.0309	0.13	0.724	0.0
A	Damage	-0.0424	0.80	0.38	0.0
A	Scratches	-0.188	1.02	0.325	0.1
C	Condition	+0.0103	0.89	0.350	0.0
C	Blood biochemistry	+0.0116	0.09	0.771	0.0
C	Cortisol	-0.000	0.00	0.987	0.0
C	Blood respiratory status	+0.0027	0.00	0.951	0.0
C	Lamellar development	-0.0454	1.03	0.317	0.1
C	Hyperplasia %	+2.78	3.43	0.075	7.7
C	Mucous cell number/mm	-0.0407	0.99	0.325	0.0
C	Clavate lamellae number/mm	+0.0186	0.44	0.51	0.0
C	Damage	+0.0385	0.55	0.461	0.0
C	Scratches	+0.144	0.44	0.509	0.0
E	Condition	-0.002	0.09	0.772	0.0
E	Blood biochemistry	-0.0141	1.37	0.25	0.9
E	Cortisol	+0.00013	0.00	0.971	0.0
E	Blood respiratory status	0.021	0.55	0.461	0.0
E	Lamellar development	-0.0217	2.60	0.117	4.8
E	Hyperplasia %	-0.47	0.16	0.690	0.0
E	Mucous cell number/mm	+0.0113	0.27	0.608	0.0
E	Clavate lamellae number/mm	+0.112	5.36	0.026	9.6
E	Damage	-0.0013	0.00	0.954	0.0
E	Scratches	+0.065	0.67	0.420	0.0
G	Condition	+0.0206	1.06	0.318	0.3
G	Blood biochemistry	+0.0050	0.01	0.918	0.0
G	Cortisol	+0.0171	1.84	0.192	4.2
G	Blood respiratory status	+0.190	5.09	**0.037**	17.7
G	Lamellar development	+0.132	3.60	0.08	14.8
G	Hyperplasia %	+2.01	1.41	0.26	2.9
G	Mucous cell number/mm	+0.095	0.87	0.364	0.0
G	Clavate lamellae number/mm	-0.0112	0.09	0.77	0.0
G	Damage	+0.0698	0.78	0.388	0.0
G	Scratches	-0.112	0.15	0.70	0.0

6.3.6 Short term effects of husbandry events

The sampling programme included three points at which the fish were likely to experience acute stress induced by husbandry events, namely at spring grading (point B versus point A), at Autumn grading (point F versus point E) and at final crowding for harvest (point G versus point H). Table 6.10-6.12 shows mean (± SE) values for all measured variables (univariate measures replaced by PCA-derived scores as appropriate) for these 3 comparisons, together with the results of T tests.

At the spring grading (Table 6.10) there were significant falls in body weight and condition, associated with significant increases in blood biochemistry, cortisol, blood respiratory status, number of clavate lamellae, overall damage and number of scratches.

Table 6.10. Mean **(± SE of an all measured variables (univariate measures replaced by PCA-derived scores as appropriate) in common carp collected before (point A) and after (point B) the spring grading. Bold type indicates significant relationships.**

Variable	Point A	Point B	T	P
Body weight	543.0 ± 23.0	456.0 ± 24.0	2.59	**0.012**
Body length	28.52 ± 0.46	27.97 ± 0.45	0.85	0.400
Condition	2.32 ± 0.05	2.00 ± 0.02	6.08	**<0.001**
Blood biochemistry	-0.67 ± 0.09	1.29 ± 0.11	-14.5	**<0.001**
Cortisol	2.13 ± 0.14	2.40 ± 0.14	- 0.68	**<0.001**
Blood respiratory status	-1.49 ± 0.19	1.40 ± 0.16	-11.64	**<0.001**
Lamellar development	-0.12 ± 0.18	-0.44 ± 0.13	1.43	0.160
Hyperplasia %	51.00 ± 4.8	59.20 ± 4.80	- 1.22	0.228
Mucous cell number/mm	1.15 ± 0.22	0.86 ± 0.17	1.06	0.300
Clavate lamellae number/mm	-1.17 ± 0.10	-0.12 ± 0.14	- 0.62	**<0.001**
Damage	-1.17 ± 0.09	-0.12 ± 0.14	- 6.19	**<0.001**
Scratches	2.27 ± 0.38	4.95 ± 0.76	- 3.17	**0.002**

At the autumn grading (Table 6.11), there were significant falls in percentage hyperplasia, associated with significant rises in blood biochemistry, cortisol, blood respiratory status, lamellar development and overall damage.

Table 6.11. Mean **(± SE) of an all measured variables (univariate measures replaced by PCA-derived scores as appropriate) in common carp collected before (point E) and after (point F) the autumn grading. Bold type indicates significant relationships.**

Variable	Point E	Point F	T	P
Body weight	2132.0 ± 124.0	2104.0 ± 69.0	0.20	0.840
Body length	45.32 ± 0.94	45.04 ± 0.51	0.25	0.800
Condition	2.19 ± 0.05	2.29 ± 0.06	-1.28	0.205
Blood biochemistry	-0.26 ± 0.07	0.64 ± 0.10	-7.54	**<0.001**
Cortisol	2.00 ± 0.02	2.36 ± 0.01	- 13.90	**<0.001**
Blood respiratory status	-0.24 ± 0.17	0.56 ± 0.17	-3.29	**0.001**
Lamellar development	-1.45 ± 0.08	0.58 ± 0.20	-9.29	**<0.001**
Hyperplasia %	24.30 ± 7.1	8.30 ± 3.1	2.05	**0.047**
Mucous cell number/mm	0.67 ± 0.13	0.67 ± 0.14	0.00	1.000
Clavate lamellae number/mm	1.02 ± 0.31	1.43 ± 0.50	- 0.69	0.49
Damage	-0.47 ± 014	0.08 ± 0.17	-2.47	**<0.016**
Scratches	1.17 ± 0.48	1.45 ± 0.35	- 0.48	0.63

At the pre-harvest crowding (Table 6.12), there were significant falls in the body weight, overall damage and (marginally) in condition factor. This was associated with significant rises in blood biochemistry, cortisol and the number of gill parasites.

Table 6.12. Mean (± SE) of all measured variables (univariate measures replaced by PCA-derived scores as appropriate) in common carp collected before (point G) and after (point H) pre-harvest crowding. Bold type indicates significant relationships.

Variable	Point G	Point H	T	P
Body weight	1750.0 ± 92	1444.0± 71	2.63	**0.013**
Body length	41.86± 0.62	40.08± 0.66	1.98	0.055
Condition	2.35± 0.05	2.22±0.05	1.76	0.088
Blood biochemistry	-1.88±0.13	1.08±0.18	-13.29	**<0.001**
Cortisol	1.91±0.04	2.73± 0.02	-21.02	**<0.001**
Blood respiratory status	0.42±0.25	0.35± 0.26	0.20	0.845
Lamellar developemnt	0.60± 0.22	0.68± 0.24	-0.26	0.800
Hyperplasia %	88.00± 5.2	72.3± 9.00	1.50	0.145
Mucous cell number (mm)	1.40± 0.28	1.55± 0.28	-0.38	0.703
Clavate lamellae number (mm)	0.25±0.10	0.15±0.11	0.68	0.503
Damage	1.75±0.21	0.67±0.25	3.28	**0.002**
Scratches	4.15 ± 0.76	3.02± 0.65	1.13	0.267

6.3.7 Long term effects of environmental conditions

The sampling programme included two periods during which the fish were expected to be exposed to sustained favourable environmental conditions, namely during the spring growth period (from point B to point C, lasting 73 days) and the autumn growth period (from point D to point E, lasting 41 days). It also included two periods during which the fish were held in sustained potentially unfavourable conditions. The first is the summer (from point C to point D, lasting 104 days), during which high temperatures and low oxygen levels were expected; in the event, these did not occur, but the fish did experience very high mortality. The second period during which the fish were held in potentially unfavorable conditions was after the autumn harvest when the fish were held in concrete ponds (from point F to point G, lasting 71 days). Table 6.13-6.16 shows mean (± SE) values for all measured variables (replaced by PCA-derived scores as appropriate) in common carp sampled at the start and end of these periods, together with the results of T tests.

6.3.7.1 Potentially favourable periods: During the spring growth period (points B-C. Table 6.13), there were significant increases in weight, length and condition factor, confirming this as a favourable period for growth. This was associated with increases in lamellar development, number of clavate lamellae, overall damage and in the number of

Argulus spp. At the same time, there were significant falls in blood biochemistry, cortisol, blood respiratory status and percentage hyperplasia.

Table 6.13. Mean (± SE) of all measured variables (univariate measures replaced by PCA-derived scores as appropriate) in common carp collected at the start (point B) and end (point C) of the favourable period of spring growth. Bold type indicates significant relationships.

Variable	Point B	Point C	T	P
Body weight	456.00 ± 24.00	1248.00± 66.00	-11.30	**<0.001**
Body length	27.97± 0.45	36.71± 0.58	-11.86	**<0.001**
Condition	2.00± 0.05	2.22±0.05	-9.28	**<0.001**
Blood biochemistry	1.29±0.11	0.03±0.15	6.88	**<0.001**
Cortisol	2.40±0.02	2.01± 0.02	13.62	**<0.001**
Blood respiratory status	1.40±0.16	-0.50± 0.16	8.35	**<0.001**
Lamellar developemnt	-0.437± 0.13	0.74± 0.16	-5.79	**<0.001**
Hyperplasia %	59.2± 4.80	13.90± 6.10	5.84	**<0.001**
Mucous cell number/mm	0.86± 0.17	0.71± 0.14	0.69	0.492
Clavate lamellae number/mm	0.17±0.06	0.71±0.10	-4.60	**<0.001**
Damage	-1.12±0.14	0.37±0.19	-2.02	**0.047**
Scratches	4.95 ± 0.76	5.11± 0.80	-0.14	0.890

During the autumn (period D-E. Table 6.14), the fish increased marginally in length, but not in weight, suggesting that this is not a particularly favourable time for growth. At the same time, there were significant increases in blood biochemistry and blood respiratory status and a continued increase in the number of *Argulus spp* per fish. During this period, condition factor, lamellar development and percentage hyperplasia all decreased.

Table 6.14. Mean (± SE) of an all measured variables (univariate measures replaced by PCA-derived scores as appropriate) in common carp collected at the start (point D) and end (point E) of the autumn growth period. Bold type indicates significant relationships.

Variable	Point D	Point E	T	P
Body weight	2086.00± 92.00	2132.00± 124.00	-0.30	0.77
Body length	43.10± 0.66	45.32± 0.94	-194	0.057
Condition	2.57±0.09	2.19±0.05	3.66	**0.001**
Blood biochemistry	-0.99±0.18	-0.26±0.07	-3.83	**<0.001**
Cortisol	1.98± 0.03	2.00± 0.02	-0.70	0.487
Blood respiratory status	-0.88± 0.16	-0.24± 0.17	-2.79	**0.007**
Lamellar development	-0.02± 0.16	-1.45± 0.08	7.81	**<0.001**
Hyperplasia %	42.40± 7.60	21.6± 6.90	2.03	**<0.049**
Mucous cell number/mm	0.76± 0.15	0.63± 0.13	0.64	0.524
Clavate lamellae number/mm	1.79±0.32	1.05±0.31	1.66	0.101
Damage	-0.45±0.10	-0.46±0.10	0.07	0.946
Scratches	2.57± 0.67	1.17± 0.49	1.96	0.096

6.3.7.2. Potentially unfavorable periods: During the summer (period C-D. Table 6.15), body weight and length increased markedly, so those fish that survived seem to have been growing well. Over this period there were also significant increases in lamellar number/length, percentage hyperplasia and number of clavate lamellae. These changes

were accompanied by decreases in blood biochemistry, lamellar development, overall damage, scratches and the number of *Argulus spp*.

Table 6.15. Mean (± SE) of an all measured variables (univariate measures replaced by PCA-derived scores as appropriate) in common carp collected at the start (point D) and end (point E) of the summer growth period. Bold type indicates significant relationships.

Variable	Point C	Point D	T	P
Body weight	1248± 66	2086±92	-7.41	**<0.001**
Body length	36.71± 0.58	43.71±0.66	-7.30	**<0.001**
Condition	2.44±0.04	2.57±0.09	-1.34	0.148
Blood biochemistry	0.66±0.18	-0.66±0.18	5.26	**<0.001**
Cortisol	2.01± 0.02	1.98±0.03	0.92	0.359
Blood respiratory status	-0.50± 0.16	-0.88± 0.16	1.68	0.096
Lamellar development	0.74± 0.16	-0.02±0.16	3.35	**0.001**
Hyperplasia %	13.90± 6.10	42.40±7.60	-2.92	**0.006**
Mucous cell number/mm	0.71± 0.14	0.76±0.15	-0.26	0.793
Clavate lamellae number/mm	0.71± 0.10	1.79±0.32	-3.23	**0.002**
Damage	0.22±0.20	-0.45±0.10	3.10	**0.003**
Scratches	5.11± 0.80	2.57±0.67	2.43	**0.017**

During the time the carp were held in the concrete holding tank (period F-G. Table 6.16), there was a significant reduction in both average weight and average length, suggesting very unfavourable conditions, possibly with larger fish being more likely to die. Over the same period, increases were seen in percentage hyperplasia, mucus cell number, overall damage and scratches. This was accompanied decreases in blood biochemistry, cortisol, clavate lamellae number and the number of *Argulus spp*.

Table 6.16. Mean (± SE) of an all measured variables (univariate measures replaced by PCA-derived scores as appropriate) in common carp collected at the start (point F) and end (point G) of the period during which they were held in a concrete tank after the autumn harvest. Bold type indicates significant relationships.

Variable	Point F	Point G	T	P
Body weight	2104.00±69.00	1750.00± 92.00	3.07	**0.004**
Body length	45.05± 0.51	41.86± 0.62	3.96	**<0.001**
Condition	2.29±0.06	2.35±0.05	-0.67	0.505
Blood biochemistry	0.64±0.62	-1.88±0.13	16.00	**<0.001**
Cortisol	2.36± 0.01	1.91± 0.04	11.78	**<0.001**
Blood respiratory status	0.56± 0.17	0.42± 0.25	0.44	0.659
Lamellar developemnt	0.58± 0.20	0.60± 0.22	-0.05	0.961
Lamellar number/length	-0.25± 0.13	0.01± 0.14	-1.39	0.173
Hyperplasia %	8.30± 3.10	88.00± 5.20	-13.27	**<0.001**
Mucus cell number/mm	0.67± 0.14	1.40± 0.28	-2.36	**0.025**
Clavate lamellae number/mm	1.43±0.50	0.25±0.09	2.31	**0.026**
Damage	0.08±0.17	1.75±0.21	-6.11	**<0.001**
Scratches	1.45± 0.35	4.15± 0.76	-3.23	**0.003**

6.3.8 Sample point, ectoparasites and welfare status

Table 6.17 shows the mean number of *Argulus spp* per fish and the percentage of fish with whitespot disease at each sampling period. The intensity of *Argulus spp* infestations varied with season, *post-hoc* testing between pre-treatment points showing that it increased significantly from point A to point C, was similar at points C and E and fell significantly from point E to point G. The number of *Argulus spp* per fish fell significantly at spring grading (A v B), over the summer period (C v D), while the fish were in the concrete holding ponds (F v G) and during pre-harvest crowding (G v H). The incidence of white spot disease was highly seasonal, with no infected fish being found in points C to G. The highest levels were seen after pre-harvest crowding and the lowest after spring grading ($Chi^2 = 10.3$. P = < 0.01).

Table 6.17. Mean (±SE) number of *Argulus* per fish and percentage of fish with whitespot disease in common carp sampled at each sampling point, together with the results of ANOVAs and Chi^2 tests.

Sample point	Mean (±SE) *Argulus*	% fish with whitespot
A	3.65 (± 0.83)	35
B	1.43 (± 0.57)	19
C	6.38 (± 0.57)	0
D	1.86 (± 0.57)	0
E	6.24 (± 0.57)	0
F	5.86 (± 0.57)	0
G	2.75 (± 0.83)	0
H	0 (± 0.83)	60
	F = 14.77. P = 0.001	Chi2 =81.83. P < 0.001

Since husbandry practice had an effect on levels of *Argulus spp* infestation, the relationship between intensity of this ectoparasitic infection and various welfare indicators was examined using regression analysis on data from the "pre-treatment" conditions only (Table 6.18). Welfare scores in fish with and without whitespot were compared by T tests using the data from samples A, B and H only (Table 6.19). A significant positive relationship was found between *Argulus spp* levels and both blood biochemistry score and cortisol level. All other relationships were non-significant. Fish with white spot were significantly shorter and in poorer condition than were those without whitespot, and also had higher blood biochemistry and cortisol scores and more mucous cells, but fewer clavate lamellae.

Table 6.18. Regression analysis of production and welfare measures in relation to levels of *Argulus* infestation in common carp sampled at points A,C,E and G). Bold type indicates significant relationships.

Variable	Regression equation	$F_{1,164}$	P	R^2(%)
Length	Y = 1.93 + 0.06X	1.41	0.24	0.2
Condition	Y = 2.12 – 0.002X	0.08	0.78	0
Blood biochemistry	Y = -0.69 + 0.07X	10.77	**0.001**	5.6
Cortisol (log)	Y = 2.03 + 0.01X	12.77	**<0.001**	6.7
Blood respiratory status	Y = -0.44 + 0.02	1.04	0.31	0
Lamellar development	Y = 0.29 + 0.23X	1.21	0.27	0.2
Hyperplasia %	Y = 39.3 – 1.34X	2.72	0.10	1.5
Mucous cell number/mm	Y = 0.89 – 0.01X	0.13	0.72	0
Clavate lamellae number/mm	Y = 1.14 – 0.0006X	0	0.99	0
Damage	Y = 0.06 + 0.002	0	0.95	0
Scratches	Y = 3.09 – 0.001	0	0.98	0

Table 6.19. Mean (+SE) production and welfare variables in carp with and without whitespot collected at sample points A, B and H.

Variable	Without whitespot	With whitespot	T	P
Length	39.7 (±0.48)	33.3 (±1.2)	4.81	**<0.001**
Condition	2.31 (0.03)	2.20 (0.05)	2.14	**0.04**
Blood biochemistry	-0.08 (0.08)	0.74 (0.20)	3.88	**<0.001**
Cortisol (log)	2.14 (0.02)	2.48 (0.05)	6.71	**<0.001**
Blood respiratory status	0.01 (0.09)	-0.13 (0.32)	0.45	0.66
Lamellar development	0.00 (0.08)	-0.04 (0.10)	0.18	0.87
Hyperplasia %	57.9 (3.8)	64.2 (7.1)	0.79	0.44
Mucous cell number/mm	0.81 (0.06)	1.44 (0.25)	2.49	**0.001**
Clavate lamellae number/mm	0.95 (0.12)	0.37 (0.11)	3.57	**0.001**
Damage	0.02 (0.08)	-0.16 (0.22)	0.77	0.48
Scratches	3.01 (0.26)	3.54 (0.77)	0.64	0.52

6.4 Discussion

6.4.1 Aims of the present study

The aims of the present study were to screen farmed common carp for several potential welfare indicators across one year of the production cycle, to explore potential influential factors such as gender and disease status and, having identified such possible confounding effects, to examine the effects of various husbandry practices on the welfare of these fish. A higher level aim was to compare the various measures taken as indicators of impaired welfare in response to stressors acting on different time scales.

6.4.2 Gender effects

Differences in the reproductive biology of males and females mean that the selective forces acting on them are different (Shine, 1989). In several kinds of animals, selection favours

larger size in males than in females. For example, successful defense of resources necessary for breeding is an important factor in reproductive success, particularly in males (DeMartini, 1988; Pollock *et al.,* 2008). Larger animals tend to win fights, so adult males are larger than adult females in number of species, including many fish. In a number of species, including bees, harvester ants, scorpionflies, damselfish, bullfrogs and iguanid lizards (Alcock, 1984; Howard, 1988; Anderson, 1994; Jaroensutasinee and Jaroensutasinee, 2001) larger males are more successful at protecting eggs (Bisazza *et al.,* 1989). In the three-spined stickleback, larger males are able to produce more sperm (Zbinden *et al.,* 2001). Higher plasma glucose levels have been reported in male European sturgeon (*Huso huso*) compared to female (Asadi *et al.,* 2006); this may reflect higher growth rate or higher food conversion efficiency (Hardy and Litvak, 2004), but may also be the result of a stronger stress response in males.

There were no significant differences in size between male and female carp in the present study, perhaps because males in this species do not defend eggs and fry or as a result of the crowding in farm situations (Britton *et al.,* 1982). In fact, very few significant gender effects were found, except that male carp were more likely to be infected by whitespot and on average had higher scores for blood respiratory status, indicating higher haematocrit, red blood cell count and haemoglobin concentrations than females. A number of studies have reported similar results for fish, with indicators of higher respiratory function in males (Gabriel *et al.,* 2004); for example, higher haematocrit scores have been reported in male roach (*Rutilus rutilus*. Kortet, 2003). Such gender-related differences may reflect an adaptive response to a more active life style in males of these species (Owens and Wilson, 1999).

The finding of higher levels of whitespot infestation in male common carp compared with females may be related to behavioural differences between the two sexes that result in greater exposure of male fish to parasitic infection. Differences in reproductive behaviour are one possibility (Reimchen and Nosil, 2001; Vainikka *et al.,* 2004), although this is perhaps an unlikely explanation in the present case as the fish in this study were not fully mature. In general, males are more susceptible to parasitism than females (Møller *et al.,* 1998; Kurtz *et al.,* 2000) and there is a negative relationship between immune functions in fish and testosterone (Vainikka *et al.,* 2004). An increase in parasite number has been reported in rainbow trout after exposure to testosterone (Buchamann and Bresiani, 1997), so it is possible that the higher white spot levels in male fish may reflect differences in circulating testosterone, even in immature fish.

6.4.3 Pond effects

The carp used in this study were held in three different growing ponds in the period between spring and autumn grading. During this period, a number of significant differences between fish held in the different ponds were reported. Other differences appeared during the study, for example in the number of *Argulus* spp and marginally clavate lamellae. These were inconsistent and hard to interpret and may have been the result of small differences in water quality and nutrient levels in the different ponds, sufficient to influence growth rates in the earliest months of culture (Green, 1992). Normally, body weight, body condition as well as blood parameters in fish are dependent on water condition, feeding programme, stress and disease (Patriche *et al.,* 2009; Yeganeh, 2011).

6.4.4 Relationship between body size and potential welfare indicators

In the present study, regression analysis identified significant positive relationships between body length (used as an indicator of overall size) and condition factor and the number of clavate lamellae. It seems that larger carp were better able to obtain and store nutrient reserves and/or to invest relatively more in tissue growth than were smaller fish; the relationship with the number of clavate lamellae may be a simple reflection of longer gills (and therefore more lamellae to be damaged) in larger fish. Significant negative relationships were found between body length and blood biochemistry, cortisol and blood respiratory status. Although it is impossible to distinguish cause and effect from such correlations, it could be that fish that are relatively unstressed, as reflected in plasma lactate, glucose and cortisol levels, and relatively less challenged in terms of investment in blood respiratory function are able to grow better than are their more stressed/challenged companions. This is in contrast to the observation that large rainbow trout have greater levels of lactate and glucose than do smaller fish (Kieffer *et al.,* 1995 and 2003), possibly because the common carp is a relatively unaggressive species and do not fight ovcrtly for access to the food needed for growth. There was also a negative relationship between body length and percentage hyperplasia, perhaps reflecting both lower stress and a need for greater exposed gill area in fast growing fish. Finally, the negative relationship between body size and number of scratches may reflect greater robustness or greater ability to avoid

damaging locations in larger fish. Reimchen, (1988) showed a similar size effect in a study on adult sticklebacks the frequency of injuries, including damaged pelvic and dorsal spines and skin lacerations (caused by predator attacks) was strongly positively related to fish body size.

Some of variables that were unrelated to body size are of interest. For example, there was no statistical association between body size and various measures of gill development, suggesting that these aspects of gill structure may be tuned to the life style of the fish concerned and/or ambient levels of dissolved oxygen (Jenjan *et al.,* in preparation, in chapters 2 and 3). The present results agree with studies by Saliu and Olonire (2007), who found no relationship between secondary lamellae number, distance between secondary lamellae or gill surface area and body length in *Chryischthys longifilis* and *Clarias anguillaris.* Also, the gill morphometric and meristic character of the gills of these species depend on the level of activity of the fish, high values being associated with an active mode of life.

6.4.5 Relationship between parasites potential welfare indicators

In the present study, the level of *Argulus spp* infestation was positively related to blood biochemistry scores and cortisol levels, but not to body size or condition. A high number of *Argulus spp* can cause osmoregulatory disturbances, indicated by increased plasma cortisol, haematocrit; plasma protein levels and reduced fish growth rate (Johnson and Albright 1992) and stress-related effects in skin and gills of fish. For example, Ruane *et al.* (1999) have shown that ectoparasite infestations can result in a stress response in the host fish. Rainbow trout (*Oncorhynchus mykiss*) fed on cortisol have a decreased parasites number (Haond *et al.,* 2003), so increases of cortisol levels may be an adaptive immune response to fish parasites. Carp with whitespot were significantly shorter and in poorer condition than were those without this parasite and they also had higher blood biochemistry and cortisol scores and more mucous cells. Cause and effect cannot be separated here, but these results suggest either that stressed fish (and in the case of whitespot, those that are in poor condition) are more vulnerable to these two parasites, or that infestation causes a stress response and in the case of whitespot, impairs condition, perhaps through an effect on food intake (Owen *et al.,* 1993).

Parasites are known to affect several features of fish physiology (Holmes and Zohar, 1990; Barber *et al.,* 2000) and can also impair health (Sures*,* 2001). Growth of yellow perch (*Perca flavescens*) infected with metacercariae of the digenean *Apophallus brevis* is lower than that of non infected individuals (Johanson and Dick, 2001). Effects of parasitic infestation on body length and condition factor might come about because parasites reduce fish feeding activity. Lemly and Esch (1983) found that the body condition of juvenile bluegill sunfish (*Lepomism acrochirus*) was negatively correlated with the intensity of infection by the trematode, *Uvulifer amblopitis*. Elevated plasma cortisol levels are a commonly observed effect of parasitic infection (Dubansky *et al.,* 2011) and cortisol is the major factor modulating changes in the health status of fish, particularly in relation to disease resistance (Ruane *et al.,* 1999).

The mucous layer in the gills of fish is potentially important in protection against disease (Roberts and Powell, 2003), being involved in many biological activities, including swimming and defence against pathogenic parasites (Calabrò *et al.,* 2005). The fact that in the present study common carp parasitized by whitespot had more mucous cells per unit of lamellar length than did uninfected fish could reflect increased mucous production when the gills of fish are irritated by whitespot.

6.4.6 Short term effects of husbandry practices

All three acute husbandry stressors (spring and autumn grading and pre-harvest crowding) were associated with significant increases in cortisol levels and in the blood biochemistry score. Various reasons have been suggested for the reduced growth observed under crowded conditions in fish, including reduced food intake and social stress (Sloman *et al.,* 2000, Trenzaddo *et al.,* 2006). It is commonly accepted that crowding acts as a chronic stressor for fish (Montero *et al.,* 1999), leading to increases metabolic rate and greater nutritional needs. The reduced food intake during crowding and increased requirement for energy, leads to a lower fish body weight, poor condition and reduced growth (van Weerd and Komen, 1998; Trenzaddo *et al.,* 2006).

The two grading operations were also associated with a rise in blood respiratory status, increased body damage and, in the spring, in the number of scratches on the body. A fall in weight and condition was seen at the spring harvest and at pre-harvest crowding. Effects of these acute stressors on gill structure were small, inconsistent and hard to interpret, with

more clavate lamellae following the spring grading and higher lamellar development scores following the autumn grading.

According to EFSA (2008) "practices at pre-harvest level (inadequate feeding or antimicrobial usage) could increase the prevalence of certain biological hazards at farm level, and may also have an effect on fish welfare and physiological condition (stress)". Clearly in the present study, short-term husbandry activities induced a strong physiological stress response in the carp sampled, in terms of cortisol release, glycogen mobilisation (increasing blood biochemistry scores) and enhanced respiratory status; all these can be seen as adaptive responses to an acute challenge. The fall in weight and condition seen in the spring grading and at pre-harvest are likely to be the result of stress-induced reduction in feeding, or possibly to a simple lack of opportunity to feed. The increased level of injury seen in all three acute stress periods could be the result of collisions with other fish, with nets and pond walls or of handling. These would presumably add to the stressors encountered by the carp. Clavate lamellae, fin damage and scratches are common problems in farmed fish (Ellis *et al.,* 2002; Ashley, 2006). In salmonids, fin damage can be caused by bites during aggressive interaction, perhaps exaggerated through the effects of stress (Turnbull *et al.,* 1996; Ellis, 2002). Carp are less aggressive than salmonids, but aggression-induced injury remains a possibility. Damage to the body surface might potentially be related to the actions of ectoparasites. Autumn grading was not associated with a change in levels of *Argulus spp*, but the number of *Argulus spp* per fish fell at spring grading and at pre-harvest crowding, so some of the skin damage observed may have been caused by dislodged fish lice.

6.4.7 Long-term effects of favourable conditions

Comparison of common carp at the beginning and end of spring showed good growth and an increasing condition factor, associated with falls in blood biochemistry, cortisol and blood respiratory status. This confirms the spring as providing favourable conditions for the carp, even though overall damage and levels of *Argulus spp* infestation were higher by the end of this period. In terms of gill structure and function, by the end of spring, the carp showed increased secondary lamellae development and decreased hyperplasia, suggesting good water quality, but they also showed higher levels of clavate lamellae. Taken together, these results lead to the conclusion that common carp enjoy good welfare during the spring.

Good growth under conditions of the warmer water temperature and more available food towards the end of spring has previously been reported in brook trout (*Salvelinus fontinalis*. Xu *et al.,* 2010). This was associated with decreases in blood glucose and lactate and cortisol (all stress indicators). The lower levels of hyperplasia found at the end of the spring growing season in the present study may reflect reduced stress, but might also be an adaptive response to increased temperatures and possibly lower oxygen levels, as decribed for crucian carp (*Carassius carassius,* Sollid *et al.,* 2005). This interpretation is supported by the observation that common carp have better developed secondary lamellae towards the end of the spring. These results agree to some extent with a study on tench (*Tinca tinca*), in which blood respiratory status was higher in spring than in winter (Guijarro *et al.,* 2003).

A number of studies have reported changes in production and physiological variables associated with breeding in fish. For example, plasma cortisol often increases in the blood of spawning fish (Wingfield and Grimm, 1977; Saha *et al.,* 2002) and Rinchard *et al.* (1993) found that some parameters of blood biochemistry were increased during the spawning season in *Gobio gobio*. This increase in blood parameters could give high resistance to infection through the spawning period (Pickering *et al.,* 1987). In caged male bluegill sunfish (*Lepomis macrochirus*) cortisol levels increased from the first day after spawning to the last day of the nesting period, while over the same period, body condition fell by about 65% (Megee *et al.,* 2006). Such effects are unlikely to be important in the present study, partly because the carp were less, rather than more, stressed at the end of the spring period and partly because few of the fish in the present study were in breeding condition.

Comparison of carp samples at the beginning and end of autumn in the present study showed that the fish increased marginally in length, but not in weight, and that this was associated with increased plasma lactate and glucose levels (blood biochemistry score) and higher blood respiratory status. Some of these effects may have been the result of increased levels of *Argulus spp* infection by the end of the study period. In terms of gill structure and function, by the end of autumn, the carp had less developed secondary lamellae and lower levels of hyperplasia. On balance, these findings suggests that autumn is not a particularly favourable time for common carp.

Changes in blood biochemistry during autumn have been recorded in other vertebrates (Storey, 1996), perhaps associated with the approach of hypoxic conditions in the winter (Nilsson et al., 1994). The decreases in secondary lamellar development observed here during the autumn could be related with changes of water temperature. Increased blood glucose and lactate suggest that the carp in the present study were stressed, although there were no changes in blood cortisol levels. This is in contrast to studies of pikeprech (*Stizostedion lucioperca*) in which blood cortisol levels decrease during autumn, leading to an increased concentration of blood protein as well as metabolism of lipid (Fatemeh *et al.,* 2008).

6.4.8 Long-term effects of unfavourable conditions

By the end of summer, when high temperatures and low oxygen levels were expected, the carp were heavier and longer than those sampled at the start of summer. At the same time, blood biochemistry scores were lower, as were levels of overall damage and the number of scratches. In terms of gill structure and function, between the start and the end of the summer period, percentage hyperplasia and the number of clavate lamellae increased and overall secondary lamellar development decreased. These changes were accompanied by a decrease in the number of *Argulus spp* per fish.

Contrary to expectations, the usual extremes of high temperature and low oxygen were not observed over the summer of this study. It may be that conditions, including food availability, were more favourable for growth than normal, so that the changes observed were the result of the fish growing well. In brook trout, it has been suggested that an increase in the quantity of feed consumed by the fish due to a temperature-induced increase in the fish metabolism in summer could cause the observed increase in fish weight and length at this time (Xu *et al.,* 2010). Similarly, the lipid and protein contents in the flesh of gilthead bream (*Sparus aurata*) and herring (*Clupea harengus*) were found to be higher in summer season than winter (Wassef and Shehata, 1991; Hamre *et al.,* 2003). The fall in the blood biochemistry score observed over the summer in the present study agrees with the results of Rocha and Branco (1998) who found low blood glucose and lactate during the summer in silver catfish (*Rhamdia quelen*). Such results could indicate that the fish were less stressed in the summer (Lermen *et al.,* 2004). Common carp studied by Chen *et al.,* (1995) showed incresed levels of blood cortisol after exposure to low temperature (4 ^{O}C).

On the other hand, in the present study there was very high mortality during the summer, so the differences could have been the result of non-random mortality by size, with smaller, more stressed fish that were in poorer physical condition being more likely to die. There is no way with the available information to distinguish between these effects and both could be at work.

In the present study, gill secondary lamellae development of common carp was decreased and hyperplasia between secondary lamellae increased during summer, both of which would tend to reduce exposed respiratory surface area. This may be the result of stress, perhaps caused by increased biomass in the ponds, but could also reflect an adaptive response to poor water quality.

During the 61 days that the carp were held in the concrete holding tanks prior to harvest, both weight and length fell markedly. At the same time, levels of damage and scratches showed marked increases, as did numbers of mucous cells and the extent of hyperplasia. The fact that cortisol levels and blood biochemistry scores fell suggest a down-regulation of the acute physiological stress response. These findings suggest that the fish were experiencing conditions that were chronically very unfavorable.

6.4.9 Consideration of potential welfare indicators

Alterations of blood cortisol levels are considered to be a primary indicator of the stress response, while the resulting mobilisation and utilisation of glucose are valuable indicators of the secondary stress response. The results of the present study suggest that common carp are very responsive to short term stress and that plasma cortisol, glucose and lactate levels are valuable indicators of such acute stress responses, likely to increase over periods of hours. According to some approaches, fish with wounds and injuries experience poor welfare, by definition, so the occurrence of recently acquired damage to the skin and fins provides a good indicator of acutely compromised welfare.

Increased production of red blood cells (reflected in increased haematocrit scores and plasma haemoglobin concentrations) represents an adaptive secondary response to a variety of challenges. Likewise, hyperplasia in a number of situations represents adaptations by the fish to defend the essential gill tissues from irritation or damage or to alter the size of the respiratory surface in the face of an increased or decreased oxygen requirements (Skidmove and Tovell, 1972). Since mucous serves to protect the gills,

increased number of mucous cells can also be seen as an adaptive secondary stress response. Thus blood respiratory status and the extent of hyperplasia and the density of mucous cells in the secondary lamellae potentially provide valuable indicators of stress responses in the medium term, perhaps changing over days and weeks.

In the short term, elevated cortisol, glucose and lactate and in the medium term elevated blood respiratory status, hyperplasia and mucous cell density probably all reflect adaptive responses to challenge, and hence potentially indicators of good welfare, defined in terms of effective functioning. However, prolonged elevation of these variables indicates exposure to chronically stressful conditions and hence probably poor welfare. This is likely to be reflected in indicators of the tertiary stress response, including production indicators of impaired welfare, such as slow growth and poor nutritional status.Long term exposure to unavoidable stress impairs immune function (Gleeson , 2007), increasing the likelihood of fish succumbing to infection and slowing the rate of recovery from injury. Thus, as well as providing an immediate measure of compromised welfare through damage to a fishes' body, the incidence of damage to fins and skin could also indirectly reflect longer term, chronic stress.

With this background, Table 6.20 summarises the changes to the various potential welfare indicators observed in the present study in response to the acute challenges of grading and crowding and the chronic challenge of unfavourable summer conditions and housing at high densities in concrete tanks. The table also compares the observed responses to those predicted in advance.

Table 6.20a. Summary of predicted and observed changes in potential welfare indicators in common carp before and after exposure to acute husbandry stressors (spring and autumn grading and final crowding). b)Summary of predicted and observed changes in potential welfare indicators in common carp before and after exposure to chronic challenges (high summer temperatures and crowding in rough concrete tanks). # = possible adaptive response to challenge. * = possible indicator of good welfare. $ = possible indicator of impaired welfare. £ = possible result of differential mortality by indicator status.

a)

Indicator	Spring and autumn grading (1 day)			Final crowding and harvest (1 day). Some mortality?	
	Expected	Observed		Expected	Observed
		Spring	Autumn		
Body weight	Fall	Fall$	No change	Fall	Fall$
Body length	No change	No change	No change	No change	Slight fall£
Condition	Fall	Fall$	No change	Fall	Slight fall$
Glucose-lactate	Sharp rise	Sharp rise#	Sharp rise#	Sharp rise	Sharp rise#
Cortisol	Sharp rise	Sharp rise#	Sharp rise#	Sharp rise	Sharp rise#
Hcrit-Hglobin	Small changes if any	Rise#	Rise#	Small changes if any	No change
Filament development	Small changes if any	No change	Rise#£	Small changes if any	No change
Hyperplasia	No change	No change	Fall#$	No change	No change
Mucous cells	No change	No change	No change	No change	No change
Clavate lamellae	Some damage	Rise$	No change	Some damage	No change
Body and fin condition	Some damage	Rise$	Rise$	Extensive damage	Fall*£

b)

Indicator	High summer temperature and low oxygen levels (several weeks). High disease-induced mortality		Rough concrete tanks at high densities(several weeks)	
	Expected	Observed	Expected	Observed
Body weight	Fall	Rise*£	Fall	Fall $
Body length	Small increase	Rise*£	Small increase	Slight fall£
Condition	Fall	No change	Fall	No change
Glucose-lactate	Stabilise at low levels or chronically elevated	Fall*£	Stabilise at low levels or chronically elevated	Fall*£
Cortisol	Stabilise at low levels or chronically elevated	No change	Stabilise at low levels or chronically elevated	Fall*£
Hcrit-Hglobin	Rise	No change	Rise	No change
Filament development	Rise	Fall # $	Fall	No change
Hyperplasia	Fall	Rise # $	Rise	Rise # $
Mucous cells	Fall	No change	Rise	Rise #$
Clavate lamellae	???	Rise $	Extensive damage	Fall*£
Body and fin condition	Rise	Fall*£	Extensive damage	Rise $

Rises in cortisol and glucose/lactate levels (as seen at grading and crowding) and haematocrit (as seen at spring grading) could reflect short-term adaptive responses to challenge and so could indicate good welfare in terms of ability of cope. Thus, measures of blood biochemistry (including glucose/lactate and cortisol) and respiratory status (reflecting red blood cell levels and so oxygen carrying capacity) are sensitive indicators of

exposure to short-term stressors, probably reflecting adaptive responses to challenge, and so good welfare.

Increased gill filament development and decreased hyperplasia, as seen during autumn grading, could be an adaptive response to increased need to extract oxygen from the water; however, the time scale is short for such morphological responses, so better survival of fish with well developed, exposed gills is also possible. The fall in gill surface area and rise in hyperplasia observed over summer and the rise in both hyperplasia and mucous cell production observed in carp after crowding in concrete tanks could be an adaptation to poor water quality, but could also be the result of differential mortality of fish with large, exposed gills in these conditions. Thus the extent of development of the respiratory surface and the extent to which this is protected by epithelial cells and mucus could be seen as adaptive responses over a slightly longer time scale to the need to take up oxygen (in cases where there is extensive exposed lamellar surface) or the need to protect the gills and the fish against poor water quality (in cases where there is reduced exposed surface). Arguablly the first condition indicates good welfare in terms of ability to adapt to challenge, while the second reflects poor welfare in medium-term to long-term exposure to poor water quality.

Increased damage to the body and fins, during grading and in fish held in concrete tanks, and increased gill damage, in spring grading and over summer, would seem clearly to indicate poor welfare. Falls in these variables could indicate good welfare, but could also be the result of enhanced mortality of injured fish. Falls in production indicators (weight, length, condition) as seen during grading and crowding and suggest stress-induced mobilisation of energy reserves and/or reduction in food intake of sufficient severity/duration to suggest impaired welfare, defined in terms of ability to cope. Increases in these same variables may imply good welfare, but may indicate enhanced mortality in fish with poor nutrient reserves, especially in the case of length, since fish do not de-grow.

6.5 Conclusions

This study emphasises the difficulty of interpreting changes in potential welfare indicators between destructive sampling sessions, as opposed to repeated measures taken from the same identified individuals, especially when it is not possible to collect accurate data on

mortality rates. However, the results allow some conclusions to be drawn about the potential effects of husbandry practice on the welfare of semi-intensively farmed common carp. According to these interpretations, at spring and autumn grading and pre-harvest crowding carp experience an intense but short-term challenge that triggers adaptive primary and secondary stress responses. These processes also result in impaired welfare in terms of a fall in weight and condition and, in the case of grading, an increase in body damage. The changes observed over the summer are impossible to interpret, given the very high disease-induced mortality rates that occurred during this period. Some at least of the changes observed during the period that the carp were held in concrete tanks could also be due to differential mortality, but the fall in body weight and the rise in hyperplasia and mucous cell number indicate poor welfare induced by chronic exposure to poor conditions. Infestation with *Argulus spp* causes a physiological stress response, but has no effect on body weight, length or condition.

Overall, the results suggest that, combined with measures of primary and secondary stress responses (cortisol, glucose/lactate and blood respiratory status), of production variables (weight, length and condition) and of physical damage, quantification of the fine structure of fish gills can throw an additional, complementary light on fish welfare, informing of changes over a medium time (5 days rather than hours on the one hand or weeks on the other) scale. Such measures can reflect both adaptive responses to immediate oxygen requirements and protective responses to exposure to poor water quality.

CHAPTER 7

GENERAL DISCUSSION

7 General discussion

7.1 Reiteration of the aims of this thesis

The aims of this thesis were to: 1) develop methods for measuring within fish species variation in the status and development of the respiratory gill surface area and to compare these to existing techniques used for this purpose; 2) discover and measure differences in the gill fine structure, using light and scanning electron microscopy; 3) connect development and fine structure of gill within-species with different tropic polymorphisms and with different behaviour coping strategy and 4) examine the correlation between gill development and microstructure and other welfare indicators in common carp exposed to different levels of temperature and oxygen, and in common carp of extensively farmed exposed to a variety of husbandry pressures through production over one year.

7.2 Quantifying variation in respiratory development and gill microstructure

The Arctic charr, brown trout and common carp used in this study all have four functional gill arches with gill filaments, all of which bear secondary lamellae. The strategy for studying variation in these respiratory structures was to collect measurements of all the variables that might contribute to the size of the respiratory surface (arch length, number and length of the gill filaments and the number, length and spacing of the secondary lamellae) for all 4 gill arches and then to use successive principal components analyses to examine the relationships among these variables and to quantify their development, allowing first for differences in body size. In other words, the data were allowed to "speak for themselves"; this is opposed to published studies in which gill surface area is estimated geometrically, from first principles.

Using the study of Arctic charr by way of example (Chapter 2), the first component for each analysis accounted for more than 70% of the total variation, had positive loadings for all variables and so represented an index of overall, length-independent variation in the relevant structure (gill arches, filaments and secondary lamellae). Principal components

analysis of these three derived variables produced an index of overall development of the respiratory surfaces in these fish that was significantly related to gill surface area estimated geometrically. Arguably, therefore the two approaches produce comparable results. However, examination of the additional components for each structure highlights additional potentially interesting independent dimensions of variability. For example, for gill arches and filaments, an additional component reflected differential development of the front and back of the head, while for the gill filaments and to some extent the secondary lamellae, other components opposed length to number, suggesting that different ways of getting larger areas may not be equivalent. Levels of asymmetry were also calculated for gill arch length, which was established to be a highly repeatable measure) using the equation FA= (XR - XL) / Max (XR, XL). Principal components analysis of asymmetry scores for all 4 gill arches identified an index of overall asymmetry, accounting for 61% of the total variance. In several fish species, levels of asymmetry in gill structures are particularly sensitive indicators of exposure to stressful conditions (Ayoada *et al.,* 2004; Jagoe and Haines 1985). The fact that levels of overall gill arch asymmetry are significantly higher in charr of the autumn spawning morph from Loch Awe, which are known from otolith analysis to have faster early rates of growth, supports this view, given the known costs of fast growth in fish (Arnott *et al.* 2006).

In addition to gill morphometrics, difference in respiratory function was also assessed by making casts of the gill cavity for salmonids (Chapter 2) and carp (Chapter 3). This method was developed and used by Okuda *et al*., (2002) to measure buccal cavity volume on cardinal fish (*Apogon doederleini*) and developed here to measure gill cavity volume as well. Still concentrating on Arctic charr, gill cavity volume estimated in this way was a significant predictor of estimated gill surface area, accounting for 35% of the variation in estimated gill surface. Estimating respiratory function by the silicon mould method is much quicker than estimating gill area based on gill morphometrics, estimated times being 2 minute and 60 minutes respectively. Although the quicker method is only a weak predictor of gill surface area, for some purposes this is sufficiently accurate; for example, differences between the spring and autumn spawning morphs of charr were significant for both gill cavity volume ($P < 0.04$) and estimated gill area ($P < 0.004$). Thus the method could be used effectively when large samples are to be processed, providing expected differences are sufficiently large.

The respiratory structures of fish, though highly complex, are much more regular in form than are those of other animal groups (Hughes and Morgan, 1973), the lungs of birds and

mammals, for example, and the tracheal systems of arthropods being much more irregular. In addition, in contrast to the effectively external position of fish gills, the respiratory structures of many other animals are often deeply embedded within the body of the animal concerned. The complex nature and embedded position of the respiratory structures in other animal groups makes estimating functional respiratory volume from morphometrics much more complicated. For example, the respiratory system of spiders consists of a pair of book lungs and poorly developed systems of respiratory tubes or tracheae. The volume of the lungs and of tracheae is often estimated by measuring their total cross-sectional area in 10 equidistiant sections from both lung and trachea (using a light box and drawing tube) and multiplying this by the distance between the sections. Using this method, Schmitz and Perry, (2002) were able to show that, while lung volume is similar in wolf spiders and the more active jumping spiders, the latter group posses a much better developed tracheal system. By way of an example from the huge mammalian literature, to estimate functional lung volume in giraffes, lung volume was estimated from a general known allometric relationship between lung mass and lung volume in mammals. Estimated dead space, calculated from standardised measurements of trachea length and trachea diameter using the geometric formula for the volume of a cylinder. Among other things, this analysis showed that the static lung volume of giraffes is smaller than expected for an animal of their body mass and that this may potentially limit aerobic capacity during exercise (Mitchell and Skinner 2011). Even with access to complex, expensive machinery, estimating lung volume in mammals remains complex and difficult. For example, in order to estimate the lumen wall area in the lungs of pigs, tubes of various known sizes were embedded in foam and scanned using high resolution computer tomography to provide references points for estimating lumen area in preserved lungs (King *et al.,* 2000).

In cases where suitable tissue was available (specifically for the common carp used in Chapter 3 and the brown trout used in Chapter 4) light and electron microscopy were used to examine and quantify variation in gill structure at the cellular level. Several measures have been used to quantify such variation, for example in studies of responses to fluctuating water quality, including oxygen and temperature (Sollid *et al.,* 2003; Turko *et al.,* 2011). In the present study, variation at the cellular level was quantified by measuring the extent to which the secondary lamellae were embedded in epithelial cells (hyperplasia), which sometimes completely fills up spaces between secondary lamellae. Percentage hyperplasia was estimated by relating the height of the layers of epithelial cells between two lamellae to the mean height to those lamellae (Figure7.1a). Higher levels of hyperplasia may serve to increase the barrier between water and blood for the diffusion of

respiratory gases and consequently decrease oxygen uptake and carbon dioxide excretion but also ion loss (Sakuragui *et al.,* 2003). Increased layers of mucous over the gills may serve a similar function, so variation in respiratory function at the cellular level was also estimated by counting the total number of mucous cells per lamella (Figure 7.1b).

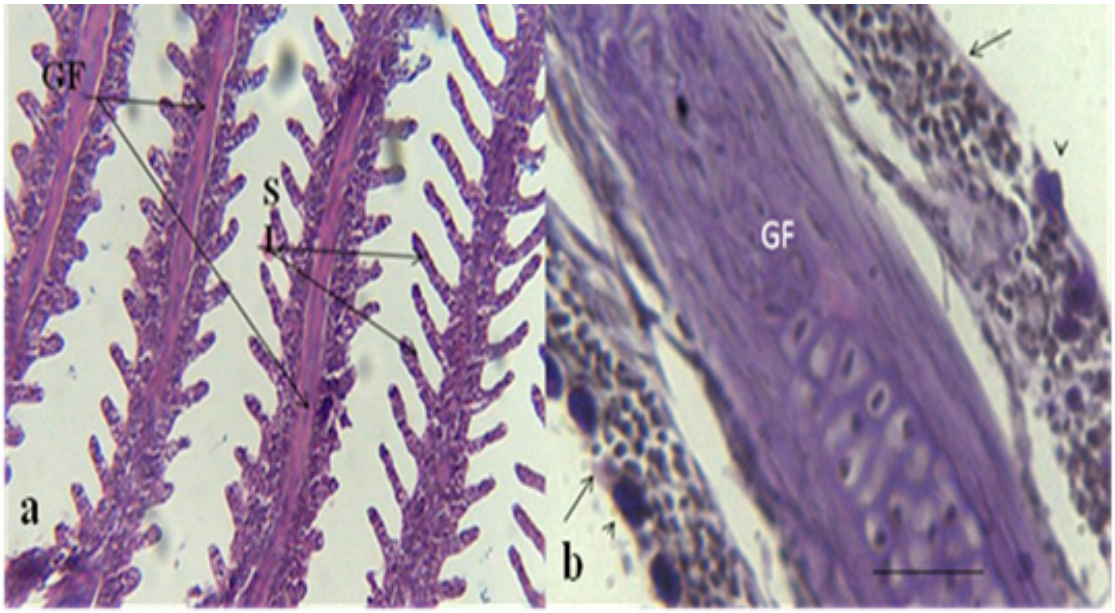


Figure 7.1a and b. Light micrograph of fish gill filament (GF) sectioned longitudinally showing gill filament (GF), gill secondary lamellae (S), gill filament (GF), mucous cell (arrowhead) and gill hyperplasia (arrows), bar= 0.4 mm (H, E and PAS stain and 100X magnification).

7.3 Sources of variation in gill development and microstructure

Defferences in the function and structure of respiratory system between different animals have been studied for many years, and "has become important in relation to comparative quantitative study of gaseous and ionic exchange" (Hughes, 1984). The animal's respiratory surfaces area divides the internal and external media, as well as forming the sites of essential gas exchange (Hughes and Morgan, 1973). At the species level the morphometric measures relate to whether the animal concerned has an active/inactive life style. Consequently the structure of the respiratory organ is probably a compromise between the need for gas exchange and a need to reduce ion and water gain or loss (Sollid *et al.,* 2003).

7.3.1 Adaptive variation in gill development

The structure and morphometrics of the gills is a compromise between the mode of life and metabolic requirements. Gill dimensions, including the length and large quantity of gill filaments, the number of gill lamellae on the filaments, and lamellar bilateral surface area, are altered by selective factors to increase gill surface area and increase oxygen uptake (Wegner *et al.,* 2010a). On the other hand, a greater number of secondary lamellae reduce the spaces between them. Studies of the gill structure and morphometrics and some respiratory properties of the blood provide important indications as to the probable mode of life of fish.

7.3.2 Species

Animals of different species usually show differences in gill morphometrics and structures. These changes might be related with the characteristic of species, the mode of life, the degree of activity and the fish habit and habitat (Hughes and Morgan, 1973). Table 7.1 summarizes some of the morphological features of the three species studied in the present thesis.

Table 7.1. A comparison of mean gill arch length, mean gill filament length, mean gill filament number, mean secondary lamellae length, mean secondary lamellae number, mean gill surface area, mean mucus cell number and mean percentage of gill hyperplasia (all these divided by body length), between the brown trout, Arctic charr and common carp used in different parts of the present thesis.

Species	Type	Body length (cm)	Body weight (gm)	Relative arch length	Relative filament length	Relative filament number	Relative lamella length	Lamellae per mm	Relative estimated gill surface area	Mucous cell number per lamella	Percentage hyperplasia
Trout	Farmed	18.11	57.63	0.27	0.21	7.80	0.009	2.2	46.21	0.08	1.96
	Wild	14.40		0.11		4.93					
	Overall	16.23		0.19		6.33					
Charr	Autumn	18.10		0.11	0.18	2.60	0.004	2.5	47.02		
	Spring	19.60		0.12	0.20	3.60	0.005	3.4	56.73		
	Overall	1 8.90		0.12	0.19	3.60	0.004	3.1	51.90		
Carp	Southern England	10.37	20.54	0.12	0.26	5.03	0.010	3.3	40.46	0.25	3.83
	Poland	27.10	789.64					2.2		0.02	0.73
	Overall	23.81	405.10					2.8		0.14	2.28

The data were not collected for the purposes of cross species comparison, and it was not possible to measure all variables for all samples. Making those species comparisons that are possible, relative arch length is somewhat higher in farmed trout compared to wild charr and carp, perhaps reflecting a more active life style in trout. Relative filament length was greater in (English) carp than in charr, perhaps representing an adaptation to generally poorer water quality. Relative filament number and relative lamella length was higher in trout and carp than in charr, perhaps reflecting adaptation for a more active lifestyle in trout, but lower oxygen levels in carp. Overall estimated gill area was higher in the two salmonid species than in carp, possibly reflecting a relatively more active life style of these species.

7.3.3 Population/provenance

For logistic reasons, the brown trout used in Chapters 3 and 4 came from wild and farmed stock respectively. Trout from these two sources were of the same approximate size, but in farmed trout the gill arches were approximately twice as long and filament numbers were some 60% higher than in wild trout, allowing for body length. Many differences have been reported between farmed and wild salmonid fishes (Norris *et al.,* 2000). These include genetic differences resulting from the process of domestication (Clifford *et al.,* 1998) and the results of differential experience during wild and captive rearing (Huntingford, 2004). Behavioural differences between farmed and wild salmonids including differences in breeding behavior (Fleming and Gross, 1993) and in aggressiveness (Fleming and Gross, 1993). The differences in gill development between the wild and farmed brown trout used in the present study could be the result of reversible adaptations to the lower oxygen levels experienced by farmed fish.

The common carp used in Chapter 3 came from a carp farm in the south of England and were transported to Glasgow for the behavioural and morphological studies, where they were housed in a recirculation system. Those use in Chapter 5 and 6 came from established farmed stocks at the Polish National Academy's Institute of Ichthyology, Chybie, Poland and were either transported a short distance from rearing pond to laboratory where they were housed in flow-though tanks (Chapter 5) or sampled directly from the holding ponds (Chapter 6). The markedly higher levels of hyperplasia and numbers of mucous cells could

indicate that the carp studied in Glasgow had experienced higher levels of stress than those studied in Poland.

7.3.4 Body size

In all of the analyses of gill structure and microstructure in this study, all variables were corrected for fish length before making comparisons between fish, since the focus of the studies was differences in gill development that were independent of overall size. It is therefore not surprising that no relationships were found between body size and the derived gill measures (size corrected) for any of the study species.

7.3.5 Gender

Gender differences were only investigated in the common carp studied in Chapter 6 and only a few significant differences were found between male and female fish. Thus, males had higher blood respiratory status than did females, indicating more blood cells generally and higher haemoglobin concentrations; a comparable result was found for roach (*Rutilus rutilus*, Kortet,2003). This might reflect higher stress levels and/or a more active mode of life in males than in females. Male carp also had heavier infestations with the protozoan ectoparasite *Ichthyothiriosis multifilis* (whitespot) than did females. The results agree with the general finding that males are more susceptible to parasitism than females, perhaps because testosterone in male fish compromises the defensive systems.

7.3.6 Trophic ecology

The study described in Chapter 2 showed that the spring spawning morph of charr from Loch Awe has a better developed respiratory structure than did the autumn morph; these differences, which are summarized in Table 7.2, could be related to the different foraging habitat of the two morphs. The spring morph, which has relatively few short gill rakers and probably specializes in large benthic prey, may experience lower oxygen levels than do the

autumn morph, which has more numerous, longer rakers and probably specializes on small prey captured in the water column. Low oxygen levels in deeper water close to substrate (Kunzmann, 1990) could explain the greater development of respiratory surfaces in the spring morph in Arctic charr. Larger gill surface areas in fish inhabiting deeper water has been described in *Pleuragramma antaracticum* (Hubold and Ekau, 1987), which live close to the bottom at 500-700 m and have large gill surface area. However, the present study is the first to identify differences in respiratory structures between tropic morphs.

Table 7.2. A comparison of habitat, growth, fluctuating asymmetry, gill raker number, gill raker length, gill surface area, gill cavity volume and buccal cavity volume between two morphs of Arctic charr.

Fish morph	Feeding habitat	Growth	Asymmetry	Raker number	Raker length	Estimated surface area	Gill cavity volume	Buccal cavity volume
Autumn	Mid-water	Fast	High	large	large	Small	small	small
Spring	Benthic	Slow	Low	small	small	Large	large	large
% difference		3%	30%	1%	18%	32%	38%	29%

The two morphs of Arctic charr also differed in levels of asymmetry in gill arch length, with the autumn form having markedly higher levels. This could be the result of faster rates of growth in the autumn morphs, especially at early stages, which could in turn be related to food availability, differences in water temperature and/or genetic variation. Fast growth is costly (Arnott et al. 2006; Jobling 1981, 1994) and this may make achieving symmetry difficult. Differences in asymmetry between morphs might also reflect differential exposure to environmental stresses (Al-Mamry *et al.,* 2011), especially if the developmental period of the gills coincides with the presence of adverse environmental events (Jawad *et al.,* 2010; Al-Mamry *et al.,* 2011). Since information on the quality of the water in which the two morphs live was not available, it is impossible to assess this possibility at present. High asymmetry values for body and gill structures in fish have been recorded in several freshwater and seawater fish species (Al-Hassan *et al.,* 1990; Jawad, *et al.,* 2001; Al-Mamry *et al.,* 2011).

7.3.7 The effects of stress coping style in respiratory function

The method used to screen common carp (Chapter 3) and brown trout (Chapter 4) for coping strategy involved monitoring their response when exposed to a novel, potentially dangerous environment. This is a well-established method for assessing risk-taking in fish

and other animal groups (Huntingford and Coyle, 2007) and in carp at least predicts stress responsiveness (Huntingford *et al.,* 2010). Emergence times over 3 successive tests were sufficiently consistent at the individual level to allow the fish to be classified as risk-takers (fish that consistently emerged from shelter quickly) and risk-avoiders (fish that consistently failed to emerge during the test). The remaining fish were classified as an intermediate group, although these fish would perhaps be better characterised as plastic, changing from slow to fast emergence over successive trials. These results suggest that common carp and brown trout classified as intermediate dependent on the novel environment test may have the special behavioural characteristic of flexibility. In chapter 3, the classification of carp into three risk-taking groups based on the novel environment test was validated by the fact that fish classified as risk-avoiders took longer than intermediate and risk-takers to settle and start foraging in a subsequent learning test (Mesquita*,* 2010).

In Chapters 3 and 4, several differences were found in the respiratory structures of fish with different coping styles, risk avoiders having relatively larger respiratory structures than risk-avoiding fish, especially in carp, with the intermediate group usually falling in between. Table 7.3 summarizes the morphological differences between fish with different coping strategies in the two study species. In carp, the differences in gill area seem to stem from differences in all the important respiratory structures, with both filaments and lamellae being fewer and shorter in risk avoiding fish. In trout, the differences in overall gill area mainly depend on differences in the number of gill filaments and, somewhat, on lamellar length. In both species, additional differences in respiratory function between coping styles arises from the fact that a greater percentage of the gill surface is exposed rather than being covered by epithelial cells and that risk-avoiding, reactive fish have more cells for producing the mucous with which the gills may be covered.

Table 7.3. Comparison of the mean respiratory variables divided body length in common carp and brown trout with three risk-taking phenotypes.

Species	Coping strategy	Filament length (mm)	Filament number/mm	Lamella length (mm)	Lamella number/mm	Gill surface area/mm^2
Carp	Risk-taking	0.29	5.12	0.020	3.84	49.40
	Intermediate	0.26	5.04	0.014	3.29	43.41
	Risk-avoiding	0.23	4.96	0.012	3.10	28.21
Trout	Risk-taking	0.21	3.91	0.010	1.95	50.71
	Intermediate	0.21	3.52	0.009	2.19	48.73
	Risk-avoiding	0.20	3.33	0.008	2.43	39.18

This study is the first to support the suggestion that development of the respiratory surface is influenced by stress coping strategy. In functional terms, the larger exposed gill surface area in proactive fish may be explained by the fact that, in carp at least, proactive fish have a higher resting metabolic rate (Huntingford *et al.,* 2010) and hence a greater need for oxygen than reactive fish. The difference in resting metabolic rate between proactive and reactive carp reported by Huntingford *et al.,* (2010) was *ca* 17%, compared to a difference in gill surface area of *ca* 42%. These results agree with the general finding that increased gill respiratory surface area is connected strongly with the activities and lives of particular species (Satora and Romek, 2010); fish with high metabolic rates usually have gill specializations that allow easy gas transfer. However the larger gill surface area required to meet the greater oxygen needs of proactive fish also represents a greater risk of ion loss in freshwater fish and uptake of pollutants. This is of fundamental biological significance because it represents an additional cost to fish adopting a proactive stress coping strategy. It is of practical significance because it suggests that proactive fish (which otherwise adapt well to intensive husbandry) may be more susceptible than reactive fish to poor water quality.

In spite of this likelihood, in Chapter 5 differences were found between proactive and reactive fish exposed at high densities to different environmental conditions. By the end of the trial, reactive fish were both shorter and lighter than proactive fish, suggesting that risk-taking, proactive fish flourish better in intensive production that do reactive fish, as might be expected if the latter are more easily stressed. Body weight and length are known to be related to water quality, feeding program and exposure to stressful conditions (Chapter 6). For just two variables, (haematocrit and lamellar number) there was an interaction between coping style and temperature. At the higher temperature, reactive fish had higher haematocrit scores and strikingly more secondary lamellae than did proactive fish. Both these differences were reversed at the lower temperature, perhaps because proactive fish need to extract more oxygen from the water and to transport this more efficiently at lower temperatures.

7.3.8 The effect of immediate environmental conditions on respiratory function

Many of the gill morphometric measures reported in this thesis were related to body length (Chapters 2,3 and 4) and so would have varied if growth rates were affected by recent environmental conditions. In addition, gill structure was directly affected by water temperature and dissolved oxygen levels (Chapter 5) and by various husbandry practices (Chapter 6). In Chapter 5, although few effects of oxygen and temperature were found on the number or size of the secondary lamellae, carp exposed to low water temperature and low oxygen levels had a higher percentage of hyperplasia and more mucus cells than those exposed to high water temperature. This could be as a stress response to low temperature and oxygen levels. Generally, these results agree with the hypothesis that fish have can remodel their gills in response to water conditions (Chapter 5) or different seasons (Chapter 6) by developing more mucus cells and more hyperplasia, as well as distances between secondary lamellae and filaments (Sollid *et al.,* 2005; Moron *et al.,* 2009). Hyperplasia may serve a defensive purpose by reducing gill respiratory function, decreasing gill surface area (Chapter 3 and 4) and affecting secondary lamellar development (Chapter 6).

7.4 Gill development and other welfare indicators

Data on potential welfare indicators collected through the study of pond-reared common carp described in Chapter 6 are easiest to understand in the case of short-term responses to specific husbandry events. For instance, the increases in plasma cortisol and glucose/lactate as seen at grading and crowding might be seen as short-term adaptive responses to challenge and so can indicate positive welfare in conditions of capability of cope. In contrast, physical damage to the fish and loss of weight and condition at grading and in fish crowded in existing tanks suggest that these husbandry practices can reduce fish welfare, as reflected in effective functioning. Biochemistry of blood, blood cortisol and blood respiratory can also give information about longer-term stress response associated with changes in condition factor and fish health (Lermen *et al.,* 2004). For example, in the present study, during the summer the carp showed relatively low blood levels of cortisol

and of lactate/ glucose, suggesting perhaps that they were not unduly stressed by the temperatures prevailing during the summer concerned. This agrees with other findings for common carp (Chen *et al.,* 1995).

The data collected on gill structure complement these commonly-used welfare indicators. The degree of growth of the gill surface and the degree to which this is occluded by epithelial cells and mucus might be seen as adaptive responses to changes in the requirement for taking up oxygen or in the need to protect the gills (and hence the fish) against low water quality. Arguably the first condition indicates positive welfare in conditions of capability to adapt to challenge, while the second condition may reflect negative welfare caused by medium to long-term exposure to water of low quality. Few if any changes in gills condition were observed that could be unequivocally understood as a direct response to grading and crowding. However changes in several gill measures were observed over the summer and in carp crowded in concrete tanks. Lack of longitudinal information on known individuals makes these data hard to identifiy, particularly since mortalities were high. In general, though, the results presented in this thesis suggest quantification of the fine structure of fish gills can be combined with measures of body size, nutrient reserves and physical injury, as well as of primary and secondary stress responses, to throw additional light on changing fish welfare over a medium time scale.

7.5 Limitations of the reported studies

The studies described in this thesis had a number of limitations, some of which were unavoidable. In Chapter 2, only frozen specimens were available, making histological studies impossible, and the sample sizes were predetermined and quite small. In addition, no data were available on the stomach contents of individual Arctic charr within the two morphs, making it impossible to carry out an analysis of gill structure in relation to trophic biology at the individual level, which would have been both powerful and interesting. In Chapter 4, to reduce the need to extensive marking and thus to minimize stress, screening of brown trout for coping strategy was carried out at a group level, providing a relative crude characterization. In addition, the fish showed some aggressive behavior in the novel environment test and screening was carried out in a laboratory shared by other researchers. Any intrinsic differences in risk-taking may have been obscured by the effects of such stressors on the behavior of the fish. Although the dataset used for the analyses reported in

Chapters 5 and 6 included detailed information on many potential welfare indicators, the gill material was restricted to histological sections through the gill filaments, limiting the analyses to measures of lamella length and number, mucous cell numbers, percentage hyperplasia as well as the incidence of parasites and lamellar damage. Finally, in the study described in Chapter 6 the fact that high mortalities occurred at several points makes it impossible to distinguish between direct effects of the relevant stressors on welfare indicators on the one hand and differential mortality on the other.

7.6 Areas for future research

An interesting point from the results is that fish were classified as having an intermediate coping strategy in Chapters 3, 4 and 5 as a result of a middling emergence times, although they may better be classified as flexible group. This group of fish may have interesting characteristics that would be worth exploring. In addition, the interesting finding that in fish classified by coping strategy, the risk-taking group had a larger gill surface area than did the risk-avoiding group. This could mean that risk-taking fish have greater exposure to pollutants in the water than do those in the risk-avoiding group. In the future, it could be interesting to examine this possibility, by offering risk-avoiding and risk-taking fish a choice of water with different oxygen levels and observe their choices.

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