Utilization of dried blood spots for assessing Dusky kob (*Argyrosomus japonicus*) glycaemia and metabolome in South African aquaculture

By

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As the candidate's supervisors, we have approved this dissertation for submission.

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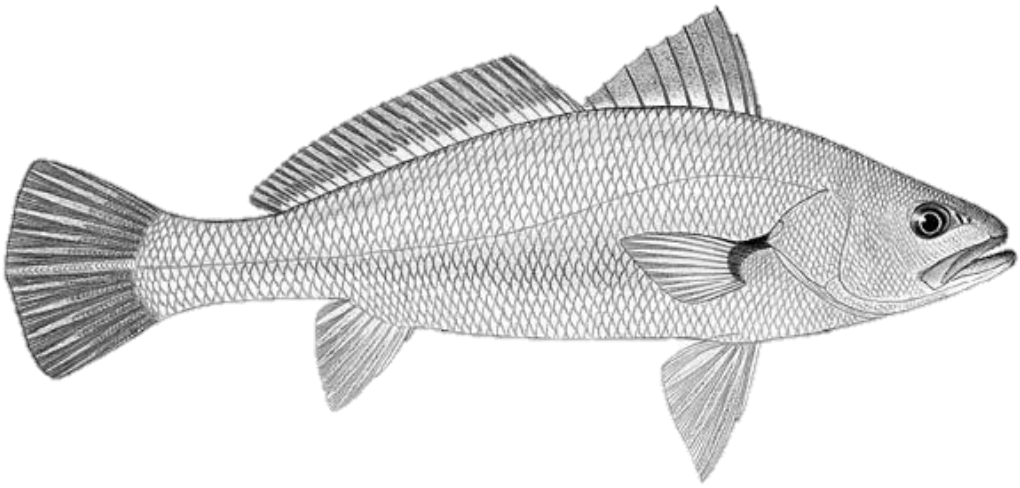

Abstract

Dusky kob (*Argyrosomus japonicus*) is an important marine fish in South African aquaculture, and has the potential to become commercially significant. The continuous growth of Dusky kob culture faces challenges with regards to fish health. There is currently a lack of on-farm tools for fish health assessment and Dusky kob are known to be prone to parasitic infections.

Fish blood glucose is a reliable indicator of biological and environmental stress; however, methods of glycaemia diagnosis are often lab based and cannot be conducted rapidly. This limits the use of glucose to assess the condition of fish on-site. Furthermore, the gill parasite *Diplectanum oliveri* continuously affects Dusky kob and the only know method of detection is microscopic analysis which requires destructive sampling. The drawbacks of fish health assessment are further amplified by the lack of reliable methods of sample collection, storage and transportation.

Conventional methods of blood sample collection have limitations, including complex protocols (collection and storage), sample degradation during storage and large volumes required for analysis. Additionally, blood samples (plasma, serum and whole blood) are often restricted to single use due to sample degradation, thus do not allow for repeated analysis. Dried blood spots (DBS) offer an alternative method of sample collection, with benefits including ease of use (collection and handling), storage and transportation. Furthermore, DBS require relatively small volumes and ensure sample integrity is maintained due to the elimination of a liquid medium thus inhibiting metabolic reactions. DBS further allow for repeated analyses across a wide range of blood parameters and numerous assays. The use of DBS is currently limited to human health research, however, can be beneficial in fish health assessment and biomarker discovery in aquaculture.

In the present study, the utility of DBS as (1) a reliable method of blood collection and (2) an effective medium for biomarker discovery in fish was investigated. Dusky kob DBS were collected from four farms namely; Mtunzini Fish Farm (currently Zini Fish Farms, KwaZulu-Natal), Oceanwise (currently Ocean Choice), Pure Ocean (both Eastern Cape) and Blue Cap (Western Cape) fish farms between February – May 2015. The use of a hand-held diabetic glucometer as a reliable tool for on farm glucose measurement

tested against laboratory based enzymatic analyses of DBS, plasma and whole blood. Lastly, the application of metabolomics analyses of Dusky kob DBS was examined as a potential for a non-destructive alternative method for the detection of parasitic infections.

A consistent over-estimation of glucose by DBS was observed, while the use of a diabetic glucometer was shown to be a reliable tool for on-farm glucose measurements. The accuracy of the glucometer was evident by the correlation with plasma glucose (R^2 = 0.973). Plasma is the recommended medium for glucose analysis since the removal of red blood cells inhibits glycolysis. Additionally, targeted metabolomic analysis of DBS by LC-MS and GC-MS identified 53 metabolites. Six amino acids (citrulline, glutamine, lysine, methionine, phenylalanine and proline) were significantly altered in accordance to parasite intensities and/or geographical distributions.

The results indicate that hand-held glucometers can be used on-farm for accurate preliminary measures of glucose, which is further beneficial for rapidity and routine analysis. Continuous measurement of glucose can aid in detecting hyper- or hypoglycaemia. The ease of use provided by DBS is essential for biomarker discovery and fish health assessment. In addition to simple storage and transportation, DBS also ensure the stability of blood parameters including metabolites. Metabolomics analysis offer an essential platform for the early detection of parasites infecting farmed fish and eliminates the need for destructive sampling. The examination of metabolites reveals compounds that are essential in the innate and adaptive immune response of fish during infection. Understanding the roles of these compounds can be used to develop and implement corrective measures.

Preface and Declarations

The work contained in this dissertation was carried out in the School of Life Sciences, University of KwaZulu-Natal (Westville Campus) from January 2015 to December 2017, under the supervision of Dr. Andre Vosloo and Dr Kevin Christison.

The study represents original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

As the candidate's supervisors, we have approved this dissertation for submission.

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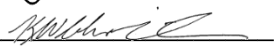
Signed:  Name: Dr Kevin W. Christison Date: 2018-03-07

DECLARATION 1 - PLAGIARISM

I, **Thabani Irvin Mdlalose** declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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    a. Their words have been re-written but the general information attributed to them has been referenced
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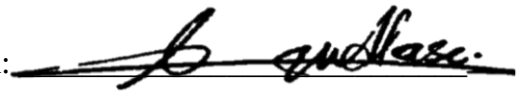
5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed: 

DECLARATION 2 - PUBLICATIONS

Publication 1 (Appendix 1):

Mdlalose, T. I, Christison, K. W, & Vosloo, A. (2017). Use of dried blood spots (DBS) to diagnose hyper-and hypoglycaemia in farmed Dusky kob *Argyrosomus japonicus* in South Africa. *Aquaculture, 473*, 43-50.

Author contributions:

Mdlalose, T. I.: Conceptualisation, experimental/laboratory work, paper write up

Christison, K. W.: Sample collection and revision

Vosloo, A.: Conceptualisation, statistical analysis, revision

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I dedicate this work to my mother, Nomasonto Joyce Mdlalose, you may not understand much of what I do, but you always keep me in your prayers and wish me well.

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Table of Contents

List of Figures

Chapter 1:

Chapter 2:

Chapter 3:

List of Tables

Chapter 1:

Chapter 3:

Chapter 1: General Introduction and Literature Review

1.1 South African Aquaculture: Brief Overview

Fish and finfish farming, often referred to as aquaculture (marine or freshwater) came into existence because of declining ocean fish stocks globally. According to Naylor *et al.* (2000) global aquaculture production doubled in size and value between 1987 and 1997, and farmed fish production currently accounts for over 25% of all fish available for human consumption. Due to the continued growth of the human population, the reliance on farmed fish production is essential to meeting nutritional (protein) demands. While global aquaculture has risen rapidly, in South Africa (SA) the growth of the industry has been slow and currently remains underdeveloped. Factors such as unfavourable climate, poor understanding of environmental regulations and sufficient affordable fish production by the marine catch fisheries sector have been indicated to be responsible for the slow growth of local aquaculture (Day *et al.*, 2016)

In 2013, the total aquaculture production in SA was 4802 tons with the marine aquaculture industry contributing 2985 tons and freshwater aquaculture production being 1816 tons (DAFF, 2014). Numerous species present the potential of being successfully produced under aquaculture conditions, however, these currently remain under research with only a few species produced commercially.

Table 1: Freshwater and marine species cultured in South African aquaculture systems as recognised by the Department of Agriculture, Forestry and Fisheries (DAFF).

Habitat	Common name	Species name	Group
Freshwater	Rainbow trout	*Onchorynchus mykiss*	Fish
	Brown trout	*Salmo trutta*	
	Mozambique tilapia	*Oreochromis mossambicus*	
	Nile tilapia	*Oreochromis niloticus*	
	Blue tilapia	*Oreochromis rendalli*	
	Sharptooth catfish	*Clarias gariepinus*	
	Common carp	*Cyprinus carpio*	
	Hairy marron	*Cherax tenuimanus*	Crustacean
Marine	Abalone (Perlemoen)	*Haliotis midae*	Mollusc
	Pacific oyster	*Crassostrea gigas*	
	Mediterranean mussel	*Mytilus galloprovincialis*	
	Black Mussel	*Chromomytilus meridionalis*	
	Dusky kob	*Argyrosomus japonicus*	Fish

1.2 *Argyrosomus japonicus* (Temminck & Schlegel, 1843)

1.2.1 General Overview

Commonly known as Dusky kob and Mulloway in South Africa and Australia respectively, *Argyrosomus japonicus* (formerly misidentified as *Argyrosomus hololepidotus* (Griffiths & Hecht, 1995)) is a marine finfish with a maximum recorded size of 75 kg (Fitzgibbon *et al.*, 2007). *A. japonicus* belongs to the Sciaenidae family comprising approximately 70 genera and 270 species globally (Silberschneider *et al.*, 2005). Sciaenids are mostly demersal fishes distributed in fresh, estuarine and marine waters in the Atlantic, Indian and Pacific Oceans. Several sciaenid species form spawning regions in estuaries and their recruitment success is

attributed to freshwater inflows to these estuaries (Griffiths, 1996). This has made sciaenids characteristic of long life spans and late maturity (Ferguson *et al.*, 2014).

Late maturity in sciaenids is an evolutionary adaptation resulting from low mortality of juveniles (Griffiths, 1996). Due to their delayed maturity Sciaenid species have suffered population declines globally, resulting mainly from overfishing targeting both adults and juveniles in spawning aggregations (Rowell *et al.*, 2008). Similar to other sciaenid species *A. japonicus* are susceptible to anthropogenic impacts due their high estuarine dependence, late maturity and elongated life span (Griffiths, 1997; Ferguson *et al.*, 2014). Reports of *A. japonicus* overfishing in South Africa have been documented, with the species classified as "recruitment overfished" (Griffiths, 1997).

1.2.2 Growth and Reproduction

Dusky kob reproduction occurs via the production of pelagic eggs approximately 938±24 µm in diameter (Silberschneider & Gray, 2008). Larvae hatch between 28-30 h post spawning at 23°C and are 2.2-2.3 mm tail length (TL) (Battaglene & Talbot, 1994). Small juveniles at length 30-150 mm TL in the wild are only found in estuaries while larger juveniles (800-1000 mm TL) predominantly occupy inshore marine waters (Mann, 2013). Growth and sexual maturity in *A. japonicus* varies in the different regions it occupies. According to Griffiths (1996) estuarine recruitment of *A. japonicus* in South Africa occurs at 20-30 mm TL, 4 weeks after hatching. However, some studies have shown that juveniles enter estuaries at 100-200 mm TL and up to up to 1 year after hatching (Silberschneider & Gray, 2008).

According to Griffiths and Hecht (1995), both sexes of *A. japonicus* have rapid growth for the first eight years, after which growth rate slows gradually. Male Dusky kob have a more rapid decrease growth rate than females. The reduction of growth rate in *A. japonicus* often signals sexual maturity. Fish have a common life history adaptation of females having a faster growth rate therefore allowing for increased egg production, thereby increasing larvae production (Roff, 1983; Griffiths & Hecht, 1995). The reduced growth rate shortly after *A. japonicus* become adult suggests that a large proportion of energy is used for reproduction (Griffiths, 1996).

1.2.3 Global Distribution

A. japonicus is a large predatory fish species distributed in both the northern and southern hemisphere. The distribution of dusky kob spreads from southern Australia, Hong Kong, southern Korea, Japan and through to southern Africa (Griffiths & Hecht, 1995; Fielder & Heasman, 2011). Dusky kob is also found in the northwest coast of India and northern Pakistan (Griffiths & Heemstra, 1995).

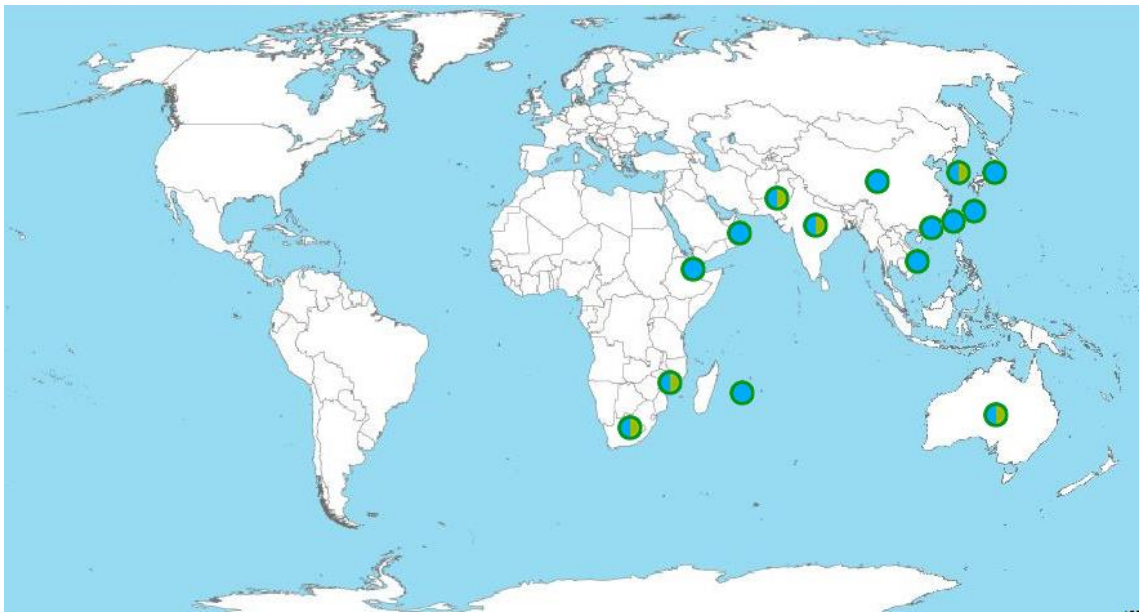

Figure 1: Global distribution of *Argyrosomus japonicus* reported from 15 countries and islands. The environment in which the species occurs is also indicated as either saltwater/marine (blue) and brackish/estuarine (green). (Source: FishBase).

In Southern Africa, wild *A. japonicus* is found from Cape Point through to southern Mozambique with the majority of the fish being abundant between Cape Agulhas and northern KwaZulu-Natal (Griffiths & Heemstra, 1995; Mann, 2013). According to Griffiths (1996) *A. japonicus* are characterized by short migration distances however adult Dusky kob from Western Cape and Eastern Cape are known to migrate to KwaZulu-Natal for spawning between winter and spring.

Palatability and large size were vital to Dusky kob becoming a reliable food source throughout its distribution globally, while it has also gained commercial and fisheries significance in South Africa and Australia. Griffiths (1996) published the first comprehensive life history description of *A. japonicus* with literature prior to that mainly focusing on the occurrence and feeding of the species. Dusky kob is abundant on the east coast of South Africa from the Cape Agulhas to

northern KwaZulu-Natal. This distribution pattern is a result of the species' preference of warmer waters. Due to declining wild stocks globally (Silberschneider *et al.*, 2009; Zilberg *et al.*, 2012), the species has been listed "Red" by the South African Sustainable Seafood Initiative (SASSI) if caught via line fishing and/or trawl.

1.2.4 Commercial Significance of Dusky kob in South Africa and Potential Challenges

Dusky kob is a commercially important fish species in South Africa and throughout its global geographical distribution. Due to the species' natural occurrence and distribution along the Indian and eastern Pacific Ocean, it has become an essential species for recreational and commercial fisheries in South Africa (Griffiths, 1996; Fitzgibbon *et al.*, 2007). This makes studying and understanding the biology of the species crucial in aquaculture and fisheries management and development. *A. japonicus* is farmed both in marine cages and on land saline ponds. Dusky kob possess attributes such as high fertility, relatively fast growth, wide range salinity tolerance and non-cannibalistic behaviour (Fitzgibbon *et al.*, 2007) which make the species suitable for aquaculture. Furthermore, dusky kob is easily marketable and sell for affordable prices (Silberschneider & Gray, 2008).

Aquaculture production of Dusky kob in S.A. increased from approximately 50 tons in 2012 to 122.5 tons in 2014, which is a record high for the South African finfish industry (DAFF, 2014). Meanwhile, the total finfish aquaculture production for Africa was 1.6 million tons in 2014 (Fisheries, 2016). The limited finfish aquaculture production in S.A. further highlights that the industry is still in development and has potential for continued growth. While advancement of aquaculture remains a priority, it essential to address challenges that may hinder growth of the industry. Viral infections on farmed fish are a common occurrence and are known to induce pathologies that result in mass mortalities. For example, commercial production of barramundi (*Lates calcarifer*) in Australia was continually affected by a picorna-like virus (Munday *et al.*, 1992). The virus induced vacuolation in the brains of fish which resulted in larvae and juvenile mass mortalities. Similar viral induced vacuolation of brain nerve cells resulting in mortality have been reported for *L. calcarifer* in India (Parameswaran *et al.*, 2008) and Red drum, *Sciaenops ocellatus*, in South Korea (Oh *et al.*, 2002).

Another major challenge for commercial aquaculture is the induction of diseases and mass mortalities due to bacterial infections. The bacterial bloom of *Aphanizomenon flos-aque* and *Microcystis aeruginosa* induced mass mortalities of silver carp (*Hypophthalmichthys molitrix*), tilapia (*O. niloticus*), catla (*Catla catla*) and common carp (*C. carpio*) on a pond culture system, in Bangladesh (Jewel *et al.*, 2003). Furthermore, fish mass mortality events are not limited to cultured populations. Over 2500 tons of wild mullet (*Liza klunzingeri*) were killed in the Arabian Gulf, in August and September 2001 (Glibert *et al.*, 2002). These deaths were mainly attributed to the bacterium *Streptococcus agalacticiae*. A major downfall for aquaculture is that disease prevalence in wild stocks can be transmitted and amplified in cultured populations (Håstein & Lindstad, 1991; Amos & Thomas, 2002; Lorenzen *et al.*, 2012). This has been observed in farmed gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) which had similar infection patterns as observed from diseased wild fish infected by *Streptococcus iniae* (Zlotkin *et al.*, 1998).

As Dusky kob commercial production is currently in its infancy, knowledge of potential diseases and their impacts is crucial for the continued advancement of the sector in S.A. aquaculture. While studies correlating fish mass mortalities due to viral and bacterial infections have been conducted for many fish species, data on the harmful effects of parasites remains highly unexplored. It is therefore essential that parasitic infections in Dusky kob are examined, together with potential secondary infections that may be induced on the fish and corrective measures be developed to ensure sustained commercial production.

1.2.4.1 Parasite Infection as an Aquaculture Problem

Parasite-host interactions can have adverse effects on the behaviour and physiology of the fish by inducing stress, causing physical damage leading to transmission of secondary infections and lead to mass mortalities of fish stocks. For example, Sheepshead minnows, *Cyprinodon variegatus*, are known hosts of *Ascocotyle panchycystis*, a heterophyid trematode. The parasites are transmitted to the fish from the snail *Litterrodinops monroensis*, where they attach to the gill epithelium and migrate to the bulbus arterious, forming dense cysts (Coleman, 1993). The formation of cysts disrupts normal blood flow in the fish, thus increased blood viscosity reduces muscular activity which in turn affects oxygen transport around the body. Contrary to this,

oxygen consumption of rainbow trout infected by *Cryptobia salmositica* did not differ significantly from non-infected fish, however, reduced aerobic scope for activity and critical swimming speed were observed 3-weeks post-infection (Kumaraguru *et al.*, 1995). Furthermore, significantly lowered blood haematocrit was observed and attributed to haemolysis induced by the parasite. The presence of parasites in fish exposes hosts to immunosuppression, secondary infections and mass mortalities (Barton & Iwama, 1991). Parasitic infections further impair the host tissue which would prompt an energy demand to mount an immune response and subsequent tissue repair (Robar *et al.*, 2011).

Known cases of health issues for Dusky kob have been documented, however, remain limited. Both juvenile and adult *A. japonicus* appear to be susceptible to infection by Monogenean parasites. Sea-cage farmed *A. japonicus* were diagnosed with *Benedenia sciaenae* infestation (Whittington, 1996), while Hayward *et al.* (2007) report gill infection by *Sciaenacotyle sciaenicola*, *Calceostoma glandulosum* and *Caligus* cf. *elongatus* (sea lice). It is therefore essential to study the potential challenges that may arise in Dusky kob farming due to parasitic infections, especially since juvenile and adult *A. japonicus* mass mortalities have been reported resulting from ectoparasitic protozoans and monogenean trematodes (PIRSA, 2003).

1.2.4.2 *Diplectanum oliveri* Williams, 1989 (Monogenea: Diplectanidae)

The continued growth of aquaculture has intensified occurrences of parasitic infections, therefore, multiple parasite-related diseases often resulting in mortalities have been reported in numerous aquaculture facilities around the world (Catalano & Hutson, 2010). Furthermore, translocation of parasites through trade of aquaculture products has been reported globally (Bondad-Reantaso *et al.*, 2005). The direct life cycles of numerous parasite taxa enable them to thrive in culture settings, thus ensures rapid reproduction and increased intensity of infection. Ectoparasites induce physical damage to their hosts, therefore exposing fish to secondary infections (Thoney & Hargis, 1991) while parasitic crustaceans have been reported to be carriers of viral and bacterial infections (Overstreet *et al.*, 2009).

Data on parasites infecting *Argyrosomus* spp. in both aquaculture and in the wild are limited, however they are prone to infection by Monogenean parasites (Pitcher, 2008). Monogenean parasites are flatworms that attach to gills, skin and fins and have been responsible for huge stock losses of farmed fish and their incidence of infection has intensified within the large scale

of global aquaculture. Commonly known as flukes, these parasites can also occur in the rectal cavity, ureter, body cavity and blood vascular system of their host (Reed *et al.*, 2009). Monogenean parasites induce pathology on their host such as erosions, hyperplasia, inflammation, haemorrhage and mucoid exudates (Dezfuli *et al.*, 2007). According to Thoney and Hargis (1991), Monogenean parasites are generally host-specific in the wild, however can infect multiple species in aquaculture settings due to the close confinement of different fish species.

Farmed *Argyrosomus regius* (meagre) have been found with *Sciaenocotyle panceri* infestations in the Mediterranean Sea (Merella *et al.*, 2009; Ternengo *et al.*, 2010). Lethargy, emaciation, gill anaemia and mortality were reported in *A. regius* because of infection by *S. panceri* following four months confinement in sea-cages (Merella *et al.*, 2009). Amin and Christison (2005) described *Neoechinorynchus (Neoechinorynchus) dorsovaginatus* n. sp. *(Acanthocephala: Neoechinorynchidae)* infecting Dusky kob from the Breede River Estuary in South Africa. *Diplectanum oliveri* Williams, 1989 (Monogenea: Diplectanidae) has been reported in both Dusky kob, *A. japonicus* and silver kob, *Argyrosomus inodorus* in South African aquaculture and is regarded as the most prevalent, resulting in fish mortalities (Christison, 2005).

While the description of the life-cycle, attachment, feeding and morphology of *D. oliveri* affecting Dusky kob, together with the potential use of praziquantel as treatment has been studied (Joubert, 2012), methods on detection of parasitic infections and general fish health assessment are yet to be established in South African aquaculture. Furthermore, understanding the consequential pathogenesis and mass mortalities from parasitic infections is not sufficient to establish corrective measures. Therefore, early detection of parasitic infections in aquaculture is essential to ensure continued production, administering of treatment and tracking disease recovery of the fish. The examination of specific and quantifiable biological responses of farmed fish challenged with an environmental stressor (i.e. parasites) could prove useful in aquatic health monitoring. These biological responses are referred to as biomarkers, and if studied sufficiently can be used in disease diagnosis, pharmaceutical and feed development and continuous health assessment.

1.4 Biomarkers in Aquaculture and Dried Blood Spots (DBS)

Biomarkers are defined as measurable biological features used for disease diagnoses, monitoring recovery and/or predicting disease prevalence (Xia *et al.*, 2013). A biomarker generally indicates normal biological function, deviation from normality and/or response of an organism to stimuli. The applications of biomarker discovery are widely utilised across fields within biology. For instance, urinary biomarkers have been used to detect kidney injury (Liangos *et al.*, 2009), while the selection of therapy for colon cancer can be made by studying mutations in the tyrosine-kinase domain of epidermal growth factor receptor (EGFR) (Ludwig & Weinstein, 2005). Furthermore, blood glucose concentration is a biomarker for monitoring diabetes, essential to indicating hyper- or hypoglycaemia (Xia *et al.*, 2013). The majority of biomarker discovery focuses on human disease diagnosis and monitoring; however, biomarkers have been applied in aquaculture as well.

1.4.1 Blood and Plasma Biomarkers in Fish

Knowledge of biomarker reference values is crucial for monitoring fish health status in aquaculture systems, and understanding how various stress inducing conditions affects blood parameters has been essential in biomarker development. For example, the use of blood and plasma was used in juvenile sobaity sea bream (*Sparidentex hasta*) to establish reference intervals for haematological and biochemical parameters (Mozanzadeh *et al.*, 2015). Reference intervals for heamotologic parameters including red blood cell count, total white blood cell count, lymphocytes, neutrophils, haemoglobin, haematocrit and erythrocyte sedimentation rate were established. Furthermore, plasma chemistry parameters such as total protein, globulin, alkaline phosphate, sodium, chloride, potassium, osmolality, glucose, cholesterol and triglyceride were also assessed for reference values. Such information is vital in elucidating how the animal channels energy into maintaining homeostasis and/or initiate an immune response, which could have detrimental effects on their growth and survival. Peres *et al.* (2014) investigated the effects of starvation on selected blood plasma biochemistry parameters in gilthead seabream (*Sparus aurata*). Juveniles unfed for 24 hours, 7 and 14 days showed a decrease of plasma protein, glucose, calcium, inorganic phosphorus and cholesterol levels.

Knowing the organismal effects of deviations in the biochemistry and physiology of animals can aid in developing suitable biomarkers for continuous health monitoring.

The use of blood parameters as biomarkers has further been applied to evaluate the effects of parental care, fish meal diets, environmental parameters, methods of killing/stunning and exercise (Li *et al.*, 2011; Zampacavallo *et al.*, 2015; Zolderdo *et al.*, 2016; Bonvini *et al.*, 2017; Di Marco *et al.*, 2017). Due to the potential presence of multiple sources of stress in aquaculture settings, it is vital to select appropriate biomarkers and methods of analyses that accurately elucidate the health status of animals. In aquaculture, minimization of production costs is vital towards continuous production and competing successfully in the market. Therefore, understanding how fish respond to various stress and developing means to counter any disturbances induced to the animals, is essential for their health and ultimately the prevention of stock losses for the farmer. Hence it is important that we establish biomarkers. Biomarker discovery is a sequential process requiring: (a) the identification of the biomarker from a small sample; (b) large scale screening and assessment; and (c) validation of the biomarker. Furthermore, the ideal biomarker is one that is significantly expressed during stress, readily quantifiable from accessible biological samples, correlates with expected outcomes and is economical, consistent and allows rapid assessment. One such biomarker that has been utilized in fish health assessment is glucose, which offers a wide range analyses of various stressors. Additionally, glucose is ideal as a biomarker since it quantifiable from blood and plasma thus eliminating the need for destructive sampling.

1.4.1.1 Glucose in Fish

As the only marine aquaculture finfish species currently showing potential of significant production in South Africa, data on Dusky kob health assessment is very limited. This could prove as a major limiting factor for the developing sector. Therefore, it is essential that we establish reference values of normal condition, particularly from blood parameters that can give an accurate measure of health and be easily and reliably quantified. Glucose is a significant parameter for human health and the diagnosis of glycaemic levels has further been simplified by the availability of hand-held diabetic glucometers. For such tools to be implemented for use in aquaculture settings, their validation against reliable laboratory based enzymatic methods of glucose quantification is essential. Examination of glucose as a biomarker for Dusky kob can

be conducted since studies on stress induction and disease diagnosis in fish using glucose provide a valuable body of knowledge.

Changes in fish blood glucose concentration in response to environmental alterations, exposure to drugs, hormones and nutritional challenges have been studied extensively (Polakof *et al.*, 2012). The feasibility of glucose as a biomarker for Dusky kob is yet to be examined. Furthermore, glucose reference ranges have to be established in order to correctly assess Dusky kob health on farm. The goal of biomarker discovery is the translation of the measured biomarker response into practical applications with rapid turnover rates in improving the health of the animal. Glucose has the potential to serve as a general biomarker for farmed dusky kob health or simply to indicate stress. Elevated glucose production in fish via gluconeogenesis and glycogenolysis (Iwama *et al.*, 1999) has been shown as a response mechanism to meet energy demands under stressful conditions. There is however, no clear consensus on the regulation of glucose metabolism in fish (Enes *et al.*, 2009; Polakof *et al.*, 2012). Nonetheless, the lack of general biomarkers of Dusky kob health is concerning and should therefore be prioritised for animal health assessment.

1.4.1.2 Metabolomics: Overview

In addition to the use of glucose for fish health assessment, recent advances in human health research has led to the development of methodologies and techniques that can quantify numerous biomarkers from small biological samples. One such tool is metabolomics, which has been used for disease diagnosis, food quality assessment and nutrition based studies, plant chemistry and environmental science applications (Bundy *et al.*, 2009; Spratlin *et al.*, 2009; Madsen *et al.*, 2010). Metabolomics is the profiling of small molecular weight compounds (metabolites) in an organ, biofluid or whole organism (Samuelsson & Larsson, 2008), therefore, offers a great tool for studying environmental effects on organism function and health. The majority of metabolomic analyses are carried out using nuclear magnetic resonance (NMR) or mass spectrometry (MS). MS is further coupled with two separation techniques namely; gas chromatography (GC) and liquid chromatography (LC). MS has advantages of reproducibility and relative sample quantification, while the major downfalls of the technique include rigorous sample preparation and destruction of sample during analysis. NMR on the other has advantages of relatively easy sample preparation, absolute quantification and the

sample is retained post analysis, however the technique is less sensitive compared to MS (Verpoorte *et al.*, 2008).

Metabolomics analyses are classified as targeted or untargeted, with the former focusing on a specific group of compounds while the latter covers a wide range of the organism's metabolome (Lankadurai *et al.*, 2013). It is without question that possibility of accurate disease diagnosis increases by studying two or more biomarkers simultaneously, therefore it stands to reason that biomarker discovery be carried through metabolomic analyses. Xia *et al.* (2013) defined the goal of biomarker discovery by metabolomics as to be able to study numerous compounds and classify between healthy and diseased individuals with optimal sensitivity and specificity.

The applications of metabolomic techniques have been utilized in both wild and cultured aquatic species. Specifically, metabolomics analyses in fish have been used to study anthropogenic effects, physiology and development; and nutrition of fish as food products. Additionally, metabolomic techniques have been modified and applied for the assessment of diseases and health monitoring in fish. Plasma metabolite spectra of farmed Atlantic salmon (*Salmo salar*) exposed to a Gram-negative bacterium (*Aeromonas salmonicida*), showed a biochemical response attributed to lipoprotein profile, choline-based residues and variations in carbohydrate concentrations (Solanky *et al.*, 2005). This demonstrates the ability of metabolomics to uncover multiple biomarkers simultaneously, which is beneficial for the development of corrective measures.

The application of metabolomics techniques in assessing fish health are quicker and provide better accuracy than conventional methods (Samuelsson & Larsson, 2008). While metabolomics offers a great tool for biomarker discovery in fish, the combined analyses of well-established methods of analyses together with recent developments is essential towards uncovering new knowledge whilst limiting the gap in literature.

1.4.1.3 Use of DBS

Both glucose and metabolomics analyses can be conducted using blood as the biological sample for biomarker analyses. While an extensive body of literature across multiple fields has extensively utilized blood as the preferred sample, the reliability of the measured responses from whole blood have been called into question. Furthermore, complex sample collection,

storage and transportation methods of whole blood are further disadvantageous towards ensuring the accuracy of results. The use of dried blood spots (DBS) as an alternative method of sample collection offers a significant improvement of sample handling and ensuring accuracy of results. Furthermore, these drops of whole blood collected on filter paper (cards) have potential applications in biomarker research (McDade *et al.*, 2007).

The use of DBS has been shown to yield comparable amounts of C-reactive protein, a biomarker for morbidity and mortality risk in humans, to plasma and serum samples (R^2 = 0.995 and 0.974, respectively) (Brindle *et al.*, 2010). Furthermore, DBS analyses were able to identify hypocortisolaemia among individuals exposed to chronic environmental stressors (Karb *et al.*, 2012). These studies show that DBS can ensure sample integrity comparable with conventional methods and further diagnose haematological anomalies. This could be vital for the examination of glucose as a suitable biomarker in farmed Dusky kob. The use of DBS further extends to metabolomics analyses, as MS based techniques (especially LC-MS) have been utilized for new-born screening since the 1960s (Li & Tse, 2010). Additionally, Déglon *et al.* (2012) reported that DBS are the preferred method of sample collection for pharmaceutical companies and clinical laboratories utilizing metabolomics. Therefore, the adaptation of reliable methods for human pharmaceutical developments in the development and validation of fish health biomarkers could be beneficial for the growth and development of aquaculture in South Africa.

1.5 Research Aims

While the culture of *A. japonicus* displays rapid growth globally, metabolic data of the species and other Sciaenidae fishes are minimal. Fitzgibbon *et al.* (2007) presented an initial study towards understanding the metabolic scope of dusky kob. The effects of hypoxia on swimming performance and metabolic scope (oxygen consumption rate (MO_2), standard metabolic rate (SMR) and active metabolic rate (AMR)) were investigated for farmed juvenile mulloway in Australia (Fitzgibbon *et al.*, 2007). Such complex parameters require intense experimental settings under controlled environments. While such data is highly valuable for the scientific community, fish farmers operating on-site require practical means of fish health assessment which in turn can have practical applications in the farming process. The necessity of understanding stress (i.e. parasitic infections) and its effect thereof experienced by farmed fish

is essential for the growth and development of the South African aquaculture industry. Furthermore, gaps in knowledge regarding valuable species could be detrimental to their existence. The current study aims to:

a) Assess the utility of DBS in the monitoring of fish health and developing biomarkers through a metabolomic approach.

The following objectives to reach the aim include:

I. Compare analytical methods (glucometer vs. enzymatic) and sample preparation and storage methods (plasma, whole blood and DBS) in quantifying blood glucose levels from farmed fish.
II. Conduct a targeted metabolomics analysis, using DBS as the biological sample to assess metabolite profiles across various farming conditions and geographic locality.

1.6 References

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

Amos, K., & Thomas, J. (2002). Disease interactions between wild and cultured fish: observations and lessons learned in the Pacific Northwest. *Bulletin-European Association of Fish Pathologists, 22*(2), 94-101.

Barton, B. A., & Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases, 1*, 3-26.

Battaglene, S. C., & Talbot, R. B. (1994). Hormone induction and larval rearing of mulloway, *Argyrosomus hololepidotus* (Pisces: Sciaenidae). *Aquaculture, 126*(1), 73-81.

Bondad-Reantaso, M. G., Subasinghe, R. P., Arthur, J. R., Ogawa, K., Chinabut, S., Adlard, R., Tan, Z., & Shariff, M. (2005). Disease and health management in Asian aquaculture. *Veterinary Parasitology, 132*(3), 249-272.

Bonvini, E., Bonaldo, A., Mandrioli, L., Sirri, R., Dondi, F., Bianco, C., Fontanillas, R., Mongile, F., Gatta, P., & Parma, L. (2017). Effects of feeding low fishmeal diets with increasing soybean meal levels on growth, gut histology and plasma biochemistry of sea bass. *Animal*, 1-8.

Brindle, E., Fujita, M., Shofer, J., & O'Connor, K. A. (2010). Serum, plasma, and dried blood spot high-sensitivity C-reactive protein enzyme immunoassay for population research. *Journal of Immunological Methods, 362*(1), 112-120.

Bundy, J. G., Davey, M. P., & Viant, M. R. (2009). Environmental metabolomics: a critical review and future perspectives. *Metabolomics, 5*(1), 3.

Catalano, R. S., & Hutson, K. S. (2010). Harmful parasitic crustaceans infecting wild arripids: A potential threat to southern Australian finfish aquaculture. *Aquaculture, 303*, 101-104.

Christison, K. W. (2005). *Diplectanum* infections in cultured kob (*Argyrosomus japonicus*) in South Africa. Paper presented at the Proceedings of the 5th International Symposium on Monogenea, Guangzhou, China.

Coleman, F. C. (1993). Morphological and physiological consequences of parasites encysted in the bulbus arteriosus of an estuarine fish, the sheepshead minnow, *Cyprinodon variegatus*. *The Journal of Parasitology*, 247-254.

DAFF. (2014). Aquaculture Yearbook. Department of Agriculture, Forestry and Fisheries, Cape Town, South Africa. pp. 39.

Day, S. B., Salie, K., & Stander, H. B. (2016). A growth comparison among three commercial tilapia species in a biofloc system. *Aquaculture International, 24*(5), 1-14.

Déglon, J., Thomas, A., Mangin, P., & Staub, C. (2012). Direct analysis of dried blood spots coupled with mass spectrometry: concepts and biomedical applications. *Analytical and Bioanalytical Chemistry, 402*(8), 2485-2498.

Dezfuli, B. S., Giari, L., Simoni, E., Menegatti, R., Shinn, A. P., & Manera, M. (2007). Gill histopathology of cultured European sea bass, *Dicentrarchus labrax* (L.), infected with *Diplectanum aequans* (Wagener 1857) Diesing 1958 (Diplectanidae: Monogenea). *Parasitology Research, 100*(4), 707-713.

Di Marco, P., Petochi, T., Marino, G., Priori, A., Finoia, M., Tomassetti, P., Porrello, S., Giorgi, G., Lupi, P., & Bonelli, A. (2017). Insights into organic farming of European sea bass *Dicentrarchus labrax* and gilthead sea bream *Sparus aurata* through the assessment of environmental impact, growth performance, fish welfare and product quality. *Aquaculture, 471*, 92-105.

Enes, P., Panserat, S., Kaushik, S., & Oliva-Teles, A. (2009). Nutritional regulation of hepatic glucose metabolism in fish. *Fish Physiology Biochemistry, 35*, 519-539.

Ferguson, G. J., Ward, T. M., Ivey, A., & Barnes, T. (2014). Life history of *Argyrosomus japonicus*, a large sciaenid at the southern part of its global distribution: Implications for fisheries management. *Fisheries Research, 151*, 148-157.

Fielder, D. S., & Heasman, M. (2011). Hatchery manual for the production of Australian bass, mulloway and yellowtail kingfish: NSW Department of Primary Industries, Port Stephens Fisheries Institute. pp.170.

FAO. (2016). The State of World Fisheries and Aquaculture (SOFIA). pp.190.

Fitzgibbon, Q., Strawbridge, A., & Seymour, R. (2007). Metabolic scope, swimming performance and the effects of hypoxia in the mulloway, *Argyrosomus japonicus* (Pisces: Sciaenidae). *Aquaculture, 270*(1), 358-368.

Glibert, P. M., Landsberg, J. H., Evans, J. J., Al-Sarawi, M. A., Faraj, M., Al-Jarallah, M. A., Haywood, A., Ibrahem, S., Klesius, P., & Powell, C. (2002). A fish kill of massive proportion in Kuwait Bay, Arabian Gulf, 2001: the roles of bacterial disease, harmful algae, and eutrophication. *Harmful Algae, 1*(2), 215-231.

Griffiths, M. H. (1996). Life history of the dusky kob *Argyrosomus japonicus* (Sciaenidae) off the east coast of South Africa. *South African Journal of Marine Science, 17*(1), 135-154.

Griffiths, M. H. (1997). Management of South African dusky kob *Argyrosomus japonicus* (Sciaenidae) based on per-recruit models. *South African Journal of Marine Science, 18*(1), 213-228.

Griffiths, M. H., & Hecht, T. (1995). Age and growth of South African dusky kob *Argyrosomus japonicus* (Sciaenidae) based on otoliths. *South African Journal of Marine Science, 16*(1), 119-128.

Griffiths, M. H., & Heemstra, P. C. (1995). A contribution to the taxonomy of the marine fish genus *Argyrosomus* (Perciformes: Sciaenidae), with descriptions of two new species from southern Africa: JLB Smith Institute of Ichthyology. pp. 40.

Håstein, T., & Lindstad, T. (1991). Diseases in wild and cultured salmon: possible interaction. *Aquaculture, 98*(1-3), 277-288.

Hayward, C. J., Bott, N. J., Itoh, N., Iwashita, M., Okihiro, M., & Nowak, B. F. (2007). Three species of parasites emerging on the gills of mulloway, *Argyrosomus japonicus* (Temminck and Schlegel, 1843), cultured in Australia. *Aquaculture, 265*(1), 27-40.

Iwama, G. K., Vijayan, M. M., Forsyth, R. B., & Ackerman, P. A. (1999). Heat shock proteins and physiological stress in fish. *American Zoologist, 39*(6), 901-909.

Jewel, M., Affan, M., & Khan, S. (2003). Fish mortality due to cyanobacterial bloom in an aquaculture pond in Bangladesh. *Journal of Biological Sciences, 6*(12), 1046-1050.

Joubert, C. J. H. (2012). The potential of commercial praziquantel formulations as "off label" treatments for *Diplectanum oliveri* (Monogenea*) infecting cultured Argyrosomus* species in the South African marine finfish aquaculture industry. (Masters), University of Western Cape, Bellville. pp. 157

Karb, R. A., Elliott, M. R., Dowd, J. B., & Morenoff, J. D. (2012). Neighborhood-level stressors, social support, and diurnal patterns of cortisol: the Chicago Community Adult Health Study. *Social Science & Medicine, 75*(6), 1038-1047.

Kumaraguru, A., Beamish, F., & Woo, P. (1995). Impact of a pathogenic haemoflagellate, *Cryptobia salmositica* Katz, on the metabolism and swimming performance of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases, 18*(4), 297-305.

Lankadurai, B. P., Nagato, E. G., & Simpson, M. J. (2013). Environmental metabolomics: an emerging approach to study organism responses to environmental stressors. *Environmental Reviews, 21*(3), 180-205.

Li, D., Fu, C., Wang, Y., Zhu, Z., & Hu, W. (2011). The hematological response to exhaustive exercise in 'all-fish'growth hormone transgenic common carp (*Cyprinus carpio* L.). *Aquaculture, 311*(1), 263-268.

Li, W., & Tse, F. L. (2010). Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. *Biomedical Chromatography, 24*(1), 49-65.

Liangos, O., Tighiouart, H., Perianayagam, M. C., Kolyada, A., Han, W. K., Wald, R., Bonventre, J. V., & Jaber, B. L. (2009). Comparative analysis of urinary biomarkers for early detection of acute kidney injury following cardiopulmonary bypass. *Biomarkers, 14*(6), 423-431.

Lorenzen, K., Beveridge, M., & Mangel, M. (2012). Cultured fish: integrative biology and management of domestication and interactions with wild fish. *Biological Reviews, 87*(3), 639-660.

Ludwig, J. A., & Weinstein, J. N. (2005). Biomarkers in cancer staging, prognosis and treatment selection. *Nature Reviews Cancer, 5*(11), 845-856.

Madsen, R., Lundstedt, T., & Trygg, J. (2010). Chemometrics in metabolomics—a review in human disease diagnosis. *Analytica Chimica Acta, 659*(1), 23-33.

Mann, B. Q. (2013). Southern African marine linefish species profiles: South African Association for Marine Biological Research. pp. 343

McDade, T. W., Williams, S., & Snodgrass, J. J. (2007). What a drop can do: dried blood spots as a minimally invasive method for integrating biomarkers into population-based research. *Demography, 44*(4), 899-925.

Merella, P., Cherchi, S., Garippa, G., Fioravanti, M. L., Gustinelli, A., & Salati, F. (2009). Outbreak of *Sciaenacotyle panceri* (Monogenea) on cage-reared meagre *Argyrosomus regius* (Osteichthyes) from the western Mediterranean Sea. *Diseases of Aquatic Organisms, 86*(2), 169-173.

Mozanzadeh, M. T., Yaghoubi, M., Yavari, V., Agh, N., & Marammazi, J. G. (2015). Reference intervals for haematological and plasma biochemical parameters in sobaity sea bream juveniles (*Sparidentex hasta*, Valenciennes 1830). *Comparative Clinical Pathology, 24*(6), 1501-1507.

Munday, B., Langdon, J., Hyatt, A., & Humphrey, J. (1992). Mass mortality associated with a viral-induced vacuolating encephalopathy and retinopathy of larval and juvenile barramundi, *Lates calcarifer* Bloch. *Aquaculture, 103*(3-4), 197-211.

Naylor, R. L., Goldburg, R. J., Primavera, J. H., Kautsky, N., Beveridge, M. C., Clay, J., Folke, C., Lubchenco, J., Mooney, H., & Troell, M. (2000). Effect of aquaculture on world fish supplies. *Nature, 405*(6790), 1017-1024.

Oh, M.-J., Jung, S.-J., Kim, S.-R., Rajendran, K., Kim, Y.-J., Choi, T.-J., Kim, H.-R., & Kim, J.-D. (2002). A fish nodavirus associated with mass mortality in hatchery-reared red drum, *Sciaenops ocellatus*. *Aquaculture, 211*(1), 1-7.

Overstreet, R. M., Jovonovich, J., & Ma, H. (2009). Parasitic crustaceans as vectors of viruses, with an emphasis on three penaeid viruses. *Integrative and Comparative Biology, 49*(2), 127-141.

Parameswaran, V., Kumar, S. R., Ahmed, V. I., & Hameed, A. S. (2008). A fish nodavirus associated with mass mortality in hatchery-reared Asian Sea bass, *Lates calcarifer*. *Aquaculture, 275*(1), 366-369.

Peres, H., Santos, S., & Oliva-Teles, A. (2014). Blood chemistry profile as indicator of nutritional status in European seabass (*Dicentrarchus labrax*). *Fish Physiology and Biochemistry, 40*(5), 1339-1347.

PIRSA. (2003). PIRSA fact sheet: mulloway aquaculture in South Australia. Retrieved from www.pir.sa.gov.au/_data/assets/pdf_file/0006/33981/mulloway.pdf. Accessed 15 May 2016.

Pitcher, G. (2008). A frontier programme for mariculture research and development. Cape Town. pp. 253

Polakof, S., Panserat, S., Soengas, J., & Moon, T. (2012). Glucose metabolism in fish: a review. *Journal of Comparative Physiology B, 182*(8), 1015-1045.

Reed, P., Francis-Floyd, R., Klinger, R., & Petty, D. (2009). Monogenean parasites of fish. *Fisheries and Aquatic Sciences. University of Florida UF, IFAS Extension. FA28, USA*, 1-4.

Robar, N., Murray, D. L., & Burness, G. (2011). Effects of parasites on host energy expenditure: the resting metabolic rate stalemate. *Canadian Journal of Zoology, 89*(11), 1146-1155.

Roff, D. A. (1983). An allocation model of growth and reproduction in fish. *Canadian Journal of Fisheries and Aquatic Sciences, 40*(9), 1395-1404.

Rowell, K., True, C., Flessa, K., & Dettman, D. (2008). Fish without water: validation and application of δ18O in *Totoaba macdonaldi* otoliths Peces sin agua: validación y aplicación de δ18O en los otolitos de *Totoaba macdonaldi*. *Ciencias Marinas, 34*(1), 55-68.

Samuelsson, L. M., & Larsson, D. G. J. (2008). Contributions from metabolomics to fish research. *Molecular Biosystems, 4*, 974-979.

Silberschneider, V., Gray, C., & Stewart, J. (2009). Age, growth, maturity and the overfishing of the iconic sciaenid, *Argyrosomus japonicus*, in south-eastern, Australia. *Fisheries Research, 95*(2), 220-229.

Silberschneider, V., & Gray, C. A. (2008). Synopsis of biological, fisheries and aquaculture-related information on mulloway *Argyrosomus japonicus* (Pisces: Sciaenidae), with particular reference to Australia. *Journal of Applied Ichthyology, 24*(1), 7-17.

Silberschneider, V., Gray, C. A., & Wales, N. S. (2005). Arresting the decline of the commercial and recreational fisheries for mulloway (*Argyrosomus japonicus*): NSW Department of Primary Industries. pp. 71.

Solanky, K. S., Burton, I. W., MacKinnon, S. L., Walter, J. A., & Dacanay, A. (2005). Metabolic changes in Atlantic salmon exposed to *Aeromonas salmonicida* detected by 1H-nuclear magnetic resonance spectroscopy of plasma. *Diseases of Aquatic Organisms, 65*(2), 107-114.

Spratlin, J. L., Serkova, N. J., & Eckhardt, S. G. (2009). Clinical applications of metabolomics in oncology: a review. *Clinical Cancer Research, 15*(2), 431-440.

Ternengo, S., Agostini, S., Quilichini, Y., Euzet, L., & Marchand, B. (2010). Intensive infestations of *Sciaenocotyle pancerii* (Monogenea, Microcotylidae) on *Argyrosomus regius* (Asso) under fish-farming conditions. *Journal of Fish Diseases, 33*(1), 89-92.

Thoney, D., & Hargis, W. (1991). Monogenea (Platyhelminthes) as hazards for fish in confinement. *Annual Review of Fish Diseases, 1*, 133-153.

Verpoorte, R., Choi, Y., Mustafa, N., & Kim, H. (2008). Metabolomics: back to basics. *Phytochemistry Reviews, 7*(3), 525-537.

Whittington, I. (1996). *Benedeniine capsalid* monogeneans from Australian fishes: pathogenic species, site-specificity and camouflage. *Journal of Helminthology, 70*(3), 177-184.

Xia, J., Broadhurst, D. I., Wilson, M., & Wishart, D. S. (2013). Translational biomarker discovery in clinical metabolomics: an introductory tutorial. *Metabolomics, 9*(2), 280-299.

Zampacavallo, G., Parisi, G., Mecatti, M., Lupi, P., Giorgi, G., & Poli, B. M. (2015). Evaluation of different methods of stunning/killing sea bass (*Dicentrarchus labrax)* by tissue stress/quality indicators. *Journal of Food Science and Technology, 52*(5), 2585-2597.

Zilberg, D., Jones, J. B., Burger, M., Nicholls, P. K., Nolan, D., Crockford, M., & Stephens, F. (2012). New pathological condition in cultured mulloway *Argyrosomus japonicus*: histopathological, ultrastructural and molecular studies. *Diseases of Aquatic Organisms, 100*(3), 219-230.

Zlotkin, A., Hershko, H., & Eldar, A. (1998). Possible transmission of *Streptococcus iniae* from wild fish to cultured marine fish. *Applied and Environmental Microbiology, 64*(10), 4065-4067.

Zolderdo, A., Algera, D., Lawrence, M., Gilmour, K., Fast, M., Thuswaldner, J., Willmore, W., & Cooke, S. (2016). Stress, nutrition and parental care in a teleost fish: exploring mechanisms with supplemental feeding and cortisol manipulation. *Journal of Experimental Biology, 219*(8), 1237-1248.

Chapter 2: Use of Dried Blood Spots (DBS) to Diagnose Hyper- and Hypoglycaemia in Farmed Dusky kob *Argyrosomus japonicus* in South Africa*

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2.1 Abstract

Biomarkers require sensitivity and rapid turnover for health assessment of individuals and populations. Blood glucose in fish shows sensitive and measurable changes in response to environmental, physiological or nutritional stressors. *Argyrosomus japonicus* is currently the only commercially produced marine aquaculture finfish species in South Africa, yet knowledge about its responses to biological and environmental stressors is limited. Herein we confirm the utility of the *Accu-Chek® Active* diabetic glucometer under field conditions to provide accurate blood glucose data for *A. japonicus*, in comparison with laboratory-based enzymatic analyses using plasma, whole blood and dried blood spots (DBS). Glucometer, whole blood and DBS glucose was correlated with plasma glucose (R^2=0.973, R^2=0.955, R^2=0.898 respectively). Whole blood glucose was consistently and significantly lower than plasma glucose, thus indicating that more complex sample preparation than storage on ice and freezing at -80°C are required to inhibit glycolysis in whole blood samples and generate accurate results. Diabetic glucometers offer a means to measure on-farm blood glucose with sufficient accuracy and rapidity. We include an analysis of a subsequent sampling from the farm, and demonstrate that through routine glucose measurements we were able to identify hypoglycaemia at the farm level, and that this corresponds to decreased condition of fish. The ease of DBS storage and stability of metabolites offer the opportunity of expanding fish health and condition monitoring by measuring multiple indicators in DBS.

Keywords: Biomarkers; Aquaculture; Blood glucose; Plasma Glucose; Dried blood spots; *Argyrosomus japonicus*

2.2 Introduction

Blood glucose in fish has been shown to respond reliably to a range of biological and environmental stimuli (Garin *et al.*, 1987; Enes *et al.*, 2009; Polakof *et al.*, 2012). Hyperglycaemia in Rainbow trout has been observed in response to increased temperature, elevated pH, high density stocking stress and hormones like cortisol, catecholamines, growth hormones, leptin and glucagon and parasite infection (Tavares-Dias *et al.*, 2007; Polakof *et al.*, 2012). Reports of hypoglycaemia are less prevalent in literature, but have been observed during food deprivation, thermal cooling, hormones like insulin, and parasitism (Eames *et al.*, 2010; Woo & Buchmann, 2012).

The antagonistic effects of glucagon (hyperglycaemia) and insulin (hypoglycaemia) suggest that glucose homeostasis in fish is similar to that of higher vertebrates. Numerous studies have been conducted in trout which demonstrate glucose sensitivity in response to various stressors (Conde-Sieira *et al.*, 2010). These changes in blood glucose levels suggest the presence of underlying pathways that regulate production or degradation of glucose in response to changes in environmental or biological conditions. The primary stress responses of fish is mediated via the hypothalamus-pituitary-adrenal (HPA) axis, and the resulting release of catecholamines and cortisol has been implicated in an array of secondary stress responses. Among others, cortisol release is associated with hepatic glycogen depletion and blood glucose elevation, aimed at fuelling metabolism under stressful conditions (Barton, 2002; Herrera *et al.*, 2009). From an energetic perspective, glucose homeostasis represents the balance between anabolism (gluco- and glyconeogenesis) and catabolism (glycolysis), further supporting the usefulness of glucose as a suitable general biomarker of fish condition (Ray & Sinha, 2014).

Biomarker research in aquaculture is essential to facilitate our understanding of biochemical and physiological processes that may be below the threshold of detection by conventional methods (McDade, 2014), yet may be used to predict animal health status. Although Dusky kob (*Argyrosomus japonicus*, variously known as Japanese meagre, Mulloway or Southern meagre) is a commercially important marine finfish species in South African aquaculture, the majority of Dusky kob farming is currently in the development stage. In 2012 the finfish sector produced 48.5 tons (2.1%) of the total SA marine aquaculture production of 2 261 tons (DAFF, 2012). Although we have a good general understanding of stress related glucose responses in fish, data are limited for *A. japonicus* and sciaenids in general. It is therefore essential to

understand hyper- and hypoglycaemia in Dusky kob generally, and specifically in the context of the farming environment.

The use of hand-held diabetic glucometers both in field and laboratory research settings has been extensively explored and dates back to over two decades. The low cost of glucometers (~US$ 30), their affordable cost per sample (~US$ 0.25) and sensitivity are attractive for practical applications in field based research. Various portable devices for blood sample analyses have been successfully used in field settings with results comparable to laboratory based methods. Iwama *et al.* (1995) evaluated the feasibility and accuracy of hand held devices for measuring glucose, in conjunction with cortisol, haemoglobin and erythrocytes from juvenile Coho salmon (*Oncorhynchus kisutch*) subjected to handling stress. Blood glucose and lactate, and plasma protein responses have been measured with handheld meters for Rainbow trout (*Oncorhynchus mykiss*) exposed to handling and confinement stress (Wells & Pankhurst, 1999). Point-of-care (POC) devices have been identified as suitable alternatives to lab-based analyses due to difficulties in storage and transportation, and have the benefit of providing immediate results (Stoot *et al.*, 2014). Generally, authors concur that hand-held glucometers vary in sensitivity and accuracy, and need to be validated against accepted methods (Eames *et al.*, 2010).

Conventional methods of biological sample collection have several limitations, including complex collection and storage protocols as well as controlled transport conditions. Furthermore, sample degradation can be a limiting factor during storage. Dried blood spots (DBS) are an alternative to the storage of blood (whole blood, serum and/or plasma) samples. DBS are whole blood droplets placed on blood cards or filter paper and allowed to dry. Blood components are then eluted in appropriate media for laboratory analyses (Lacher *et al.*, 2013). DBS are currently widely used in new-born screening programs to identify metabolic disorders (Mei *et al.*, 2001; McDade *et al.*, 2007). Furthermore, the use of DBS has been key to the diagnosis of various blood parasites (Hsiang *et al.*, 2010; Hwang *et al.*, 2012). Advantages of using DBS involve ease of use in non-clinical environments and can be done by non-professionals. Unlike traditional protocols for blood collection, DBS do not require immediate freezing or refrigeration, are easy to store and can be transported as non-hazardous goods (McDade *et al.*, 2007; Lacher *et al.*, 2013). The use of DBS in fish health assessment has not been explored, thus the present study provides a first analysis of DBS accuracy and efficiency to further improve farmed fish health monitoring and biomarker research.

Routine blood glucose monitoring in fish farms may aid in assessing current, and predicting future, health status. This study represents a first attempt to develop protocols for non-destructive blood collection and rapid-turnaround blood glucose analyses. We report a comparison of analytical methods (glucometer vs. enzymatic) and sample preparation and storage methods (plasma, whole blood, and DBS) in quantifying blood glucose levels from farmed fish. Plasma glucose provides a more reliable measure of glycaemic levels and was chosen as reference method. Furthermore, we discuss the usefulness of DBS as a convenient medium of sample collection and storage, and provide some future directions for implementing these findings.

2.3 Materials and Methods

2.3.1 Sample Collection

The blood samples were collected from the Mtunzini Fish Farm located on the north coast of KwaZulu-Natal, South Africa, in May 2015. The farm uses pond culture and consists of a total of 52 ponds. Thirty animals were collected from six ponds with five animals collected from each pond. Fish were sampled in batches of five fish at time from a single pond. It took between 5-10 minutes to collect all biological material from a single fish. Fish were kept in aerated water from the corresponding pond until removed for blood collection.

In order to assess glucose concentrations in representative size ranges on the farm, we used fish between 64.5 g and 1348.5 g (average 435.1±64.77 g, 275.1±14.18 mm standard length). Blood (0.3 – 1 mL, scaled to fish size) was collected from the caudal vein immediately anterior to the caudal peduncle using a heparinized 1 mL syringe and a 21G x 1½" hypodermic needle. Each animal was weighed and the standard and total lengths were measured.

Blood glucose was immediately measured using a diabetic glucometer (*Accu-Chek® Active*, Roche) which measures glucose transformation in the blood sample using a PQQ-dependent (pyrroloquinoline quinone) glucose dehydrogenase reaction. Thirty blood samples were analysed in duplicate to determine within-sample variability: coefficient of variance (CoV) ranged between 0 (first and second analysis the same) and 11.7%, with an average CoV of 2.61 ± 0.027% (Fig. 1).

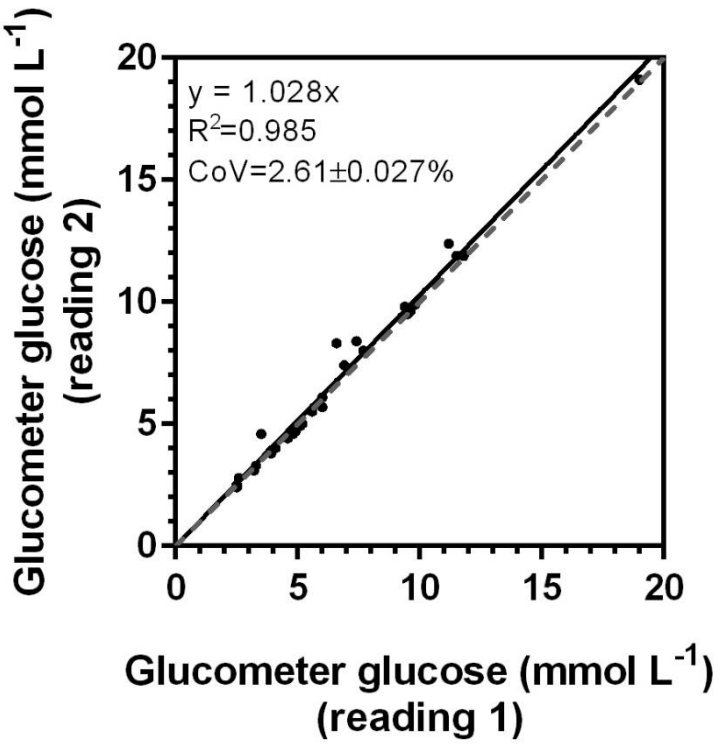

Figure 8: Within-sample variability of blood glucose as measured by hand-held glucometer. Coefficient of variance was 2.61±0.027% (range 0.0 - 11.7%, based on N=30). The dotted lines represent a line of unity.

Apart from immediate glucose analysis by glucometer, blood was prepared in three different ways to compare the accuracy of the different blood storage methods (Fig. 2). Firstly, blood plasma was obtained by centrifugation (Hawksley Haematospin 1300, 10 min, 13,000 ×g). The plasma was separated from the packed cells and stored on ice. Secondly, drops of whole blood (five ~20 µL spots, 13 mm diameter) were spotted on Whatman 903 specimen collection cards for subsequent glucose analyses. The cards were air-dried at room temperature in a dry rack (Whatman 903 Dry Rack). Once dried, the blood cards were stored in an air tight plastic container with desiccant (Sigma Dri-Can®). Thirdly, the remaining whole blood was transferred to 1.5 mL microcentrifuge tubes. Plasma and whole blood samples were immediately placed on ice, and transported to the University of KwaZulu-Natal, Westville Campus, Durban, for laboratory analyses. After 6 h on ice, whole blood and plasma samples

were stored at -80°C and blood cards were stored at 4°C for 14 days before enzymatic analyses for D-glucose were conducted.

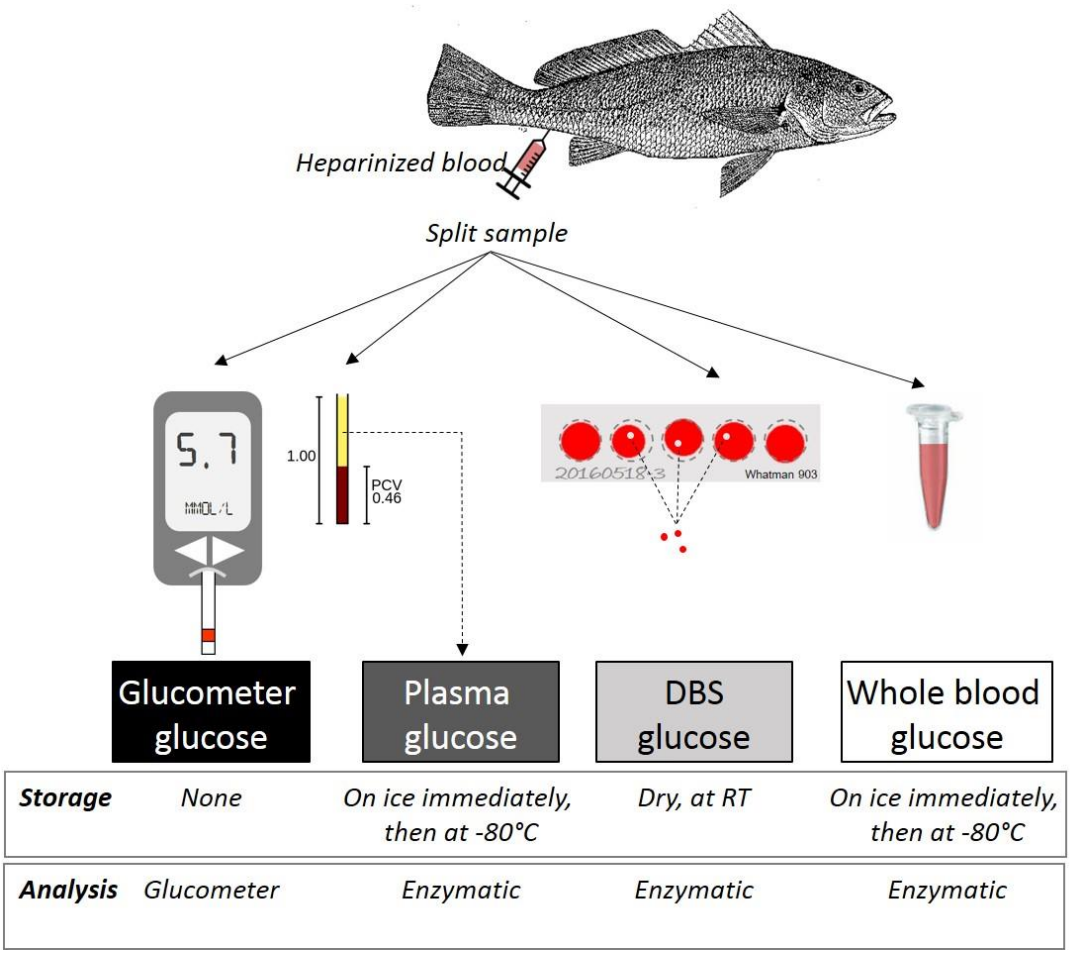

Figure 9: Overview of sampling procedure, with each heparinized blood sample (N=30) prepared for glucose analysis with different storage (immediate, plasma, DBS or whole blood) and analysis methods (glucometer or enzymatic kit).

2.3.2 Dried Blood Spot Analyses

Filter card discs (3 mm diameter) were manually punched from DBS in triplicates and transferred to 96 well plates. The volume of blood represented by the 3 mm disc was quantified by pipetting known volumes of blood onto filter cards and analysing the resulting diameter of the blood spot by image analysis (ImageJ 1.49, NIH, USA). Based on the regression analysis of blood volume (in µL) against DBS area, the 3 mm diameter punches represented a blood volume of 5 µL.

Although several extraction buffers (5% w/v trichloroacetic acid, Rattenbury *et al.* (1989); 2.5% sulphosalicyclic acid, Parker *et al.* (1997)) have been used to elute glucose from filter cards, we used Millipore water (Shigeto *et al.*, 2011) due to the method being recently shown as efficient. Each disc was eluted with 100 µL deionized water (resistivity 18.2 MΩ.cm) using constant shaking for 1 hour. Elution efficiency was tested for 1, 2 and 3 hour extraction periods, but after no significant differences were found (paired t-test: $p > 0.05$), the protocol was standardized using 1 h elution time. Thereafter 10 µL of eluent was analysed in triplicate for D-glucose using the GOPOD enzymatic kit for glucose (K-GLUC D-Glucose Assay Kit, Megazyme). Absorbance was measured at 510 nm (BIO-Tek Powerwave XS multiwell reader) using KC4 (version 3.4 rev. 21) for data acquisition. Glucose in DBS eluent was quantified against a standard curve constructed from glucose standards of 1, 5, 25, 50 and 100 mmol L^{-1} (also in triplicate) analysed simultaneously on each 96 well plate.

2.3.3 Plasma and Whole Blood Analysis

After two weeks in -80°C storage, plasma and whole blood samples were thawed to room temperature and briefly centrifuged. As described above, triplicate 10 µL samples were used for the enzymatic analyses, except that glucose was quantified against a D-glucose standard as per kit specification. Whole blood sample colour correction was done against 10 µL whole blood samples in dH_2O.

2.3.4 Comparison to Subsequent Sampling Event

The results from the May 2015 Mtunzini samples were compared to a second sampling approximately six months later (November 2015). Fish collection, blood sampling and blood glucose analysis by glucometer (*Accu-Chek® Active,* Roche) was carried out as described above.

2.3.5 Statistical analyses

Data were analysed using Graphpad Prism (version 6). Linear regression was used to determine the regression equation and Pearson-squared correlation. Between-method comparisons were analysed using ANOVA and post-hoc Tukey multiple comparisons test ($p<0.05$) after

normality testing (Shapiro-Wilk normality test). Between-sampling event comparisons were analysed using an unpaired t-test with Welch's correction for unequal variances ($P<0.05$) after normality testing (Shapiro-Wilk).

2.4 Results

As fish were brought from ponds in batches of five and maintained in aerated water before sampling, it was important to establish whether this procedure affected the blood glucose concentrations. To this end the blood glucose of the fish were pooled in their sequence number (1 to 5) for analysis. ANOVA was not significant ($F_{(4, 25)}=0.493$, $P=0.7408$), indicating that the blood glucose concentration was not significantly affected from the time being collected from ponds and being processed for sampling. Similarly, Grutter and Pankhurst (2000) reported no significant differences in plasma glucose levels of *Hemigymnus melapterus* at time intervals of up to 5 minutes between capture and blood sampling. The glucose values reported here do not represent basal, resting values, as the handling preceding the analyses may have elevated blood glucose.

2.4.1 Between-Method Analysis

Blood glucose concentrations obtained from glucometer (8.02 ± 0.664 mmol L^{-1}), enzymatic analysis of plasma (7.47 ± 0.663 mmol L^{-1}), and dried blood spots (9.39 ± 0.568 mmol L^{-1}) were not significantly different from one another (analysis of variance, $F_{(3, 113)}=10.37$, $P< 0.0001$). Whole blood glucose (4.74 ± 0.490 mmol L^{-1}) analysed by enzymatic kit, was significantly lower than measured by the other methods (Fig. 3).

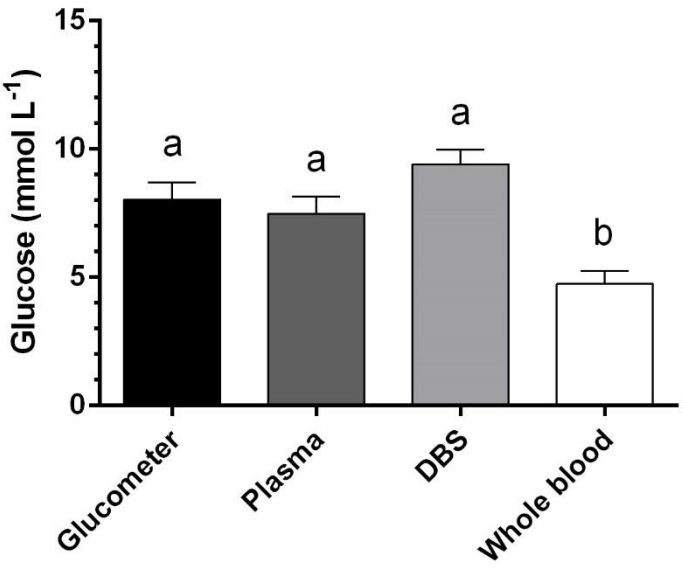

Figure 10: Glucose in farmed Dusky kob, *A. japonicus* (mean ± S.E.M., N=30), analysed on-farm by glucometer, or enzymatically in the lab using different sample storage methods (plasma, DBS and whole blood). On-farm measurements using glucometers were as reliable as lab-based measurements using blood plasma or dried blood spots. Whole blood glucose was significantly lower compared to the other methods. Different letters denote significance at $P<0.05$, ANOVA ($F_{(3, 113)} = 10{,}37$, $P < 0{,}0001$), Tukey's multiple comparisons test.

The regression analyses of the different glucose analysis methods are presented in Fig. 4. Plasma glucose was chosen as the independent variable as it is the preferred method for human health studies (Working Group on Selective Electrodes, 2001). Rapid separation of plasma from actively respiring blood cells prevents glucose depletion after sample collection (Sacks *et al.*, 2011) and best reflects the organismal glucose concentration. The best correlations with plasma glucose was from glucose as measured by diabetic glucometer ($y=1.043x$, $R^2=0.973$) and from DBS ($y=1.236x$, $R^2=0.898$).

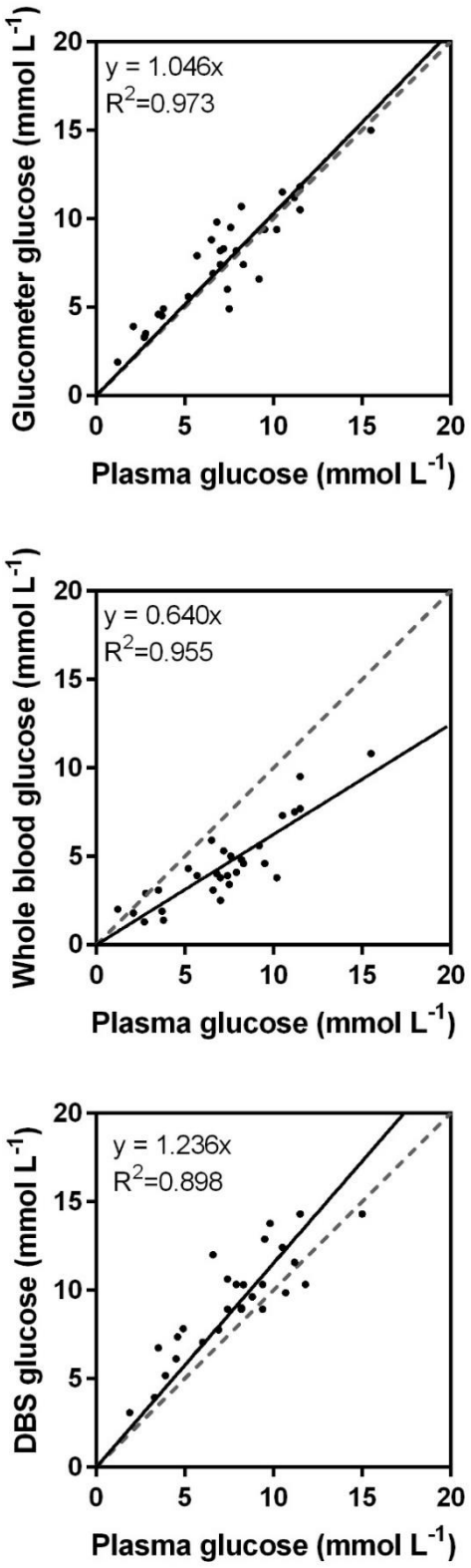

Figure 11: Linear regressions of glucometer, whole blood and DBS glucose versus plasma glucose (reference method). Dotted lines represent a line of unity.

2.4.2 Between-Sampling Analysis

Blood glucose (4.67 ± 0.361 mmol L^{-1}), measured by glucometer from Dusky kob in November 2015, was significantly lower (P<0.0001) compared to fish sampled in May 2015 (as reported above, 8.02 ± 0.664 mmol L^{-1}). The fish were of similar average size (342.3 ± 47.13 g, n=30, compared to 435.1 ± 64.77 g, n=30 respectively) (Fig. 5).

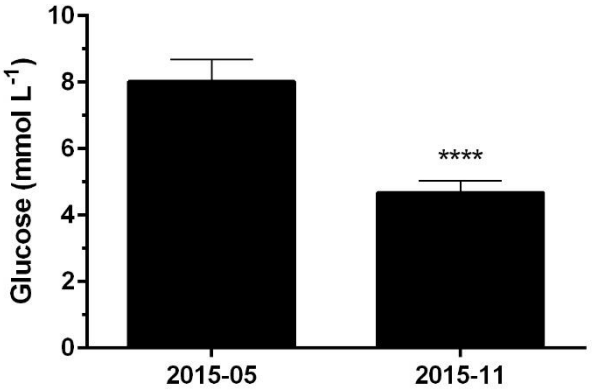

Figure 12: Dusky kob blood glucose (mean ± S.D.) as measured by glucometer from Mtunzini Fish Farm in May 2015 (N=30) and November 2015 (N=28). Statistical significance indicated (t-test, P<0.0001).

2.5 Discussion

2.5.1 Between Method Comparison

This study shows that issues of continued glycolysis (and indeed other pathways of the intermediary metabolism by using stored whole blood) can be addressed by using dry blood spots. DBS offer an attractive alternative blood storage method since they absorb and dry the sample; consequently, there is no liquid medium for metabolic pathways to occur. Von Schenck *et al.* (1985) reported no significant difference in glucose concentration measure between DBS (5.31 mmol L^{-1}) and venous blood (5.27 mmol L^{-1}) from 32 human subjects. Furthermore, DBS can easily and rapidly be prepared on-farm and transported to laboratories for enzymatic analyses, making sampling in remote areas possible.

Glucometer, whole blood and DBS glucose correlated well with plasma glucose, with correlation coefficients (R^2) of 0.898 or better (Fig. 4). Lacher *et al.* (2013) found good correlation between human whole blood and DBS glucose (R^2=0.81), with the DBS having significantly lower mean glucose (6.08 mmol L^{-1} compared to 6.34 mmol L^{-1} blood glucose; $p < 0.01$). The authors ascribed this difference to variations in the volume of each blood spot and subsequently the elution efficiency which led to reduced sensitivity of the DBS method. In contrast, here we report a slightly better correlation coefficient (R^2=0.898) with average DBS glucose not significantly different from plasma glucose (Fig. 3). As the correlation equation (y=1.236x) suggests that DBS may overestimate plasma glucose concentration by approximately 12%, this error should be of greater significance at higher glucose concentrations. In the absence of blood glucose reference ranges, blood glucose data were arbitrarily grouped as low (0-8 mmol L^{-1}), medium (8-12 mmol L^{-1}) and high (12-18 mmol L^{-1}) to determine the extent of the variation between methods in each range. In the low and medium blood glucose ranges, all three methods yield comparable concentrations. In the high concentration range, DBS over-estimates plasma glucose by 26% ($P<0.05$), which exceeds the recommended 20% (Eames *et al.*, 2010) required for implementation. However, in this high range, the diagnosis of hyperglycaemia would be accurate regardless of whether it is overestimated (Fig. 6).

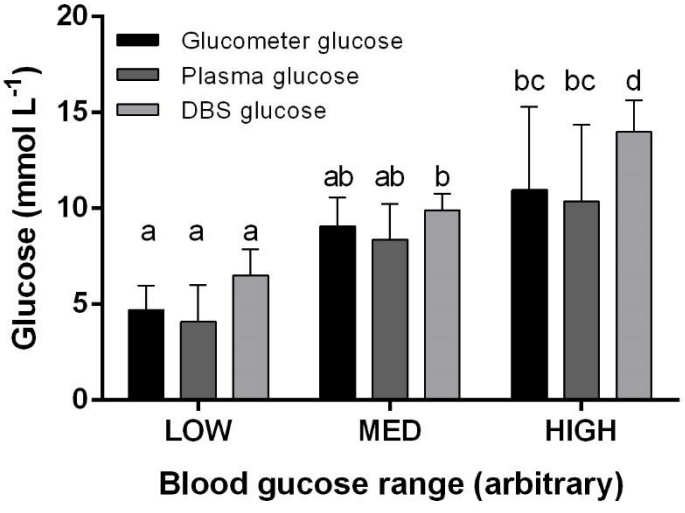

Figure 13: Grouping of blood glucose values as low, medium or high (using arbitrary cut-offs of 8 and 12 mmol L^{-1}) demonstrates that DBS (compared to plasma glucose) may over-estimate blood glucose at elevated levels ($P<0.05$, two-way ANOVA, Tukey multiple comparisons). The mean difference of 3.638 mmol L-1 represents a 26% over-estimation of blood glucose using DBS.

2.5.2 Accuracy of DBS – and Downstream Benefits

According to McDade *et al.* (2007), DBS were considered disadvantageous due to the lack of comprehensive assay development and validation and also the small volumes available to work with. A large body of recent research has allayed this concern (McDade, 2014), and this cautionary note has to be balanced against the benefit of using DBS as a useful method for blood sample storage, without complex handling, storage and transport requirements. DBS also add the benefit that multiple parameters can be measured ranging from pathogen diagnostics and individual metabolites to comprehensive targeted and untargeted metabolomic and proteomic profiles (Martin *et al.*, 2013; Zukunft *et al.*, 2013; Miller *et al.*, 2015), therefore providing information about the general physiological condition of the fish.

DBS have been widely used for human disease diagnosis through the use of various laboratory techniques. The use of DBS has led to development and validation of alternative sampling and diagnostic protocols for the Chikungunya Virus (CHIKV) where the viral RNA was effectively extracted from the blood spots, amplified and quantified by real-time RT-PCR (Andriamandimby *et al.*, 2013). Holguín *et al.* (2013) used the Architect Chagas assay on dried blood samples to detect the presence of immunoglobin G due to *Trypanosoma cruzi* infection of patients in Madrid. Such studies often compare the sensitivity and specificity of DBS in comparison to conventional serological methods. Furthermore, DBS can be used in non-laboratory and non-clinical environments and can be collected by any individual, which could prove useful for fish farmers should they require regular monitoring of stress and health status of their fish.

2.5.3 Usefulness of Hand-Held Glucometers

Glucometer glucose did not differ significantly from plasma glucose suggesting that routine blood glucose measurements using the *Accu-Chek® Active* diabetic glucometer are comparable to laboratory based enzymatic analyses. Iwama *et al.* (1995) reported glucometer levels two times higher than those measured by a laboratory-based assay in juvenile Coho salmon for 24 h following a 30 seconds handling stressor. In a study evaluating a handheld meter in stressed and unstressed Channel catfish (*Ictalurus punctatus*) fingerlings, glucose values 30% lower

compared to levels measured by laboratory methods were reported (Beecham *et al.*, 2006). Glucose concentrations measured with diabetic glucometers may vary with some degree of accuracy compared values determined by thorough laboratory-based methodology, therefore should be treated as relative measurements rather than absolute values until the accuracy has been confirmed.

2.5.4 Concerns with whole blood sampling

As expected, whole blood glucose was significantly lower than measured by the other methods (Fig. 3), confirming that whole blood is a poor medium for glucose analyses when samples are stored over time. According to Voss *et al.* (1992), whole blood samples remain stable for 1 week and at least 1 year at 4°C and -70°C respectively, however, in the present study immediate placement on ice (6 h) and subsequent storage of the blood in -80°C (2 weeks) proved ineffective in preventing glycolytic depletion of glucose. It is recommended that an effective glycolysis inhibitor (e.g. citrate buffer, fluoride, iodoacetate) be added to the blood sample if immediate freezing is impossible (Tonyushkina & Nichols, 2009; Sacks *et al.*, 2011). Glycolysis will continue in the 1-2 h it takes for iodoacetate and fluoride to cross cell membranes (Tonyushkina & Nichols, 2009). However, if the intention is to use blood for metabolomics analysis, this step needs to be considered carefully as citrate addition may skew results of some tricarboxylic acid (TCA or Krebs) cycle intermediates.

2.5.6 Blood Glucose and the Stress Response

The link between handling stress, elevated cortisol as a primary effect, and increased plasma glucose as a secondary effect has been reviewed extensively (Barton, 2002; Martinéz-Porchas *et al.*, 2009; Ellis *et al.*, 2012), and the sensitivity of blood glucose to single and repeated handling stress has been demonstrated in several fish species (Jentoft *et al.*, 2005; Butcher *et al.*, 2007). In contrast, Meagre (*A. regius*) has been shown to exhibit a low cortisol, and consequently low blood glucose, response after acute stress (Samaras *et al.*, 2015). Although it has not been confirmed, our data suggest that *A. japonicus* may have a similar low blood glucose response and that the sampling method employed in this study did not differentially affect the blood glucose measurements between different fish. It is unclear whether the

preceding handling may have elevated blood glucose, and this aspect requires further assessment. A low blood glucose response would be a very beneficial physiological feature of *A. japonicus*, as reliable blood glucose measurements can be generated in the farming environment without sophisticated techniques like aortal cannulation (Deng *et al.*, 2000).

2.5.7 Between-Sampling Comparisons

The results of the between-sampling comparison demonstrate the ability of hand-held glucometers to identify a farm-level effect, using a relatively small number of fish. Hypoglycaemia during sub-optimal feeding appears to be a general response in fish (Chavin & Young, 1970; Groff & Zinkl, 1999; Eames *et al.*, 2010), and can manifest even at elevated cortisol levels typical of starved fish (Peterson & Small, 2004; Eslamloo *et al.*, 2016). The low blood glucose in fish sampled in November corresponded to (a) slightly poorer condition than for the May sampling (body mass (Mb) vs. length (L) ratio calculated as Fulton's Condition Index (Mb/L^3): 1.11 ± 0.018 vs 1.08 ± 0.023, not significant) and (b) significantly reduced hepatosomatic index (liver mass/Mb*100): 2.21 ± 0.144 vs 1.35 ± 0.078, $P<0.0001$) (Christison and Vosloo, unpublished data). These data demonstrate that the ability to sample fish non-destructively and measure blood glucose levels in real time provides a useful avenue for fish farmers to generate information that may inform decision making. In the farming environment, the day-to-day variability in blood glucose in fish will be dependent on an interplay of a range of factors that have been demonstrated as glucose modulators, including general stress, density-dependent stress, parasite load, feeding ration, time since feeding and water quality (temperature, pH, DO etc.). The fact remains that blood glucose is a general biomarker, and should be supplemented by other, more specific biomarkers, as well as continuous environmental and management data. Ideally, a well-chosen suite of biomarkers should be developed to further analyse and diagnose fish condition in the farming context. Regardless, blood glucose remains a useful and easily measured first response in farmed fish.

2.5.8 Where to Now: Deriving A Glucose Reference Range for Farmed Dusky Kob

The major limiting factor in implementing routine blood glucose as a monitoring tool in aquaculture is the absence of a reference range for blood glucose. A brief literature search

revealed that only one previous study (Butcher *et al.*, 2007) reported blood glucose for *A. japonicus*, in response to angling stress. The blood glucose values reported in this study are the first for *A. japonicus* in the farming context. As a first step toward determining whether a blood glucose reference range is possible, a summary of blood glucose data from other sciaenids (Shi drum, *Umbrina cirrosa* (Ballarin *et al.*, 2004), Yellow croaker, *Pseudosciaena crocea* (Cheng *et al.*, 2013) and Meagre, *A. regius* (Chatzifotis *et al.*, 2010; Fanouraki *et al.*, 2011; McGrath *et al.*, 2011; Vargas-Chacoff *et al.*, 2014; Samaras *et al.*, 2015; Barata *et al.*, 2016; Millán-Cubillo *et al.*, 2016)) is provided (Fig. 7). In general, blood glucose levels from farm or field-based studies appear to be higher and more variable than from controlled laboratory studies.

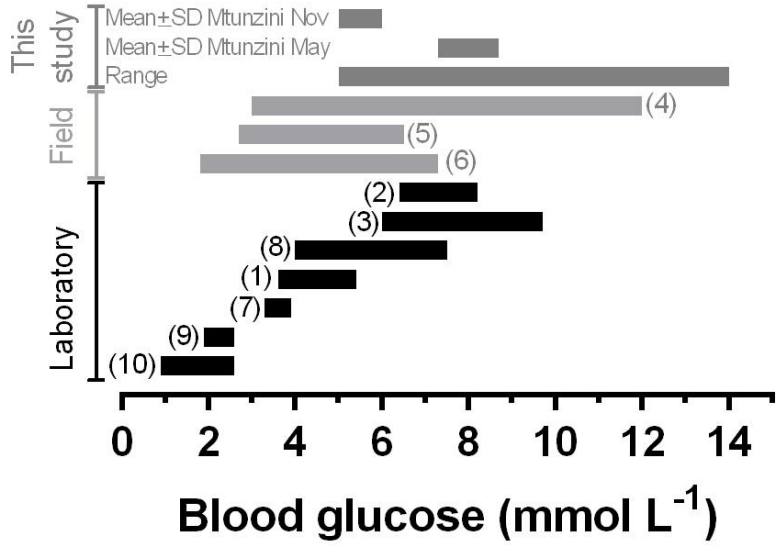

Figure 14: Blood glucose measurements in Sciaenidae reported from laboratory (black bars) of field studies (grey bars) shows large variation. 1 *Umbrina cirrosa* (Ballarin et al., 2004), 2 *Argyrosomus regius* (Chatzifotis et al., 2010), 3 *A. regius* (Fanouraki et al., 2011), 4* *Pseudosciaena crocea* (Cheng et al., 2013), 5 *A. regius* (Vargas-Chacoff et al., 2014), 6 *A. regius* (Samaras et al., 2015), 7 *A. regius* (Barata et al., 2016), 8* *A. regius* (Millán-Cubillo et al., 2016), 9 *A. regius* (McGrath et al., 2011), 10 *A. japonicus* (Butcher et al., 2007). Data from this study are presented as the range as well as the mean ± S.D. for the two sampling events.

* *In instances where numerical data were not provided, blood glucose was estimated from graphs.*

Against this backdrop it is essential that samples for deriving reference ranges are collected in ways that limit pre-analytical variability, e.g. fasting vs. non-fasting, fish capture and handling, blood sampling site and sample handling (Friedrichs *et al.*, 2012). Of the methods reported here, validated hand-held glucometers and sample storage on DBS, followed by laboratory

analysis, have the greatest potential to provide large data sets required for deriving reference ranges, with minimal impact on the fish or the farms.

After blood glucose analysis, statistical procedures are used to determine the reference interval (RI). The blood glucose RI (e.g. using farmed *A. japonicus* in South Africa as a delimited sample population) is derived by non-parametric methods, and is bounded by the 2.5^{th} and 97.5^{th} fractiles of the data set of reference values, with corresponding 90% confidence intervals around these cut-offs (Friedrichs *et al.*, 2012). Fish presenting blood glucose levels outside of the RI can then be diagnosed at hypo- or hyperglycaemic. With large data sets, it becomes possible to confirm age- and size dependent RIs using the same statistical methods as in human medicine (see Mazzaccara *et al.* (2008) as an example).

The benefit of using DBS in deriving reference ranges is the fact that the same DBS can be used for further analyses, as the punch volume represents a fraction of the total DBS volume. In this manner, a single DBS sampling program provides the opportunity for deriving reference ranges for multiple biomarkers, providing the detection limit and stability of each analyte is satisfactory. Coupled to targeted and untargeted mass spectrometry methods (Zukunft *et al.*, 2013; Alfaro & Young, 2016), a large amount of biological information becomes available for an individual, allowing the refinement of the RI to Decision Thresholds (Friedrichs *et al.*, 2012) that define the boundaries of the physiological transitions into hypo- and hyperglycaemia more accurately than statistical methods.

2.6 Conclusions

This study was a first attempt for assessing the usefulness of blood glucose as a biomarker for research on farmed South African Dusky kob, *A. japonicus*. Firstly, blood glucose can be assessed on-site with sufficient accuracy using diabetic glucometers, offering fish farmers a simple yet valuable initial assessment of general fish condition in real time. Secondly, DBS offer a promising alternative to conventional blood sample storage methods, with the added benefit of providing a matrix for further assessments using metabolomics to supplement single end-point measurements like blood glucose. Blood glucose is thus a useful first biomarker for assessing fish health status and condition in the farming context, as reduced blood glucose was supported by morphometric indices like the hepatosomatic index. Implementation of these findings requires a defined glucose reference range for farmed Dusky kob for accurate

diagnosis of hypo- and hyperglycemia. Furthermore, a comprehensive understanding of glucose metabolism in *A. japonicus* both in normal and stressful conditions is required in order to evaluate and interpret results from routine blood glucose analyses. The present study serves as a basis for future biomarker research in *A. japonicus* farmed in South African aquaculture. The ease of DBS storage and transport offers the opportunity to add value to a wide range of studies on environmental, nutritional and biological stressors on South African aquaculture fish species.

2.7 Acknowledgements

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2.8 References

Alfaro, A. C., & Young, T. (2016). Showcasing metabolomic applications in aquaculture: a review. *Reviews in Aquaculture, 0,* 1-18. doi:10.1111/raq.12152

Andriamandimby, S. F., Heraud, J.-M., Randrianasolo, L., Rafisandratantsoa, J. T., Andriamamonjy, S., & Richard, V. (2013). Dried-blood spots: a cost-effective field method for the detection of chikungunya virus circulation in remote areas. *PLoS Neglected Tropical Diseases, 7*(7), e2339. doi:10.1371/journal.pntd.0002339

Ballarin, L., Dall'Oro, M., Bertotto, D., Libertini, A., Francescon, A., & Barbaro, A. (2004). Haematological parameters in *Umbrina cirrosa* (Teleostei, Sciaenidae): a comparison between diploid and triploid specimens. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 138*(1), 45-51. doi:http://dx.doi.org/10.1016/j.cbpb.2004.02.019

Barata, M., Soares, F., Aragão, C., Almeida, A. C., Pousão-Ferreira, P., & Ribeiro, L. (2016). Efficiency of 2-phenoxyethanol and Clove Oil for Reducing Handling Stress in Reared Meagre, *Argyrosomus regius* (Pisces: Sciaenidae). *Journal of the World Aquaculture Society, 47*(1), 82-92. doi:10.1111/jwas.12245

Barton, B. A. (2002). Stress in Fishes: A Diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids. *Integrative and comparative biology, 42*(3), 517-525. doi:10.1093/icb/42.3.517

Beecham, R. V., Small, B. C., & Minchew, C. D. (2006). Using portable lactate glucose meters for catfish research: acceptable alternatives to established laboratory methods? *North American Journal of Aquaculture, 68*(4), 291-295. doi:10.1577/A05-074.1

Butcher, P. A., Broadhurst, M. K., Reynolds, D., Reid, D. D., & Gray, C., A. . (2007). Release method and anatomical hook location: effects on short-term mortality of angler-caught *Acanthopagrus australis* and *Argyrosomus japonicus*. *Diseases of aquatic organisms, 74*(1), 17-26.

Chatzifotis, S., Panagiotidou, M., Papaioannou, N., Pavlidis, M., Nengas, I., & Mylonas, C. C. (2010). Effect of dietary lipid levels on growth, feed utilization, body composition and serum metabolites of meagre (*Argyrosomus regius*) juveniles. *Aquaculture, 307*(1–2), 65-70. doi:http://dx.doi.org/10.1016/j.aquaculture.2010.07.002

Chavin, W., & Young, J. E. (1970). Factors in the determination of normal serum glucose levels of goldfish, *Carassius auratus* L. *Comparative Biochemistry and Physiology, 33*(3), 629-653.

Cheng, Z., Mai, K., Ai, Q., Li, Y., & He, Z. (2013). Effects of dietary pregelatinized corn starch on growth, apparent digestibility, and digestive enzyme activity of large yellow croaker fingerlings (*Pseudosciaena crocea*). *The Israeli Journal of Aquaculture, 65.2013.843*.

Conde-Sieira, M., Aguilar, A. J., López-Patiño, M. A., Míguez, J. M., & Soengas, J. L. (2010). Stress alters food intake and glucosensing response in hypothalamus, hindbrain, liver, and Brockmann bodies of rainbow trout. *Physiology & Behavior, 101*(4), 483-493. doi:http://dx.doi.org/10.1016/j.physbeh.2010.07.016

DAFF. (2012). *Aquaculture yearbook 2012, South Africa*. Retrieved from Cape Town:

Deng, D. F., Refstie, S., Hemre, G.-I., Crocker, C. E., Chen, H. Y., Cech, J. J., & Hung, S. S. O. (2000). A new technique of feeding, repeated sampling of blood and continuous collection of urine in white sturgeon. *Fish physiology and biochemistry, 22*(3), 191-197. doi:10.1023/a:1007803307617

Eames, S. C., Philipson, L. H., Prince, V. E., & Kinkel, M. D. (2010). Bloodsugar measurement in zebrafish reveals dynamics of glucose homeostasis. *Zebrafish, 7*(2), 205-213. doi:10.1089/zeb.2009.0640

Ellis, T., Yildiz, H. Y., Lopez-Olmeda, J., Spedicato, M. T., Tort, L., Overli, O., & Martins, C. I. (2012). Cortisol and finfish welfare. *Fish physiology and biochemistry, 38*(1), 163-188. doi:10.1007/s10695-011-9568-y

Enes, P., Panserat, S., Kaushik, S., & Oliva-Teles, A. (2009). Nutritional regulation of hepatic glucose metabolism in fish. *Fish physiology and biochemistry, 35*(3), 519-539. doi:10.1007/s10695-008-9259-5

Eslamloo, K., Morshedi, V., Azodi, M., & Akhavan, S. R. (2016). Effect of starvation on some immunological and biochemical parameters in tinfoil barb (*Barbonymus schwanenfeldii*). *Journal of Applied Animal Research*, 1-6. doi:10.1080/09712119.2015.1124329

Fanouraki, E., Mylonas, C. C., Papandroulakis, N., & Pavlidis, M. (2011). Species specificity in the magnitude and duration of the acute stress response in Mediterranean marine fish in culture. *General and Comparative Endocrinology, 173*(2), 313-322. doi:http://dx.doi.org/10.1016/j.ygcen.2011.06.004

Friedrichs, K. R., Harr, K. E., Freeman, K. P., Szladovits, B., Walton, R. M., Barnhart, K. F., & Blanco-Chavez, J. (2012). ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. *Veterinary Clinical Pathology, 41*(4), 441-453. doi:10.1111/vcp.12006

Garin, D., Rombaut, A., & Freminet, A. (1987). Determination of glucose turnover in sea bass *Dicentrarchus labrax*. Comparative aspects of glucose utilization. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, 87*(4), 981-988.

Groff, J. M., & Zinkl, J. G. (1999). Hematology and clinical chemistry of cyprinid fish. Common carp and goldfish. *Veterinary Clinics of North America: Exotic Animal Practice, 2*(3), 741-776.

Grutter, A. S., & Pankhurst, N. W. (2000). The effects of capture, handling, confinement and ectoparasite load on plasma levels of cortisol, glucose and lactate in the coral reef fish *Hemigymnus melapterus*. *Journal of Fish Biology, 57*(2), 391-401. doi:10.1111/j.1095-8649.2000.tb02179.x

Herrera, M., Vargas-Chacoff, L., Hachero, I., Ruíz-Jarabo, I., Rodiles, A., Navas, J. I., & Mancera, J. M. (2009). Physiological responses of juvenile wedge sole *Dicologoglossa cuneata* (Moreau) to high stocking density. *Aquaculture Research, 40*(7), 790-797. doi:10.1111/j.1365-2109.2008.02162.x

Holguín, A., Norman, F., Martín, L., Mateos, M. L., Chacón, J., López-Vélez, R., & Pérez-Molina, J. A. (2013). Dried Blood as an Alternative to Plasma or Serum for

Trypanosoma cruzi IgG Detection in Screening Programs. *Clinical and Vaccine Immunology, 20*(8), 1197-1202. doi:10.1128/CVI.00221-13

Hsiang, M. S., Lin, M., Dokomajilar, C., Kemere, J., Pilcher, C. D., Dorsey, G., & Greenhouse, B. (2010). PCR-based pooling of dried blood spots for detection of malaria parasites: optimization and application to a cohort of Ugandan children. *Journal of Clinical Microbiology, 48*(10), 3539-3543. doi:10.1128/jcm.00522-10

Hwang, J., Jaroensuk, J., Leimanis, M. L., Russell, B., McGready, R., Day, N., . . . Imwong, M. (2012). Long-term storage limits PCR-based analyses of malaria parasites in archival dried blood spots. *Malaria Journal, 11*, 339-339. doi:10.1186/1475-2875-11-339

Iwama, G. K., Morgan, J. D., & Barton, B. A. (1995). Simple field methods for monitoring stress and general condition of fish. *Aquaculture Research, 26*(4), 273-282. doi:10.1111/j.1365-2109.1995.tb00912.x

Jentoft, S., Aastveit, A. H., Torjesen, P. A., & Andersen, Ø. (2005). Effects of stress on growth, cortisol and glucose levels in non-domesticated Eurasian perch (*Perca fluviatilis*) and domesticated rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 141*(3), 353-358. doi:http://dx.doi.org/10.1016/j.cbpb.2005.06.006

Lacher, D. A., Berman, L. E., Chen, T.-C., & Porter, K. S. (2013). Comparison of dried blood spot to venous methods for hemoglobin A1c, glucose, total cholesterol, high-density lipoprotein cholesterol, and C-reactive protein. *Clinica Chimica Acta, 422*, 54-58. doi:http://dx.doi.org/10.1016/j.cca.2013.03.032

Martin, N. J., Bunch, J., & Cooper, H. J. (2013). Dried blood spot proteomics: surface extraction of endogenous proteins coupled with automated sample preparation and mass spectrometry analysis. *Journal of the American Society for Mass Spectrometry, 24*(8), 1242-1249. doi:10.1007/s13361-013-0658-1

Martinéz-Porchas, M., Martínez-Córdova, L. R., & Ramos-Enriquez, R. (2009). Cortisol and Glucose: Reliable indicators of fish stress? *Pan-American Journal of Aquatic Sciences, 4*(2), 158-178.

Mazzaccara, C., Labruna, G., Cito, G., Scarfò, M., De Felice, M., Pastore, L., & Sacchetti, L. (2008). Age-Related Reference Intervals of the Main Biochemical and Hematological Parameters in C57BL/6J, 129SV/EV and C3H/HeJ Mouse Strains. *PLoS One, 3*(11), e3772. doi:10.1371/journal.pone.0003772

McDade, T. W. (2014). Development and validation of assay protocols for use with dried blood spot samples. *American Journal of Human Biology, 26*(1), 1-9. doi:10.1002/ajhb.22463

McDade, T. W., Williams, S., & Snodgrass, J. J. (2007). What a drop can do: Dried blood spots as a minimally invasive method for integrating biomarkers into population-based research. *Demography, 44*(4), 899-925. doi:10.1353/dem.2007.0038

McGrath, S. P., Broadhurst, M. K., Butcher, P. A., & Cairns, S. C. (2011). Fate of three Australian teleosts after ingesting conventional and modified stainless- and carbon-steel hooks. *ICES Journal of Marine Science, 68*(10), 2114-2122. doi:10.1093/icesjms/fsr151

Mei, J. V., Alexander, J. R., Adam, B. W., & Hannon, W. H. (2001). Use of filter paper for the collection and analysis of human whole blood specimens. *The Journal of Nutrition, 131*(5), 1631S-1636S.

Millán-Cubillo, A. F., Martos-Sitcha, J. A., Ruiz-Jarabo, I., Cárdenas, S., & Mancera, J. M. (2016). Low stocking density negatively affects growth, metabolism and stress pathways in juvenile specimens of meagre (*Argyrosomus regius*, Asso 1801). *Aquaculture, 451*, 87-92. doi:http://dx.doi.org/10.1016/j.aquaculture.2015.08.034

Miller, M. J., Kennedy, A. D., Eckhart, A. D., Burrage, L. C., Wulff, J. E., Miller, L. A. D., . . . Elsea, S. H. (2015). Untargeted metabolomic analysis for the clinical screening of inborn errors of metabolism. *Journal of Inherited Metabolic Disease, 38*, 1029-1039. doi:10.1007/s10545-015-9843-7

Parker, D. R., Bargiota, A., Cowan, F. J., & Corrall, R. J. M. (1997). Suspected hypoglycaemia in out patient practice: accuracy of dried blood spot analysis. *Clinical Endocrinology, 47*(6), 679-683. doi:10.1046/j.1365-2265.1997.3061130.x

Peterson, B. C., & Small, B. C. (2004). Effects of fasting on circulating IGF-binding proteins, glucose, and cortisol in channel catfish (*Ictalurus punctatus*). *Domestic Animal Endocrinology, 26*(3), 231-240. doi:http://dx.doi.org/10.1016/j.domaniend.2003.10.005

Polakof, S., Panserat, S., Soengas, J., & Moon, T. (2012). Glucose metabolism in fish: a review. *Journal of Comparative Physiology B, 182*(8), 1015-1045. doi:10.1007/s00360-012-0658-7

Rattenbury, J. M., Herber, S. M., & Price, K. J. (1989). Dried blood spot glucose measurement in hyperinsulinaemic hypoglycaemia. *Clinical Biochemistry, 22*(4), 301-303. doi:http://dx.doi.org/10.1016/S0009-9120(89)80022-0

Ray, S. N. C., & Sinha, R. C. (2014). Serum cortisol and glucose: reliable bioindicators of stress in fish, *Labeo rohita*. *International Journal of Innovative Science, Engineering & Technology, 1*(8), 6-17.

Sacks, D. B., Arnold, M., Bakris, G. L., Bruns, D. E., Horvath, A. R., Kirkman, M. S., . . . Nathan, D. M. (2011). Guidelines and recommendations for laboratory analysis in the diagnosis and management of diabetes mellitus. *Diabetes Care, 34*(6), e61-e99. doi:10.2337/dc11-9998

Samaras, A., Papandroulakis, N., Costari, M., & Pavlidis, M. (2015). Stress and metabolic indicators in a relatively high (European sea bass, *Dicentrarchus labrax*) and a low (meagre, *Argyrosomus regius*) cortisol responsive species, in different water temperatures. *Aquaculture Research*, n/a-n/a. doi:10.1111/are.12800

Shigeto, S., Katafuchi, T., Okada, Y., Nakamura, K., Endo, F., Okuyama, T., . . . Okumiya, T. (2011). Improved assay for differential diagnosis between Pompe disease and acid α-glucosidase pseudodeficiency on dried blood spots. *Molecular Genetics and Metabolism, 103*(1), 12-17. doi:http://dx.doi.org/10.1016/j.ymgme.2011.01.006

Stoot, L. J., Cairns, N. A., Cull, F., Taylor, J. J., Jeffrey, J. D., Morin, F., . . . Cooke, S. J. (2014). Use of portable blood physiology point-of-care devices for basic and applied research on vertebrates: a review. *Conservation Physiology, 2*(1). doi:10.1093/conphys/cou011

Tavares-Dias, M., Ruas de Moraes, F., Onaka, E. M., & Rezende, P. C. B. (2007). Changes in blood parameters of hybrid tambacu fish parasitized by *Dolops carvalhoi* (Crustacea, Branchiura), a fish louse. *Veterinarski Arhiv, 77*(4), 355-363.

Tonyushkina, K., & Nichols, J. H. (2009). Glucose meters: a review of technical challenges to obtaining accurate results. *Journal of diabetes science and technology (Online), 3*(4), 971-980.

Vargas-Chacoff, L., Ruiz-Jarabo, I., Páscoa, I., Gonçalves, O., & Mancera, J. M. (2014). Yearly growth and metabolic changes in earthen pond-cultured meagre *Argyrosomus regius*. *Scientia Marina, 78*(2), 193-202. doi:10.3989/scimar.03965.06B

Von Schenck, H., Lönnström, L., & Engström, M. (1985). Quality control of reflectometric determinations of glucose in dried blood spots on filter paper. *Clinical Chemistry, 31*(5), 706-709.

Voss, E. M., Cembrowski, G. S., Clasen, B. L., Spencer, M. L., Ainslie, M. B., & Haig, B. (1992). Evaluation of capillary collection system for HbA1c specimens. *Diabetes Care, 15*(5), 700-701.

Wells, R. M. G., & Pankhurst, N. W. (1999). Evaluation of Simple Instruments for the Measurement of Blood Glucose and Lactate, and Plasma Protein as Stress Indicators in Fish. *Journal of the World Aquaculture Society*, *30*(2), 276-284. doi:10.1111/j.1749-7345.1999.tb00876.x

Woo, P. T. K., & Buchmann, K. (Eds.). (2012). *Fish Parasites: Pathobiology and Protection*. London: CABI. pp. 395.

Working Group on Selective Electrodes, S. D. (2001). IFCC recommendation on reporting results for blood glucose. *Clinica Chimica Acta*, *307*(1–2), 205-209. doi:http://dx.doi.org/10.1016/S0009-8981(01)00431-4

Zukunft, S., Sorgenfrei, M., Prehn, C., Möller, G., & Adamski, J. (2013). Targeted metabolomics of dried blood spot extracts. *Chromatographia*, *76*(19), 1295-1305. doi:10.1007/s10337-013-2429-3

Chapter 3: Metabolomics analyses of cultured Dusky kob, *Argyrosomus japonicus* (Temminck & Schlegel, 1843) in South African aquaculture.

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3.1 Abstract

Global aquaculture continuously suffers from the effects of parasitic infections in fish. These infections induce physical damage, secondary disease exposure and mass mortalities to their hosts. Conventional methods of parasite detection in fish require destructive sampling and provide no corrective measures for containment and/or treatment of the infections. Metabolomics analyses have potential applications for biomarker discovery, disease diagnosis and continuous health assessment in aquaculture. The present study evaluates liquid chromatography (LC) and gas chromatography (GC) mass spectrometry (MS) techniques on dried blood spots (DBS) from farmed Dusky kob (*Argyrosomus japonicus*) infected by the monogenean parasite *Diplectanum oliveri*. Targeted metabolite (amino acids, organic acids and acylcarnitines) analyses, combined with haematology (blood glucose and packed cell volume) and necropsy (condition factor and hepatosomatic index) were utilized. A total of 53 metabolites were detected from *A. japonicus* DBS, with six amino acids (citrulline, glutamine, lysine, methionine, phenylalanine and proline) showing significant differences between fish with high and low parasite intensities (ANOVA; $p < 0.05$). Principal components analyses (PCA) based on all detected metabolites showed 43.5% variability between the fish, which corresponded with the geographical distributions of the sampling sites. Glutamine was upregulated in parasite-infected fish, possibly due to its role in the innate and adaptive immune responses during infection, especially for the proliferation of leukocytes. We propose improvement of current biomarker discovery techniques by conducting global untargeted metabolite profiling of farmed Dusky kob. Furthermore, the use of other analytical platforms (e.g. NMR spectroscopy) and the incorporation of tissue sample analyses can be useful for fish health assessment in aquaculture as this may uncover specific biomarkers thereby reducing the use of large sample sizes.

Keywords: *Argyrosomus japonicus*, Dusky kob, Monogenea, *Diplectanum oliveri*, metabolomics, amino acids, organic acids, acylcarnitines, biomarkers.

3.2 Introduction

Metabolomics is the global profiling of metabolites obtained in a cell, biofluid, tissue or whole organism and can be used to examine changes in metabolite concentrations as a result of stress induction (Samuelsson & Larsson, 2008). Some study areas utilizing metabolomics include human disease diagnostics, human nutrition, environmental sciences, plant chemistry and food quality assessment (Bundy *et al.*, 2009; Spratlin *et al.*, 2009; Madsen *et al.*, 2010). Literature on fish metabolomics further covers anthropogenic effects on both wild and cultured fish, physiology and development and assessment of fish products for human consumption. Furthermore, metabolomics studies evaluating diseases and health monitoring in fish are numerous. For example, Solanky *et al.* (2005) used ^{1}H-NMR based techniques to profile metabolite changes in cultured Atlantic salmon (*Salmo salar*) exposed to a Gram-negative bacterium (*Aeromonas salmonicida*). Using principal component analysis (PCA) to examine plasma metabolite spectra, the results showed a biochemical response from the infected salmon which was attributed to lipoprotein profile, choline-based residues and variations in carbohydrate concentrations. The use of metabolomics analyses in monitoring fish health have been shown to be quicker and more accurate than conventional methods (Samuelsson & Larsson, 2008). While metabolomics research has focused on many fish species with various stressors, the effects of parasites on fish, particularly in aquaculture, are yet to be examined from an "omics" perspective.

The feasibility of using metabolomics analyses for the detection of parasitic infections in farmed fish offers a valuable tool for biomarker discovery and fish health assessment. Fish aquaculture faces a challenge with regards to parasite infections, which may result in various diseases, reduced growth rates, mass mortalities and elevated costs for their containment (Thoney & Hargis, 1991). While various parasite taxa induce pathology in aquaculture, monogenean parasites are regarded as the most prevalent in fish culture and pose a serious threat in production (Thoney & Hargis, 1991). These ectoparasites have a direct life cycle, often inhibiting the gills, skin, fins and oral cavity of their host (Buchmann & Bresciani, 2006).

Several monogeneans in the genus *Diplectanum* and their associated pathology on fish have been examined and appear to have similar effects. *Diplectanum aequans* is known to infect cultured European sea bass, *Dicentrarchus labrax*, attaching to the gills of their host (Oliver, 1977). The parasite induces epithelial hyperplasia and haemorrhage around its haptor (point of attachment) and hamuli (point of insertion), respectively. Furthermore, Dezfuli *et al.* (2007)

reported white mucoid exudate around the sites of attachment while the epithelium of the primary and secondary lamellae showed erosion and inflammation. Similarly, *Diplectanum sciaenae* infecting broodstock meagre (*Argyrosomus regius*) in Spain caused significant gill pathology (Andree *et al.*, 2016), including epithelial hyperplasia, fusion of gill lamellae and congested lamellar capillaries. These studies were conducted using microscopic analyses, which often lack practical applicability in improving fish health as they require destructive sampling and produce biological findings which fail to indicate early disease induction.

Parasitology analyses capture the endpoint of the effects of parasites in studying gill histopathology. This is mainly because the detection of parasites in fish require observational studies, essentially, microscopic analyses requiring destructive sampling. Furthermore, the lack of knowledge on physiological responses of fish leave no room for corrective measures and containment strategies to be put in place. Early detection of parasitic infections and subsequent physiological responses by the hosts can aid in the development and improvement of pharmaceutical treatments and feed, biomarker discovery and continuous assessment of fish health and improve farming conditions to manage challenges associated with increased production.

The use of metabolomics analyses in aquaculture offers a suitable method for the early detection of stress induction on fish. The two most widely used analytical methods in metabolomics are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectrometry. MS is further coupled with two separation techniques: liquid chromatography (LC) or gas chromatography (GC). This increases the sensitivity of MS, therefore ensures accurate metabolite identification and quantification (Samuelsson & Larsson, 2008). The advantages of MS include small sample volumes and identification of numerous metabolites. Currently, most metabolomics analyses focus on human health however, numerous metabolomics studies on fish have been conducted in research areas including ecotoxicology, disease diagnostics and nutrition.

In a study to evaluate the applicability of metabolomics an ecotoxicology, Samuelsson *et al.* (2006) investigated the effects of contraceptive oestrogen ethinylstradiol (EE_2) on rainbow trout. Changes in the metabolites were observed in fish exposed to 10 ng/L of EE_2, with vitellogenin, alanine, phospholipids and cholesterol being the most significant. Santos *et al.* (2009) studied the effect of copper exposure (3.2 – 128 µg of Cu/L) for 4 days on the metabolome of the stickleback (*Gasterostous aculeatus*). Significant alterations in metabolite

concentrations were observed from 10 µg of Cu/L and higher. Stentiford *et al.* (2005) combined histopathological, proteomic and metabolomic analyses in an attempt to identify biomarkers of liver tumours from the common dab (*Limanda limanda*). The study found 4 metabolites (potential empirical formulae: $C_{31}H_{45}N_1O_4$, $C_{33}H_{47}NO_4$, $C_{31}H_{39}N_3O_5$ and $C_{28}H_{29}N_7O_8$) that could discriminate between diseased and non-tumorous tissue. This signifies the need for multidisciplinary analyses for accurate disease diagnosis and health assessment in aquaculture.

The effects of monogenean parasites on gill histopathology are well studied, therefore contribute extensive knowledge on fish health assessment, however, these findings require corroboration by evaluating associated physiological responses of infected fish. The applications of metabolomics analyses in aquaculture are beneficial over conventional methods of assessing infection since it allows for multidisciplinary studies. According to Alfaro and Young (2016) applications of metabolomics have focused mostly on four key areas: hatchery production, diet and nutrition, post-harvest quality control, and disease and immunology. Furthermore, the ability of metabolomics to elucidate immune responses and disease-resistance mechanisms allows for the development of corrective measures such as pharmaceutical treatment strategies (Low *et al.*, 2017). For example, a GC/MS based assessment of dead and surviving Crucian carps, *Carassius carassius*, infected with *Edwardsiella tarda* identified elevated palmitic acid and reduced D-mannose as significant metabolites for survival (Guo *et al.*, 2014). The study also found that reduced fructose and mannose metabolism, and elevated unsaturated fatty acid biosynthesis were the major pathways in ensuring survival. These findings show that physiological responses to environmental stress (i.e. bacterial or viral infections) induced on aquaculture species can be elucidated by the broad-scale metabolite profiling (Young *et al.*, 2017).

Argyrosomus japonicus (Dusky kob) is a commercially important fish species in South African aquaculture. The species is farmed on land in saline ponds or recirculating aquaculture systems (RAS), possessing attributes such as high fertility, relatively fast growth, high salinity and thermal tolerance and non-cannibalistic behaviour (Fitzgibbon *et al.*, 2007). While Dusky kob often thrive under varied environmental conditions, their responses to stress has not been examined and the effects induced by stress are unknown. Dusky kob are however, known to be prone to parasitic infections, particularly from the gill parasite *Diplectanum oliveri* which has been identified from both farmed and wild Dusky kob (*A. japonicus*) in South Africa and Australia (Williams, 1989; Christison, 2005; Hayward *et al.*, 2007). To our knowledge, specific pathology associated with this parasite has not been reported and physiological responses to

parasitic infections have not been examined in Dusky kob, although reports of other *Diplectanum* infecting other sciaenid species indicate localized cellular disruption due to the penetration of the hamuli deep into the epithelial cells (Oliver, 1977; Dezfuli *et al.*, 2007; Joubert, 2012; Andree *et al.*, 2016). The extent to which these disruptions cause functional disturbances of gas exchange, osmoregulation or primary and secondary metabolic response are unknown.

In the present study, we evaluated the applicability of metabolomics in identifying biomarkers for Dusky kob with *D. oliveri* infection by conducting targeted metabolic analysis of organic acids, amino acids and acylcarnitines. We investigated the effectiveness of metabolomics analyses of a small sample size on different groups of metabolites against analyses of a larger sample size on a single targeted metabolite group. We also assess if Dusky kob farmed in ponds or RAS from different geographic locations show differences in metabolite concentrations. Furthermore, we discuss the usefulness of dried blood spots as means for sample collection and storage.

3.3 Materials and Methods

3.3.1 Sample Collection

Dusky kob were sampled from four farms namely; Mtunzini Fish Farm (currently Zini Fish Farms, KwaZulu-Natal), Oceanwise (currently Ocean Choice), Pure Ocean (both Eastern Cape) and Blue Cap (Western Cape) fish farms between February – May 2015. The Mtunzini fish farm uses pond culture while the other farms produce fish in RAS. Ethical clearance was provided under UKZN AREC 030/016.

Figure 1: Geographical distributions of Mtunzini (KwaZulu-Natal), Oceanwise, Pure Ocean (both Eastern Cape) and Blue Cap (Western Cape) fish farms. Mtunzini, Oceanwise and Pure Ocean are located along the Indian Ocean coastline, whereas Blue Cap is situated along the Atlantic Ocean.

In the present study, the use of an anesthetic prior to sampling of the fish was avoided as this could potentially induce a physiological response thus resulting in false positives and/or negatives in the data. Six fish from each farm were sampled for blood since standard MS protocols are optimized for dried blood spot (DBS) analyses. Additional blood was collected and stored for hematology analyses (DBS, blood glucose and packed cell volume). Fish were further sampled for tissues essential for necropsy (liver and spleen) and parasitology (gills) analyses.

3.3.1.1 Haematology Analyses

Approximately 1 mL of blood was collected from the caudal vein immediately anterior to the caudal peduncle using a heparinized 1 mL syringe and a 21G x 1½" hypodermic needle. Each animal was weighed and the standard and total lengths were measured.

DBS: Drops of whole blood (five ~20 µL spots, 13 mm diameter) were spotted on Whatman 903 specimen collection cards. The cards were air-dried at room temperature in a dry rack (Whatman 903 Dry Rack). Once dried, the blood cards were stored in an air tight plastic container with desiccant (Sigma Dri-Can®). The cards were transported to the University of KwaZulu-Natal and subsequently stored at 4°C before being transported to North West University, Potchefstroom, South Africa for metabolomics analyses. Blood glucose concentration and packed cell volume (PCV) were measured as described by Mdlalose *et al.* (2017).

3.3.1.2 Necropsy Analyses

The necropsy (condition factor and hepatosomatic index (HSI) analyses) of all samples were conducted on-farm immediately post-sampling. To assess the overall condition of the fish we used the Fulton's condition factor (K), calculated using the formula: $K = \frac{W}{L^3} * 100$. The formula quantifies the condition of fish by taking into consideration weight of the fish in grams (W) and standard length in centimetres (L). To measure the energy reserve capacity of the fish we used the hepatosomatic index (HSI), which is the ratio of liver weight to body weight. The HSI was calculated as $HSI = \frac{\text{liver weight (g)}}{\text{body weight (g)}} * 100$ (Chellappa *et al.*, 1995).

3.3.1.3 Parasitology Analyses

Half gill samples were collected in histology cassettes and stored in approximately 100 mL of 10% buffered neutral formalin (BNF). The gill samples were transported to the National Marine Research Aquarium (Seapoint, Cape Town) for parasite counts. For this study, only the left or right gill was used from each fish. Juvenile and adult gill flukes were identified and counted using a dissecting microscope. Counts were made from each gill filament and the total for only the half gill count was taken into account as it was assumed that parasitic prevalence would be similar on both gills.

3.3.2 Organic Acids Extraction and Derivatization (GC/MS)

Five 3 mm spots (approximately 7 µL blood) were cut from the dried blood spots into 1.5 mL Eppendorf microcentrifuge tubes. Ultrapure water (1.5 mL) and 3 mm stainless steel beads were transferred to the tubes and samples were homogenized (Retsch MM 400, 30 Hz, 4 min). The homogenates were then transferred to a Kimax tube. The original tube was rinsed with 500 µL of ultrapure water which was also added to the Kimax tube. Thereafter 100 µL of the internal standard (3-phenylbutyric acid, Sigma-Aldrich, cat #78243) was added. A volume of 230 µL 5 mol/L HCl was added to the sample thus acidifying to pH 1. The samples were allowed to mix for 30 minutes on a rotatory wheel. The mixture was centrifuged for 3 minutes at 728 x g. Once the centrifugation was completed, the sample formed an upper organic phase and a lower aqueous phase. This organic phase was transferred to a new clean Eppendorf tube. Diethylether (3 mL) was added to the aqueous phase remaining in the previous tube and centrifuged for 3 minutes at 728 x g (as above). After centrifugation, the organic phase (diethylether) was removed and added to the ethylacetate (organic phase) previously transferred to the new tube. Two spatula of sodium sulphite were added to the ethylacetate/diethylether mixture to facilitate removal of residual water. The sample was thereafter centrifuged for 5 minutes at 728 x g (as above). Organic (upper) and aqueous (lower) phases were formed post centrifugation and the organic phase was transferred to a new clean tube. The organic solvents were evaporated under nitrogen at 37°C.

Derivatization of organic acids was carried out using Bis(trimethlysilyl)-triflouroacetamid (BSTFA), trimethylchlorosilane (TMCS) and pyridine. Derivatization reagents (40 µL BSTFA, 7 µL TMCS and 7 µL pyridine) were added to the dried extracts using Hamilton syringes. The extracts were then incubated at 85°C for 45 minutes and thereafter transferred to vials for GC-MS analyses.

3.3.2.1 GC-MS Analysis

Organic acid spectrometry was based on Reinecke *et al.* (2012) using the Aligent 7890A GC-MS system with a mass selective detector (model 5970C), an HP 5970C MS and Chemistation (Revision E.02.00). Sample introduction was conducted using a fused-silica capillary column (DB-1MS UI, 30 m, 2.50 µm i.d., 0.25 µm film thickness). An initial temperature of 60°C was maintained for 2 min and then increased by 5°C/min to 120°C. Temperature was subsequently

increased to 295°C (7°C/min) and maintained for 2 min. The carrier gas used was Helium at a constant 1 ml/min flow rate. SCAN (50-600 amu) positive ion monitoring was used to generate mass spectra of GC peaks at 70 eV. The temperature for the MS source was 230°C while the quadrupole was kept at 150°C.

3.3.3 Amino Acids and Acylcarnitines Analysis (LC MS/MS)

Amino acids and acylcarnitines analysis followed a standardized procedure used for newborn metabolic screening. Fish DBS discs (3 mm) were punched into 96 micro-well plates using an automated Blood Spot Distributer. Thereafter, standards and blank spots not containing blood were added into additional wells.

Internal and External controls (Centre for Disease Control and Prevention controls (CDC's)) were also punched to the plates according to a predefined protocol. An internal standard was added to each well (except blanks) (Eppendorf 12 channel multipipettor). Elution of the amino acids and acylcarnitines from DBS discs was carried out with constant shaking for 1 hour. Sample eluent was subsequently transferred to new polypropylene 96-well plates. The samples were evaporated to dryness under nitrogen in a fume hood for 20 minutes at 45°C.

Butanolic-HCl (100 µL) was added to each well, where after derivatization was carried out by covering the micro-well plates and heating for 45 minutes at 60°C. Thereafter the samples were allowed to dry under nitrogen at 40°C. A volume of 100 µL acetonitrile (water 50% (v/v), 0.1% formic acid) was added to each well. The plates were covered with aluminium foil, placing the shiny surface on the inside. The samples were mixed by shaking for 10 minutes prior to LC-MS/MS analysis.

3.3.3.1 LC-MS Analysis

The spectrometry was conducted according to George *et al.* (2010) and the LC and MS conditions are given in Table 1 and Table 2. MS was conducted with the Aligent 6460 Triple Quadrupole LC/MS using three modes: neutral loss scan, precursor ion scan and multiple reaction monitoring (MRM). Samples were analysed in sequential order based on the three modes, where the injection parameters were automatically altered to suit the mode in use.

Table 1: LC conditions as described by George *et al.* (2010)

LC Run conditions	
Column	None
Injection volume	5 µl
Autosampler temperature	6°C
Needle wash	Flush port (50:25:25 H_2O, IPA:MeOH:H_2O, 5 sec)
Mobile phase	A = H_2O + 0.1 formic acid B = methanol + 0.1 formic acid
Analysis time	1.8 min
Flow rate	0.5 ml/min
Isocratic Analysis	A = 20%, B = 80%

Table 2: Mass spectrometry conditions as described by George *et al.* (2010)

MS Conditions 6460	
Ion mode	Positive, ESI
Drying gas temperature	300°C
Sheath Gas temperature	300°C
Drying gas flow	5 L/min
Nebulizer pressure	60 psi
Capillary voltage	4000 V
Charging voltage	2000 V
MRM acquisition	Q1 peak and Q2 peak widths = 0.7 m/z
Delta EMV	200 V

The neutral loss scan analysed only amino acids at 140 to 270 m/z in the first quadrupole, thereafter set at a fixed 102 m/z in the third quadrupole. The total scan time was 300 ms at 9 eV. The precursor ion scan was used to analyse acylcarnitines. The first quadrupole scans at 210 to 510 m/z while the third quadrupole is fixed at 85 m/z. The scan time was 510 ms with the collision energy of 25 eV. MRM was used to analyse both acylcarnitines and amino acids. Each amino acid and acylcarnitine was analysed with a specific mass-to-charge ratio (m/z) in the first and second quadrupole, based on the precursor ion and the most intense product ion of the compound.

3.3.4 Multivariate Statistical Analyses

Multivariate analyses were conducted between farms for anatomical measures (mass, tail length and standard length), HSI, haematology parameters (PCV and glucose) and metabolites (amino acids). Data were analysed using GraphPad Prism version 7 (GraphPad Software, Inc). All data were normally distributed (Kolmogorov-Smirnov test, $P < 0.05$) and one-way ANOVA followed by Tukey's multiple comparisons test ($P < 0.05$) was performed.

3.3.5 Metabolomic Data Analysis

Exploratory analyses for the detected metabolites across all fish samples were conducted using Metaboanalyst version 3.0 (Xia *et al.*, 2015). Following normalization and log transformation of data sets, One-way ANOVA and post-hoc analysis (Fisher's LSD) were performed on all detected metabolites. A principal component analysis (PCA) was used as an unsupervised method to explain total variation within the data and to determine separation patterns based on significant loadings (principal components). Significant compounds reported from Metaboanalyst (One-way ANOVA) were further analysed in GraphPad Prism similarly to necropsy data analyses as described above.

3.3.6 Pathway Analysis

To create a metabolic map, Kyoto Encyclopedia of Genes and Genomes (KEGG) ID's for the metabolites were identified using the human metabolome database (http://www.hmdb.ca/) (Wishart *et al.*, 2012). Only the amino acids and some organic acids were identified, while no

acylcarnitines from the present study were found. The KEGG ID's were used to create a metabolite template list for Pathway Projector, using the format: KEGG ID, COLOUR, SIZE, COMPOUND NAME, SIZE (i.e. C00041, blue, 25, Alanine, 50). Thereafter the network was generated using Pathway Projector (www.g-language.org/PathwayProjector/) (Kono *et al.*, 2009).

3.4 Results

3.4.1 Necropsy Analyses

Conducting necropsy analyses allows for screening of general fish condition. The fish were of comparable sizes and no juveniles were used, however, samples from Blue Cap were significantly larger (mass = 608.0 g) compared to other farms (Table 3). A significantly lower condition factor was observed for fish from Oceanwise (Table 3). A common measure of fish health is glucose concentration (Eames *et al.*, 2010), which in the present study did not differ significantly between farms. However, the glucose concentration for Oceanwise fish was low (3.7 mmol/L) and this correlated with a significantly lower condition factor (0.93). The HSI for Mtunzini fish samples was significantly higher (2.8%) compared to fish from Oceanwise and Pure Ocean, however did not differ significantly from fish sampled in Blue Cap.

Table 3: Various biological parameters measured from sampled fish (n = 6; mean ± S.E.M). Different letters denote significant differences within columns (One-way ANOVA; Tukey's multiple comparisons test ($P < 0.05$)). Different letters indicate significant differences in each column.

Farm	Mass (g)	SL (mm)	TL (mm)	Glucose (mmol/L)	K (g/mm^3)	PCV (%)	HSI (%)
Mtunzini	300.8 ± 122.01^{c}	238.0 ± 28.4^{a}	276.2 ± 33.01^{ac}	6.2±1.3	1.16 ± 0.04^{b}	33.1 ± 1.4	2.8 ± 0.31^{a}
Oceanwise	331.9 ± 103.54^{c}	271.5 ± 30.7^{ab}	308.7 ± 35.35^{ac}	3.7±0.7	0.93 ± 0.03^{a}	32.9 ± 2.7	1.2 ± 0.15^{b}
Pure Ocean	161.0 ± 25.80^{a}	215.0 ± 9.7^{a}	230.0 ± 14.14^{ab}	5.8±0.5	1.29 ± 0.05^{b}	29.5 ± 2.2	1.7 ± 0.23^{b}
Blue Cap	608.0 ± 77.62^{b}	346.7 ± 15.7^{b}	361.7 ± 12.02^{c}	6.8±2.0	1.25 ± 0.05^{b}	31.9 ± 3.0	1.9 ± 0.18^{ab}

*SL = Standard length; TL = Tail length K = Condition factor; PCV = Packed cell volume; HSI = Hepato-somatic index

3.4.2 Parasitic Infections (only *D. oliveri*)

Parasites were more prevalent in fish from Mtunzini (918 parasites in half gill set) relative to Oceanwise and Pure Ocean, whereas no parasites were observed in fish from Blue Cap (Table 4). The parasites were also found in gills of fish from Oceanwise and Pure Ocean, however these were minimal at 7 and 3 parasites respectively, from all fish.

Table 4: Gill parasite counts from Dusky kob (n = 6 fish) conducted via microscopy. Only *D. oliveri* was recorded on the gills of infected fish where either the left or right gill (half gill set) was used for quantification. Significance is denoted by letter (One-way ANOVA; Tukey's multiple comparisons test ($P < 0.05$)). n =5 was used for Oceanwise

Farm	No. of parasites (per half gill)	Mean ± SEM
Mtunzini	918[a]	183.6 ± 43.76
Oceanwise	7	1.4 ± 1.4
Pure Ocean	3	0.6 ± 0.6
Blue Cap	0	0

3.4.3 Metabolomics Analyses

MS analyses conducted in this study represent a first attempt at using metabolomics analyses in farmed *A. japonicus.* A total of 53 metabolites (25 organic acids, 8 acylcarnitines and 20 amino acids) were identified and quantified between GC-MS and LC-MS/MS analyses. This is consistent with most vertebrate metabolism as serum metabolite profiles are often dominated by amino acids. However, from the 53 metabolites, only 21 (5 organic acids, 6 acylcarnitines and 15 amino acids) were significantly different between two or more farms (Supplementary data, Table 1A). To explore the effectiveness of the methods we conducted principal components analyses as an unsupervised method. We compared a small sample size (N = 22) using multiple metabolite groups (Figure 2) against a larger sample size (N = 120) using only amino acid concentrations (Figure 3).

Metabolite profiles of fish with high parasite intensity (i.e. Mtunzini) were not significantly different from non-parasitized fish (Figure 2 and 3). While distinct separation of the fish did not correspond to differences in parasite load, patterns resembling the geographical distribution of the farms were observed, especially when exploring a large sample size as the combined principal components had a higher variation (59.8%) (Figure 3) compared to a smaller sample size (43.5%) (Figure 2). Blue Cap is in the Western Cape, Mtunzini is in KwaZulu-Natal (KZN) while both Oceanwise and Pure Ocean are located in the Eastern Cape. The coast of South Africa generally has temperature gradient of cold water (16-18°C) in the Western Cape to warmer water in KZN (22-26°C), with Eastern Cape having intermediary temperature ranges (18-22°C).

The PCA plots also tentatively elucidate differences in farming conditions. Mtunzini fish farm uses pond culture and the samples clearly group closer together (Figure 3), separated from the other farms which use recirculating aquaculture systems. The pond culture used at Mtunzini also relies on brackish water from the Mlalazi River estuary, with obvious differences (*e.g.* temperature, salinity, turbidity) from seawater used in recirculatory systems.

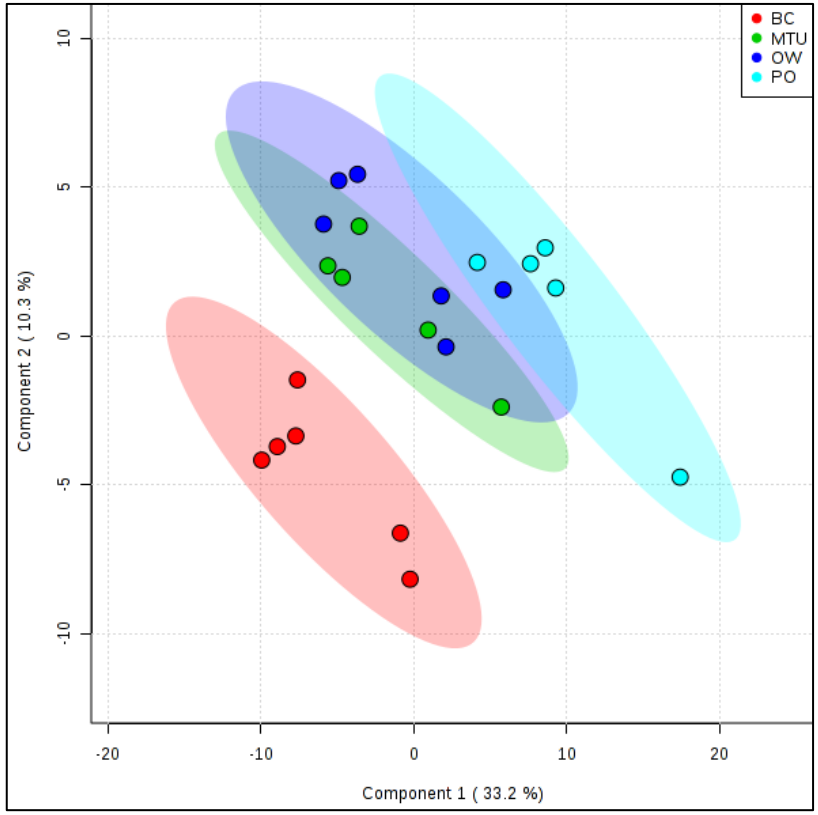

Figure 2: Principal components analyses scores plot for fish sampled from Blue Cap (BC), Mtunzini (MTU), Oceanwise (OW) and Pure Ocean (PO) fish farms (n = 6 per farm*). The samples were clustered based on the combination of amino acids (N = 20), acylcarnitines (N = 7) and organic acids (N = 26) concentrations.

* denotes that n = 5 was used for Pure Ocean and Mtunzini

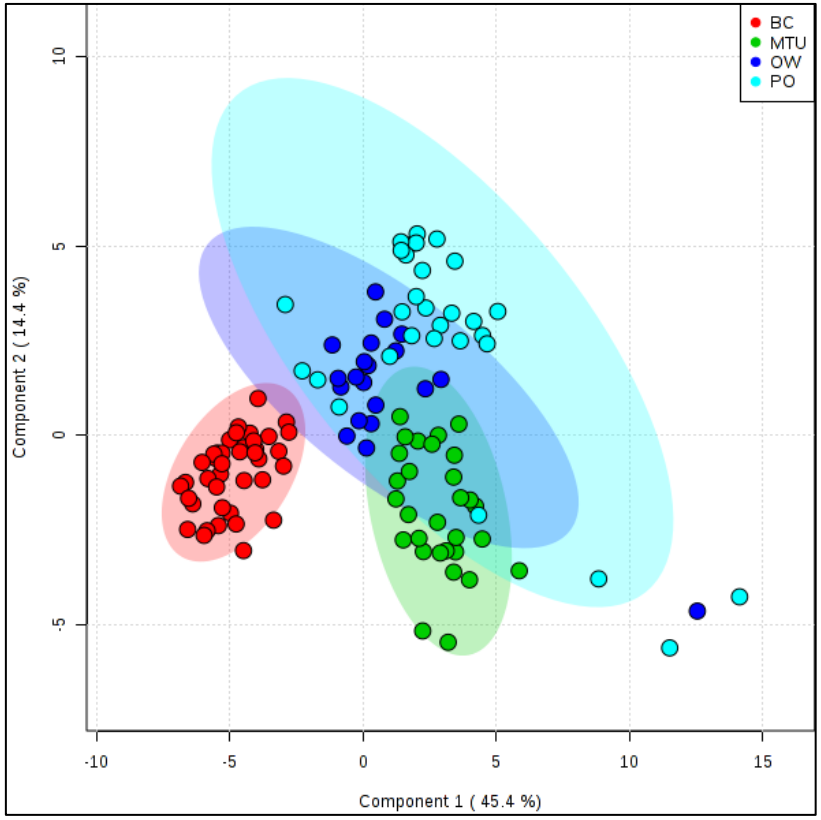

Figure 3: Principal components analyses scores plot for amino acid concentrations in DBS of fish sampled from Blue Cap (BC; N = 40), Mtunzini (MTU; N = 30), Oceanwise (OW; N =20) and Pure Ocean (PO; N = 30) fish farms.

Numerous fish species such as zebrafish have been studied extensively, resulting to the development metabolic maps based on genomic, proteomic and/or metabolic data. Knowledge on the metabolic pathways of fish have enabled their application as model species for human based research. Using some of the identified metabolites, we constructed an arbitrary Dusky kob metabolic network (Figure 4). The compounds were identified using KEGG, however some of the compound ID's were not identified since the human metabolome was used. Significant differences in amino acids were observed mostly in the compounds involved in the TCA and urea cycles (Figure 4).

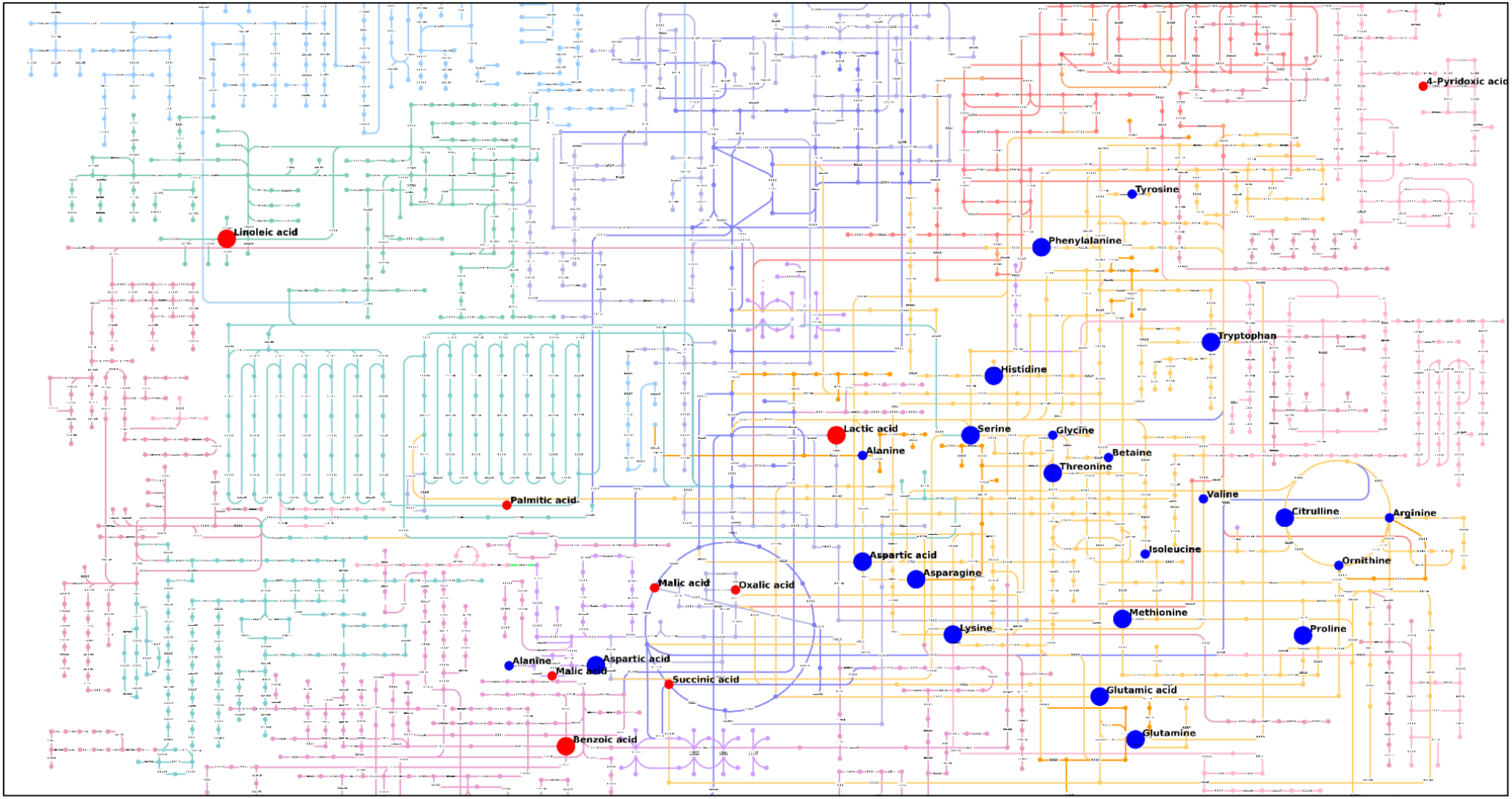

Figure 4: Metabolomic profile of farmed *Argyrosomus japonicus.* The profile was created using Pathway Projector (www.g-language.org/PathwayProjector/) based on all identified metabolites. Significantly altered compounds are denoted in larger points. Most amino acids (blue) and few organic acids (red) are shown while acylcarnitines were not obtained from KEGG human metabolome database (Only C18 acylcarnitine was present).

There were only six amino acids (citrulline, glutamine, lysine, methionine, phenylalanine and proline) that showed significant differences which can potentially indicate different levels parasite prevalence on the fish or geographical / husbandry differences. Glutamine (282.3 ± 23.36 µmol/L), lysine (318.1 ± 3.78 µmol/L) and proline (3404 ± 194.3 µmol/L) concentrations were significantly higher in the fish sampled from Mtunzini compared to the rest of the farms, while from Blue Cap had significantly elevated methionine (180.4 ± 12.12 µmol/L) and phenylalanine (322 ± 28.35 µmol/L) (Figure 5).

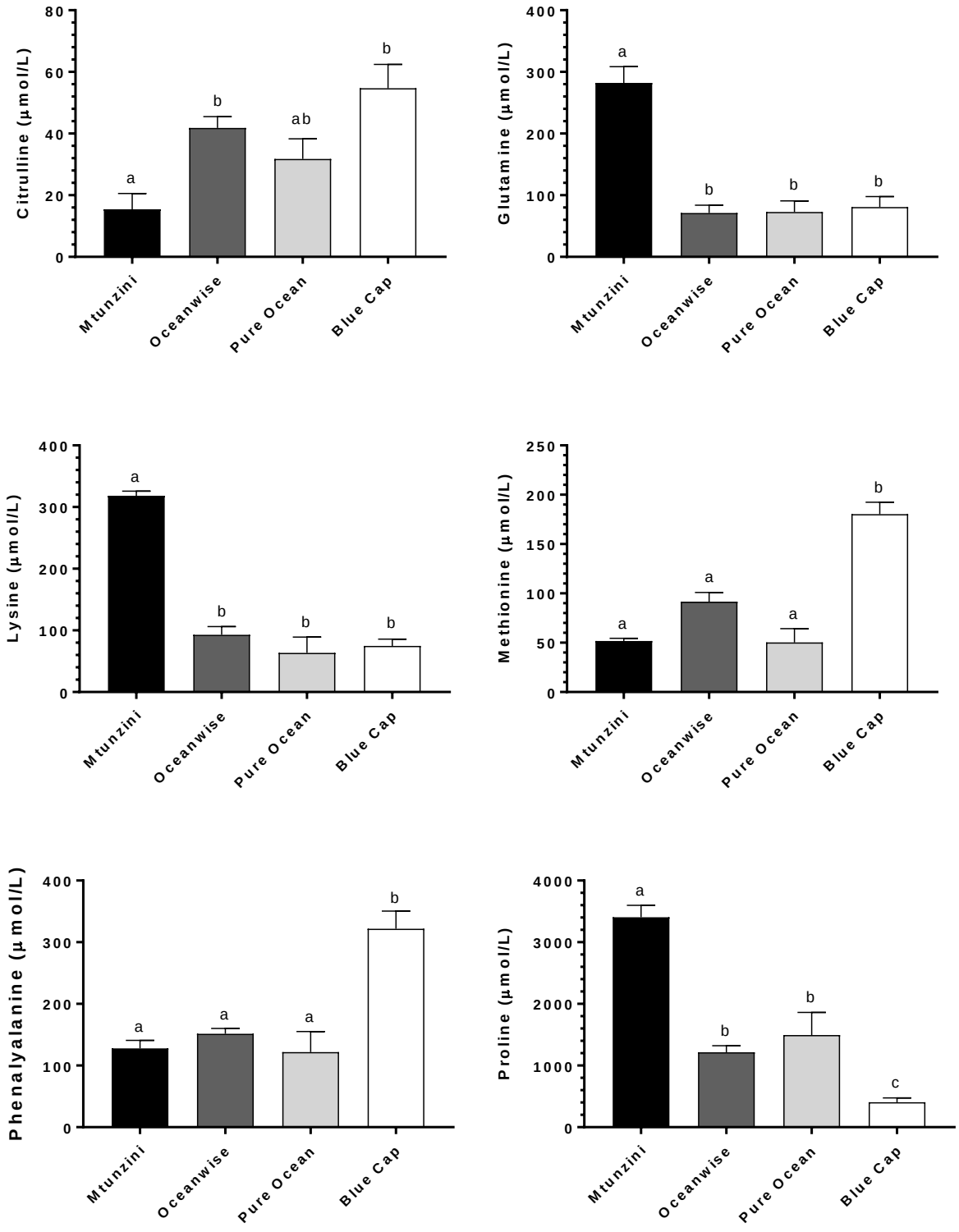

Figure 5: Amino acid concentrations in DBS of farmed Dusky kob, *A. japonicus* (mean ± S.E.M., N=5), from four farms with variable parasite prevalence and different geographic distribution. Letters denote significant differences at $P < 0.05$, ANOVA and Tukey's multiple comparisons test.

Using the significant amino acids (Figure 5), pathway analyses were conducted to determine biochemical pathways influenced by these compounds. The compounds were identified using KEGG and pathway topology and over-representation analyses were conducted in Metaboanalyst, using zebrafish (*Danio rerio*) as a model organism. The analyses revealed seven significant ($P < 0.05$) pathways that the amino acids were involved in (Table 5). Pathways with the most impact included phenylalanine, tyrosine and tryptophan biosynthesis (0.5), phenylalanine metabolism (0.4074), alanine, aspartate and glutamine metabolism (0.1498) and cysteine and methionine metabolism (0.1036) (Table 5), see also Figure 5. Although aminoacyl-tRNA biosynthesis and arginine and proline metabolism had the highest significance, their impact values were 0.0 and 0.0989 respectively.

Table 5: Summary of pathway analysis conducted using the six significant amino acids (citrulline, glutamine, lysine, methionine, phenylalanine and proline). Total denotes the number of all compounds involved in the pathway; Hits, denote the matched number of compounds from the uploaded data (i.e. six amino acids); p-value is calculated from the enrichment analysis; Impact is defined as the pathway impact value calculated from pathway topology analysis. (Letters in brackets indicate pathways as they appear in Figure 6).

Pathway	Total	Hits	P-value	Impact
Aminoacyl-tRNA biosynthesis (a)	67	5	$1.56E^{-6}$	0.0
Arginine and proline metabolism (b)	43	3	$5.77E^{-4}$	0.0989
Phenylalanine, tyrosine and tryptophan biosynthesis (c)	4	1	0.0178	0.5
D-Glutamine and D-glutamate metabolism	5	1	0.0222	0.0
Biotin metabolism (d)	5	1	0.0222	0.0
Nitrogen metabolism (e)	9	1	0.0397	0.0
Phenylalanine metabolism (f)	11	1	0.0484	0.4074
Alanine, aspartate and glutamate metabolism (g)	24	1	0.1030	0.1498
Cysteine and methionine metabolism	29	1	0.1233	0.1036
Pyrimidine metabolism (h)	41	1	0.1705	0.0
Purine metabolism (i)	66	1	0.2620	0.0

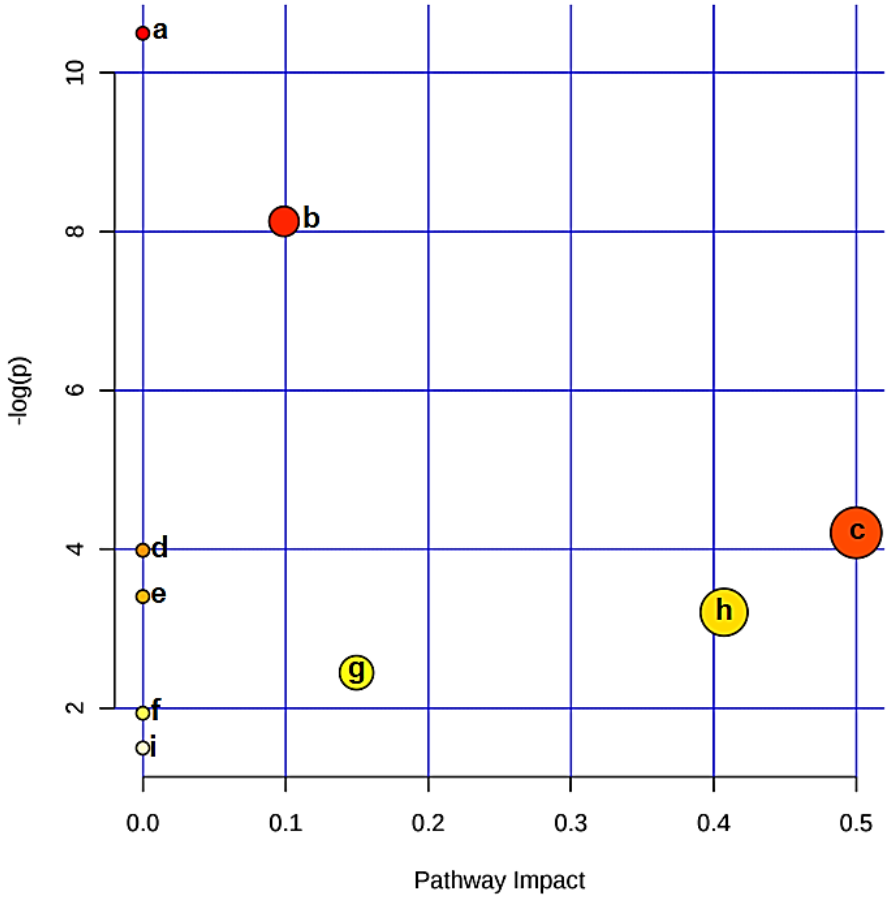

Figure 6: Pathway enrichment analysis of the six significant metabolites (citrulline, glutamine, lysine, methionine, phenylalanine and proline). The represented pathways are Aminoacyl-tRNA biosynthesis (a); Arginine and proline metabolism (b); Phenylalanine, tyrosine and tryptophan biosynthesis (c); Biotin metabolism (d); Nitrogen metabolism (e); Phenylalanine metabolism (f); Alanine, aspartate and glutamate metabolism (g); Pyrimidine metabolism (h) and Purine metabolism (i).

3.5 Discussion

3.5.1 The Use of DBS and Metabolomic Analyses for Fish Health Assessment

Using three previously validated targeted methods, metabolite analyses of DBS from farmed Dusky kob identified 53 metabolites in the present study. Using targeted mass spectrometry analyses and multivariate data analyses we were able to elucidate patterns of geographical distributions of the farms where fish were sampled (Figure 2 and Figure 3). This can potentially

be useful to elucidate differences in farming conditions and environmental parameters which may have severe effects on the fish. The use of metabolomics in fish (and other aquatic species) has been shown to be essential in animal health assessment (Samuelsson & Larsson, 2008). The application of metabolomics analyses has been further demonstrated in other aquatic organisms. Dove *et al.* (2012) used ^{1}H nuclear magnetic resonance spectroscopy (NMR) to study potential biomarkers in whale sharks (*Rhincodon typus*) where PCA analyses could resolve between healthy and unhealthy individuals. Homarine and trimethylamine oxide (TMAO) were subsequently confirmed as significantly decreased in diseased whale sharks. These studies show the effectiveness of metabolomics analysis and PCA as a screening method to distinguish animals based on their health status.

The observed grouping of the samples can be attributed to variance within all metabolite concentrations measured and how their changes are related to the observed parasite load (Figure 5). While the only stressor examined was parasite load, numerous environmental factors such as solvents, pesticides and endocrine-disrupting chemicals have been shown to influence changes in the fish metabolome (Samuelsson & Larsson, 2008). This can be used as a guide in enhancing the scope of future studies by incorporating the effects of water quality parameters, different regions from which the farms are located and different culture systems on the metabolome of Dusky kob. There is a paucity of literature on metabolite responses to parasitic infections of fish. Therefore, it remains unclear whether a strong parasite infection signal can be studied through the metabolome profile of fish.

3.5.2 Amino Acids in Fish

Fish are known to require similar essential dietary amino acids (EAA: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) as required by most animals (Ketola, 1982). Furthermore, fish and other aquatic organisms have conditionally essential amino acids (CEAA) namely cysteine, glutamine, hydroxyproline, proline and taurine (Li *et al.*, 2009). In the present study, all other essential amino acids (EAA) were determined with significant differences between farms, except for isoleucine, leucine and valine (Supplementary data, Table S2). The lack of detection of EAA in high concentrations using metabolomics is limiting towards understanding biomarkers and is often attributed to several factors. Mass spectrometry techniques (LC and GC) require sample derivatization which leads to non-detection of several compounds (Zhang *et al.*, 2012). Furthermore, LC-

MS/MS techniques have also been demonstrated to determine more amino acids when plasma or serum samples are analysed (Becker *et al.*, 2012), as opposed to the present study in which DBS were examined.

Despite limitations in the methodology and use of DBS, several EAA (phenylalanine, methionine and lysine) and dispensable amino acids (glutamine and citrulline) are known to respond to stress induction in fish were observed (Figure 5). The observed parasite intensity (Table 4) tentatively corresponds with the measured amino acid concentrations (Figure 5), however is contradictory to PCA analyses (Figure 2 and 3). This demonstrates that alterations in amino acids occur individually depending on their roles in the physiology of the fish (Table 6).

Table 6: The six significant amino acids from this study, and their known roles in the physiology, biochemistry and metabolism of several fish species, including the biological samples they were measured from and stress (or condition) induced for the analyses.

Amino acid	Stressor/Condition	Matrix	Function	Species	Reference
Citrulline	Oxidative stress	Erythrocytes	Anti-apoptotic function	Common carp	Li *et al.* (2013)
	Neurotoxicity	Plasma	Source of arginine	Channel catfish	Buentello and Gatlin (2001)
Glutamine	Exogenous ammonia	Liver; Muscle and Intestine	Ammonia removal	Rainbow trout	Anderson *et al.* (2002)
	Dietary supplementation		Regulate food intake	Japanese flounder	Kim *et al.* (2003)
	Oxidative stress	Erythrocytes	Anti-apoptotic function	Common carp	Li *et al.* (2013)
Lysine	Dietary supplementation		Lipid transporter on mitochondrial membrane	Various fishes	Harpaz (2005)
	Diet, temperature and stocking density		Enhance growth	Israeli carp	Viola *et al.* (1992)
	Dietary lysine deficiency	Muscle	Increase metabolic rate	Pacu	Abimorad *et al.* (2017)
	Dietary supplementation		Enhance growth; Feed conversion; Reduce fat levels	Rainbow trout	Cheng *et al.* (2003b)
Methionine	Dietary supplementation	Intestinal tissue	Induce larval intestinal maturation	Sea bass	Péres *et al.* (1997)
	Salinity	Plasma and Muscle	High energy storage; antioxidant	Arctic charr	Bystriansky *et al.* (2007)
	Dietary supplementation		Lipid transporter on mitochondrial membrane	Various fishes	Harpaz (2005)
Phenylalanine	Tube feeding		Influence metamorphosis	Sole	Pinto *et al.* (2009)
	Hormone exposure	Muscle	Influence pigmentation	Japanese flounder	Yoo *et al.* (2000)

	Albino mutants	Head samples	Influence pigmentation	Rainbow trout	Boonanuntanasarn *et al.* (2004)
	Hydrostatic pressure	Brain tissue	Neurotransmitters that modulate stress responses	European flounder	Damasceno-Oliveira *et al.* (2007)
Proline	Dietary supplementation	Vertebrae	Enhance growth; collagen function	Atlantic salmon	Aksnes *et al.* (2008)
	Dietary supplementation		Enhance growth; collagen synthesis	Various fishes	Li and Wu (2017)
	Oxidative stress	Erythrocytes	Anti-apoptotic function	Common carp	Li *et al.* (2013)
	Streptococcus agalactiae infection	Liver	Reduce mortality	Tilapia	Zhao *et al.* (2015)

3.5.3 Significance of Variable Amino Acid Responses

Glutamine and citrulline were significantly reduced in the Senegalese sole, *Solea senegalensis* due to high stocking density (14 kg m^{-2}) (Costas *et al.*, 2008). Furthermore, reduced plasma citrulline concentration was found in *S. senegalensis* exposed to handling stress (Aragao *et al.*, 2008). Mortality of farmed tilapia due to elevated temperatures (25°C and 30°C) has been reported to correlate with significantly lower L-proline concentration (Zhao *et al.*, 2015).

These variable amino acid responses demonstrate the value of metabolomics in discovering reliable metabolic features that can subsequently be developed as biomarkers of health in fish. The lack of research on the effects of parasites on their fish hosts in relation to fluctuations in amino acids, limits the conclusions that can be drawn based on the results of this study. Furthermore, understanding amino acid responses is essential for health assessment of farmed fish and could be useful in other fields including drug and feed development. Amino acids play a major role in the regulation of metabolic pathways responsible for growth, reproduction and resistance to environmental stressors and pathogenic organisms.

3.5.3.1 Role of Glutamine in the Immune Response

The free α-amino acid glutamine occurs in abundance in fish muscle and plasma. Purine and pyrimidine nucleotide synthesis are largely dependent on glutamine, while this amino acid is also essential for the regulation of acid-base balance in the body (Li *et al.*, 2009). Glutamine in fish is a major substrate for leukocyte proliferation, thus is important to the immune response (Buentello & Gatlin, 1999). In the present study glutamine was significantly elevated in fish from Mtunzini (Figure 5), which were also most highly infected by *D. oliveri* (Table 4).

Although ecto-parasitic infections are known to induce physical damage on fish, studies have shown elevated responses from the host's immune system. The immune response of various fish species post infection by monogenean parasites has been demonstrated through changes in gene expression (Lindenstrøm *et al.*, 2003; Lindenstrøm *et al.*, 2004; Matejusová *et al.*, 2006; Kania *et al.*, 2007). Dash *et al.* (2014) reported significant alterations of innate immune response factors (antiprotease activity, natural agglutinin level and lysosome activity) in *Labeo rohita* parasitized by the monogenean *Dactylogyrus catlaius.* Additionally, immune-related gene expression in the gill was significantly upregulated for β2-microglobulin, major histocompatibility complex (I and II) and tumour necrosis factor-α, while downregulated

expression of superoxide dismutase and interleukin 1β was observed in the kidney. Furthermore, various fish species have shown cellular and humoral immune responses against infection by monogenean parasites. For example, the production of immunoglobulin in the blood of carp against *Dactylogyrus vastator* (Vladimirov, 1971) and tiger puffer producing antibodies due to *Heterobothrium okamotoi* infection (Wang *et al.*, 1997) are examples of specific humoral responses against monogenean parasites. These findings show that although ectoparasite infection although external, induces an innate and acquired immune response from their fish hosts. Rubio-Godoy (2007), however, suggested that immunoglobulin production only served as a partial protection measure against parasitic infection, therefore extensive research on fish immune response due to parasites is essential.

The most abundant amino acid in the muscle and blood is glutamine, serving significant roles in the synthesis of purines, pyrimidines and nucleotides, production of amino sugars and proteins and mostly as an energy source (Newsholme, 2001; Li *et al.*, 2009). High glutamine utilization by lymphocytes during stress suggests that the amino acid is essential to the immune response of the organism (Calder & Yaqoob, 1999). For example, mice fed casein diets supplemented with 40 g glutamine/kg had a 30% mortality which was significantly lower than that of the control (80% mortality) following infection by *Staphylococcus aureus* (Suzuki *et al.*, 1993). Several studies have shown similar effects in the immune response and survival of rats during stress (Salleh *et al.*, 1991; Inoue *et al.*, 1993; Naka *et al.*, 1996).

Mammalian leukocytes have a high utilization rate of glutamine during injury and infection (Newsholme, 2001). This amino acid is essential to immune cells, especially lymphocytes, macrophages and neutrophils during stress. In contrast, the significance of glutamine in fish lymphocytes is not well defined. Several studies on the role of glutamine in improving fish immunity and stress tolerance have shown that the amino acid is mostly essential for leukocyte proliferation, modulating cytokine and nitric oxide (NO) production (Li *et al.*, 2009). Juvenile red drum, *Sciaenops ocellatus*, fed enhanced dietary glutamine for seven weeks showed significantly higher serum lysosome activity, elevated intra- and extracellular superoxide anion production by macrophages (Cheng *et al.*, 2011) while enhanced neutrophil oxidative radical production was observed in juvenile hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) fed similar diets for eight weeks (Cheng *et al.*, 2012). *In vitro* amino acid utilization by channel catfish immune cells demonstrated that glutamine supplementation resulted in the proliferation of naïve T- and B-lymphocytes (Pohlenz *et al.*, 2012). Glutamine requirement for lymphocytes and macrophages *in vitro* can be replaced by hydrolysable glutamine dipeptides (Calder &

Yaqoob, 1999; Zheng *et al.*, 2006), however cannot be substituted by glutamic acid or glucose (Wilmore & Shabert, 1998). These findings demonstrate the significance of glutamine in immune cell proliferation, thus serving as source of energy and stimulation for the host immune response during stress.

The upregulated glutamine concentration in Mtunzini (Figure 5) may correlate with increased immune function required to combat the high parasite load (Table 4). Confirmation of this correlation however requires support from white blood cell counts or other immunological responses. Nonetheless, at Mtunzini, parasite load and resultant immune upregulation is not so extreme as to deplete circulating glutamine in the blood, and cause subsequent immunodeficiency. This is further substantiated by the fish being in good condition (Table 3). In salmonids, a K-value of 1.40 and higher signals a fish in good condition while fish with poor condition are indicated by a K-value of 1.00 and below (Barnham & Baxter, 2003). The lowered plasma glutamine concentrations observed in the other farms are most likely the result of demand for glutamine (by the liver, kidney, gut and immune system) exceeding the supply (from the diet and from muscle). Lardon *et al.* (2013) reported decreased concentrations of glutamine and glutamate in the crucian carp (*Carassius carassius*) exposed to anoxia for 1 day and 1 week. This observation was related to decreased activity of the tricarboxylic acid (TCA) cycle and increased concentrations of the neurotransmitter gamma aminobutyric acid (GABA). It has been suggested that lowered plasma glutamine concentration contributes, at least in part, to the immunosuppression which accompanies such situations (Calder & Yaqoob, 1999).

3.5.3.2 Lysine and Proline

Wang *et al.* (2015) reported significantly elevated lysine and proline concentrations from tongue sole (*Cynoglossus semilaevis*), due to increased salinity, suggesting that some EAA and free amino acids are essential for osmotic regulation in fish. In contrast to expected low concentrations of lysine and proline in fish sampled from brackish water ponds in Mtunzini, a significant accumulation of both amino acids was observed (Figure 5). One explanation for this increase in lysine and proline could be that the fish were exposed to elevated salinity at the time of sampling. Incidentally, a higher collagen content was reported to correlate with elevated hydroxyproline concentrations in the marine crab *Scylla serrata* (Sivakumar *et al.*, 2000). According to Li *et al.* (2009) numerous amino acids in fish are well defined with regards to their synthesis, pathways and downstream effects however proline synthesis in fish has not

been examined extensively. Evidence of the importance of proline has been demonstrated in other aquatic animals. Vosloo *et al.* (2013) suggested that L-proline supplementation of feed in abalone (*Haliotis midae*) reduced the energy demands of antioxidant production and resulted less DNA damage, which can have long term effects such as chromosome damage and subsequent apoptosis. The lack of understanding the importance of the accumulation or degradation of amino acids as an immune response in fish is a challenge for researchers.

Lysine: Elevated from Feed Supplementation?

In the present study, fish from Mtunzini had elevated concentrations of lysine, proline and glutamine (Figure 5). The observed increased lysine is potentially due to dietary supplementation as it is an EAA. Elevated dietary lysine has been shown to have effects on other amino acids and compounds, particularly in the urea cycle. Berge *et al.* (1998) reported a reduction of arginine and ornithine as a result of elevated plasma lysine in Atlantic salmon fed a lysine supplemented diet. Furthermore, Rainbow trout fed elevated dietary lysine showed reduced arginine degradation (Kaushik & Fauconneau, 1984). The accumulation of arginine is known to be characterized by the inhibition of arginase activity. This has been preciously demonstrated by *In vitro* analyses of arginase in Atlantic salmon, where arginase activity was inhibited due to the addition of lysine (Berge *et al.*, 1998). In teleost fish, arginase can further be synthesized by converting citrulline via arginosuccinate (Mommsen *et al.*, 2001). Based on this and KEGG pathways, we can construct a mechanism of elevated lysine and its downstream effects observed for the Mtunzini fish (Figure 7).

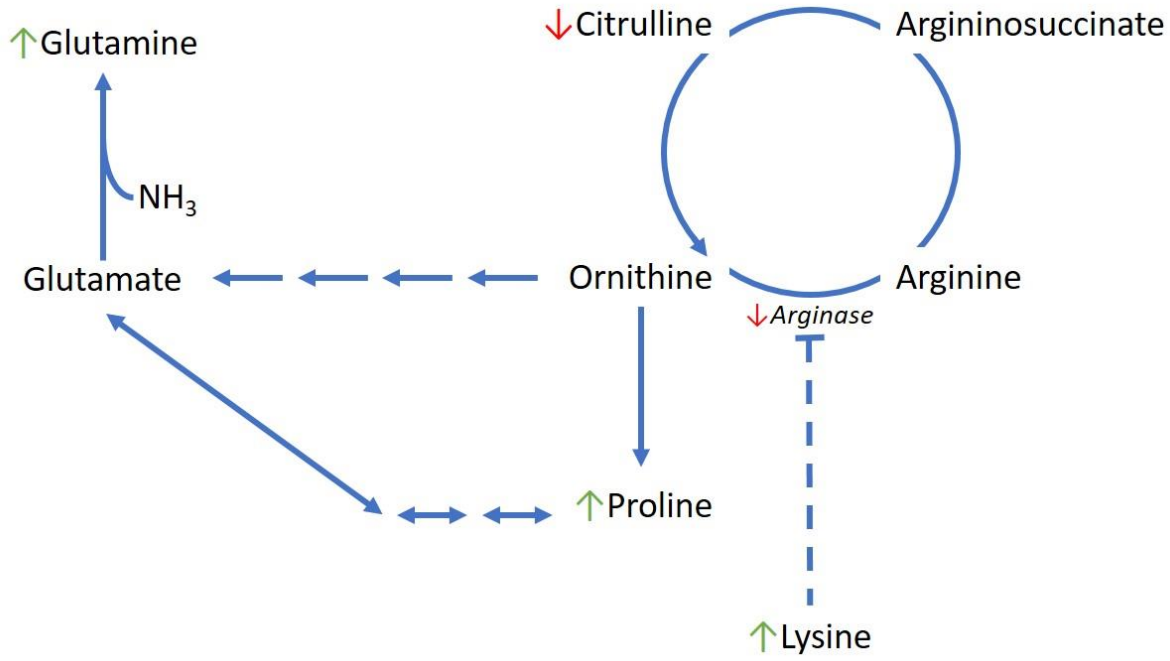

Figure 7: A serendipitous pathway due to lysine elevation from Mtunzini fish. The mechanism shows potential interactions and effects of lysine on arginine, proline and glutamine as observed in the present study.

The elevated lysine and subsequent interaction with compounds in the urea cycle can potentially explain the observed accumulation of proline and glutamine in the present study (Figure 5 and 7). The synthesis of proline in animals occurs from glutamine, glutamate, ornithine and arginine (Wu *et al.*, 2008), with maximal arginine-derived proline production occurring in the absence of proline oxidase (Wu *et al.*, 2011). Glutamine, a by-product of arginine was significantly elevated in Mtunzini fish (Figure 5) whereas arginine was not altered significantly between farms in the present study. This is in accordance with observations in channel catfish fed an arginine supplemented diet, where no significant increase in plasma arginine was observed, however arginine by-products ornithine, glutamine and glutamate were significantly elevated (Pohlenz *et al.*, 2014). These observations suggest that lysine enhances glutamine and proline production for their associated benefits. For example, dietary glutamine supplementation improves growth and survival of the tongue sole by upregulating the expression of the heat shock protein 70 (HSP70) and enhancing the antioxidant capacity of the fish (Liu *et al.*, 2015). Meanwhile proline has significant roles in cellular metabolism and physiology including glutamine and protein synthesis; and regulation of gene expression and cell proliferation (Wu *et al.*, 2011). Furthermore, both glutamine and proline have protective

functions against hydroxy radicals by inhibiting oxidative damage and apoptosis of fish erythrocytes (Li *et al.*, 2013). These studies highlight the need for understanding dietary amino acid requirements in fish, particularly for Dusky kob. Furthermore, analysing the downstream effects of elevated EAAs can aid in biomarker development and fish health assessment.

3.5.4 Dietary Amino Acids Requirements

A major aspect of aquaculture production is the supplementation of feed with dietary amino acids and proteins. Supplemented feeds not only benefit the animals in providing essential amino acids that they cannot synthesize in adequate quantities, but also improves nutritional value and growth rates, enhances immunity and improves overall quality of flesh. According to Wilson (2002), fish have protein requirements of up to 55% (two to four times) higher than other vertebrates. This demonstrates the inefficient utilization of protein by fish. In the present study, different responses were observed for lysine and methionine (Figure 5). Lysine was significantly higher in fish from Mtunzini while methionine was significantly reduced compared to fish from other farms. Both lysine and methionine are considered as limiting amino acids in fish diets and therefore have adverse effects on the growth rate and health of the fish (Li *et al.*, 2009). The nutritional and physiological benefits of lysine supplemented feed in fish have been studied extensively, for example Cheng *et al.* (2003a) reported reduced excretion of ammonia and soluble phosphorus in rainbow trout consuming feed-grade lysine, while an enhanced immune response was also shown in Jian carp fed dietary lysine supplemented aquafeed (Zhou, 2005). These findings highlight the need for extensive research on species-specific amino acid requirements in fish and how they influence physiological responses in the intensive aquaculture environment.

3.5.5 Fatty Acids in Fish

Fish are universally regarded as an important food source, providing essential human nutrients such as omega-3 fatty acids. Lipids (and their fatty acid constituents) are known to be vital for fish physiology and occur in abundance (Tocher, 2003) serving as a predominant source of energy for routine metabolism. Therefore, understanding lipid and fatty acid metabolism in fish is essential for the growth and development of aquaculture. The reported abundance of lipids and fatty acids in literature does not coincide with the findings of the present study. Here we

report only linoleic acid (9,12-octadecadienoic acid) (Supplementary data, Table 1A) as the only significant polyunsaturated fatty acid (PUFA). The poor representation of fatty acids is likely due to the use of DBS, as it may be an unfavourable source for lipid extraction. Leaver *et al.* (2008) reported that highly unsaturated fatty acids (HUFA) are primarily deposited in the flesh of fish. Changes in plasma fatty acids can however reflect short-term disturbances, as Samuelsson *et al.* (2006) found in rainbow trout exposed to EE_2. Several studies on Atlantic salmon have demonstrated the liver to be a significant source of fatty acids (Ruyter *et al.*, 2000; Montero *et al.*, 2001) and thus a better sampling matrix using metabolomics to understand fatty acid metabolism.

The use of DBS for MS analyses of fatty acids appears to be a limiting factor as evident by the under-representation of fatty acids in this study. This limitation however, is not observed in human based studies as extensive research has effectively quantified fatty acids through DBS analyses. Amino, organic and fatty acids have been detected using optimized methods of extraction and quantification for newborn screening studies (Zytkovicz *et al.*, 2001; Chace *et al.*, 2003). Luginbühl *et al.* (2016) further reported the effectiveness of DBS in detecting the presence of alcohol in the blood by quantifying fatty acid ethyl esters using LC-MS/MS. Furthermore, long chain polyunsaturated amino acids (LC-PUFA) remain intact in DBS and can be quantified after 6 months' storage at -28°C (Pupillo *et al.*, 2016). These studies are evidence that optimization of DBS protocols in the present study and incorporation of liver and muscle tissue analyses can be beneficial in determining the presence of more metabolites, thus allowing for better understanding of Dusky kob physiology.

3.6 Conclusions

Metabolomic analyses conducted in the present study uncovered multivariate data patterns of metabolite composition from dried blood spots of farmed Dusky kob in South Africa. These patterns have the potential to be utilized as biomarkers since they indicate that geographical distributions (therefore the effects environmental parameters) can be assigned with some degree of certainty (Figure 2 and Figure 3). MS analyses revealed significant amino acid compounds that vary in concentration seemingly meaningful with regards to parasite load (Figure 5) especially when taking into account their biological roles. These compounds show the potential to be significant biomarkers, however they require validation using controlled

experimentation. The numerous biological data extracted through metabolomics in this study further elucidate the benefit of utilizing DBS as means of sample collection and storage which can be achieved under non-clinical (field) settings, where conventional methods are difficult to implement.

The exploratory analysis by PCA of Dusky kob metabolite spectra showed clustering patterns that were influenced heavily by 21 metabolites (Supplementary data, Table 1A). These compounds and several more proved useful in outlining important biochemical pathways that are influenced by either the upregulation or degradation of metabolites in Dusky kob (Figure 4). Detailed analyses of the pathways and endpoint effects at the organism level are essential in the development of validated animal health assessment measures. Furthermore, knowledge of essential amino acids and fatty acids for Dusky kob are important towards biomarker development and fish health monitoring.

Although targeted metabolomic approaches were successful in identifying 53 compounds in this study, there was minimal evidence correlating with observed parasitic infection trends. Therefore, no conclusions can be drawn with certainty regarding the effects of parasite on the physiology of Dusky kob. Several factors including extraction protocols, sensitivity of the techniques and biological sample used could have negatively influenced the observed metabolome spectra. Given the knowledge present on fish amino acid and fatty acid metabolism, metabolite compounds composition in Dusky kob are not expected to differ from other vertebrates. To ensure that the full spectrum of metabolites is studied and understood for the species, several biological samples (i.e. muscle, liver, etc.) need to be analysed. Furthermore, the use of untargeted metabolomic analyses and different metabolomics platforms (i.e. NMR spectroscopy) are fundamental to giving new insights on the general scope of Dusky kob metabolite profile.

We expected to observe differential metabolite responses corresponding to varied parasite intensity, however we identified clustering patterns that give a geographical signature. Furthermore, the methods were not able to identify significant fatty acids which are essential to understanding how diseased animals respond to the energy demands caused by stress induction in aquaculture systems. We have further demonstrated the usefulness of DBS for blood collection and storage and their wide range applicability in biomarker discovery (i.e. blood glucose and metabolomics). Overall, we have conducted a first exploratory analysis of

metabolomics techniques in farmed Dusky kob, which has shown to be an essential tool towards biomarker analyses and fish health assessment.

Table S1: Metabolite concentrations (µmol/L) in dried blood spots of Dusky kob from four farms

Farm	Mtunzini					Oceanwise					Pure Ocean					Blue Cap				
Sample I. D	M11	M12	M21	M24	M30	O11	O12	O14	O15	O17	P3	P11	P13	P28	P30	B15	B18	B20	B26	B30
Lactic acid, (2TMS)	5,2	4,8	11,6	12,4	17,3	26,4	23,1	18,7	28,1	13,6	7,3	10,4	10,1	3,1	11,7	8,3	9,2	4,5	3,9	16,5
Octadecanoic-Acid	3,5	4,9	27,2	8,5	8,9	5,8	6,9	4,1	5,4	9,5	7,6	4,9	3,4	5,8	7,3	8,1	25,7	14,7	8,8	6,1
9-Octadecenoic-Acid	2,7	3,8	37,7	8,5	4,7	0,9	0,8	0,8	1,8	1,6	2,1	0,6	0,6	2,8	2,0	1,7	7,0	5,5	2,9	1,4
2,3-Dihydroxybutane	13,7	14,6	0,7	3,5	0,3	8,5	2,2	8,0	0,1	0,1	0,1	2,8	4,3	2,9	0,4	0,4	0,2	0,5	0,1	6,9
Succinic-Acid	2,3	2,9	8,1	2,2	2,2	4,9	4,9	3,7	6,8	5,0	3,6	3,7	3,0	3,2	3,6	1,8	11,1	3,3	7,0	3,3
Benzoic-Acid	0,8	0,7	0,3	0,9	0,3	1,1	1,2	0,9	0,2	0,4	0,1	1,4	1,3	0,9	0,6	0,4	0,2	0,3	0,3	0,7
9,12-Octadecadienoic-Acid	0,4	1,0	8,5	3,0	1,6	0,4	0,2	0,3	0,4	0,9	0,4	0,1	0,1	0,4	0,3	1,0	3,6	0,9	0,8	0,5
3,4-Dihydroxybutyric-Acid	0,6	1,0	1,7	0,6	0,7	0,7	0,3	0,6	1,6	0,5	0,5	0,4	0,4	0,8	0,5	0,5	2,3	0,8	1,0	0,4
Tetradecanoic-Acid	0,6	0,9	4,8	1,3	0,8	0,5	0,5	0,4	0,7	0,9	1,2	0,8	0,6	2,2	1,7	0,7	2,4	2,5	1,0	0,6
Hexanoic-Acid	0,7	0,4	0,4	0,4	0,0	0,7	0,7	0,6	0,1	0,1	0,0	0,7	0,7	0,2	0,0	0,1	0,1	0,1	0,1	0,6
Hexadecanoic acid	0,3	0,3	1,9	0,6	0,7	0,1	0,1	0,1	0,3	0,1	0,2	0,1	0,1	0,3	0,2	0,5	0,5	0,3	0,2	0,1
Palmitelaidic-Acid	0,5	0,6	6,4	1,8	1,8	0,1	0,1	0,1	0,4	0,2	0,6	0,2	0,2	1,8	1,2	1,6	1,2	0,9	0,4	0,2
Nonanoic-Acid	0,3	0,5	0,3	0,5	0,2	0,4	0,4	0,4	0,2	0,1	0,1	0,5	0,3	0,6	0,3	0,3	0,3	0,1	0,2	0,4
9-Octadecenoic acid, TMS ester	0,4	0,7	5,4	8,5	0,9	0,3	0,2	0,2	0,5	0,5	0,6	0,3	0,2	1,0	0,7	0,3	1,7	1,2	0,6	0,3
Palmitic-Acid	8,9	12,5	76,2	20,0	17,8	8,6	10,0	7,3	11,1	12,9	16,8	10,6	8,6	19,0	18,9	12,1	43,2	26,0	16,5	10,7
1,4-Butanediol, bis-TMS	1,5	1,9	1,5	0,9	0,6	2,1	1,7	1,2	0,8	0,6	0,6	1,3	1,8	1,4	0,8	0,6	1,3	0,5	0,7	1,2
1,2-Butanediol	0,7	1,0	0,0	0,0	0,0	1,0	0,8	0,7	0,0	0,0	0,0	0,8	0,8	0,2	0,0	0,0	0,0	0,0	0,0	1,0
Isobutyric-Acid	0,2	0,3	0,6	0,3	0,1	0,3	0,3	0,2	0,2	0,3	0,2	0,4	0,3	0,2	0,3	0,1	0,5	0,1	0,5	0,2
Glutaconic-Acid	0,0	0,1	0,2	0,0	0,1	0,1	0,1	0,1	0,1	0,2	0,1	0,1	0,1	0,1	0,1	0,1	0,4	0,1	0,2	0,1
Malic-Acid	0,8	0,9	1,9	0,7	0,6	1,6	1,4	1,2	1,7	1,3	0,5	1,1	0,4	0,7	0,7	0,7	3,1	0,9	1,9	1,0
4-Pyridinecarboxylic-Acid	0,4	0,3	0,0	0,0	0,0	0,8	1,0	0,3	0,1	0,1	0,0	1,2	0,4	0,0	0,0	0,0	0,0	0,1	0,1	0,2

Monostearylglycerol	0,3	0,3	1,5	8,5	0,0	0,2	0,2	0,2	2,4	0,4	1,0	0,7	0,3	3,4	8,3	0,0	0,0	0,9	0,8	0,2
Glycolic acid, (2TMS)	0,6	1,2	3,3	1,3	1,8	0,9	0,6	0,7	1,5	0,5	0,6	1,0	0,6	0,9	0,5	1,2	2,2	0,6	1,1	0,5
Oxalic acid	0,0	0,2	0,5	0,1	0,1	0,2	0,2	0,1	0,2	0,2	0,1	0,2	0,1	0,1	0,2	0,1	0,5	0,1	0,4	0,2
Malonic-Acid	19,6	10,5	0,0	1,0	0,5	14,6	21,2	18,2	0,5	0,0	0,4	27,1	16,2	1,1	0,4	0,4	0,0	0,0	0,0	15,9
C0	10,2	10,4	11,0	10,5	10,1	13,5	18,3	11,5	13,3	12,3	16,9	10,4	11,9	10,5	9,6	11,2	12,6	14,2	14,4	16,4
C2	4,8	4,9	3,5	7,3	2,8	8,2	7,3	3,8	5,0	5,4	1,4	2,0	5,3	2,5	1,3	2,7	1,3	0,8	0,7	1,1
C3	0,8	0,8	0,5	1,3	0,4	0,1	0,1	0,4	0,4	0,1						0,0				
C4	0,1	0,1	0,0	0,1	0,1	0,1	0,2	0,2	0,2	0,1	0,2	0,1	0,3	0,0	0,1	0,0	0,0	0,0	0,0	0,0
C5	0,0	0,0		0,0		0,1	0,1	0,1	0,1	0,1	0,6	0,1	0,1	0,1	0,1	0,1	0,0	0,1	0,1	0,1
C8	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,0	0,1	0,1	0,1	0,1	0,1
C10	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,1	0,1	0,0	0,0	0,0	0,0	0,0	0,1	0,0	
C16	0,1	0,1	0,0	0,0	0,0	0,1	0,1	0,1	0,0	0,1	0,0	0,0	0,1	0,1	0,0	0,0	0,0	0,0	0,1	
Alanine	318	397	346	364	354	784	743	330	584	647	54,2	262	504	300	77,4	429	275	224	221	296
Arginine	3,5	2,5	2,0	3,6	2,0	1,5	2,8	0,7	0,9	1,3	1,0	2,8	0,5	0,7	1,6	1,5	1,0	1,0	1,1	1,9
Asparagine	4,8	4,4	2,7	3,8	3,8	2,7	2,7	1,1	2,9	1,7		4,5	4,7	4,4	11,2	1,2	1,0	0,8	0,9	0,8
Aspartic acid	11,3	18,2	7,4	10,5	6,5	5,9	3,7	1,7	8,5	12,8	2,6	1,9	0,5	0,6	0,8	7,3	15,0	4,6	15,5	20,9
Citruline	5,7	3,4	21,5	15,5	31,2	42,2	48,8	29,5	39,2	49,5	31,3	50,1	28,2	38,9	10,5	41,1	58,2	83,2	49,2	41,7
Glutamic acid	107	138	105	135	86,2	251	250	65,9	134	217	29,9	61,5	42,3	61,9	15,9	311	230	154	243	409
Glutamine	316	300	289	324	179	69,1	98,1	24,1	83,5	80,5	87,6	134	54,7	60,7	27,6	52,7	69,0	147	74,7	60,6
Glycine	421	358	368	341	616	1111	879	235	844	902	24,0	358	499	881	96,2	301	304	169	142	361
Histidine	47,1	48,7	58,2	40,2	66,7	67,1	40,6	42,8	80,2	71,0	13,2	82,3	35,7	90,5	16,1	2,6	2,0	3,2	2,3	3,2
Isoleucine	258	355	395	393	374	439	486	240	415	495		492	215	308	57,2	555	437	458	237	305
Lysine	322	316	308	325	194	77,0	109	27,3	94,7	89,5	92,6	156	61,0	69,6	30,9	63,0	81,1	164	86,9	68,6
Methionine	47,9	45,9	49,9	53,1	61,6	86,0	106	70,0	75,9	119	24,8	86,2	56,2	71,6	13,1	182	210	156	149	202
Ornithine	2,8	2,7	2,2	3,1	3,3	3,0	3,7	0,9	3,7	2,3	2,1	7,8	2,9	3,8	0,9	2,4	1,8	1,7	1,2	1,3
Phenylalanine	100	17	136	102	129	154	155	127	168	219	160	307	97,3	189	40,3	404	301	367	244	291
Proline	2665	3576	3774	3608	3396	1437	887	1007	1353	1065		394	2212	1260	1018	679	397	316	302	333
Serine	151	130	138	156	264	1277	1535	143	1276	1872	20,5	73,5	184	448	58,8	514	410	178	377	1051
Threonine	54,9	75,3	81,7	67,8	70,2	157	225	90,0	153	168	18,0	51,2	86,6	90,0	23,3	184	168	124	186	327
Tryptophan	349	454	401	430	300	906	826	242	520	750	117	227	246	235	83,8	814	502	308	396	645

Tyrosine	90,6	197	720	213	110	117	119	88,9	129	182	63,4	154	58,9	100	27,5	174	179	217	102	169
Valine	137	146	189	202	219	284	341	199	276	352		457	174	227	40,3	462	457	569	387	467

Table S2: Significant compounds identified by ANOVA and Fisher's LSD post-hoc analysis. (FDR = false discovery rate; MTU = Mtunzini; BC = Blue Cap; PO = Pure Ocean; OW = Oceanwise)

Compounds	f-value	p-value	log 10	FDR	Fisher's LSD
Histidine	55,80	0,00	8,590	0,00	MTU-BC; OW-BC; PO-BC
C_3	42,81	0,00	7,670	0,00	MTU-BC; OW-BC; PO-BC
Glutamic acid	19,82	0,00	5,210	0,00	BC-MTU; BC-PO; MTU-PO; OW-PO
Aspartic acid	14,68	0,00	4,360	0,00	BC-PO; MTU-PO; OW-PO
Methionine	13,92	0,00	4,210	0,00	BC-MTU; BC-OW; BC-PO; OW-MTU; OW-PO
Glutamine	13,14	0,00	4,060	0,00	MTU-BC; OW-BC; MTU-OW; MTU-PO; OW-PO
C_2	13,12	0,00	4,050	0,00	MTU-BC; OW-BC; PO-BC; OW-PO
Threonine	12,52	0,00	3,930	0,00	BC-MTU; BC-PO; OW-MTU; OW-PO
C_4	12,30	0,00	3,900	0,00	MTU-BC; OW-BC; PO-BC
Lysine	11,97	0,00	3,820	0,00	MTU-BC; OW-BC; MTU-OW; MTU-PO; OW-PO
Tryptophan	10,78	0,00	3,560	0,00	BC-MTU; BC-OW; BC-PO; OW-MTU; OW-PO
Citrulline	7,23	0,00	2,660	0,01	BC-MTU; OW-MTU; PO-MTU
Serine	6,55	0,00	2,460	0,01	BC-PO; OW-MTU; OW-PO
Alanine	5,70	0,01	2,200	0,02	OW-BC; MTU-PO; OW-PO
Proline	5,17	0,01	2,030	0,03	BC-MTU; BC-OW; BC-PO; OW-MTU; OW-PO
Lactic acid, 2TMS	4,72	0,01	1,870	0,05	OW-BC; OW-MTU; OW-PO
Phenylalanine	4,30	0,02	1,730	0,06	MTU-BC; OW-BC; MTU-OW; MTU-PO; OW-PO
Arginine	3,60	0,03	1,470	0,10	MTU-BC; MTU-OW; MTU-PO
Tyrosine	3,54	0,04	1,450	0,10	BC-PO; MTU-PO; OW-PO
2,3-Dihydroxybutane	3,20	0,05	1,320	0,13	BC-PO; MTU-PO
9,12-Octadecadienoic-Acid	3,17	0,05	1,310	0,13	MTU-BC; PO-BC

3.9 References

Abimorad, E. G., Ducatti, C., Castellani, D., Jomori, R. K., Portella, M. C., Sartori, M. M. P., & Carneiro, D. J. (2017). Dietary lysine deficiency increases the incorporation rate of nitrogen-free compounds in muscle of pacu (*Piaractus mesopotamicus*). *Aquaculture, 479*, 522-527.

Aksnes, A., Mundheim, H., Toppe, J., & Albrektsen, S. (2008). The effect of dietary hydroxyproline supplementation on salmon (*Salmo salar* L.) fed high plant protein diets. *Aquaculture, 275*(1), 242-249.

Alfaro, A. C., & Young, T. (2016). Showcasing metabolomic applications in aquaculture: a review. *Reviews in Aquaculture, 0*, 1-18.

Anderson, P., Broderius, M., Fong, K., Tsui, K., Chew, S., & Ip, Y. (2002). Glutamine synthetase expression in liver, muscle, stomach and intestine of *Bostrichthys sinensis* in response to exposure to a high exogenous ammonia concentration. *Journal of Experimental Biology, 205*(14), 2053-2065.

Andree, K., Roque, A., Duncan, N., Gisbert, E., Estevez, A., Tsertou, M., & Katharios, P. (2016). *Diplectanum sciaenae* (Van Beneden & Hesse, 1863)(Monogenea) infecting meagre, *Argyrosomus regius* (Asso, 1801) broodstock in Catalonia, Spain. A case report. *Veterinary Parasitology: Regional Studies and Reports, 1*, 75-79.

Aragao, C., Corte-Real, J., Costas, B., Dinis, M. T., & Conceição, L. (2008). Stress response and changes in amino acid requirements in Senegalese sole (*Solea senegalensis* Kaup 1858). *Amino Acids, 34*(1), 143-148.

Barnham, C. A., & Baxter, A. F. (2003). Condition factor, K, for salmonid fish: Department of Primary Industries. 1-3, ISSN 1440-2254.

Becker, S., Kortz, L., Helmschrodt, C., Thiery, J., & Ceglarek, U. (2012). LC–MS-based metabolomics in the clinical laboratory. *Journal of Chromatography B, 883*, 68-75.

Berge, G. E., Sveier, H., & Lied, E. (1998). Nutrition of Atlantic salmon (*Salmo salar*); the requirement and metabolic effect of lysine. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 120*(3), 477-485.

Boonanuntanasarn, S., Yoshizaki, G., Iwai, K., & Takeuchi, T. (2004). Molecular cloning, gene expression in albino mutants and gene knockdown studies of tyrosinase mRNA in rainbow trout. *Pigment Cell & Melanoma Research, 17*(4), 413-421.

Buchmann, K., & Bresciani, J. (2006). Monogenea (Phylum Platyhelminthes). *Fish Diseases and Disorders, 1*, 297-344.

Buentello, J., & Gatlin, D. (2001). Plasma citrulline and arginine kinetics in juvenile channel catfish, *Ictalurus punctatus*, given oral gabaculine. *Fish Physiology and Biochemistry, 24*(2), 105-112.

Buentello, J. A., & Gatlin, D. M. (1999). Nitric oxide production in activated macrophages from channel catfish (*Ictalurus punctatus*): influence of dietary arginine and culture media. *Aquaculture, 179*(1), 513-521.

Bundy, J. G., Davey, M. P., & Viant, M. R. (2009). Environmental metabolomics: a critical review and future perspectives. *Metabolomics, 5*(1), 3-21.

Bystriansky, J., Frick, N., & Ballantyne, J. (2007). Intermediary metabolism of Arctic char *Salvelinus alpinus* during short-term salinity exposure. *Journal of Experimental Biology, 210*(11), 1971-1985.

Calder, P., & Yaqoob, P. (1999). Glutamine and the immune system. *Amino Acids, 17*(3), 227-241.

Chace, D. H., Kalas, T. A., & Naylor, E. W. (2003). Use of tandem mass spectrometry for multianalyte screening of dried blood specimens from newborns. *Clinical Chemistry, 49*(11), 1797-1817.

Chellappa, S., Huntingford, F., Strang, R., & Thomson, R. (1995). Condition factor and hepatosomatic index as estimates of energy status in male three-spined stickleback. *Journal of Fish Biology, 47*(5), 775-787.

Cheng, Z., Buentello, A., & Gatlin, D. M. (2011). Effects of dietary arginine and glutamine on growth performance, immune responses and intestinal structure of red drum, *Sciaenops ocellatus*. *Aquaculture, 319*(1), 247-252.

Cheng, Z., Gatlin, D. M., & Buentello, A. (2012). Dietary supplementation of arginine and/or glutamine influences growth performance, immune responses and intestinal morphology of hybrid striped bass (*Morone chrysops* × *Morone saxatilis*). *Aquaculture, 362*, 39-43.

Cheng, Z. J., Hardy, R. W., & Usry, J. L. (2003a). Effects of lysine supplementation in plant protein-based diets on the performance of rainbow trout (*Oncorhynchus mykiss*) and apparent digestibility coefficients of nutrients. *Aquaculture, 215*(1), 255-265.

Cheng, Z. J., Hardy, R. W., & Usry, J. L. (2003b). Plant protein ingredients with lysine supplementation reduce dietary protein level in rainbow trout (*Oncorhynchus mykiss*) diets, and reduce ammonia nitrogen and soluble phosphorus excretion. *Aquaculture, 218*(1), 553-565.

Christison, K. W. (2005). *Diplectanum* infections in cultured kob (*Argyrosomus japonicus*) in South Africa. Paper presented at the Proceedings of the 5th International Symposium on Monogenea, Guangzhou, China.

Costas, B., Aragão, C., Mancera, J. M., Dinis, M. T., & Conceição, L. E. (2008). High stocking density induces crowding stress and affects amino acid metabolism in Senegalese sole *Solea senegalensis* (Kaup 1858) juveniles. *Aquaculture Research, 39*(1), 1-9.

Damasceno-Oliveira, A., Fernández-Durán, B., Gonçalves, J., Serrão, P., Soares-da-Silva, P., Reis-Henriques, M. A., & Coimbra, J. (2007). Effects of cyclic hydrostatic pressure on the brain biogenic amines concentrations in the flounder, *Platichthys flesus*. *General and Comparative Endocrinology, 153*(1), 385-389.

Dash, P., Kar, B., Mishra, A., & Sahoo, P. (2014). Effect of *Dactylogyrus catlaius* (Jain 1961) infection in *Labeo rohita* (Hamilton 1822): Innate immune responses and expression profile of some immune related genes. *Indian Journal of Experimental Biology, 52*(3), 267-280.

Dezfuli, B. S., Giari, L., Simoni, E., Menegatti, R., Shinn, A. P., & Manera, M. (2007). Gill histopathology of cultured European sea bass, *Dicentrarchus labrax* (L.), infected with *Diplectanum aequans* (Wagener 1857) Diesing 1958 (Diplectanidae: Monogenea). *Parasitology Research, 100*(4), 707-713.

Dove, A. D., Leisen, J., Zhou, M., Byrne, J. J., Lim-Hing, K., Webb, H. D., Gelbaum, L., Viant, M.R., Kubanek, J., & Fernández, F. M. (2012). Biomarkers of whale shark health: a metabolomic approach. *PLoS One, 7*(11), e49379.

Eames, S. C., Philipson, L. H., Prince, V. E., & Kinkel, M. D. (2010). Blood sugar measurement in zebrafish reveals dynamics of glucose homeostasis. *Zebrafish, 7*(2), 205-213.

Fitzgibbon, Q., Strawbridge, A., & Seymour, R. (2007). Metabolic scope, swimming performance and the effects of hypoxia in the mulloway, *Argyrosomus japonicus* (Pisces: Sciaenidae). *Aquaculture, 270*(1), 358-368.

George, M. P., Presser, D., Szczesniewski, A., Yu, C., McCain, K., & Zhang, J. (2010). Simultaneous analysis of amino acids and acylcarnitines in dried blood spots. Retrieved from USA: www.aligent.cpm/chem/qqq. Accessed 11 May 2015.

Guo, C., Huang, X.Y., Yang, M.J., Wang, S., Ren, S.T., Li, H. & Peng, X.X. (2014). GC/MS-based metabolomics approach to identify biomarkers differentiating survivals from death in crucian carps infected by *Edwardsiella tarda*. *Fish & Shellfish Immunology*, *39*(2), pp.215-222.

Harpaz, S. (2005). L-carnitine and its attributed functions in fish culture and nutrition—a review. *Aquaculture, 249*(1), 3-21.

Hayward, C. J., Bott, N. J., Itoh, N., Iwashita, M., Okihiro, M., & Nowak, B. F. (2007). Three species of parasites emerging on the gills of mulloway, *Argyrosomus japonicus* (Temminck and Schlegel, 1843), cultured in Australia. *Aquaculture, 265*(1), 27-40.

Inoue, Y., Grant, J. P., & Snyder, P. J. (1993). Effect of glutamine-supplemented intravenous nutrition on survival after *Escherichia coli*-induced peritonitis. *Journal of Parenteral and Enteral Nutrition, 17*(1), 41-46.

Joubert, C. J. H. (2012). The potential of commercial Praziquantel formulations as "off label" treatments for *Diplectanum oliveri* (Monogenea) infecting cultured *Argyrosomus* species in the South African marine finfish aquaculture industry. (M.Sc.), University of the Western Cape, Bellville. pp. 157.

Kania, P., Larsen, T. B., Ingerslev, H. C., & Buchmann, K. (2007). Baltic salmon activates immune relevant genes in fin tissue when responding to *Gyrodactylus salaris* infection. *Diseases of Aquatic Organisms, 76*(1), 81-85.

Kaushik, S., & Fauconneau, B. (1984). Effects of lysine administration on plasma arginine and on some nitrogenous catabolites in rainbow trout. *Comparative Biochemistry and Physiology Part A: Physiology, 79*(3), 459-462.

Ketola, H. G. (1982). Amino acid nutrition of fishes: requirements and supplementation of diets. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 73*(1), 17-24.

Kim, S. K., Takeuchi, T., Yokoyama, M., & Murata, Y. (2003). Effect of dietary supplementation with taurine, β-alanine andGABA on the growth of juvenile and fingerling Japanese flounder *Paralichthys olivaceus*. *Fisheries Science, 69*(2), 242-248.

Kono, N., Arakawa, K., Ogawa, R., Kido, N., Oshita, K., Ikegami, K., Tamaki, S., & Tomita, M. (2009). Pathway projector: web-based zoomable pathway browser using KEGG atlas and Google Maps API. *PLoS One, 4*(11), e7710.

Lardon, I., Nilsson, G. E., Stecyk, J. A., Vu, T. N., Laukens, K., Dommisse, R., & De Boeck, G. (2013). 1H-NMR study of the metabolome of an exceptionally anoxia tolerant vertebrate, the crucian carp (*Carassius carassius*). *Metabolomics, 9*(2), 311-323.

Leaver, M. J., Bautista, J. M., Björnsson, B. T., Jönsson, E., Krey, G., Tocher, D. R., & Torstensen, B. E. (2008). Towards fish lipid nutrigenomics: current state and prospects for fin-fish aquaculture. *Reviews in Fisheries Science, 16*(sup1), 73-94.

Li, H.-T., Feng, L., Jiang, W.-D., Liu, Y., Jiang, J., Li, S.-H., & Zhou, X.-Q. (2013). Oxidative stress parameters and anti-apoptotic response to hydroxyl radicals in fish erythrocytes: protective effects of glutamine, alanine, citrulline and proline. *Aquatic Toxicology, 126*, 169-179.

Li, P., Mai, K., Trushenski, J., & Wu, G. (2009). New developments in fish amino acid nutrition: towards functional and environmentally oriented aquafeeds. *Amino Acids, 37*(1), 43-53.

Li, P., & Wu, G. (2017). Roles of dietary glycine, proline, and hydroxyproline in collagen synthesis and animal growth. *Amino Acids, 50*(1), 1-10.

Lindenstrøm, T., Buchmann, K., & Secombes, C. (2003). *Gyrodactylus derjavini* infection elicits IL-1β expression in rainbow trout skin. *Fish & Shellfish Immunology, 15*(2), 107-115.

Lindenstrøm, T., Secombes, C. J., & Buchmann, K. (2004). Expression of immune response genes in rainbow trout skin induced by *Gyrodactylus derjavini* infections. *Veterinary Immunology and Immunopathology, 97*(3), 137-148.

Liu, J., Mai, K., Xu, W., Zhang, Y., Zhou, H., & Ai, Q. (2015). Effects of dietary glutamine on survival, growth performance, activities of digestive enzyme, antioxidant status and hypoxia stress resistance of half-smooth tongue sole (*Cynoglossus semilaevis* Günther) post larvae. *Aquaculture, 446*, 48-56.

Low, C. F., Rozaini, M., Musa, N., & Syarul Nataqain, B. (2017). Current knowledge of metabolomic approach in infectious fish disease studies. *Journal of Fish Diseases, 40*(10), 1267-1277.

Luginbühl, M., Schröck, A., König, S., Schürch, S., & Weinmann, W. (2016). Determination of fatty acid ethyl esters in dried blood spots by LC–MS/MS as markers for ethanol intake: Application in a drinking study. *Analytical and Bioanalytical Chemistry, 408*(13), 3503-3509.

Madsen, R., Lundstedt, T., & Trygg, J. (2010). Chemometrics in metabolomics—a review in human disease diagnosis. *Analytica Chimica Acta, 659*(1), 23-33.

Matejusová, I., Felix, B., Sorsa-Leslie, T., Gilbey, J., Noble, L. R., Jones, C. S., & Cunningham, C. O. (2006). Gene expression profiles of some immune relevant genes from skin of susceptible and responding Atlantic salmon (*Salmo salar* L.) infected with *Gyrodactylus salaris* (Monogenea) revealed by suppressive subtractive hybridisation. *International Journal for Parasitology, 36*(10), 1175-1183.

Mdlalose, T., Christison, K., & Vosloo, A. (2017). Use of dried blood spots (DBS) to diagnose hyper-and hypoglycaemia in farmed Dusky kob *Argyrosomus japonicus* in South Africa. *Aquaculture, 473*, 43-50.

Mommsen, T. P., Moon, T. W., & Plisetskaya, E. M. (2001). Effects of arginine on pancreatic hormones and hepatic metabolism in rainbow trout. *Physiological and Biochemical Zoology, 74*(5), 668-678.

Montero, D., Robaina, L., Socorro, J., Vergara, J., Tort, L., & Izquierdo, M. (2001). Alteration of liver and muscle fatty acid composition in gilthead seabream (*Sparus aurata*) juveniles held at high stocking density and fed an essential fatty acid deficient diet. *Fish Physiology and Biochemistry, 24*(1), 63-72.

Naka, S., Saito, H., Hashiguchi, Y., Lin, M.-T., Furukawa, S., Inaba, T., Fukushima, R., Wada, N., Muto, T. (1996). Alanyl-glutamine-supplemented total parenteral nutrition improves survival and protein metabolism in rat protracted bacterial peritonitis model. *Journal of Parenteral and Enteral Nutrition, 20*(6), 417-423.

Newsholme, P. (2001). Why is L-glutamine metabolism important to cells of the immune system in health, postinjury, surgery or infection? *The Journal of Nutrition, 131*(9), 2515S-2522S.

Oliver, G. (1977). Pathogenic effect of attachment of *Diplectanum aequans* (Monogenea, Monopisthocotylea, Diplectanidae) to the gills of *Decentrarchus labrax* (Pisces, Serranidae). *Parasitology Research, 53*(1), 7-11.

Péres, A., Cahu, C., & Infante, J. Z. (1997). Dietary spermine supplementation induces intestinal maturation in sea bass (*Dicentrarchus labrax*) larvae. *Fish Physiology and Biochemistry, 16*(6), 479-485.

Pinto, W., Figueira, L., Dinis, M. T., & Aragão, C. (2009). How does fish metamorphosis affect aromatic amino acid metabolism? *Amino Acids, 36*(2), 177-183.

Pohlenz, C., Buentello, A., J Helland, S., & Gatlin, D. M. (2014). Effects of dietary arginine supplementation on growth, protein optimization and innate immune response of channel catfish *Ictalurus punctatus* (Rafinesque 1818). *Aquaculture Research, 45*(3), 491-500.

Pohlenz, C., Buentello, A., Mwangi, W., & Gatlin, D. M. (2012). Arginine and glutamine supplementation to culture media improves the performance of various Channel catfish immune cells. *Fish & Shellfish Immunology, 32*(5), 762-768.

Pupillo, D., Simonato, M., Cogo, P. E., Lapillonne, A., & Carnielli, V. P. (2016). Short-term stability of whole blood polyunsaturated fatty acid content on filter paper during storage at −28°C. *Lipids, 51*(2), 193-198.

Reinecke, C. J., Koekemoer, G., Van der Westhuizen, F. H., Louw, R., Lindeque, J. Z., Mienie, L. J., & Smuts, I. (2012). Metabolomics of urinary organic acids in respiratory chain deficiencies in children. *Metabolomics, 8*(2), 264-283.

Rubio-Godoy, M. (2007). Fish host-monogenean parasite interactions, with special reference to Polyopisthocotylea. *Advances in the Immunobiology of Parasitic Diseases. Trivandrum: Research Signpost*, 91-109.

Ruyter, B., Rosjo, C., Einen, O., & Thomassen, M. (2000). Essential fatty acids in Atlantic salmon: effects of increasing dietary doses of n-6 and n-3 fatty acids on growth, survival and fatty acid composition of liver, blood and carcass. *Aquaculture Nutrition, 6*(2), 119-128.

Salleh, M., Ardawi, M., & Majzoub, M. F. (1991). Glutamine metabolism in skeletal muscle of septic rats. *Metabolism, 40*(2), 155-164.

Samuelsson, L. M., Förlin, L., Karlsson, G., Adolfsson-Erici, M., & Larsson, D. J. (2006). Using NMR metabolomics to identify responses of an environmental estrogen in blood plasma of fish. *Aquatic Toxicology, 78*(4), 341-349.

Samuelsson, L. M., & Larsson, D. G. J. (2008). Contributions from metabolomics to fish research. *Molecular Biosystems, 4*, 974-979.

Santos, E. M., Ball, J. S., Williams, T. D., Wu, H., Ortega, F., Van Aerle, R., . Katsiadaki, I., Falciani, F., Viant, M.R., & Chipman, J. K. (2009). Identifying health impacts of exposure to copper using transcriptomics and metabolomics in a fish model. *Environmental Science & Technology, 44*(2), 820-826.

Sivakumar, P., Suguna, L., & Chandrakasan, G. (2000). Molecular species of collagen in the intramuscular connective tissues of the marine crab, *Scylla serrata*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 125*(4), 555-562.

Solanky, K. S., Burton, I. W., MacKinnon, S. L., Walter, J. A., & Dacanay, A. (2005). Metabolic changes in Atlantic salmon exposed to *Aeromonas salmonicida* detected by 1H-nuclear magnetic resonance spectroscopy of plasma. *Diseases of Aquatic Organisms, 65*(2), 107-114.

Spratlin, J. L., Serkova, N. J., & Eckhardt, S. G. (2009). Clinical applications of metabolomics in oncology: a review. *Clinical Cancer Research, 15*(2), 431-440.

Stentiford, G., Viant, M., Ward, D., Johnson, P., Martin, A., Wenbin, W., Cooper, H.J., Lyons, B.P., & Feist, S. (2005). Liver tumors in wild flatfish: a histopathological, proteomic, and metabolomic study. *OMICS: A Journal of Integrative Biology, 9*(3), 281-299.

Suzuki, I., Matsumoto, Y., Adjei, A. A., Asato, L., Shinjo, S., & Yamamoto, S. (1993). Effect of a glutamine-supplemented diet on response to methicillin-resistant *Staphylococcus aureus* infection in mice. *Journal of Nutritional Science and Vitaminology, 39*(4), 405-410.

Thoney, D., & Hargis, W. (1991). Monogenea (Platyhelminthes) as hazards for fish in confinement. *Annual Review of Fish Diseases, 1*, 133-153.

Tocher, D. R. (2003). Metabolism and functions of lipids and fatty acids in teleost fish. *Reviews in Fisheries Science, 11*(2), 107-184.

Viola, S., Lahav, E., & Arieli, Y. (1992). Response of Israeli carp, *Cyprinus carpio* L., to lysine supplementation of a practical ration at varying conditions of fish size, temperature, density and ration size. *Aquaculture Research, 23*(1), 49-58.

Vladimirov, V. (1971). The immunity of fishes in the case of *Dactylogyrosis*. *Parasitologiya, 5*, 51-58.

Vosloo, A., Laas, A., & Vosloo, D. (2013). Differential responses of juvenile and adult South African abalone (*Haliotis midae* Linnaeus) to low and high oxygen levels. *Comparative Biochemistry and Physiology, Part A, 164*, 192-199.

Wang, G., Kim, J.-H., Sameshima, M., & Ogawa, K. (1997). Detection of antibodies against the monogenean *Heterobothrium okamotoi* in tiger puffer by ELISA. *Fish Pathology, 32*(3), 179-180.

Wang, G., Xu, K., Tian, X., Dong, S., & Fang, Z. (2015). Changes in plasma osmolality, cortisol and amino acid levels of tongue sole (*Cynoglossus semilaevis*) at different salinities. *Journal of Ocean University of China, 14*(5), 881-887.

Williams, A. (1989). Some monogenean parasites of the genera *Calceostoma* van Beneden, 1852 and *Diplectanum* Diesing, 1858 from *Argyrosomus hololepidotus* (Lacepède, 1802)(Sciaenidae: Teleostei) in Western Australia. *Systematic Parasitology, 14*(3), 187-201.

Wilmore, D. W., & Shabert, J. K. (1998). Role of glutamine in immunologic responses. *Nutrition, 14*(7), 618-626.

Wilson, R. P. (2002). Amino acids and proteins. *Fish nutrition, Third Edition.* pp. 143-179.

Wishart, D. S., Jewison, T., Guo, A. C., Wilson, M., Knox, C., Liu, Y., Djoumbou, Y., Mandal, R., Aziat, F., Dong, E., & Dong, E. (2012). HMDB 3.0—the human metabolome database in 2013. *Nucleic Acids Research, 41*(D1), D801-D807.

Wu, G., Bazer, F., Datta, S., Johnson, G., Li, P., Satterfield, M., & Spencer, T. (2008). Proline metabolism in the conceptus: implications for fetal growth and development. *Amino Acids, 35*(4), 691-702.

Wu, G., Bazer, F. W., Burghardt, R. C., Johnson, G. A., Kim, S. W., Knabe, D. A., . Li, P., Li, X., McKnight, J.R., Satterfield, M.C., & Satterfield, M. C. (2011). Proline and hydroxyproline metabolism: implications for animal and human nutrition. *Amino Acids, 40*(4), 1053-1063.

Xia, J., Sinelnikov, I. V., Han, B., & Wishart, D. S. (2015). MetaboAnalyst 3.0—making metabolomics more meaningful. *Nucleic Acids Research, 43*(W1), W251-W257.

Yoo, J. H., Takeuchi, T., Tagawa, M., & Seikai, T. (2000). Effect of thyroid hormones on the stage-specific pigmentation of the Japanese flounder *Paralichthys olivaceus*. *Zoological Science, 17*(8), 1101-1106.

Young, T., Kesarcodi-Watson, A., Alfaro, A. C., Merien, F., Nguyen, T. V., Mae, H., Le, D.V., & Villas-Bôas, S. (2017). Differential expression of novel metabolic and immunological biomarkers in oysters challenged with a virulent strain of OsHV-1. *Developmental & Comparative Immunology, 73*, 229-245.

Zhang, A., Sun, H., Wang, P., Han, Y., & Wang, X. (2012). Modern analytical techniques in metabolomics analysis. *Analyst, 137*(2), 293-300.

Zhao, X.-l., Han, Y., Ren, S. T., Ma, Y. M., Li, H., & Peng, X. X. (2015). L-proline increases survival of tilapias infected by *Streptococcus agalactiae* in higher water temperature. *Fish & Shellfish Immunology, 44*(1), 33-42.

Zheng, Y.-M., Li, F., Zhang, M.-M., & Wu, X.-T. (2006). Glutamine dipeptide for parenteral nutrition in abdominal surgery: a meta-analysis of randomized controlled trials. *World Journal of Gastroenterology: WJG, 12*(46), 7537.

Zhou, X. (2005). Use of synthetic lysine in fish feeds: a review on research and application. *Feed Ind, 27*(2), 1-7.

Zytkovicz, T. H., Fitzgerald, E. F., Marsden, D., Larson, C. A., Shih, V. E., Johnson, D. M., . . . Grady, G. F. (2001). Tandem mass spectrometric analysis for amino, organic, and fatty acid disorders in newborn dried blood spots. *Clinical Chemistry, 47*(11), 1945-1955.

Chapter 4: General Conclusions

This study presented a first examination of the utilization of DBS for analysing blood glucose concentration and the metabolome of farmed South African Dusky kob. First, we evaluated the potential of successfully quantifying glycaemia in farmed fish using a diabetic glucometer, DBS, whole blood and plasma (Chapter 2). The results indicated that Dusky kob blood glucose can be measured accurately on farm using a diabetic glucometer. Furthermore, DBS proved to be an effective method for collecting blood due to ease of use, maintaining sample integrity and simple storage and transportation protocols. Moreover, DBS offer an extended utilization for further assessment of blood parameters by more complex methodologies.

Secondly, targeted LC-MS and GC-MS analyses were conducted on DBS of farmed Dusky kob collected from four farms along the coast of South Africa (Chapter 3). The metabolomics analyses combined with principal components analysis revealed patterns corresponding to the geographical distribution of the farms. We also identified six amino acids (citrulline, glutamine, lysine, methionine, phenylalanine and proline) that show differences in concentrations which seem to correspond with parasite intensity. Additionally, the successful identification of 53 metabolites from DBS further confirm that the method of sample collection can be beneficial in the discovery of biomarkers.

The results presented herein require further analyses and validation, so as to establish certainty of their usefulness as biomarkers of fish health. The observed hypo- and hyperglycaemia for Dusky kob in the present study requires supplementation from reference values. Furthermore, extensive understanding of glucose metabolism in Dusky kob is essential to expand the significance of the findings presented. While we successfully identified numerous compounds from metabolomics analyses, patterns corresponding to parasite intensity did not emerge. Potentially, more meaningful metabolite patterns can be elucidated by untargeted analyses and/or the utilization of a different metabolomics platform i.e. NMR.

Overall, the present study is a first attempt at discovering biomarkers from farmed Dusky kob. The work presented here provides a basis for the research and development of fish

health assessment measures in South African aquaculture. We propose improvements from the present study by incorporating controlled experiments, where environmental and biological parameters can be kept constant. Furthermore, we propose an extensive exploration of the use of metabolomics techniques for fish health assessment. This will ensure the determination of reference ranges which can then be used in disease diagnosis thus preventing potential mass mortalities. Lastly, the incorporation of additional biochemical, haematological, molecular and physiological parameters in studying fish health biomarkers, can be beneficial towards the development of fish health assessment protocols and setting up corrective measures that ensures continued growth of the aquaculture industry.

Appendices

Appendix 1: Research Publication (Chapter 2)

Aquaculture 473 (2017) 43–50

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Use of dried blood spots (DBS) to diagnose hyper- and hypoglycaemia in farmed dusky kob *Argyrosomus japonicus* in South Africa

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Argyrosomus japonicus

ABSTRACT

Biomarkers require sensitivity and rapid turnover for health assessment of individuals and populations. Blood glucose in fish shows sensitive and measurable changes in response to environmental, physiological or nutritional stressors. *Argyrosomus japonicus* is currently the only commercially produced marine aquaculture finfish species in South Africa, yet knowledge about its responses to biological and environmental stressors is limited. Herein we confirm the utility of the *Accu-Chek® Active* diabetic glucometer under field conditions to provide accurate blood glucose data for *A. japonicus*, in comparison with laboratory-based enzymatic analyses using plasma, whole blood and dried blood spots (DBS). Glucometer, whole blood and DBS glucose was correlated with plasma glucose ($R^2 = 0.973$, $R^2 = 0.955$, $R^2 = 0.898$ respectively). Whole blood glucose was consistently and significantly lower than plasma glucose, thus indicating that more complex sample preparation than storage on ice and freezing at −80 °C are required to inhibit glycolysis in whole blood samples and generate accurate results. Diabetic glucometers offer a means to measure on-farm blood glucose with sufficient accuracy and rapidity. We include an analysis of a subsequent sampling from the farm, and demonstrate that through routine glucose measurements we were able to identify hypoglycaemia at the farm level, and that this corresponds to decreased condition of fish. The ease of DBS storage and stability of metabolites offer the opportunity of expanding fish health and condition monitoring by measuring multiple indicators in DBS.

1. Introduction

Blood glucose in fish has been shown to respond reliably to a range of biological and environmental stimuli (Garin et al., 1987; Enes et al., 2009; Polakof et al., 2012). Hyperglycaemia in Rainbow trout has been observed in response to increased temperature, elevated pH, high density stocking stress and hormones like cortisol, catecholamines, growth hormones, leptin and glucagon and parasite infection (Tavares-Dias et al., 2007; Polakof et al., 2012). Reports of hypoglycaemia are less prevalent in literature, but have been observed during food deprivation, thermal cooling, hormones like insulin, and parasitism (Eames et al., 2010; Woo and Buchmann, 2012).

The antagonistic effects of glucagon (hyperglycaemia) and insulin (hypoglycaemia) suggest that glucose homeostasis in fish is similar to that of higher vertebrates. Numerous studies have been conducted in trout which demonstrate glucose sensitivity in response to various stressors (Conde-Sieira et al., 2010). These changes in blood glucose levels suggest the presence of underlying pathways that regulate production or degradation of glucose in response to changes in environmental or biological conditions. The primary stress response in fish is mediated via the hypothalamus-pituitary-adrenal (HPA) axis, and the resulting release of catecholamines and cortisol has been implicated in an array of secondary stress responses. Among others, cortisol release is associated with hepatic glycogen depletion and blood glucose elevation, aimed at fuelling metabolism under stressful conditions (Barton, 2002; Herrera et al., 2009). From an energetic perspective, glucose homeostasis represents the balance between anabolism (gluco- and glyconeogenesis) and catabolism (glycolysis), further supporting the usefulness of glucose as a suitable general biomarker of fish condition (Ray and Sinha, 2014).

Biomarker research in aquaculture is essential to facilitate our understanding of biochemical and physiological processes that may be below the threshold of detection by conventional methods (McDade, 2014), yet may be used to predict animal health status. Although Dusky kob (*Argyrosomus japonicus*, variously known as Japanese meagre, Mulloway or Southern meagre) is a commercially important marine finfish species in South African aquaculture, the majority of Dusky kob farming is currently in the development stage. In 2012 the finfish sector produced 48.5 tons (2.1%) of the total SA marine aquaculture production of 2261 tons (DAFF, 2012). Although we have a good general understanding of stress related glucose responses in fish, data are limited

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for *A. japonicus* and sciaenids in general. It is therefore essential to understand hyper- and hypoglycaemia in Dusky kob generally, and specifically in the context of the farming environment.

The use of hand-held diabetic glucometers both in field and laboratory research settings has been extensively explored and dates back to over two decades. The low cost of glucometers (~US$ 30), their affordable cost per sample (~US$ 0.25) and sensitivity are attractive for practical applications in field based research. Various portable devices for blood sample analyses have been successfully used in field settings with results comparable to laboratory based methods. Iwama et al. (1995) evaluated the feasibility and accuracy of hand held devices for measuring glucose, in conjunction with cortisol, haemoglobin and erythrocytes from juvenile Coho salmon (*Oncorhynchus kisutch*) subjected to handling stress. Blood glucose and lactate, and plasma protein responses have been measured with handheld meters for Rainbow trout (*Oncorhynchus mykiss*) exposed to handling and confinement stress (Wells and Pankhurst, 1999). Point-of-care (POC) devices have been identified as suitable alternatives to lab-based analyses due to difficulties in storage and transportation, and have the benefit of providing immediate results (Stoot et al., 2014). Generally, authors concur that hand-held glucometers vary in sensitivity and accuracy, and need to be validated against accepted methods (Eames et al., 2010).

Conventional methods of biological sample collection have several limitations, including complex collection and storage protocols as well as controlled transport conditions. Furthermore, sample degradation can be a limiting factor during storage. Dried blood spots (DBS) are an alternative to the storage of blood (whole blood, serum and/or plasma) samples. DBS are whole blood droplets placed on blood cards or filter paper and allowed to dry. Blood components are then eluted in appropriate media for laboratory analyses (Lacher et al., 2013). DBS are currently widely used in new-born screening programs to identify metabolic disorders (Mei et al., 2001; McDade et al., 2007). Furthermore, the use of DBS has been key to the diagnosis of various blood parasites (Hsiang et al., 2010; Hwang et al., 2012). Advantages of using DBS involve ease of use in non-clinical environments and can be done by non-professionals. Unlike traditional protocols for blood collection, DBS do not require immediate freezing or refrigeration, are easy to store and can be transported as non-hazardous goods (McDade et al., 2007; Lacher et al., 2013). The use of DBS in fish health assessment has not been explored, thus the present study provides a first analysis of DBS accuracy and efficiency to further improve farmed fish health monitoring and biomarker research.

Routine blood glucose monitoring in fish farms may aid in assessing current, and predicting future, health status. This study represents a first attempt to develop protocols for non-destructive blood collection and rapid-turnaround blood glucose analyses. We report a comparison of analytical methods (enzymatic-glucometer vs. enzymatic-spectrophotemetric) and sample preparation and storage methods (plasma, whole blood, and DBS) in quantifying blood glucose levels from farmed fish. Plasma glucose provides a more reliable measure of glycaemic levels and was chosen as reference method. Furthermore, we discuss the usefulness of DBS as a convenient medium of sample collection and storage, and provide some future directions for implementing these findings.

2. Materials and methods

2.1. Sample collection

The blood samples were collected from the Mtunzini Fish Farm located on the north coast of KwaZulu-Natal, South Africa, in May 2015. The farm uses pond culture and consists of a total of 52 ponds. Thirty animals were collected from six ponds with five animals collected from each pond. Fish were sampled in batches of five fish at time from a single pond. It took between 5 and 10 min to collect all biological material from a single fish. Fish were kept in aerated water from the corresponding pond until removed for blood collection.

In order to assess glucose concentrations in representative size ranges on the farm, we used non-anaesthetized fish between 64.5 g and 1348.5 g (average 435.1 ± 64.77 g, 275.1 ± 14.18 mm standard length). Blood (0.3–1 mL, scaled to fish size) was collected from the caudal vein immediately anterior to the caudal peduncle using a heparinized 1 mL syringe and a 21G × 1½ in. hypodermic needle. Each animal was weighed and the standard and total lengths were measured. The blood used in this study represent incidental samples, as fish were subsequently sacrificed for necropsy and parasite assessment as per animal health management requirements of the farming permit.

Blood glucose was immediately measured using a diabetic glucometer (*Accu-Chek® Active*, Roche) which measures glucose transformation in the blood sample using a PQQ-dependent (pyrroloquinoline quinone) glucose dehydrogenase reaction. Thirty blood samples were analysed in duplicate to determine within-sample variability: coefficient of variance (CoV) ranged between 0 (first and second analysis the same) and 11.7%, with an average CoV of 2.61 ± 0.027% (Fig. 1).

Apart from immediate glucose analysis by glucometer, blood was prepared in three different ways to compare the accuracy of the different blood storage methods (Fig. 2). Firstly, blood plasma was obtained by centrifugation (Hawksley Haematospin 1300, 10 min, 13,000 × *g*). The plasma was separated from the packed cells and stored on ice. Secondly, drops of whole blood (five ~20 μL spots, 13 mm diameter) were spotted on Whatman 903 specimen collection cards for subsequent glucose analyses. The cards were air-dried at room temperature in a dry rack (Whatman 903 Dry Rack). Once dried, the blood cards were stored in an air tight plastic container with desiccant (Sigma Dri-Can®). Thirdly, the remaining whole blood was transferred to 1.5 mL microcentrifuge tubes. Plasma and whole blood samples were immediately placed on ice, and transported to the University of KwaZulu-Natal, Westville Campus, Durban, for laboratory analyses. After 6 h on ice, whole blood and plasma samples were stored at −80 °C and blood

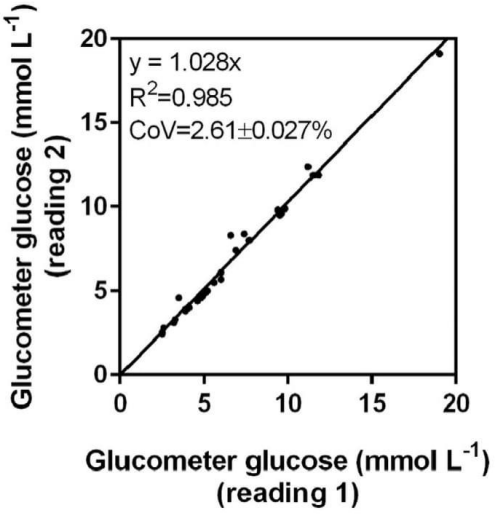

Fig. 1. Within-sample variability of *A. japonicus* blood glucose based on two subsequent measurements using a hand-held glucometer (*Accu-Chek® Active*). The average coefficient of variance (CoV) between the first and second measurements was 2.61 ± 0.027% (range 0.0–11.7%, based on $N = 30$ different fish). The dotted line represents a line of unity, and the solid line is described by the regression equation $y = 1.028\times$, $R^2 = 0.985$.

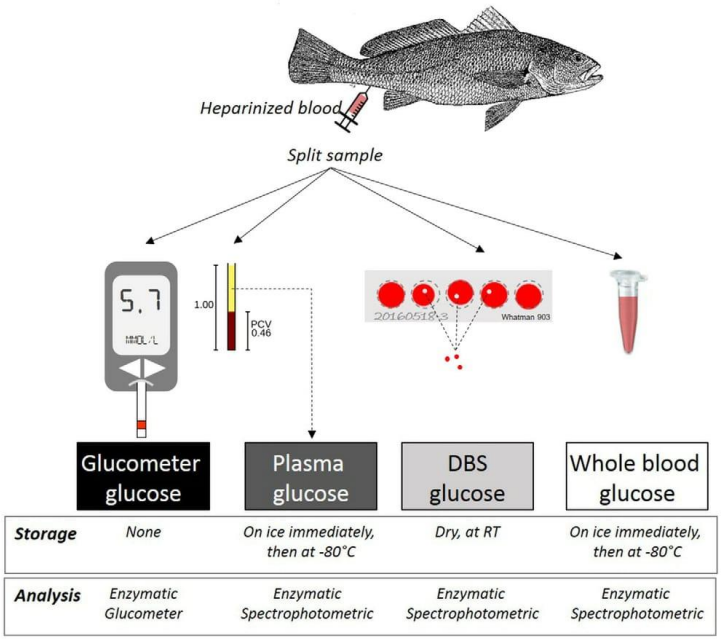

Fig. 2. Overview of *A. japonicus* blood sampling and processing procedure. Each heparinized blood sample ($N = 30$) was analysed immediately using a glucometer (*Accu-Chek® Active*), centrifuged to obtain plasma, spotted onto Whatman 903 filter cards to produce dried blood spots (DBS) or stored as whole blood. Plasma, DBS and whole blood glucose were subsequently analysed in the laboratory by enzymatic-spectrophotometric kit (Megazyme).

cards were stored at 4 °C for 14 days before enzymatic-spectrophotometric analyses for D-glucose were conducted.

2.2. Dried blood spot analyses

Filter card discs (3 mm diameter) were manually punched from DBS in triplicates and transferred to 96 well plates. The volume of blood represented by the 3 mm disc was quantified by pipetting known volumes of blood onto filter cards and analysing the resulting diameter of the blood spot by image analysis (ImageJ 1.49, NIH, USA). Based on the regression analysis of blood volume (in μL) against DBS area, the 3 mm diameter punches represented a blood volume of 5 μL.

Although several extraction buffers (5% w/v trichloroacetic acid, Rattenbury et al. (1989); 2.5% sulphosalicyclic acid, Parker et al. (1997)) have been used to elute glucose from filter cards, we used Millipore water (Shigeto et al., 2011) due to the method being recently shown as efficient. Each disc was eluted with 100 μL deionized water (resistivity 18.2 MΩ·cm) using constant shaking for 1 h. Elution efficiency was tested for 1, 2 and 3 h extraction periods, but after no significant differences were found (paired *t*-test: $P > 0.05$), the protocol was standardized using 1 h elution time. Thereafter 10 μL of eluent was analysed in triplicate for D-glucose using the GOPOD enzymatic kit for glucose (K-GLUC D-Glucose Assay Kit, Megazyme). Absorbance was measured at 510 nm (BIO-Tek Powerwave XS multiwell reader) using KC4 (version 3.4 rev. 21) for data acquisition. Glucose in DBS eluent was quantified against a standard curve constructed from glucose standards of 1, 5, 25, 50 and 100 mmol L^{-1} (also in triplicate) analysed simultaneously on each 96 well plate.

2.3. Plasma and whole blood analysis

After two weeks in −80 °C storage, plasma and whole blood samples were thawed to room temperature and briefly centrifuged. As described above, triplicate 10 μL samples were used for the enzymatic analyses, except that glucose was quantified against a D-glucose standard as per kit specification. Whole blood sample colour correction was done against 10 μL whole blood samples in dH_2O.

2.4. Comparison to subsequent sampling event

The results from the May 2015 Mtunzini samples were compared to a second sampling approximately six months later (November 2015). Fish collection, blood sampling and blood glucose analysis by glucometer (*Accu-Chek® Active*, Roche) was carried out as described above.

2.5. Statistical analyses

Data were analysed using Graphpad Prism (version 6). Linear regression was used to determine the regression equation and Pearson-squared correlation. Between-method comparisons were analysed using ANOVA and post-hoc Tukey multiple comparisons test ($P < 0.05$) after normality testing (Shapiro-Wilk normality test). Between-sampling event comparisons were analysed using an unpaired *t*-test with Welch's correction for unequal variances ($P < 0.05$) after normality testing (Shapiro-Wilk).

3. Results

As fish were brought from ponds in batches of five and maintained in aerated water before sampling, it was important to establish whether this procedure affected the blood glucose concentrations. To this end the blood glucose of the fish were pooled in their sequence number (1 to 5) for analysis. ANOVA was not significant ($F_{(4,\ 25)} = 0.493$, $P = 0.7408$), indicating that the blood glucose concentration was not significantly affected from the time being collected from ponds and being processed for sampling. This is an important observation, as Grutter and Pankhurst (2000) reported no significant differences in plasma glucose levels of *Hemigymnus melapterus* at time intervals of up to 5 min between capture and blood sampling, while Wells and Pankhurst (1999) found a steady increase in blood glucose for up to an hour after handling in *Oncorhynchus mykiss*. The glucose values reported here do not represent basal, resting values, as the handling preceding the analyses may have elevated blood glucose.

3.1. Between-method analysis

Blood glucose concentrations obtained from glucometer (8.02 ± 0.664 mmol L^{-1}), enzymatic-spectrophotometric analysis of plasma (7.47 ± 0.663 mmol L^{-1}), and enzymatic-spectrophotometric analysis of dried blood spots (9.39 ± 0.568 mmol L^{-1}) were not significantly different from one another (analysis of variance, $F_{(3,\ 113)} = 10.37$, $P < 0.0001$). Whole blood glucose (4.74 ± 0.490 mmol L^{-1}) analysed by enzymatic kit in the laboratory, was significantly lower compared to the other methods (Fig. 3).

The regression analyses of the different glucose analysis methods are presented in Fig. 4. Plasma glucose was chosen as the independent variable as it is the preferred method for human health studies (Working Group on Selective Electrodes, S.D., 2001). Rapid separation of plasma from actively respiring blood cells prevents glucose depletion after sample collection (Sacks et al., 2011) and best reflects the organismal glucose concentration. The best correlations with plasma glucose was from glucose as measured by diabetic glucometer ($y = 1.043\times$, $R^2 = 0.973$) and from DBS ($y = 1.236\times$, $R^2 = 0.898$).

3.2. Between-sampling analysis

Blood glucose (4.67 ± 0.361 mmol L^{-1}), measured by glucometer from Dusky kob in November 2015, was significantly lower ($P < 0.0001$) compared to fish sampled in May 2015 (as reported above, 8.02 ± 0.664 mmol L^{-1}). The fish were of similar average size (342.3 ± 47.13 g, $n = 30$, compared to 435.1 ± 64.77 g, $n = 30$ respectively) (Fig. 5).

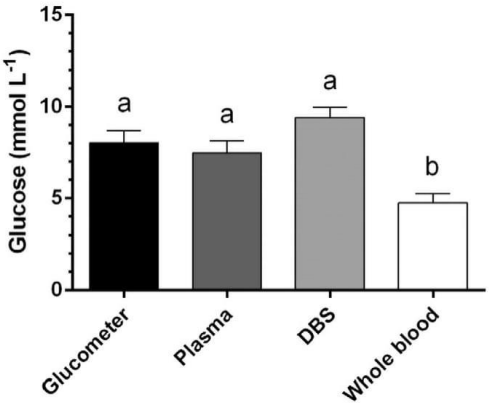

Fig. 3. Glucose in farmed Dusky kob, *A. japonicus* (mean ± S.E.M., $N = 30$), analysed on-farm by glucometer (*Accu-Chek® Active*), or by enzymatic-spectrophotometric kit (Megazyme) in the lab using different sample storage methods (plasma, DBS and whole blood). On-farm measurements using glucometers were as reliable as lab-based measurements using blood plasma or dried blood spots. Whole blood glucose was significantly lower compared to the other methods. Different letters denote significance at $P < 0.05$, ANOVA ($F_{(3,\ 113)} = 10.37$, $P < 0.0001$), Tukey's multiple comparisons test.

4. Discussion

4.1. Between method comparison

This study shows that issues of continued glycolysis (and indeed other pathways of the intermediary metabolism by using stored whole blood) can be addressed by using dry blood spots. DBS offer an attractive alternative blood storage method since they absorb and dry the sample; consequently, there is no liquid medium for metabolic pathways to occur. Von Schenck et al. (1985) reported no significant difference in glucose concentration measure between DBS (5.31 mmol L^{-1}) and venous blood (5.27 mmol L^{-1}) from 32 human subjects. Furthermore, DBS can easily and rapidly be prepared on-farm and transported to laboratories for enzymatic analyses, making sampling in remote areas possible.

Glucometer, whole blood and DBS glucose correlated well with plasma glucose, with correlation coefficients (R^2) of 0.898 or better (Fig. 4). Lacher et al. (2013) found good correlation between human whole blood and DBS glucose ($R^2 = 0.81$), with the DBS having significantly lower mean glucose (6.08 mmol L^{-1} compared to 6.34 mmol L^{-1} blood glucose; $P < 0.01$). The authors ascribed this difference to variations in the volume of each blood spot and subsequently the elution efficiency which led to reduced sensitivity of the DBS method. In contrast, here we report a slightly better correlation coefficient ($R^2 = 0.898$) with average DBS glucose not significantly different from plasma glucose (Fig. 3). As the correlation equation ($y = 1.236\times$) suggests that DBS may overestimate plasma glucose concentration by approximately 12%, this error should be of greater significance at higher glucose concentrations. In the absence of blood glucose reference ranges, blood glucose data were arbitrarily grouped as low (0–8 mmol L^{-1}), medium (8–12 mmol L^{-1}) and high (12–18 mmol L^{-1}) to determine the extent of the variation between methods in each range. In the low and medium blood glucose ranges, all three methods yield comparable concentrations. In the high concentration range, DBS over-estimates plasma glucose by 26% ($P < 0.05$), which exceeds the recommended 20% (Eames et al., 2010) required for implementation. However, in this high range, the diagnosis of hyperglycaemia would be accurate regardless of whether it is overestimated (Fig. 6).

4.2. Accuracy of DBS – and downstream benefits

According to McDade et al. (2007), DBS were considered disadvantageous due to the lack of comprehensive assay development and validation and also the small volumes available to work with. A large body of recent research has allayed this concern (McDade, 2014), and this cautionary note has to be balanced against the benefit of using DBS as a useful method for blood sample storage, without complex handling, storage and transport requirements. DBS also add the benefit that multiple parameters can be measured ranging from pathogen diagnostics and individual metabolites to comprehensive targeted and untargeted metabolomic and proteomic profiles (Martin et al., 2013; Zukunft et al., 2013; Miller et al., 2015), therefore providing information about the general physiological condition of the fish.

DBS have been widely used for human disease diagnosis through the use of various laboratory techniques. The use of DBS has led to development and validation of alternative sampling and diagnostic protocols for the Chikungunya Virus (CHIKV) where the viral RNA was effectively extracted from the blood spots, amplified and quantified by real-time RT-

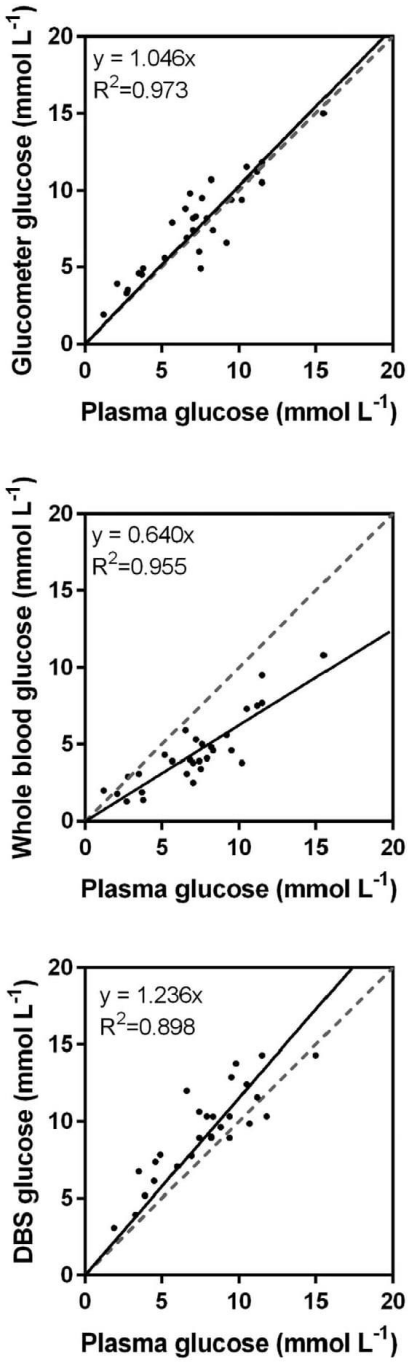

Fig. 4. Linear regressions of glucometer (*Accu-Chek® Active*), whole blood and DBS glucose versus plasma glucose (reference method) in farmed Dusky kob, *A. japonicus*. Dotted lines represent a line of unity, and the solid lines are described by the regression equation in each panel.

PCR (Andriamandimby et al., 2013). Holguín et al. (2013) used the Architect Chagas assay on dried blood samples to detect the presence of immunoglobin G due to *Trypanosoma cruzi* infection of patients in Madrid. Such studies often compare the sensitivity and specificity of DBS in relation to conventional serological methods. Furthermore, DBS can be used in non-laboratory and non-clinical environments and can be collected by any individual, which could prove useful for fish farmers should they require regular monitoring of stress and health status of their fish.

4.3. Usefulness of hand-held glucometers

Glucometer glucose did not differ significantly from plasma glucose suggesting that routine blood glucose measurements using the *Accu-Chek® Active* diabetic glucometer are comparable to laboratory based enzymatic-spectrophotometric analyses. Iwama et al. (1995) reported glucometer levels two times higher than those measured by a laboratory-based assay in juvenile Coho salmon for 24 h following a 30 seconds handling stressor. In a study evaluating a handheld meter in stressed and unstressed Channel catfish (*Ictalurus punctatus*) fingerlings, glucose values 30% lower compared to values measured by laboratory methods were reported (Beecham et al., 2006). Glucose concentrations measured with diabetic glucometers may vary with some degree of accuracy compared values determined by thorough laboratory-based methodology, therefore should be treated as relative measurements rather than absolute values until the accuracy has been confirmed.

4.4. Concerns with whole blood sampling

As expected, whole blood glucose was significantly lower than measured by the other methods (Fig. 3), confirming that whole blood is a poor medium for glucose analyses when samples are stored over time. According to Voss et al. (1992), whole blood samples remain stable for 1 week and at least 1 year at 4 °C and −70 °C respectively, however, in the present study immediate placement on ice (6 h) and subsequent storage of the blood in −80 °C (2 weeks) proved ineffective in preventing glycolytic depletion of glucose. It is recommended that an effective glycolysis inhibitor (e.g. citrate buffer, fluoride, iodoacetate) be added to the blood sample if immediate freezing is impossible (Tonyushkina and Nichols, 2009; Sacks et al., 2011). Glycolysis will continue in the 1–2 h it takes for iodoacetate and fluoride to cross cell membranes (Tonyushkina and Nichols, 2009). However, if the intention is to use blood for metabolomics analysis, this step needs to be considered carefully as citrate addition may skew results of some tricarboxylic acid (TCA or Krebs) cycle intermediates.

4.5. Blood glucose and the stress response

The link between handling stress, elevated cortisol as a primary effect, and increased plasma glucose as a secondary effect has been reviewed extensively (Barton, 2002; Martinéz-Porchas et al., 2009; Ellis et al., 2012), and the sensitivity of blood glucose to single and repeated handling stress has been demonstrated in several fish species (Jentoft et al., 2005; Butcher et al., 2007). In contrast, meagre (*A. regius*) has been shown to exhibit a low cortisol, and consequently low blood glucose, response after acute stress (Samaras et al., 2015). Although it has not been confirmed, our data suggest that *A. japonicus* may have a similarly low blood glucose response and that the sampling method employed in this study did not differentially affect the blood glucose measurements between different fish. It is unclear whether the preceding handling may have elevated blood glucose, and this aspect requires

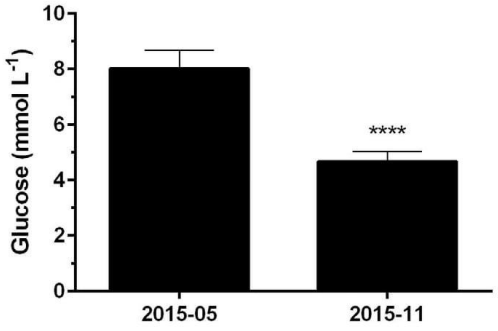

Fig. 5. Blood glucose (mean ± S.D.) in farmed Dusky kob (*A. japonicus*) from Mtunzini Fish Farm as measured by glucometer in May 2015 ($N = 30$) and November 2015 ($N = 28$). Statistical significance indicated (*t*-test, $P < 0.0001$).

further assessment. A low blood glucose response would be a very beneficial physiological feature of *A. japonicus*, as reliable blood glucose measurements can be generated in the farming environment without sophisticated techniques like aortal cannulation (Deng et al., 2000).

4.6. Between-sampling comparisons

The results of the between-sampling comparison demonstrate the ability of hand-held glucometers to identify a farm-level effect, using a relatively small number of fish. Hypoglycaemia during sub-optimal feeding appears to be a general response in fish (Chavin and Young, 1970; Groff and Zinkl, 1999; Eames et al., 2010), and can manifest even at elevated cortisol levels typical of starved fish (Peterson and Small, 2004; Eslamloo et al., 2016). The low blood glucose in fish sampled in November corresponded to (a) slightly poorer condition than for the May sampling (body mass (Mb) vs. length (L) ratio calculated as Fulton's Condition Index (Mb/L^3): 1.11 ± 0.018 vs 1.08 ± 0.023, not significant) and (b) significantly reduced hepatosomatic index (liver mass / Mb * 100): 2.21 ± 0.144 vs 1.35 ± 0.078, $P < 0.0001$ (Christison and Vosloo, unpublished data). These data demonstrate that the ability to sample fish non-destructively and measure blood glucose levels in real time provides a useful avenue for fish farmers to

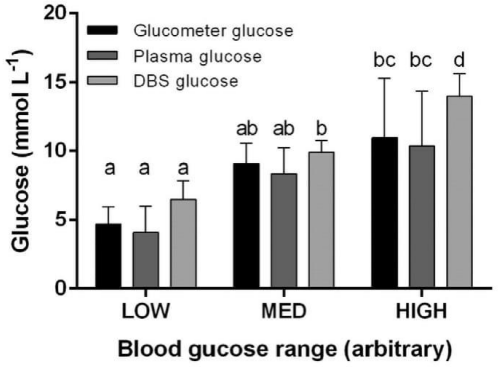

Fig. 6. Grouping of blood glucose values in farmed Dusky kob (*A. japonicus*) as low, medium or high (using arbitrary cut-offs of 8 and 12 mmol L^{-1}) demonstrates that DBS (compared to plasma glucose) may over-estimate blood glucose at elevated levels ($P < 0.05$, two way ANOVA, Tukey multiple comparisons). The mean difference of 3.638 mmol L^{-1} represents a 26% over-estimation of blood glucose using DBS compared to glucometer and plasma glucose measurements.

generate information that may inform decision making. In the farming environment, the day-to-day variability in blood glucose in fish will be dependent on an interplay of a range of factors that have been demonstrated as glucose modulators, including general stress, density-dependent stress, parasite load, feeding ration, time since feeding and water quality (temperature, pH, DO etc.). The fact remains that blood glucose is a general biomarker, and should be supplemented by other, more specific biomarkers, as well as continuous environmental and management data. Ideally, a well-chosen suite of biomarkers should be developed to further analyse and diagnose fish condition in the farming context. Regardless, blood glucose remains a useful and easily measured first indicator in farmed fish.

4.7. Where to now: deriving a glucose reference range for farmed Dusky kob

The major limiting factor in implementing routine blood glucose as a monitoring tool in aquaculture is the absence of a reference range for blood glucose. A brief literature search revealed that only one previous study (Butcher et al., 2007) reported blood glucose for *A. japonicus*, in response to angling stress. The blood glucose values reported in this study are the first for *A. japonicus* in the farming context. As a first step toward determining whether a blood glucose reference range is possible, a summary of blood glucose data from other sciaenids (Shi drum, *Umbrina cirrosa* (Ballarin et al., 2004), Yellow croaker, *Pseudosciaena crocea* (Cheng et al., 2013) and meagre, *A. regius* (Chatzifotis et al., 2010; Fanouraki et al., 2011; McGrath et al., 2011; Vargas-Chacoff et al., 2014; Samaras et al., 2015; Barata et al., 2016; Millán-Cubillo et al., 2016)) is provided (Fig. 7). In general, blood glucose levels from farm or field-based studies appear to be higher and more variable than from controlled laboratory studies.

Against this backdrop it is essential that samples for deriving reference ranges are collected in ways that limit pre-analytical variability, e.g. fasting vs. non-fasting, fish capture and handling, blood sampling site and sample handling (Friedrichs et al., 2012). Of the methods reported here, validated hand-held glucometers and sample storage on DBS, followed by laboratory analysis, have the greatest potential to provide large data sets required for deriving reference ranges, with minimal impact on the fish or the farms.

After blood glucose analysis, statistical procedures are used to determine the reference interval (RI). The blood glucose RI (e.g. using farmed *A. japonicus* in South Africa as a delimited sample population) is derived by non-parametric methods, and is bounded by the 2.5th and 97.5th fractiles of the data set of reference values, with corresponding 90% confidence intervals around these cut-offs (Friedrichs et al., 2012). Fish presenting blood glucose levels outside of the RI can then be diagnosed at hypo- or hyperglycaemic. With large data sets, it becomes possible to confirm age- and size dependent RIs using the same statistical methods as in human medicine (see Mazzaccara et al. (2008) as an example).

The benefit of using DBS in deriving reference ranges is the fact that the same DBS can be used for further analyses, as the punch volume represents a fraction of the total DBS volume. In this manner a single DBS sampling program provides the opportunity for deriving reference ranges for multiple biomarkers, providing the detection limit and stability of each analyte is satisfactory. Coupled to targeted and untargeted mass spectrometry methods (Zukunft et al., 2013; Alfaro and Young, 2016), a large amount of biological information becomes available for an individual, allowing the refinement of the RI to Decision Thresholds (Friedrichs et al., 2012) that define the boundaries of the physiological transitions into hypo- and hyperglycaemia more accurately than statistical methods.

5. Conclusions

This study was a first attempt for assessing the usefulness of blood glucose as a biomarker for research on farmed South African Dusky kob, *A. japonicus*. Firstly, blood glucose can be assessed on-site with

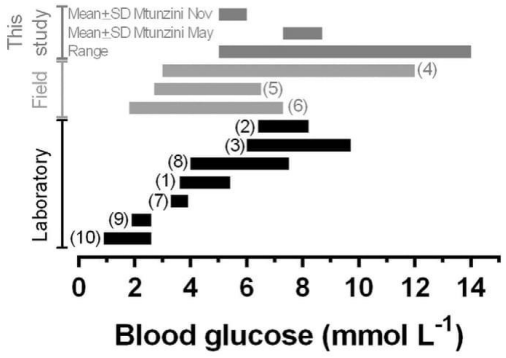

Fig. 7. Blood glucose measurements in Sciaenidae reported from laboratory (black bars) or field studies (grey bars) show large variation. 1 *Umbrina cirrosa* (Ballarin et al., 2004), 2 *Argyrosomus regius* (Chatzifotis et al., 2010), 3 *A. regius* (Fanouraki et al., 2011), 4* *Pseudosciaena crocea* (Cheng et al., 2013), 5 *A. regius* (Vargas-Chacoff et al., 2014), 6 *A. regius* (Samaras et al., 2015), 7 *A. regius* (Barata et al., 2016), 8* *A. regius* (Millán-Cubillo et al., 2016), 9 *A. regius* (McGrath et al., 2011), 10 *A. japonicus* (Butcher et al., 2007). Data from this study are presented as the range as well as the mean ± S.D. for the two sampling events. *In instances where numerical data were not provided, blood glucose was estimated from graphs.

sufficient accuracy using diabetic glucometers, offering fish farmers a simple yet valuable initial assessment of general fish condition in real time. Secondly, DBS offer a promising alternative to conventional blood sample storage methods, with the added benefit of providing a matrix for further assessments using metabolomics to supplement single end-point measurements like blood glucose. Blood glucose is thus a useful first biomarker for assessing fish health status and condition in the farming context, as reduced blood glucose was supported by morphometric indices like the hepatosomatic index. Implementation of these findings requires a defined glucose reference range for farmed Dusky kob for accurate diagnosis of hypo- and hyperglycemia. Furthermore, a comprehensive understanding of glucose metabolism in *A. japonicus* both in normal and stressful conditions is required in order to evaluate and interpret results from routine blood glucose analyses. The present study serves as a basis for future biomarker research in *A. japonicus* farmed in South African aquaculture. The ease of DBS collection, storage and transport offers the opportunity to add value to a wide range of studies on environmental, nutritional and biological stressors on South African aquaculture fish species.

Acknowledgements

This study was financially supported by the Marine Living Resources Fund of the South African Department of Agriculture, Forestry and Fisheries. The support of Mtunzini Fish Farm is acknowledged.

References

Alfaro, A.C., Young, T., 2016. Showcasing Metabolomic Applications in Aquaculture: A Review. (Reviews in Aquaculture, in press) http://dx.doi.org/10.1111/raq.12152.
Andriamandimby, S.F., Heraud, J.-M., Randrianasolo, L., Rafisandratantsoa, J.T., Andriamamonjy, S., Richard, V., 2013. Dried-blood spots: a cost-effective field method for the detection of chikungunya virus circulation in remote areas. PLoS Negl. Trop. Dis. 7, e2339.
Ballarin, L., Dall'Oro, M., Bertotto, D., Libertini, A., Francescon, A., Barbaro, A., 2004. Haematological parameters in *Umbrina cirrosa* (Teleostei, Sciaenidae): a comparison between diploid and triploid specimens. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 138, 45–51.
Barata, M., Soares, F., Aragão, C., Almeida, A.C., Pousão-Ferreira, P., Ribeiro, L., 2016. Efficiency of 2-phenoxyethanol and clove oil for reducing handling stress in reared meagre, *Argyrosomus regius* (Pisces: Sciaenidae). J. World Aquacult. Soc. 47, 82–92.
Barton, B.A., 2002. Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. Integr. Comp. Biol. 42, 517–525.
Beecham, R.V., Small, B.C., Minchew, C.D., 2006. Using portable lactate glucose meters for catfish research: acceptable alternatives to established laboratory methods? N. Am. J. Aquac. 68, 291–295.
Butcher, P.A., Broadhurst, M.K., Reynolds, D., Reid, D.D., Gray, C.A., 2007. Release method and anatomical hook location: effects on short-term mortality of angler-caught *Acanthopagrus australis* and *Argyrosomus japonicus*. Dis. Aquat. Org. 74, 17–26.
Chatzifotis, S., Panagiotidou, M., Papaioannou, N., Pavlidis, M., Nengas, I., Mylonas, C.C., 2010. Effect of dietary lipid levels on growth, feed utilization, body composition and serum metabolites of meagre (*Argyrosomus regius*) juveniles. Aquaculture 307, 65–70.
Chavin, W., Young, J.E., 1970. Factors in the determination of normal serum glucose levels of goldfish, *Carassius auratus* L. Comp. Biochem. Physiol. 33, 629–653.
Cheng, Z., Mai, K., Ai, Q., Li, Y., He, Z., 2013. Effects of dietary pregelatinized corn starch on growth, apparent digestibility, and digestive enzyme activity of large yellow croaker fingerlings (*Pseudosciaena crocea*). The Israeli Journal of Aquaculture. 65 (2013), 843.
Conde-Sieira, M., Aguilar, A.J., López-Patiño, M.A., Míguez, J.M., Soengas, J.L., 2010. Stress alters food intake and glucosensing response in hypothalamus, hindbrain, liver, and Brockmann bodies of rainbow trout. Physiol. Behav. 101, 483–493.
DAFF, 2012. Aquaculture Yearbook 2012, South Africa. Department of Agriculture, Forestry and Fisheries, Cape Town, p. 60.
Deng, D.F., Refstie, S., Hemre, G.-I., Crocker, C.E., Chen, H.Y., Cech, J.J., Hung, S.S.O., 2000. A new technique of feeding, repeated sampling of blood and continuous collection of urine in white sturgeon. Fish Physiol. Biochem. 22, 191–197.
Eames, S.C., Philipson, L.H., Prince, V.E., Kinkel, M.D., 2010. Blood sugar measurement in zebrafish reveals dynamics of glucose homeostasis. Zebrafish 7, 205–213.
Ellis, T., Yildiz, H.Y., Lopez-Olmeda, J., Spedicato, M.T., Tort, L., Overli, O., Martins, C.I., 2012. Cortisol and finfish welfare. Fish Physiol. Biochem. 38, 163–188.
Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A., 2009. Nutritional regulation of hepatic glucose metabolism in fish. Fish Physiol. Biochem. 35, 519–539.
Eslamloo, K., Morshedi, V., Azodi, M., Akhavan, S.R., 2016. Effect of starvation on some immunological and biochemical parameters in tinfoil barb (*Barbonymus schwanenfeldii*). J. Appl. Anim. Res. 1–6.
Fanouraki, E., Mylonas, C.C., Papandroulakis, N., Pavlidis, M., 2011. Species specificity in the magnitude and duration of the acute stress response in Mediterranean marine fish in culture. Gen. Comp. Endocrinol. 173, 313–322.
Friedrichs, K.R., Harr, K.E., Freeman, K.P., Szladovits, B., Walton, R.M., Barnhart, K.F., Blanco-Chavez, J., 2012. ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. Vet. Clin. Pathol. 41, 441–453.
Garin, D., Rombaut, A., Freminet, A., 1987. Determination of glucose turnover in sea bass *Dicentrarchus labrax*. Comparative aspects of glucose utilization. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 87, 981–988.
Groff, J.M., Zinkl, J.G., 1999. Hematology and clinical chemistry of cyprinid fish. Common carp and goldfish. Veterinary Clinics of North America: Exotic Animal Practice 2, 741–776.
Grutter, A.S., Pankhurst, N.W., 2000. The effects of capture, handling, confinement and ectoparasite load on plasma levels of cortisol, glucose and lactate in the coral reef fish *Hemigymnus melapterus*. J. Fish Biol. 57, 391–401.
Herrera, M., Vargas-Chacoff, L., Hachero, I., Ruíz-Jarabo, I., Rodiles, A., Navas, J.I., Mancera, J.M., 2009. Physiological responses of juvenile wedge sole *Dicologoglossa cuneata* (Moreau) to high stocking density. Aquac. Res. 40, 790–797.
Holguín, A., Norman, F., Martín, L., Mateos, M.L., Chacón, J., López-Vélez, R., Pérez-Molina, J.A., 2013. Dried blood as an alternative to plasma or serum for *Trypanosoma cruzi* IgG detection in screening programs. Clin. Vaccine Immunol. 20, 1197–1202.
Hsiang, M.S., Lin, M., Dokomajilar, C., Kemere, J., Pilcher, C.D., Dorsey, G., Greenhouse, B., 2010. PCR-based pooling of dried blood spots for detection of malaria parasites: optimization and application to a cohort of Ugandan children. J. Clin. Microbiol. 48, 3539–3543.
Hwang, J., Jaroensuk, J., Leimanis, M.L., Russell, B., McGready, R., Day, N., Snounou, G., Nosten, F., Imwong, M., 2012. Long-term storage limits PCR-based analyses of malaria parasites in archival dried blood spots. Malar. J. 11, 339.
Iwama, G.K., Morgan, J.D., Barton, B.A., 1995. Simple field methods for monitoring stress and general condition of fish. Aquac. Res. 26, 273–282.
Jentoft, S., Aastveit, A.H., Torjesen, P.A., Andersen, Ø., 2005. Effects of stress on growth, cortisol and glucose levels in non-domesticated Eurasian perch (*Perca fluviatilis*) and domesticated rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 141, 353–358.
Lacher, D.A., Berman, L.E., Chen, T.-C., Porter, K.S., 2013. Comparison of dried blood spot to venous methods for hemoglobin A1c, glucose, total cholesterol, high-density lipoprotein cholesterol, and C-reactive protein. Clin. Chim. Acta 422, 54–58.
Martin, N.J., Bunch, J., Cooper, H.J., 2013. Dried blood spot proteomics: surface extraction of endogenous proteins coupled with automated sample preparation and mass spectrometry analysis. J. Am. Soc. Mass Spectrom. 24, 1242–1249.
Martinéz-Porchas, M., Martínez-Córdova, L.R., Ramos-Enriquez, R., 2009. Cortisol and glucose: reliable indicators of fish stress? Pan-American Journal of Aquatic Sciences 4, 158–178.
Mazzaccara, C., Labruna, G., Cito, G., Scarfò, M., De Felice, M., Pastore, L., Sacchetti, L., 2008. Age-related reference intervals of the main biochemical and hematological parameters in C57BL/6J, 129SV/EV and C3H/HeJ mouse strains. PLoS One 3, e3772.
McDade, T.W., 2014. Development and validation of assay protocols for use with dried blood spot samples. Am. J. Hum. Biol. 26, 1–9.
McDade, T.W., Williams, S., Snodgrass, J.J., 2007. What a drop can do: dried blood spots as a minimally invasive method for integrating biomarkers into population-based research. Demography 44, 899–925.
McGrath, S.P., Broadhurst, M.K., Butcher, P.A., Cairns, S.C., 2011. Fate of three Australian teleosts after ingesting conventional and modified stainless- and carbon-steel hooks. ICES J. Mar. Sci. 68, 2114–2122.

Mei, J.V., Alexander, J.R., Adam, B.W., Hannon, W.H., 2001. Use of filter paper for the collection and analysis of human whole blood specimens. J. Nutr. 131, 1631S–1636S.
Millán-Cubillo, A.F., Martos-Sitcha, J.A., Ruiz-Jarabo, I., Cárdenas, S., Mancera, J.M., 2016. Low stocking density negatively affects growth, metabolism and stress pathways in juvenile specimens of meagre (*Argyrosomus regius*, Asso 1801). Aquaculture 451, 87–92.
Miller, M.J., Kennedy, A.D., Eckhart, A.D., Burrage, L.C., Wulff, J.E., Miller, L.A.D., Milburn, M.V., Ryals, J.A., Beaudet, A.L., Sun, Q., Sutton, V.R., Elsea, S.H., 2015. Untargeted metabolomic analysis for the clinical screening of inborn errors of metabolism. J. Inherit. Metab. Dis. 38, 1029–1039.
Parker, D.R., Bargiota, A., Cowan, F.J., Corrall, R.J.M., 1997. Suspected hypoglycaemia in out patient practice: accuracy of dried blood spot analysis. Clin. Endocrinol. 47, 679–683.
Peterson, B.C., Small, B.C., 2004. Effects of fasting on circulating IGF-binding proteins, glucose, and cortisol in channel catfish (*Ictalurus punctatus*). Domest. Anim. Endocrinol. 26, 231–240.
Polakof, S., Panserat, S., Soengas, J., Moon, T., 2012. Glucose metabolism in fish: a review. J. Comp. Physiol. B. 182, 1015–1045.
Rattenbury, J.M., Herber, S.M., Price, K.J., 1989. Dried blood spot glucose measurement in hyperinsulinaemic hypoglycaemia. Clin. Biochem. 22, 301–303.
Ray, S.N.C., Sinha, R.C., 2014. Serum cortisol and glucose: reliable bioindicators of stress in fish, *Labeo rohita*. International Journal of Innovative Science, Engineering & Technology 1, 6–17.
Sacks, D.B., Arnold, M., Bakris, G.L., Bruns, D.E., Horvath, A.R., Kirkman, M.S., Lernmark, A., Metzger, B.E., Nathan, D.M., 2011. Guidelines and recommendations for laboratory analysis in the diagnosis and management of diabetes mellitus. Diabetes Care 34, e61–e99.
Samaras, A., Papandroulakis, N., Costari, M., Pavlidis, M., 2015. Stress and metabolic indicators in a relatively high (European sea bass, *Dicentrarchus labrax*) and a low (meagre, *Argyrosomus regius*) cortisol responsive species, in different water temperatures. Aquac. Res. (n/a–n/a).
Shigeto, S., Katafuchi, T., Okada, Y., Nakamura, K., Endo, F., Okuyama, T., Takeuchi, H., Kroos, M.A., Verheijen, F.W., Reuser, A.J.J., Okumiya, T., 2011. Improved assay for differential diagnosis between Pompe disease and acid α-glucosidase pseudodeficiency on dried blood spots. Mol. Genet. Metab. 103, 12–17.
Stoot, L.J., Cairns, N.A., Cull, F., Taylor, J.J., Jeffrey, J.D., Morin, F., Mandelman, J.W., Clark, T.D., Cooke, S.J., 2014. Use of portable blood physiology point-of-care devices for basic and applied research on vertebrates: a review. Contemp. Phys. 2.
Tavares-Dias, M., Ruas de Moraes, F., Onaka, E.M., Rezende, P.C.B., 2007. Changes in blood parameters of hybrid tambacu fish parasitized by *Dolops carvalhoi* (Crustacea, Branchiura), a fish louse. Veterinarski Arhiv. 77, 355–363.
Tonyushkina, K., Nichols, J.H., 2009. Glucose meters: a review of technical challenges to obtaining accurate results. Journal of Diabetes Science and Technology (Online) 3, 971–980.
Vargas-Chacoff, L., Ruiz-Jarabo, I., Páscoa, I., Gonçalves, O., Mancera, J.M., 2014. Yearly growth and metabolic changes in earthen pond-cultured meagre *Argyrosomus regius*. Sci. Mar. 78, 193–202.
Von Schenck, H., Lönnström, L., Engström, M., 1985. Quality control of reflectometric determinations of glucose in dried blood spots on filter paper. Clin. Chem. 31, 706–709.
Voss, E.M., Cembrowski, G.S., Clasen, B.L., Spencer, M.L., Ainslie, M.B., Haig, B., 1992. Evaluation of capillary collection system for HbA1c specimens. Diabetes Care 15, 700–701.
Wells, R.M.G., Pankhurst, N.W., 1999. Evaluation of simple instruments for the measurement of blood glucose and lactate, and plasma protein as stress indicators in fish. J. World Aquacult. Soc. 30, 276–284.
Woo, P.T.K., Buchmann, K., 2012. Fish Parasites: Pathobiology and Protection. CABI, London.
Working Group on Selective Electrodes, S.D., 2001. IFCC recommendation on reporting results for blood glucose. Clin. Chim. Acta 307, 205–209.
Zukunft, S., Sorgenfrei, M., Prehn, C., Möller, G., Adamski, J., 2013. Targeted metabolomics of dried blood spot extracts. Chromatographia 76, 1295–1305.

Appendix 2: Conference Outputs

1. Aquaculture Association of Southern Africa (ASSA) Conference: 28-30 September 2015, Polokwane, South Africa

a) Poster Presentation:

METABOLOMIC ANALYSES FROM DRIED BLOOD SPOTS OF CULTURED DUSKY KOB *ARGYROSOMUS JAPONICUS* INFECTED BY THE GILL PARASITE *DIPLECTANUM OLIVERI* (MONOGENEA)

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INTRODUCTION

- Accumulation of parasites in fish farms is favoured by the increase in water temperature
- *Diplectanum oliveri* considered the most infectious parasite in the South African finfish aquaculture industry.

Fish hosts endure pathological effects
a. ulcerative skin lesions
b. anaemia
c. gill necrosis

- The aims of this project were (1) to validate the use of dried blood spots in aquaculture research and (2) to correlate metabolite concentrations with parasite load

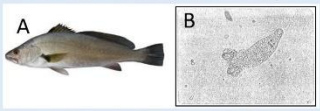

Figure 1: A. *Argyrosomus japonicus* (Dusky kob). **B.** Monogenean gill parasite *Diplectanum* sp. (100X)

METHODS

Dusky kob were collected from Oceanwise (N=30), Pure Ocean (N=30), Blue Cap (N=40) and Mtunzini (N=30) fish farms. Each animal was weighed and the standard and total lengths were measured. Blood (0.3 – 1 mL, scaled to fish size) was collected from the caudial vein in the tail region using a heparinized 1 mL syringe and 20 g needle. Packed cell volume (PCV) assessment was conducted using a battery operated micro-centrifuge. Blood spots were collected for targeted metabolomics analyses by spotting drops of blood (five ~20 µL spots) on Whatman 903 specimen collection cards. Metabolomics analyses were conducted at North-West University /DTI Metabolomics Platform. Amino acids were extracted and analysed by LC MS/MS. A principal components analyses was performed on the 19 amino acids studied using MetaboAnalisyst 3.0 (Xia *et al.*, 2015). Gill samples were collected and fixed in 10% formalin for parasite analyses. The gills, liver and spleen were weighed for each animal and thereafter gill-somatic, hepatosomatic and spleen-somatic indices were calculated.

Figure 2: (A) A fish being dissected for sample collection. (B) Blood spots on Whatman 903 card placed on micro-centrifuge for PCV analyses.

RESULTS AND DISCUSSION

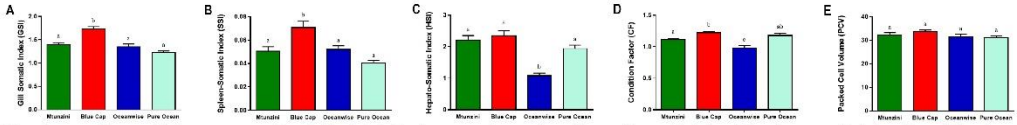

Figure 3: Gill, spleen and liver weights are expressed in relation to total body mass and are referred to as gill somatic index (A), spleen-somatic index (B) and Hepato-somatic index (C) respectively. Condition factor (CF) showed significant variability between the farms (D). Packed cell volume (PCV, %) of *A. japonicus* from all farms did not differ significantly (E).

Reduced red blood cell count in fishes has been attributed to parasite infection. Shahi *et al.* (2013) reported significant reduction in PCV by infected *Carassus carassus* (24,85 vs 29,00 from control) and *Schizothorax curvifrons* (24,85 vs 62,8 from control). In the present study, no significant differences were observed in PCV between the farms however a control group is required to establish a normal PCV range for farmed *A. japonicus*.

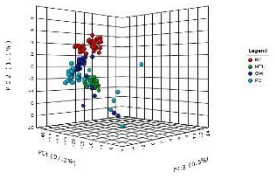

Figure 4: Three dimensional PC scores plot for *A. japonicus* corresponding to amino acids analyses.

Table 1: The first principal component (PC 1) explains 97,2% of the variation across all samples and is defined by these amino acids

Amino Acid	PC 1 score
Alanine	0,279
Proline	0,328
Serine	0,269
Isoleucine	0,278
Valine	0,267
Tryptophane	0,286
Glysine	0,278

- PCA separated farms clearly, indicating variability in underlying metabolic processes (Werth *et al.*, 2010).
- Amino acid variability in the present study can be attributed to various factors i.e.
  - parasite infection,
  - nutrition and
  - blood spot quality/variability

Figure 5: Blood cards showing good blood spots (A), hemolysis (B) and coagulation (C). An SOP has since been developed to ensure high quality sample collection.

CONCLUSIONS

- The use of DBS provides an alternative method for non destructive sampling and convenient sample collection, storage and transportation (Aim 1)
- This study presents preliminary results for amino acid analyses however these are to be supervened by correlations to parasite load (Aim 2)
- Integration of biological responses and welfare requirements of farmed *A. japonicus* in South Africa is essential for health monitoring of the fish.

Acknowledgements: We thank Pure Ocean aquaculture, Oceanwise, Blue Cap and Mtunzini Fish Farms for providing study animals and North-West University for metabolomic analyses.

References:
1. Shahi, N., Yousuf, A. R., Rather, M. I., Ahmad, F., & Yaseen, T. (2013). First report of blood parasites in fishes from Kashmir and their effect on the haematological profile. *Open Vet J*, 3, 89-93.
2. Werth, M. T., Halouska, S., Shortridge, M. D., Zhang, B., & Powers, R. (2010). Analysis of metabolomic PCA data using tree diagrams. *Analytical biochemistry*, 399(1), 58-63.
3. Xia, J., Sinelnikov, I. V., Han, B., & Wishart, D. S. (2015). MetaboAnalyst 3.0—making metabolomics more meaningful. *Nucleic acids research*, gkv380.

b) Oral Presentation: Abstract

CAN ON-FARM BLOOD GLUCOSE ANALYSE BE USED TO DIAGNOSE HYPER- AND HYPOGLYCEMIA IN FARMED KOB *ARGYROSOMUS JAPONICUS*?

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Blood glucose is commonly studied as a stress indicator for hyper- or hypoglycaemia. Inconsistencies in sample preparation for lab based glucose analyses may cause incorrect predictions of fish health condition. Requirements for sample preparation and accurate analysis of blood glucose may be circumvented by the use of a diabetic glucometer. Here we report a first attempt to correlate blood glucose data using a commercially available glucometer with lab-based enzymatic analyses. Blood glucose concentrations fish from the Mtunzini Fish Farm were measured on-farm using a diabetic glucometer. Whole blood and plasma samples were collected for enzymatic analyses. In the lab, a D-glucose enzymatic kit was used for whole blood and plasma analyses. The results were compared to on-farm blood glucose. On-farm blood glucose correlated well with lab plasma glucose ($y = 0,9282x + 1,083$, $R^2 = 0.857$), and were not significantly different based on a sample size of 30. Lab-based enzymatic analysis of whole blood glucose consistently underestimated farm-based data by 40%? Whole blood sample degradation has been previously reported, with 7% glucose depletion per hour of storage. It is recommended that blood for glucose analyses be drawn from the subject after 8 hours of fasting and blood samples must be preserved in citrate buffer to avoid glycolysis. Plasma glucose is more stable, plus it can be preceded by a battery operated micro-centrifuge step that also provides an assessment of packed cell volume (PCV). This method paves the way for future farm-based monitoring of fish condition, however requires refinement.

2. World Aquaculture Conference: 26-30 June 2017, Cape Town, South Africa

Oral Presentation: Abstract

UNTARGETED METABOLOMICS ANALYSES OF CULTURED DUSKY KOB *Argyrosomus japonicus* IN SOUTH AFRICAN AQUACULTURE

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The dusky kob (*Argyrosomus japonicus*) is known to suffer infections from the gill monogenean parasite *Diplectanum oliveri*. These monogenean parasites have adverse effects including epithelial hyperplasia and haemorrhage around attachment points and erosion and inflammation of gill lamellae. These often lead to secondary infections, reduced growth rates and mass mortality of fish stocks. To our knowledge, the physiological impacts of *D. oliveri* on farmed dusky kob have not been examined. The present study aims to identify and quantify metabolites in dusky kob which in turn can be used in applications of fish health assessment.

To achieve this objective, we applied GC-MS and LC-MS/MS based metabolomics analyses on fish dried blood spots (DBS) collected from four farms. The farms represent different parasite prevalence, geographical locations (KwaZulu-Natal, Eastern Cape and Western Cape) and farming conditions (RAS and ponds). A principal components analysis (PCA) was used as a reduction technique to cluster the samples based on similarities in metabolite concentrations. Using the identified metabolites, we produced a metabolomic profile for farmed dusky kob. Furthermore, we examined biological indices (hepato-somatic index and spleen somatic index), haematology parameters (blood glucose and packed cell volume) and condition factor from the fish.

A total of 25 organic acids, 20 amino acids and 8 acylcarnitines were identified with 6 amino acids (Citrulline, Glutamine, Lysine, Methionine, Phenylalanine and Proline) showed significant differences between the farms. The PCA produced a 43.5% variability (PC 1 and PC 2) which can be attributed to correlations in metabolite concentrations between the fish.

While significant differences in metabolite concentrations were observed and variations in metabolite correlations clustered the farms together (PCA), these observations cannot be attributed with certainty to parasitic infections only. Farming conditions, geographical locations and environmental factors have to be taken into consideration. Metabolomics is a valuable platform for fish health assessment as it may aid in early disease detection, biomarker development and implementation of corrective measures in farms.

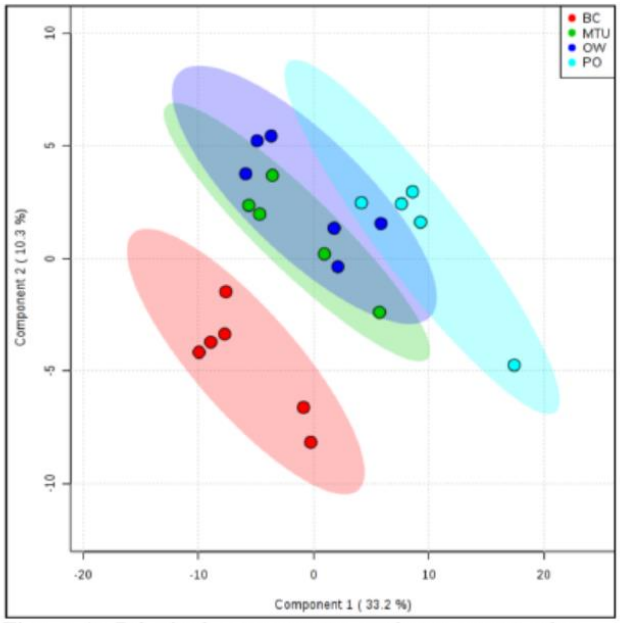

Figure 1: Principal components analyses scores plot for fish sampled from Blue Cap (BC), Mtunzini (MTU), Oceanwise (OW) and Pure Ocean (PO) fish farms.

Table 1: Various biological parameters measured from sampled fish. (CF = Condition factor; PCV = Packed cell volume; HSI = Hepato-somatic index).

Farm	Mass (g)	Glucose (mmol/L)	CF	PCV (%)	HSI
Mtunzini	432.1	6.2	1.16	33.1	2.8
Oceanwise	395.2	3.7	0.93	32.9	1.2
Pure Ocean	273.7	5.8	1.29	29.5	1.7
Blue Cap	882.8	6.8	1.25	31.9	1.9

Appendix 3: Ethical Clearance

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

30 August 2016

Dr Andre Vosloo
School of Life Sciences
Westville Campus

Dear Dr Vosloo,

Protocol reference number: AREC/030/016
Project title: Biomarker development for SA finfish culture

Full Approval – Research Application

With regards to your revised application received on 11 August 2016. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 30 August 2017.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

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Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Academic Leader Research: Professor Samson Mukaratirwa
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Jessica Light
Cc Mtunzini Fish Farm
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