GENETIC APPROACHES TO THE ANALYSIS OF BODY COLOURATION IN NILE TILAPIA (*Oreochromis niloticus* L.)

A thesis submitted for the Degree of Doctor of Philosophy

Amy Halimah Rajaee

Institute of Aquaculture, University of Stirling

Stirling, Scotland, UK

April 2011

DECLARATION

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

Signature of Candidate.....

Signature of Supervisor.....

Signature of Co-supervisor.....

Date.....

ABSTRACT

Body colouration in tilapia is an important trait affecting consumer preference. In the Nile tilapia (*Oreochromis niloticus*), there are three colour variants which are normal (wild type), red and blond. In some countries, the red variant is important and reaches higher prices in the market. However, one major problem regarding red tilapia culture is their body colouration which is often associated with blotching (mainly black but also red) which is undesirable for the consumer. The overall aim of this work was to expand knowledge on various aspects of body colouration in Nile tilapia using genetic approaches. The results of this research are presented as four different manuscripts. The manuscripts (here referred as Papers) have either been published (Paper IV) or are to be submitted (Paper I, II and III) in relevant peer reviewed journals.

Paper I and II investigated the inheritance of black blotching and other body colour components of the red body colour. Specifically, Paper I consisted of two preliminary trials (Trial 1 and 2), to look at the ontogeny of black blotching and body colour components over a period of six months. Trial 1 investigated the effect of tank background colour (light vs dark) on black blotching and other body colour components and was carried out using a fully inbred (all female) clonal red line. Trial 2 was carried out using mixed sex fish and was aimed to investigate the association of black blotching with the sex of the fish. The results from this study were used to guide the experiment described in Paper II. Sixteen red sires with various levels of black and red blotching were crossed to clonal females and the inheritance of blotching and other body colour components were investigated using parent-offspring regressions. The results showed no significant heritability for black blotching and body redness, but a significant

correlation for body redness and black blotching was found in female offspring at one sampling point suggesting that attempts to increase body redness may increase black blotching, as had been hypothesized.

Paper III was divided into two parts. The first objective was to map the blond locus onto the tilapia linkage map and the second was to investigate the interaction of the blond and red genes on black blotching using the blond-linked markers to distinguish different blond genotypes in heterozygous red fish (i.e. RrBlbl or Rrblbl). In the blond fish, the formation of melanin is almost blocked via much reduced melanophores and this feature may be able to help reducing the black blotching in red tilapia. Two intraspecific families (*O. niloticus*) and one interspecific family (*O. aureus* and *O. niloticus*) were used as mapping families and the blond locus was located in LG5. Four out of eight markers were successfully used to assess the interaction of blond on red blotched fish. The blond gene did not significantly reduce the area of blotching but did reduce the saturation (paler blotching) and enhanced the redness of body colour in the Rrblbl fish compared to the RrBlbl group.

Finally, Paper IV aimed to find out the effect of male colouration on reproductive success in Nile tilapia. A choice of one wild type male and one red male was presented to red or wild type females and these fish were allowed to spawn under semi-natural spawning conditions. Eggs were collected from the female's mouth after spawning and paternity was assessed using microsatellite genotyping and phenotype scoring. No significant departures from equal mating success were observed between the red and wild type males, however there was a significant difference between the red and wild

type females in the frequency of secondary paternal contribution to egg batches. The results suggest that mating success of wild type and red tilapia is approximately equal.

The results from this research help to broaden our knowledge and understanding on the aspects of body colouration in Nile tilapia and provide fundamental information for further research.

ACKNOWLEDGEMENT

First of all, I would like to express my humble gratitude to Almighty Allah (swt) for guiding me to complete this dissertation. Peace and blessings be upon Muhammad, His servant and messenger.

I would like to take this opportunity to record my appreciation to both my supervisors, Dr. David J. Penman and Prof. Brendan McAndrew for their continuous guidance and support all throughout the journey of my PhD study. Dave and Brendan, thank you for helping me right from the start, until the end, and I am very grateful for all the advices and comments you both had given me.

My special thanks also goes to Ms. Ann Gilmour, who has been teaching me the molecular techniques and for taking such good care of me during the early years in this foreign country. I would also like to thank Dr. John Taggart for assisting my molecular work, as well as to all colleagues in the molecular laboratory. Milton, Sofia, Asmund, Xiao and Oscar, thank you for all your help and for the friendships.

It is also impossible for me to complete my study without the help of Keith Ranson and William Hamilton. Thank you for all the assistance during my work in the tropical facility and it will always be remembered. I would like to thank Denny Conway, Brian Howie, Jacqui Ireland, Charlie, Jane, Dr. Saini and Christos for your kind help during my research.

I would also like to thank University Putra Malaysia and Ministry of Higher Education Malaysia for funding my research.

To all my dearest friends, Rania, Laura, Mairi, Sofia, Sophie, Oi, Sara, Polly, Xell, Milton, Sean, Greta and the list goes on…my highest regard goes to all of you. Many thanks for being a friend and for making this PhD journey more pleasant. Not forgotten to my friends back in Malaysia, especially to Fatehah and Siti Rahmah, I hope our friendship will last, regardless of wherever we are.

Last but not least, I would like to give my sincere thanks to my parents, Mr. Rajaee and Mdm. Tamah; my sisters Nordiana, Azlinawati and Edazarena, as well as my brother Noh Alhadi for having faith in me and supporting me all this time. To my fiancé, Alif Asraf, thank you for your patience and for the continuous support. May Allah swt. bless us all always.

TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1. General Introduction

1.1 Tilapia history and current state of culture

Tilapia is a common name use to describe a group of African Cichlid fishes belonging to the tribe Tilapiine (Trewavas, 1983). There are mainly four genera; *Tilapia*, *Oreochromis*, *Sarotherodon* and *Danakilia*, each are classified according to their reproductive behaviour. Tilapias of the genus *Tilapia* are substrate spawners while *Sarotherodon* and *Oreochromis* are mouthbrooders.

Tilapias have become globally important aquatic species in many tropical and sub-tropical countries worldwide. It has been known as the 'aquatic chicken' due to its remarkable success as a farmed fish. Fast growth rate, adaptability to a wide range of culture conditions, disease resistance and a high demand as a food source are some of the desirable qualities which makes this fish so popular in aquaculture. The importance of tilapia culture is confirmed by continuous reviews and manuals published over the years (e.g. Balarin and Hatton, 1979; Jauncey and Ross, 1982; Balarin and Haller, 1982; Guerrero, 1987; Tave, 1988; Popma and Green, 1990; Perschbacher, 1992; Suresh and Kwei-Lin, 1992; Beveridge and McAndrew, 2000; El-Sayed, 2006; Lim and Webster, 2006; and Morrison *et al*., 2006) as well as proceedings from major international symposia (e.g. Pullin and Lowe-McConnell, 1982; Fishelson and Yaron, 1983; Pullin *et al.*, 1988; Fitzsimmons, 1997; Fitzsimmons and Carvalho, 2000; Bolivar *et al*., 2004; Contreras-Sanchez and Fitzsimmons, 2006; and Elghobashy *et al*., 2008).

Tilapia is now the second most cultured group species after the carps (FAO, 2008b) surpassing the salmonids group. World tilapia production has been led by

China as the major producer (also exporter), followed quite distantly by Egypt and other countries like Philippines, Indonesia, Thailand, Taiwan and Brazil (FAO, 2008a). In 2008, the production for tilapia exceeded 2.7 million tonnes and this almost tripled the world production back in 1999 which was merely about one million tonnes (FAO, 2008b). Tilapia as a food fish has tremendous demand, especially in the USA, which is the major importer for tilapia (FAO, 2008a) mainly due to insufficient local production to satisfy market demand.

Despite being native to Africa, tilapia is mostly cultured in Asia where culture underwent three developmental phases; (i) cultured on a small scale with slow development from 1950-1980; (ii) immense increase of over 300% production in the period of 1981-1991 and (iii) great development and improvement in culture especially in selective breeding programmes from 1992 until the present (El-Sayed, 2006). Amongst all tilapia species, Nile tilapia (*Oreochromis niloticus*) is the most popular and well-studied due to its commercial value (McAndrew, 2000). This species has become dominant in tilapia culture due to its spectacular growth performance in compared to other tilapia species. World aquaculture production for the Nile tilapia alone reached two million tonnes in 2007 (FAO, 2007) covering about 83% of the total production for the tilapia group (FAO, 2008b). Besides *O. niloticus*, the hybrid group and the blue tilapia (*Oreochromis aureus*) are also important in aquaculture (Baroiller and Toguyeni, 2004).

To date, improvement of tilapia broodstocks has been one of the major foci for tilapia culture. Growth rates, body colour, cold- and salt-tolerance and production of all-male offspring are some of the important traits that have been focused on. Males are mostly favoured in tilapia culture, due to faster growth rate compared to females,

leading to production of all-male (monosex) populations and due to overcrowding problems created by reproduction following maturation in mixed sex groups. Production of monosex population through hormonal and genetic manipulation has been of major importance, however the latter is not straightforward since sex determination in tilapia is proven to be more complex than the simple monofactorial system (XX/XY); this subject has been recently reviewed by Baroiller *et al.* (2009). Body colour is also an important trait, in respect of market demand; i.e. red tilapia can reach higher market prices compared to the wild type in some countries where it is preferred (Pullin, 1983; McAndrew *et al*., 1988; Romana-Eguia and Eguia, 1999; Ng and Hanim, 2007). The resemblance of this red strain to some marine species like red snapper and some sea bream is one of the factors that attract consumers (Popma and Masser, 1999; Moralee *et al.*, 2000), hence establishing them for aquaculture. It was presumably the introduction of this red variant that made red tilapia popular in some countries which showed lack of interest in this species before. Nonetheless, red tilapia culture has issues with their 'colour quality' where the red phenotypes are often associated with some black spots (also known as blotches) which reduce their attractiveness. This topic is discussed in more detail in the next section.

The great acceptance of tilapia as a commercial farmed species has led to major genetic improvement programs to improve their culture performance. Many of these programs were implemented and promoted in Asia, where tilapia is mostly cultured. The production of the GIFT strain (Genetically Improved Farm Tilapia) implemented by World Fish Center (formerly known as ICLARM – International Center for Living Aquatic Resources Management) in collaboration with some other co-partners such as AKVAFORSK (Institute of Aquaculture Research of Norway) and UNDP (United

Nations Developments Programme) has brought tilapia culture forward by using improved stocks through selective breeding. The project which was initially based in the Philippines focused on increasing the growth performance, along with other important traits. A GIFT-derived strain, the GST™ (GenoMar Supreme Tilapia) has been further improved by selective breeding to exploit optimum genetic gain for important traits in tilapia. A major change applied in this project was the application of DNA fingerprinting as an identification system to replace the conventional physical tags (El-Sayed, 2006). Other genetic improvement programs have as well been carried out on a smaller scale, such as GET-EXCEL, FAC-selected (FaST) or IDRC (Bolivar and Newkirk, 2002) and SEAFDEC-selected (Basiao and Doyle, 1999). Impacts, benefits and issues with GIFT had been well reviewed by Eknath and Hulata (2009) who also discussed the status of genetic resources used in Nile tilapia, documenting a range of topics such as characterization and conservation of genetic variation, evaluation and utilization of genetic diversity in Nile tilapia.

1.2 Application of genetics in aquaculture

1.2.1 Genetic markers

The application of genetic markers in aquaculture and fisheries research has made it possible to better understand and extract valuable information for a variety of studies directly at the molecular level. To date, genetic markers have been used extensively in studies on genetic variation in wild and captive populations studies and aspects of selection programs such as identification of strains and species, parentage

analysis, genome mapping, inbreeding and sex identification. Genetic markers can be divided into two classes, Type 1 markers and Type 2 markers. Type 1 markers consist of actual genes of known function (coding sequence) while Type 2 are of anonymous genomic segments. Allozymes are one example of Type 1 markers while popular Type 2 markers are Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and microsatellites (Gjedrem, 2009). Genetic markers can be either co-dominant or dominant depending on their mode of inheritance. For co-dominant markers such as microsatellites, allozymes and RFLPs it is possible to distinguish between homozygous and heterozygous individuals.

Microsatellites, also known as simple sequence repeat (SSR), are one of the most popular genetic markers currently used in aquaculture research. Microsatellites consist of short tandem repeat sequences, usually of 1-6 base pairs and are mostly located in non-coding regions. Some of the advantages of microsatellites include high level of polymorphism, easy amplification using PCR, small samples required for analysis and ubiquity throughout genomes. Microsatellites are also co-dominant markers which makes them very useful in pedigree studies (Ferguson *et al*., 1995; Reece, 2003; Chistiakov *et al*., 2006). On the other hand, the downside of microsatellites is they are more expensive to genotype compared to SNPs markers (Glaubitz *et al*., 2003). Microsatellites also tend to have artefact bands, especially with dinucleotide repeats, probably due to polymerase slippage during PCR making the allele-scoring process more complex. Non-amplifying alleles (null alleles) caused by mutations at primer sites could also lead to false homozygotes, resulting in genotyping errors (Ferguson *et al*., 1995). In spite of these weaknesses, microsatellites are the most

commonly used types of marker in aquaculture and fisheries studies (Liu and Cordes, 2004). Applications of microsatellites along with their genomic distribution, evolution and function in fish genetics have been extensively reviewed by Chistiakov *et al*. (2006). A number of reviews on the role and application of genetic markers in aquaculture and fisheries field have also been published quite recently (Okumus and Ciftci, 2004; Liu and Cordes, 2004; Lo Presti *et al*., 2009; Chauhan and Rajiv, 2010), acknowledging their importance in current research.

1.2.2 Linkage Mapping

Before beginning any sequencing process for a genome, it is essential to create a framework of the genome first. This can be done by genetic and physical mapping. Physical mapping is a process of assembling DNA segments on its physical location on a chromosome by *in situ* hybridization. Meanwhile genetic mapping or linkage mapping is a process of assigning DNA markers along the chromosomes based on their recombination frequency (Fletcher *et al*., 2007). Linkage maps are based on Mendel's law of segregation, where genes or markers that segregate together are very likely to be inherited together and are placed on the same chromosome (Fincham, 1994). Only polymorphic genetic markers are useful in map construction (i.e. the marker needs to be heterozygous at least in one parent). In a linkage map, each chromosome should be represented by one linkage group once sufficient markers have been analysed. Unlike physical maps, linkage maps do not give accurate physical measurements since recombination frequency is not constant throughout the genome. The frequency of crossing-over is usually high in the region near the telomeres but decreases near the

centromeres. Variation in recombination fractions and map length also usually occur between different sexes, for example as observed in the zebrafish (Singer *et al*., 2002) as well as in the rainbow trout (Sakamoto *et al*., 2000). The standard map unit is called the centiMorgan (cM) where 1 cM refers to 1% chance of recombination (Fletcher *et al*., 2007). This map distance does not have any universal relationship with the actual physical distance between markers (Lynch and Walsh, 1998). The process of constructing a linkage map has been well explained by Danzmann and Garbi (2007) with some examples of linkage map construction in some aquaculture species.

The most commonly used method in producing linkage maps is by converting the observed recombination frequencies into an additive map using a mapping function. Mapping functions are used since only odd numbers of recombinants can be observed, but not double and other even numbers of recombinants (Gjedrem, 2009). Two of the mapping functions widely used in present research is the Haldane and Kosambi mapping functions. In Haldane, crossovers are assumed to occur at random and independent over the whole chromosome while Kosambi assumed that the crossover in a region influences the frequency of crossovers in other regions (Lynch and Walsh, 1998).

Before markers can be assigned into linkage groups, the recombination frequencies between markers need to be calculated and tested for linkage. The chance for detection of true linkage depends on the sample size number, number of markers used and the observed recombination rate of any two markers. Based on these two values, a LOD (Logarithm of Odds) score is calculated and a certain threshold is applied to decide if the markers are truly linked. Usually, a higher LOD score will be applied if the map is constructed from scratch. In general, it is recommended that a

LOD score threshold higher than 4.0 is used to be on the safe side (Danzmann and Garbi, 2007), but a value of 2.0 is usually the minimum acceptability (Fincham, 1994). In determining order of markers, the higher the LOD score between any two markers, the most likely those markers are to be regarded as next to each other (Danzmann and Garbi, 2007). In today's research, a number of softwares have been designed to help with linkage map construction (Table 1). Each of these softwares have their own constraints and strengths, but pair-wise LOD scores and two-point recombination frequencies should be the same across packages, although it is expected that the order of markers and map distances could vary due to the algorithms used (Danzzman and Garbi, 2007).

Linkage maps provide essential information for genetic studies, particularly in identifying QTL (quantitative trait loci). Linked markers for particular QTL then can be applied towards marker assisted selection (MAS) (Poompuang and Hallerman, 1997). Detection of a QTL region is usually followed up by either comparative genomic or by a process called positional cloning, where the QTL region will be mapped to a smaller region until the gene(s) responsible for the trait are identified (Lynch and Walsh, 1998).

Table 1: Some of the popular softwares used for linkage map construction (adapted from Danzmann and Gharbi, 2007)

Software	Platform	Populations
CARTHAGENE	PC, UNIX	F2 backcross RIL outcross
CRIMAP	PC, UNIX	Pedigree
JOINMAP	PC	F2 backcross RIL DH Outcross
LINKMFX	PC	Outcross
MAPMAKER	PC, MAC, UNIX	F2 backcross RIL DH
MAPMANAGER	PC, MAC	F2 backcross RIL

F2=F2 intercross; RIL=Recombinant inbred lines; DH=double haploids

1.2.3 Tilapia genome

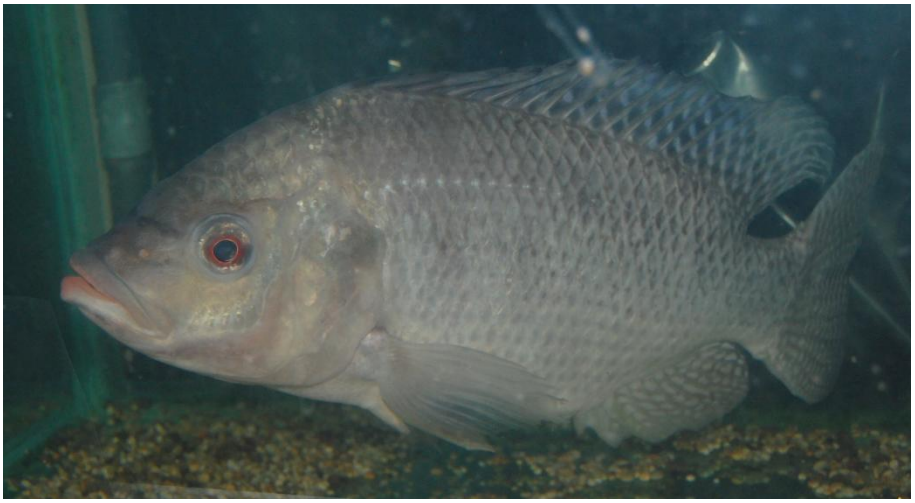
Tilapia is a good model species for genetic analysis due to their ability to breed all year and their short time to achieve maturity. For *Oreochromis spp.*, the first genetic linkage map was constructed using haploid progeny (Kocher *et al*., 1998) which was later refined using an F2 interspecies hybrid population (Lee *et al.*, 2005). Several other studies attempting to find the sex-determining genes which can be helpful in producing monosex populations have also been published (Lee *et al*., 2003; Lee *et al*., 2004; Karayucel *et al.*, 2004; Ezaz *et al*., 2004; Cnaani and Kocher, 2008) with evidence for cue of these genes being linked to the red colour (Karayucel *et al.*, 2003; Lee *et al.*, 2005). A BAC (Bacterial Artificial Chromosome) physical map of Nile tilapia has also been constructed (Katagiri *et al.*, 2005; see also review on physical

mapping in Nile tilapia by Martins *et al.,* 2004). Sequencing project for the tilapia genome is now currently in progress (Broad Institute, 2011).

1.2.4 Genetics of colour variants in Oreochromis niloticus

McAndrew *et al.* (1988) reported three colour variants in the Nile tilapia (Stirling strain originating from Lake Manzala, Egypt) which are normal, red and blond (Figure 1). Normal is the usual wild type pigmented colour whilst red showed pigmentation unlike the wild type with no obvious black pigmentation either on the skin or in the peritoneum. Blond are described as having a lack of pigmentation on the skin although the normal stripes can be faintly seen with an unpigmented peritoneum. The red tilapia is often associated with black blotching (thus this phenotype termed as blotched).

The red body colour in *O. niloticus* is controlled by a dominant allele where both homozygous *RR* and heterozygous *Rr* produce red body colour. On the other hand, blond is controlled by a separate locus, and the phenotype is only seen in homozygous recessives (Scott *et al*., 1987). Normal body colour is recessive to red but dominant over blond.



(a) Wild type

(b) Blond

(c) Red

Figure 1: Colour variants in *O. niloticus*

Up to now, genetics underlying the blotched phenotype are still poorly understood. It is predicted that the red gene is associated with black blotching since the blotched phenotype can only be expressed in its presence (McAndrew *et al.*, 1988). A study on the Thai and Egyptian red tilapia strain by Hussain (1994) also showed that the blotched phenotype is epistatic to the '*R*' gene. It had been suggested the recessive '*r*' allele is associated with blotching since Rr individuals are generally more blotched than RR; hence *Rr* heterozygotes are the carriers for black blotches. However, this is not absolutely true since some *Rr* fish have been produced with no apparent melanophore blotching and some *RR* fish have higher degree of blotching compared to *Rr* individuals (McAndrew *et al*., 1988). Another possibility is that the blotched phenotype may be controlled at a secondary locus, where their presence maybe masked in the wild type due to the primary colour locus (McAndrew *et al*., 1988; Mather *et al*., 2001; Garduno Lugo *et al*., 2004).

The blotch pattern can also appear in a way where the melanophores are replaced by red pigments (red blotches). These blotches usually appear deep red in colour (McAndrew *et al.*, 1988) and are described as red chromatophores and only shown on the body of the red fish (Avtalion and Reich, 1989). It was suggested that these are 'unpigmented' melanophores and are replaced by erythrophores (McAndrew *et al*., 1988). However, no histological evidence has been carried so far to verify the differences in cell types found in the red and black blotches.

A study by Hilsdorf *et al.* (2002) explained the circumstances of melanophore appearance in the embryos of the red and wild type tilapia. Melanophores first appearance can be seen on the embryo over the yolk sac, at ~40 hours post-fertilization in the wild type fish (at 26-28°C). Further melanophores can be seen in the yolk sac of

wild type larvae 5 days after fertilization, while many red larvae are still melanophore-free. At seven days after fertilization, the melanophore start to be seen on the body, with irregular distribution and mostly appear on top of the head. At this stage, wild type and red fish with a high degree of blotching cannot be clearly distinguished and can only be differentiated after day 9 through the structure of the distribution pattern. In wild type, the distribution is more uniform and with cells of a homogenous size meanwhile the melanophores are more intense with larger and diffuse pigmented cells in the red fry.

Blond, another mutant colour in *O. niloticus* was first described by Scott *et al*., (1987). The blond phenotype can be identified at first pigmentation stage (approximately 36-hours post-fertilization) due to the lack of pigmentation of the fertilized eggs compared with the wild type. At the first feeding stage, the blond fry show an overall lack of pigmentation over the whole body (Scott *et al.,* 1987). Blond is described as having a khaki-like appearance (Tave, 1991) or pale colour with reduced melanin granules (McAndrew *et al.,* 1988). The usual vertical stripes found in wild type fish can only be seen faintly. Sometimes, normal coloured fish are capable of showing lighter colouration similar to the blond which makes the identification of blond fish confusing. The easiest way to recognize blond is under anaesthesia, during stress condition or during spawning phase. The wild type tends to change their hue to darker colouration while blond tends to be much paler and showed no usual marked stripes as in the wild type (Scott *et al.,* 1987). Histology for blond showed almost no pigmentation on the stratum spongiosum level but some black pigments could be seen scattered in the hypodermis with a layer of iridophores (McAndrew *et al.,* 1988). In the peritoneum, the blond fish also showed no sign of pigmentation and this is totally

contrary to the normal wild type where the membrane is almost black (Scott *et al.*, 1987). Since the melanin granules seems to be almost completely blocked or removed within the blond fish, it has been suggested that the blond gene might be useful to produce lighter-coloured fish where it could be beneficial in clearing the black blotching in the red tilapia (McAndrew *et al.*, 1988). Mapping this locus onto the existing linkage map of tilapia (Lee *et al.*, 2005) will help to enhance the map to be more comprehensive for future reference. Flanking markers for blond are needed to help in studies on red and blond interaction, since blond is recessive and its presence would be masked under red. Mapping the blond locus would also be helpful to establish whether this is the same locus as red, which is located in linkage group 3 (Lee *et al.*, 2005).

Studies on expression of colour patterns and its inheritance are essential to develop skills in breeding and management of fish which will allow them to be developed as a potential genetic model and to improve this trait for aquaculture. Understanding the mechanisms that underly this process and interactions between some colour variants may help us to overcome problems such as malpigmentation in which can help to improve some commercial farm species. Some colour variants may appear the same in different species but the genetic mechanism that underlies them may differ.

1.2.5 Red tilapia culture

Red tilapia usually refers to hybrid between two or maybe more species of tilapia that are selectively breed for red colour morph. The term 'red' generally refers to a range of colour variants due to absence or lack of normal black pigmentation on the skin in comparison with the wild type fish (McAndrew *et al*., 1988; Hilsdorf *et al*., 2002). Some of the commercial red strains have genetic material from up to four different species, largely dominated by *O. mossambicus* and *O. niloticus*, where the origin of the red variant could be either of those two species (Behrends *et al*., 1982; McAndrew *et al*., 1988). Hybridization of the red mutant with other species occurred to improve its performance to suit culture conditions. It was predicted that the red variant comes from *O. mossambicus*, but due to poor growth rate of this species, farmers started to cross-breed them with *O. niloticus* for a faster growth rate. These hybrids were then crossed with other species such as *O. aureus* and *O. urolepis hornorum* to obtain other desirable traits such as tolerance towards cold water (*O. aureus*) and salinity. This has subsequently resulted in confusion in determining the origin of each strain.

Table 2: Some of the popular red tilapia strains used in aquaculture

Strain	Suspected Origin of Species	Reference	Colour Inheritance
Thai Red	*O. mossambicus* x *O. niloticus*	Galman and Avtalion (1983); Hussain (1994)	RR= Red Rr = Red rr = Wild type
Philippine Red	*O. mossambicus-hornorum* hybrid x *O. niloticus*	Galman *et al.* (1988); Koren *et al.* (1994); Reich *et al.* (1990)	
Stirling red	*O. niloticus*	McAndrew *et al.* (1988)	
Taiwanese Red	*O. mossambicus* x *O. niloticus*	Galman and Avtalion (1983); Liao and Chang (1983); Huang *et al.* (1988)	RR = Pink Rr = Red rr = Wild type
Fijian Red	*O. niloticus* X *O. mossambicus*	Mather *et al.* (2001)	
Florida Red	*O. mossambicus* x *O. urolepis hornorum*	Behrends *et al.* (1982); Watanabe *et al.* (2002)	Unknown
Israeli ND56	*O. niloticus* x *O. aureus*	Hulata *et al.* (1995)	Unknown

Preference for red tilapia exists in certain markets most probably driven by cultural bias towards fish colouration, as can be seen in some countries in South East Asia and South America. Within these countries the prices of red tilapia is usually higher than the wild morph and can reach up to twice as much. For example, premium price is paid for red tilapia in most of the urban market in the Philippines (Romana-Eguia and Eguia, 1999), in Fiji (Mather *et al*., 2001) and Mexico (Garduno Lugo *et al.*, 2003). In Puerto Rico, price paid per serving in the restaurant for red tilapia can be

equal to the silk snapper (*Lutjanus vivanus*) which is a popular marine food fish in this area (Head *et al.*, 1994). In Thailand, red tilapia is also use by the Chinese as a sacrificial offering (Bangkok Post, 2010). Such preference has limited the market demand and commercial production of other types of tilapia in these areas. For example, in Malaysia, red tilapia consists of 85% of total tilapia culture (Ng and Hanim, 2007) due to consumer preferences and bias against the wild morph.

One of the major focuses in red tilapia culture is to understand the basis for colouration and improve their colour quality. Several studies on this subject include Behrends *et al.* (1982);, McAndrew *et al.* (1988), Avtalion and Reich (1989), Tave *et al.* (1989), Hussain (1994), Majumdar *et al.* (1997), Mather *et al.* (2001), Hilsdorf *et al.* (2002) and Garduno-Lugo *et al.* (2004). One of the major issues with red tilapia culture is the black melanin blotching which negatively affect their marketability (Mather *et al.*, 2001). The blotched pattern can appeared either in scattered patches or segregated group of large melanophores and might covered any areas on the fish including the peritoneum (McAndrew *et al.*, 1988). Red tilapia with blotching is not of as high value as the uniform red and consumers usually associate this with damaged or infected fish.

Several studies attempted to improve red body colour by using mass selection. A study by Mather *et al.* (2001) used mass selection on Fijian red hybrid tilapia *(O. niloticus* x *O. mossambicus*) to produce red fish with reduced blotching. Comparison of fish in three selection lines; control (C), high-selection (H; top 30% red fish) and low-selection (L; top 50% red fish) for black blotching on red individuals were tested for three generations. Results from this study showed significant reductions of black blotching between C line and the selection lines (H and L) in generation 2 and 3. However, there was no significant difference between the two selections lines within

each generation. When data was compiled across generations, significant reduction of black blotching was seen in both H and L line, but not in the C line, suggesting mass selection may have helped to produce red fish with reduced blotching. The results were also tested for growth performance, where mass selection seems to not affect growth performance, as only generation 3 showed reduced growth in all lines which was suggested could be due to unusual cold weather conditions.

Another study by Garduno Lugo *et al.* (2004) also applied mass selection technique in red *O. niloticus* to obtain red fish with reduced blotching after five generations. Selections for red individuals were done at two different stages, when fry were only 3 g and also before reproduction stage. There was significant reduction on the degree of blotching from fish in the first generation to fifth generation, where wild type (rr genotypes) were consistently removed from each generation, eliminating most of the recessive alleles.

Production of red fish free from blotches through mass selection however needs particular paired mating and as mentioned in Garduno-Lugo *et al*., (2004), it primarily depends on two major factors which are the genetics control of the colour (Tave, 1986) and the selection pressure applied towards blotching (Mather *et al.,* 2001). As in the case of Fijian hybrids in the study described above, significant results from mass selection will only be seen if all wild type individuals are removed from data sets, and if not, improved body colour (i.e. reduced blotching) can only be seen in the first generation, but not in the subsequent generations. Since red Fijian hybrids are of heterozygous Rr genotype, whilst the RR is pink and rr is wild type, the recessive alleles will always be inherited in every generation, hence limiting the application of

mass selection. The homozygous RR pink in this hybrid are avoided to be used in breeding programs due to low hybrid vigour.

1.3 Genetics of pigmentation

Fish colours have long fascinated humans. From biologists, fish enthusiasts and farmers to the end users who are the customers, fish colouration has been taken seriously as a factor that is important not only for the commercial value, but also as a model for genetic studies. It is well known that fish can change their colours according to different situations such as during stimulation and courtship (Fujii, 1969). It is also known that colour plays a vital role in survival, in order to avoid predators and competitors or increase the chances of feeding or reproduction. The responsible aspects for these colour changes are specialized pigment cells called chromatophores.

To date, several papers and book chapters by Fujii (1969, 1993a, 1993b, and 2000) have been the main references for fundamental information on pigmentation and colouration in fish. These publications have been cited in many other reviews with a more developed research explaining details and in-depth work within this topic. Recent studies have focused on the role of pigments, their regulation and motility especially in morphological and physiological colour changes in fish as well as pigment synthesis pathways. The latest reviews on this topic include Fujii (2000), Kelsh (2004), Braasch *et al*. (2008), Kelsh and Parichy (2008) and Leclercq *et al*. (2010). More research has been published in the last decade providing interesting findings for this research area. To name a few, Kelsh *et al*. (2000) used the embryo of the zebrafish to investigate the genetics behind melanophore development, Quigley and Parichy (2002) also used

zebrafish to look at the formation of pigment patterns, Sugimoto (2002) studied morphological colour changes in fish, Kimler and Taylor (2002) described mechanisms of pigmentary organelle transport in fish xanthophores and melanophores, Lamoureux *et al*. (2005) at pigment pattern in medaka embryo and Logan *et al*. (2006) at the regulation of melanophores in zebrafish. The number of articles published on pigmentation in fish over the last few years shows the continuing importance of this topic.

There are some terminologies used to describe or categorize pigment cells or types and first these terms must be understood correctly to avoid confusion. Pigment cells, also known as chromatophores, are the most common way to classify each type of pigment cells, basically according to the colour of the pigment they contain. There are six groups of chromatophores; melanophores, erythrophores, xanthophores, leucophores iridophores and cyanophores, the last being the most recently recognized pigment cell (Bagnara *et al*., 2007). Each chromatophore contains different pigment organelles which are called chromatosomes. The terminology used to describe pigmentation is outlined in Table 3.

Chromatophores are usually 'dendritic' cells (i.e. they have the ability to disperse and aggregate). Melanophores, erythrophores, xanthophores, leucophores and cyanophores are all dendritic cells and they all contain light-absorbing pigments (e.g. carotenoid, melanin) except for leucophores. Meanwhile for iridophores, they are usually non-dendritic although it was also reported that sometimes they can develop dendritic processes, as observed by Iga and Matsuno (1986) in gobiid fishes, Fujii *et al*. (1991) in the dark sleeper fish and in the paddlefish (Zarnescu, 2007). Leucophores and

iridophores both contain colourless pigments and are light-reflecting cells although their mechanism of light reflection is different from each other (Fujii, 1993a).

Table 3: Types of chromatophores

Chromatophore (Pigment Cell)	Chromatosome (Pigment Organelle)	Pigment	Cell type	Colour
Melanophore	Melanosome	Melanin	Light-absorbing	Black-brown
Erythrophore	Erythrosome	Carotenoids/Pteridines	Light-absorbing	Red
Xanthophore	Xanthosome	Carotenoids/Pteridines	Light-absorbing	Orange-yellow
Leucophore	Leucosome (refractosome)	Guanine-crystals	Light-reflecting	White/creamy
Iridophore	Iridosome	Mainly guanine, can as well contain hypoxanthine, uric acid or adenine	Light-reflecting	Silver/metallic
Cyanophore	Cyanosome	unknown	Light-absorbing	Electric blue

Source: Fujii (1993a); Fujii, (2000)

Melanophores, or termed as melanocytes for mammals and birds (Braasch *et al*., 2008), are the brown and black pigment cells and one of the most commonly found chromatophores especially in the dermis (Fujii, 2000). Melanophores can be easily found in the skin in any area that has shades of black-brownish colour and play a key role in rapid colour changes in fish (Fujii, 1993a). They contain melanin pigments and the melanisation process takes place in the organelle. Sometimes, the melanisation process is not complete and this immature pigment organelle is then called a premelanosome. Premelanosomes are more usually found in the epidermis rather than dermis. Melanophores can also appear without having the melanisation process and such melanophores, known as 'amelanotic melanophores', are colourless and have been detected in the 'orange-red' and white varieties of the medaka (Hama and Hiyama,

1966; Sugimoto *et al*., 1985; Fujii, 1993a). It was predicted that these non-melanised melanophores are related to the state of tyrosinase enzyme activities in the melanosome, where tyrosinase may be inhibited in the amelanotic melanophores (Hama and Hiyama, 1966). Melanophores are usually the largest chromatophores, with its organelle having a diameter of about 0.5 µm and the organelle being round or slightly ellipsoid. Because of their large size and position just below the iridophores, melanophores usually expand their dendritic activities resulting in melano-iridophore complexes and this gives rise to a combined organelle called a melaniridosome (Fujii, 1993a). Such cases have been reported among others by Kaleta (2009) in three species of salmonid; brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta m. fario*) and rainbow trout (*Oncorhynchus mykiss*).

Erythrophores are the reddish components usually found in the dermal level of fish skin. They are usually motile and slightly smaller than melanophores (Fujii 1993a). Basically, the composition and morphological features of erythrophores are very similar to xanthophores, the yellow-orange pigmented cells (Fujii, 1993a) and both of these chromatophores types are usually described together in much of the literature. Erythrophores and xanthophores are mostly present in the dermis, although in some exceptional cases, xanthophores also have been observed in the epidermis as reported by Obika and Meyer-Rochow (1990) in Antarctic blenny (*Trematomus bernacchii*) and in *Sparus aurata* (Ferrer *et al*., 1999). The pigments contributing to the red-orange-yellow colouration are pteridines and carotenoids. Both pigments may appear together in the same cell (but not in the same organelle; at least not yet reported) and their organelles can be classified either according to the pigment colour (erythrosomes for red and xanthosomes for yellow-orange) or as pterinosomes and carotenoid vesicles

(carotenoid droplets) based on the chemical content (Matsumoto, 1965; Fujii, 1993a). Pterinosomes are usually round, surrounded by a single membrane and contain thin fibril structures (Takeuchi and Kajishima, 1972). Carotenoid vesicles, on the other hand, have a hardly noticeable membrane and sometimes resemble oil droplets (Fujii, 1993a). Combinations of these two pigments in a cell are sometimes referred as xantho-erythrophore (Goodrich *et al*., 1941) and result in ranges of colour from pale yellow to red, depending on the ratio between pigments as well as the carotenoids and pteridines it contains (Bagnara and Matsumoto, 2006). Pteridines and carotenoids are fat- and water-soluble pigments respectively and may be lost during sample preparation for histological observation by light microscopy (Le Douarin and Kalcheim, 1999), making their identification sometimes difficult compared to other chromatophores.

Xanthosomes can reach the same size as melanosomes, but with a less uniform size and shape, as reported in the zebrafish by Hirata *et al.* (2003). Meanwhile Nakamura *et al*. (2010) suggested that there are two types of xanthophores in the Japanese flounder, early-appearing xanthophores which have smaller cell size and late-appearing xanthophores which are larger. However, this suggestion needs a further confirmation, since there is a chance that the cell sizes differ due to different response of the cell against treatment by anaesthetic and stress. Xanthophores have an important role in the formation of stripes in the adult pigment pattern in zebrafish mutants. In the larval stages, a reduced amount of xanthophores has no interference with melanophore stripes pattern structure; however at the adult stage partial absence of xanthophores usually leads to a disrupted stripe pattern (Odenthal *et al*., 1996).

Leucophores are light-reflecting cells containing small crystals. Usually surrounded by a double membrane, the organelles (leucosomes) have a spherical or

ellipsoidal shape and are able to disperse light rays in all directions and wider wavelengths (Takeuchi, 1976; Fujii, 1993a; Fujii, 2000). Leucophores usually have similar size to other light-absorbing pigment cells; sometimes they can be larger although with less dendritic processes (Fujii, 1993a). Up to now, leucophores have only been found in teleosts (Fujii, 1993b). Leucophores are usually motile, and as reported by Fujii *et al.,* (1997), due to their optical properties, their response is one of the most complicated to be quantitatively measured. Since leucophores as well as iridophores usually coexist alongside with other light-absorbing pigment cells, motile activities of other chromatophores often disturb the analyses of leucophores. Leucophores contain guanine crystals which could be purines or colourless pteridines (Oliphant and Hudon, 1993).

Iridophores, usually found on the side and belly area, contain light-reflecting crystals that usually result in the silvery areas of skin. They can be in very condensed stacks, giving no space for other chromatophores. The main compound in iridophores is guanine, but they can also contain purines, namely hypoxanthine and uric acid (Fujii, 1993a). These platelets are colourless however due to interaction with light, they result in silvery or metallic colour depending on their spacing and orientation (Kasukawa and Oshima, 1987; in Fujii, 1993a). Iridophores can be of two types; static or mobile. Static iridophores usually consists of thick stacks while mobile iridophores usually consist of dendritic structures (Iga *et al*., 1987; Fujii *et al*., 1991).

1.4 Colouration and mate choice

In many fish species, males usually have more embellished traits compared to females such as brighter colours and elongated fins. This is considered to be a result of sexual selection as first suggested by Darwin (1871). Sexual selection consists of two main mechanisms; (i) intrasexual competitions where males will have to compete to win the females and (ii) intersexual mate preference where females choose which males to mate with (Reichard *et al*., 2005). The latter is also known as female mate choice, where females set their preference based on direct or indirect cues shown by males (Darwin, 1871; Andersson, 1994). This section will discuss mainly the involvement of colours in mate choice towards reproductive success in fish, taking into account some relevant aspects related to it. Constraints and variation within female preferences, together with studies which did not find similar results are also discussed if information is obtainable, as well as implications of colour as genetic indicators.

To date, studies on mate choice on the basis of colouration in fish could be seen from three different perspectives; preference for carotenoid-based colour, melanin-based colour and colour-assortative mating. Female preference for brighter colour that is carotenoid-based has been convincingly shown in two species, the guppy, *Poecilia reticulata* (Houde and Hankes, 1997; Karino and Shinjo, 2004; Karino and Urano, 2008) and the three-spine stickleback, *Gasterosteus aculeatus* (Milinski and Bakker, 1990; Braithwaite and Barber, 2000). Female sticklebacks showed preference for males with a more intense red colouration under laboratory experiments (Milinski and Bakker, 1990; Braithwaite and Barber, 2000) as well as under natural environments (Bakker and Mundwiler, 1994). The red colouration is developed on the males' lower throat during the breeding season, as part of their nuptial colouration. However, some constraints

applied to this preference. Milinski and Bakker (1992) reported that the cost of travel (time and distance) for females between bright and dull males can reduce female selectivity. The degree of difference of the red intensity between males is also important since preference for red can only be seen if the magnitude of difference is great (Braithwaite and Barber, 2000). Female preferences for redness may also vary between populations (McKinnon, 1995) and other factors apart from nuptial colouration also contribute to mating success (see Cubillos and Guderley, 2000). Many of these factors could be the reasons why preference for red was not observed in some studies as in Heuschele *et al.* (2009). As described in Barber *et al.* (2001), brighter red colouration in males is predicted to be a sign for better resistance towards parasite infection (in this study, *Schistocephalus solidus*). Offspring from brighter males were reported to be more parasite resistant compared to offspring from dull males, although somehow it negatively affects their growth rate. This supports the findings from Milinski and Bakker (1990) where the intensity of red colouration in male sticklebacks reduced if they were infected by parasites.

In the guppy, female preferences were influenced by the brightness of orange spot patterns in males, although this condition differed between populations. A study by Houde and Hankes (1997) investigated two populations of guppies from natural populations in Trinidad, the Yarra and Paria which differed in the degree of orange colouration, the former being less orange. Females from both these populations showed strong preferences for the Paria males which have stronger orange colouration. Karino and Shinjo (2004) also found significant results for female preferences towards male orange spot patterns in the feral guppy in Japan. It was suggested in an earlier study that female preferences in guppies varied among populations (Endler and Houde, 1995).

Female preferences could also be influenced by longer fins in males (Bischoff *et al*., 1985) and black spots (Endler and Houde, 1995), with mismatched results on the degree of preferences for males' orange spots. Houde and Endler (1990) tested female preferences for males' orange spots in some populations of guppy with various degree of male orange colouration. Females coming from populations with a higher degree of orange colouration in males showed strong preferences, whilst in populations where males were less orange, females showed weaker or no preference. This condition is certainly contradicted by the study of Houde and Hankes (1997) explained earlier, and this variation could be due to the existence of polymorphism within female preferences as can also be seen in the eastern mosquitofish which is discussed later in this section. Brightness of the orange spot patterns in male could be a signal for males' health status, where infected males had reduced intensity in their nuptial colouration (Houde and Torio, 1992).

Apart from these two model species, female preferences for carotenoid-based colour in males also exist in some other species, although again these may be population-specific. This includes the green swordtail (*Xiphophorus helleri*) from Jalapa, where females showed a significant preference for the red morph males compared to the black morph (Franck *et al*., 2003). In this population, females are uniformly black striped, but in males, the lateral stripe could be black (black morph) or red (red morph). The lateral stripes, also known as the 'sword', consist of a set of elongated ventral fin rays towards the caudal fin (Johnson and Basolo, 2003). Maan *et al*. (2004) investigated female preferences in a cichlid species from Lake Victoria, *Pundamilia nyererei* under both laboratory trials and in natural field. Males in this species have dorsally reddish and laterally yellowish colouration. Mate choice studies

in both environments suggested that redness in males was the most important criteria to influence female preferences, the more intense the better chance for mating success. Even under natural environments, where other signals of mating choice exist, the red colouration was still the primary factor for mating success. This finding was confirmed by another study by Maan *et al*. (2010) in two different populations of *P. nyererei.*

Apart from carotenoid-based colour, melanin-based colour can also influence female preferences. Bisazza and Pilastro (2000) investigated female preferences in the eastern mosquitofish, *Gambusia holbrooki*, towards males with melanistic spots and found variation in female preferences. In this species, melanistic spots are linked to the Y-chromosome and only expressed in males. Two populations of the eastern mosquitofish, a feral population in Italy and another from Florida used in this study showed contradictive results, in which the feral populations from Italy did not show preference for melanistic males, but the population from Florida did. Melanistic males were frequently found in the Florida population but were absent in the Italian population which made possible for the authors to carry out tests for imprinting and rare-male effects. However, both theories are found to be non-significant.

A study by Fernandez and Morris (2008) found significant variation in female *Xiphophorus cortezi* preference towards spotted caudal (Sc) melanin pattern in males. In two out of three populations of *X. cortezi* used in this study, females showed strong preference for the Sc melanin pattern in males. However in the third population, in contrast females showed preference for non-Sc males. Further investigation showed that females of the first two populations had very low percentages of Sc themselves, while in the third, females showed higher levels of Sc. The Sc melanin pattern in this species serves as an indicator that they were carrying *Xmrk* oncogene (*Xiphophorus*

melanoma receptor kinase) which is responsible for the expression of the Sc melanin pattern in *X. cortezi*. Individuals need at least one copy of the *Xmrk* gene to express the Sc phenotype, although sometimes such individuals may show a lack of the Sc pattern. A double copy of *Xmrk* is reported to result in reduced viability which could lead to shorter life-span. The authors then suggested that the Sc melanin pattern expressed in this species is a way of acknowledging that they are carriers for *Xmrk*, hence avoid mating with each other as a way to reduce the chances for offspring to inherit a double copy of the *Xmrk* oncogene.

To date, reported studies related to colour-assortative mating are mainly from the African cichlids, perhaps because of their richness and diversity in colouration (Kocher, 2004). Between species that are closely related, little difference can be seen from their morphological and ecological aspects; however body colour and nuptial colouration are usually strongly diverged (Seehausen and van Alphen, 1999). The term colour-assortative or colour-based mating in this context is used when mating preferences are sorted by males (or females) colour pattern. Among the species that has been focused on are some populations of *Tropheus spp*. (Salzburger *et al*., 2006; Egger *et al*., 2008), *Pseudotropheus zebra complex* (Couldridge and Alexander, 2002; Knight and Turner, 2004; Blais *et al*., 2009), *Pundamilia pundamilia* and *P. nyererei* (Haesler and Seehausen, 2005; Verzijden and ten-Cate, 2007; Stelkens *et al*., 2008), *Rhampochromis longiceps* and *R. chiliangli* (Genner *et al*., 2007) and *Metriaclima zebra* (Jordan, 2008). Most of these studies reported female preference for conspecific male colouration (Seehausen and van Alphen, 1998; Couldridge and Alexander, 2002; Salzburger *et al*., 2006; Genner *et al*., 2007; Egger *et al*., 2008), or for males which resemble the conspecific males (Couldrige and Alexander, 2002; Stelkens *et al*., 2008)

although this preference can be altered by some restrictions such as water turbidity (Maan *et al*., 2010), masked light conditions (Seehausen and van Alphen, 1998) or if nuptial colouration between males are quite similar (Knight and Turner, 2004).

Cichlids are perhaps the most species-rich family, with around 2500 known species and over 1500 species found in the African Great Lakes (Seehausen *et al*., 1999). Famous for their diversity in colouration, it is thought that part of the rapid speciation process within the cichlids could be caused by sexual selection based on colour patterns (Dominey, 1984). Female preferences for conspecific males or other males that resemble them suggest that colour patterns may act as an indicator for species identification, especially for closely related species. Hybridization in the natural environment is quite rare although it may occur in certain situations such as in the absence of conspecific males (Couldridge and Alexander, 2002). This, in addition to a marked degree in assortative mating (Salzburger *et al*., 2006) suggest that colour-assortative mating is vital for early step for species formation as well as maintaining variety of colour morphs and main genetic pedigrees (Sturmbauer and Meyer, 1992 in Salzburger *et al*., 2006). On the other hand, it is expected that other factors such as heritability of female preferences (Haesler and Seehausen, 2005), random mating by hybrid females (Stelkens *et al*., 2008) and polymorphism in female preferences (van der Sluijs *et al*., 2007) may contribute some disruptions towards colour-assortative mating process.

Female preferences for carotenoid-based, melanin-based and other colour-based mating provide substantial information towards the importance of colouration in mate choice studies. Understanding the contribution of female preference to evolution is very complicated without the basic knowledge of how the selection currently affects the

female preferences especially in the natural environment. Although laboratory trials have contributed to suggestions and theories, this information would be best if confirmed under natural conditions.

1.5 Aim and outline of thesis

The general aim of this thesis was to gain understanding on various aspects of body colouration in Nile tilapia using genetic approaches. It was also anticipated that this thesis could provide fundamental information for further research aiming to improve the quality of body colour in the Nile tilapia, especially the red colouration. The outline of this thesis and connections between chapters are summarized in Figure 2. **Chapter 3** consisted of two preliminary analyses for Chapter 4 and aimed to look at the ontogeny of blotching and body colour components. Trial 1 studied the effect of tank colour background (light versus dark background; using clonal line fish) and Trial 2 investigated the association of black blotching with the sex of the fish (using outbred fish). **Chapter 4** investigated the inheritance of blotching and other body colour components in red Nile tilapia using red sires with different levels of blotching (black and red) crossed with fully inbred clonal line red females. Correlations between colour components were measured and heritability was estimated using sire-offspring regressions. **Chapter 5** is divided into two parts; The first one focused on mapping the blond locus onto the tilapia linkage map (Lee *et al*., 2005). Two intraspecific families of *O. niloticus* and one interspecific family of crosses between *O. aureus* and *O. niloticus* were used for mapping. The second part of this chapter investigated the interaction between the blond and red genes on blotching in Nile tilapia. Using blond-linked molecular markers from the mapping study, marker-assisted selection was applied to differentiate heterozygous red fish at the blond locus (RrBlbl or Rrblbl). Image analysis was performed on blotching on both groups of fish to assess the effect of blond gene on blotching. **Chapter 6** investigated the effect of male body colour on mating success in Nile tilapia. Red or wild type females were presented with a choice

of one wild type and one red male and allowed to spawn under semi-natural conditions. Microsatellite markers were used for paternity analyses. The results were then discussed with regard to spawning in aquaculture and natural environments. Finally, **Chapter 7** discusses the results from this thesis regarding their implications and importance towards aquaculture.

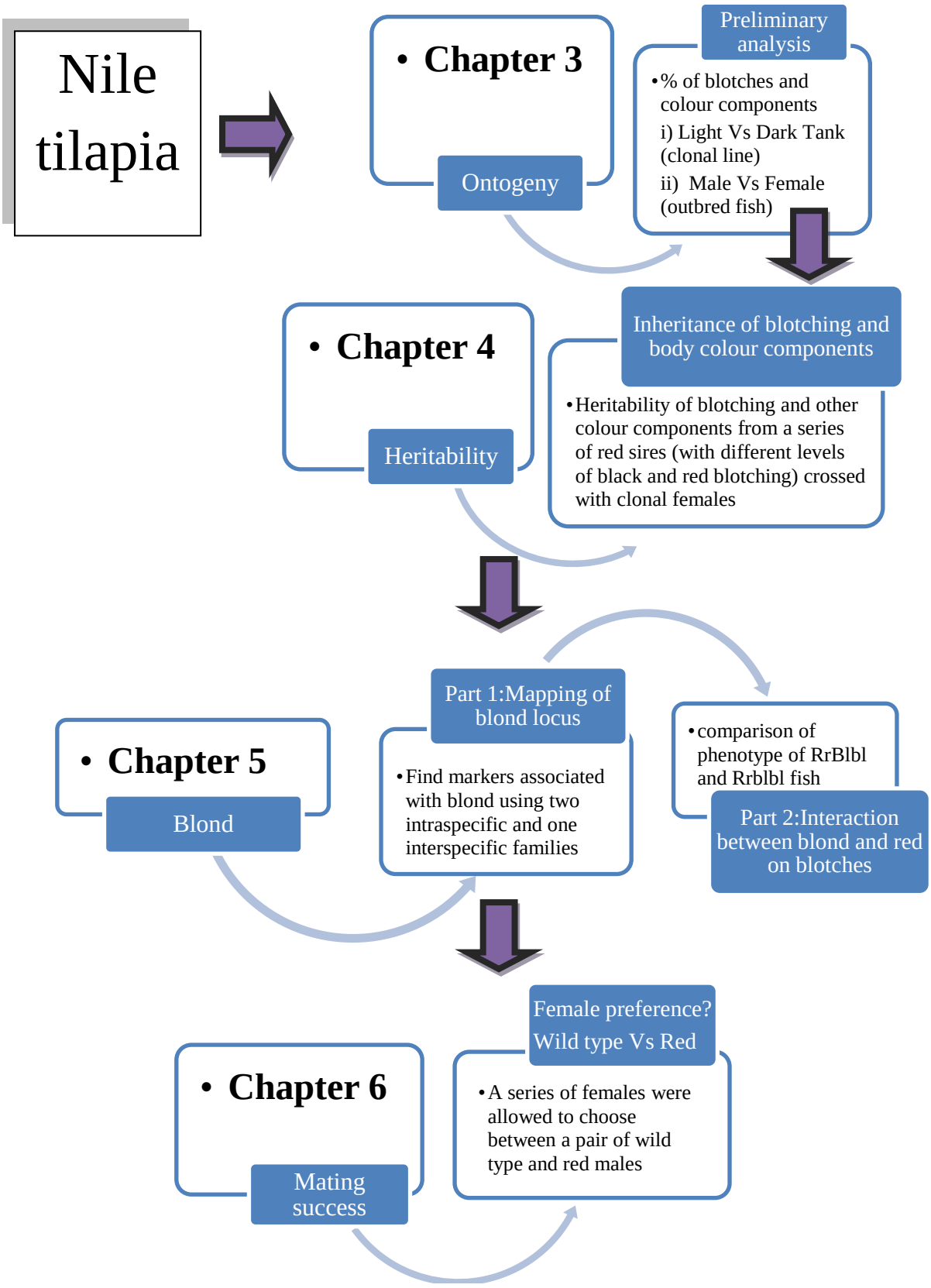


Figure 2: Schematic representation of the research frame

CHAPTER 2

MATERIALS AND METHODS

Chapter 2 – Materials and Methods

This chapter only includes the materials and methods used to carry out the image analysis. Other protocols and procedures used in this study are described in the relevant sections of the experimental chapters.

1.1 Photography set up

The photography took place in the wet laboratory within the Tropical Aquarium Facilities in the Institute of Aquaculture, University of Stirling. The camera was attached to a tripod through a custom made extension that made it possible for the camera to take photos vertically. The height of the tripod from the floor was 75cm. A ruler and an 18% gray card were placed on a gray tray (42 cm x 31 cm) in a translucent white tent (75cm x 75cm x 75cm). The ruler and the gray card were required for post-processing of the images. The fish to be photographed was also placed in this same tray after being anaesthetized. The camera was set to manual mode with a focal length of 35 or 55mm (depends on experiment), shutter speed of 1/25 and aperture of F5.6. A focal length of 35mm was used in Chapters 3 and 4 whilst focal length of 55mm was used for the research described in Chapter 5. An image of each side of the fish was taken using a wireless remote control and images were stored in a 3008 x 2000 pixel format on a 'high' quality setting (RAW compression of 12-bit). These images were saved in a digital memory card before transfer into a personal computer for subsequent analysis.

1.2 Image processing

All images taken were saved in the raw format which were NEF files (Nikon Exchange Format). The white balance of these images was first standardized according to the 18% gray card using Nikon ViewNX™ software (version 1.5.2, Nikon® Corporation) before further analysis was carried out. This alteration was done using the quick adjustment tool for white balance by taking a '3x3' average of the gray card area using the *'dropper tool'* provided within the software. The edited image was then saved and converted into *tiff* format (16bit) using the *'convert files'* tool. These adjusted images were then used for all subsequent analysis.

1.3 Image analysis

Image analysis was carried out using a freeware Java based program developed by the National Institute of Health (NIH), USA called ImageJ (version 1.43s, available at http://rsb.info.nih.gov/ij) with an additional plugin called 'RGBMeasure'. This plugin need to be installed first by downloading the Java file from http://rsbweb.nih.gov/ij/plugins/rgb-measure.html and instructions for installation are given within the mentioned website. In the following section, the analysis part is described as a step by step procedure. Manipulation of this software to do such type of image analysis is developed by the author of this thesis.

1.3.1 Image set up and setting scale in ImageJ

Step 1: To be able to measure the colour components within the image, the image file which was first opened as a 16-bit image needed to be to change to 'RGBcolor' type. This was done by selecting the *'Image'* menu, going to *'Type'* and selecting *'RGBColor'* from the list (Figure 1). A new image file was opened and the previous 16-bit image was closed.

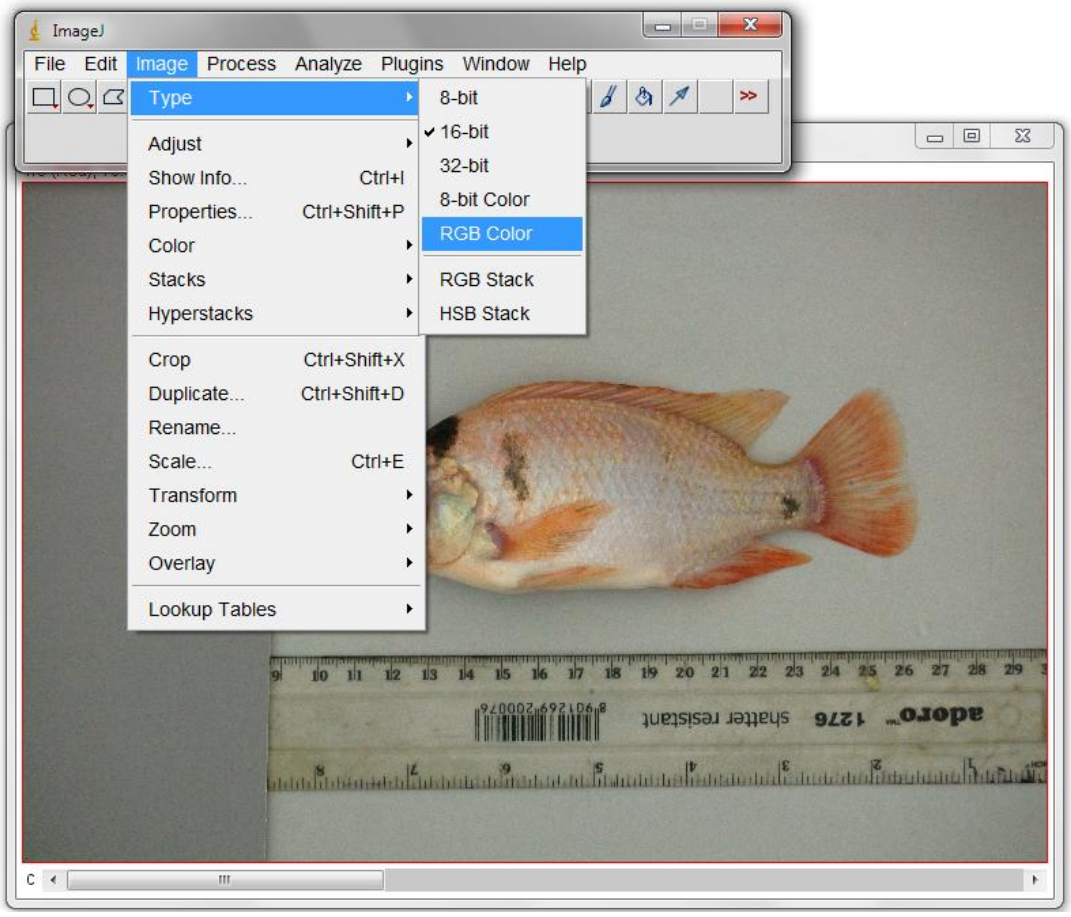


Figure 1: Changing image type

Step 2: Setting the scale for the image was usually done on the first image and then set as *'global'* for subsequent images. To set the scale, first, a straight line was drawn using the *'straight line'* toolbar , between two points of known distance (based on

the ruler within the image). The image was 'zoomed in' if required to focus on the ruler using the *'magnifying tool'* and left-clicking on the image (or right-clicking to decrease magnification) (Figure 2). After the line was drawn, the *'Analyze'* menu and *'Set Scale'* were selected (Figure 3).

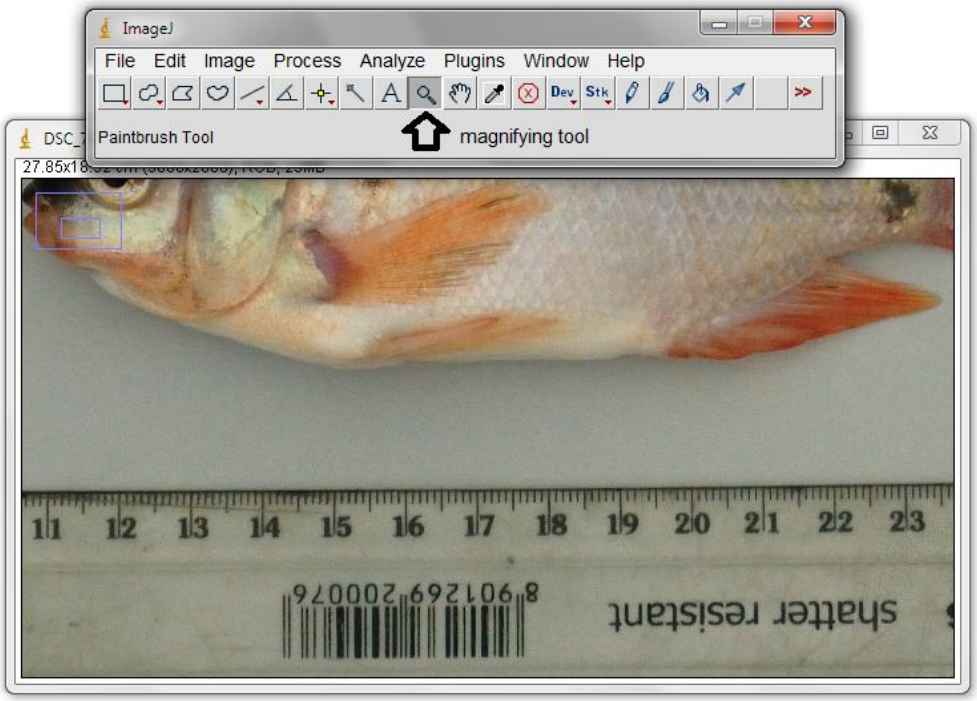


Figure 2: Magnification on image using the magnifying tool (left-click to increase, right-click to decrease)

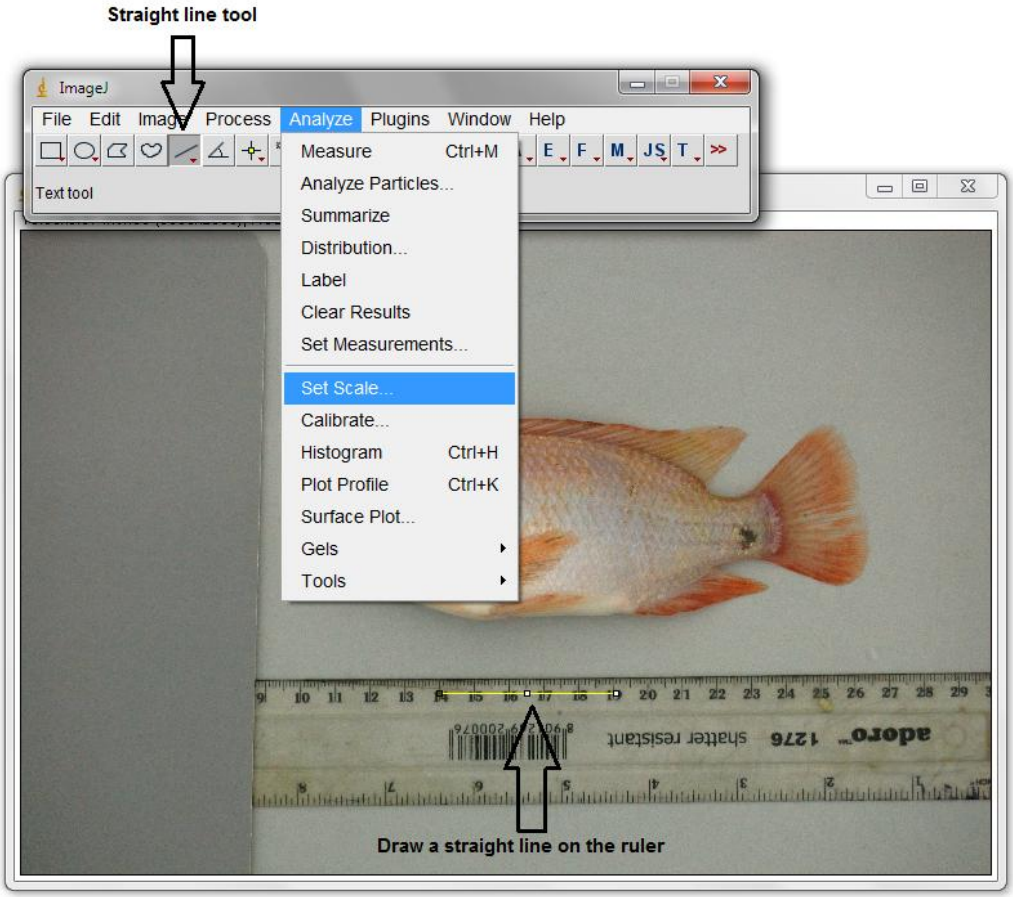


Figure 3: Setting scale in ImageJ

Step 3: In the *'Set Scale'* window, the length of the line in pixels was displayed. The *'Known Distance'* and the *'Unit of Length'* was filled in and the *'Global'* box was checked to apply the same scale for further images (Figure 4). The scale in pixels per length unit was shown at the bottom of the window and this could be taken down for future reference.

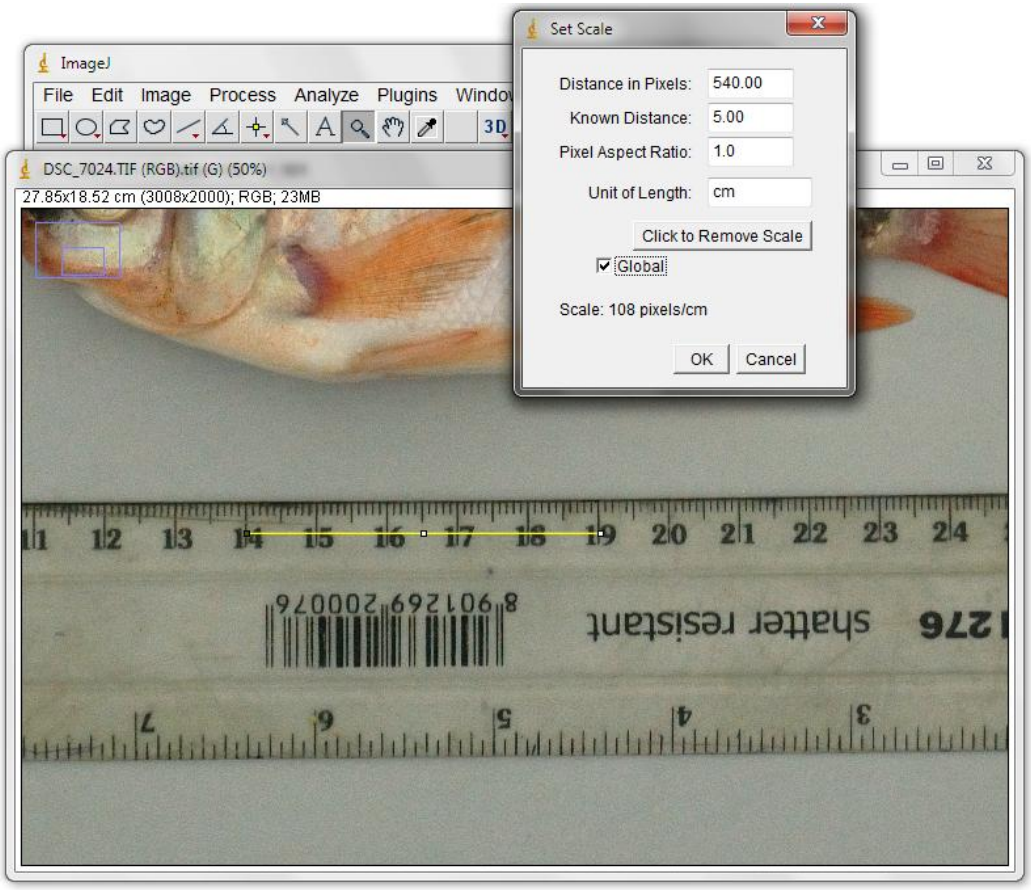


Figure 4: Set Scale window in ImageJ

Step 4: To check either the scale was correct, another line on the ruler was drawn and by selecting the *'Analyze'* menu and choosing *'Measure'* (Figure 5), a *'Result'* window was opened (Figure 6).

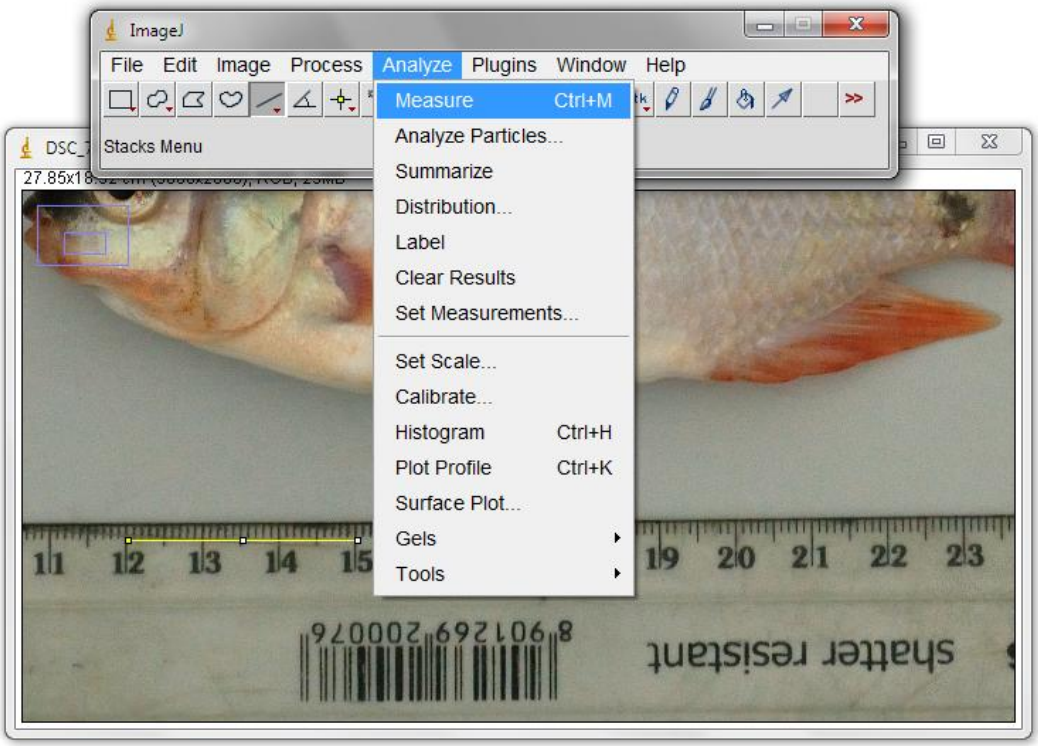


Figure 5: Measuring known length to check the scale

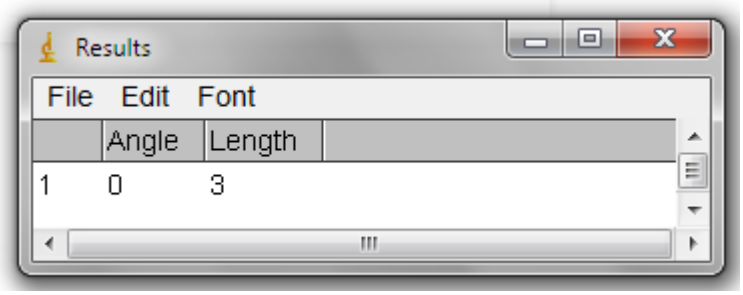


Figure 6: Sample of the 'Result' Window

1.3.2 Selecting and outlining region of interest (ROI)

Step 5: The next step was to select the region of interest. From the *'Analyze'* menu, *'Tools'* was selected and then *'ROI Manager'*. A new window called *'ROI Manager'* was opened. Using the *'Elliptical' toolbar* , the outline of the eye area of the fish was drawn and added to *'ROI Manager'* using the *'Add'* button in the *'ROI Manager'* (Figure 7).

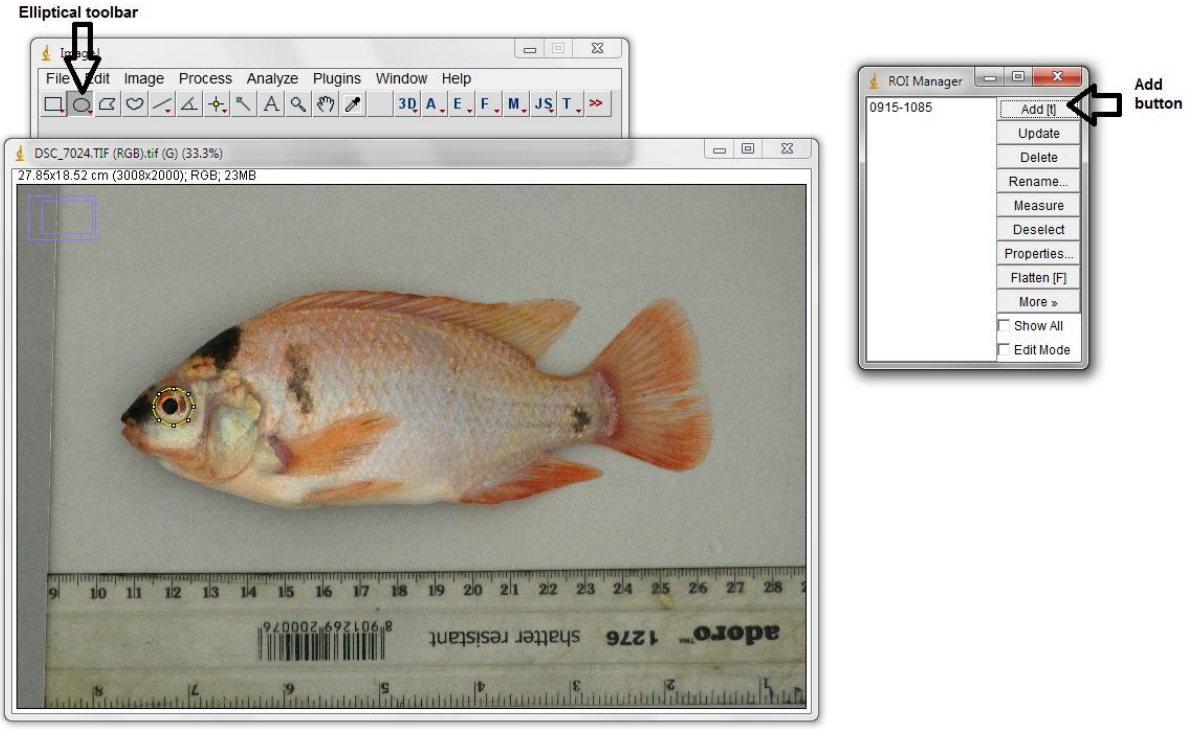


Figure 7: Adding region of interest (ROI)

Step 6: Applying the same technique in the previous step but using the *'Freehand Selection'* toolbar  , outlines of the pelvic and pectoral fins (Figure 8) as well as the whole area of the fish (excluding dorsal, anal and caudal fins) were drawn and added to the *ROI manager* respectively (Figure 9).

Figure 8: Outlines of the eye, pectoral and pelvic fins

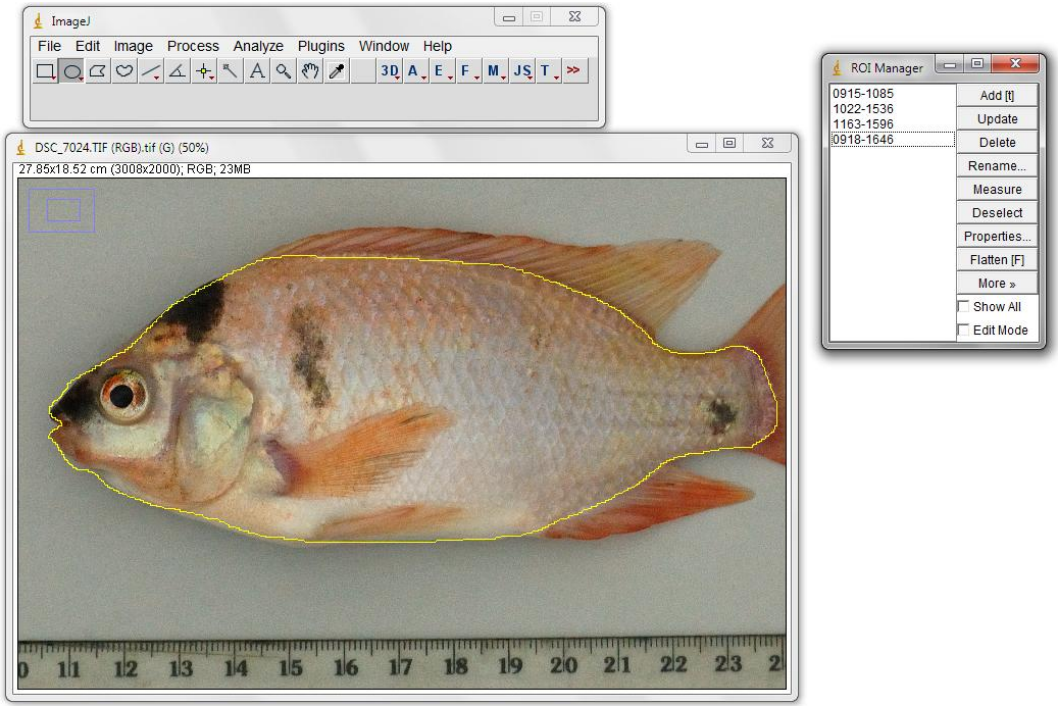


Figure 9: Outlining the body of fish

Step 7: A more refine outlines was achieved by selecting the region from the *ROI Manager* and right-clicking the *'Elliptical'* toolbar and selecting *'Selection Brush Tool'* (Figure 10).

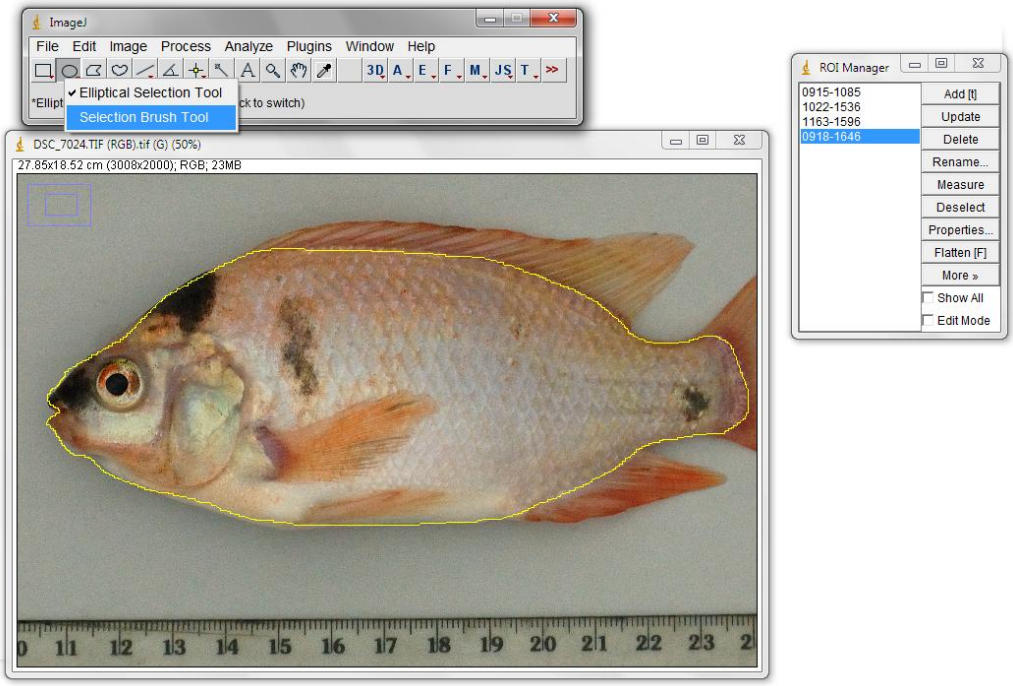


Figure 10: Changing the 'Elliptical' toolbar into 'Selection Brush Tool'

Step 8: By double clicking the *'Selection Brush Tool'*  , a new window was opened (Figure 11). The size of the brush depended on the number entered. This could be adjusted and changed depending on the need. This 'brush' was used to edit the outline wherever needed. The *'update'* button in the *ROI Manager* was clicked after the modification was made (Figure 12). Right clicking the toolbar reverted the 'brush' tool into *'Elliptical'* toolbar again.

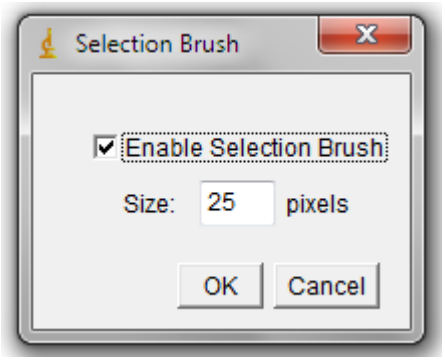


Figure 11: Selection brush option

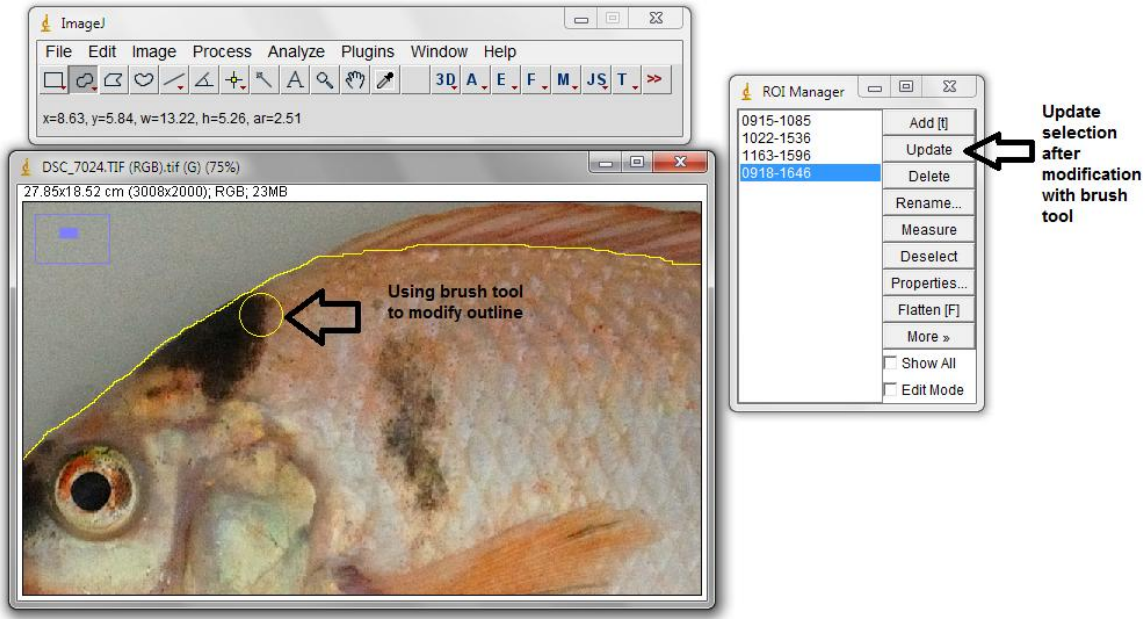


Figure 12: Applying brush tool to get a perfect outline

1.3.3 Combining and saving ROI

Step 9: The *'ROI'* areas for the eye and the fins were selected from the list (multiple selection was made using the 'shift' button) and combined by right clicking and selecting *'combine'* from the list (Figure 13).

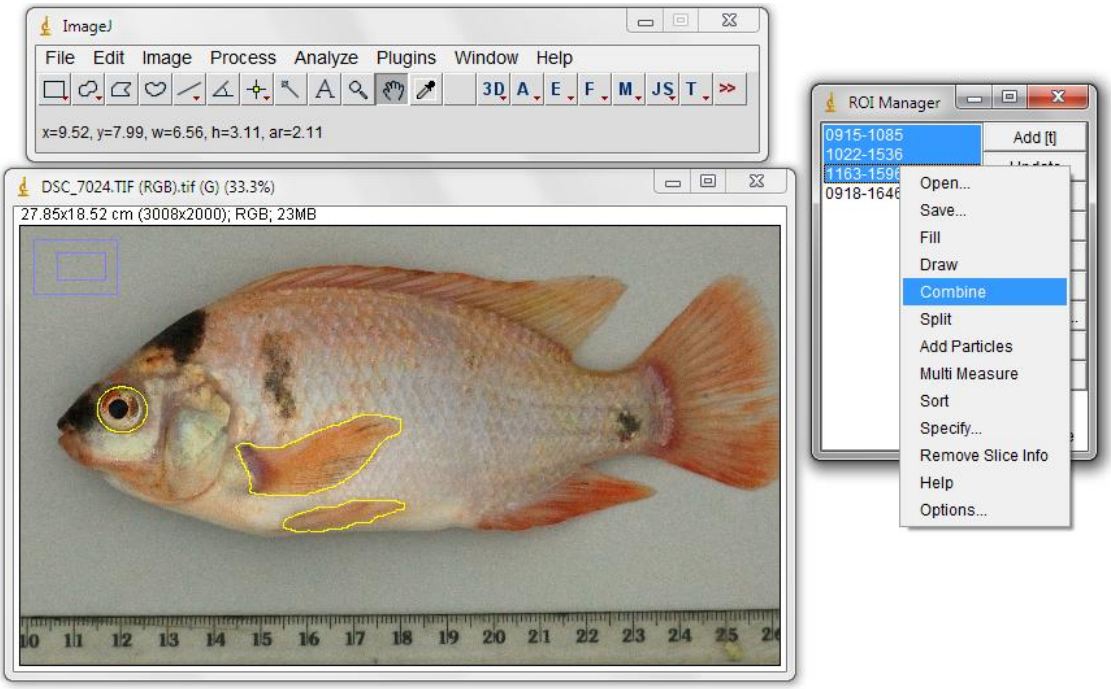


Figure 13: Combining ROI

Step 10: The *'Add'* button on the *ROI Manager* was clicked to add the new *'ROI'* to the list. The previous three (separate) *'ROI'* on the list were deleted. The new combined *'ROI'* and the body outline were saved as different filenames (Figure 14).

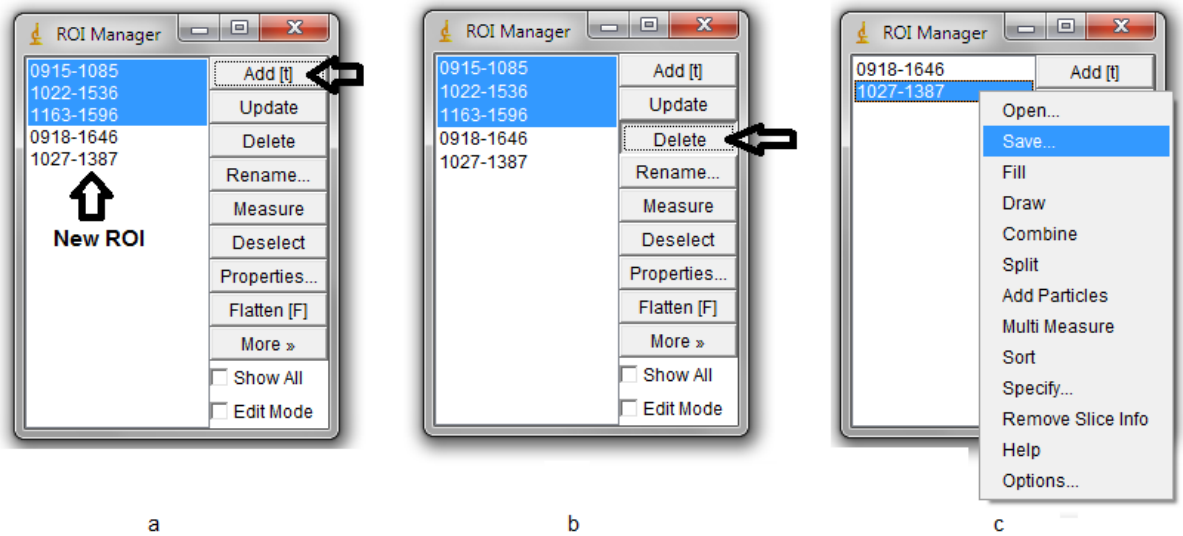


Figure 14: (a) Adding (b) Deleting and (c) Saving ROI

1.3.4 Quantifying blotched areas

Step 11: This step was omitted if the fish did not have any blotches. To begin, the '*wand tool'*  on the toolbar was double clicked. A window opened and the mode was changed into '8-connected' and the tolerance to '10'. This was a suitable tolerance and mode to select the blotches (Figure 15). The wand tool helped to find edges and trace shapes.

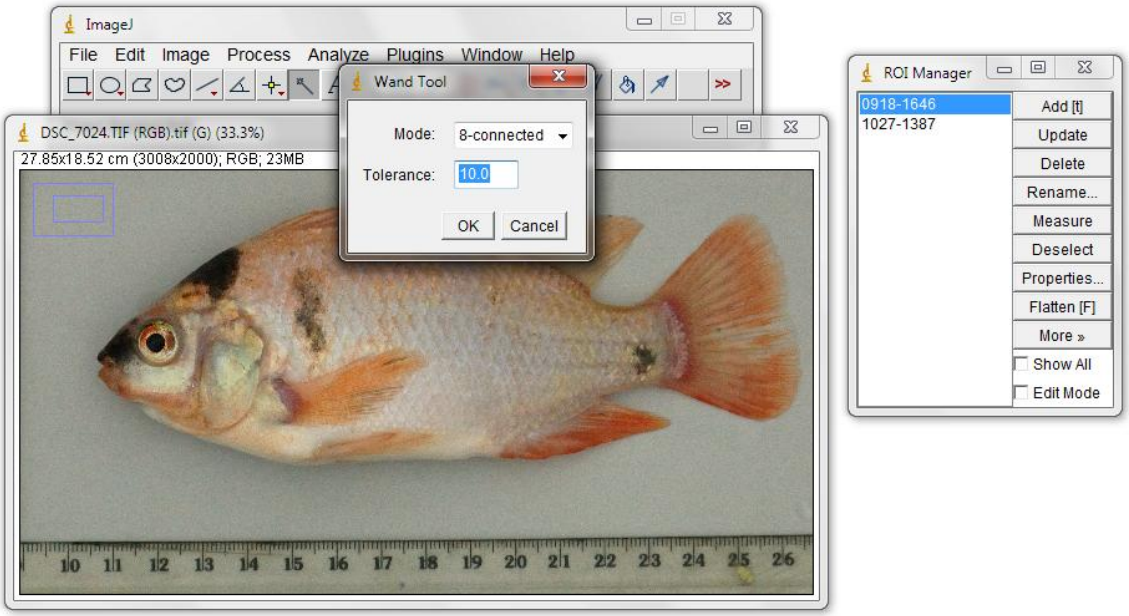


Figure 15: Setting the wand tool

Step 12: The wand tool was pointed and clicked to the black blotched areas resulted in the blotched area being selected. This selected area was added to the *ROI Manager* and the surrounding area of the blotches were added to *ROI Manager* until a satisfying selection was made (Figure 16). These *'ROIs'* were combined as explained in Step 9.

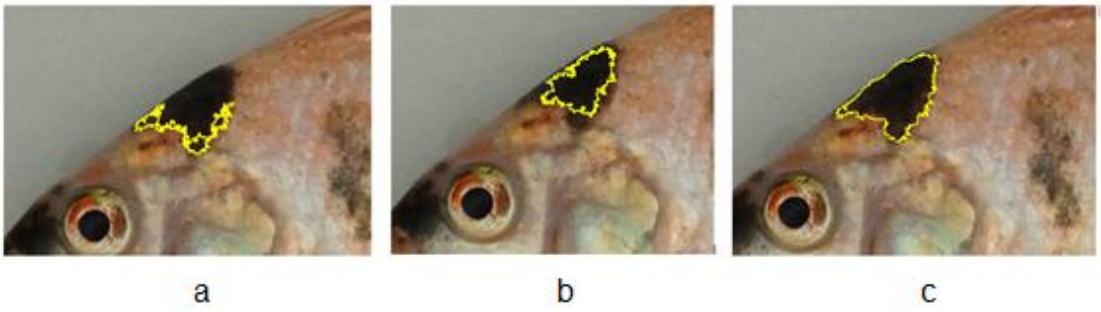


Figure 16: (a) (b) Selecting regions containing the black blotches and (c) Combined regions of black blotches

Step 13: This step was repeated for other regions of black blotches on the body. All of them were combined for easier measurements afterwards (Figure 17). This *'ROI'* was then saved.

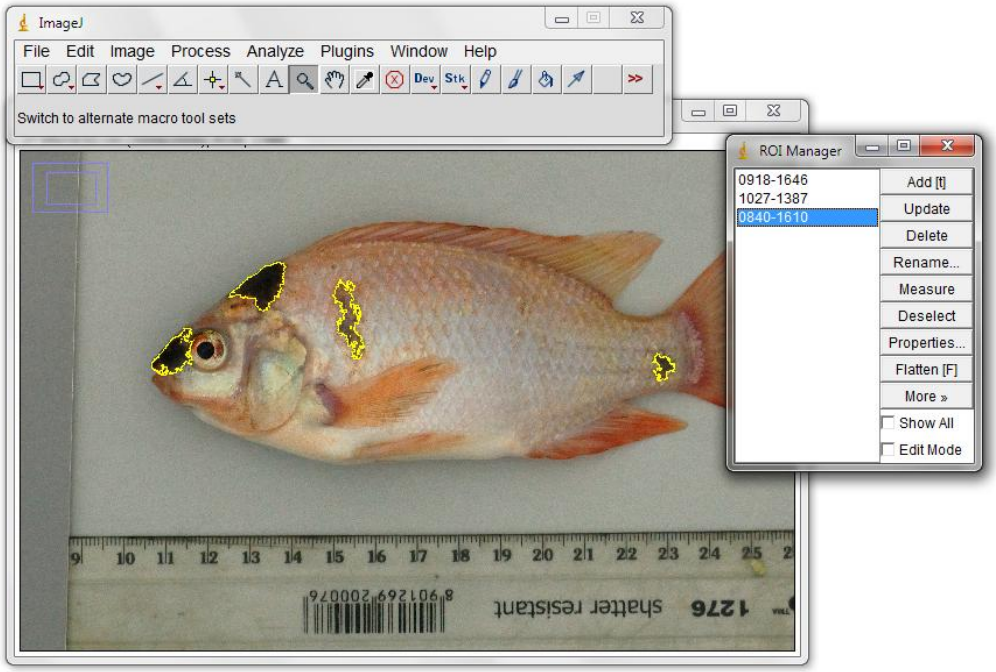


Figure 17: Combined regions of black blotches

1.3.5 Measuring area and colour components

Step 14: Measurements parameters were set by selecting *'Analyze'* on the menu bar then *'Set Measurements'*. The three measurements needed were *'Area'*, *'Standard Deviation'* and *'Mean Gray Value'* (Figure 18).

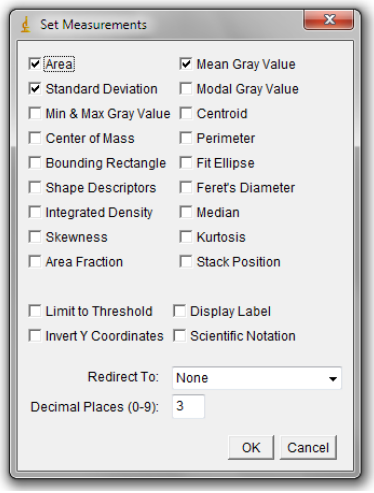


Figure 18: Set measurements window

Step 15: The combined *'ROI'* for the eye, pectoral and pelvic fins from the *ROI Manager* list were selected to be 'cleared' before further analysis could be done on the blotches area (go to *'Edit'* and select *'Clear'* from the list) (Figure 19).

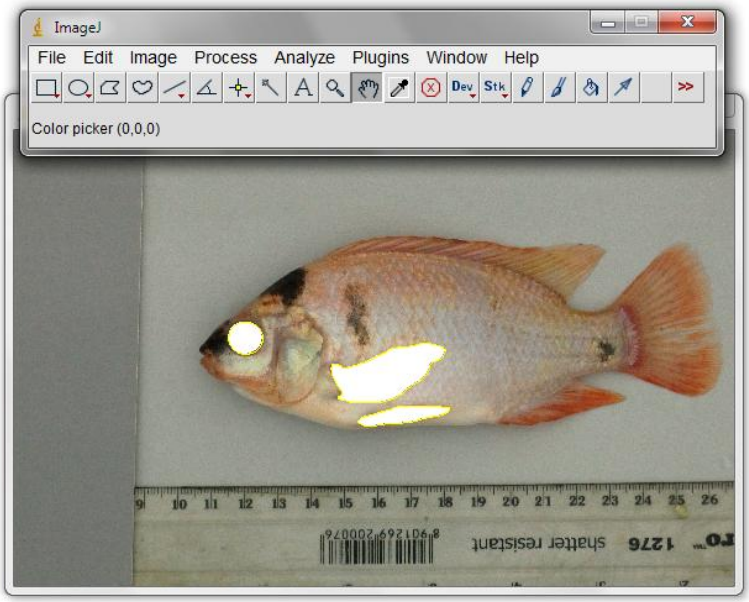
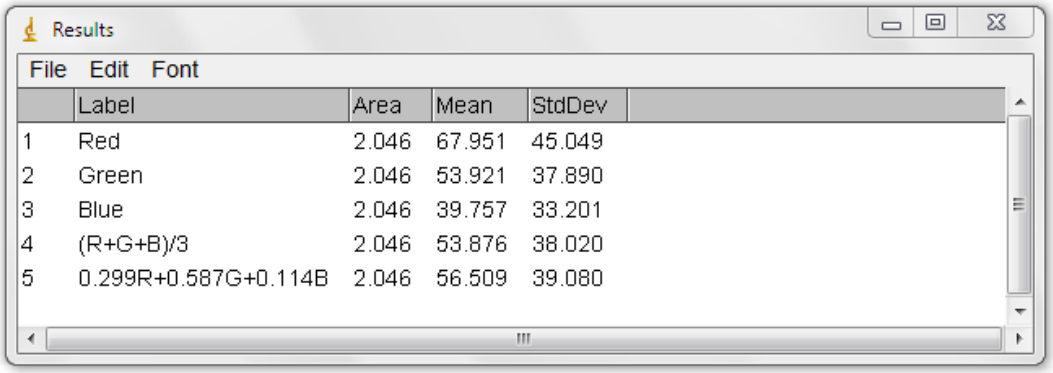


Figure 19: Clearing the ROI

Step 16: To get the measurements of the blotches, together with the colour components, the *'ROI'* for blotches was selected from the *ROI Manager*. From the *'Plugins'* on the menu bar, *'Analyze'* and then *'Measure RGB'* were selected. This gave the mean and standard deviation values for each RGB component and two intensity (brightness) measurements, one with just a normal average of RGB and another with a weighed equation (Figure 20). These results were copied and pasted into Microsoft Excel.



	Label	Area	Mean	StdDev
1	Red	2.046	67.951	45.049
2	Green	2.046	53.921	37.890
3	Blue	2.046	39.757	33.201
4	(R+G+B)/3	2.046	53.876	38.020
5	0.299R+0.587G+0.114B	2.046	56.509	39.080

Figure 20: Results table for RGB measurement

Step 17: Next, the region for the blotches was cleared before measuring the red background. Step 15 was repeated to clear the blotched regions (Figure 21).

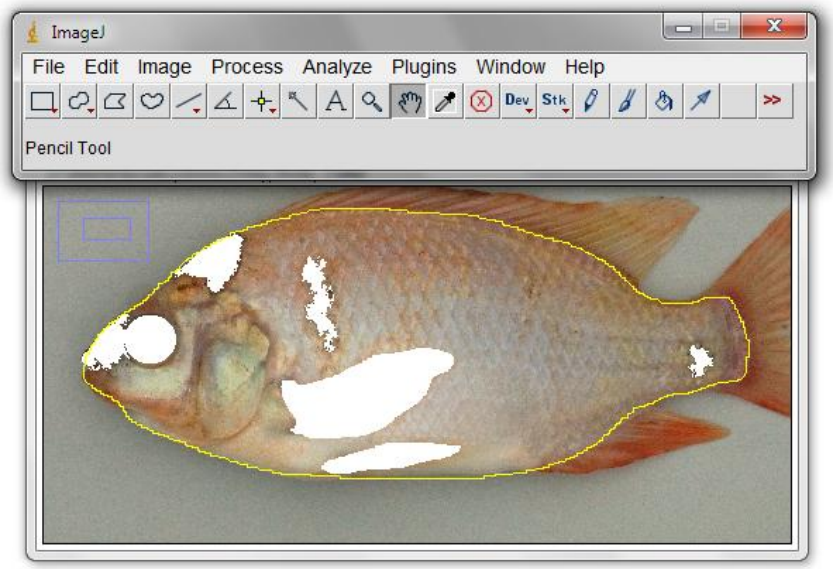


Figure 21: Clearing ROI of the blotches

Step 18: To exclude the 'cleared' area from the body colour measurement, the *ROI* for the body outline was selected. *'Edit'* and *'Clear Outside'* (Figure 22).

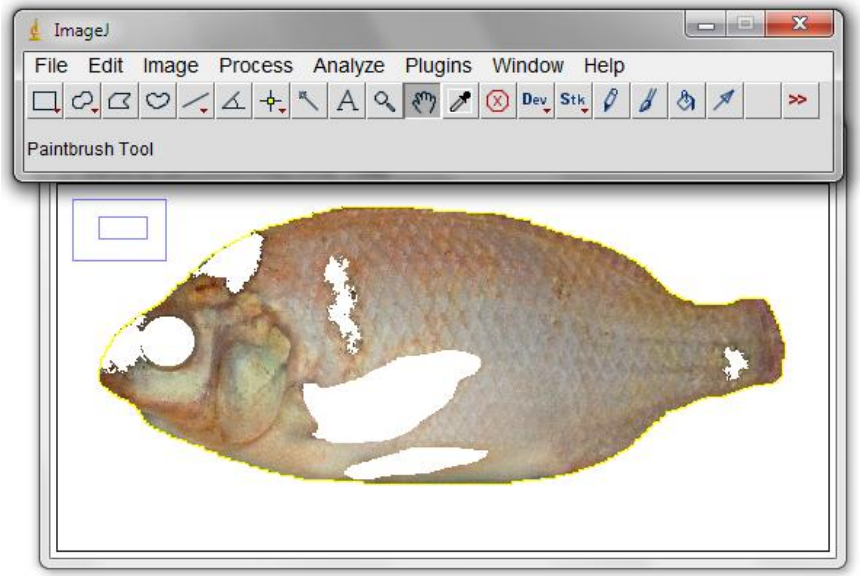


Figure 22: Clearing the outside region of the body perimeter

Step 19: Using the wand tool (refer to Step 11 and Step 12), the tolerance was increased to 15~20. Clicking within the body perimeter selected the body area and this was added to the *ROI Manager*. This step was repeated multiple times until the whole area of the body was selected but avoiding including the 'cleared' area within the selection (Figure 23). The technique was relatively similar in selecting the region for the blotches (refer to Steps 12 and 13). All the '*ROIs'* (refer to Steps 9 and 10) were combined and if necessary, the 'brush' tool (refer to Steps 7 and 8) was used to refine the selection. The combined area should cover the 'coloured' region only (Figure 24).

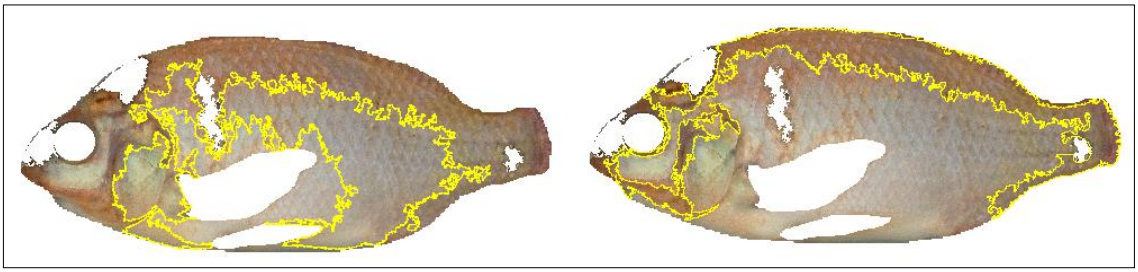


Figure 23: Selecting the area within the body outline

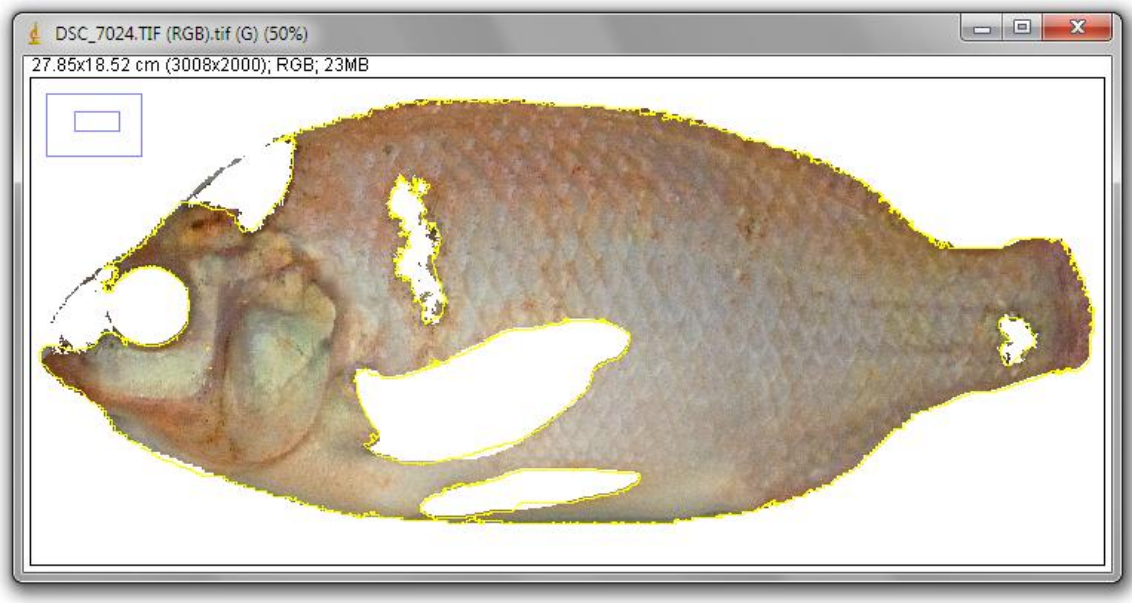


Figure 24: Combined ROI within the body area but excluding the 'cleared' region

Step 20: The combined *ROI* was saved and Step 16 was used to measure the area and the colour components (Figure 25).

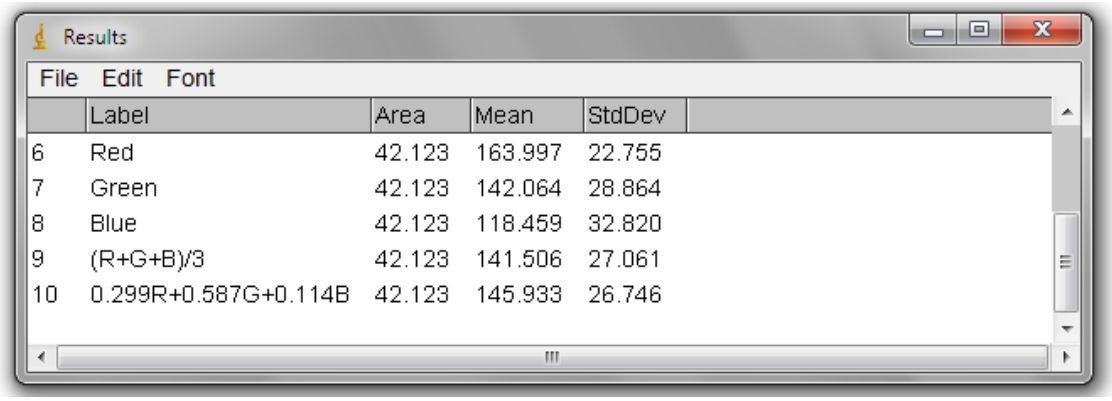


Figure 25: Result table for the body area and its colour components

The percentages of the body area occupied by the blotches was calculated by the equation:

$$Percentages\ of\ blotches = a / (a+b) \times 100$$

where a = area of blotches (refer to Figure 20); b = area within the body outline (refer to Figure 25).

Redness (for body colour), brightness and saturation (for both body colour and blotches) were calculated based on the RGB values (Red, Green, Blue) given in the results table. The formulae to calculate these parameters are given below;

$$Redness = R/(R+G+B)$$

$$Brightness = 0.299R+0.587G+0.114B$$

$$Saturation = Maximum\ (R,G,B) - Minimum\ (R,G,B)$$

(e.g. if R=250, G=180, B=200; Saturation = [Max-Min] = 250-180 = 70).

CHAPTER 3

Paper I: Preliminary analysis of blotching and body colour components in red Nile tilapia (*Oreochromis niloticus*): an ontogeny analysis

Status: To be submitted to a relevant peer-reviewed journal

Contributions:

The present manuscript was compiled and written in full by the author of this thesis. Fish rearing, sampling, lab and statistical analyses were carried out by the candidate. The other co-authors contributed towards the experimental design, statistical analyses, guidance and editing of the manuscript.

Preliminary analysis of blotching and body colour components in red Nile tilapia (*Oreochromis niloticus*): an ontogeny analysis

Amy H. Rajaee, Brendan J. McAndrew and David J. Penman*

Institute of Aquaculture, School of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland, United Kingdom

*Author to whom correspondence should be addressed. Tel.:+44(0)1786467901;

Fax: +44(0)1786 472133; email: d.j.penman@stir.ac.uk

Abstract

Red tilapia culture has become increasingly popular in some countries where there is a cultural preference for this body colouration. However, a common problem often associated with red tilapia is the appearance of varying proportions of blotched phenotypes (mainly black but also red blotching) which can make such fish less valuable than the pure red individuals. Knowledge of the mechanism and inheritance of this trait is still poorly understood. This study investigated the ontogeny of blotching and body colour components using image analysis. Two trials were set up – Trial 1 looked at the level of blotching within a fully inbred clonal line using two different tank background colours (dark green and light grey) and Trial 2 used outbred crosses to find any association of blotching with fish sex. Image analysis of fish was carried out every four weeks up to six times from the age of 3 to 8 months old. Within the clonal line, the level of blotching reached up to 3% of the body area and 65% of clonal fish used in the study showed some blotching. Ontogeny of the blotches and body colour components showed quite a different pattern between the clonal line and the outbred fish. The level of blotching and its colour components were not affected by tank background colour and did not differ between the sexes. Other body colour components, however, varied significantly between males and females, especially for the Green (G) and Blue (B) components over the six month period, and for the Red (R) component for age 5 months old for the tank background colours. These trials served as a preliminary study before further analysis on the inheritance of the blotched trait.

Keywords: red tilapia, blotches, image analysis, ontogeny, clonal line

1. Introduction

Body colouration is one of the traits of interest in many aquaculture species as this can affect market value. In many aquaculture species, attempts to improve body colour are carried out by manipulating feed (Gouveia *et al.*, 2003; Chatzifotis *et al*., 2011) and control the environment such as light and tank background (Han *et al*., 2005; Doolan *et al*., 2007; Pavlidis *et al*., 2008).

The red body colour in the Nile tilapia (*Oreochromis niloticus*) is a mutant colour controlled by a single dominant allele (McAndrew *et al*., 1988). Red tilapia manage to achieve higher prices in certain markets due to their appearance resembling some marine species such as the red snapper (Romana-Eguia and Eguia, 1999). However, the red colour is often associated with spots or blotches (mainly black but also red) which makes them less attractive and affects their market prices. In *O. niloticus*, these blotches are hypothesized to be mainly associated with the heterozygous red fish which carry the Rr genotype but the genetics underlying this trait are still not fully understood (McAndrew *et al.*, 1988). For red tilapia, attempts to improve body colour have been carried out through mass selection (Mather *et al.*, 2001; Garduno Lugo *et al.*, 2004) but this does not explain the mode of inheritance for the blotched phenotype.

In order to measure heritability of this trait, environmental factors that may have influence on the trait first need to be studied. This study was carried out to investigate: (a) the level of blotching within a fully inbred all-female red clonal line using different tank background colours; (b) association of the blotching with the sex of fish; and (c) the ontogeny of the blotches as well as the colour components of the red body

colouration. Trials were divided into two categories; firstly using a fully inbred clonal line fish and secondly, using mixed sex red *O. niloticus*. The results from this study were expected to provide preliminary information before a further study on the inheritance of blotches could be set up.

2. Materials and methods

2.1 Facilities and experimental set up

All trials were carried out in the tropical aquarium facility of the Institute of Aquaculture, University of Stirling. Fish used for all trials were produced by in vitro fertilization and fertilized eggs were incubated up to first-feeding stage in down-welling incubators before being transferred to experimental tanks. All trials were conducted using circular plastic (food grade) tanks with lids (radius = 19cm; height = 30cm; volume = 23 litre; mean flow rate = 2.14 litre/min) in a recirculating system at 27^0C. Fish were fed twice a day with trout pellets (Skretting, UK).

Females from a fully inbred clonal red line (isogenic line) were used in Trial 1. This line was produced by one generation of mitotic gynogenesis followed by meiotic gynogenesis from a mitotic gynogenetic female and hormonal masculinisation of some of the meiotic gynogenetic progeny. The production of gynogenetic was achieved by fertilising eggs with UV irradiated milt (no viable paternal DNA) and suppression of the first cleavage or second meiotic division by late pressure or heat shock. The full description on producing the mitotic gynogens and the production of inbred clones was described by Hussain *et al.* (1991) and Sarder *et al*. (1999).

2.2 Experimental design

2.2.1 Trial 1: Effect of tank background colour on blotching

This trial was carried out using fully inbred clonal line fish, reared in two different tank background colours (light vs dark). The tanks, originally opaque white in colour were painted using spray paint and were tested for any effect of fading or wear off before used for experiments. The tanks were painted dark green (for dark background treatment) and light grey (for light background treatment). These colours were chosen based on the range of normal colours of manufactured tanks used in most hatchery and fish facilities. Three replicates were used for each tank colour with 29 fish per tank (total 6 tanks).

For the ontogeny analysis, ten fish were randomly selected from each tank and were PIT-tagged for identification and fin biopsied for DNA verification for clonal status. Measurements of standard length and weight were recorded every four weeks. Digital images of the fish were also taken after the measurements for image analysis. This ontogeny analysis was carried out over a period of six months (July to December).

2.2.2 Trial 2: Effect of sex on blotching

This trial was carried out using mixed-sex homozygous red Nile tilapia. Five initial crosses from different pairs of broodstock were produced and sacrificed at three months old to check for the ratio of male:female. Using parents which gave variation in blotching within the batches and an approximately 1:1 ratio of male and female, a second set of crosses (n=3 families; total 3 tanks; one tank per cross) were produced. Fish were reared in opaque white colour tanks with each tank consisted of 40 randomly selected fish.

At three months old, twenty fish from each tank were randomly selected and PIT-tagged. Digital images and measurements of standard length and weight were taken every four weeks over a period of six months (August to January). Fish were sexed at the end of the trial and recorded. A total 60 fish (20 per each tank) were used for the trial.

2.3 Verification of the clonal line

DNA from the clonal line was extracted using REALPURE DNA extraction kit (REAL laboratories, Spain) using fin tissues (approximately 0.3cm^2 per sample) digested in 3µl of proteinase K (10mg/ml) in the presence of 75 µl cell lysis solution and incubated overnight at 55^{o}C. Subsequently, 3 µl of DNA-free RNAase (2mg/ml) was added to each sample which was incubated for one hour at 37^{o}C. Samples were then brought to room temperature and 45 µl of protein precipitate solution was added to each tube. All samples were agitated by vortexing prior to centrifuging at 3570xg for 20 minutes at 4^{o}C. 50 µl of the supernatant was then transferred into a new eppendorf tube containing 75 µl isopropanol. The DNA pellets were precipitated by centrifuging at 3570xg for 10 minutes at 4^{o}C. The solution was then poured off and the tube was washed by adding 150 µl of 70% ethanol and further centrifuged at 3570xg for 10 minutes at 4^{o}C. The solution was once again poured off and the tubes were air-dried for about 40 minutes to make sure the ethanol was completely evaporated. The DNA pellets were resuspended in 40 µl of TE buffer (1 mM Tris, 0.01 mM EDTA, pH 8.0) and left overnight in room temperature to dissolve before used. DNA concentration was quantified using a spectrophotometer (Nanodrop, ND-1000) and diluted to 60~100 ng/µl with TE buffer for PCR amplifications.

PCR was carried out using three microsatellite markers selected from the tilapia linkage map (Lee *et al.*, 2005). These markers, UNH985, UNH104 and GM258 were previously used with unrelated samples and showed high level of polymorphism. PCR conditions were as described in Rajaee *et al.* (2010) and sizing of PCR products was accomplished using a CEQ 8800 Genetic Analysis System automated DNA sequencer (Beckman Coulter). Allele sizes were analysed using CEQ8800 fragment analysis software.

2.4 Image analysis

Fish was first anaesthetized with 10% benzocaine at 1ml per litre. Digital images of each fish were taken using a DSLR (digital single-lens reflex) camera (Nikon D40). The camera was set to manual mode with a focal length of 35mm, shutter speed of 1/25 and an aperture of F5.6. The camera was held by a tripod with a custom made extension so that the camera was able to take photos vertically. Fish that had been anaesthetized were placed individually on a gray tray within a translucent white tent. A ruler and an 18% gray card (as a standard reference for white balance) were included in every image. An image of each side of fish was taken. Images were stored in a 3008 x 2000 pixel format on a 'high' quality setting (RAW compression of 12-bit). The white balance of each image was standardized according to the 18% gray card using Nikon software, ViewNX™ version 1.5.2 before the images were converted to tiff format. Images were then analysed using ImageJ software (version 1.43; http://rsb.info.nih.gov/ij; with plugin for RGB-measure) for measurements of body colour and area covered by black blotches. Images were first transformed from '16-bit' to 'RGB Color' using this software to measure colour intensity. The scale was set in

the first image by drawing a straight line on the ruler and set as 'global scale' for subsequent images. The area covered by black blotches was selected using the wand tool (8-connected mode, tolerance of 10.0). Body colour intensity was measured after the area of eye, fins and black blotches was cropped.

2.5 Analysis of blotching and colour components

The black blotched area was calculated as a percentage using the ratio of area covered by blotches over total body area (excluding fins and eyes). One limitation to the quantification of the blotched area was the difficulty to measure accurately on the area close to the dorsal or ventral lines of the fish due to the digital images being only two-dimensional (left and right sides). Blotches on these areas appeared darker due to the angle of the blotched area relative to the camera. Colour components for the blotches as well as the body colour were measured using R (red), G (green) and B (blue) values. For both the blotches and body colour components, brightness and saturation were calculated from the RGB values using transformation equations to the HSV colour space (hue, saturation and value). Saturation was calculated by deducting the minimum value within the RGB components from the maximum value of the components (e.g. if R=250, G=180, B=200; Saturation = [max-min] = 250-180 = 70). Brightness was calculated with the equation, *Brightness (value) = 0.299R+0.587G+0.114B*, meanwhile redness of body colour was measured using the equation *Redness = R/(R+G+B).* For body colour, the redness component is usually positively correlated with the saturation component. Interpretation of the RGB components towards redness, brightness and saturation elements of body colour is illustrated in Figure 1.

Low redness

Redness = 0.353
Brightness =186.203
Saturation=20.783
R=195.842
G=183.458
B=175.059

High Redness

Redness = 0.408
Brightness = 149.676
Saturation = 57.061
R=179.917
G=139.481
B=122.856

Low brightness

Redness = 0.374
Brightness = 160.124
Saturation = 34.914
R=177.773
G=154.487
B=142.89

High Brightness

Redness = 0.345
Brightness =192.968
Saturation = 14.104
R= 198.236
G= 192.000
B= 184.132

Low saturation

Redness = 0.346
Brightness = 186.494
Saturation = 15.647
R=192.003
G=185.658
B=176.356

High saturation

Redness = 0.387
Brightness = 175.388
Saturation = 51.697
R=200.359
G=167.858
B=148.662

Figure 1: Examples for contribution of RGB components towards redness, brightness and saturation component of the body colour.

2.6 Statistical analysis

Statistical analysis was carried out using PASW Statistics (SPSS) v.18 and data sets were initially checked for a normal distribution using the one-sample Kolmogorov-Smirnov test, as well as for homogeneity of variances using Levene's test. Data for standard length, weight, percentages of blotches and each of the colour components were compared between groups for each sampling point (age) using a nested ANOVA for Trial 1 and a two-way ANOVA for Trial 2. A three-way repeated measures ANOVA manipulated by a General Linear Model (GLM) was used to test the effect of tank colour, age and replicates (for Trial 1) and age, family and sex (for Trial 2). Association of parameters of the colour components were tested using Pearson product-moment correlation.

3. Results

3.1 Trial 1:

Clonal fish used were genotyped using three microsatellite markers which showed no ambiguity about their status. All markers were homozygous and all individuals showed the same allele for each locus. The level of blotching within this clonal line reached up to 3% of the whole body area and 39 out of 60 clonal fish (65%) had some blotching on their body. The mean weight and length between dark and light tanks were not significantly different at sampling 1 (age 3 months old) as well as at every other sampling points (Table 1).

Table 1: Comparison of standard length and weight between dark and light tanks in Trial 1 (nested ANOVA; n refers to number of replicates).

Trial 1	Dark tanks (n=3)	Light tanks (n=3)
Standard Length (mm)	Mean ± SD	Mean ± SD
3 months old	109 ± 8.1	119 ± 8.2
4 months old	113 ± 9.3	123 ± 9.8
5 months old	116 ± 11.1	127 ± 11.4
6 months old	120 ± 11.6	131 ± 12.7
7 months old	124 ± 11.9	135 ± 14.5
8 months old	131 ± 11.0	139 ± 14.1
Body Weight (g)		
3 months old	47.5 ± 12.4	65.1 ± 13.5
4 months old	50.4 ± 13.2	68.1 ± 17.9
5 months old	55.8 ± 16.7	75.6 ± 23.4
6 months old	60.3 ± 17.8	81.3 ± 25.7
7 months old	68.0 ± 19.7	90.5 ± 30.6
8 months old	80.2 ± 18.9	99.5 ± 30.2

3.1.1 Effect of tank background colour on blotching

Figures 2 and 3 show ontogenic changes of the blotches and body colour components respectively. No significant difference was observed in the level of blotching between the fish from different tank background colours and none of the colour components of the blotches differed significantly between the two groups at any of the sampling points. For body colour components, the R and G components were significantly different at the age of 5 and 8 months old meanwhile the B component was significantly different at 8 months old. The brightness of body colour was also significantly higher in the dark tanks at 5 and 8 months old but no differences were observed for the saturation component. A significantly higher value of redness was observed in the light tanks at 8 months old.

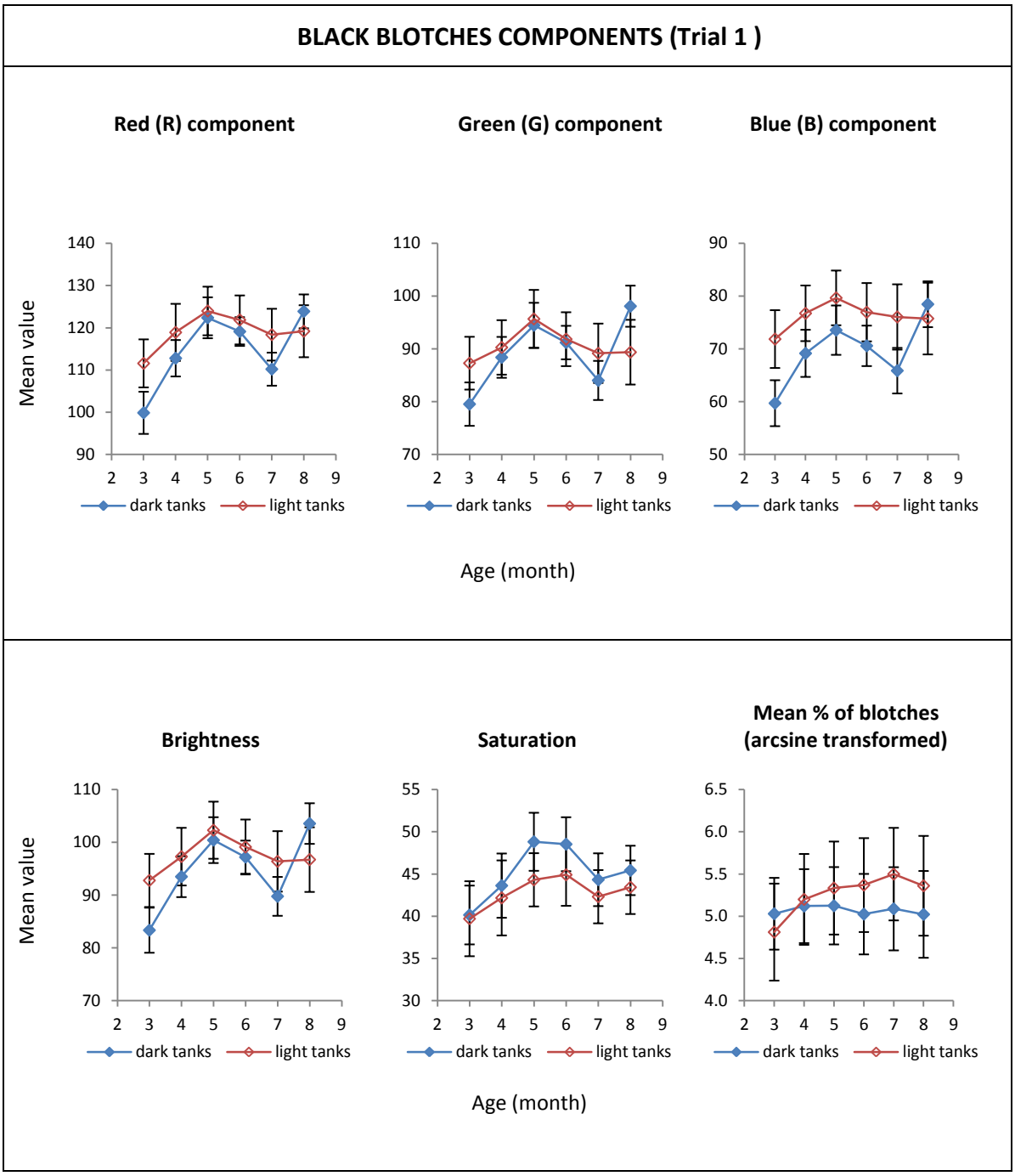


Figure 2: Colour components of the black blotches and mean area (%) of black blotches in the Nile tilapia fully inbred clonal line between light and dark tanks tested with nested ANOVA (Trial 1). Values are expressed as mean±SE (Light tanks, n=3; Dark tanks, n=3; n refers to number of replicates). No significant differences were observed at any age between light and dark tanks.

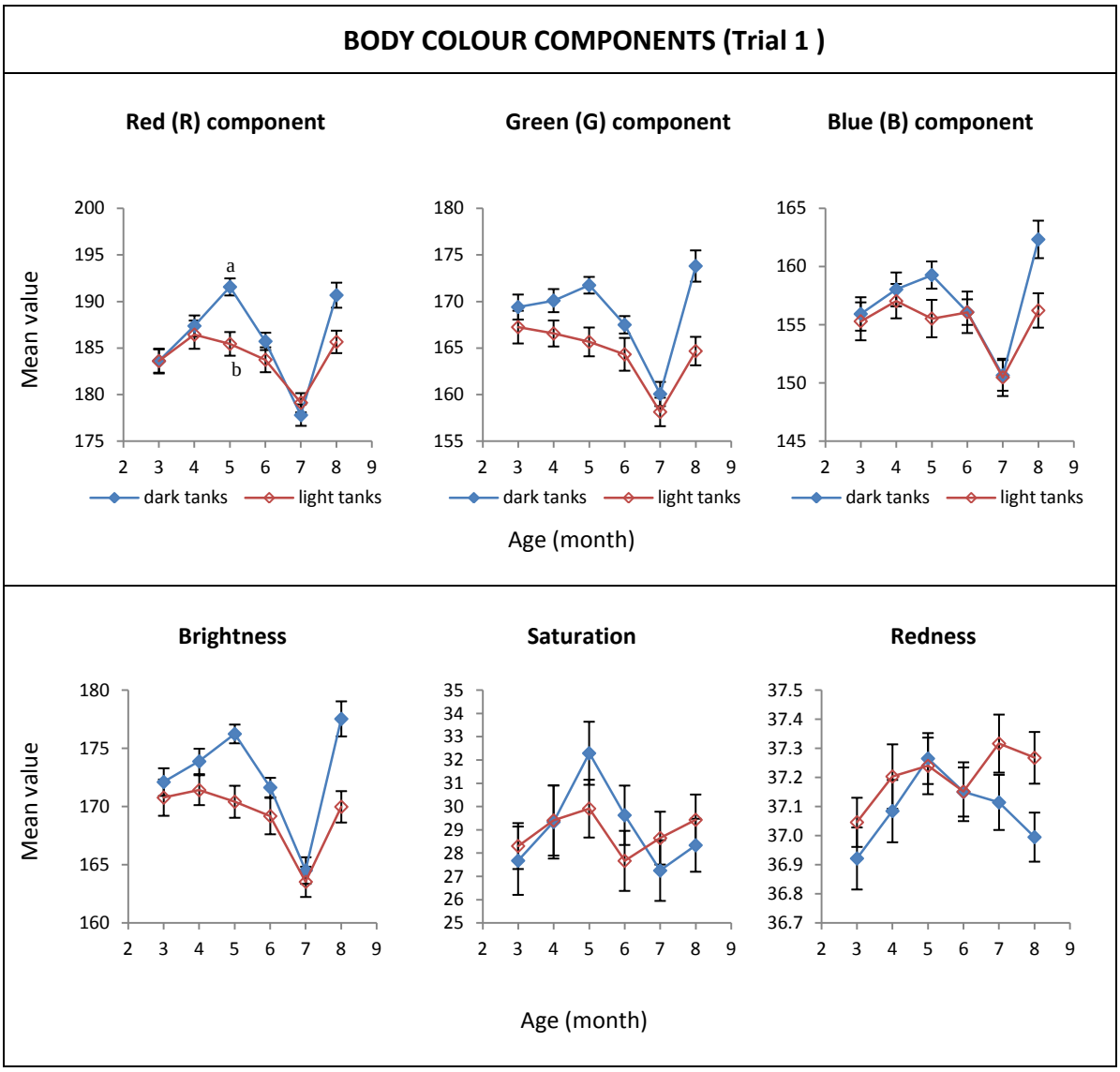


Figure 3: Body colour components in the Nile tilapia fully inbred clonal line between light and dark tanks tested with nested ANOVA (Trial 1). Values are expressed as mean±SE (Light tanks, n=3; Dark tanks, n=3; n refers to number of replicates). Different lower case letters indicated significant differences between tank colour within the sampling point (age), in which P<0.05.

Three-way repeated measures ANOVA showed that tank background colour was not a significant factor for all parameters measured.. Replicates (which was nested under tank background colour) showed a significant effect for all parameters measured (P<0.05) except for percentages of blotches. On the other hand, for the black blotches components, age was not a significant factor for the percentages of blotches, Blue and

Saturation components as well as for the Saturation and Redness components of the body colour but was significant for all other parameters ($P<0.05$). Interaction between age and tank colour was significant for Red, Green and Brightness components of the black blotches ($P<0.05$) and was not significant for the rest of the parameters measured. Correlations between colour components of the blotches and body colour were all significant and were positively correlated (Table 2).

Table 2: Correlations between colour components between light and dark tanks in Trial 1

Body colour components

		Light Tanks (n=179)	**Dark Tanks (n=180)**
Variable 1	Variable 2	Pearson Correlation, r	Pearson Correlation, r
Red	Green	0.734*	0.735*
Red	Blue	0.683*	0.547*
Green	Blue	0.927*	0.908*

Blotches colour components

		Light Tanks (n=102)	**Dark Tanks (n=132)**
Variable 1	Variable 2	Pearson Correlation, r	Pearson Correlation, r
Red	Green	0.903*	0.894*
Red	Blue	0.806*	0.726*
Green	Blue	0.961*	0.924*

*P<0.001

3.2 Trial 2

For Trial 2, the level of blotching reached up to 8.5% of the whole body area with 30 out of 60 experimental fish (50%) having blotching on their body. The weight and length between male and female fish differed significantly during sampling 1 (age 3 months old) and at every other sampling points (age 4-8 months old) during the trials (Table 3). This significant difference was not surprising as it is well known in tilapia

culture that male fish are usually larger than females (e.g. Hussain, 2004). Family (represented by tank) is however not a significant factor for both weight and length.

Table 3: Comparison of standard length and weight between male and female fish in Trial 2 (2-way ANOVA). Values are expressed as mean±SD.

Trial 2	**Tank 1 (n-20)**		**Tank 2 (n=20)**		**Tank 3 (n=20)**	
Mean Standard Length (mm)	Female	Male	Female	Male	Female	Male
3 months old	93±6.2	102±10.3	101±9.9	106±9.9	95±10.8	106±7.5
4 months old	99±10.6	111±13.9	108±10.4	113±8.0	99±10.9	115±11.1
5 months old	107±11.3	120±10.1	114±9.2	120±7.8	105±11.7	124±11.3
6 months old	114±9,5	130±8.8	119±8.8	125±8.1	111±13.3	132±11.7
7 months old	122±9.6	141±8.8	125±8.9	132±7.8	117±8.0	141±12.1
8 months old	127±9.5	147±8.4	129±7.0	135±9.0	123±14.0	149±11.4
Mean Body Weight (g)						
3 months old	31.5±8.5	36.7±10.7	40.6±10.5	42.6±10.3	29.2±2.5	39.6±9.2
4 months old	37.9±13.5	48.4±15.5	47,7±10.2	51.1±12.4	33.1±9.5	51.4±13.5
5 months old	47.4±15.0	61.1±13.7	53.8±8.9	58.4±12.5	41.2±13.2	65.9±15.3
6 months old	61.1±17.1	81.4±18.1	65.8±11.3	69.8±13.6	50.9±17.4	82.9±19.5
7 months old	70.8±17.7	96.9±20.4	74.3±14.2	77.2±13.5	59.4±19.3	98.1±21.7
8 months old	76.9±16.7	113.4±21.7	79.6±12.3	84.9±17.5	66.3±21.4	111.2±22.2

3.2.1 Effect of sex on blotching

The level of blotching in males and females was not significantly different and there was also no significant difference for any of the colour components of the blotches (Figure 4). For body colour, the Red component was only significantly different at 4 months old (Female>Male) meanwhile the Green, Blue and Brightness components were significantly different between males and females for all sampling points throughout the trial (Female>Male) (Figure 4). The Saturation component was only significantly different at 6 and 8 months old (Male>Female) while Redness was

significantly higher in the male fish for all sampling points except for at the age of 4 and 5 months.

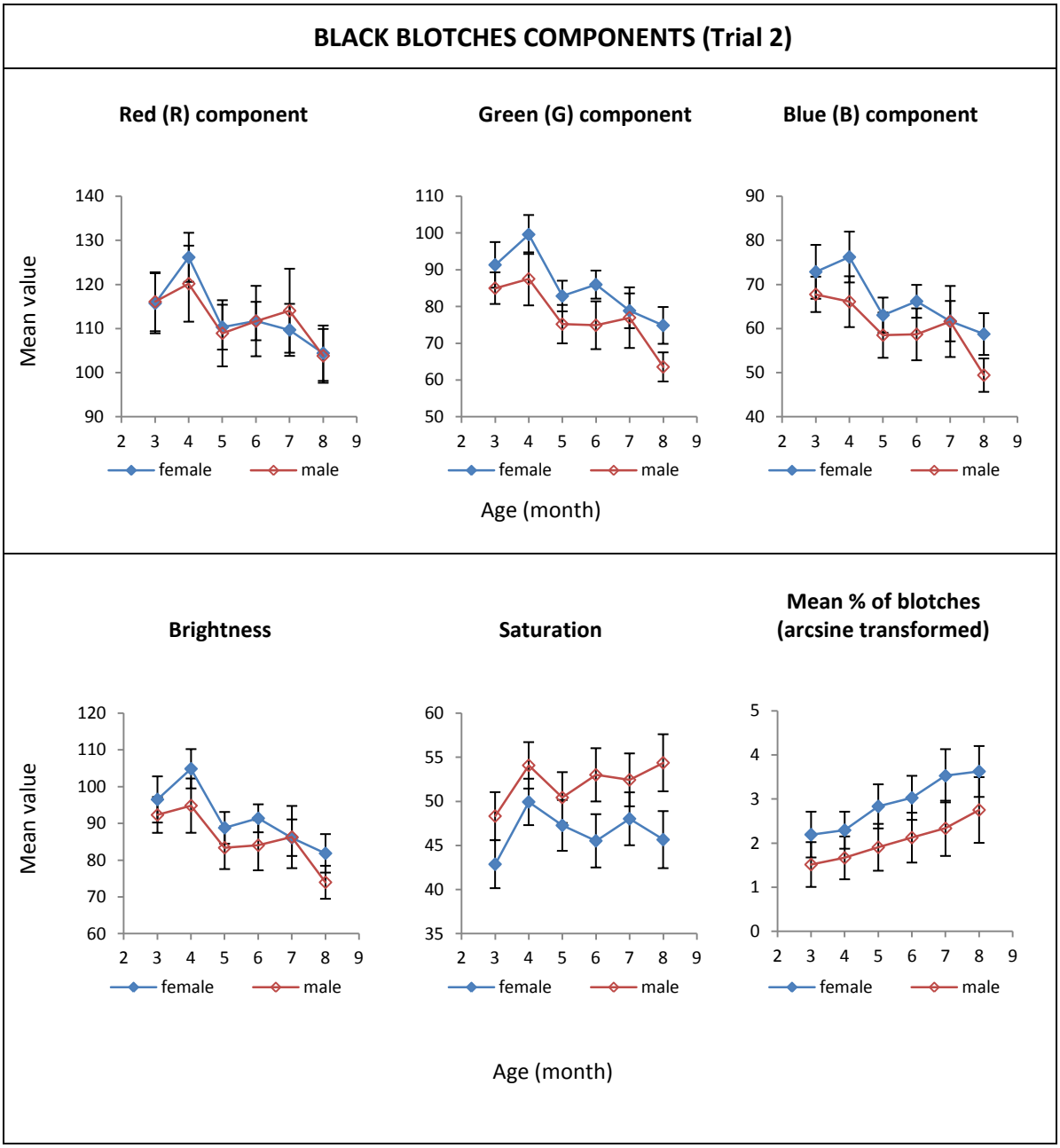


Figure 4: Colour components of the black blotches and mean area (%) of black blotches between male and female fish tested with 2-way ANOVA (Trial 2). Values are expressed as mean±SE (Females, n=18; Males, n=12; n refers to number of fish with blotches used in the analysis). No significant differences were observed at any age between males and females.

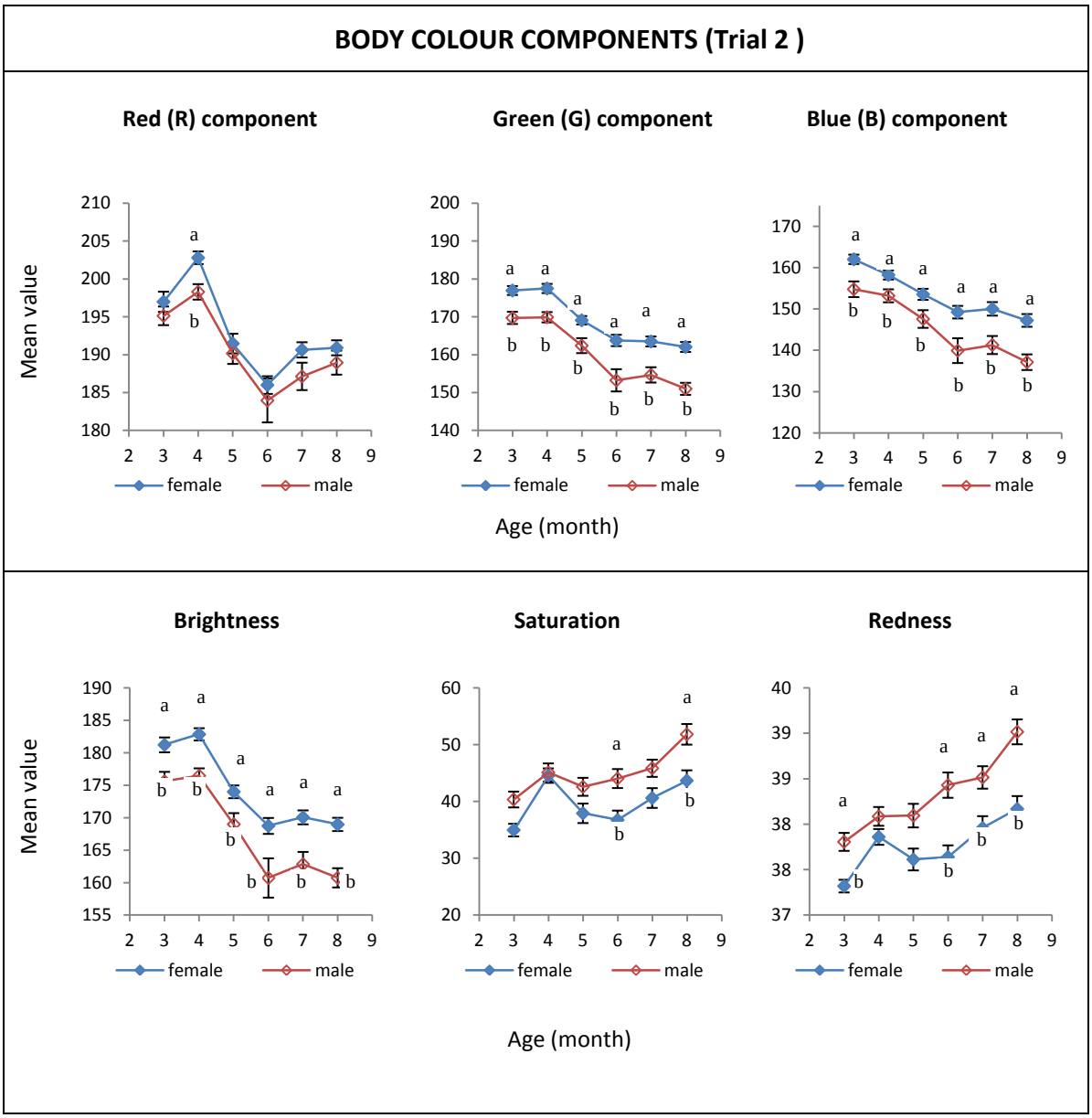


Figure 5: Body colour components of male and female fish tested with 2-way ANOVA (Trial 2). Values are expressed as mean±SE (Female, n=30; Male, n=30; n refers to total number of female and male fish from 3 different crosses). Different lower case letters indicate significant differences between male and female within the sampling point (age) in which $P<0.05$.

A three-way repeated measured ANOVA showed that age was a significant factor for all parameters measured. For the black blotches components, the sex of the fish was not a significant factor for any of the colour components or the level of blotching and there was no interaction between age and sex. For body colour components, sex of the fish was significant for all the colour components but there were

no significant interactions between age and sex of the fish for all the components. For weight and length parameters, fish sex was a significant factor and there was a significant interaction between age and fish sex for both length and weight parameters. On the other hand, family was a significant factor for all components measured except for the Green and Brightness components of the body colour parameters, although no significant interactions were detected between age and family. Correlations between colour components of the blotches and body colour were all significant and were positively correlated (Table 4).

Table 4: Correlations between colour components between females and males in Trial 2

Body colour components

		Females (n=180)	**Males (n=180)**
Variable 1	Variable 2	Pearson Correlation, r	Pearson Correlation, r
Red	Green	0.633*	0.849*
Red	Blue	0.422*	0.749*
Green	Blue	0.904*	0.939*

Blotches colour components

		Females (n=108)	**Males (n=72)**
Variable 1	Variable 2	Pearson Correlation, r	Pearson Correlation, r
Red	Green	0.849*	0.94*
Red	Blue	0.749*	0.894*
Green	Blue	0.939*	0.973*

*P<0.001

3.3 Ontogeny of blotching and colour components

The area covered by blotches did not significantly change over the period of six months in both Trial 1 and 2. On a further observation on the ontogeny of the blotches, the saturation of the blotches in some fish changed or disturbed at certain stages (Figure

6). The saturation of the black blotches in some fish can be extremely reduced at certain stages (age) but returned to its original state or became more saturated afterwards. This occurrence was however not associated with any treatment or the sex of fish but only observed in several fishes.

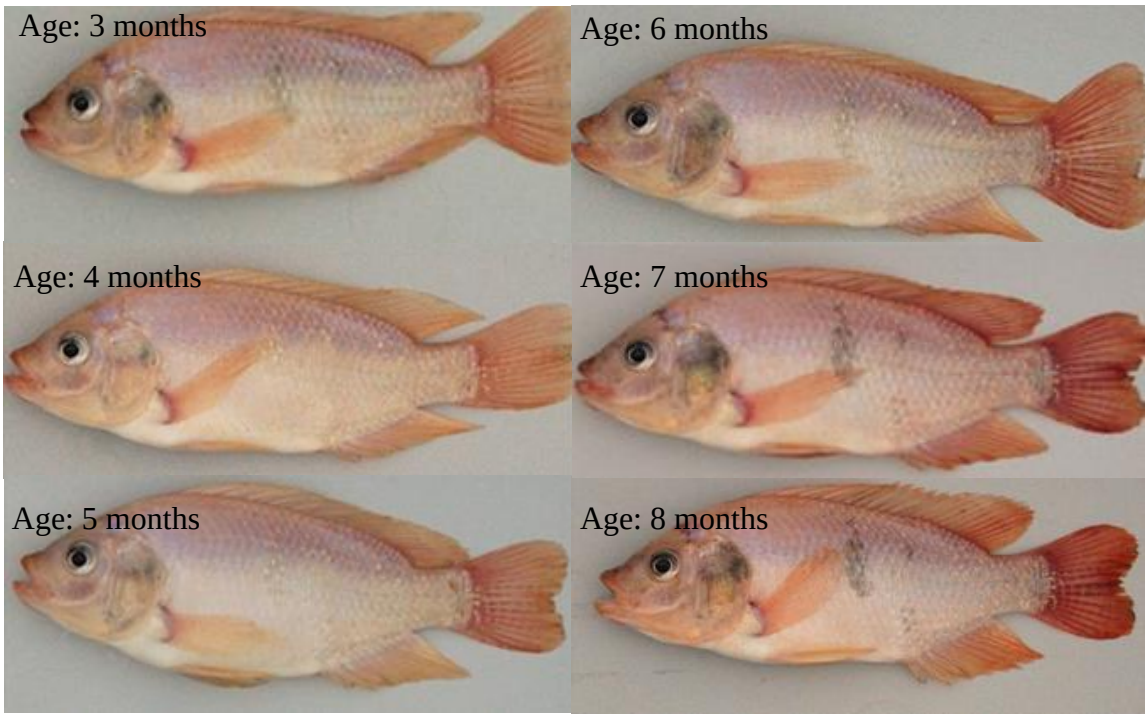


Figure 6: Ontogeny of black blotches for the same fish from age 3 months to 8 months old. The black blotches became less vivid starting at the age of 4 months but became intense again at the age of 6 months.

4. Discussion

The colour components of the black blotches showed different patterns between Trials 1 and 2. Different patterns were also observed for the body colour components and none of these patterns fit into a simple model. In Trial 1, the body colour components reduced from the age of 5 months old to 6 months (except for the Blue component of light tanks) but increased again from 7 to 8 months old (except for Redness component). For Trial 2, the RGB components of body colour reduced from

the age of 4 months until 6 months old (except for the B components which reduced starting from the age of 3 months) and increased slightly afterwards. When compared together, in both trials, the colour components reduced in the month of November (since Trial 2 was started a month later than Trial 1). However, we did not manage to associate this reduction to any factor such as changes in pellet size or tank position and the fish were reared in a closed system with constant photoperiod and temperature. Correlations between all colour components of the blotches and the body colour were significant, suggesting that these components are closely related to each other. All the pairwise combinations of the colour components between tank colours and between males and females showed positive relationships.

In this present study, the results suggested that level of blotching and the colour components of the blotches are not affected by the tank background colour used in this study and did not differ significantly between males and females. Colour components of the body colouration however did differ between males and females suggesting they should be analysed separately in further studies on mixed sex red tilapia. Tank background colour may affect the body colour components but did not affect the redness value (significant difference only found at the age of 8 months old when all RGB components were significantly different). Age was however strongly associated with the colour parameters and since this did not fit a simple model, predictions are difficult to make. It is suggested that for further study, image analysis sampling needs to be carry out at least at two sampling points to avoid bias in the data sets. The trials in this study were carried out to provide information and guidance for a further study on the inheritance of blotching in the red Nile tilapia. It was anticipated that results from this study would allow assessment of the effects of factors such as tank background

colour (Trial 1) and sex (Trial 2). For Trial 1, it is essential to look at the effect of tank background colour since for the future main experiment, a set of parents which may have been reared in various tank background colours will be used to produce offspring, and thus this may affect offspring-parent correlations. The specific tank colour used in this experiment was chose since these broodstock (for the future experiment) was reared in tanks consist of these two colours, hence justifying the choice of colours used in this experiment. The trials also looked at the ontogeny of the blotches within a clonal line and within outbred crosses. A fully inbred clonal line was used in Trial 1 with the assumption that the genetic variance between the individuals is zero within the line. Therefore all variance observed between them is due to environmental factors (Koedprang *et al.*, 2000).

The influence of environmental variance is difficult to separate from genetically-based variance but this has been made possible by the production of isogenic (genetically identical) fish. The use of clonal fish in studies on estimation of phenotypic variance are not only applied by direct study of the clones but also by crossing the clones with outbred individuals. Apart from tilapia, clonal fish have also been produced in other aquaculture species such as the common carp, *Cyprinus carpio* (Bongers *et al*., 1998), rainbow trout, *Oncorhynchus mykiss* (Young *et al*., 1995) and red sea bream, *Pagrus major* (Kato *et al*., 2002). The production of clones in fishes has been extensively reviewed by Komen and Thorgaard (2007).

The most comprehensive study on ontogeny of pigment pattern in fish apart from the zebra fish has mainly been carried out in the flatfishes (see review by Bolker and Hill, 2000) although data presented were mostly at the cellular level. Pigment development in the flatfishes changes at two phases, the first during larval development

and second at metamorphosis. At the larval stages, melanophores consist in large dendritic cells while at metamorphosis stages, a smaller type of melanophores developed on the ocular side (Bolker *et al*., 2005). Pigmentation development are very much related to environmental aspects, since malpigmentation such as albinism only usually occurred under commercial hatchery conditions but is quite rare in the wild. Some of the environmental factors that has been studied includes diets and light intensity (Venizelos and Benetti, 1999) as well as bottom substrates (Ottesen and Strand, 1996). Flatfish are known to have the ability to alter their colouration in order to blend with their background, however, there are only a small number of studies reported on long term morphological changes (a slower process of colour changing).

Morphological background adaptation may result in degeneration of melanophores on a light background or the formation of new melanophores on a dark background (Sugimoto, 1993, 2000, 2002). In a study by Van der Salm *et al*. (2005), Mozambique tilapia (*Oreochromis mossambicus)* from a black background were consistently darker than fish reared in lighter background. Fish from the black background also had higher plasma cortisol levels, indicating that the fish were more stressed compared to fish from the lighter background. Association of dark tank colour with higher level of plasma cortisol has also been reported for rainbow trout, *Oncorhynchus mykiss* (Green *et al*., 1991), the red porgy (*Pagrus pagrus),* (Rotllant *et al.,* 2003) and Australian snapper *(Pagrus auratus)* (Doolan *et al.*, 2008). On the other hand, a study by Kittilsen *et al*. (2009) revealed that melanin-based spots in salmon are a stress-response indicator. The authors reported the non-spotted fish had significantly higher post-stress cortisol levels than spotted fish, revealing that the spotted phenotype is a way to identify stress-sensitive and stress-resistant individuals. Some other studies

on melanin-based colouration, although more focused on behavioural and sexual selection, include the Eastern mosquitofish, *Gambusia holbrooki* (Bisazza and Pilastro, 2000) and some Poecilids species (Tobler *et al*., 2006). Association of melanin-based colouration with behavioural syndromes in vertebrates was recently reviewed by Ducrest *et al*. (2008).

Interpretation of colour changes in fish is a complicated subject especially on given the difficulties to express colour differences in a way that correlates with human vision. To our knowledge, the present study was the first to report on ontogenic changes on blotching and skin colour parameters in red tilapia. Therefore, further studies are needed to incorporate and validate the results presented here.

Acknowledgement

This study was carried out as part of the PhD study of the first author, funded by the Ministry of Higher Education Malaysia and University Putra Malaysia. The authors would like to thank Keith Ranson and William Hamilton for helping with the fish welfare, and to Denny Conway and Brian Howie for assistance with the photography.

CHAPTER 4

Paper II: Analyses of heritability of blotching and other body colour components in red Nile tilapia (*Oreochromis niloticus* L.)

Status: To be submitted to a relevant peer reviewed journal

Contributions:

The present manuscript was compiled and written in full by the author of this thesis. Fish rearing, sampling, lab and statistical analyses were carried out by the candidate. The other co-authors contributed towards the experimental design, statistical analyses, guidance and editing of the manuscript.

Analyses of heritability of blotching and other body colour components in red Nile tilapia (*Oreochromis niloticus* L.)

Amy H. Rajaee, Brendan J. McAndrew, David J. Penman*

Institute of Aquaculture, School of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland, United Kingdom

*Author to whom correspondence should be addressed. Tel.:+44(0)1786467901; Fax: +44(0)1786 472133; email: d.j.penman@stir.ac.uk

Abstract

The external colouration of fish is an important commercial trait which influences consumer buying decisions, thereby determining the economic value of the fish. The emergence of the red variants of tilapia has strongly boosted interests of consumers towards this species in many countries. However, the red colouration is often tainted with blotching, which considerably affect their appearances and the market price. Genetic control of this trait is still poorly understood, leading to difficulty to overcome this problem. This study was to investigate the heritability of blotching and other colour components of the body colouration in the red Nile tilapia and their correlations. A series of red males with variation in blotching were crossed to females from an isogenic clonal line and associations of the sire-offspring regression for the level of blotching and colour components were studied. The sire-offspring regressions were individually plotted using data at ages 42 weeks and 46 weeks and data on overlapped sampling period. Heritabilities for the area of black blotches and the colour components of the black blotches were not significantly different from zero ($P>0.05$). Brightness component of the body colour showed negative correlation during sampling 2 (age 46 weeks) for both female and male offspring (Female: $r = -0.555$, $P=0.026$; Male: $r = -0.636$, $P=0.011$) but estimation for other body colour components were not significantly different from zero. A significant correlation was observed for body redness and area of black blotches for female offspring on data of overlapped sampling period ($r= 0.696$; $P=0.008$) but this was not significant for the sires ($r=0.247$; $P=0.34$) or the male offspring ($P>0.05$ for all sampling points). Correlations between colour components in the sires and the offspring were slightly different especially for the redness and saturation components suggesting there could be different mechanisms of

colour elements between sires and offspring. The results were discussed in more depth towards future efforts in improving the red body colouration in the Nile tilapia.

Keywords: red tilapia, blotching, heritability

1. Introduction

Tilapia are now the second most important cultured group of fish species after carps (FAO, 2008) and are widely cultured in the world due to their tolerance and adaptability to a broad range of environments. Culture of the red tilapia has been of growing importance due to preference for the red colouration in certain countries which is probably driven by cultural bias. The popularity of red tilapia culture has increased mainly in South East Asia and South America and usually reaches higher prices than the wild morph (Head *et al*., 1994; Romana-Eguia and Eguia, 1999; Mather *et al*., 2001; Garduno-Lugo *et al*., 2004; Ng and Hanim, 2007).

Red tilapia are genetic mutants in the genus *Oreochromis* and a red mutant in a pure strain of *O. niloticus* originally from Lake Manzala (Egypt) has been developed in the Institute of Aquaculture, Stirling. This has since been referred to as the red Stirling strain. The genetics of this red strain and some other colour variants were described by McAndrew *et al.* (1988). As with many other red tilapia strains, one common problem that is usually associated with the red tilapia is the appearance of irregular black spots (blotching) which negatively affect their appearance.

To date, the genetics underlying the blotch trait is still not fully understood. This trait was hypothesized to be associated with the heterozygous red fish which carry the Rr alleles since Rr fish displayed a higher level of blotching compared to RR individuals, with more isolated patterns. This observation however was not absolutely true since some RR fish had higher levels of blotching than Rr fish (McAndrew *et al*., 1988).

Prior to the study of inheritance described here, two preliminary studies were carried out using clonal line and mixed sex fry to look at the ontogeny of blotching and associated body colour components as well as to measure environmental variance. The present work described here was carried out to investigate the heritability of blotching and other associated body colour components in the red Nile tilapia (Stirling strain) and their correlations.

2. Materials and methods

2.1 Fish and experimental design

Fish used in this study were from the red Stirling strain and the experiment was carried out in the tropical aquarium facility of the Institute of Aquaculture, University of Stirling. A total of sixteen homozygous red males with various level of black and red blotching, ranging from none to a maximum degree of mixed red and black blotching that could be found from the stock, were selected as sires. These males were varied in size and age (ranging between 200g until 600g). Each of these males was crossed to a clonal female by in vitro fertilization (four clonal females were use to provide eggs for all the crosses). The crosses were produced in the period of January to March 2010, depending on the fish spawning time and each cross was kept in isolation (one cross per tank). The clonal females used in this study were as described in the previous chapter (Chapter 3, section 2.1). Fertilized eggs were incubated up to the first-feeding stage in down-welling incubators before being transferred to experimental tanks. Progenies were reared in circular plastic (food grade) tanks with lids (radius=19cm; height=30cm; volume=23litres; mean flow rate=2.14 litre/min) in a

recirculating system at 27°C. Fish were fed with trout pellets (Skretting, UK) twice a day. At around 3 months old, the number of fish in each tank was reduced to 20~30. Digital images of the sires were taken once, prior to milt stripping, and two images were taken for the offspring, at 42 and 46 weeks old. The sires were PIT-tagged for identification and returned to their stock tanks after use. The offspring were returned to their own tanks after the first image analysis was carried out. The fish were killed and sexed after taking the second image.

2.2 Image analysis

From a total of 16 crosses, twelve fish were randomly selected from each cross for the colour image analysis with the exception of two crosses (n=7 and n=11 due to mortality). Fish were PIT-tagged for identification (after anaesthetizing with 10% benzocaine at 1ml/litre) and measurements of standard length and weight were taken for each fish. Image analysis was carried out as described in the previous chapter (Chapter 3, section 2.4).

Since some of the crosses were produced at different times (section 2.1), digital images for these crosses were taken at different times of the year, according to their age. From the preliminary trials carried out earlier (Chapter 3), all colour components decreased in November and either increased or decreased again in December. Since the colour parameters did not fit a simple model and were hard to predict, the image analysis sampling here was at two different sampling points between November to January (for the offspring) to avoid complete bias in the data sets. For the sires, since

their ages differed and they were previously reared in various tanks (different environments), digital images were taken only once.

2.3 Analysis of blotching and colour components

The red blotched area was difficult to measure due to a more complex patchy structure compared to the black blotches. Hence, only the black blotched area was quantified, as a percentage using the ratio of area covered by black blotches over total body area (excluding all fins and eyes). One limitation to this analysis was quantification of blotched areas close to the dorsal or ventral lines of the fish could not be quantified so accurately since digital images were only two-dimensional. Blotches on these areas appeared darker due to the angle of the blotched area relative to the camera.

Colour components of the black blotches and the background body colour (which included the red blotched area) were measured using RGB (red, green, blue) values. RGB is an additive colour model with numeric value between 0-255 where minimum RGB value (0,0,0) equals to black and maximum RGB value (255,255,255) equals to white. Since RGB is an additive colour model, the parent-offspring regression was presented using exploitation of all the three values rather than a single RGB component. For both the blotches and body colour components, brightness and saturation were calculated from the RGB values using transformation equations to the HSV colour space (hue, saturation and value). Saturation was calculated by deducting the minimum value within the RGB components from the maximum value of the components (e.g. if R=250, G=180, B=200; Saturation = [max-min] = 250-180 = 70). Brightness was calculated with the equation, *Brightness (value)* =

0.299R+0.587G+0.114B. This weighted equation was used to match the way human perceived colour since human eyes are more sensitive to green than red and blue (Ferreira and Rasband, 2010). Redness of body colour was also measured using the equation *Redness = R/(R+G+B).* From the preliminary analysis carried out earlier (Chapter 3), body colour components were significantly different for females and males, hence were analysed separately in this study. Since red blotches were not able to be quantified due to complex structures, correlation of area of black blotching with body redness was carried out instead since body redness included the area of red blotching but excluded the black blotched area.

2.4 Data analysis

The narrow-sense heritability (h^2) which represents the fraction of total phenotypic variation (V_P) attributable to additive genetic effects (V_A) was estimated directly from the slope of the parent-offspring regression. Since only single parent was used, $h^2 = 2b_{O|P}$ where the regression slope, $b_{O|P} = ½ (V_A/V_P)$ (Lynch and Walsh, 1998).

The parent-offspring regression was plotted according to age (at two sampling points). A third regression line was plotted using crossed-sampling data to avoid the possibility of batch effects due to different sampling time in which a graph was plotted for each parameter against time of sampling and data for sampling point on the 29th Nov 2010 which included records from 13 crosses were used for the regression plot. Figure 1 showed plotted data for the black blotches component for all the crosses based on the time of sampling. The same graph was plotted for the body colour components (graph not shown), separately for females and males, and data on the 29th Nov 2010 were used for the regression plot.

Correlations between colour components in offspring and in sires were tested using Pearson product-moment correlation. Statistical analysis was carried out using PASW Statistics (SPSS) v.18 and data sets were initially check for a normal distribution using the one-sample Kolmogorov-Smirnov test. Data for percentages of blotches and redness were transformed using arcsine square root transformation prior to analysis.

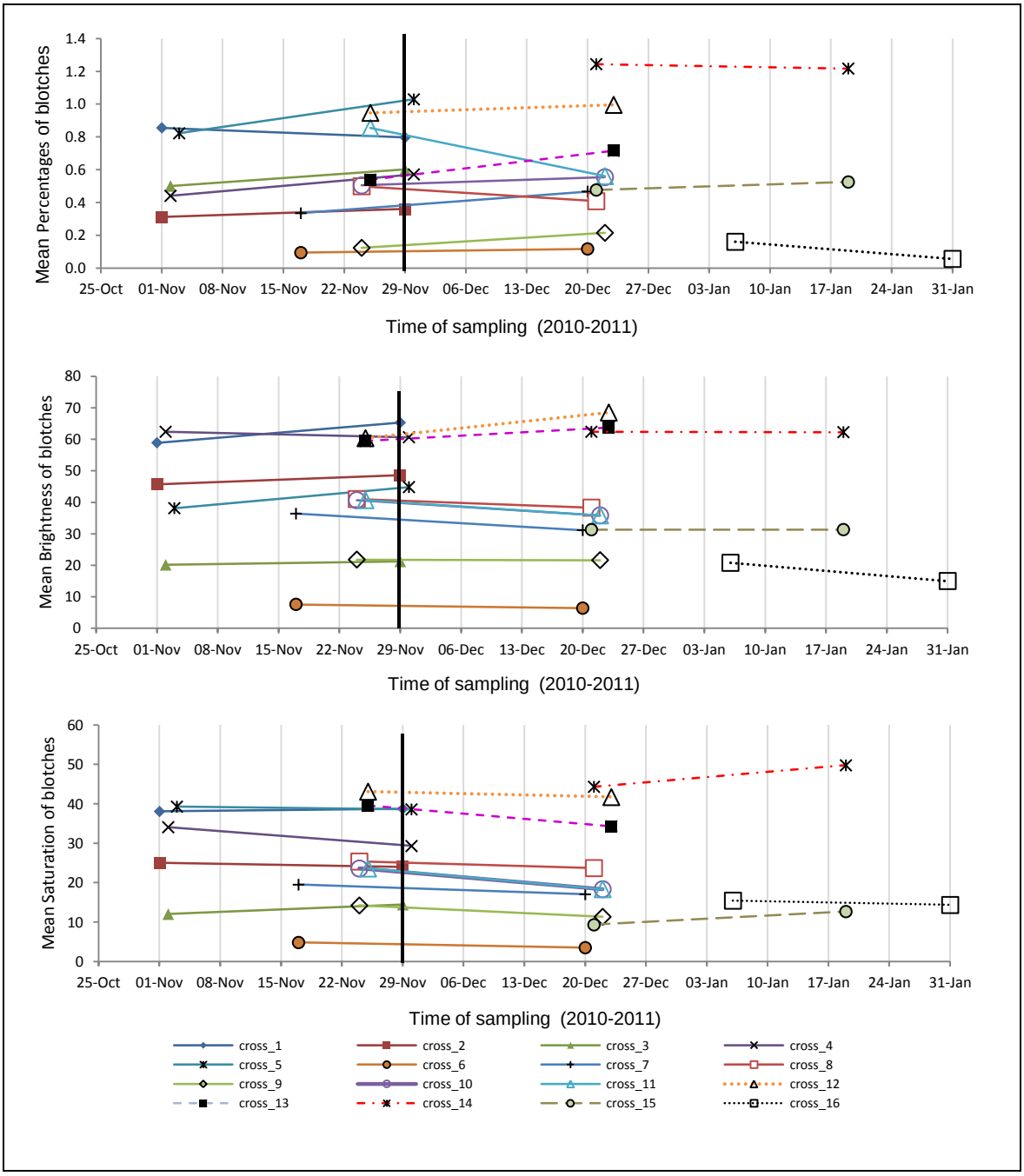


Figure 1: Mean percentages, brightness and saturation of the black blotches for each cross at time of sampling. Thick vertical line on the 29th Nov shows 13 families with overlapping sampling periods and these data (interpolated where necessary) were used for plotting parent-offspring regression to fix any batch effect due to different sampling point.

3. Results

3.1 Variation in blotching and red body colour

Table 1 shows the level of black blotching within offspring from each cross and their sires. The black blotching in the offspring reached up to 5.1% and in the sire up to 7.3% (of total body area) and was most usually found on top of the head, snout and dorsal part of the body. Some fish also showed blotching on the fins however this was not quantified in this study. Variation in black blotching in the offspring is shown in Figure 2.

Table 1: Percentages of black blotches within sire and offspring within each cross. For each cross, n=12 except for cross #7 (n=7) and cross #15 (n=11). Number of female and male used per cross were subjected to random sampling.

Cross	No of fish		No of fish with black blotches	Range of black blotches at 42 weeks (%)	% of black blotches within sire
	Females	Males			
#1	11	1	10	0.1-2.5	3.8
#2	6	6	7	0.1-1.0	0.5
#3	6	6	3	0.3-4.8	1.0
#4	9	3	9	0.2-1.6	2.2
#5	8	4	9	0.4-2.5	1.6
#6	8	4	1	1.1	0
#7	3	4	3	0.4-1.2	0.3
#8	11	1	5	0.4-3.4	7.3
#9	1	11	3	0.4-3.4	1.6
#10	5	7	6	0.3-1.7	1.4
#11	7	5	7	0.3-5.1	2.7
#12	11	1	11	0.3-5.1	0.3
#13	12	0	10	0.2-1.5	0.8
#14	5	7	11	0.1-3.5	0
#15	9	2	4	0.5-2.4	0.7
#16	9	3	4	0.5-2.0	0.9

Figure 2: Variances in black blotching in the red Nile tilapia

For the red blotched area, although this was not quantified in this study, some of the offspring showed very define clustered red blotch (Figure 3). In cross #3, most of the offspring had this type of red blotching, but in the sire the blotching was more diffuse. There were also various levels of background red colour observed within the offspring ranging from uniform faint pink colour to reddish with scattered diffuse blotching (Figure 4). In crosses #6 and #15, the sire was pale pink in colour and most of the progeny seemed to resembled this phenotype, although in other crosses, there was variation among the offspring.

Figure 3: Red blotches with more clustered structure in the offspring. Fish with this type of blotching had very few black blotches.

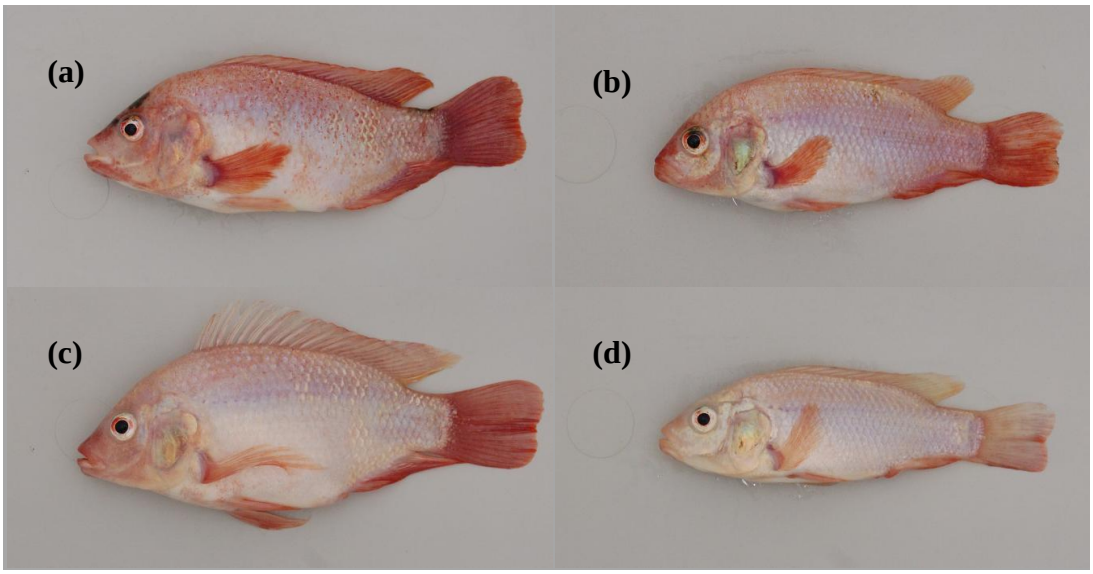


Figure 4: Variation of red colouration in the offspring. a) red with small and scattered red blotches; b) red with no blotches; c) pale pink but the fins are reddish and d) very pale pink including the fins

3.2 Parent-offspring regression

The heritabilities for the mean percentages of the black blotches, brightness and saturation of the blotches at any of the sampling points or during the overlapped sampling period were not significantly different from zero (Figure 5). From the preliminary trials carried out earlier, body colour components for females and males were significantly different, hence were regressed separately in this study. No significant correlations for body redness and saturation were observed (Fig.6, Fig.8) at any of the sampling points or during the overlapped sampling period but there was a significant negative correlation for brightness at the second sampling time (age 46 weeks) for both male and female offspring (Figure 7). Negative heritabilities (usually assumed as zero) indicated that the trait was non-additive (Lynch and Walsh, 1998).

Body redness and black blotches area was not significantly correlated for the sires and both males and females offspring at any of the sampling points (Figure 9). However, when data on the overlapped sampling period was used, a significant positive correlation was observed for female offspring ($r=0.696$; $P=0.008$) but was not significant for the males offspring ($r=0.502$; $P=0.096$). This could be due to a lower number of males in each cross (Table 1) which would gave a less precise estimation of mean value. When data for body redness of females and males offspring were combined, a significant correlation with the area of blotches was also observed for the overlapped sampling period ($r=0.588$; $P=0.034$, graph not shown).

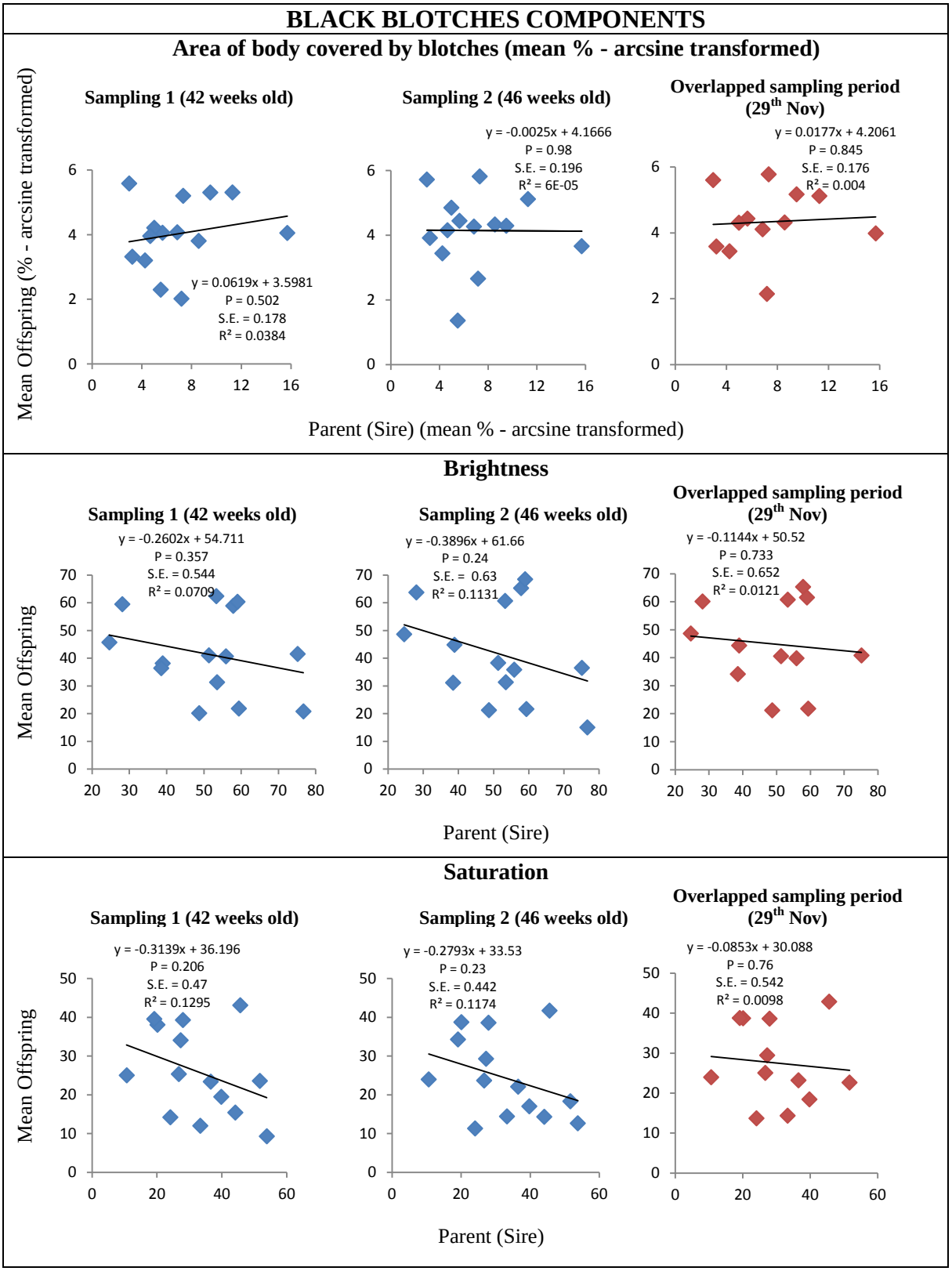


Figure 5: Parent-offspring regression for black blotches components (mean percentages, brightness and saturation for black blotches). Sampling 1 and 2 were plotted using data from 16 crosses at age 42 weeks and 46 weeks old respectively meanwhile overlapping sampling period on the 29th was plotted using data from 13 crosses. For each cross, n=12 fish except for two crosses where n=7 and n=11 respectively.

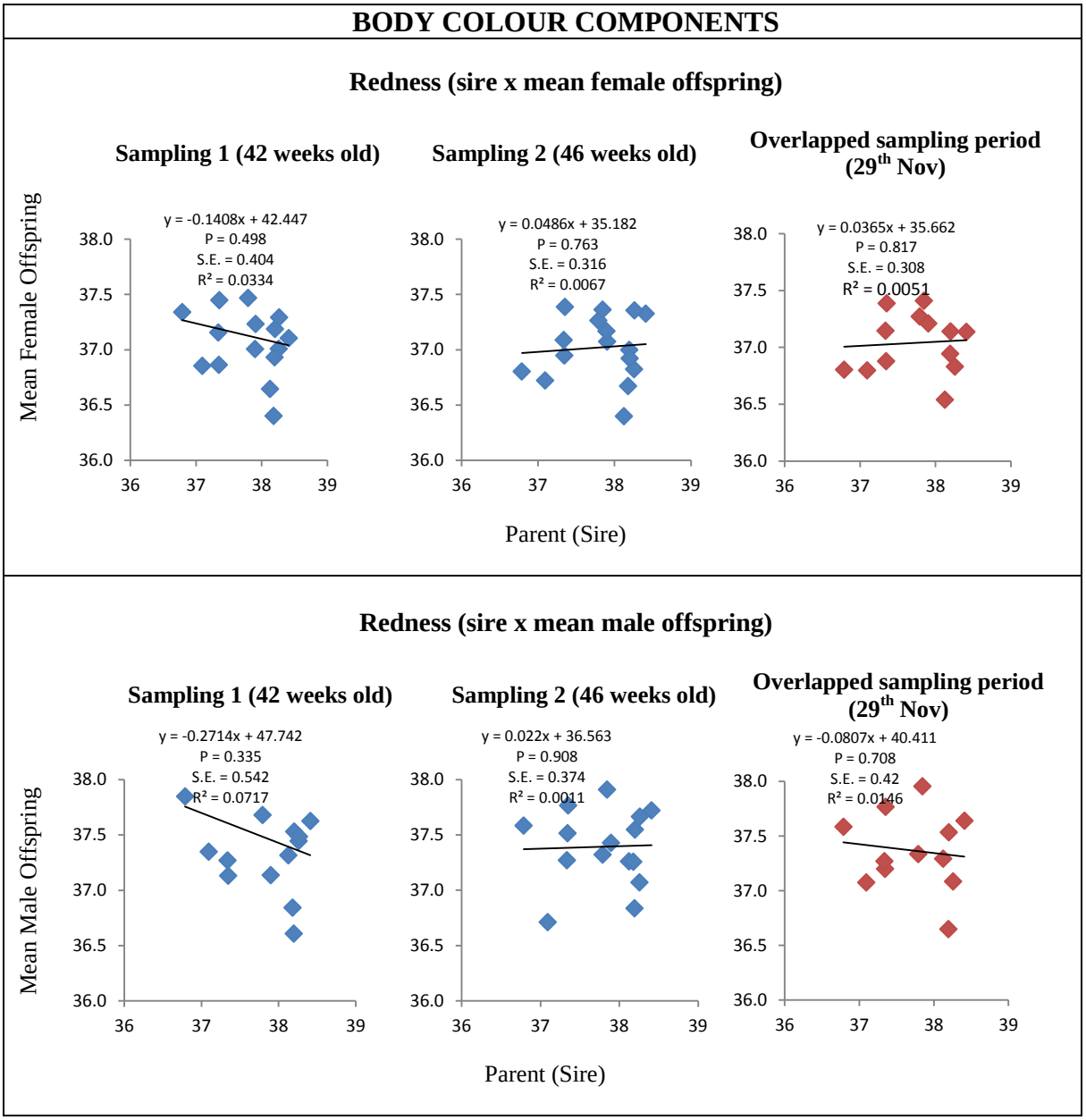


Figure 6: Parent-offspring regression for body redness component. Sampling 1 and 2 were plotted using data from 16 crosses (15 crosses for males) at age 42 weeks and 46 weeks old respectively meanwhile overlapping sampling period on the 29th was plotted using data from 13 crosses (12 crosses for males offspring). For females offspring, 2 crosses had n<5 (maximum females per cross, n=12) and for males, 7 crosses had n<5 (maximum males per cross, n=11). One of the crosses had no males (hence the lower number of crosses for males).

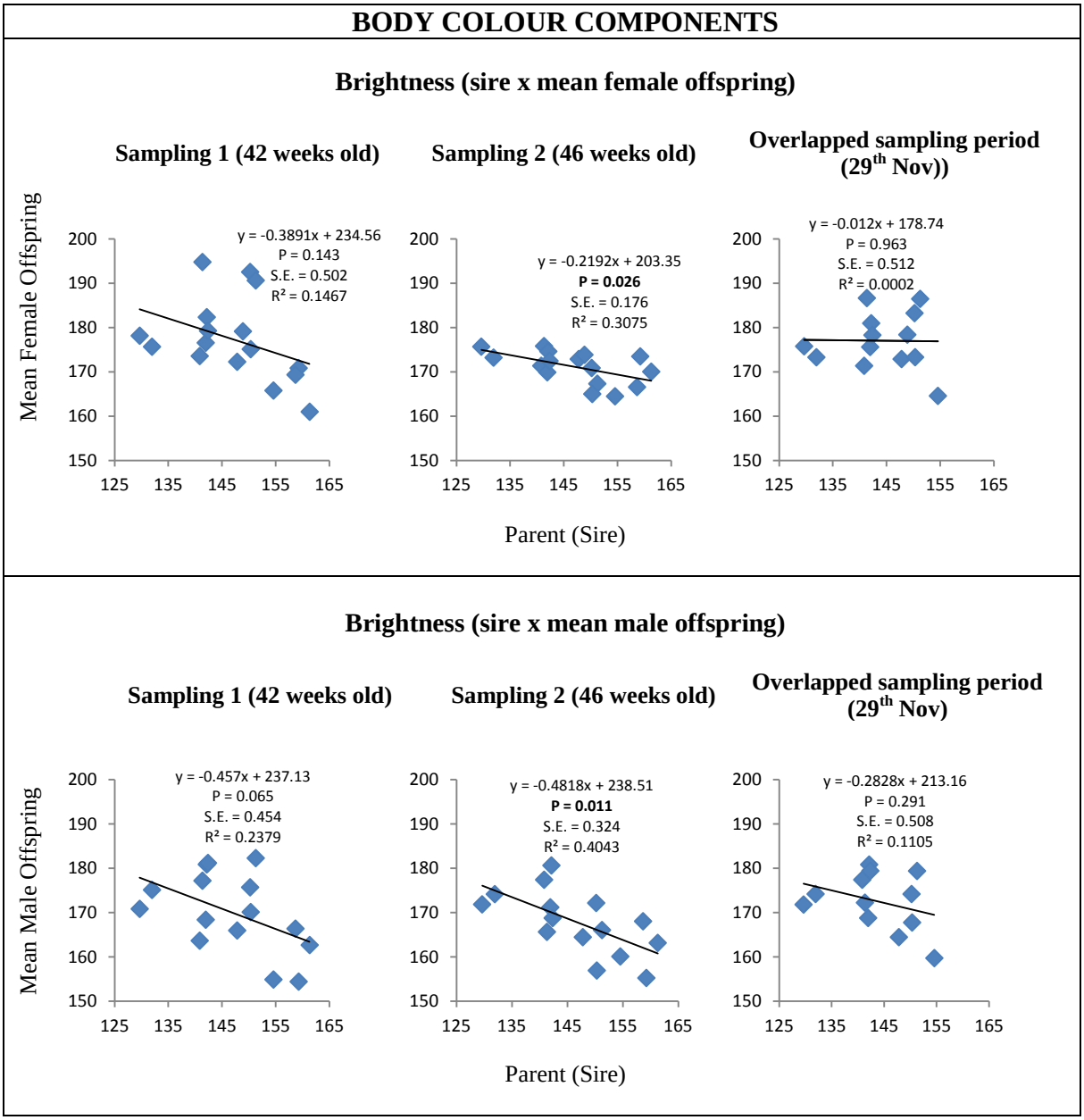


Figure 7: Parent-offspring regression for body brightness component. Sampling 1 and 2 were plotted using data from 16 crosses (15 crosses for males) at age 42 weeks and 46 weeks old respectively meanwhile overlapping sampling period on the 29th was plotted using data from 13 crosses (12 crosses for males offspring). For females offspring, 2 crosses had n<5 (maximum females per cross, n=12) and for males, 7 crosses had n<5 (maximum males per cross, n=11). One of the crosses had no males (hence the lower number of crosses for males).

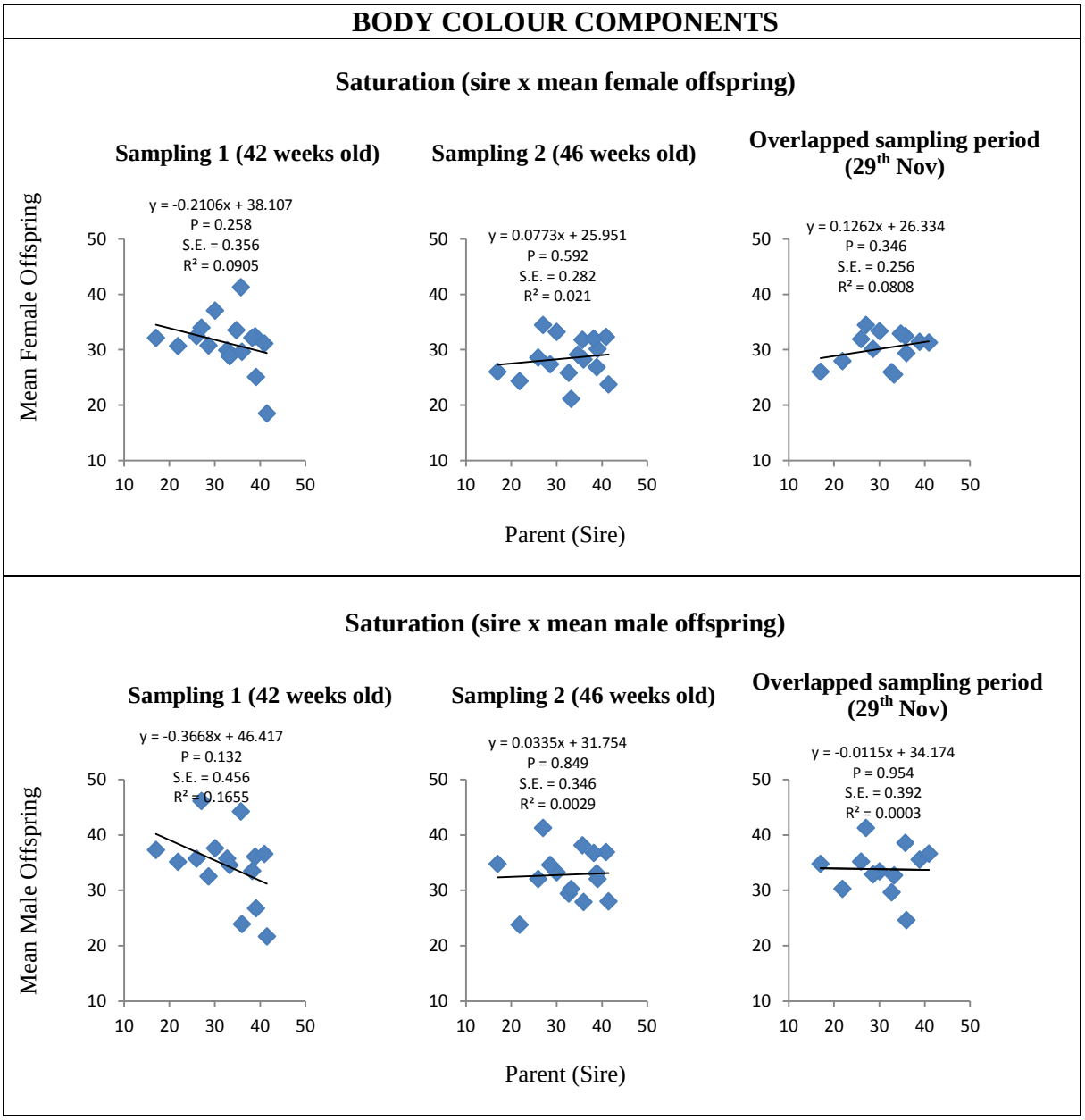


Figure 8: Parent-offspring regression for body saturation component. Sampling 1 and 2 were plotted using data from 16 crosses (15 crosses for males) at age 42 weeks and 46 weeks old respectively meanwhile overlapping sampling period on the 29th was plotted using data from 13 crosses (12 crosses for males offspring). For females offspring, 2 crosses had n<5 (maximum females per cross, n=12) and for males, 7 crosses had n<5 (maximum males per cross, n=11). One of the crosses had no males (hence the lower number of crosses for males).

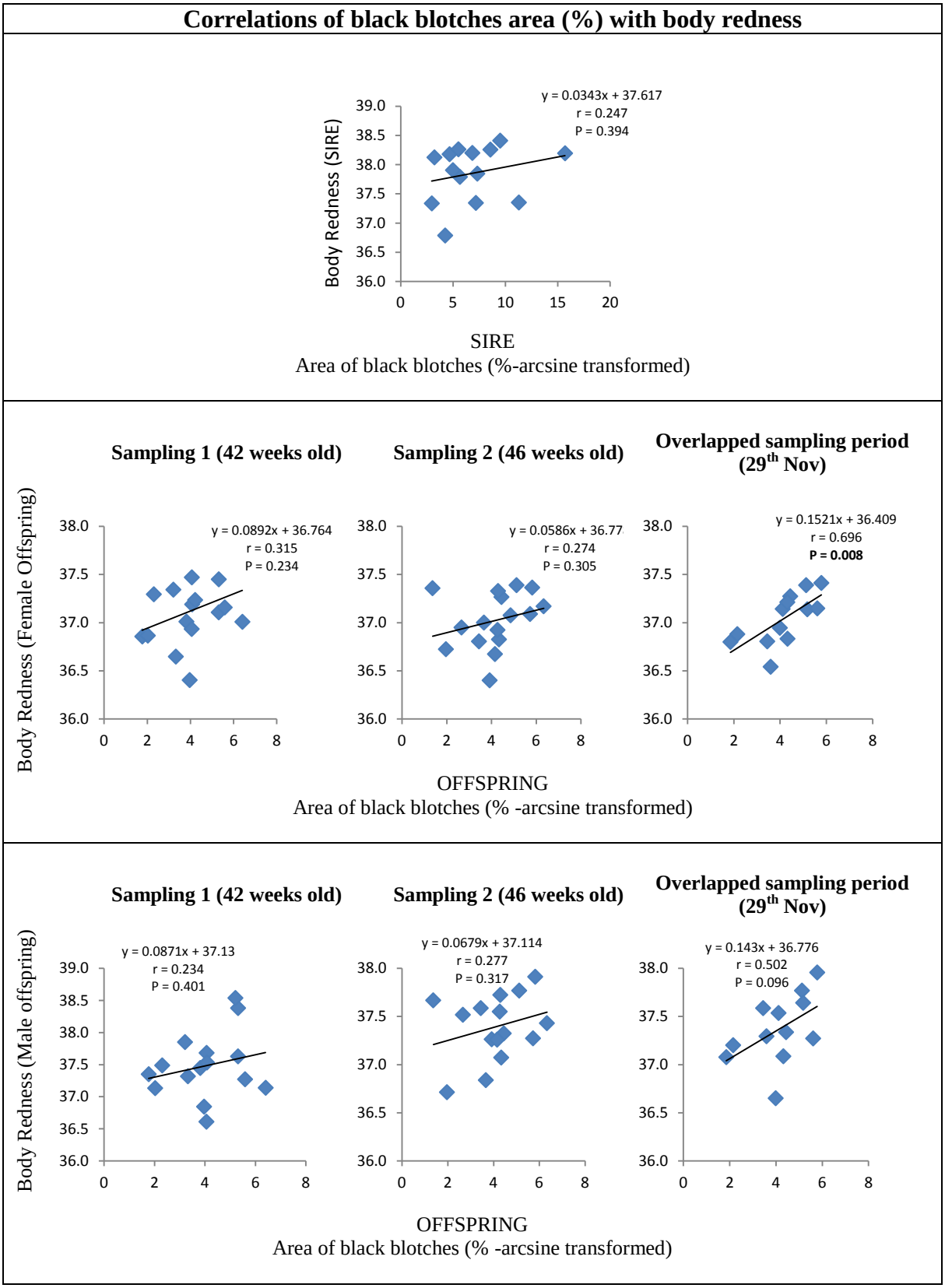


Figure 9: Correlations between area of black blotches with body redness in the sires, females offspring and males offspring. Correlation for sires was plotted using data from 14 individuals. Sampling 1 and 2 were plotted using data from 16 crosses (15 crosses for males) at age 42 weeks and 46 weeks old respectively meanwhile overlapping sampling period on the 29th was plotted using data from 13 crosses (12 crosses for males offspring).

In Table 2, correlations between all colour components in the sires and offspring for blotches and body colour based on data on the overlapping sampling period (on 29th Nov) are shown. Results for the correlations of the colour components based on these data (for the offspring) were in agreement with data on sampling 1 (age 42 weeks) and sampling 2 (age 46 weeks) (data not shown).

Correlations between RGB components in the blotches were significant in both parents and offspring, indicating that the colour components (for parent and offspring independently) were closely related to each other and had positive relationships. However, correlation between brightness and saturation component was only significant in the offspring, but not in the sires. For body colour components, all RGB components were significantly related to each other within the sires, females and males offspring with positive relationships. The same applied for brightness component, where it was significantly related to each of the RGB components (positive correlation) within the sires and the offspring. On the other hand, redness component was not related to any of the RGB components for the sires, but was negatively correlated to the G and B components in both female and male offspring. For saturation component, the R value had a significant positive correlation within the sires but in the offspring (both males and females), a significant negative correlation was found with the B component. Redness and brightness component was also significant in the offspring (both male and female showed negative correlations) but not significant in the sires. Finally, redness and saturation component was significantly correlated in both sires and offspring with positive correlation.

Table 2: Correlations between colour components of the blotches and body colour in sires and offspring (female and male separately for body colour components). For sires, correlation was plotted using individual data (n referred to number of individuals) while for offspring mean data from each cross was used (n referred to number of crosses). Correlations were calculated using data on the overlapped sampling period on the 29th Nov. ***** signified P<0.05

Blotches colour components		**Sires (n=14)**	**Offspring (n=13)**
Variable 1	Variable 2	Pearson's Correlation, r	Pearson's Correlation, r
Red	Green	**0.871***	**0.994***
Red	Blue	**0.753***	**0.976***
Green	Blue	**0.958***	**0.989***
Brightness	Saturation	0.52	**0.912***

Body colour components		**Sires (n=16)**	**Female offspring (n=13)**	**Male offspring (n=12)**
Variable 1	Variable 2	Pearson's Correlation, r	Pearson's Correlation, r	Pearson's Correlation, r
Red	Green	**0.896***	**0.922***	**0.880***
Red	Blue	**0.762***	**0.888***	**0.785***
Green	Blue	**0.875***	**0.988***	**0.978***
Redness	Red	0.288	-0.433	-0.380
	Green	-0.115	**-0.743***	**-0.771***
	Blue	-0.366	**-0.807***	**-0.867***
Brightness	Red	**0.951***	**0.953***	**0.921***
	Green	**0.987***	**0.996***	**0.995***
	Blue	**0.883***	**0.979***	**0.961***
Saturation	Red	**0.539***	-0.087	-0.064
	Green	0.232	-0.451	-0.519
	Blue	-0.135	**-0.551***	**-0.668***
Redness	Brightness	-0.006	**-0.685***	**-0.709***
Redness	Saturation	**0.916***	**0.929***	**0.941***
Brightness	Saturation	0.307	-0.378	-0.441

4. Discussion

Correlations of colour components for the males and females offspring were in agreement (no differences observed between males and females). Differences in the colour elements between sires and offspring indicated that the colour elements within older fish (parents) were slightly different from younger fish (offspring) mainly on the redness and saturation components. On the other hand, the parents were previously reared in various environments which may have involved different tank background colours. From our previous preliminary trials, tank background colour had a significant effect on the Red (R) component which could be one of the reasons for these differences. Skin pigmentation is affected by the increases or decreases in the number of dermal chromatophores which can be influence by background adaptation as described by Sugimoto (2002).

Different results for the brightness component of body colour and correlation of body redness with area of black blotches during sampling 1, 2 and overlapped sampling period suggested that the mechanism of body colouration can be exploited by reasons such as fish age or time of sampling. The significant correlations found in the present study, although not conclusive since only significant at one point (either at age 46 weeks or at the overlapped sampling period) should not be simply ignored since it did suggest that the components could be correlated. Significant correlation of the body redness and area of black blotches indicated that attempt to increase redness in the body colouration through selective breeding may result in more black blotches being inherited since these two components were correlated. This indeed would be a challenge in the attempt to improve the body colouration in red tilapia.

McAndrew *et al*. (1988) reported that the area covered by black blotches in the red phenotype can reached up to 24.6% of the skin. Stock fish used in this present study did not have high blotching rate due to selection against blotching in the stock (Ranson, personal comm.), as well as the fish used in the present study being RR homozygous, which usually had less blotching. One of the interesting observations was that the red blotching in some of the progeny was very defined and clustered. The red blotches in the parent fish were not as defined as this although it is not known either this structure would change over time, perhaps explaining why such observations were not made in the parents.

The design of parent-offspring regression in this study is one of the simplest to estimate heritability since only the phenotypic value of the sire was used while the genetic contribution from the dams was considered to be constant across the families due to the use of clonal line. Sire-offspring regression is more reliable than dam-offspring since the latter is usually associated with maternal effects, hence justifying the use of four clonal females as the dams. Heritability is a function of both the genetic and environmental variance where it is restricted to a particular population, therefore the results presented here only applied to our current population of study.

Body colour was related with physiological processes such as sexual maturity and breeding (Svensson *et al.,* 2005), where in this present study, the fish already reached maturation when the analyses were carried out. This somehow may provide noise in the data analysis. Tilapia usually reached sexual maturation as early as 3 months old, when it attain a body weight around 40-60g (Hussain, 2004). Therefore, perhaps by carrying the image analysis to an earlier stage would be recommended, although tagging smaller fish may be a tricky task.

In *Xiphophorus* species, the melanistic pigmentation patterns (the tailspot locus) are controlled by at least six different sex-linked and autosomal loci. In some species such as *X. maculatus*, the phenotypic expression of the tailspot pattern can be altered by two modifier loci, which interacts with a particular allele at the tailspot locus. Nevertheless, the modifier alleles apparently do no interact with each other and do not affect on the expression of other tailspot alleles (Borowsky, 1984).

Inheritance of colour pattern in the koi carp (C*yprinus carpio*) provides one example of how complex colour inheritance can be. To date, genetic control of many colour patterns in the koi carp are still not fully understood and some studies provided contradictive results and need further investigation (David *et al*., 2004). Gomelsky *et al*. (1996, 1998) suggested that the black spots in white-red koi variant is controlled by a single dominant gene which is completely independent of the gene responsible for red colour. However, this gene is only responsible for the presence or absence for the black spots while the patterns are suggested to be under control of multiple genes. Koi carp is known to have a wide variation in colouration and patterns even when the broodstock are of similar colouration or pattern.

There have been few studies on the heritability of skin colour and spots in fish. In the rainbow trout (*Onchorhynchus mykiss*), estimated heritability for skin colour and spots were 0.47 and 0.61 respectively (Kause *et al*., 2003). In the brown trout (*Salmo trutta*), heritability of the red spots was 0.73 (Blanc *et al*., 1994). Some other studies reported on the heritability of skin colour although these studies were more focused on genetics of sexual selection. In the three-spined stickleback, estimated heritability for the intensity of red male colouration was 0.23 (Bakker, 1993) meanwhile in the male

guppies, estimated heritabilities of 0.58-0.79 were reported for the total number, size and brightness of orange spots (Brooks and Endler, 2001).

Although no significant heritability was observed in this study, it is anticipated that the results would help to improve understanding of the mechanism of blotching and other body colour components in the red tilapia. There have been very few studies done to explore this trait, despite its importance in improving body colouration in the red tilapia.

Acknowledgement

This study was carried out as part of the PhD study of the first author, funded by the Ministry of Higher Education Malaysia and University Putra Malaysia. The authors would like to thank Denny Conway and Brian Howie for assistance with the photography.

CHAPTER 5

Paper III: Linkage mapping of the *blond* locus in Nile tilapia (*Oreochromis niloticus* L.) and preliminary analysis on its effect on blotching in red tilapia

Status: To be submitted to a relevant peer reviewed journal

Contributions:

The present manuscript was compiled and written in full by the author of this thesis. Fish rearing and sampling were carried out by the candidate. Laboratory and statistical analyses for linkage mapping in family 1 is carried out by the candidate meanwhile for family 2 and 3 were carried out by Dr. V.P. Saini and C. Palaiokostas respectively with assistance from the candidate. Supervisors (Dr. David Penman and Prof. Brendan McAndrew) provided assistance with the experimental design, guidance and editing of the manuscript.

Linkage mapping of the *blond* locus in Nile tilapia (*Oreochromis niloticus* L.) and preliminary analysis on its effect on blotching in red tilapia

Amy H. Rajaee, Christos Palaiokostas, Ved P. Saini[1], Brendan J. McAndrew and David J. Penman*

Institute of Aquaculture, School of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland, United Kingdom

[1]Present address: Aquaculture Research Unit, Directorate of Research, MPUAT Udaipur, 313001, Rajasthan, India

*Author to whom correspondence should be addressed. Tel.:+44(0)1786467901;

Fax: +44(0)1786 472133; email: d.j.penman@stir.ac.uk

Abstract

Colour blotching of the skin in red tilapia is undesired by tilapia producers since it affects their marketability. This study was carried out to discover if the homozygous recessive blond gene reduces or removes blotching in red tilapia by studying the interaction between the red and blond genes. Genome wide mapping was first carried out to find molecular markers that are linked to blond. These markers were used to identify the genotype of individual fish for the blond and red from the specifically designed crosses so the effect of recessive blond gene on blotched fish could be assessed. Two sets of mapping families (intraspecific and interspecific) were used for the mapping and the blond locus was successfully located in LG5. Six markers were significantly linked to blond in the intraspecific family and eight markers in the interspecific family. The blond locus is closely linked to UNH309, UNH169 and GM017 and linkage maps containing the blond locus were successfully constructed. Four out of eight markers were successfully used to assess the interaction of blond on red blotched fish. The blond gene did not significantly reduce the area of blotching but did reduce the saturation (paler blotching) and enhanced the redness of body colour in the Rrblbl fish compared to the RrBlbl group.

Keywords: tilapia, linkage mapping, blond, red, blotching, microsatellite

1. Introduction

McAndrew *et al*. (1988) reported three colour variants in the Nile tilapia which are normal (wild type), red and blond. The normal wild type colouration of Nile tilapia (*Oreochromis niloticus*) is dark grey in colour with vertical stripes containing melanophores. The blond (or bronze) tilapia can be confused with the normal wild type colour although its greyish shades of colour are paler with fainter stripes than the wild type (Scott *et al.*, 1987). The blond phenotype can be distinguished easily under anaesthesia or exposing them to a darker background as the wild type usually darkens, while the blond tend to stay paler than the wild type. The red variant are fish which showed no signs of normal pigmentation but in the heterozygous state, they usually were associated with black blotching (thus this phenotype termed as blotched). McAndrew *et al.* (1988) reported that melanin granules in blond fish are smaller than those in the wild type and the darker regions of blotched fish. In contrast to wild type, red and blotched fish which have both iridophores and melanophores in the peritoneum (below the serosal membrane), only iridophores are present in the blond fish.

Placing a fish on a light background will lead to the activation of their MCH (melanin concentrating hormone) and inhibition of alpha-MSH (melanin stimulating hormone) which leads to concentration of pigment and paling of skin. In contrast, MSH will be activated and alpha-MSH will be released if the fish is placed on a dark background, leading to a dispersion of pigments in the dermal melanophores and skin darkening (Rotllant *et al*., 2003). In blond tilapia, the formation of melanin seems to be almost blocked by the blond gene, although this mechanism is still not fully understood (McAndrew *et al*., 1988). They are still able to respond to environmental changes by skin lightening and darkening, suggesting the melanin cells are still responding to nerve

impulses. However, skin darkening is much reduced compared to that seen in wild type fish. This feature may help in reducing the black blotches or spots which are very commonly associated with red tilapia. Blotching in red tilapia is undesired and negatively affects their marketability (Mather *et al*., 2001).

Since blond is recessive and its appearance may be masked by red, a study on the interaction between these genes would be assisted by molecular markers linked to the blond locus. An existing linkage map of the tilapia has been published by Lee *et al.* (2005) and linkage mapping for this study is carried out using markers from this linkage map. The objectives of this study are (i) to map the blond locus onto the existing linkage map of tilapia; and (ii) to study the effect of blond on blotching in red Nile tilapia.

2. Materials and methods

2.1 Experiment 1: Linkage mapping of blond locus

2.1.1 Mapping families

Two intraspecific families (*Oreochromis niloticus* blond female x wild type male; backcrossed to a blond female) and one interspecific family (*O. aureus* female x *O. niloticus* blond male; backcrossed to a blond *O. niloticus* male) were produced for mapping the blond locus. The offspring from these crosses were scored either as blond (blbl) or wild type (Blbl). The interspecific family was produced to provide additional polymorphic markers for the linkage map. Caudal fin biopsies were collected from the

offspring when they were at least 3 months old and kept in 95% ethanol for DNA extraction.

2.1.2 Genome-wide scanning for blond polymorphism

A total of 65 microsatellite markers from the tilapia linkage map (Lee *et al.,* 2005), spaced at approximately 10-15 cM apart were tested. Initial mapping was carried out on one intraspecific family using bulked segregant analysis (BSA) (Michaelmore *et al.*, 1991). DNA from 23 blond and 23 wild type individuals were pooled together to give a blond pool and wild type pool respectively for the polymerase chain reaction (PCR). After loci associated with blond were identified from BSA, PCRs were carried out using individual samples with markers from the linkage group concerned. A total of 43 individuals for each intraspecific family and 37 individuals for the interspecific family were analysed.

2.2 Experiment 2: Preliminary analysis on effects of blond on blotching

2.2.1 Breeding families

A homozygous red fish (RRBlBl) was crossed with a blond fish (rrblbl) and two progeny from this cross (RrBlbl) were backcrossed to an non-sib blond (rrblbl) (Figure 1). The phenotypes of these backcross progeny were expected to consist of red:wild type:blond with the ratio of 2:1:1 (where red consists of two genotypes RrBlbl:Rrblbl in a 1:1 ratio). All the wild type and blond progeny were discarded at about 3 months old when body colours could be clearly scored. These two families had a total of 34 red progeny (19 from Family 1 and 15 from Family 2). At the age of 6 months old, digital

images of each fish were taken under anaesthesia (10% benzocaine; 1ml/litre). Standard length and weight were recorded before the fish were PIT-tagged and fin-biopsied for DNA extraction.

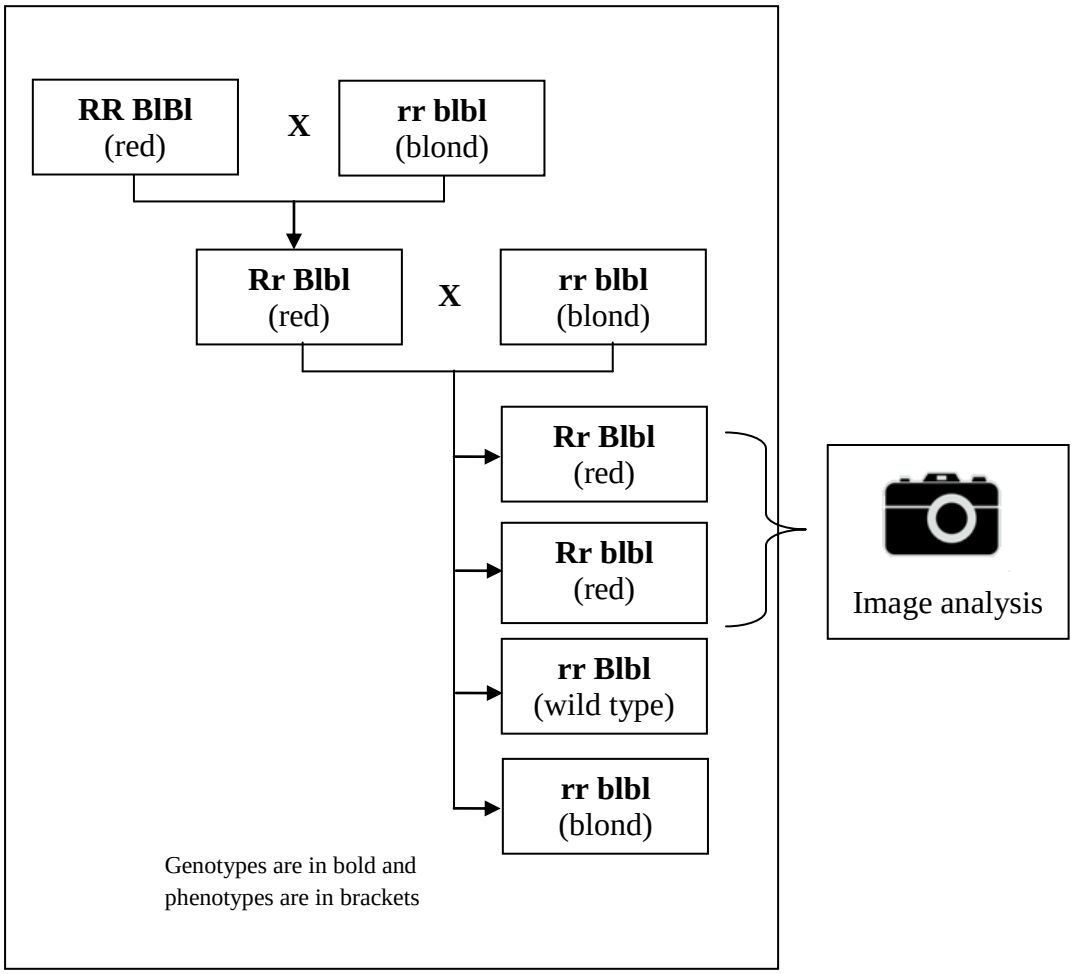


Figure 1: Breeding family for Experiment 2

2.2.2 Image analysis

Digital images of each fish were taken using a DSLR (digital single-lens reflex) camera (Nikon D40). The camera was set to manual mode with a focal length of 55mm, shutter speed of 1/25 with an aperture of F5.6. The camera was held by a tripod with a custom made extension so that the camera was able to take photos vertically.

Anaesthetized fish were placed individually on a gray tray within a translucent white tent. A ruler and an 18% gray card (as a standard reference for white balance) were included in every image. An image of each side of the fish was taken. Images were stored in a 3008 x 2000 pixel format on a 'high' quality setting (RAW compression of 12-bit). The white balance of each image was standardized according to the 18% gray card using Nikon software, ViewNX™ version 1.5.2 before the images were converted to tiff format. Images were then analysed using ImageJ software (version 1.43; http://rsb.info.nih.gov/ij; with plugin for RGB-measure) for measurements of body colour and area covered by black blotches. Images were first transformed from '16-bit' to 'RGB Color' using this software to measure colour intensity. The scale was set in the first image by drawing a straight line on the ruler and set as 'global scale' for subsequent images. Black blotched areas were selected using the wand tool (8-connected mode, tolerance of 10.0). Body colour intensity was measured after the areas of the eye, fins and black blotches were cropped.

2.2.3 RrBlbl and Rrblbl determination

A total of eight markers were tested to distinguished RrBlbl and Rrblbl individuals. These microsatellite markers were selected based on linkage to the blond locus derived from Experiment 1.

2.3 DNA extraction

All DNA samples were extracted from ethanol-preserved tissue using the REALPURE DNA extraction kit (REAL laboratories, Spain) using 1.5ml eppendorf tubes. Four biopsy punches of the fry tails (approximately 0.5 cm^2 of tissue in total)

were digested in 3 µl of proteinase K (10mg/ml) in the presence of 75 µl cell lysis solution during overnight incubation at 55^{o}C. Subsequent to this, 3 µl of DNA-free RNAase (2mg/ml) was added for each sample with additional one hour incubation at 37^{o}C. Samples were then brought to room temperature and 45 µl of protein precipitate solution was added to each tube to precipitate protein residues. All samples were agitated by vortexing prior to centrifuging at 3570xg for 20 minutes at 4^{o}C. 50 µl of the supernatant was then transferred into a new eppendorf tube containing 75 µl isopropanol. The DNA pellets were precipitated by centrifuging at 3570xg for 10 minutes at 4^{o}C. The solution was then poured off and the tubes were washed by adding 150 µl of 70% ethanol and further centrifuged at 3570xg for 10 minutes at 4^{o}C. Finally, the solution was poured off once again and the tubes were air-dried for about 40 minutes to make sure the ethanol was completely evaporated. The DNA pellets were resuspended in 40 µl of TE buffer (1 mM Tris, 0.01 mM EDTA, pH 8.0) and left overnight in room temperature before used. DNA concentration was quantified using a spectrophotometer (Nanodrop, ND-1000) and diluted to 60~100 ng/µl for PCR amplifications.

2.4 PCR and genotyping

All PCRs were carried out using the tailed primer method (Boutin-Ganache *et al.,* 2001). Three types of "tail" were used: Godde (catcgctgattcgcacat), CAGtag (cagtcgggcgtcatca) and M13R (ggataacaatttcacacagg). The "tail" was added to the 5'-end of either the reverse or forward primer, depending on the marker sequence (a primer starting with 'G' is preferably avoided). Another labelled primer, containing the complementary sequence to the tail was then used to label the PCR products. These

primers were labelled with different fluorescent dyes for each type of tail for easier identification of products during multiplex genotyping.

PCR was carried out in a 15 µl reaction in a benchtop thermocycler (T gradient thermocycler, Whatman Biometra, Goettingen, Germany) containing 1X PCR buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 0.5 units of KLEAR Taq enzyme (Thermo Scientific, UK), 0.2 pmol of tailed primer, 4 pmol of non-tailed primer, 4 pmol of labelled primer and 60~100 ng genomic DNA. PCR conditions were as follows: one cycle of pre-denaturation of 15 minutes at 95^{o}C; 30 cycles of denaturation of 1 minute at 95 ^{o}C, annealing for 1 minute and 1 min of extension at 72 ^{o}C; followed by a final extension step of 30 min at 72^{o}C. If the primers required different annealing temperatures, the PCR was broken down into 5 cycles with the lower annealing temperature and 25 cycles with the higher annealing temperature. PCR products were first checked by electrophoresis in 1.2% agarose gels with 0.5X TAE buffer (Tris-acetate-EDTA), stained with 2% ethidium bromide. Sizing of PCR products was accomplished using CEQ 8800 Genetic Analysis System automated DNA sequencer (Beckman Coulter) and allele sizes were analysed with CEQ8800 software.

2.5 Statistical analysis

2.5.1 Experiment 1

Contingency chi-square analysis was used to test for absence or presence of linkage between each marker and the blond locus. Linkage analysis between markers was then calculated using CRI-MAP based upon maximum likelihood scores at LOD $\geq$ 3.0. Linkage maps were constructed using MapChart (ver. 2.2).

2.5.2 Experiment 2

Comparisons of standard length and weight between RrBlbl and Rrblbl fish were carried out using t-tests. Percentages of black blotches and redness of body colour (calculated using the equation R/(R+G+B)) were tested using the Mann Whitney U test after arcsine square root transformation of the data. Brightness, saturation and each of the colour components for body colour and the black blotches were also compared using the Mann Whitney U test. Brightness was calculated using the equation *Brightness = 0.299R+0.587G+0.114B* and saturation was calculated by deducting the maximum value within the RGB components to the minimum value of the components (e.g. if R=250, G=180, B=200; saturation = (max-min) = 250-180 = 70). Association of each body colour component was tested using Pearson product-moment correlation.

3. Results

3.1 Experiment 1

From the bulked segregant analysis of the markers from the first set of linkage groups (LG1 – LG13), two markers from LG5 showed association with blond leading to further analysis using all markers from this linkage group. A total of eleven out of thirteen markers were tested using individual samples. Table 1 showed chi-square analysis of linkage between each marker and the blond locus. Of eleven markers tested, six markers were significantly associated with the blond phenotype in all families plus three additional markers in the interspecific family. Two of these additional markers (GM636 and GM012) were not polymorphic in the intraspecific families while UNH980 did not show a significant association. Markers showing the strongest

association with blond were UNH309, GM017 and UNH169 in the intraspecific families and UNH309, UNH169 and GM636 in the interspecific family.

Two markers, GM021 and GM537 did not show any linkage to any other markers in any of the families, hence were excluded from the final construction of the linkage maps. Marker GM636 failed to link accurately during the construction of the linkage map therefore was also excluded. Marker GM006 was linked to UNH309, GM017 and UNH169 in both intra- and interspecific families as well as with UNH980 in the interspecific family. In the intraspecific families, marker UNH980 was only linked with UNH169 while in the interspecific family it was only linked with GM006. The blond locus was tightly linked with GM017, UNH309 and UNH169 in the intraspecific families and with UNH309 and UNH169 in the interspecific family. The markers that showed the highest LOD score were GM017 and UNH309 (intraspecific) and UNH169 and UNH309 (interspecific).

Table 1: Association between blond locus and markers in Linkage Group 5 (LG5).

Markers in LG5	Intraspecific *(O. niloticus)*				Interspecific *(O.aureus x O. niloticus)*	
	Family 1		Family 2		Family 3	
	χ^2	p	χ^2	p	χ^2	p
GM012	NP	-	NP	-	9.4	0.024
UNH927	17.3	3.210×10^{-5}	23.0	1.605×10^{-6}	16.7	4.370×10^{-5}
UNH884	10.1	0.001	14.5	1.368×10^{-4}	14.3	1.580×10^{-4}
UNH309	40.0	2.539×10^{-10}	32.7	1.060×10^{-8}	34.0	1.981×10^{-7}
GM017	40.0	2.539×10^{-10}	32.7	1.060×10^{-8}	20.2	6.838×10^{-6}
GM537	0.1	0.75	1.4	0.485	5.6	0.135
UNH169	36.4	6.273×10^{-8}	33.1	3.005×10^{-7}	34.0	1.981×10^{-7}
GM636	NP	-	NP	-	34.0	1.981×10^{-7}
UNH980	0.0	1.000	0.1	0.744	23.9	2.554×10^{-5}
GM021	3.4	0.337	2.0	0.577	6.9	0.075
GM006	15.7	0.001	21.2	9.733×10^{-5}	22.3	5.710×10^{-5}

NP = non-polymorphic (homozygous for at least one of the parents)

Linkage maps were constructed using eight (intraspecific) and nine (interspecific) loci including the blond locus (Figure 2). Distances between markers were calculated in centimorgans (cM). The intraspecific map did not include marker GM012 since it was not polymorphic (homozygous for at least one of the parents). The major difference between these two maps was the marker GM017 which was closely linked to blond (~2.5cM) in the intraspecific map but was much further away (~37.5 cM) in the interspecific map. The blond locus appears to be approximately 2.5 cM from UNH309 and GM017 in the intraspecific families and 7.5 cM from UNH309 and UNH169 in the interspecific family.

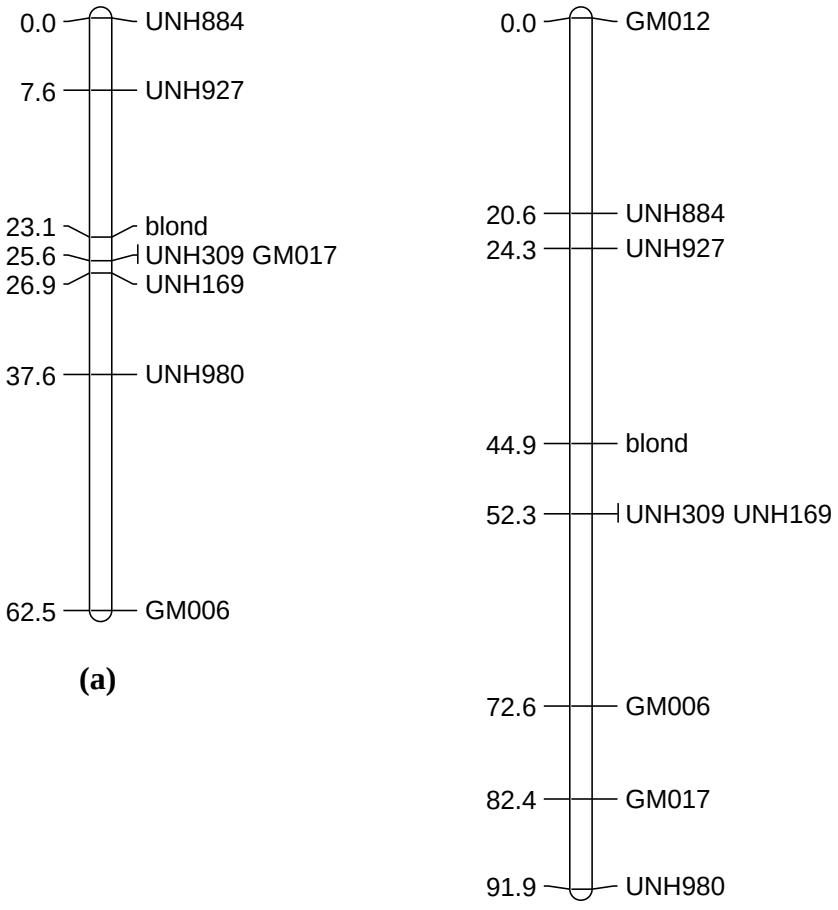


Figure 2: Chromosome 5
(a) Linkage Map of Family 1&2 combined (intraspecific)
(b) Linkage Map of Family 3 (interspecific)

3.2 Experiment 2

Eight markers which were significantly linked to blond in the interspecific family were used to distinguish between RrBlbl and Rrblbl fish. Since the sire and dam were both homozygous in Family 2, only samples from Family 1 were considered for analysis. Of eight markers tested, six markers were polymorphic (Table 2). Based on alleles from parents and grandparents, six out of nineteen individuals were concluded to be RrBlbl.

Table 2: Genotype results for Experiment 2. Bold signified RrBlbl individuals. Alleles segregating for blond (blbl) were shaded. Based on results in Exp. 1, the blond locus was tightly linked with UNH309, GM017 and UNH169, hence these markers were the best to differentiate RrBlbl and Rrblbl individuals.

Sample No.	Markers from Linkage Group 5												Genotype
	GM012		UNH927		UNH309		GM017		UNH169		GM636		
♀ (rr blbl)	240	256	216	216	200	200	164	164	172	172	212	212	
♂ (Rr Blbl)	240	240	216	220	200	204	164	166	154	172	212	218	
#1	240	256	216	216	200	204	164	166	154	172	212	218	**Rr Blbl**
#2	240	240	216	220	200	200	164	164	172	172	212	212	Rr blbl
#3	240	240	216	220	200	200	164	164	172	172	212	212	Rr blbl
#4	240	256	216	216	200	204	164	166	154	172	212	218	**Rr Blbl**
#5	240	256	216	220	200	200	164	164	172	172	212	218	Rr blbl
#6	240	256	216	216	200	204	164	166	154	172	212	218	**Rr Blbl**
#7	240	256	216	216	200	200	164	164	172	172	212	212	Rr blbl
#8	240	256	216	220	200	200	164	164	172	172	212	212	Rr blbl
#9	240	240	216	216	200	204	164	166	154	172	212	218	**Rr Blbl**
#10	240	256	216	220	200	204	164	166	154	172	212	218	**Rr Blbl**
#11	240	240	216	220	200	204	164	166	154	172	212	218	**Rr Blbl**
#12	240	256	216	216	200	200	164	164	172	172	212	212	Rr blbl
#13	240	256	216	220	200	200	164	164	172	172	212	212	Rr blbl
#14	240	256	216	220	200	200	164	164	172	172	212	212	Rr blbl
#15	240	256	216	220	200	200	164	164	172	172	212	212	Rr blbl
#16	240	256	216	216	200	200	164	164	172	172	212	212	Rr blbl
#17	240	256	216	216	200	200	164	166	172	172	212	212	Rr blbl
#18	240	240	216	220	200	200	164	164	172	172	212	212	Rr blbl
#19	240	240	216	220	200	200	164	164	172	172	212	212	Rr blbl

T-tests showed that length and weight of the two groups were not significantly different (data not shown). Table 3 showed the comparisons of all the tested colour components. The area of blotching in the two groups did not differ although the red (R) component of the blotches was slightly higher in the Rrblbl group (P=0.048) and a significant increase in the saturation of the blotches for Rrblbl group was observed (P=0.005). Brightness and other colour components of the blotches were not significantly different.

Table 3: Comparison between RrBlbl and Rrblbl group using Mann-Whitney U test

Components for body colour	RrBlbl (n=6)		Rrblbl (n=13)	
	Mean	SE	Mean	SE
Red (R)	189.14	2.52	191.02	1.80
Green (G)	172.69	3.11	169.81	2.27
Blue (B)	150.49	1.96	145.17	1.96
Redness ; R/(R+G+B)	37.42*	0.25	37.92*	0.45
Brightness	175.08	2.83	173.35	1.97
Saturation	38.65*	1.27	45.85*	1.64
Components for black blotches	RrBlbl (n=6)		Rrblbl (n=11)	
	Mean	SE	Mean	SE
Percentages of black blotches	13.26	3.08	9.83	1.85
Red (R)	101.90*	8.28	123.79*	5.44
Green (G)	78.63	8.78	90.37	6.08
Blue (B)	57.34	6.33	57.28	3.72
Brightness	83.16	8.14	96.58	5.45
Saturation	44.56*	5.08	66.49*	4.02

*$P < 0.05$

For body colour, higher saturation value = redder

For black blotches, higher saturation value = less black

For body colour, the redness of the Rrblbl group was significantly higher. There was a significant increase in the saturation level in the Rrblbl group but no other parameters differed significantly between the two groups although the B component was marginally significant. Correlations between colour components were all significant (Table 4) except for R and B components for the blotches in the RrBlbl group.

Table 4: Correlations between colour components in RrBlbl and Rrblbl individuals

for body colour		**RrBlbl** (n=6)	**Rrblbl** (n=13)
Variable 1	Variable 2	Pearson Correlation, r	Pearson Correlation, r
Red	Green	0.930*****	0.773*****
Red	Blue	0.888*****	0.620*
Green	Blue	0.926*****	0.830*****

for black blotches		**RrBlbl** (n=6)	**Rrblbl** (n=11)
Variable 1	Variable 2	Pearson Correlation, r	Pearson Correlation, r
Red	Green	0.902*****	0.914*****
Red	Blue	0.790	0.674*****
Green	Blue	0.969*****	0.840*****

*****P<0.05

4. Discussion

An association between blond and LG5 markers was found from scanning approximately half of the tilapia genome using BSA. Further analysis allowed mapping of the blond locus in LG5 which also established that blond is a separate locus from red (red is in LG3; Lee *et al*., 2005). From the published tilapia linkage map (Lee *et al*., 2005), there were 13 microsatellite markers and three Type 1 markers in LG5. Two out of these 13 microsatellites failed to amplify during PCR (UNH817 and UNH149) and another two failed to show any linkage with other markers (GM021 and GM537), therefore were excluded from the analysis. LG5 constructed from these results, especially the intraspecific families, was in fairly close agreement with the one produced by Lee *et al*. (2005). The major difference between the two maps was the position of GM017. It was closer to the blond locus in the intraspecific but more distant in the interspecific, which may have been due to differences in types of mapping panel and number of samples used. In linkage map construction, finding differences in the

order of markers is not unusual even between mapping families of the same species. Given the fact that these two maps were from different types of mapping panel (intra- and interspecies), a certain degree of difference maybe expected. However, the linkage map from Lee *et al*. (2005) was constructed using F2 hybrids (also *O. aureus* and *O. niloticus*) but the intraspecific map from our results was more similar to this existing map. More progeny were used to construct the intraspecific map (n=83) and the map of Lee *et al*. (2005) (a minimum of n=70) than the interspecific map (n=37). Therefore, we would have more confidence in the very similar results of the first two maps.

From the results, it was proposed that the blond locus lies in the middle of LG5 in the interval of UNH309 to UNH927 with a relatively large interval (15.5 cM) between blond and UNH927. In the tilapia linkage map (Lee *et al*., 2005), the *cski* gene was located within this interval and coincidently, *c-ski1* was responsible for the orange blotch (OB) phenotype in the cichlid *Metriaclima zebra* (Streelman *et al*., 2003). The closest markers to the blond locus were UNH309, UNH169 and GM017 (<4cM) in the intraspecific map and GM309 and UNH169 (<8 cM) in the interspecific map. In this study, we tested eight markers that showed significant association with blond from the mapping to assist in determining the RrBlbl and Rrblbl individuals. The final discrimination of RrBlbl and Rrblbl individuals was carried out using 5 markers, as the marker GM012 was more distant from the blond locus. On the other hand, marker UNH927 (since it was on the other side of the blond locus) provide additional information to identify individuals where no recombination occurred between blond locus and adjacent markers.

As mentioned earlier, we hypothesized that blond might help to remove or reduce blotching in red fish. The saturation of the blotches was significantly higher in

the Rrblbl group, suggesting they were less black than RrBlbl fish. For the RGB colour model, brightness is the average value of RGB, the higher the value, the brighter it is. Black is considered as having zero brightness (R=0, G=0, B=0) meanwhile white is the maximum brightness (R = 255, G = 255, B= 255). Brightness is the term used to explain how light and dark a colour is meanwhile saturation is a measurement of colour purity, or vibrancy of the colour. Colour is more saturated if the differences between RGB components is bigger, but if the values of RGB components are close to each other, the colour is said to be less saturated.

Lower saturation values means a colour is less vivid, or less pure which means more gray is present within the colour. The brightness of a colour can change without changing the saturation and vice versa, as long as the colour attributes are maintained (e.g. brightness can change but maintain the same saturation if all colour components increase by the same amount). Saturation does not affect brightness, however, brightness does usually affect the saturation of a colour, for example if approaching white or black, saturation must decrease. Maximum saturation is somewhere in the midtone region, and adjusting the tonality from this region towards white or black will reduce the saturation that a given colour can display. This mean that lower saturation value can either mean nearer to white or nearer to black. Black is considered as not having any colour (absorbing all light spectrum but not reflecting any, although technically, black does reflect some light). In measuring colour nearer to black (in this case the black blotches), when no differences in the brightness level were seen but the saturation was significantly different, this suggested that the group with higher saturation (Rrblbl) contained some hue (colour) attributes, hence they were less black. It was anticipated that when the blotches were paler, the background red colour of the

skin interfered with the appearance of the blotches and contributed towards the saturation value. This result was supported as a significant difference of the R component of the black blotches was observed (higher in the Rrblbl), suggesting a different colour attribute within the blotches of this group. When comparing with the naked eye, the blotches in the Rrblbl fish were more faded although the differences were not very dramatic. This was presumably as a result of reduction of melanophore sizes of the blotches in the Rrblbl fish compared with RrBlbl, although this would need further investigation.

As for the background body colour, a marginally significant increase in redness value was seen in the Rrblbl fish compared to the RrBlbl fish. This was further supported by a higher saturation value in the Rrblbl group, suggesting that the redness was more vivid within this group than the RrBlbl. This result was rather unexpected since we did not predict that the redness of body colour would be affected by the interaction of blond and red, although the impact of this was also not very obvious if compared by naked eye.

Correlation analyses between colour components were significant for body colour and the black blotches, suggesting that these components were closely related with each other. Only the R x B component for the intensity of black blotches was not significant for the RrBlbl fish, which was in contrast with Rrblbl fish. This could be caused by the number in the RrBlbl group which was lower than the Rrblbl. All the pairwise combinations of the colour components had a positive relationship suggesting that they have the same features.

The use of digital images for this type of study is suitable since the analysis is done using the whole image plus having the flexibility to apply other analyses such as area measurement. This method is also inexpensive but careful steps need to be taken in setting up the photography. Interpretation of colour variation is a complicated procedure, mainly related to the way that colour is perceived by humans. In our study, we used the RGB (red, green, blue) colour system which is an additive colour model and it was suitable for our use since this colouring system also appears to be reliable as an indicator for natural colouration (Tlusty, 2005) and corresponds to human vision (Hancz *et al*., 2003).

The fact that blond showed smaller melanophores contributes to the idea of using this gene to reduce the appearance of melanin blotching on red fish (McAndrew *et al.,* 1988). However, although the blotches were paler in the Rrblbl group, presumably due to reduction of the size of melanophores, the effect was not sufficient to improve the appearance of the red fish. On the other hand, the number of samples used for the second part of the study was rather small, due to unexpected mortality caused by technical problems in the rearing system. However, the data provided should be enough to serve as a preliminary analysis.

Acknowledgement

This study was part of the PhD study of the first author, funded by the Ministry of Higher Education Malaysia and University Putra Malaysia. The authors would like to thank Keith Ranson and William Hamilton for helping with the fish welfare, and Denny Conway and Brian Howie for assistance with the photography.

CHAPTER 6

Paper IV: The effect of male colouration on reproductive success in Nile tilapia (*Oreochromis niloticus*)

Status: Published in Aquaculture (2010) **308**:S119-S123

Contributions: The present manuscript was compiled and written in full by the author of this thesis. Fish rearing, sampling, lab and statistical analyses were carried out by the candidate. The other co-authors contributed towards the experimental design, statistical analyses, guidance and editing of the manuscript.

The effect of male colouration on reproductive success in Nile tilapia (*Oreochromis niloticus*)

Amy H. Rajaee[a], Felicity A. Huntingford[b], Keith J. Ranson[a], Brendan J. McAndrew[a], David J. Penman[a,*]

[a]Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK

[b]University of Glasgow, Institute of Biomedicine and Life Sciences, Glasgow G12 8QQ, Lanark, Scotland, UK

*Corresponding author: Tel.: + 44 (0) 1786 467901; Fax: + 44 (0) 1786 472133; E-mail: d.j.penman@stir.ac.uk

Abstract

Red tilapia stocks have been developed for aquaculture from rare colour mutations. This study was carried out to discover if male body colour (wild type or red) influences mating success in the Nile tilapia (*Oreochromis niloticus*), by allowing females to choose between wild type and red males under semi-natural spawning conditions. In a series of eight trials, ten females (wild type females in four trials, red in the other four) were placed in an arena tank with two size-matched males, one wild type and one red. Each trial ran for five weeks, during which fish were allowed to spawn and eggs were collected from the mouths of the females after spawning. Paternity was assessed using microsatellite genotyping and phenotype scoring. Where both males contributed to a batch of fry from a single female, the batch was allocated to the "primary" sire for statistical analysis. No significant departures from equal mating success as the primary sire were observed between red and wild type males. However, there was a significant difference between the red and wild type females in the frequency of secondary paternal contribution to egg batches. These results are discussed with reference to spawning of Nile tilapia in natural and aquaculture environments.

Keywords: reproductive success, mate choice, colour, assortative mating, Nile tilapia, *Oreochromis niloticus*

1. Introduction

Colouration in fish plays important roles in many different aspects of behaviour, for example in species recognition (Couldridge and Alexander, 2002; Salzburger *et al*., 2006), sexual selection for incipient speciation (Seehausen *et al*., 1999; Knight and Turner, 2004) or as an indicator for 'good genes' (Barber *et al*., 2001). It is generally agreed that development and elaboration of male morphological traits such as colour patterns and ornaments in many species including fish are the results of sexual selection (Turner, 1993; Andersson, 1994). Female mate choice for such traits has been reported in several species, for example where females prefer males with brighter orange colouration in the guppy, *Poecilia reticulata* (Houde and Hankes, 1997; Karino and Shinjo, 2004), or males with longer fins in the green swordtail, *Xiphophorus helleri* (Basolo, 1990). However, female preferences vary, e.g. among populations (Houde and Endler, 1990; Endler and Houde, 1995, Bisazza and Pilastro, 2000), which could be due to genetic variation, developmental trajectories or environmental factors (Jennions and Petrie, 1997) which are difficult to measure.

African cichlids are famous for their diversity and richness of morphology and colour patterns (Kocher, 2004; Turner, 2007). Sexual selection has been suggested to play a major role in speciation (Seehausen, 2000; Knight and Turner, 2004) and colour patterns have been reported to be important traits for female mate choice (Seehausen and van Alphen, 1998; Couldridge and Alexander, 2002). Mate choice trials have shown that females mate assortatively (preferring to mate with males from their own population or species) in cichlids from Lake Malawi and a satellite lake (Knight and Turner, 2004; Genner *et al*., 2007).

Mutations affecting body colour have arisen in at least two species of tilapias, *Oreochromis mossambicus* and *O. niloticus* (McAndrew *et al*., 1988). Red tilapias are used widely in aquaculture and this has increased the value of the fish in certain markets (Popma and Masser, 1999). The red body colour mutation in the Stirling stock of *O. niloticus* (originating from Lake Manzala, Egypt) is controlled by a dominant allele in a single gene. It is thus possible to generate homozygous red and homozygous wild type fish from the same population, where the only consistent difference between the two types is at the red locus. The objective of the present study was to test whether female *O. niloticus* showed assortative mating when presented with a choice between wild type and red males under semi-natural spawning conditions.

Several possible outcomes of these trials were hypothesized. Firstly, as the result of sexual selection, wild type males might be expected to be preferred by females (both wild type and red). Secondly, based on imprinting, females might tend to mate with males which are more familiar to them. Since the female fish used for the trials were reared with other fish of the same phenotype only (wild type and red fish were reared and maintained separately before the experiment), it might be expected that the females would choose males of the same colour. Thirdly, mating success of red males could be higher if males with more intense red colouration are more favourable among females. Similarly, under the rare-male effect theory, females could be more attracted with males which are unfamiliar or novel to them as has been reported in feral guppies (Hughes *et al*., 1999).

2. Materials and methods

2.1 Facilities and *O. niloticus* broodstock

All mate choice trials were carried out in the tropical aquarium facilities of the Institute of Aquaculture, University of Stirling. The adult fish used in this study (homozygous wild type or homozygous red: McAndrew *et al*., 1988) were bred from stocks maintained in the facilities. Trials were carried out in a circular arena tank (radius = 1.05 m; height = 0.50 m; volume = 1.73 m^3; flow rate = 4.14 litre/minute) in a recirculating system at 27^oC. Fish were fed twice a day with trout pellets (Skretting, UK). All adult fish were PIT-tagged (Passive Integrated Transponder) for identification and a fin biopsy was removed from the soft-rayed part of the dorsal fin and preserved in 95% ethanol for DNA analysis.

2.2 Experimental design

Each trial involved ten females (either all wild type or all red, depending on the trial) and two males, one wild type and one red. The males in each trial were closely size-matched (mean difference in length = 2.2%; weight = 5.8%) with body weight of at least 9% more than the mean weight of the females. Females were transferred into the experimental tank a day earlier than the males. Each trial ran for 5 weeks, during which the fish were left to spawn naturally in the arena tank. Standard length and weight of all adult fish were taken before and after every trial.

A total of eight trials were carried out (wild type females in four trials, red in the other four), alternating between trials involving wild type and red females. Different males were used for every trial, from a variety of different families and avoiding

repeating a pairing of males from the same families (additionally, males were not taken from the same families as females used in these trials). Each set of ten females (and replacements from the same source if necessary) was used for two trials.

2.3 Egg or larvae collections and fry rearing

Observation of females to detect those carrying egg clutches was carried out daily and eggs were removed immediately from such females. The clutch was removed by netting the female, opening its mouth gently and allowing eggs or larvae to drop into a pail of water. The PIT-tag number of the female was recorded prior to returning it to the experimental tank. Eggs were then transferred into downwelling incubators until they hatched and the fry had absorbed the yolk sac. Batches of fry were grown on until at least three weeks post-hatch (approximately 15 mm in total length) for clear scoring of colour (red or wild type). Batches of fry where <10 fry survived to this stage were eliminated from the dataset.

2.4 DNA extraction

DNA was isolated from ethanol-preserved tissue using the REALPURE DNA extraction kit (REAL laboratories, Spain). Some modifications were made to the manufacturer's instructions in order to work efficiently with 96 well PCR (Polymerase Chain Reactions) plates. Up to two fin biopsy punches (approximately 0.5 cm^2 of fin tissue in total) or fry tails were digested by 3 µl of proteinase K (10mg/ml) in the presence of 75 µl cell lysis solution during overnight incubation at 55^oC (Nguyen Huu Ninh, pers. comm.). DNA concentration was quantified using a spectrophotometer (Nanodrop, ND-1000) and diluted to 60-100 ng/µl for PCR amplifications.

2.5 Microsatellites and PCR amplifications

Microsatellite markers (GM150, GM258, GM526, UNH104, UNH982, UNH985 and UNH995) were chosen from the tilapia genetic linkage map (Lee *et al*., 2005). All PCRs were performed using the tailed primer method (Boutin-Ganache *et al*., 2001). A standard primer sequence or "tail" was added to the 5'-end of either the reverse or forward primer, depending on the marker. Additional universal primer labelled with fluorescent dye (which has the tail sequence) was used to label the PCR products. PCR was carried out in 15 µl reactions containing 1X PCR buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 0.5 units Taq enzyme (Thermo Scientific, UK), 4 pmol of reverse or forward primer (non-tailed), 0.2 pmol of tailed primer, 4 pmol of labelled primer and 60-100 ng genomic DNA. PCR conditions were as follows: one cycle of 2 min at 95^{o}C, 30 cycles of 1 min at 95 ^{o}C, 1 min at 57 ^{o}C, 1 min at 72 ^{o}C, with a final extension step of 30 min at 72 ^{o}C. PCR products were first checked by electrophoresis in 1.2% agarose gels with 0.5X TAE buffer, stained with 2% ethidium bromide. Sizing of PCR products was accomplished using CEQ 8800 Genetic Analysis System automated DNA sequencer (Beckman Coulter) and allele sizes were analysed with CEQ8800 software.

2.6 Genotyping and paternity analyses

Paternity analyses were determined through microsatellite genotyping and phenotype scoring. Red colour is controlled by a single autosomal dominant allele, therefore offspring from a wild type female sired by a wild type male should be all wild type unless both parents were heterozygous for the bronze (blond) locus, where a quarter of the offspring were expected to be blond (Mc Andrew *et al*., 1988). On the

other hand, offspring from a wild type female sired by a red male should be all red. For offspring from wild type females, since paternity confirmation could be based on phenotype scoring, a small number of fry (minimum of 4 per batch) were also genotyped to confirm the results based on colour phenotype. For offspring of red females, a minimum of 10 fry per batch were taken randomly and genotyped at between two to four loci until paternity could be assigned without ambiguity.

Where both males contributed to a batch of fry from a single female, the batch was allocated to the "primary" sire (i.e. the father of the majority of the offspring in that batch) for the initial statistical analysis (comparison of the mating success of wild type and red males). In spawnings by wild type females even a single fry fathered by the "secondary" male could be detected through phenotype scoring. However, for red females, estimation of the contribution could only be based on number of samples that were genotyped (a minimum of 10, hence a minimum of 10% contribution by the secondary male).

2.7 Statistical analysis

Chi-squared tests were use to compare the mating success of wild type and red males, with a null hypothesis of equal mating success (1:1). Chi-square analysis was initially carried out on data from each single trial (apart from trial 7, where only three spawnings occurred). Fisher's exact test was then used to compare data from trials involving the same set of females. Data were pooled where no significant differences were detected, and chi-square analysis was carried out to compare pooled data against the 1:1 equal mating success null hypothesis. This process was repeated with trials

involving sets of females of the same colour, females of the two different colours and finally all trials.

To compare the frequencies of secondary male contribution, the data from wild type females and red females was split into two groups: batches with < 10% contribution from the secondary male and batches with ≥ 10% contribution (due to the minimum detection level of 10% in batches from red females). Contingency chi-square analysis was used to compare the groups. Data from the two sets of wild type females were also compared in a similar manner, but without the restriction of the 10% detection threshold (based on phenotypic scoring of all surviving fry, so secondary male contribution could be detected down to 1/n, where n = number of surviving fry: the distribution of the number of surviving fry was similar between these two sets of females, so could be eliminated as a potential source of bias).

3. Results

A total of 88 egg batches were produced throughout the whole experiment, five of which were eliminated from the analysis due to low numbers of surviving fry. Of the eight trials, one (Trial 7) gave only three egg batches, while the others gave a minimum of eight. A summary of the number of spawnings produced and the primary sires for each trial is given in Table 1. None of the individual trials showed a significant departure from the null hypothesis (1:1 ratio of wild type and red males), although Trial 7 could not be tested due to the low number of spawnings. Subsequent analyses based on hierarchically pooling data from different trials (Table 2) did not show any significant differences between trials involving the same female sets, between female

sets of the same body colour or between females of different body colours, or any departures from 1:1 among the pooled data.

Table 1: Number of spawnings and paternity in arena spawning trials in *O. niloticus.*

Trial No.	Dam Phenotype	No. of batches predominantly sired by		Total no. of spawnings	$\chi^2_{(1:1)}$ *	*P*
		Wild type ♂	Red ♂			
1	Wild type	9	6	15	0.267	0.606
2	Red	4	6	10	0.100	0.752
3	Wild type	3	6	9	0.444	0.505
4	Red	4	5	9	0.000	1.000
5	Red	7	10	17	0.235	0.628
6	Wild type	4	4	8	0.125	0.724
7	Red	3	0	3	-	-
8	Wild type	3	9	12	2.088	0.149
	Total	37	46	83		

* calculated with Yates' correction

Table 2: Comparisons between *O. niloticus* spawning trials and analyses of pooled data. For each set of trials being compared (column 1), a heterogeneity χ^2 test was first carried out to compare the proportions of primary sires (see data in Table 1), then if non-significant the data was pooled and compared to the null hypothesis of equal mating success of red and wild type males.

Female group	**Fisher's Exact Test one-tailed *P***	**Pooled data**			**χ^2 (1:1)**	
		Wild type	**Red**	**Total**	**χ^2**	***P***
Wild type ♀♀$_1$ (Trial 1 + Trial 3)	0.200	12	12	24	0.000	1.000
Wild type ♀♀$_2$ (Trial 6 + Trial 8)	0.251	7	13	20	1.800	0.180
All Wild type ♀♀ (Wild type ♀♀$_1$+Wild type ♀♀$_2$)	0.244	19	25	44	0.818	0.366
Red ♀♀$_1$ (Trial 2 + Trial 4)	0.350	8	11	19	0.474	0.491
Red ♀♀$_2$ (Trial 5 + Trial 7)	0.105	10	10	20	0.000	1.000
All Red ♀♀ (Red ♀♀$_1$+Red ♀♀$_2$)	0.432	18	21	39	0.231	0.631
All ♀♀ (Wild type + Red)	0.689	37	46	83	0.976	0.323

Contributions from both males were detected in 26 out of the 83 fry batches. Of these, 17 had at least 10% of fry being fathered by the secondary sire, up to a maximum of 40.5%. Five out of 44 batches (11%) from wild type females and 12 out of 39 batches (30%) from red females had more than 10% of fry sired by the secondary sire (Fig. 1), which was significantly different (χ^2 = 4.780, df = 1, P = 0.029). Fourteen out of 24 batches (58%) from wild type female set 1 showed some contribution from the secondary male, while three out of 20 batches (15%) from wild type female set 2 showed some contribution from the secondary male: this was marginally non-significant (χ^2 = 3.727, df = 1, P = 0.054).

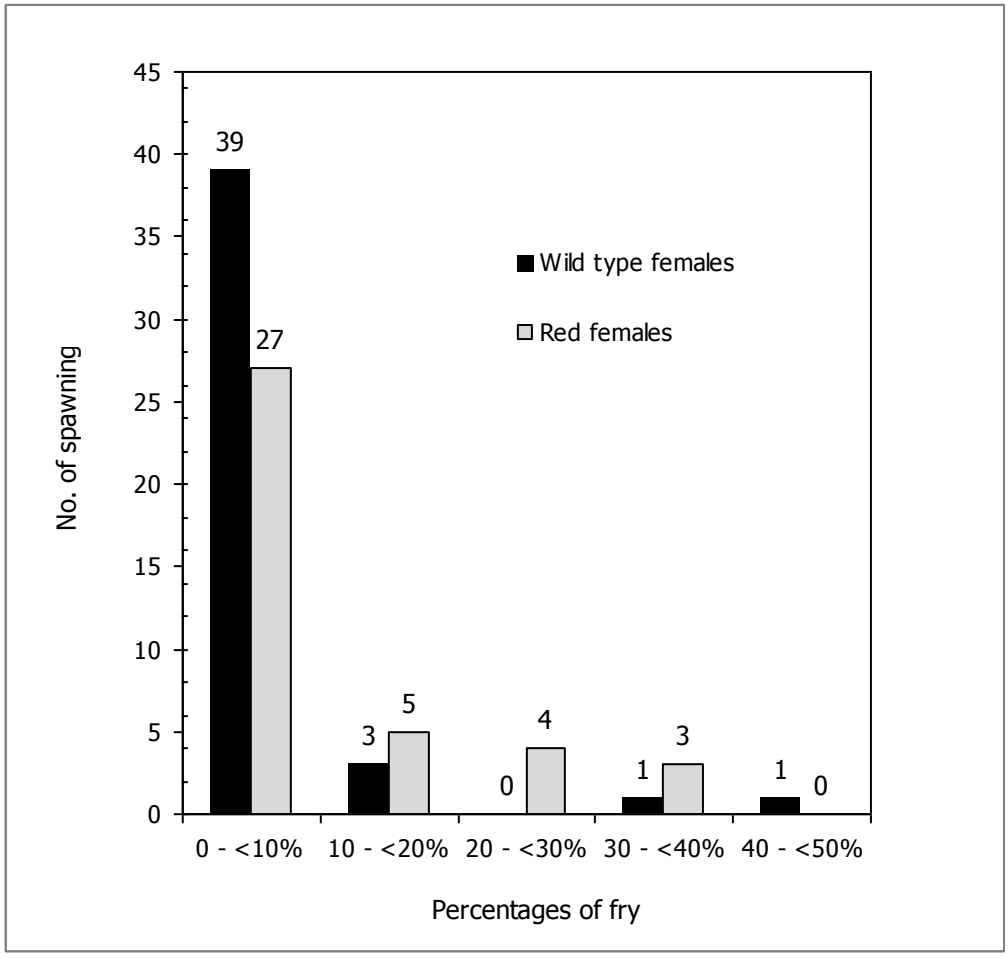


Figure 1: Percentages of fry sired by 'secondary' sire in spawnings by wild type and red female *O. niloticus*.

4. Discussion

The trials carried out in this study showed no significant difference in mating success between wild type and red males as the primary sire, suggesting that in Nile tilapia under the conditions of our study, red or wild-type colouration in males does not influence female preferences. However, the results indicate that there were differences between the wild type and red females in terms of the frequency of secondary male paternity (which occurred more often in batches of eggs from red females than from wild type females), and also suggest that there may have been differences in secondary male paternity between different groups of wild type females.

Colour-based mating may be of importance between species or populations in at least some species of cichlids, for example in the *Pseudotropheus zebra* complex, where colour pattern is the major component for species recognition (Couldridge and Alexander, 2002). Four closely-related species of the *P. zebra* complex were tested under laboratory mating and females showed significant preference in the amount of time spent with conspecific males. When conspecific males were absent, females preferred heterospecific males which showed similar colour pattern, resembling the conspecific males. Other studies on Lake Victoria cichlids were also consistent with this study (Seeuhausen, 1997; Seehausen and van Alphen, 1998; Seehausen *et al*., 1999), confirming the role of colour pattern in species recognition among closely related species.

In some species, males with more intense red colouration or carotenoid-based colour are more preferred by females. For example, in *Pundamilia nyererei*, a cichlid species from Lake Victoria of East Africa, females showed preference for male redness

under laboratory mating as well as in the field (Maan *et al*., 2004). Similar observations were also made in some population of guppies (Houde and Endler, 1990; Endler and Houde, 1995).

The experimental design used in this study could also test for a rare-male effect where the same group of females were given a choice of different males, one which is of their own phenotype and another which is unfamiliar to them. Since the stock fish used in this study were reared and maintained separately according to their phenotype before the trial took place, we can assume that each phenotype was unfamiliar with the other, in this case, red is unfamiliar with wild type, and vice versa. The rare-male effect was demonstrated in guppies, where females showed a preference for novel males and avoided mating with males that they were familiar with (Hughes *et al*., 1999). This hypothesis, however, would lead to the opposite effect to mate preference based on imprinting, where females choose males based on familiarity or prior experience (Breden *et al*., 1995; Verzijden and ten Cate, 2007). The results of the present study did not fit with either of these theories: neither wild type nor red females showed any significant preference towards males (as primary sires) based on body colour.

Although the results presented in this study show no clear evidence for colour-assortative mating in Nile tilapia, it is not necessary to conclude that colour is not be of importance for mating within this species, as other factors may also influence the role of colour in mate choice. Several possibilities that might influence mating success were controlled in these trials, for example, kinship or relatedness was minimized as far as possible by using males from different families, males were size-matched in all trials and nest size/shape was not a factor due to the absence of spawning substrate. It is possible that the absence of spawning nests reduced male territoriality and thus made it

harder for females to choose one male or the other. This may also be reflected in the multiple paternity observed, although a high frequency of multiple paternity was also observed by Fessehaye *et al*. (2006), where Nile tilapia were spawning in hapas with the hapa floor resting on the bottom of the ponds, which may have allowed nest-building by males.

The observed difference in the frequency of secondary paternity between red and wild type females was not predicted and appears to represent a more subtle level of differences in mating behaviour. Multiple paternity appears to be fairly common in *Oreochromis* (e.g., Hulata *et al*., 1981; Fessahaye *et al*., 2006, 2009) but it is not clear why body colour might influence this.

The outcome of this study is rather surprising given the strong sexual selection for colour pattern in many species of fish and particularly in cichlids. It would be interesting to carry out similar trials in more natural conditions to see if female preference for male body coloration would be observed in Nile tilapia. However, although the spawning conditions in the current study were not "natural", they are certainly relevant to aquaculture, where many hatcheries use tanks or hapas for breeding. The results of this study may also have implications for the survival of red tilapia escapees from aquaculture: it might be assumed that these would have lower survival than wild-type tilapia due to higher predation and lower mating success, but the present study suggests that mating success in mixed feral populations may be comparable to that of wild type tilapia. This would, however, have to be investigated in more natural spawning habitats than that used in the present study.

Acknowledgements

This study was part of the PhD study of the first author, funded by the Ministry of Higher Education Malaysia and University of Putra Malaysia.

CHAPTER 7

GENERAL DISCUSSION

Chapter 7 – General Discussion

The general aim of this research was to gain understanding on different aspects of body colouration in the Nile tilapia using genetic approaches as well as to provide fundamental information for further research intending to improve the red body colouration. To achieve these objectives, three main approaches were carried out. The first was by investigating the blotching and some other body colour components in the red Nile tilapia. An ontogeny analysis was carried out using clonal line and mixed sex fish and association of blotching and other body colour components with sex and tank background colour were tested. The level of blotching within the clonal line was also investigated. Using a series of crosses between red males and clonal fish, heritability of the blotching and body colour components were studied using parent-offspring regression. The second approach was by mapping the blond gene and use the linked-marker to study the effect of interaction between red and blond on blotching in red fish. Finally, the effect of body colouration on male reproductive success was tested under semi-natural spawning conditions using wild and red Nile tilapia. The outcomes of this study are discussed including the potential importance of each approach to aquaculture.

Analysis of blotching and other body colour components in the red Nile tilapia

In red tilapia culture, body colouration is one of the important traits to increase their value and improve their acceptance. Colour appearance has been one of the major factors that influence customer acceptance, as in many other species such as salmon, red sea bream and red porgy. Skin pigmentation in fish is a complex process involving cellular, genetic, physiological and environmental factors. Environmental factors are

responsible for physiological changes (reviewed by Fujii, 2000) and morphological changes (Sugimoto, 2002) in skin colouration which involve hormonal and neural signals resulting in pigments dispersion/aggregation or increase/decrease of chromatophores. Genetic influences on skin pigmentation can either be due to one or a few loci according to Mendelian inheritance or polygenic which usually have additive effects on the phenotype. Under monogenic control, the environmental effects are usually minimal and hence provide stable colour phenotypes compared to polygenic control which usually changes during various development stages, especially the colour intensity.

In this study, we investigated the factors influencing blotching and other body colour components in red tilapia, on which information is still scarce. An ontogeny analysis of the blotching and body colour components (Chapter 3 – Paper I) over a six month period starting from the age of 3 months suggested that the area covered by the blotches did not significantly change in this period. Between males and females and between tank background colours, the level of blotching were not significantly different, indicating sex and the tank colour used in this study may not have influenced the level of blotching. These results suggested that the possible effect of environmental factors (tank colour) in the red parents used in the heritability study (Chapter 4 - Paper II), in which these red fish were previously reared in a range of different background colours, was probably minimal. On the other hand, the redness component of male body colouration were always significantly higher than the females (not significant at the age of 4 and 5 months), adding one more advantage towards the production of monosex male population in red tilapia culture.

The ontogeny analysis carried out in this study was to observe the changes in colour pattern through time (developmental stages) and to suggest a suitable time to carry out image analysis. Body colour intensity were related with various physiological processes such as during sexual maturity and breeding (McLennan, 1995; Kodric-Brown, 1998; Svensson *et al*., 2005) leading to differences in pigmentation between males and females. In some species such as the salmonids, the deposition of carotenoids (which is an important source for flesh colouration) from the muscle to the skin and eggs occurred during sexual maturation in which this process usually starts earlier in the males than the females (Tacon, 1981; Leclercq *et al.*, 2010). Due to such reasons, recorded images need to be taken at a suitable time to avoid bias caused by these factors. However, since tilapia are asynchronous spawners, with variable inter-spawning interval, ranging from 7 days to a month or more (Martinez-Chavez, 2008), so it is very hard to standardized a fixed time to undertake the image analysis.

As mentioned in earlier chapters, one limitation to this study was the inability to measure the red blotches due to their generally diffuse shapes. Although some of the offspring showed red blotching which was very defined and concentrated, this was not the case for most of the red blotched areas observed. Due to this limitation, we were unable to study the correlation between the black and the red blotched areas. Instead, we studied the correlation between overall body redness and the black blotched area which showed a significant positive correlation at one of the sampling points but only for female offspring. This does suggest that any attempts to increase body redness may result in increasing black blotching in the red fish. In the present study, when measuring body colour components, the numbers of fish represented per family were variable (since data were divided between males and females). This unbalanced data

may influence the estimation of genetic parameters and consequently the accuracy of estimation of heritability or correlation values.

From the results, heritability for blotching was not significantly different from zero indicating it was not an additive trait. Blotching was assumed to be hypostatic to the red gene, since it can only be expressed in the red fish but was completely suppressed in the wild type (McAndrew *et al*., 1988). The use of clonal line for this study was for convenience, as it provided constant contributions from the mother for all the crosses and allowed observation of the inheritance of this trait from one parent only. Parent-offspring regressions are usually of three types, two single parent-offspring regressions (plotting offspring mean versus either the father or mother trait phenotypic variance) or midparent-offspring regression in which the offspring mean is plotted against the mean of their parent. Single-parent regressions are usually restricted to fathers since mother-offspring regressions are usually biased due to maternal effect contributions (Lynch and Walsh, 1998). Perhaps a more complex mating design should be used, for example the partial diallel crosses which will allow estimation on other variance components such as variance within and between crosses. More crosses should also be used to reduce the error and increase the possibility to detect significant correlations.

As reviewed by some authors, pigment pattern formation in teleosts at a given developmental stage is often under strong genetic control (Parichy, 2006; Mills and Patterson, 2009). In the zebrafish (*Danio rerio*), adult pigment patterns is either arise by reorganization of the early larval pigments to suit the adult patterns or it could comprise of two separate populations of cells (Parichy, 2006). Pigmentation development in the summer flounder (*Paralichthys dentatus*) also consists of two

developmental stages, in which at the metamorphosis stage, a second wave of melanophores development occurred (Bolker *et al*., 2005). Similar observations in the Japanese flounder has also been reported (Matsumoto and Seikai, 1992). Genetic control is often the principal factor for fish colouration, however, environmental and hormonal factors also play a role especially in morphological changes. Environmental aspects such as background colour (Doolan *et al*., 2008), diets (Dickey-Collas, 1993) and light intensity (Pavlidis *et al*., 2008) need to be controlled before recording or measuring skin colouration. Taking the measurement when physiological processes such as sexual maturity and smolting stages occurred should also be avoided since colour expression may be altered during this processes. In relation with this present study, a further approach to tackle these physiological processes needs to be achieved, perhaps by incorporating the information of reproductive stages while measuring the variation of pigmentation, although this may be complex to apply. In addition, pigment analyses by HPLC (chromatographic system) may serve as a good addition towards the study to identify pigments and their concentrations.

Interaction of blond and red towards blotching: Genetic mapping of the blond locus

Blond (also known as bronze) is a colour mutant with much reduced melanophores, first reported by Scott *et al*. (1987). This mutant colour is inherited as a simple autosomal recessive and it was hypothesized that this may help to reduce the appearance of blotching in red fish. In this study, linkage mapping of this gene was able to establish the red and blond as separate loci in the tilapia linkage map (Chapter 5

– Paper III). The gene responsible for red phenotype was previously reported to be in LG3 (Lee *et al*., 2005), meanwhile blond was found to be in LG5 in the present study. The closest marker from the intraspecific map was about 2.5 cM to one side of the blond locus and 15.5 cM to the other side. Six microsatellite markers were significantly associated with the blond locus and five of these were used to distinguish RrBlbl and Rrblbl fish. However, preliminary analysis on the interaction between red and blond on blotching resulted in only slight fading of the blotches in Rrblbl fish and the effect was not sufficient to improve the appearance of the red fish. The potential of blond for commercial purposes may be an advantage due to no 'bruised' appearance after death. However, there has been very few reports on this mutant colour, despite its potential to be developed commercially. Scott *et al.* (1987) suggested that it is possible that the blond may be at a behavioural drawback due to the incapability of the individual fish to display social patterning, but to date no studies were reported to our knowledge. The blond may as well be more visible to predators due to lighter body colouration. Further identification of the blond gene could be performed by a comparative genomic approach looking for synthenic regions of LG5 with the medaka genome.

The effect of male colouration on reproductive success in Nile tilapia

The importance of colouration in mate choice has been observed in many species of fish. In African cichlids, colouration is one of the important traits for female mate choice (Seehausen and van Alphen, 1998) and a major component for species recognition (Couldridge and Alexander, 2002). In this study, preference for male colouration was tested using a series of red and wild type females under semi-natural

spawning conditions (Chapter 6 – Paper IV). The study did not reveal any preference for colouration although secondary paternity between red and wild type females showed a more subtle level of variance in mating behaviour. Multiple paternity in Nile tilapia culture occurs fairly commonly (Fessehaye *et al.*, 2006; 2009) under commercial breeding condition although there is no apparent association with body colouration. Given the strong sexual selection for colour pattern usually observed in many species of fishes, especially the cichlids, it was quite surprising that this study did not find any preference for colouration. Although the study was carried out under semi-natural spawning conditions, this is a normal practice in tilapia culture as hatcheries use tanks or hapas to carry out breeding. The existence of red tilapia in the wild is rarely reported, therefore it is presumed that escapees from aquaculture may be subject to lower survival due to higher predation or lower reproductive success. The results from this study indicated that mating success in mixed feral populations may be similar to the wild type although this would need to be confirmed through further investigations under more natural spawning habitats. In addition, it will be also interesting to study the mating success in the blond fish against red and wild type.

CONCLUSIONS

The present research project improved our understanding on different aspects of body colouration in the Nile tilapia, in particular on the fundamental aspects of the blotching phenotype about which information was very scarce. By providing background knowledge, it is anticipated that the present findings will be able to facilitate further research towards commercial application as well as to enhance fundamental knowledge.

Some drawbacks of this work, mainly on the analysis of blotching and body colour components, which are still inconclusive, require more investigations to further incorporate and validate the current findings. Future work may include a different approach on the colour analysis or perhaps using a more complex mating design to include measurements of other variance components.

Using genetic approaches, such as the use of molecular markers to assist study on genes interaction and for paternity analyses were very helpful and made it possible to carry such study at the molecular level. Future work could include the search of candidate gene responsible for blond which will supply an additional piece of information for the tilapia genome.

REFERENCES

REFERENCES

Andersson, M. 1994. Sexual Selection. Princeton University Press, NJ.

Avtalion, R. R., Reich, L. 1989. Chromatophore inheritance in red tilapias. *The Israeli Journal of Aquaculture Bamidgeh* **41**: 98-104.

Bagnara, J. T., Matsumoto, J. 2006. Comparative anatomy and physiology of pigment cells in nonmammalian tissues. In: *The Pigmentary System*. Blackwell Publishing Ltd. (pp. 11-60).

Bagnara, J. T., Fernandez, P. J., Fujii, R. 2007. On the blue coloration of vertebrates. *Pigment Cell Research* **20**: 14-26.

Bakker, T.C.M. 1993. Positive genetic correlation between female preference and preferred male ornament in stickleback. *Nature* **363**:255-257.

Bakker, T. C. M., Mundwiler, B. 1994. Female mate choice and male red coloration in a natural three-spined stickleback (*Gasterosteus aculeatus*) population. *Behavioral Ecology* **5**: 74-80.

Balarin, J. D., Haller, R. D. 1982. The intensive culture of tilapia in tanks, raceways and cages: In *Recent Advances in Aquaculture* (ed. by J. F. Roberts, R. J. Muir), Croom Helm Ltd., London & Canberra (pp. 265-355).

Balarin, J. D., Hatton, J. P. 1979. Tilapia: A Guide to their Biology and Culture in Africa. *Pisces Press, University of Stirling, Scotland*: (147p)

Bangkok Post. 2010. http://www.bangkokpost.com/business/economics/203228/a-fish-for-all-reasons

Barber, I., Arnott, S.A., Braithwaite, V.A., Andrew, J., Huntingford, F.A. 2001. Indirect fitness consequences of mate choice in sticklebacks: offspring of brighter males grow slowly but resist parasitic infections. *Proceedings of the Royal Society B* **268**:71-76.

Baroiller, J. F., D'Cotta, H., Bezault, E., Wessels, S., Hoerstgen-Schwark, G. 2009. Tilapia sex determination: Where temperature and genetics meet. *Comparative biochemistry and physiology. Part A, Molecular and Integrative Physiology* **153**: 30-38.

Baroiller, J. F., Toguyeni, A. 2004. The Tilapiini tribe: environmental and social aspects of reproduction and growth. In: *Fisheries and Aquaculture, Encyclopedia of Life Support Systems (EOLSS), Developed under the Auspices of the UNESCO* (ed. by P. Safran). Eolss Publishers, Oxford, UK.

Basiao, Z. U., Doyle, R. W. 1999. Test of size-specific mass selection for Nile tilapia, *Oreochromis niloticus* L., cage farming in the Philippines. *Aquaculture Research* **30**: 373–378.

Basolo, A.L. 1990. Female preference for male sword length in the green swordtail, *Xiphophorus helleri* (Pisces: Poeciliidae). *Animal Behaviour* **40**:332-338.

Behrends, L. L., Nelson, R. G., Smitherman, R. O., Stone, N. M. 1982. Breeding and culture of the red-gold colour phase of tilapia. *Journal of World Mariculture Society* **13**: 210-220.

Beveridge, M. C., McAndrew, B. J. (Eds.). 2000. *Tilapias: Biology and Exploitation*. Dordrecht: Kluwer Academic Press.

Bisazza, A., Pilastro, A. 2000. Variation of Female Preference for Male Coloration in the Eastern Mosquitofish *Gambusia holbrooki.* Behaviour Genetics **30**(3):207-212.

Bishoff, R. J., Gould, J. L., Rubenstein, D. I. 1985. Tail size and female choice in the guppy (*Poecilia reticulata*). *Behavioral Ecology and Sociobiology* **17**: 253-255.

Blais, J., Plenderleith, M. Rico, C., Taylor, M. I., Seehausen, O., van Oosterhout, C., Turner, G. F. 2009. Assortative mating among Lake Malawi cichlid fish populations is not simply predictable from male nuptial colour. *BMC Evolutionary Biology* **9**:53.

Blanc, J-M., Chevassus, B., Krieg, F. 1994. Inheritance of the number of red spots on the skin of the brown trout. *Aquatic Living Resources* **7**:133-136.

Bolivar, R. B., Newkirk, G. F. 2002. Response to within family selection for body weight in Nile tilapia (*Oreochromis niloticus*) using a single-trait animal model. *Aquaculture* **204**: 371-381.

Bolivar, R., Mair, G., Fitzsimmons, K. 2004. New Dimensions in Farmed Tilapia: In *Proceedings of the Sixth International Symposium on Tilapia in Aquaculture*. American Tilapia Association, Aquaculture CRSP, and Ministry of Agriculture, Philippines. Manila, Philippines. (p. 854).

Bolker, J. A., Hill, C. R. 2000. Pigmentation development in hatchery-reared flatfishes. *Journal of Fish Biology* **56**:1029-1052.

Bolker, J. A., Hakala, T. F., Quist, J. E. 2005. Pigmentation development, defects, and patterning in summer flounder (*Paralichthys dentatus*). *Zoology* **108**:183-193.

Bongers, A. B. J., Sukkel, M., Gort, G., Komen, J., Richter, C.J.J. 1998. Development and use of genetically uniform strains of common carp in experimental animal research. *Laboratory Animals* **32**:349-363.

Borowsky, R. 1984. The Evolutionary Genetics of *Xiphophorus*. In: *Evolutionary Genetics of Fishes* (ed. By B.J. Turner). Plenum Press, New York (pp. 235-310).

Boutin-Ganache, I., Raposo, M., Raymond, M., Deschepper, C.F., 2001. M13-tailed primers improved the readability and usability of microsatellites analyses performed with two different allele-sizing methods. Biotechniques 31, 24-28.

Braasch, I., Volff, J.-N., Schartl, M. 2008. The evolution of teleost pigmentation and the fish-specific genome duplication. *Journal of Fish Biology* **73**: 1891-1918.

Braithwaite, V. A., Barber, I. 2000. Limitations to colour-based sexual preferences in three-spined sticklebacks (*Gasterosteus aculeatus*). *Behavioral Ecology and Sociobiology* **47**: 413-416.

Breden, F., Novinger, D., Shubert, A. 1995. The effect of experience on mate choice in the Trinidad guppy, *Poecilia reticulata. Environmental Biololgy of Fish* **42:**323-328.

BroadInstitute. 2011. http://www.broadinstitute.org/models/tilapia.

Brooks, R., Endler, J. A. 2001. Female guppies agree to differ: Phenotypic and genetic variation in mate-choice behaviour and the consequences for sexual selection. *Evolution* **55**:1644-1655.

Chatzifotis, S., Vaz Juan, I., Kyriazi, P., Divanach, P., Pavlidis, M. 2011. Dietary carotenoids and skin melanin content influence the colouration of farmed red porgy (*Pagrus pagrus*). 2009. *Aquaculture Nutrition* **17**:e90-e100.

Chauhan, T., Rajiv, K. 2010. Molecular markers and their applications in fisheries and aquaculture. *Advances in Bioscience and Biotechnology* **01**: 281-291.

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

Cnaani, A., Kocher, T. D. 2008. Sex-linked markers and microsatellite locus duplication in the cichlid species *Oreochromis tanganicae*. *Biology Letters* **4**: 700-703.

Contreras-Sanchez, W., Fitzsimmons, K. 2006. Tilapia, Sustainable Aquaculture from the New Millennium. In: *Proceedings of the Seventh International Symposium on Tilapia in Aquaculture. American Tilapia Association*. Aquaculture CRSP (p. 389).

Couldridge, V.C., Alexander, G.J. 2002. Color patterns and species recognition in four closely related species in Lake Malawi cichlid. *Behavioural Ecology* **13**:59-64.

Cubillos, E. R. 2000. Analysis of the factors related with mate choice and reproductive success in male three-spined sticklebacks. *Journal of Fish Biology* **56**: 1201-1216.

Danzmann, R. G., Gharbi, K. 2007. Linkage Mapping in Aquaculture Species. In: *Aquaculture Genome Technologies* (ed. by Zhanjiang John Liu), Blackwell Publishing Ltd. (pp. 139-167).

Darwin, C. 1871. The descent of Man, and selection in relation to sex. Murray, London.

David, L., Rothbard, S., Rubinstein, I., Katzman, H., Hulata, G., Hillel, J., Lavi, U. 2004. Aspects of red and black color inheritance in the Japanese ornamental (Koi) carp (*Cyprinus carpio* L.). *Aquculture* **233**:129-147.

Dickey-Collas, M. 1993. The occurrence of juvenile pigmentation abnormalities in plaice *Pleuronectes platessa* larvae fed on enriched and unenriched Artemia salina nauplii. *Journal of Fish Biology* **42**:787–795.

Dominey, W. J. 1984. Effects of sexual selection and life history on speciation: species flocks in African cichlids and Hawaiian Drosophila. In *Evolution of fish species flocks* (ed. A. A. Echelle & I. Kornfield), pp. 231–250. Orono, ME: University of Maine at Orono Press.

Doolan, B. J., Booth, M. A., Jones, P. L., Allan, G. L. 2007. Effect of cage colour and light environment on the skin of Australian snapper *Pagrus auratus* (Bloch & Schneider, 1801). *Aquaculture Research* **38**:1395-1403.

Doolan, B. J., Allan, G. F., Booth, M. A., Jones, P. L. 2008. Effects of cage netting colour and density on the skin pigmentation and stress response of Australian snapper *Pagrus auratus* (Bloch & Schneider, 1801). *Aquaculture Research* **39**:1360-1368.

Ducrest, A-L., Keller, L., Roulin, A. 2008. Pleiotropy in the melanocortin system, coloration and behavioural syndromes. *Trends in Ecology and Evolution* **23**(9):502-510.

Egger, B., Obermuller, B., Eigner, E., Sturmbauer, C., Sefc, K. M. 2008. Assortative mating preferences between colour morphs of the endemic Lake Tanganyika cichlid genus Tropheus. *Hydrobiologia* **615**: 37-48.

Eknath, A. E., Hulata, Gideon. 2009. Use and exchange of genetic resources of Nile tilapia (*Oreochromis niloticus*). *Reviews in Aquaculture* **1**: 197-213.

El-Sayed, A. M. 2006. *Tilapia Culture*. CABI Publishing.

Elghobashy, H., Fitzsimmons, K., Diab, A. S. 2008. From the Pharaohs to the Future. In: *Proceedings of the Eighth International Symposium on Tilapia in Aquaculture* (ed. by H. Elghobashy, K. Fitzsimmons, & A. S. Diab). Egypt Ministry of Agriculture, Cairo, Egypt (p. 1447).

Endler, J.A., Houde, A.E. 1995. Geographic variation in female preferences for male traits in *Poecilia reticulata*. *Evolution* **49**:456-468.

Ezaz, M. T. Harvey, S. C., Boonpakdee, C., Teale, A. J., McAndrew, B. J., Penman, D. J. 2004. Isolation and physical mapping of sex-linked AFLP markers in Nile tilapia (*Oreochromis niloticus*, L.). *Marine Biotechnology* **6**: 435-445.

FAO. 2007. *Fishery and Aquaculture Statistics*. Rome, Italy.

FAO. 2008a. *The state of World Fisheries and Aquaculture*. Rome, Italy.

FAO. 2008b. *World aquaculture production by species groups*. Rome, Italy.

Ferguson, A., Taggart, J. B., Prodohl, P. A., McMeel, O., Thompson, C., Stone, C., McGinnity, P., Hynes, R. A. 1995. The application of molecular markers to the study and conservation of fish populations, with special references to *Salmo*. *Journal of Fish Biology* **47**: 103-126.

Fernandez, A. A., Morris, M. R. 2008. Mate choice for more melanin as a mechanism to maintain a functional oncogene. *Proceedings of the National Academy of Sciences of the United States of America* **105**: 13503-13507.

Ferreira, T. A., Rasband, W. 2010. The ImageJ User Guide Version 1.43. 189p.

Ferrer, C., Solano, F. & Zuasti, A. 1999. Ultrastructural and biochemical analysis of epidermal xanthophores and dermal chromatophores of the teleost *Sparus aurata*. *Histology and Histopathology* **14**: 383-390.

Fessehaye, Y., El-bialy, Z, Rezk, M.A., Crooijmans, R., Bovenhuis, H., Komen, H. 2006. Mating systems and male reproductive success in Nile tilapia (*Oreochromis niloticus*) in breeding hapas: a microsatellite analysis. *Aquaculture* **256**:148-158.

Fessehaye, Y., Bovenhuis, H., Rezk, M.A., Crooijmans, R., van Arendonk, J.A.M., Komen, H. 2009. Effects of relatedness and inbreeding on reproductive success of Nile tilapia (*Oreochromis niloticus*). *Aquaculture* **294**:180-186.

Ferrer, C., Solano, F., Zuasti, A. 1999. Ultrastructural and biochemical analysis of epidermal xanthophores and dermal chromatophores of the teleost *Sparus aurata*. *Histology and Histopathology* **14**: 383-390.

Fincham, J. R. S. 1994. *Genetic Analysis: Principles, Scope and Objectives*. Blackwell Science Ltd., Great Britain.

Fishelson, L., Yaron, Z. 1983. International Symposium on Tilapia in Aquaculture. Tel Aviv University Press, Tel Aviv.

Fitzsimmons, K. 1997. Tilapia Aquaculture. In: *Proceedings of the Fourth International Symposium on Tilapia in Aquaculture* (ed. by K. Fitzsimmons). Northeast Regional Agricultural Engineering Service. Ithaca, New York (p. 808).

Fitzsimmons, K., Carvalho, J. 2000. Tilapia Aquaculture in the 21st Century. In: *Proceedings of the Fifth International Symposium on Tilapia in Aquaculture*. Ministry of Agriculture Brazil and Aquaculture CRS. Rio de Janeiro (p. 682).

Fletcher, H. L., Hickey, G. I., Winter, P. 2007. *Genetics: Instant Notes*. Taylor and Francis Group. Oxon, United Kingdom.

Franck, D., Muller, A., Rogmann, N. 2003. A colour and size dimorphism in the green swordtail (population Jalapa): female mate choice, male-male competition, and male mating strategies. *Acta ethologica* **5**: 75-79.

Fujii, R. 1969. Chromatophores and pigments. In: *Fish Physiology Volume III* (ed. by W. S. Hoar & D. J. Randall). Academic Press, New York (p. 307).

Fujii, R. 1993a. Coloration and chromatophores. In: *The Physiology of Fishes* (ed. by D. H. Evans). CRS Press, Boca Raton, Florida (pp. 535-562).

Fujii, R. 1993b. Cytophysiology of fish chromatophores. *International Review of Cytology* **143**: 191-255.

Fujii, R. 2000. Review: The regulation of motile activity in fish chromatophores. *Pigment Cell Research* **13**: 300-319.

Fujii, R., Hayashi, H., Toyohara, J. & Nishi, H. 1991. Analysis of the reflection of light from motile iridophores of the dark sleeper, *Odontobutis obscura*. *Zoological Science* **8**: 461-470.

Fujii, R., Yamada, T., Fujita, T. & Oshima, N. 1997. An improved photoelectric method for recording motile responses of indvidual leucophores. *Zoological Science* **14**: 549-558.

Galman O. R., Avtalion, R. R. 1983. A preliminary investigation of the characteristics of red tilapias from the Philippines and Taiwan. In: *Proceedings of the International Symposium on Tilapia in Aquaculture* (ed. by L. Fishelson and Z. Yaron). Nazareth, Israel, Tel Aviv University, Tel Aviv. (pp. 291-301).

Galman, O. R., Moreau, J., Avtalion, R. R. 1988. Breeding characteristics and growth performances of Philippine Red tilapia. In: *The Second International Symposium on Tilapia in Aquaculture* (ed. by R. S. V. Pullin, T, Bhukasawan, K. T. Tonguthai & J. L. McLean). *ICLARM Conference Proceedings*, Manila. (pp.169-175).

Garduno-Lugo, M., Granados-Alvarez, I., Olvera-Novoa, M. A., Munoz-Cordova, G. 2003. Comparison of growth, fillet yield and proximate composition between

Stirling Nile tilapia (wild type) (*Oreochromis niloticus*, Linnaeus) and red hybrid tilapia (Florida red tilapia x Stirling red *O. niloticus*) males. *Aquaculture Research* **34**: 1023-1028.

Garduno-Lugo, M., Munoz-Cordova, G., Olvera-Novoa, M. 2004. Mass selection for red colour in *Oreochromis niloticus* (Linnaeus 1758). *Aquaculture Research* **35**: 340-344.

Genner, M.J., Nichols, P., Carvalho, G.R., Robinson, R.L., Shaw, P.W., Smith, A., Turner, G.F. 2007. Evolution of a cichlid fish in a Lake Malawi satellite lake. *Proceedings of the Royal Society B* **274**:2249-2257.

Goodrich, H. B., Hill, G. A., Arrick, M. S. 1941. The chemical identification of gene-controlled pigments in *Platypoecilus* and *Xiphophorus* and comparisons with other tropical fish. *Genetics* **26**: 573-586.

Gomelsky, B., Cherfas, N. B., Ben-Dom, N., Hulata, G. 1996. Color inheritance in ornamental (Koi) carp (*Cyprinus carpio* L.) inferred from color variability in normal and gynogenetic progenies. *Israelli Journal of Aquaculture Bamidgeh* **48**: 219–230.

Gomelsky, B., Cherfas, N. B., Hulata, G. 1998. Studies on the inheritance of black patches in ornamental (Koi) carp. *Israelli Journal of Aquaculture Bamidgeh* **50**: 134–139.

Gouveia, L., Rema, P., Pereira, O., Empis, J. 2003. Colouring ornamental fish (*Cyprinus carpio* and *Carassius auratus*) with microalgal biomass. *Aquaculture Nutrition* **9**:123-129.

Green, J.A., Baker, B.I. 1991. The influence of repeated stress on the release of melanin concentrating hormone in the rainbow trout. *Journal of Endocrinology* **128:**261-266.

Guerrero, R. D. 1987. *Tilapia Farming in the Philippines*. Manila, Philippines.

Haesler, M. P., Seehausen, O. 2005. Inheritance of female mating preference in a sympatric sibling species pair of Lake Victoria cichlids: implications for speciation. *Proceedings of the Royal Society B* **272**: 237-45.

Hama, T., Hiyama, Y. 1966. Amelanotic Melanophore in Color Varieties of the Medaka, *Oryzias latipes*. **42**: 929-933.

Han, D., Xie, S., Lei, W., Zhu, X., Yang, Y. 2005. Effect of light intensity on growth, survival and skin colour of juvenile Chinese longsnout catfish (*Leiocassis longirostris* Gunther). *Aquaculture* **248**:299-306.

Hancz, C., Magyary, I., Molnar, T., Sato, S., Horn, P., Taniguchi, N. 2003. Evaluation of color intensity enhanced by paprika as feed additive in goldfish and koi carp using computer-assisted image analysis. *Fisheries Science* **69**:1158-1161.

Head, W. D., Zerbi, A., Watanabe, W. O. 1994. Preliminary observations on the marketability of saltwater-cultured Florida Red tilapia in Puerto Rico. *Journal of the World Aquaculture Society* **25**:432-441.

Heuschele, J., Mannerla, M., Gienapp, P., Candolin, U. 2009. Environment-dependent use of mate choice cues in sticklebacks. *Behavioral Ecology* **20**: 1223-1227.

Hilsdorf, A. W. S., Penman, D. J., Farias, E. C., McAndrew, B. J. 2002. Melanophore appearance in wild and red tilapia embryos. *Pigment Cell Research* **15**: 57-61.

Hirata, M., Kei-ichiro, N., Kanemaru, T., Shibata, Y., Kondo, S. 2003. Pigment cell organization in the hypodermis of zebrafish. *Developmental Dynamics* **227**: 497-503.

Houde, A.E., Endler, J.A. 1990. Correlated evolution of female mating preferences and male color patterns in the guppy *Poecilia reticulata*. *Science* **248**:1405-1408.

Houde, A.E., Hankes, M.A. 1997. Evolutionary mismatch of mating preferences and male colour patterns in guppies. *Animal Behaviour* **53**:343-351.

Houde, Anne E., Torio, A. J. 1992. Effect of parasitic infection on male color pattern and female choice in guppies. *Behavioral Ecology* **3**: 346-351.

Huang, C. M., Chang, S. L., Cheng, H. J., Liao, I. C. 1988. Single gene inheritance of red body coloration in Taiwanese Red tilapia. *Aquaculture* **74**: 227-232

Hughes, K.A., Du, L., Rodd, F.H., Reznick, D.N. 1999. Familiarity leads to female mate preference for novel males in the guppy, *Poecilia reticulata*. *Animal Behaviour* **58**:907-916.

Hulata, G., Rothbard, S., Avtalion, R.R. 1981. Evidence for multiple paternity in *Sarotherodon* broods. *Aquaculture* **25**:281-283.

Hulata, G., Karplus, I., Harpaz, S. 1995. Evaluation of some red tilapia strains for aquaculture: growth and color segregation in hybrid progeny. *Aquaculture Research* **26** (10):765-771.

Hussain, M. G. 1994. Genetics of body color inheritance in Thai and Egyptian Red tilapia strains. *Asian Fisheries Science* **7**: 215-224.

Hussain, M. G. 2004. Farming of Tilapia: Breeding Plans, Mass Seed Production and Aquaculture Techniques. *Momin Offset Press, Bangladesh* (149p).

Hussain, M.G., Penman, D. J., McAndrew, B. J. 1993. Suppression of first cleavage in the Nile tilapia, *Oreochromis niloticus* L. – A comparison of the relative effectiveness of pressure and heat shocks. *Aquaculture* **111**:263-270.

Iga, T., Matsuno, A. 1986. Motile iridophores of a freshwater goby, *Odontobutis obscura*. *Cell and Tissue Research* **244**: 165-171.

Jauncey, K., Ross, B. 1982. *A Guide to Tilapia Feeds and Feeding*. Scotland, UK: Institute of Aquaculture, University of Stirling.

Jennions, M.D., Petrie, M. 1997. Variation in mate choice and mating preferences: A review of causes and consequences. *Biological Reviews* **72**:283-327.

Johnson, J. B., Basolo, A. L. 2003. Predator exposure alters female mate choice in the green swordtail. *Behavioral Ecology* **14**: 619-625.

Jordan, R. C. 2008. Color-Based Association Among Heterospecifics in Lake Malaŵi Rock-Dwelling Cichlids. *Ethology* **114**: 272-278.

Kaleta, K. 2009. Morphological analysis of chromatophores in the skin of trout. *Bull. Vet. Inst. Pulawy* **53:**117-121.

Karayucel, I., Ezaz, M. T., Karayucel, S., McAndrew, B. J., Penman, D. J. 2004. Evidence for two unlinked "sex reversal" loci in the Nile tilapia, *Oreochromis niloticus*, and for linkage of one of these to the red body colour gene. *Aquaculture* **234**: 51-63.

Karino, K., Shinjo, S. 2004. Female mate preference based on male orange spot patterns in the feral guppy *Poecilia reticulata* in Japan. *Ichthyological Research* **51**:316-320.

Kasukawa, H., Oshima, N. 1987. Divisionistic generation of skin hue and the change of shade in the scalycheek damselfish, *Pomacentrus lepidogenys*. *Pigment Cell Research* **1**: 152-157.

Katagiri, T., Kidd, C., Tomasino, E., Davis, J. T., Wishon, C., Stern, J. E., Carleton, K. L., Howe, A. E., Kocher, T. D. 2005. A BAC-based physical map of the Nile tilapia genome. *BMC Genomics* **6**: 89.

Kato, K., Hayashi, R.,Yuasa, D.,Yamamoto, S., Miyashita, S., Murata, O., Kumai, H., 2002. Production of cloned red sea bream, *Pagrus major*, by chromosome manipulation. *Aquaculture* **207**:19–27.

Kause, A., Ritola, O., Paananen, T., Eskelinen, U., Mantysaari, E. 2003. Big and beautiful? Quantitative genetic parameters for appearance of large rainbow trout. *Journal of Fish Biology* **62:**610-622.

Kelsh, R. N. 2004. Review: Genetics and Evolution of Pigment Patterns in Fish. *Pigment Cell Research* **17**: 326-336.

Kelsh, R. N., Parichy, D. M. 2008. Pigmentation. In: *Fish larval physiology* (ed. by R. N. Finn & B. G. Kapoor). Science Publishers, Enfield, NH. (pp. 27-49).

Kelsh, R. N., Schmid, B. & Eisen, J. S. 2000. Genetic analysis of melanophore development in zebrafish embryos. *Developmental biology* **225**: 277-93.

Kimler, V. A., Taylor, J. D. 2002. Morphological studies on the mechanisms of pigmentary organelle transport in fish xanthophores and melanophores. *Microscopy Research and Technique* **58**: 470-80.

Kittilsen, S., Schjolden, J., Beitnes-Johansen, I., Shaw, J. C., Pottinger, T. G., Sorensen, C., Braastad, B. O., Bakken, M., Overli, O. 2009. Melanin-based skin spots reflect stress responsiveness in salmonids fish. *Hormones and Behaviour* **56**:292-298.

Knight, M.E., Turner, G.F. 2004. Laboratory mating trials indicate incipient speciation by sexual selection among populations of the cichlid fish *Pseudotropheus zebra* from Lake Malawi. *Proceedings of the Royal Society B* **271**: 675-680.

Kocher, T. D., Lee, W. J., Sobolewska, H., Penman, D. J., McAndrew, B, J. 1998. A genetic linkage map of a cichlid fish, the tilapia (*Oreochromis niloticus*). *Genetics* **148**: 1225-1232.

Kocher, T.D. 2004. Adaptive evolution and explosive speciation: The cichlid fish model. *Nature Reviews Genetics* 5:288-298.

Kodric-Brown, A. 1998. Sexual dichromatism and temporary color changes in the reproduction of fishes. *American Zoologist*. **38**:70-81.

Koedprang, W., Ohara, K. Taniguchi, N. 2000. Genetic and environmental variances on growth and reproductive traits of silver crucian carp *Carrassius langsdorfii* using communal and separate rearing system. *Fisheries Sciences* **66**:1092-1099.

Komen, H., Thorgaard, G. H. 2007. Androgenesis, gynogenesis and the production of clones in fishes: A review. *Aquaculture* **269**:150-173.

Koren, A., Pruginin, Y., Hulata, G., 1994. Evaluation of some red tilapia strains for aquaculture: I. Color inheritance. *Israelli Journal of Aquaculture Bamidgeh* **46** (1): 9–12

Lamoreux, M. L., Kelsh, R. N., Wakamatsu, Y., Ozato, K. 2005. Pigment pattern formation in the medaka embryo. *Pigment Cell Research* **18**: 64-73.

Le Douarin, N., Kalcheim, C. 1999. *The Neural Crest*. Cambridge University Press.

Leclercq, E., Taylor, J. F., Migaud, H. 2010. Morphological skin colour changes in teleosts. *Fish and Fisheries* **11**: 159-193.

Lee, B. Y., Penman, D. J., Kocher, T. D. 2003. Identification of a sex-determining region in Nile tilapia (*Oreochromis niloticus*) using bulked segregant analysis. *Animal Genetics* **34**: 379-83.

Lee, B. Y., Hulata, G., Kocher, T . D. 2004. Two unlinked loci controlling the sex of blue tilapia (*Oreochromis aureus*). *Heredity* **92**: 543-9.

Lee, B. Y., Lee, W. J., Streelman, J. T., Carleton, K. L., Howe, A. E., Hulata, G., Slettan, A., Stern, J. E., Terai, Y., Kocher, T. D. 2005. A second-generation genetic linkage map of tilapia (*Oreochromis spp*.). *Genetics* **170**:237-44.

Liao, I. C., Chang, S. L. 1983. Studies on the feasibility of red tilapia culture in saline water. *Proceedings of the First International Symposium on Tilapia in Aquaculture.* pp. 524–533.

Lim, C. E., Webster, C. D. (Eds). 2009. *Tilapia biology, culture and nutrition*. The Haworth Press Inc., New York.

Liu, Z. J., Cordes, J. F. 2004. DNA marker technologies and their applications in aquaculture genetics. *Aquaculture* **238**: 1-37.

Lo Presti, R., Lisa, C., Di Stasio, L. 2009. Molecular genetics in aquaculture. *Italian Journal of Animal Science* **8**: 299-313.

Logan, D. W., Burn, S. F., Jackson, I. J. 2006. Regulation of pigmentation in zebrafish melanophores. *Pigment Cell Research* **19**: 206-13.

Lynch, M., Walsh, B. 1998. Genetics and Analysis of Quantitative Traits. *Sinauer Associates, Sunderland, M.A.* (980p).

Maan, M.E., Seehausen, O., Soderberg, L., Johnson, L., Ripmeester, E.A.P., Mrosso, H.D.J., Taylor, M.I., van Dooren, T.J.M., van Alphen, J.J.M. 2004. Intraspecific sexual selection on a speciation trait, male coloration, in the Lake Victoria cichlid *Pundamilia nyererei*. *Proceedings of the Royal Society B* **271**:2445-2452.

Maan, M. E., Seehausen, O., Van Alphen, J. J. M. 2010. Female mating preferences and male coloration covary with water transparency in a Lake Victoria cichlid fish. *Biological Journal of the Linnean Society* **99**: 398-406.

Majumdar, K. C., Nasaruddin, K., Ravinder, K. 1997. Pink body colour in Tilapia shows single gene. *Aquaculture*: 581-589.

Martinez-Chavez, C. C. 2008. Photic entrainment and onset of puberty in Nile tilapia *Oreochromis niloticus niloticus*. PhD Thesis. University of Stirling, Scotland, UK. (196p).

Mather, P. B., Lal, S. N., Wilson, J. 2001. Experimental evaluation of mass selection to improve red body colour in Fijian hybrid tilapia (*Oreochromis niloticus* x *Oreochromis mossambicus*). *Aquaculture Research* **32**:329-336.

Matsumoto, J. 1965. Studies on fine structure and cytochemical properties of erythrophores in swordtail, *Xiphophorus helleri*, with special reference to their pigment granules (Pterinosomes). *The Journal of Cell Biology* **27**: 493-504.

Matsumoto, J., Seikai, T. 1992. Asymmetric pigmentation and pigment disorders in pleuronectiformes (flounders). *Pigment Cell Research* ***S*2:**275–282.

McAndrew, B. J. 2000. Evolution, phylogenetic relationships and biogeography. In: *Tilapias; Biology and Exploitation* (ed. by M. C. M. Beveridge & B. J. McAndrew). Kluwer Academic Publishers (pp. 1-32).

McAndrew, B. J., Roubal, F. R., Roberts, R. J., Bullock, A. M., McEwen, I. M. 1988. The genetics and histology of red, blond and associated colour variants in *Oreochromis niloticus*. *Genetica* **76**:127-137.

McKinnon, J. 1995. Video mate preferences of female three-spined sticklebacks from populations with divergent male coloration. *Animal Behaviour* **50**: 1645-1655.

McLennan, D. A. 1995. Male mate choice based upon female nuptial coloration in the brook stickleback, *Culaea inconstans* (Kirtland). *Animal Behaviour*. **50**: 213-221.

Michelmore, R. W., Paran, I., Kesseli, R. V. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. *Proceedings of National Academy of Sciences, USA* **88**: 9828-9832.

Milinski, M., Bakker, T. C. M. 1992. Costs Influence Sequential Mate Choice in Sticklebacks, *Gasterosteus aculeatus*. *Proceedings of the Royal Society B* **250**: 229-233.

Milinski, M., Bakker, T. C. M. 1990. Female sticklebacks use male colouration in mate choice and hence avoid parasitized males. *Nature* **344**: 330-333.

Mills, M.G., Patterson, L. B. 2009. Not just black and white: pigment pattern development and evolution in vertebrates. *Seminars in Cell and Developmental Biology* **20:**72–81.

Moralee, R. D., van der Bank, F. H., van der Waal, B. C. W. 2000. Biochemical genetic markers to identify hybrids between the endemic *Oreochromis mossambicus* and the alien species, *O. niloticus* (Pisces: Cichlidae). *Water, South Africa* **26**: 263-268.

Morrison, C., Fitzsimmons, K., Wright, J. R. 2006. *Atlas of Tilapia Histology*.

Nakamura, M., Seikai, T., Aritaki, M., Masuda, R., Tanaka, M., Tagawa, M. 2010. Dual appearance of xanthophores, and ontogenetic changes in other pigment cells during early development of Japanese flounder *Paralichthys olivaceus*. *Fisheries Science* **76**: 243-250.

Ng, W. K., Hanim, R. 2007. Performance of genetically improved Nile tilapia compared with red hybrid tilapia fed diets containing two protein levels. *Aquaculture Research* **38**: 965-972.

Obika, M., Meyer-Rochow, V. B. 1990. Dermal and epidermal chromatophores of the Antarctic teleost *Trematomus bernacchii*. *Pigment Cell Research* **3**:33–37.

Odenthal, J., Rossnagel, K., Haffter, P., Kelsh, R. N., Vogelsang, E., Brand, M., van Eeden, F. J. M., Furutani-Seiki, M., Granato, M., Hammerschmidt, M., Heisenberg, C. P., Jiang, Y. J., Kane, D. A, Mullins, M. C., Nusslein-Volhard, C. 1996. Mutations affecting xanthophore pigmentation in the zebrafish, *Danio rerio*. *Development* **123**: 391-398.

Okumus, I., Ciftci, Y. 2004. Fish Population Genetics and Molecular Markers : II-Molecular Markers and Their Applications in Fisheries and Aquaculture. *Turkish Journal of Fisheries and Aquatic Sciences* **3**: 51-79.

Ottesen, O. H., Strand, H. K. 1996. Growth, development, and skin abnormalities of halibut (*Hippoglossus hippoglossus* L.) juveniles kept on different bottom substrates. *Aquaculture* **146**:17-25.

Parichy, D. M. 2006. Evolution of danio pigment pattern development. *Heredity* **97**:200–210.

Pavlidis, M., Karkana, M., Fanouraki, E., Papandroulakis, N. 2008. Environment control of skin colour in red porgy, *Pagrus pagrus*. *Aquaculture Research* **39**:837-849.

Perschbacher, P. 1992. A review of seawater acclimation procedures for commercially important euryhaline tilapias. *Asian Fisheries Science* **5**: 241-248.

Poompuang, S., Hallerman, E. M. 1997. Toward detection of quantitative trait loci and marker-assisted selection in fish. *Reviews in Fisheries Science* **5**: 253-277.

Popma, T. J., Green, B. W. 1990. Sex reversal of tilapia in earthen ponds: aquacultural production manual. In: *Research and Development Series 35*. Auburn University, Alabama (p. 15).

Popma, T., Masser, M. 1999. Tilapia: Life history and biology. Southern Regional Aquaculture Center Publication No. 283.

Pullin, R. S. V. 1983. Choice of tilapia species for aquaculture: In *International Symposium on Tilapia in Aquaculture* (ed. by L. Fishelson & Z. Yaron). Tel Aviv, Tel Aviv University Press (pp. 64-76).

Pullin, R. S. V., Bhukaswan, T., Tonguthai, K., Maclean, J. L. 1988. The Second International Symposium on Tilapia in Aquaculture. In: *ICLARM Conference Proceedings 15*. Department of Fisheries, Bangkok, Thailand and International Center For Living Aquatic Resources Management, Manila, Philippines.

Pullin, R. S. V., Lowe-McConnell, R. H. 1982. The biology and culture of tilapias. In *ICLARM Conference Proceedings 7*. International Center For Living Aquatic Resources Management (ICLARM), Manila, Philippines.

Quigley, I. K., Parichy, David M. 2002. Pigment pattern formation in zebrafish: a model for developmental genetics and the evolution of form. *Microscopy research and technique* **58**: 442-55.

Rajaee, A. H., Huntingford, F. A., Ranson, K. J., McAndrew, B. J., Penman, D. J. 2010. The effect of male colouration on reproductive success in Nile tilapia (*Oreochromis niloticus*). *Aquaculture* **308**:S119-S123.

Reich, L., Don, J., Avtalion, R. R. 1990. *Inheritance of the red color in tilapias. Genetica* **80**:195-200.

Reichard, M., Bryja, J., Ondrackova, M., Davidova, M., Kaniewska, P., Smith, C. 2005. Sexual selection for male dominance reduces opportunities for female mate choice in the European bitterling (*Rhodeus sericeus*). *Molecular ecology* **14**: 1533-42.

Romana-Eguia, M. R. R., Eguia, R. V. 1999. Growth of five Asian red tilapia strains in saline environments. *Aquaculture* **173**:161-1.

Rottlant, J., Tort, L., Montero, D. Pavlidis, M., Martinez, M., Wendelaar Bonga, S. E., Balm, P. H. M. 2003. Background colour influence on the stress response in cultured red porgy *Pagrus pagrus. Aquaculture* **223**:129-139.

Sakamoto, T., Danzmann, R G, Gharbi, K., Howard, P., Ozaki, a, Khoo, S. K., Woram, R. a, Okamoto, N., Ferguson, M. M., Holm, L. E., Guyomard, R., Hoyheim, B. 2000. A microsatellite linkage map of rainbow trout (*Oncorhynchus mykiss*) characterized by large sex-specific differences in recombination rates. *Genetics* **155**:1331-45.

Salzburger, W., Niederstatter, H., Brandstatter, A., Berger, B., Parson, W., Snoeks, J., Sturmbauer, C. 2006. Colour-assortative mating among populations of *Tropheus moori*, a cichlid fish from Lake Tanganyika, East Africa. *Proceedings of the Royal Society B* **273**:257-266.

Sarder, M. R., Penman, D. J., Myers, J. M., McAndrew, B. J. 1999. Production and propagation of fully inbred clonal lines in the Nile tilapia (*Oreochromis niloticus* L.). *Journal of Experimental Zoology* **284**(6):675-685.

Scott, A. G., Mair, G. C., Skibinski, D. O. F., Beardmore, J. A. 1987. Blond a useful new genetic marker in the tilapia *Oreochromis niloticus* (Linnaeus). *Aquaculture and Fisheries Management* **18**: 159-165.

Seehausen, O. 1997. Distribution of and reproductive isolation among color morphs of a rock- dwelling Lake Victoria cichlid (*Haplochromis nyererei*). *Ecology Freshwater Fish* **6**:59-66.

Seehausen, O. 2000. Explosive speciation rates and unusual species richness in haplochromine cichlid fishes: Effects of sexual selection. *Advances in Ecological Research* **31**:237-274.

Seehausen, O., van Alphen, J.J.M. 1998. The effect of male colouration on female mate choice in closely related Lake Victoria cichlids (*Haplochromis nyererei* complex). *Behavioural Ecology and Sociobiology* **42**:1-8.

Seehausen, O., van Alphen, J. J. M. 1999. Can sympatric speciation by disruptive sexual selection explain rapid evolution of cichlid diversity in Lake Victoria? *Ecology Letters* **2**: 262-271.

Seehausen, O., van Alphen, J.J.M., Lande, R. 1999. Colour polymorphism and sex ratio distortion in a cichlid fish as an incipient stage in sympatric speciation by sexual selection. *Ecology Letters* **2**:367-378.

Singer, A., Perlman, H., Yan, Y., Walker, C., Corley-Smith, G., Brandhorst, B. Postlethwait, J. 2002. Sex-specific recombination rates in zebrafish (*Danio rerio*). *Genetics* **160**: 649-57.

Stelkens, R. B., Pierotti, M. E. R., Joyce, D. A, Smith, A. M., van der Sluijs, I., Seehausen, O. 2008. Disruptive sexual selection on male nuptial coloration in an experimental hybrid population of cichlid fish. *Philosophical transactions of the Royal Society of London B*, **363**: 2861-70.

Streelman, J. T., Albertson, R. C., Kocher, T. D. 2003. Genome mapping of the orange blotch colour pattern in cichlid fishes. *Molecular Ecology* **12**: 2465-2471.

Sturmbauer, C., Meyer, A. 1992. Genetic divergence, speciation and morphological stasis in a lineage of African cichlid fishes. *Nature* **358**: 578-581.

Sugimoto, M. 1993. Morphological color changes in the medaka, *Oryzias latipes*, after prolonged background adaptation - I. Changes in the population and morphology of melanophores. *Comparative Biochemistry and Physiology A* **104** (3):513–518.

Sugimoto, M. 2002. Morphological colour changes in fish: Regulation of pigment cell density and morphology. *Microscopy Research and Technique* **58**:496–503.

Sugimoto, M., Uchida, N., Hatayama, M. 2000. Apoptosis in skin pigment cells of the medaka, *Oryzias latipes* (Teleostei), during long-term chromatic adaptation: The role of sympathetic innervation. *Cell and Tissue Research* **301**:205–216.

Suresh, A. V., Kwei-Lin, C. 1992. Tilapia culture in saline waters: A review. *Aquaculture* **106**: 201-226.

Svensson, P. A., Forsgren, E., Amundsen, T., Skold, H. N. 2005. Chromatic interaction between egg pigmentation and skin chromatophores in the nuptial coloration of female two-spotted gobies. *The Journal of Experimental Biology*. **208**:4391-4397.

Tacon, A. G. J. 1981. Speculative review of possible carotenoid function in fish. *The Progressive Fish-Culturist* **43**(4):205-208.

Takeuchi, I. K. 1976. Cell and Tissue Electron Microscopy of Two Types of Reflecting Chromatophores (Iridophores and Leucophores) in the Guppy , *Lebistes reticulatus* Peters. *Cell and Tissue Research* **173**: 17-27.

Takeuchi, I. K., Kajishima, T. 1972. Fine structure of goldfish xanthophore. *Journal of anatomy* **112**: 1-10.

Tave, D. 1986. *Genetics for Fish Hatchery Managers*. AVI Publishing Company Westport, Connecticut.

Tave, D. 1988. Genetics and breeding of Tilapia: A review. In: *The Second International Symposium on Tilapia in Aquaculture Volume 15* (ed. by R.S.V. Pullin, T. Bhukaswan, K. Tonguthai, & J. L. Mclean). *ICLARM Conference Proceedings* (Bangkok, Thailand). pp. 288-293.

Tave, D., Rezk, M., Smitherman, R. O. 1989. Genetics of Body Color in Tilapia mossambica. *Journal of the World Aquaculture Society* **20**: 214–222.

Tlusty, M. 2005. Use of digital colour analysis to assess variation within individual adult American lobsters (*Homarus americanus*) and the process of addition of colour in white lobsters. *New Zealand Journal of Marine and Freshwater Research* **39**:571-580.

Tobler, M., Plath, M., Burmeister, H., Schlupp., I. 2006. Black spots and female association preferences in a sexual/ asexual mating complex (Poecilia, Poeciliidae, Teleostei). *Behavioural Ecology and Sociobiology* **60**: 159-165.

Trewavas, E. 1983. Tilapiine Fishes of the Genera *Sarotherodon, Oreochromis* and *Danakilia*. *British Museum (Natural History),* London.

Turner, G.F. 1993. Teleost mating behaviour. In: Pitcher, T.J. (Ed.), Behaviour of Teleost Fishes, 2nd edition. Chapman and Hall, London, 307-331.

Turner, G.F. 2007. Adaptive radiation of cichlid fish. *Current Biology* **17**(19): R827-R831.

Verzijden, M. N., ten Cate, C. 2007. Early learning influences species assortative mating preferences in Lake Victoria cichlid fish. *Biology Letters* **3**: 134-6.

van der Salm., A. L., Spanings, F. A. T., Gresnigt., R., Wendelaar Bonga, S. E., Flik, G. 2005. Background adaptation and water acidification affect pigmentation and stress physiology of tilapia, *Oreochromis mossambicus. General and Comparative Endocrinology* **144**:51-59.

van der Sluijs, I., van Alphen, J. J. M., Seehausen, O. 2007. Preference polymorphism for coloration but no speciation in a population of Lake Victoria cichlids. *Behavioral Ecology* **19**: 177-183.

Venizelos, A., Benetti, D. D. 1999. Pigment abnormalities in flatfish. *Aquaculture* **176**:181-188.

Verzijden, M.N., ten Cate, C. 2007. Early learning influences species assortative mating preferences in Lake Victoria cichlid fish. *Biology Letters* **3**:134-136.

Young, W. P., Wheeler, P. A., Thorgaard, G. H. 1995. Asymmetry and variability of meristic characters and spotting in isogenic lines of rainbow trout. *Aquaculture* **137**:67-76.

Watanabe, W. O., Losordo, T. M., Fitzsimmons, K., Hanley, F. 2002. Tilapia Production Systems in the Americas: Technological Advances, Trends, and Challenges. *Reviews in Fisheries Science* **10**(3&4): 465-498.

Zarnescu, O. 2007. Ultrastructure of the skin melanophores and iridophores in paddlefish, *Polyodon spathula*. *Micron* **38**: 81-84.

APPENDICES

Appendix 1: Selected markers from tilapia linkage map (Lee *et al*., 2005) for genome wide scanning including all markers in LG5 with their annealing temperatures

No.	Linkage Group (LG)	Marker	Annealing temperature (^{o}C)	No.	Linkage Group (LG)	Marker	Annealing temperature (^{o}C)	No.	Linkage Group (LG)	Marker	Annealing temperature (^{o}C)
1	LG 1	GM633	57	26		GM206	55-60	51		UNH899	57-61
2		UNH985	57	27		GM553	55-60	52		GM386	58
3		UNH931	57	28		GM509	57-60	53	LG 8	GM027	55
4		UNH148	57	29	LG 5	UNH817	60	54	LG 9	UNH843	60
5		UNH995	60	30		UNH884	57-59	55		UNH886	57-60
6		UNH104	57	31		UNH309	59-61	56		GM343	56
7		GM258	57	32		UNH980	57	57		UNH132	57-60
8		UNH846	57	33		GM012	51-57	58		GM062	52-56
9	LG 2	GM420	56	34		UNH149	50	59	LG 10	UNH994	57-60
10		GM096	58-60	35		UNH927	55-57	60		UNH960	60
11		UNH860	57	36		GM017	54-60	61		GM080	53
12		UNH854	57	37		GM537	54-57	62		GM472	54-57
13		UNH159	56	38		UNH169	47-51	63	LG 11	UNH990	60
14	LG 3	GM354	57	39		GM636	57-59	64		UNH192	52-56
15		GM271	60	40		GM021	52	65		GM215	52-55
16		clcn5	57	41		GM006	56	66		GM399	56
17		UNH971	57	42	LG 6	GM673	57	67		UNH878	55-57
18		GM150	57	43		UNH948	55-57	68		UNH979	57-60
19		GM128	60	44		UNH908	55-59	69	LG 12	GM377	55-58
20		GM526	57	45		GM321	56-58	70		UNH874	55-57
21		UNH982	57	46		UNH968	57-61	71		UNH1009	58
22	LG 4	GM470	59	47		GM440	52-54	72		UNH189	51
23		UNH124	47-52	48	LG 7	GM205	48-52	73	LG 13	GM373	52-55
24		UNH952	55-59	49		UNH973	58	74		UNH954	57
25		UNH170	53-61	50		UNH914	55-57				

Appendix 2: LOD and recombination values for each marker pair in Family 1& Family 2. Below diagonal are LOD scores and above diagonal are recombination fractions. Only LOD score > 3.0 is showed.

	Marker		UNH927	UNH884	UNH309	GM017	UNH169	UNH980	GM021	GM537	GM006	Blond	GM012	GM636
1	UNH927	Fam. 1		0.08	0.18	0.18						0.18		
		Fam. 2		0.08	0.18	0.18	0.18					0.13		
2	UNH884	Fam. 1	6.85											
		Fam. 2	6.85									0.20		
3	UNH309	Fam. 1	3.47				0.03				0.02	0		
		Fam. 2	3.47			0	0				0.10	0.05		
4	GM017	Fam. 1	3.47				0.03				0.02	0		
		Fam. 2	3.47		11.74		0				0.10	0.05		
5	UNH169	Fam. 1			9.71	9.71		0.05				0.03		
		Fam. 2	3.47		11.74	11.74					0.09	0.05		
6	UNH980	Fam. 1					8.29							
		Fam. 2									0.15			
7	GM021	Fam. 1												
		Fam. 2												
8	GM537	Fam. 1												
		Fam. 2												
9	GM006	Fam. 1			3.05	3.05						0.02		
		Fam. 2			6.09	6.09	13.17	4.40				0.15		
10	Blond	Fam. 1	3.47		11.74	11.74	9.71				3.05			
		Fam. 2	4.95	3.05	8.29	8.29	8.29				4.40			
11	GM012	Fam. 1												
		Fam. 2												
12	GM636	Fam. 1												
		Fam. 2												

Appendix 3: LOD and recombination for each marker pair in Family 1&2 (combined) and Family 3. Below diagonal are LOD scores and above diagonal are recombination fractions. Only LOD score > 3.0 is showed.

	Marker		UNH927	UNH884	UNH309	GM017	UNH169	UNH980	GM021	GM537	GM006	Blond	GM012	GM636
1	UNH927	Fam 1+2		0.08	0.18	0.18	0.19					0.15		
		Fam 3		0.03	0.15		0.15					0.15	0.18	0.15
2	UNH884	Fam 1+2	13.69			0.25	0.26					0.23		
		Fam 3	7.97		0.18		0.18						0.15	0.18
3	UNH309	Fam 1+2	6.94	3.94		0	0.01				0.15	0.03		
		Fam 3	3.77	3.05		0.12	0.01				0.17	0		0.07
4	GM017	Fam 1+2	6.94	3.94	23.48		0.01				0.15	0.03		
		Fam 3			4.59		0.12				0.15	0.12		0.12
5	UNH169	Fam 1+2	6.29	3.48	21.15	21.15		0.14			0.22	0.04		
		Fam 3	3.77	3.05	17.60	4.59					0.18	0		0.09
6	UNH980	Fam 1+2					9.57							
		Fam 3									0.11			
7	GM021	Fam 1+2												
		Fam 3												
8	GM537	Fam 1+2												
		Fam 3												
9	GM006	Fam 1+2			8.79	8.79	10.49	-				0.17		
		Fam 3			6.35	3.54	5.68	6.13				0.09		0.15
10	Blond	Fam 1+2	8.33	4.96	19.42	19.42	17.92				7.37			
		Fam 3	3.77		9.93	4.59	9.93				5.27			
11	GM012	Fam 1+2												
		Fam 3	3.05	3.77										
12	GM636	Fam 1+2												
		Fam 3	3.77	3.05	12.11	4.59	11.05				7.07			