The quantification of external colour changes during sexual transition in the protogynous spotty wrasse *Notolabrus celidotus*

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“The sea, once it casts its spell, holds one in its net of wonder forever”

Jacques Cousteau

Abstract

Hermaphroditism in fish has been well documented. Protogynous hermaphrodites (female to male sex change) are normally found in a polygamous social hierarchy. The male is the dominant within the group, followed by the largest female and it is common to observe aggressive behaviour as an exercise of dominance. Most of the studies relating to sex change used tropical species as models, however, little is known about temperate species. In this research, *Notolabrus celidotus,* a temperate protogynous wrasse, was used to investigate sex change. The overall goal was to develop an accurate methodology to assist with colour analysis of an external landmark. Ten males and ten females had pictures taken from the whole body. Digital photography and computational image analysis programs were used to help quantify the colour. Results were found through circular statistics, as hue is read as an angle. It was found that the anal fin presented the most significant mean variation of hue, within the body (female = 95.353 ± 3.16°; male = 141.138 ± 17.23°). The technique was then used to quantify the colour changes of the anal fin as individuals transitioned from female to male. Histological examination was also analysed and classified according to the transitional phase of every fish. The developed technique was able to track the colour changes and allocate them into distinct sexual transition stages. Statistical analysis showed that major colour changes began at the mid-transitional stage and progressed until the terminal phase male was achieved. The histological analysis of the gonads contributed to the study demonstrating internal changes through each sexual stage, additionally showing connection between the changes internally and externally. Through the development of a method to quantify external colour in relation to sexual state, this work creates opportunities for future studies. Investigations of sex change in this species can now be conducted without the need for invasive or destructive sampling to identify sex changing individuals. Further to this, individuals that are at different stages of sexual transition may be identified and selected for sampling by their external colour. Overall, this study was able to quantify and allocate external colour changes into distinct stages that map the restructure of the gonads during sex change.

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Chapter 1

General introduction

1.1 Sex change and hermaphroditism

To enhance reproductive success and individual fitness, sexual patterns in fish had to have important evolutionary adaptations in relation to sexual patterns. These patterns evolved into two sex strategies, gonochorism or hermaphroditism. The former and most commonly found within fish was defined as the existence of testes or ovaries in one individual through its lifetime, where sex reversal is not possible by natural means (Chan, 1970). Although it is important to note that the final sexual state may not be reflected by the initial gonadal development (Devlin & Nagahama, 2002). For example, some gonochoristic animals are intersex, having both ovarian and testis tissue at the same time, when immature; developing sex differentiation at later stages, such as the Nassau grouper (*Epinephelus status*), once believed to be a protogynous hermaphrodite species (Sadovy & Colin, 1995). The latter sexual strategy has been of steadily growing interest, especially within fish, where animals with this condition have the capability to change sex once or multiple times during their life. Thus, improving reproductive success within each fish species.

1.1.1 Hermaphroditism

Since the early work of Atz (1969) and Reinboth (1970) (cited by Warner (1975), cases of hermaphroditism in teleost fish have increased from 18 families throughout five orders (Warner 1975), to 27 families in seven orders, approximately 6% of all teleost families, with possible evidence for an additional 31 genera from 21 families of 6 orders (Sadovy de Mitcheson & Liu, 2008). Two types of hermaphroditism have been found in teleost fishes, simultaneous (synchronous) and sequential hermaphroditism. The former, is characterised by individuals that are functionally male and females at the same time, such as many fish species from the bass family (Serranidae), e.g. harlequin bass (*Serranus tigrinus*) (Pressley, 1981). In sequential hermaphroditic species, the individual starts its life with a specific gender (primary sex) and at some stage in life, change to the opposite sex (secondary sex). If the individual is born as male and change to a female, it is called protandry, conversely if born as a female and change to male is called protogyny (Warner, 1975). A third type of sequential hermaphroditism called bidirectional sex change, where sex change from female to male and male to female (Kuwamura & Nakashima, 1998).

Protogynous fish species are the most dominant in sequential hermaphroditism, with at least 15 out of 27 families confirmed (see Sadovy de Mitcheson & Liu, 2008 for review). In general, protogynous species are commonly found in a polygamous social hierarchy, where the larger males tend to monopolize mating with female, by defending spawning territory or controlling a harem (Warner, 1984). Within the population structure of social protogynous species, there are several primary males living within the harem. They delay their colour changes to sneak into the harem of the male to fertilise the female eggs. Due to the delayed colour changes, the identification of primary males can be difficult and may only be visible by gonadal histology, where the testes of the primary males appear similar to gonochoristic species (Reinboth, 1970; Robertson & Warner, 1978; Warner & Robertson, 1978; Munday *et al*., 2006b). However, in labrids, there are cases where the primary males may have larger testis than secondary males (Buxton, 1990). These primary males may form up to 50% of a population group (Robert & Warner, 1978). However, the proportions can be much lower, as was observed in the spotty (*Notolabrus celidotus*) which primary males were composed from 15% to 10% of the population (Jones, 1980).

One of the species extensively studied is the tropical labrid bluehead wrasse (*Thalassoma bifasciatum*). This protogynous species forms a social hierarchy and has been a classic model for sex change. With this species, the loss of a dominant male (TP) can usually trigger the process of sex change in the largest female of the harem. This involves changes in the behaviour, anatomy and external coloration of the fish (Warner & Swearer, 1991).

1.1.2 Social Controlled sex change

Many protogynous species can be induced to change sex by influencing key external stimuli such as social structure. The induction of sex change in sequential hermaphrodite fish species, does not occur automatically with the loss of a dominant member of the group. Rather through a specific stimulus, be it social reorganization or a behavioural event (Ross, 1990). In captivity, sex change induction can be achieved by artificial and social controlled induction. Artificial induction to sex change, involves the addition to an external stimulus, such as an aromatase inhibitor (AI). This can drastically induce the reduction of estradiol-17β (E_2), a key sex steroid for maintaining the female state. Nozu *et al* (2009) worked on a short-term AI treatment on the three-spot wrasse (*Halichoeres trimaculatus*) and were able to fully achieve complete sex change in a 5 days period. AI was also tested in the protogynous honeycomb grouper (*Epinephelus merra*) which again resulted in success in a fully complete sex change (Bhandari *et al*, 2004).

During social experiments of protogynous species, whenever there is a male within the group, sex change is suppressed among females, thus the sequence of events that stimulates sex transformation cannot be completed (Shapiro, 1988). However, if the male dies, the dominant and largest female, ‘alpha-female’, would rapidly start acting like the dominant male initiating the process of sex change (Shapiro, 1988; Robertson & Choat, 1974). Socially controlled sex change in teleost fishes is a dramatic example of adaptive reproductive plasticity (Lamm *et al.*, 2015), in which the dominant sex changer has to go through a cascade of behavioural, physiological and morphological changes naturally (Godwin, 2009). Social controlled experiments to induce sex change is commonly done in species that live among a harem group or those with rigid dominance (Fishelson, 1970; Shapiro, 1979). This type of experiment has been done since the 1970s (Fishelson, 1970), with the removal of the dominant fish, being the trigger point to initiate the process of sexual transitioning. The precision of this response has been verified on several occasions (Robertson, 1972; Fricke & Fricke, 1977; Shapiro, 1979; Shapiro, 1981; Ross *et al.*, 1983; Warner & Swearer, 1991). Social controlled induction of sex change can be separated into two types. The first, group size and group sex ratio may be the proximate determinants of sex change, which occurs in monandric and sexually dimorphic species (Shapiro, 1979). In the second type, induction depends on the relative size ratios within the social unit, and in contrast to the former system, this occurs in diandric and sexually monomorphic species (Ross *et al*., 1983; Ross *et al*., 1990; reviewed in Ross, 1990). Overall, social induction has proven to be an efficient way to study sex change, given the points that stimulate the transition.

1.1.3 Behavioural changes

Behaviour often differs greatly between the sexes (Shapiro, 1981) and behavioural change may be the first indication of sexual transition in an individual. The behaviour of hierarchical sequential hermaphrodite groups can be related into the concept of the ‘behavioural syndrome’ (Sih *et al*., 2004) or ‘animal personality’ (Dingemanse *et al*., 2010) referring to the correlation between behaviour of individuals across time and contexts (e.g. correlations among feeding, antipredator, mating, aggressive, and dispersal behaviours) (Sprenger *et al*., 2012).Therefore, in a group, some individuals are more aggressive, more active or bold, while others are generally less aggressive, active or bold (Sih *et al*., 2004). In protogynous species, such as the *L. dimidiatus*, the dominant male has a constant urge to behave aggressively towards the rest of the group, especially the largest females. By showing dominance, the male prevents the possibility of the largest female from an earlier change of sex. This ensures the stability and social ranking of the group (Kuwamura, 1984). In the same study, social ranking and dominance was also observed between the females, where the largest female would behave aggressively towards the smaller ones. Thus, dominating them and consequently, suppressing any tendency to change sex.

Changing behaviour has been recognised with the first process in the female-male transformation. In the protogynous rock hind grouper (*Epinephelus adscensionis*) the largest female showed increased aggression toward smaller individuals as soon as 2 days after male removal. This aggression continued to increase over the following weeks of gonadal transition (Kline *et al*., 2011). For the protogynous *T. bifasciatum*, behavioural analysis during sex change in the wild, showed an increase of aggressive behaviour by the largest female towards the others within minutes from the male removal (Warner & Swearer, 1991). This highlights the importance of behavioural analysis during the process of sex change.

1.1.4 Physiological changes

The physiological process of protogyny sex change can be separated into different phases, such as early transition phase (ET) and late-transition phase (LT). As seen in behavioural changes, the entire transition may be rapid, taking just 8 days, in the case of the *T. bifasciatum* (Warner & Swearer, 1991) to several weeks, as observed in the ballan wrasse (*Labrus bergylta*) (Muncaster *et al*., 2013). Physiological sex change in this chapter will be separated into gonadal sex changes and endocrine sex changes.

1.1.4.1 Gonadal sex change

As sex change for protogynous fish progresses, internal transformations begin to occur. As females, the ovaries, once with no detectable testicular tissues, are progressively restructuring into a totally functional testis (Nakamura *et al*., 1989). During the female phase, as the mature female is in its breeding season, the ovaries are filled with previtellogenic oocytes (PVO). As the male dies or is removed from the harem, gonadal restructuring of the dominant female begins (ET), with PVOs starting to degenerate through phagocytosis, by microphages cells, a process known as atresia. Leydig cells start to develop on the inner part of the changing lamellae. This is followed by the proliferation of spermatogonia. At LT, spermatogonia increases in volume to almost 75% of the ovarian lamellae. At this stage almost all atretic PVOs are absorbed, and gonad is changed to testis containing different development stages of spermatogenic cells. At the conclusion of the Terminal Phase (TP) transition, the fish has become a fully functional male and spermatogonia, as well as all stages of spermatogenesis is fully occupy the gonad, with no viable oocytes remaining (Nakamura *et al*., 1989; Bhandari *et al*., 2003; Muncaster *et al*., 2013).

1.1.4.2 Endocrine sex change

In sequential hermaphrodite fishes, sex steroid hormones are important mediators of sexual and aggressive behaviour (Godwin *et al*., 2000), also to achieve maturation. Aggressive behaviour in sex change fish species, are linked to the production and secretion of arginine vasotocin (AVT) and in *T. bifasciatum*, the aggressively dominant and courteous male are known to produce almost three times the amount of AVT than the females (Godwin *et al*., 2000).

The sequence of sex change events involving sex steroids starts *via* the Hypothalamic-pituitary-gonad (HPG) axis (Nakamura, 1989; Frisch, 2004). During the breeding season, whilst still in the female phase, the feminine sex steroids, E_2, are produced in high concentration (Muncaster *et al*., 2010). This helps maintain ovary functions as well as avoiding inhibiting the start of ET phase (Bhandari *et al*., 2005; Kojima *et al*., 2008). At the commencement of sex change, as ET take effect, there is a rapid drop in the sex steroids, E_2, which is linked to the degeneration of PVO. As E_2 continues to drop, the male sex steroids, 11KT, levels start to rise. The importance of 11KT in male reproduction has been suggested in numerous occasions (Fostier *et al*., 1983; Hourigan *et al*., 1990; Nagahama, *et al*., 1994; Bhandari *et al*., 2003). At the LT stage, there is a rapid increase in the levels of 11KT until reaching its peak, which was linked to the proliferation of spermatogenic cells in the gonads (Bhandari *et al*., 2003). In males, 11KT has been linked to male secondary characteristics, such as aggressive and sexual behaviours, and also to physiological attributes such as body markings and gonadal rearrangements, by influencing spermatogenesis (Borg, 1994)

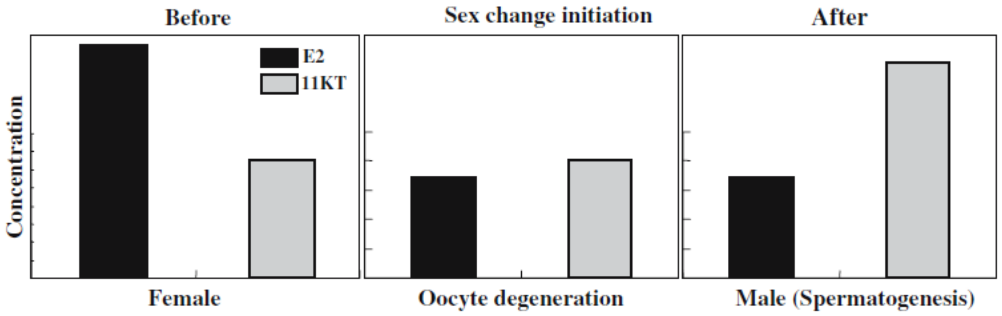


Figure 1-1**:** A diagram representing the transition between sex hormones during protogynous sex change. Starting as a mature female, through the sex changing stages, and finally a terminal phase male (Nakamura *et al*., 2005).

1.1.5 External changes

External coloration and patterns are the simplest way to differentiate species. In labrids, where sexual dimorphism is common, skin coloration is one of the most reliable ways to distinguish male from female. There are many studies which visually describes the skin colouration of sequential hermaphrodite fish species. Roede (1972) made a descriptive observation of seven tropical labrids species at different stages of their life, where external colour and marking were described between male-male and male-female change overs. Also, in tropical species, Shapiro (1981) made a descriptive analysis of colour changes during the female-male transition of the *Anthias squamipinnis*. Choat (1965) made a thorough description of the colour patterns of *N. celidotus* as females, males, and during transition between sexes. However, the quantitative assessment of colours during sex change has been overlooked over the years. Ohta *et al* (2008) attempted to address this by analysing colour changes and linking these to the relative concentration of the sex steroids. During sex change, there are many landmarks that can be seen as reliable areas to identify genders or transitional phase. Each species has its own distinctive patterns and colours where the assessment of colour is done. For example, it was found that the anal fin in the *P. sieboldi*, is the area of the body that had the greatest colour change during sex change (Ohta *et al*., 2008).

1.2 Sex determination and differentiation in fish

From the moment that an egg is fertilized, sex determination plays a vital role in the sexual characteristics of an organism (Kobayashi *et al*., 2012). However, sex determination is more labile in fish and presents a complex pattern of variables and mechanisms utilized among different fish orders (Devlin & Nagahama, 2002). Some of the variables are caused by anthropogenic activities, that ultimately affects the aquatic habitat, such as pollution, changes in water temperature, and exogenous steroids (Devlin & Nagahama, 2002). Sexual determination varies greatly in fish and in those that have XX and XY determined genes the key players tend to be *Sox9* and *Wnt4* in the ovarian development and *Sry* and *Dmrt1* in testis formation (Zarkower, 2001; Nagahama, 2005; Chen et al., 2015).

Sex steroids also play an important role in sex determination via three mechanisms: genotypic, temperature and behaviour (Devlin & Nagahama, 2002; Godwin *et al*., 2003). At the genotypic level, protogynous species are linked to high levels of endogenous estrogen which acts as an ovarian inducer. Although exogenous androgen influence has been linked to the masculinization of the female, endogenous androgen has no effect on natural testicular differentiation (Nakamura *et al*., 2003). Temperature sensitive sex determination can be caused by the increase or decrease of water temperature. Sex determination is controlled by the activity of many biochemical pathways involving different proteins, such as transcription factors and receptors. Temperature can drastically influence the structure and function of proteins. Thus, a change in water temperature encountered by fish could alter sex determination and influence the development of gender (Devlin & Nagahama, 2002). A study on the effects of temperature on ovarian steroid production was undertaken in the common carp (*Cyprinus carpio*). It was found that the optimal temperature for production of testosterone and estradiol was 24 ºC. Conditions below or over the optimal physiological temperature result in a sharp drop on the sex hormones production (Manning & Kime, 1984). For protogynous fish species, the behavioural sex determination after sexual maturation leads to understanding of alternative phenotypes within the same sex. Also, this can explain the phenotypic reorganisation in adulthood. The latter is linked to changes in circulating sex steroids, E_2 to 11-ketotestosterone (11KT) and sex change (Oliveira, 2005). Sex steroids play an important role in sex determination and gonadal differentiation. However, exogenous factors may also have a direct influence in the sex of the individual.

1.3 Colour formation

The mechanism underpinning the beautiful and vast display of colours and patterns, as well as the ability to change colour in fish are highly complex and have important evolutionary attributes. The colours displayed in the skin is determined by integumental chromatophore cells (Nery & de Lauro Castrucci, 1997). These pigmented cells are able to rearrange themselves by dispersion and aggregation within the skin (physiological colour change) (Fujii, 2000). In addition, an increase or decrease in their density (morphological colour change) may occur (Fujii, 1993). Physiological colour changes are known to be rapid, within minutes depending on the species (Nilsson-Sköld *et al*., 2016), and the pigmented cells associated with it differs from morphological colour changes by being motile. Physiological colour changes are extremely important for animals. Camouflage and background adaptation have a major influence on hunting and also on avoiding been hunted, making this ability a major driving force for colour change in many species. A study on female pipefish revealed that the ornamental coloration was shut off whenever predators invaded the mating area (Fuller & Berglund, 1996). However, physiological colour changes also play an important role in the mating and reproductive behaviour, social interaction, together with thermoregulation and UV protection (Nilsson-Sköld *et al*., 2016). For example, in the Southern pygmy perch (*Nannoperca australis*), males develop red and black nuptial coloration on their fins and bodies prior to breeding (Morrongiello *et al*., 2010). In juvenile guppies (*Poecilia reticulata*), specific eye coloration may be linked to the aggressiveness of the animal, as the eye colour patterns can change rapidly. During an aggressive encounter, the winning fish immediately darkened his eyes and the loser lightened his (Martin & Hengstebeck, 1981). Finally, in zebrafish, the shift in melanosomes was found to be an effective “sunscreen” against UV damaging properties (Mueller & Neuhauss, 2014). Physiological colour changes are rapid due to hormonal activity on these cells and neural signal (Sugimoto, 1993), with the latter being much faster, as images collected by the lateral eyes, has its information transmitted to the central nervous system, where it is processed and instantly sent to chromatophores, via nervous connection (Fujii, 2000). In the female two-spotted goby, the release of the hormone MSH and prolactin in the system were linked to the increase of brightness of the colour orange, a nuptial coloration (Nilsson Sköld *et al*., 2008). Also, physiological results from the motile chromatophores, erythrophores, found in the skin of the swordtail fish (*Xiphophorus hellerii*) and the squirrelfish (*Holocentrus ascensionis*) indicated that the pigment translocation of this cell is controlled by the sympathetic nerve system (Negishi & Obika, 1980; Luby-Phelps & Porter, 1982).

The change of density of chromatophores within the skin, are known to be linked with slow colour change, or morphological colour change. This type of colour change can be defined as a variation in skin pigment concentrations and, or, alterations in the morphology, density and distribution of chromatophores in the three-dimensional organisation of the integuments (Chavin, 1969; Leclercq *et al.*, 2010). The process is considered slow, occurring over weeks or months through diet and environmental condition and during two different life stages, e.g. immature/nuptial, larvae/juvenile and juvenile/adult (Leclercq *et al.*, 2010). Colour change through nutrition and environmental condition fluctuations are called proximate factors of morphological colour changes (Leclercq *et al.*, 2010). This is a body response to what is occurring in the environment, as a way to adapt to the new condition. Nutrition can affect the skin pigmentation. Seikai (1989) found that flatfish fed with wild zooplankton had normal skin pigmentation, whereas the same animals fed with Brazilian *Artemia nauplii* and rotifers, had albinism achieved. An experiment with environmental changes has been done on zebrafish (*Oryzias latipes*). Several individuals were split and placed into two different background, one dark and the other white. The animals adapted to the dark background had a much darker body whereas, conversely, those adapted to a white background had a much lighter body colour (Sugimoto, 2002). The colour changes during two life stages have been identified as ultimate factors of morphological colour change (Leclercq *et al.*, 2010). The red coris wrasse (*Coris gaimard*) undergo through a dramatic colour transformation as it changes from juveniles to adults (Nilsson-Sköld *et al.*, 2016). Also, in dimorphic sex change species, the transition between genders, can also be considered as morphological colour changes. One example is the protogynous bamboo leaf wrasse (*Pseudolabrus sieboldi*), which during sexual transitioning, changes the colour of the anal and dorsal fin (Ohta, 2008). Another example is the species in which the study is focused (*N. celidotus*), which also undergo through morphological colour changes in the anal fin, as well as throughout the body (Jones, 1980).

1.4 Digital photography and computer analysis for colour quantification

Since photography became digitalized, it facilitated the studies aimed to quantify colours. In the past, scientists were limited to mainly spectroradiometry and spectrophotometry. Both are instruments that are able to precisely quantify light energy at individual wavelengths within the spectrum or light absorption by a sample and used as methods for colour measurements through light absorption. Although these methods are still being used, for their wide spectral range and objectivity, when multiple readings are needed it can become very time consuming and expensive (Svensson & Nilsson-Sköld, 2011). It also requires a static subject, as the probe needs to be close to or touching the colour patch (Stevens *et al*., 2007). Alternatively, the modernization of photography into the digital era has improve the flexibility and image precision of digital photography. There are many advantages of digital photography when compared with spectrometry. In particular, the ability to use powerful and complex image processing algorithms to analyse spatial patterns of a place or object without having to reconstruct each point sample (Stevens *et al*., 2007). Other advantages are the fast speed to acquire data, especially when dealing with tissues prone to rapid colour change. It allows measurements of pre-defined areas to be used as anatomical landmarks on the animal (Svensson & Nilsson-Sköld, 2011). Powerful software programs have also revolutionised image analyses. Programs such as Adobe Photoshop (Adobe Systems Inc.) and ‘Image J’ (Sun Microsystems, Inc.) can be used to standardise photography, ensuring the same colour condition for each photograph.

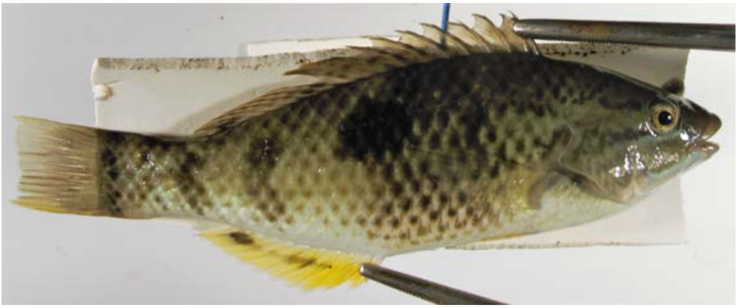
1.5 *Notolabrus celidotus* (Spotty)

Notolabrus (formerly *Pseudolabrus*) *celidotus* (Bloch & Schneider, 1801), commonly known as Spotty or Paketi, in Māori, is part of the labridae (wrasse) family. The species is one of the most abundant wrasse species found throughout New Zealand, and arguably the most studied, even amongst other New Zealand labrids. Much of the ecology of this species has been studied within a single decade by one researcher (Jones, 1980; Jones & Thompson, 1980; Jones, 1981; Jones, 1983; Jones, 1984a; Jones, 1984b; Jones, 1984c; Jones, 1988).

Notolabrus celidotus is an endemic species that inhabit harbours, estuaries and reefs with low wave exposure. The highest population densities are found in shallow waters, between 0-5 m, however some may be found at depths below 25 m (Denny, 2005). The habitat can vary between juveniles and adults. Juveniles prefer to be aggregated in sheltered spots, such as amongst macroalgae, and crevasses, which are usually patchy. Hence, they are dispersed into loose schools. Adults are more randomly dispersed in the habitat, as shelter dependence seems to decrease with growth (Jones, 1984a).

Typical of labrids, *N. celidotus* is a carnivorous species, where the diet is size-specific, changing as it grows. The diet of the juveniles consists mainly of gammarids and copepods (Jones, 1984a). As the fish grows, it starts feeding on larger invertebrates (e.g. bivalves and gastropods) and foraging in larger areas, seeking microhabitats in which prey items are more constant. Diet has been described by Jones, (1984a). Choat (1965) made a detailed anatomical description of this species at three stages, the initial phase (IP), terminal phase (TP) and transitional phase (Table 1).

Table 1-1: External description of *N. celidotus* as a breeding female (IP); during sexual transition (transition phase); and as male, when sex change is complete, or terminal phase (TP). Anatomical detail was observed by Choat (1965). Each picture corresponds to the sexual stage in life. (Fish: IP 14cm, TP 16cm and the male is 18cm total length)

Body description	Picture
IP: General body colouration range from pale whitish grey to yellowish green, with a black mark on the scales, which becomes more pronounced towards the posterior of the body. Contains a large black spot below the 7th and 9th spine; The anal fin contains 3 anterior pointy, hard spines followed by 10 soft rays, interconnected by tissue, the colour is yellow and can vary from bright yellow to pale yellow, it also contains two black spots between the 1st and 4th ray and 6th to 9th ray; Dorsal fin, characterised by 9 spines (anterior) and 11 rays (posterior) has its colouration is pale to dusky, with little variation, dark small spots can also occur between the rays; tail and pectoral fins vary from pale translucent yellow to colourless.	
Transitional phase: commonly seeing at specimens ranging from 140mm and 180mm in length (tip of moth to the tip of the centre of the tail fin). At this phase, there is a natural transition from female to male, where male markings are starting to appear but female marking still remains. The black scales on the body slowly starts to fade, and the black large spot also starts to fade away, with the blotchy black mark starting to appear; the yellow colouration of the anal fin from the females also starts to fade into TP characteristics. The anal fin colouration and markings might be pointed as the main distinction during sexual transitioning.	
TP: The body colouration is similar to the females, although the black scales are less pronounced. Blue ocelli also appear throughout the body as spot and as broken and unbroken lines from the jaw to the rim of the eye; The large spot at the body of the females, also disappears and a blotch black spot appears at the bottom of the dorsal; The anal fin loses the two black spots but display a median longitudinal yellowish, sometimes grey, stripe, on a pale whitish green background. Reddish orange markings can also appear just below and above the stripe, however it might be linked to the breeding season, although there is no published information on it. This reddish orange colouration sometimes is also seeing on the sides of the abdomen; The dorsal fin also loses its dark spots, and the colouration is usually pale yellowish green; The tail and pectoral fins colouration has no changes between male and female (IP - TP).	

N. celidotus are social animals in which hierarchical organization is commonly seen (Jones, 1983). They are monandric, meaning that only a percentage of the females in each hierarchic group will change to a TP male. In this community there are also a number of small females that transition early, without colour changes. These female mimics will opportunistically rush in and inseminate the eggs released by females during courtship with larger dominant terminal phase males (Jones, 1980). The hierarchical organization also means that the males are highly territorial, and aggressive towards the group. Large females can also become highly aggressive towards the smaller females from the group, socially inhibiting their maturation, which gives rise to the "alpha female" of the harem. If the male of a captive group is removed, then the alpha female undergoes sex change (Jones & Thompson, 1980). This socially induced sex change has also been described in the tropical labrid fish *Labroides dimidiatus* (Robertson, 1972).

The spawning season of *N. celidotus* has a duration of 5 months, from July until early December (Jones & Thompson, 1980), where the male will breed with multiple females. A mating system classified as 'resource defence polygyny' since the TP males defending territory are the one accounting for most of the spawning (Emlen & Oring, 1977). In the wild, during the breeding season, the females swim into deeper areas of the reefs to spawn, which is consistently occupied by TP males. The most successful of the TP males are the ones that reside in their spawning territory for longer periods, spend more time swimming, courting and showing aggressive behaviour towards IP males, and less time feeding during the spawning season (Jones, 1981).

1.6 Thesis research

1.6.1 Aims and objectives

The overall goal of this study is to develop an accurate methodology that will assist in the colour analysis of an external landmark to give an indication of the sexual state as fish transition from female to male. With the new methodology, it will be possible to track and point the stage of sex change an individual is going through. The study will also, analyse histologically the gonads before, during and after the sex change process. The two areas of study are an important part of the gender transitioning and might assist future studies in the *N. celidotus* and other temperate and tropical protogynous teleost fishes.

1.6.2 Thesis format

This thesis is composed of four chapters. Chapter 1 is the introduction, discussing sex change in fish, especially in protogynous species, such as the *N. celidotus* or spotty. Skin colour formation and colour quantification using digital photography are also discussed, as well as the general characteristics of the spotty. Chapter 2 involves the development of a technique to quantify skin colours in the spotty in both, female and male individuals. Chapter 3 uses the technique used in the previous chapter to analyse the skin colour changes as the fish changes sex from female to male, also showing the sex change by histological analysis. Chapter 4 discussed the second and third chapters and their findings using current literature, also the limitations of the study and possible future studies in the area of research.

Chapter 2

Developing a systematic colour measurement in the protogynous *N. celidotus*

2.1 Introduction

2.1.1 Definition of colour

Colour may be defined as “the property of an object of producing different sensations on the eye as a result of the way it reflects or gives out light” (Soanes *et al.*, 2006). Colour is formed by a visible light, which is a radiation in form of electromagnetic waves captured by an animal eye (Ohta & Robertson, 2005). As humans, colours begin to be visible at 400 nm (violet) and ends at 700 nm (red) and anything below or above these range fades into darkness (Endler, 1990, Waldman, 2002). In the late 1600s, Isaac Newton experimentally demonstrated that white light, as sunlight, was composed of seven colours: violet, blue, indigo, green, yellow, orange and red. In general, colour is produced whenever white light is modified by reflection or transmission by an object (Ohta & Robertson, 2005). The colour markings of an animal are dependent upon light reflectance and ambient light spectrum. These two factors can vary according to season, weather, macro and microhabitat (i.e. water depth) and time of the day (Endler, 1990). These markings are exceptionally important for communication, camouflage and thermoregulation (Stuart-Fox & Moussalli, 2009).

2.1.2 Different colours spaces and conventions

To the human eye, colour is perceived as a brain reaction to a specific visual stimulus (Yasir & Quin, 2009). These visual stimuli depend on three classes of receptors found in the retina. These receptors have three different electromagnetic wavelength-sensitivity, a short (blue), a medium (green) and a long (red) wavelength-sensitivity cone (Roorda & Williams, 1999). These three primary colours can be combined, creating all of the colours found in the spectrum. The individual amount of red, green and blue (RGB), can be defined as tristimulus values to create the RGB colour model. In this system, each primary colour can have 256 levels. When viewed digitally, the values range from 0 (absence of colour) to 255 (pure colour). This allows colours across the spectrum to be defined quantitatively.

By selecting a point in a coloured picture, software packages such as Adobe Photoshop (Adobe Systems Inc.) can calculate each tristimulus value. The combination of the three primary colours values then correspond to a specific colour of the spectrum. With that said, if all values of red, green and blue light are at their maximum values at the same time (red = 255, green = 255, and blue = 255) it produces pure white, conversely, in the absence of all primary colours, it produces pure black. When all the three colours are have the same value (R = 128, G = 128, B = 128) , then neutral grey is reached. For any other colour, the variation in the input from each of the three primary colours will be the determinant of the final colour (Figure 2.1). As an example, bright red values on RGB are R = 246, G = 20 and B = 50 values. One of the problems using RGB colour model, is the dependency on external factors, e.g. sensitivity of the camera, and illumination (Leon *et al.*, 2006). As perceived by the human eye, the primary colours, RGB, are the base of all colours visible in the spectrum. The mixing of the three colours at different values, determines the final colour. Therefore, caution needs to be taken when using RGB for colour measurement due to its dependency on external factors (Yam & Papadakis, 2004).

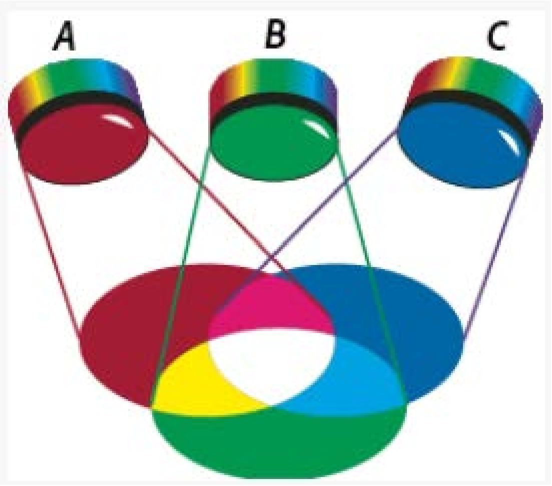


Figure 2-1: The primary colours, red (A), green (B) and blue (C). (Adobe Photoshop)

Each colour, also known as hue, found in the visible spectrum is also dependent on saturation and brightness. Hue represents the colour, indicated by the wavelength of light reflected from an object (Pitcher *et al.*, 2007). It is measured as an angular direction in degrees ranging from 0° to 360° on a colour wheel. In common use, hue is known by the colour visible to human, such as red (c. 0°), yellow (c. 60°), green (c. 120°) and blue (c. 240°). Saturation measures the strength or 'purity' of the colours, also called 'chroma'. It is represented as a percentage where 0% is a shade of grey and 100% is fully saturated. Brightness is also measured as percentage ranging from 0% (black) to 100% (white), and is related to the lightness or darkness of the colour. Together the hue – saturation – brightness (HSB) colour model measures the intensity related to coloration (Skarstein & Folstad, 1996). It is important to note that hue is also dependent on saturation and brightness. Thus, the lower the saturation the duller the hue will be. Also, the colour effect provided by saturation and hue is lost as brightness is reduced. For example, a brightness value of 0% will yield black. Conversely, the higher the saturation and brightness, the more pure a hue colour will be (Figure 2.2).

As with RGB, the HSB model has problems that need consideration. One of the problems was described by Poynton (1999). HSB is based on RGB components. This makes the colour model device dependent, since the colour ranges of RGB model varies according to the display device and computer program (Poynton, 1999; Yam & Papadakis, 2004). For example, an image appears darker on a

Windows system than on a Mac OS computer. This is due to the standard RGB colour space being darker in Windows than in Mac OS (Adobe Systems, 2002).

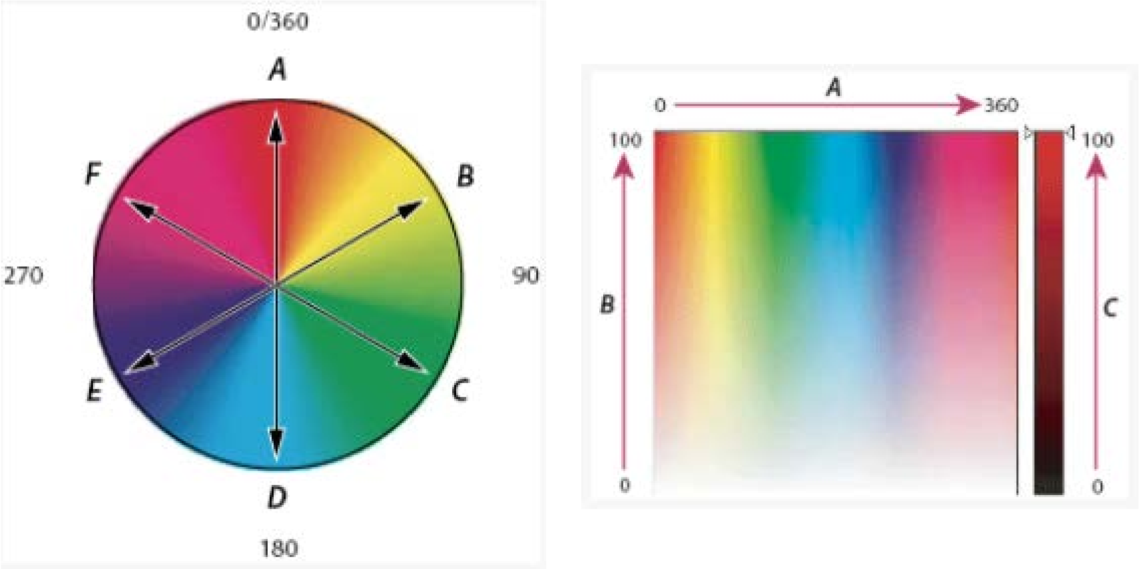


Figure 2-2: Standard colour wheel showing the angular hue values (e.g. 0°, 90°) (right). Beside is a visual interpretation of hue (A), saturation (B) and brightness (C) (left) (Adobe Photoshop).

Evaluation of colour via human perception might be complicated as visual colour is perceived differently from person to person. To resolve this problem, many systems and formulae were developed to mitigate differences in human perception (Robertson, 1977). Created in 1976 by the International Lighting Commission (Commission International de l'Eclairage, CIE), CIE L*a*b* has been chosen due to its standardized and uniform colour spaces, as well as it is similar to human perception (Chen *et al.*, 2004; Kuehni, 2003). CIE L*a*b* works through a formula recommended "in the interest of uniformity of usage" (CIE, 1976). The formula firstly needs a transformation from RGB to tristimulus X, Y, Z. Then

$L^* = 116\ (Y/Y_n)^{0.333} - 16,$

$a^* = 500\ [(X/X_n)^{0.333} - (Y/Y_n)^{0.333}],$

$b^* = 200\ [(Y/Y_n)^{0.333} - (Z/Z_n)^{0.333}],$

where Xn, Yn, Zn are the tristimulus values of the nominally white object colour stimulus (Kuehni, 2003). There are three channels in the CIE L*a*b* model, the lightness channel (L*), the a* channel and the b* channel. The L* channel describes the relative lightness from pure black (0) to pure white (255). The a* channel is related to the balance between pure green (0) to pure magenta (255) with neutral grey as 127.5. Finally, the b* channel which is the balance between pure blue (0) to pure yellow (255) with neutral grey as 127.5. However, it is common to have the L*a*b* values transformed and ranging between -100 to 100 for a* and b* with 0 as neutral grey, and L* from 100 to 0, (Svensson & Nilsson-Sköld, 2011). Digital software such as Adobe Photoshop use these conversions automatically. In L*a*b*, colour is represented by the hue-angle a* – b* plane (Pavlidis *et al*., 2006) (Figure 2.3). Overall, CIE L*a*b*, was created with the main objective to standardise and homogenise the colour spaces, thereby creating a robust colour analysis.

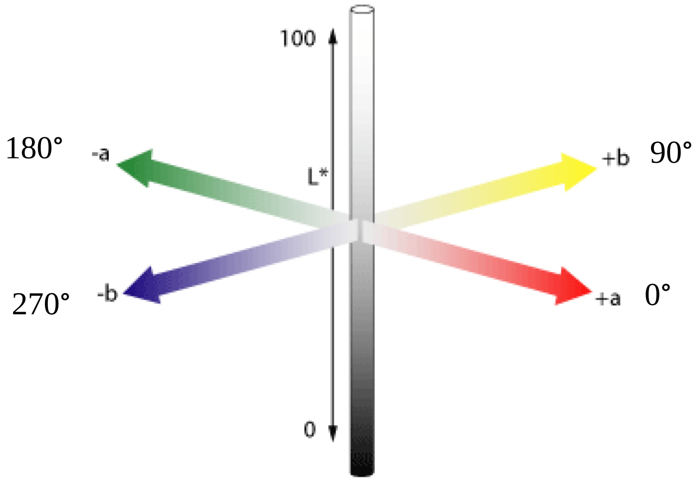


Figure 2-3: Graphical representation of the CIE Lab colour model. (L) Represents lightness and is measured in %, the closer to 0% the darker the colour is. (a) And (b) are the hue represented by four colours (-a) green, (+a) red, (-b) blue and (+b) yellow.

Hue is measured in angle. CIE L*a*b* creates an interesting model to choose when working with colours, because a* and b* channels can be transformed into a singular value of hue. The hue angle (*H*°) in the a* – b* plane, increases in an anti-clockwise rotation in the a*, b* axis, where 0° corresponds to the colour red, 90° to yellow, 180° to blue, and 270° to green (Pavlidis *et al*., 2006). Knowing the hue is an angle for ab, trigonometry laws can be applied, where the first, red-yellow quadrant, (+a, +b) would be positive signed values. For the second, yellow-green quadrant (-a, +b) and third, green-blue quadrant (-a, -b), calculations for hue angle should be: hue = 180+ Arc tan(b/a); and the fourth, blue-red quadrant (+a, -b) should be calculated as: hue = 360+ Arc tan(b/a) (Figure 2.4) (Francis, 1975; McLellan *et al*., 1994).

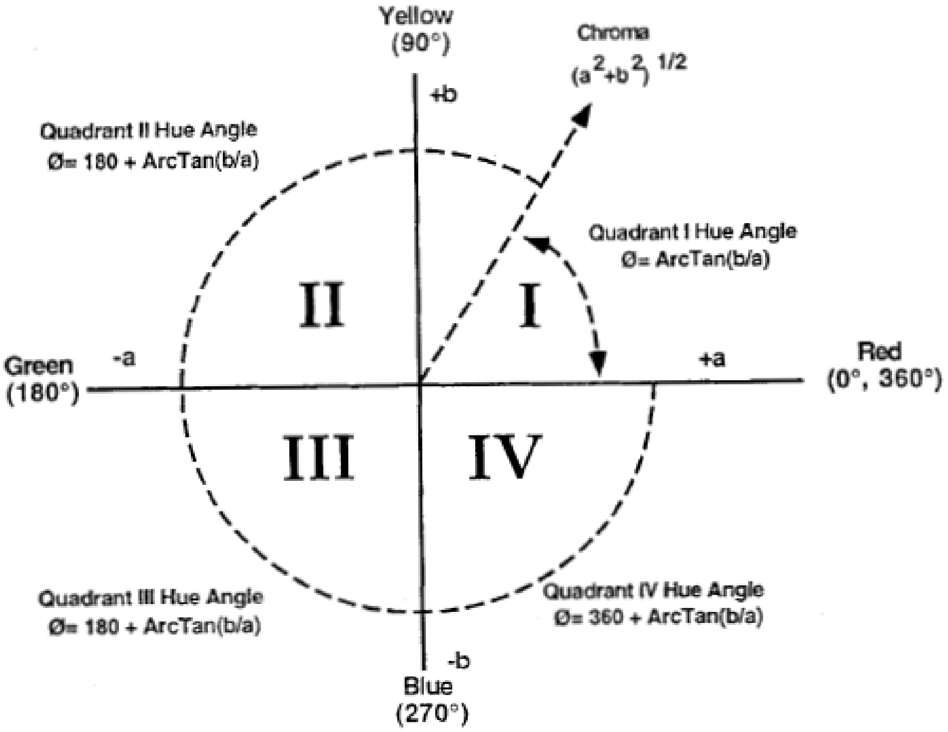


Figure 2-4: L*a*b* system diagram, showing the four quadrants and their trigonometrical formulae (McLellan *et al.*, 1994).

2.1.3 Colours in fish

In the animal kingdom, skin colours play an important role in life. In fish, the vast colour variation and the ability to change skin colouration are linked to vital physiological and behavioural information. Information such as gender, age, maturity, and breeding season are conveyed (Choat, 1965; Inouye *et al.*, 2001; Grether, 2005). Skin colour also has evolutionary advantages, e.g. camouflage and sexual communication. Furthermore, it is well-known that change in coloration can be a body response to the surrounding environment (Abbot, 1973; Sugimoto, 2002). Colours and colour changes, therefore, are important evolutionary adaptations. The subtlest changes in hues can determine the efficacy of survival and communication with conspecific (Fujii, 2000).

In fish, colours are formed by pigmented chromatophore cells. These specialised cells are able to generate colours by the scattering and reflection of light. Colour is generated by intracellular structures with refractive indices different from the cytoplasmic matrix (Fujii, 1993; Fujii, 2000). There are five main types of chromatophores that have been linked to fish: melanophores, erythrophores, xanthophores, iridophores and leucophores (Table 2.1). The first three chromatophores are categorised as light-absorbing cells, whereas the latter two are light-reflecting (Fujii, 2000). Inside of each chromatophore, there are pigmented organelles that by aggregation and dispersion have a direct link to the skin colouration. Each pigmented organelle contains the unique pigment which will give the colour characteristics to the cell. For example, melanophores contain brown to black pigmented organelles called melanosomes (Fujii, 1993). Their aggregation or dispersion will effectively contribute to a pattern or animal colouration (Figure 2.5).

Table 2-1: Five types of chromatophores found in teleost. New chromatophores have been identified however due to the lack of information, they were not added to this table. An adaptation from Nilsson Sköld *et al*. (2016).

Chromatophore	**Organelle**	**Pigment**	**Colour**
Melanophores	Melanosomes	Eumelanin	Black, brown; light absorption
Erythrophores	Erythrosome; carotenoid droplet	Pteridines; carotenoids	Orange to red; light absorption
Xanthophores	Xanthosomes	Pteridines; carotenoids	Yellow to orange; light absorption
Iridophores	Reflecting platelet	Purines, especially guanine platelet crystals	Structural colours and iridescence. For example: glittering blue, green, and silver; light reflection
Leucophores	Leucosomes	Mainly uric acids	White; light reflection

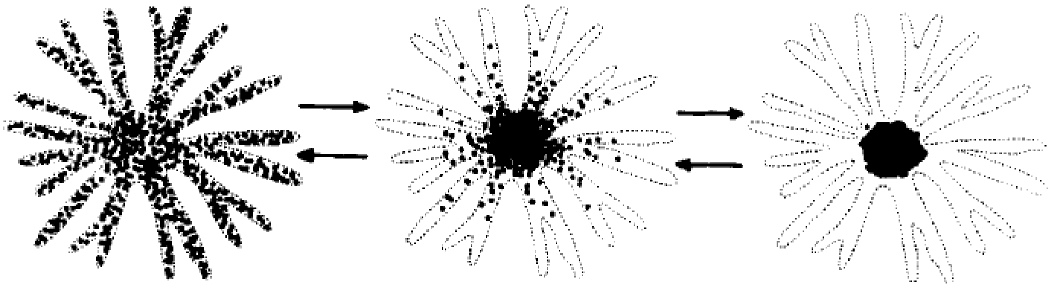


Figure 2-5: A diagram showing the aggregation and dispersion of the pigmented organelles in a dendritic chromatophore. Dendritic, because it resembles the morphologies of neurons. Such chromatophores include melanophores, xanthophores, erythrophores, and leucophores (Fujii, 1993).

Visually, external colouration and patterns are the simplest way to differentiate species. In labrids, where sexual dimorphism is common, skin coloration is one of the most reliable methods to distinguish male from female. There are many studies characterizing the markings of sequential hermaphrodite fish species. Roede (1972) made a descriptive observation of seven tropical labrid species at different stages of their life, where he noticed external colour and marking was described between male-male and male-female transitions. Choat (1965) made a detailed description of the colour patterns of the temperate wrasse *N. celidotus*. Through visual observations, it was possible to distinguish different stages in life as well as the gender of each individual. The distinguishable traits were: the colouration of the anal fin; a large round dark spot on the side (female); four dark traverse bars on the posterior portion of the body (female); and the appearance of bright blue broken or unbroken line through the body (male) (*sensu* Choat, 1965). Overall, visual observations of external markings have proved to be a simple but reliable way to differentiate species or gender, especially labrids.

The digitalisation of photography and image analysis have revolutionized the way colour measurement can be achieved. In the past, research was limited mainly to spectroradiometry and spectrophotometry as methods for colour measurement. Both are time consuming and expensive processes. However nowadays, speed to obtain data is much greater, as well as the standardisation efficiency of photography through computer software (Svensson & Nilsson-Sköld, 2011). Villafuerte and Negro (1998) found digital image analysis to be an affordable way of colour quantification. The use of digital photography and image analysis for skin colour quantification have being used since late 1990s (e.g. Kilner, 1997).

Since then, there were many studies using this methodology. The study scanned the red eye lore of live male and female red-legged partridges. The RGB values of the first picture was taken and used to adjust the remaining pictures, standardising the images. Deutsch (1997) used digital image analysis to quantify colour. The study aimed to examine the colour variations found in male rock-dwelling cichlids and the functionality of these variations in terms of adaptation, reinforcement or sexual selection between and within species complexes. Hue, saturation and brightness (HSB) were assessed at five different body locations. Meakin & Qin (2012) analysed HSB and RGB in the juvenile King George whiting (*Sillaginodes punctate*), to identify the effect of light intensity on the colour change. Ohta *et al*. (2008) measured fin hue in the protogynous wrasse *Pseudolabrus sieboldie* across different sexual stages, which was then linked to the change of sexual steroids. Finally, the CIE L*a*b* was used as a method to compare the chromaticity parameters in the red skin Sparidae (Pavlidis *et al*., 2006). The use of digital photography and image analysis have proved to be an affordable and efficient method to quantify colours. However important steps have to be considered to ensure the accuracy of colour measurement (Stevens *et al*., 2007).

The aims of this chapter were the attempt to develop a new methodology for measuring the skin colouration of *N. celidotus*, through digital photography and computational image analysis, using RGB, HSB and CIE L*a*b* colour models. Targeting the external differences between males and females at different parts of the body, with the objective to find the most reliable area for the measurement of colour. And ultimately developing a systematic methodology for assessing colour in this sex change species.

2.2 Methodology

2.2.1 Fishing procedure

During the period of 27/09/17 to 30/10/17, a total of 84 *N. celidotus* (72 females and 12 males) were caught using sabiki hooks (size 7), from 2 different boat ramp locations within Sulphur Point, Tauranga Harbour. Both areas are well sheltered against strong swells and currents.

- Fishing area 1: Sulphur Point boat ramp (37.66'91"S, 176.16'59"E), an area with frequent movement of boats going in and out of the water.
- Fishing area 2: Sanford's boat ramp (37°40'17.95"S, 176°10'18.60"E). Many fish species usually habitat this area, as there is a dock for commercial fishing boats as well as a fish processing factory. Most of the largest individuals used in this study were caught at this location.

As the fish were caught, they were placed into an 80 L transporting tank containing fresh sea water and a portable aerator to maintain oxygen saturation and reduce ammonia build-up. This ensured that the animals would stay healthy for the short period, between fishing and transporting to the aquaculture facility at Toi-Ohomai Institute of Technology, Tauranga. On arrival, the animals were placed into a 1600 L seawater tank at similar temperature as their natural habitat. The fish could then acclimate to captivity over 2 weeks, during which time they were fed whole green shell mussels. No excessive noise or handling were allowed in order to minimise the stress of the new environment. Also, several housings made of PVC as well as rocks were added, so the fish could hide. This study was reviewed by animal ethics commission (Protocol #994)

2.2.2 Captivity and animal health care

The acclimatization tank was designed to be a closed, recirculating system, where water is pumped out from the tank continuously, passing through a chiller (set at 17 °C), a UV filter and a pressure biofilter, before moving back to the tank. The tank also contained a protein skimmer. Once a week the protein skimmer and the biofilter were thoroughly cleaned and water exchanged. Water quality was tested every 3-4 days determining pH, nitrite (NO_2), nitrate (NO_3), ammonia (NH_3), salinity, temperature and dissolved oxygen. Fish were fed every 1-2 days with whole mussels for a period of 20 minutes. After feeding, the uneaten food was manually removed, and bottom of the tank cleaned. During the early stages when the fish were in the acclimatization tank, there was an increase of ammonia, followed by nitrite and nitrate levels. The increase is normal for a recirculating system as the biofilter adjusts to the extra nitrogen loading. All the parameters were below harmful concentrations (pH between 7 and 8; $NH_3 < 1$ppm; $NO_2 < 0.5$ ppm; and $NO_3 < 40$ ppm).

2.2.3 Photography procedure

10 females and 10 males of sizes ranging from 140mm to 220mm, were randomly selected and individually transferred to a 10 L seawater tank containing the anaesthetic 2-phenoxyethanol (0.6 ml/L, Sigma-Aldrich New Zealand Co.). The time of exposure was approximately 2 minutes, depending on the size of the fish. 2-phenoxyethanol has proven to be an efficient anaesthetic in several fish species (Sehdev *et al*., 1963; Barton & Helfrich, 1981; Weyl *et al*., 1996; Tsantilas *et al*., 2006). Once anesthetized, the fish was removed from the bath tank, the excess water was blotted using clean absorbent paper and finally, placed onto a raised platform added to the base of the camera stand. Photographs were taken of the full body on its lateral position, exposing the dorsal, tail and anal fin alongside of a colour calibration chart. The same position also exposed other important features that separates female from male (i.e. large round spot on females and blue ocelli on males). As soon as the photographs were taken, the fish was placed on a recovery fresh seawater tank, for 5-10 min before transferring back to the acclimatization tank.

2.2.4 Photography

2.2.4.1 Camera

All photography was conducted using a Canon PowerShot G11 (Canon Inc.) on Macro setting and RAW file format as recommended for colour imaging by Stevens *et al*. (2007).

2.2.4.2 Camera stand

The camera was fixed to a purposely built stand containing two LED light (200° beam, lumino flux: 4000 K, colour rendering index 83) sources, fixed to each side of the stand (Polaroid MP-4 Land Camera, Model 44-31). The camera was positioned at 36cm from the base of the stand and 25.5 cm above the fish and the colour calibration chart. The base of the stand had a light grey colour which was used as the background. Before the start of the experiment, different background colour was tested (green, blue, black, white, and light grey). Two main issues were found as soon as preliminary photographs were taken. One of the problems encountered was the colour pattern of the fins, which loses much of its brightness from the base to the tip. The second was the lighting and the background choice. Initial tests found that the white background did not affect the colouration of the fins, however as soon as the images were standardized, the light over-exposed much of the image, around the fins. To reduce this over-exposure of the images, it was decided to test the other background colours, which significantly reduced the problem. However, brighter colours such as the green and blue, or even black background, affected the colouration of the fins. This was especially evident in the anal fin where much of the sexually dimorphic colour change is observed. Finally, it was found that a light grey background had the best results as they did not affect the colouration of the fin and the image did not blow-out, caused by overexposure, which results in a complete loss of highlight detail. The stand was enclosed inside a dark room, where the only light was from the stand light sources, therefore, avoiding any changes in light condition.

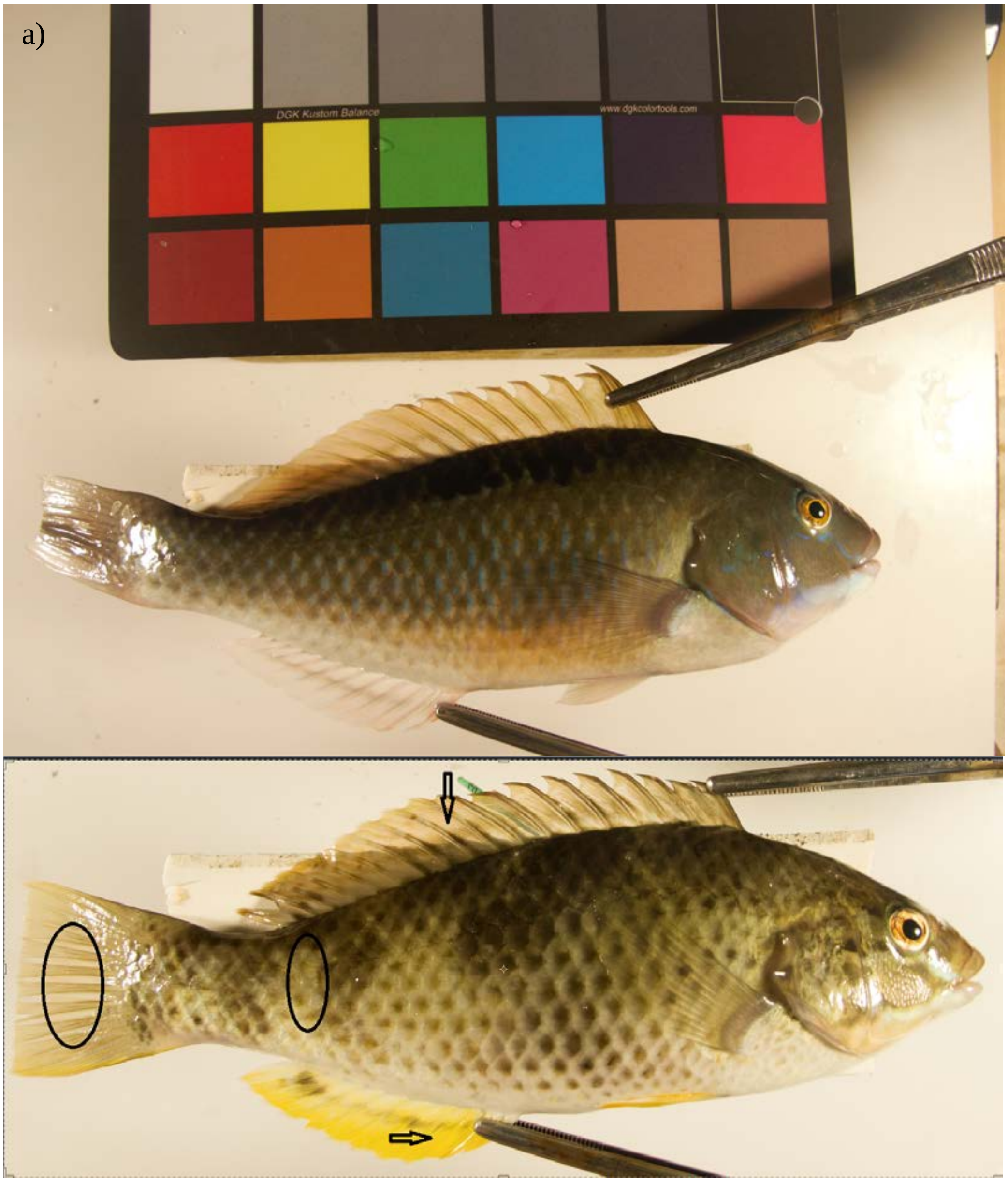
2.2.4.3 Photo calibration

A photo calibration chart (DGK Colour Tools DKK, http://dgkcolortools.com) containing 12 variations of RGB, plus white, black and 4 different shades of grey, was placed on top of a small platform raised 10.5 cm from the base of the camera stand (Figure 2.6). The animal was placed beside of the chart, also on a second, platform of similar height. Raising the fish was necessary to avoid shaded areas underneath the fish that could affect colour quantification results.

Figure 2-6: Photo calibration chart used in the experiment. (20x14cm)

2.2.5 Image analysis

All photos were analysed using the Adobe Photoshop CC 2018 (Adobe System Inc.) software. To ensure all photographs had the same colour standards, the following steps were essential. Firstly, the values of black and white of the colour calibration chart had to be adjusted, by adjusting the input of red, green and blue. This was done by selecting “Image” followed by “Adjustment” then “Levels”. At this point, black and white values were adjusted at minimum of 5 and maximum 245 for red, followed by green, then blue inputs. This procedure was done for each individual picture. Through this equalization process the classical hue for the yellow square of the colour chart was always 60°. The classical hue of yellow was listed as it can be used as comparison with the yellow anal fin of females. The “eyedropper” tool was then selected, to finally determine the HSB, RGB and CIE L*a*b* values for a standardized area of the anal, dorsal, tail and finally the posterior part of the side of the body (Figure 2.7a). For the anal fin, the landmark selected was the integument between the 1st and the 2nd anterior ray, as this appears to be the most consistent in colour. Other areas of the anal fin presented great variation in the yellow colouration as well as the presence of two dark spots that also varied in size according to each individual. The anal fin is made of 3 anterior spines, which are hard and pointy, followed by 9 rays, which are much softer and blends with the fin (Figure 2.7b). The landmark used for the dorsal fin was again between the 1st and the 2nd anterior ray. For the tail fin, the area selected was in the centre, avoiding the edges of the fins. Finally, the area selected for the body was in between where the dorsal and the anal fin finish, as there are some black markings across the area for the females, where the males are paler. All landmarks were selected due to the existence of sexually dimorphic variations in these locations. The pectoral fins were excluded on this basis. The blue ocelli found on the head and body of the males were excluded as the intensity varied greatly between individuals of the same sex and often with season.



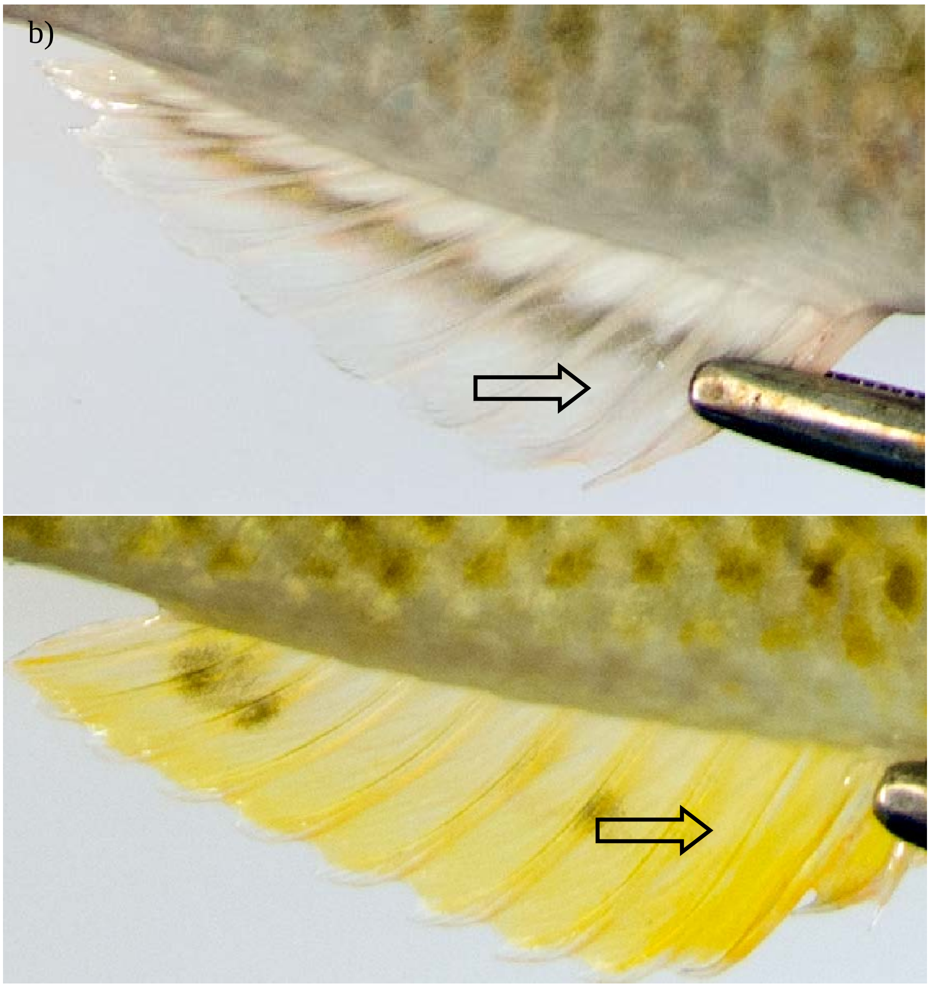


Figure 2-7: a) Photo of a male spotty (*N. celidotus*) compared with the female spotty. The landmarks proposed for female *N. celidotus* were the same proposed landmarks for the male. b) One of the most visible differences between male and female is the anal fin. The arrow on the female anal fin, indicates the area of analysis, between the 1st and the 2nd anterior ray, avoiding the black spot, seeing beside of the arrow. *N. celidotus* have three anterior spines, followed by 9 rays.

2.2.6 Accuracy of the procedure

Before the experiment could start, a single picture of the colour calibration chart was taken, and its image standardized and analysed using the Image analysis methodology, described before. The aim was to ensure the precision of the photography, achieved by calculating the variability between each point of the colour square. Four colour squares were chosen (red, yellow, green, and blue) for this test. In each of these colour squares, 10 points were randomly selected and a coefficient of variance (CV) for HSB, RGB, and CIE Lab calculated to assess variability and precision of the colour analysis.

2.2.7 Statistical analysis

After all photographs were standardized, the data was analysed using SPSS 20 statistical software package (SPSS Inc., Chicago, IL) and Microsoft Excel 2013. Visually, the anal fin is the main landmark to differentiate males from females. Therefore, the anal fin was used to demonstrate how different the colouration is between the sexes. By using SPSS, it was possible to generate three-dimensional graphs, which was designed to show the differences between male and female for the three colour models. For a better representation of the graph, all values were scaled to the minimum and maximum values found for each parameters of the colour models (e.g. red 0 – 255, brightness 0 – 100%). The difference in the anal fin colouration between sexes were also tested by analysing the relationship between brightness, L*, and the chromaticity coordinates (a*, b*). The choice of L*a*b* was simply based on the uniformity and standardization of the colour measurements. An important consideration to make is that the chromaticity coordinates, which is also attributed to hue, is a circular variable where numerical values can be identical such as 0° and 360°. Therefore, classical statistics do not necessarily accommodate circular data. Following Pavlidis and colleagues (2006) recommendations, it was decided to incorporate circular statistics for colour measurement. The angular hue (*H*°ab) was calculated to analyse all the body landmarks. The Rayleigh *z* test was applied to test the uniformity of the circular distribution (Zar, 1999) between each body part, female versus male. In other words, to check if the colour angle follows a distribution pattern or are just randomly distributed across the angles of a circle. Trigonometrical laws were applied to find the radians. For the *H*° of HSB, the radians were calculated from the degree angle of hue given by Photoshop. In Excel, a simple transformation from degrees can be calculated with the formula =RADIANS (x°), where x is the hue in degrees. *H*° of RGB was found by transforming RGB into a singular hue value by the formula proposed in the algorithm of Gardner (2007) (*sensu* Meakin & Qin, 2011):

(IF(Min=Max,0,IF(R=Max,(G-B)/(Max-Min),IF(G=Max,2+(B-R)/(Max-Min),
4+(R-G)/(Max-Min))))*60)

where ‘Max’ and ‘Min’ represent the largest and smallest red (R), green (G) and blue (B) values obtained for each data set. The above formula was also calculated using Excel cells. Then, *H*° for the a*b* coordinates were found by converting a* and b* coordinate values into a singular hue value, by the formula *H*°ab = Arc tan (b^*/a^*). By finding the radians, the *H*°, sine, and cosine could then be calculated. At that point, *H*°RGB and *H*°a*b* were all compared to the HSB hue, given by Photoshop. A circular statistic also means that a classical average and standard deviation (SD) cannot be applied. Therefore, the average and SD were calculated following Zar (1999) recommendations. Finally, a correlation test was done to compare classical hue to the angular hue form a*b* and from RGB transformations. Thus, indicating which transformation was the most reliable to measure skin colouration of *N. celidotus*.

2.3 Results

2.3.1 Accuracy test

The precision of the colour analysis varied according to the colour model used when sampled from the four colour chart squares. The CV of HSB and L*a*b were less than 5% while RBG had the greatest CV (253.86 %) (Table 2.2).

Table 2-2: Test of variability of colours taken from a digital camera.

	H			S			B		
Filter	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
Red	0.00	0.00	0.00	99.70	0.67	0.68	80.90	0.32	0.39
Yellow	60.00	0.00	0.00	95.20	0.92	0.97	99.90	0.32	0.32
Green	96.90	0.32	0.33	66.80	0.79	1.18	75.80	0.42	0.56
Blue	211.30	0.48	0.23	73.30	0.48	0.66	99.00	0.00	0.00

Filter	R			G			B		
	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
Red	206.60	1.17	0.57	0.50	1.27	253.86	0.70	1.57	223.86
Yellow	254.90	0.32	0.12	254.90	0.32	0.12	12.00	2.26	18.84
Green	114.10	1.45	1.27	193.20	0.79	0.41	64.10	1.52	2.38
Blue	67.50	1.43	2.12	156.30	1.77	1.13	253.20	0.92	0.36

Filter	L*			a*			b*		
	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
Red	51.30	0.48	0.94	77.40	0.52	0.67	67.00	0.00	0.00
Yellow	97.90	0.32	0.32	-16.00	0.00	0.00	103.10	0.32	0.31
Green	69.30	0.48	0.70	-60.50	0.53	-0.87	58.10	0.57	0.98
Blue	60.80	0.63	1.04	-13.70	0.48	-3.53	-61.40	0.52	-0.84

SD: standard deviation; CV: coefficient of variance

2.3.2 Colour comparison between male and female

A CV < 5% set was determined an acceptable target of precision, therefore the next step was to compare the male with the female anal fin colouration. The comparison was firstly analysed through three, 3-D graphs. For each graph, a colour model (HSB, RGB, CIE L*a*b*) was used to compare sexes, with all the graphs clearly demonstrating colour differences. For HSB, due to the nature of the colours between males and females there was an expected difference in hue values. For the males, the hue had a mean angular value of 105.27 ± 25.37° with the saturation mean of 3.40 ± 1.01% between the 10 individuals. Meaning that the colour of the anal fin on males were dull. Whereas the mean hue for the females was 57.23 ± 2.61°, with the saturation of 95.40 ± 7.97%. Thus, the yellow colour of the females was 4.60% short of been fully "pure" (100%). Both genders presented the brightness values above 95% (Figure 2.8a). For RGB colour models, the filter blue was the main differential of the colour between the sexes. Males presented much higher levels of blue colouration (238.96 ± 9.58) compared with the females (11.47 ± 20.23). The great difference in levels of blue, separated the yellow (female) anal fin colour, from the whitish male anal fin (Figure 2.8b). Differences between gender colouration was also observed through CIE L*a*b*. Vector b* presented the most variance between female (99.72 ± 5.27) and male (2.80 ± 1.52). Thus, the vector b* could be considered the main component to separate sex in the *N. celidotus* (Figure 2.8c).

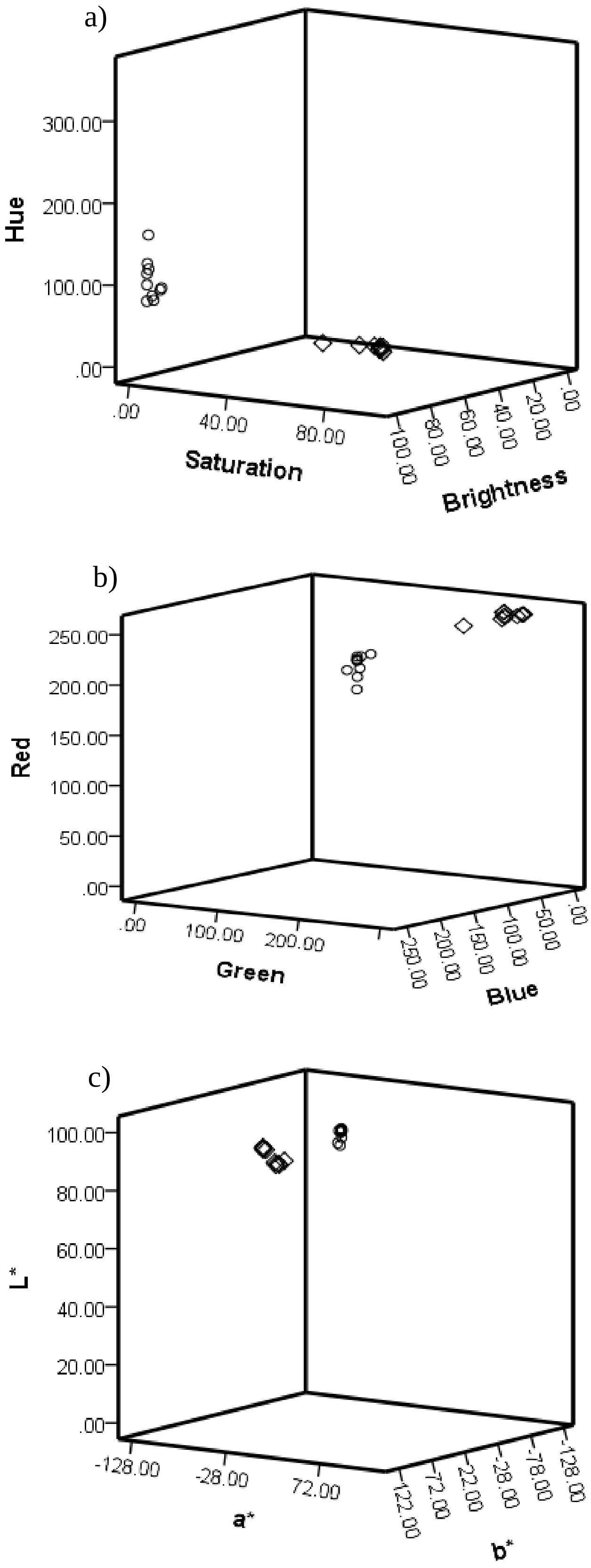


Figure 2-8: Comparison of colour of anal fin from female (◊) and male (○) *N. celidotus*, using a) HSB, b) RGB, and c) CIE L*a*b* colour models.

When the CIE L*a*b* parameter relationships were compared, vector b* was the main factor to differentiate between male and female external colouration in the anal fin. Again, due to the large variance between male and female, as previously mentioned. The relationship between L* (female = 94.75 ± 2.65; male = 97.17 ± 2.00) and vector a* (female = -9.52 ± 6.16; male = -3.45 ± 1.10) showed male and female groups all clustered together (Figure 2.9a). However, as vector b* (female = 99.72 ± 5.27; male = 2.80 ± 1.52) was added into the relationship, the male and female group became distinct (Figure 2.9b, c).

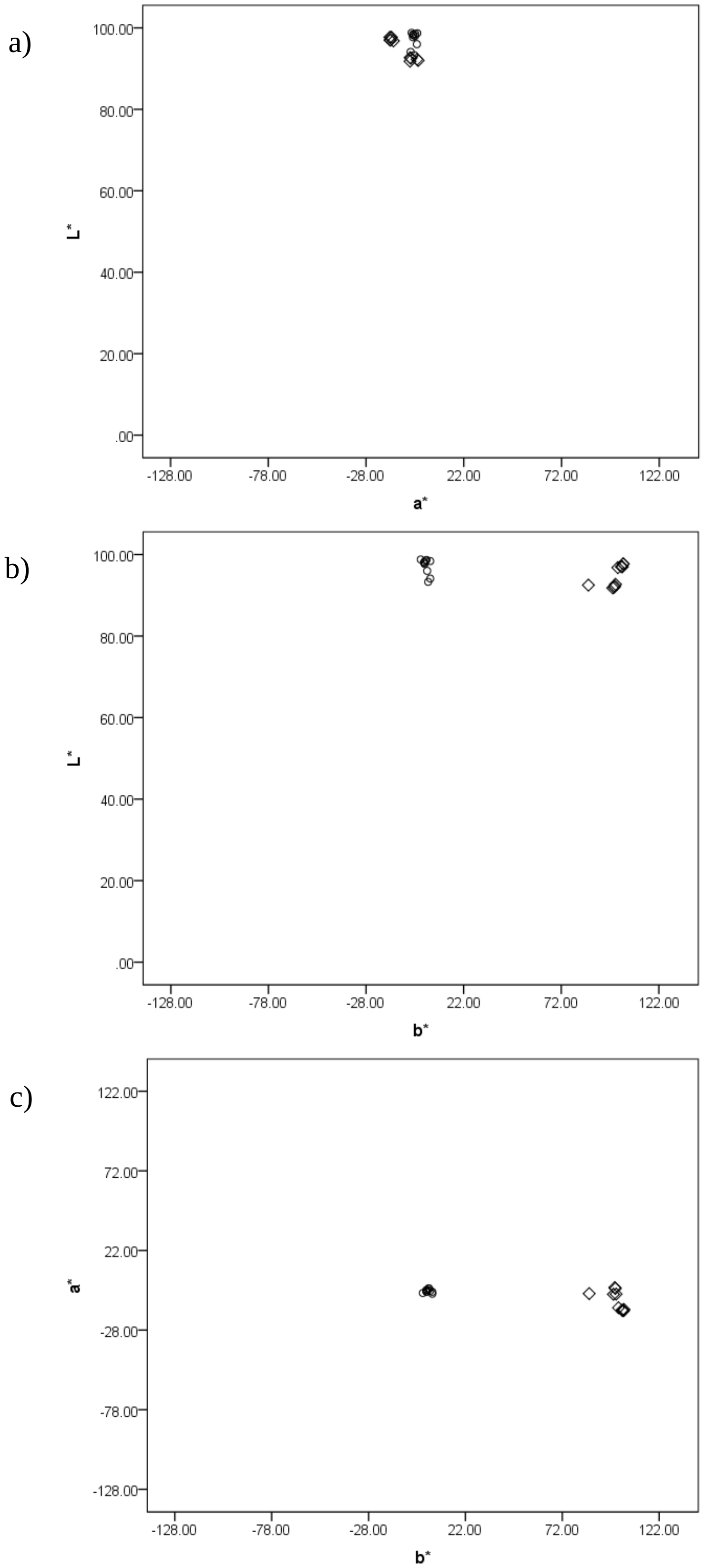


Figure 2-9: Relationship between measurements comparing the anal fin of female (◊) and males (○) *N. celidotus*. a) chromaticity coordinates between L* and a*; b) chromaticity coordinate between L* and b*; c) chromaticity coordinates between a* and b*.

2.3.3 Circular statistics to measure colour

As previously mentioned, vectors a* and b* are angular coordinates of hue. These can be converted into a singular hue value (*H*°ab) (Pavlidis *et al*, 2006). Thus, circular statistics were adopted. To test the circular statistics, four body landmarks, well known to be different from male to female were tested using *H*°a*b*. This is commonly read in radians, but can be transformed into degrees, using trigonometrical laws (see Figure 2.4 for the formulae), for easier interpretation. As suspected, the anal fin (female *H*°a*b* = 95.353° ± 3.16°; male *H*°a*b* = 141.138° ± 17.23°) presented the most visible colour variation between gender (Figure 2.10). All body parts had significant differences between the means (Watson-Williams F-Test (WW-Test = $p < 0.05$), as well as uniformity of the means (Rayleigh Test = $p < 0.05$, $N = 10$) (Table 2.3).

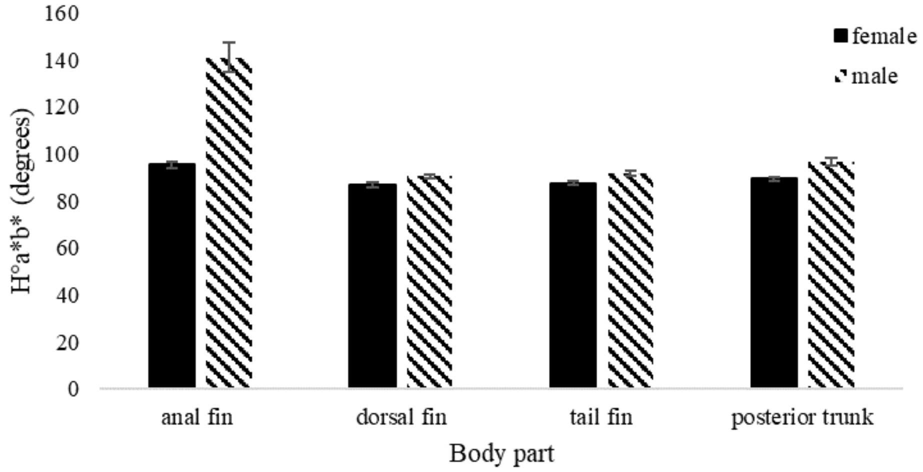


Figure 2-10: Body part comparison using hue angle of a* and b* coordinates in *N. celidotus*. (Error Bars are Standard Deviations n=10 males and 10 females).

Table 2-3: Comparison of significance of the mean *H*°a*b* (WW F-Test) for the four body landmarks as well as their uniformity (Rayleigh Test).

Body part	Mean H°		SD		SE		Rayleigh Test		WW F-Test
	female	male	female	male	female	male	female	male	
anal fin	95.353°	141.138°	3.156°	17.231°	1.175°	6.412°	<0.001	<0.001	<0.001
dorsal	86.744°	90.5°	2.785°	2.433°	1.037°	0.906°	<0.001	<0.001	<0.05
tail fin	87.667°	91.655°	2.61°	2.929°	0.972°	1.09°	<0.001	<0.001	<0.05
Posterior trunk	89.37°	96.699°	2.59°	3.832°	0.964°	1.426°	<0.001	<0.001	<0.001

2.3.4 Anal fin as a potential landmark

With anal fin having been accepted as the most reliable body landmark to study colour differences between male and female, there was the possibility to exploit the angular hue (*H*°) for each of the colour models. Curiously, the mean *H*° (radians) found using HSB, RGB, and a*b* for the females returned the same results to both classical descriptive and circular statistics. Conversely for males all values were different (Table 2.3). Mean angular hue values, in degrees, also had similar values between the two statistical models, and again, for the females, this was the case for RGB and HSB angular hue only. The males presented the greatest differences in the angular mean between both statistical analyses (Table 2.4).

Table 2-4: Radians mean values and standard deviation (SD) for the angular hue (H°), estimated by classical descriptive statistics ($_1$Hue) and circular statistics ($_2$Hue) for 10 males and 10 females *N. celidotus*.

	Radians					
	Female			Male		
Fish #	*H*° HSB	*H*°RGB	*H*°ab	*H*° HSB	*H*°RGB	*H*°ab
1	0.95	0.95	-1.55	2.20	2.22	-0.44
2	1.03	1.03	-1.43	2.08	2.06	-0.46
3	1.05	1.05	-1.43	1.74	1.71	-0.73
4	1.04	1.04	-1.42	1.59	2.77	-0.86
5	1.05	1.04	-1.42	2.82	3.58	0.00
6	0.94	0.94	-1.56	1.40	2.84	-1.03
7	0.97	0.97	-1.51	1.65	1.66	-0.76
8	0.97	0.97	-1.52	1.40	1.39	-1.03
9	1.04	1.04	-1.42	1.98	1.18	-0.56
10	0.95	0.95	-1.51	1.52	1.50	-0.89
Mean $_1$Hue	1.00	1.00	-1.48	1.84	2.09	-0.67
Mean $_2$Hue	1.00	1.00	-1.48	-1.32	-1.10	-0.68
SD $_1$Hue	0.05	0.05	0.06	0.44	0.76	0.32
SD $_2$Hue	0.04	0.04	0.06	0.42	0.73	0.30

Mean $_1$Hue and $_1$SD were estimated according to the formula: Mean $_1H^\circ=\Sigma H^\circ/N$ and $_1$SD= $[\Sigma(H^\circ-Mean\ H^\circ)\ /\ (N-1)]^{1/2}$. Mean $_2$Hue and $_2$SD was estimated according to the formula: Mean H°= Arc tan$[(X/ r)/(Y/r)$ and SD= $[(-2\ ln(r)]^{1/2}$; where X=$\Sigma(\cos H^\circ)\ /N$; $r=(X2+Y2)^{1/2}$; Y=$\Sigma(\sin H^\circ)\ /N$; H°ab= Arc tan (b^*/a^*), in radians; N = number of observations.

Table 2-5: Mean values and standard deviation (SD) for the angular hue (*H*°), estimated by classical descriptive statistics ($_1$Hue) and circular statistics ($_2$Hue) for 10 males and 10 females *N. celidotus*.

	Degrees					
	Female			Male		
Fish #	*H*° HSB	*H*°RGB	*H*°ab	*H*° HSB	*H*°RGB	*H*°ab
1	54.30	54.16	91.10	125.80	127.06	154.65
2	58.80	58.98	97.89	119.10	118.21	153.43
3	60.00	60.00	98.22	99.90	97.74	138.24
4	59.70	59.78	98.57	91.30	158.60	130.97
5	59.90	59.86	98.66	161.60	205.15	180.00
6	54.00	53.96	90.75	80.10	162.93	120.96
7	55.70	55.69	93.20	94.70	95.35	136.70
8	55.50	55.57	93.10	80.00	79.75	121.22
9	59.80	59.64	98.64	113.20	67.47	148.17
10	54.60	54.48	93.40	87.00	86.22	129.23
Mean $_1$Hue	57.23	57.21	95.35	105.27	119.85	141.36
Mean $_2$Hue	57.23	57.21	84.65	255.51	242.99	38.86
SD $_1$Hue	2.61	2.64	3.33	25.36	43.79	18.17
SD $_2$Hue	2.48	2.51	3.16	23.98	42.06	17.23

Where *X*=cos and *Y*=sin. The transformation of the M $_2$Hue follows trigonometrical laws, in which to determining the quadrant: Sin +, Cos + : the mean angle is computed directly. Sin +, Cos -: the mean angle = 180 –θr. Sin -, Cos -: the mean angle = 180 + θr. Sin -, Cos + : the mean angle = 360 -θr.

Finally, the strength of the relationship between hue values was tested. Where *H*°HSB, given by Photoshop in degrees, served as a base to the new transformed angular hue values. For the females, *H*°RGB had a strong correlation to *H*°HSB (r^2=0.99) as well as *H*°ab in relation to *H*°HSB (r^2=0.97). For the males, the strength of the relationship between *H*°RGB was poor (r^2=0.24) compared to *H*°HSB and *H*°ab (r^2=0.99).

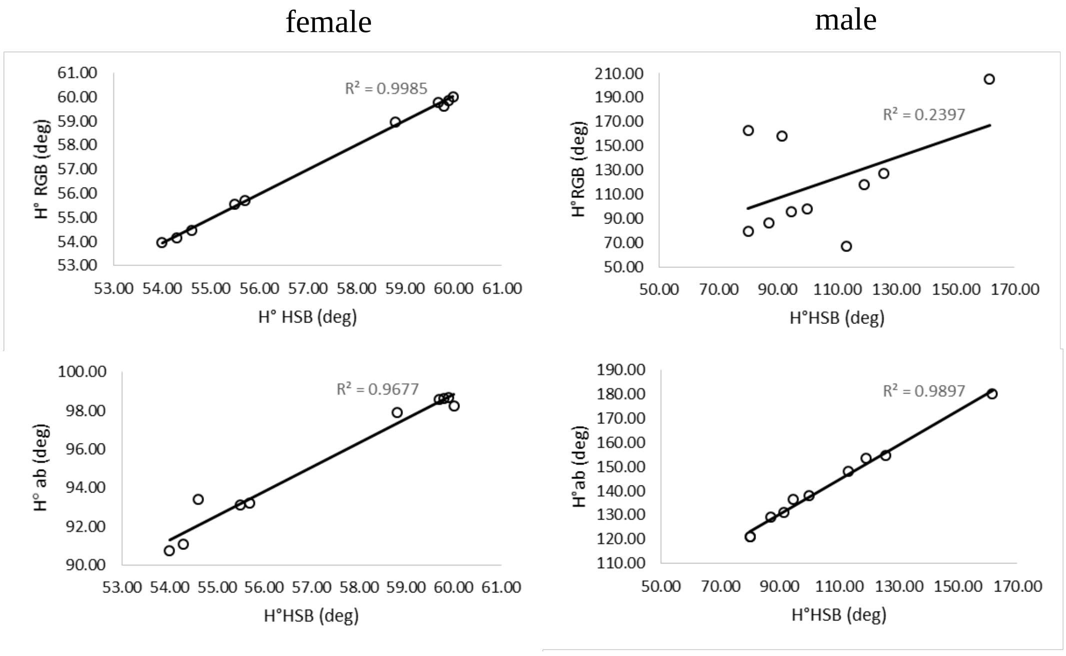


Figure 2-11: The relationship between calculated Hue values and digitally derived values from the HSB colour model for male and female *N. celidotus*.

2.4 Discussion

This study investigated an alternative approach to measure the skin colouration of *N. celidotus* through digital photography and computational image analysis. A total of 10 males and 10 females were photographed and the images analysed for colour quantification. Visually, the difference in skin colouration and colour patterns between gender are easily observed in the *N. celidotus* (*see* review by Choat (1965)). However, no one has ever attempted to compare male and female external colouration through colour quantification in this species.

The colour models, HSB, RGB and CIE L*a*b*, were plotted and analysed individually to identify colour differences in the anal fin for both genders. For the first part of the study, simple descriptive statistical analysis was used to quantify colour of the anal fin. In the anal fin of *N. celidotus*, females are known to have yellow colouration, and as sex changes, the fin becomes a pale whitish green colour (Choat, 1965). Therefore, distinct differences were already expected. It is worth remembering that HSB, although it directly gives the hue value, is a human-vision specific colour space, and it is unlikely to be accurate (Steven *et al.*, 2007). Poynton (1999), also argued that HSB is based on RGB components (device dependent). Thus, making both colour models impractical for conveyance of accurate colour information. Despite the inaccuracy of colour quantification inherent in HSB and RGB models, the variation in colours from male and female were noticeable. CIE L*a*b*, on the other hand, due to its uniformity, standardisation and device-independence, could be considered as the most reliable colour model measurement, providing consistent colour, regardless of the input or output device such as digital camera, scanner, monitor, and printer (Yam & Papadakis, 2004). Due to its reliability in colour quantification, the relationship between the filters were further tested. Although, lightness and hue (a* and b*) were tested, the strongest relationship was between the hue-angle in the a* – b* plane. One of the reasons was that lightness relates to a variation from white to black and had little variation between male and female markings (Figure 2.9). Furthermore, the possibility to transform both angles into a singular value, arises as proposed by Pavlidis *et al.* (2006). The relationship between L*a*b* parameters were also tested to analyse the skin colour in the blue-footed booby (*Sula nebouxii*) seabird (Velando *et al.*, 2006). Of the three colour models specified, CIE L*a*b* was suggested to be the best for colour quantification. Based on uniformity, standardisation and device-independence comparison.

Measurement and comparison of hue might seem straightforward when using Adobe Photoshop and statistical analysis. Many published studies on animal skin have only used the classical statistical approach, without considering that hue is an angle (Davenport & Bradshaw, 1995; Deutsch, 1997; Ellis *et al.*, 1997; Gouveia *et al.*, 2003). Therefore, to compare means of hue, circular statistics should be considered (Pavlidis *et al.*, 2006). In this chapter, the classical statistical methods were only considered to compare colour from scatterplot graphs. The results revealed a distinctive colour difference between genders. However, if colour measurements are to be based on a unit from a circular scale then it is logical that circular statistics should be used for data analysis.

Body landmarks that yielded quantifiable differences in colour between male and female spotty were successfully identified. It was found that the anal fin was the most reliable mark to differentiate sex. Circular statistical analysis also showed that the anal fin colouration was the easiest way to separate male from female.

In another protogynous wrasse, *Pseudolabrus sieboldin*, the anal fin was also the body part that presented the most notable change. Colour change of the male morph was associated with increases in the circulating plasma androgen 11 ketotestosterone (Ohta *et al*., 2007). Also noticed by Ohta *et al* (2007), all other body landmark tested had little difference between genders. Furthermore, results from the anal fin values calculated showed a strong correlation between hue derived from HSB and calculated *H*°ab. This implies that the values of *H*°ab, can be associated to the values given by Adobe Photoshop. Few other, published papers used *H*°ab as colour measurement (Pavlidis *et al*., 2006; Kalinowski *et al*., 2005; Erikson & Misimi, 2008). Ultimately, *H*°ab was successfully used to compare body marks colouration in the *N. celidotus*, specially the anal fin.

In the present study, it was determined a reliable key landmark showing clear differences in colour between male and females. The best method to quantify this was using circular statistics since hue is angle-based values. By developing a methodology to quantify colour in the spotty, it is possible now to analyse the full process of the anal fin colour change during sexual transitioning from female to male.

Chapter 3

Colour changes and histological analysis of sex change in the temperate protogynous wrasse *Notolabrus celidotus*

3.1 Introduction

Protogyny sex change in wrasses is common and has been documented several times (Atz, 1965; Robertson, 1972; Robertson & Choat, 1974; Robertson & Warner, 1978; Warner & Robertson, 1978; Jones, 1980; Nakamura *et al.*, 1989; Sadovy de Mitcheson & Liu, 2008; Muncaster *et al.*, 2013). The most accepted explanation for this sexual strategy is based on the “Size advantage model” hypothesis (Guiselin, 1969). By transitioning from female to male late, it allows the animal to grow, leading to an increase in reproductive success (Robertson, 1972; Warner, 1975; Warner et al., 1975; Warner, 1988; Kazancıoğlu & Alonzo, 2010). Size advantage also leads to higher hierarchical ranking and social dominance in harems (Robertson, 1972; Robertson & Choat, 1974; Kuwamura, 1984). Dominance is commonly attained by aggressive behaviour towards the smaller individuals from the group (Robertson & Choat, 1974; Kuwamura, 1984). Dominance status can be simply measured by serial removal technique. A group of fish are put together, the fish that monopolises food and territory is termed dominant. By removing the dominant individual, another will take its place, becoming the second dominant, and so on until group is ranked (Metcalfe *et al.*, 1989; Huntingford & Garcia de Leaniz, 1997). Similar experiments have also been used to induce natural sex change in wrasses (Fishelson, 1970; Robertson, 1972; Fricke & Fricke, 1977; Shapiro, 1979; Shapiro, 1981; Ross *et al.*, 1983; Warner & Swearer, 1991). The dominance in protogynous wrasses are extremely important to sex change induction and maintenance of the harem. Sex change will only be triggered by the removal of the dominant male from the rest of the harem

(Shapiro, 1988; Robertson & Choat, 1974). Although sex change in wrasses are well documented, not much has been focused in temperate species.

Notolabrus (former *Pseudolabrus*) *celidotus* Bloch & Schneider, 1801, commonly known as Spotty or Paketi, in Māori, is part of the labridae (wrasse) family. The species is endemic to New Zealand and commonly found in shallow harbours, estuaries and reefs with low wave exposure (Denny, 2005). Like the other wrasses, they are protogynous and monandric (not all females will become males) (Jones, 1980). Living in a hierarchical group, where the male dominates all the females in the harem (Jones, 1983). Spawning season starts late July until early December (Jones & Thompson, 1980). They are polygynous, and the male will breed multiple times during the spawning season. Whenever the male dies, the dominant female will change sex and assume his place as a polygynous male (Robertson & Choat, 1974; Ross *et al*., 1983; Shapiro, 1979). *N. celidotus* are dimorphic and its colour patterns can be used to distinguish the sex or if the animal is transitioning between gender (Choat, 1965). Although this species is one of the most studied wrasses in New Zealand, most of the research has been focused on the internal changes (e.g. gonadal analysis) and excluded research on skin colour and external patterns changes.

The process of protogynous sex change is commonly related to environmental and social cues. When the socio-environmental cues are met, visual stimulus is initiated (Ross *et al*., 1983), triggering a cascade of physiological events through the brain-pituitary-gonad (BPG), until transition is completed. The specific details of sex change may vary according to each species, however, there are general steps to the transitional process (Muncaster *et al*.,2013). The earliest sign of sex change in a protogynous individual is the display of male dominant behaviour. Behavioural changes are rapid, occurring within minutes from the male removal (Robertson, 1972; Warner & Swearer, 1991; Godwin, 1994). As dominance is achieved, gonadal restructuring starts by degeneration of oocytes in the ovary. The process is followed by spermatogenesis until a functional testis forms (Warner & Swearer, 1991; Bhandari *et al*., 2003; Muncaster *et al*., 2013). The gonadal restructuring is linked to the exchange of serum steroid hormones. At female phase, during the breeding season, estradiol-17β (E_2) levels are elevated. As sex change starts, there is a decrease in E_2 levels, which declines furthermore during later stages. Meanwhile, 11-ketotestosterone (11-KT) levels increases reaching its peak as transition is complete (Nakamura *et al*., 2003; Bhandari *et al*., 2003; Ohta *et al*., 2008; Muncaster *et al*., 2013). The steroids and gonadal changes have also been linked to the dimorphic changes between sexes (Ohta *et al*., 2008). These dimorphic changes are an essential part of sex change as visual stimulus is needed to initiate the transition. However, the role of sex steroids has been the most researched.

The aim of this study is to investigate whether external colour change can be associated to the stage of gonadal transition. This was achieved by digital photography and image analysis for the external changes, and histological examination of the gonad at different stages of sex change.

3.2 Methods

3.2.1 Sampling procedure

During the period of 4 months (November – March), all fishes from treatment group tanks were sampled. Fish were firstly euthanised through an overtime exposure to the anaesthetic, 2-phenoxyethanol (0.6 ml/L, Sigma-Aldrich New Zealand Co.). When the animal was deceased, the sampling procedure could continue. Straight after death, body length, weight and digital photographic pictures were taken from the full body (same methodology used in Chapter 2). The colour markings used for this study were not affected after death as pictures were taken straight after been removed from anaesthetic tank. For endocrine analysis, blood was collected from the caudal vein using a 25-gauge needle attached to a, 1 ml, heparinised syringe (heparin sodium, Sigma-Aldrich New Zealand Co.). Then inserted into a micro centrifuge (Gyrozen Mini), spinning at 3000 rpm for 3 minutes, in which red blood cells were separated of the plasma. Plasma was finally collected and fast frozen in dry ice and stored at -80°C. The whole brain and the pituitary gland were also collected. An incision was made just above the eye, until the rear end of the head exposing the brain, which was carefully removed, followed by the pituitary gland. They were also stored at -80°C. Another small portion of the gonad (left area) was also collected, as well as head kidney, liver and flesh. These were not used in this experiment but will be useful for future analysis.

3.2.2 Histology

Gonads were removed, weighted and the right side thinly sliced (1 mm) at the rear middle and front part. Gonad tissues were fixed in either Bouins (male and transition gonads) or neutral buffered formalin (female gonads). Bouins was chosen due to the great resolution of condensed chromatin packing used to identify the stages of spermatozoid and gonad development (Muncaster *et al.*, 2013). The tissues were preserved in the solution for no longer than 24 hours, before been placed into 70% ethanol (C_2H_6O). These were serially dehydrated using ethanol and xylene and then paraffin embedded. Histological 3 µm thick sections were stained with haematoxylin and eosin on glass slides. The stage of sexual state was classified according to presence or absence of female and male germ cells using light microscopy.

3.2.3 Photography and digital analysis

The anal fins images and digital analysis were taken using the same technique developed in chapter two. Where the chromatic coordinates a* and b* (CIE, 1976) were used to calculate the hue angular value for the anal fin. 10 points within the first and the second anterior ray were randomly selected, avoiding the dark spots and centre line. Values for a* and b* were found using Adobe Photoshop CC 2018 (Adobe System Inc.).

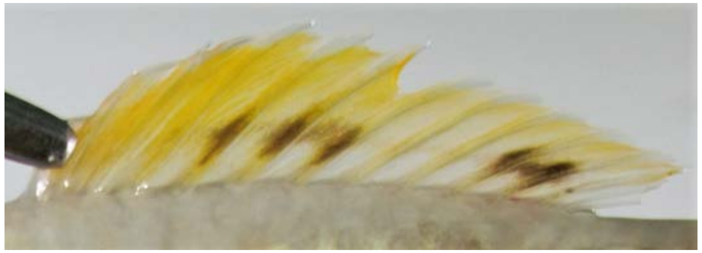
3.2.4 Statistical analysis

Statistical analysis was carried out with Oriana version 4 (2018 Kovach Computing Services, Isle of Anglesey, UK). The program is based on circular statistics, as described in Chapter 2. Watson-Williams F-Test was performed to identify significant variations between the hue angle means of the anal fin at different sex change stages.

3.3 Results

The anal fin colouration showed visible changes during the transition from female to male. During the transition, there was a distinct discolouration of the anal fin, from yellow to a pale white green hue (Table 3.1). Another feature was the reduction of the two spots also visible in the females. As transition progressed, the two spots started to lose their shape and colour, becoming diffuse, until both markings became absent. Later in the transition, a centre line appeared from the anterior to the posterior part of the fin, where the two spots were previously located.

Table 3-1: Visual description of the anal fin before, during and after the sex change is complete in *N. celidotus*.

Description	Pictures
NBF (non-breeding females): Yellow throughout, although mid-section may be clear/patchy. Two dark spots - front 1/3 and last third of fin (front spot is mostly located between the 1st to the 3rd anterior ray and the last spot is commonly located from 6th to 9th ray). There were individuals, however, with elongated spots and others with no or very small front spot.	
ET (early transition): Anal fin still yellow, however, the rear 2/3 of the fin appears to lose its yellow pigmentation. Front third remains slightly yellower. The two spots are still visible but the dark colouration in the front spot starts to fade at the fin membrane (between the rays), but not at the rays. Last fin appears to have no changes on its shape and colour.	
MT (mid-transition): The yellow colouration has faded significantly throughout the fin, however, there may be some dull yellow patches at the anterior part, mostly invested at the spines and rays. The front spot is now diffused and becoming transparent. A mid-line starts to develop where the spot once was prominent and elongates all the way to the last spot. The last spot has decline in size, but it is still dense and dark, manly at the rays.	

LT (late transition): In most of the individuals the yellow colouration has now diffused, becoming a pale white green colour. The front spot has now fully disappeared, and the mid-line has become visible. The last spot has declined more and now has the width of the mid-line. In some individuals, the last spot has also completely diffused.

TP (terminal phase): No visible yellow colouration. No visible spots and the mid-line are evident. The colour of the mid-line may range from yellow to grey, also some can be tinged red. However mostly identified at breeding months.

The change in colouration of the anal fin was analysed and compared at different stages of the sexual transition (Figure 3.1 & 3.2). During NBF and ET stage, there were no significant differences in hue angle (Watson-Williams test, $p > 0.05$). However, as the fish became mid transitional the mean anal fin hue, 108.35 ± 6.53°, became significantly ($p < 0.05$) paler. The changes in angular hue from female to male is demonstrated as bar graph of hues with error bars and significance stars (Figure 3.2). The mean fin hue of LT fish 114.34 ± 5.86° was not significant different to the previous stage but significant different to the next stage ($p < 0.05$). Interestingly, there was no hue angle significance between one stage and the following.

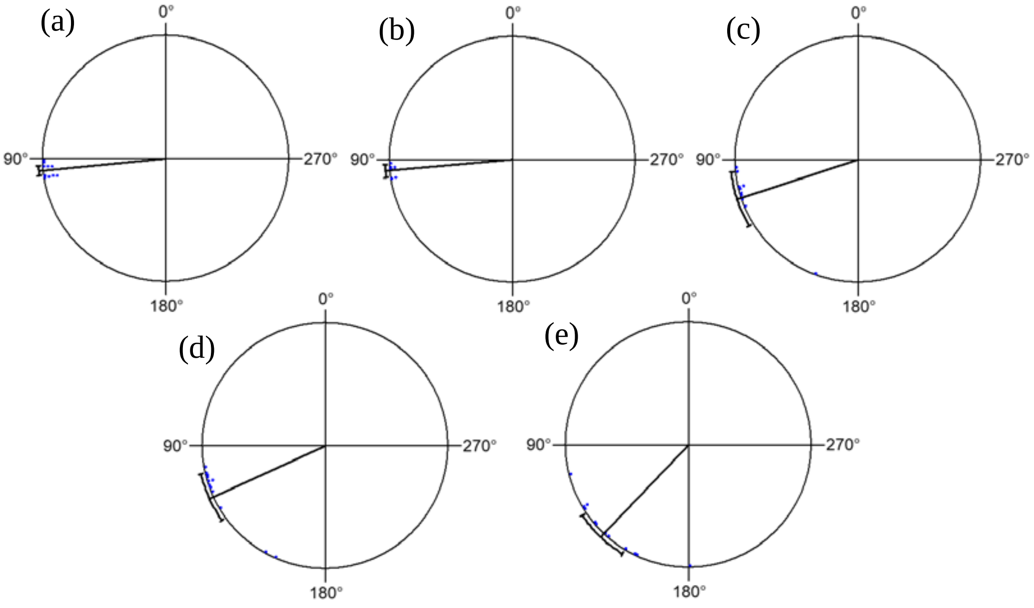


Figure 3-1: Angular hue changes during sexual transitioning. a) breeding female; b) early transition (ET); c) mid transition (MT); d) late transition (LT); e) terminal phase male (TP). Bars indicates the means of the angles and standard deviation from female to male.

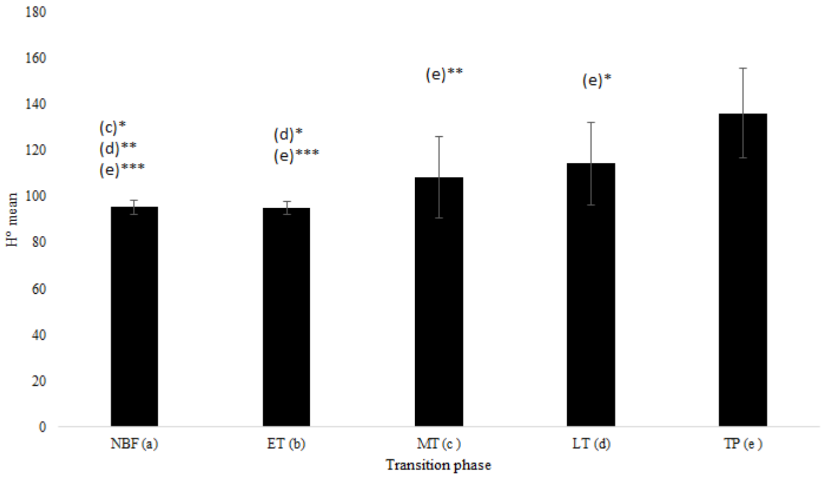


Figure 3-2: The change in angular hue from female (NBF) to TP male. Watson-Williams Test was added to identify any significant angular value variation within the means. Where $p < 0.05$ *, $p < 0.01$ **, and $p < 0.001$ ***. Error bars are Standard Deviations (n>20).

Fish showed a range of transitional stages throughout the course of the experiment. They had all the five transitional stages, used in the colour changes (NBF, ET, MT, LT, and TP). As the anal fin colour began to change, the gonads also began the transformation (Figure 3.3).

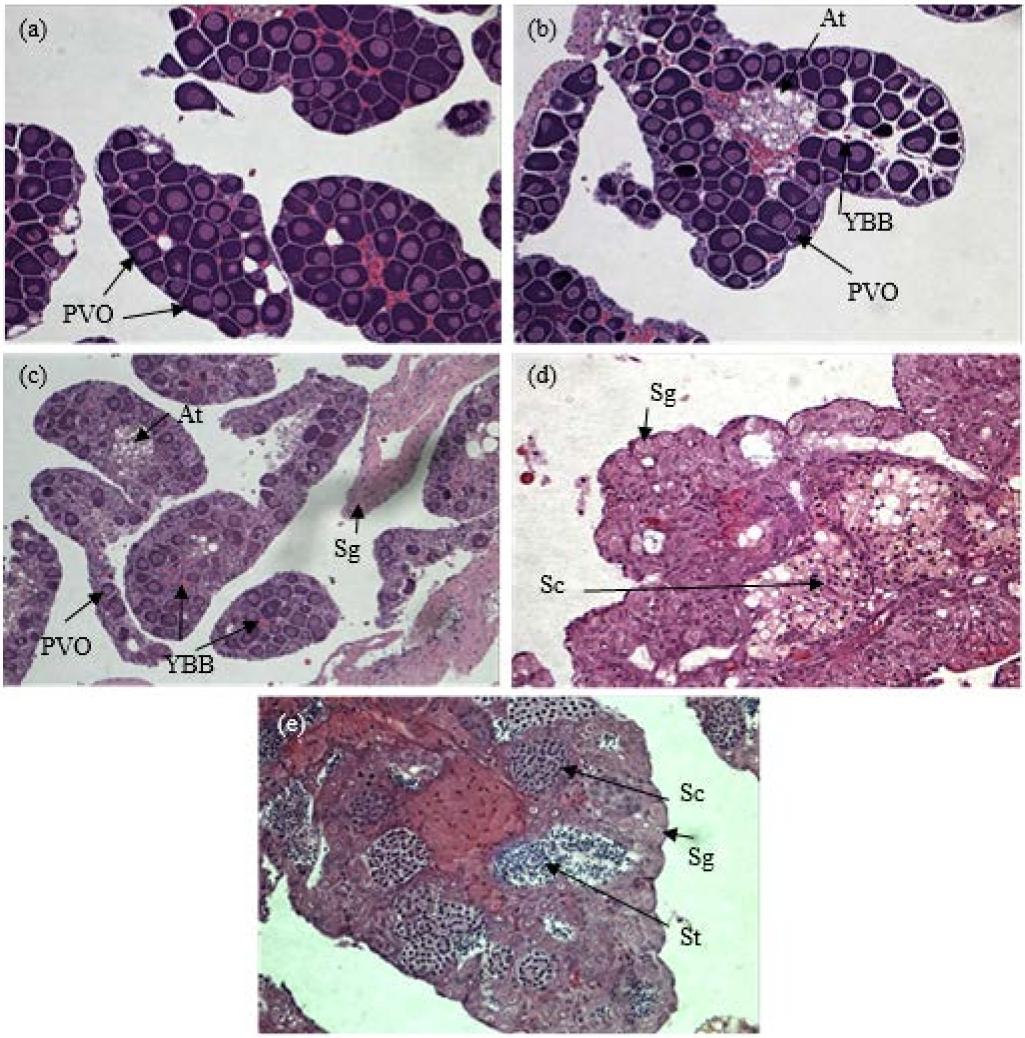


Figure 3-3**:** gonadal development during sex change in mature N. celidotus: (a) gonad at female (NBF) stage, (b) early transition, (c) mid transition, (d) late transition and (e) terminal phase (male). Microscope zoom = 20X (a, b and c); 40X (d and e). At, atresia; PVO, previtellogenic oocyte; Sc, spermatocytes; Sg, spermatogonia; St, spermatids; YBB, yellow brown body.

The descriptive analysis of the gonads was also made for easier interpretation (Table 3.2).

Table 3-2: Descriptive analysis of the *N. celidotus* gonads during all stages of sex change.

Gonad stage	Description
Female, non-breeding female (NBF)	At female stage, the ovaries were made of ovarian lamellae which extend into the lumen. The non-breeding females had many pre vitelogenic oocytes (PVOs); some MPG, yellow brown bodies (YBB), SCs, GCs in pockets. During breeding season, mature ovaries have full vitellogenic or hydrated oocytes. Followed by atresia of unovulated oocytes.
Early transition (ET)	There are still many PVOs in the gonad, many are healthy, others are in atresia, being degenerate by phagocytes. Yellow brown bodies (YBB) are also visible due to degraded oocytes. The YBB are also dominated by melanomacrophages. Spermatogonial proliferation starts to occur at the transforming lamellae.
Mid-transition (MT)	PVOs are becoming scarce as atresia continues to occur. Spermatogonia starts to become dominant in the transforming gonad. Spermatogia proliferation appear at the periphery of the lamellae where large areas of YBB can be seen.
Late transition (LT)	PVOs are no longer visible in the new gonad. Different stages of spermatogenesis are occurring at the same time. Spermatogenic cysts evident in lamellae periphery and spermatogonia becomes dominant over other germ cells. Further spermatogonia proliferation appears in the large YBB areas.
Terminal phase (TP)	Sex change is complete and now the individual has become a fully functional male. Spermatogenesis and spermiation are evident and active in all areas of the gonads. The lobular structure is evident.

3.4 Discussion

This study investigated the relationship between external colour change and gonadal stage of transition. Digital photography and image analysis were conducted aiming at the anal fin, which was established with the most colour difference between genders (*see* Chapter 2). This part of the study was able to quantify the colour of the anal fin as sex changed progressed. As a female, the fins presented a yellow hue, with little variation between the tones of yellow. The fin has also two dark spots, one at the anterior region and the other at the posterior region. As ET stage started, the yellow colouration began to fade. There are also little changes in the dark spot, with only the front beginning to reduce in size. However, results showed no significant changes. At MT, the anal fin had significantly lost its yellow hue, becoming translucent, especially in the centre and posterior part of the fin. The anterior spot had now reduced in size even further with some individuals already missing the front spot completely. A centre line began to appear, although still not fully visible. By LT stage, as the fish has almost completed the full process of sex change, the anal fin had lost the, yellow pigmentation and anterior dark spot become more diffused. Centre line at this stage began to generate colour, varying from yellow to grey. Finally, at TP stage, the yellow pigmentation and both spots had completely disappeared, leaving only a pale white green colouration and a long strip located in the centre of the fin. The discoloration of the anal fin during sexual transitioning, from yellow to a pale white green colour as well as the diffusion of the two dark spots, supports the descriptive analysis of the *N. celidotus* body, made by Choat (1965). The use of the anal fin for colour quantification was also used in the protogynous wrasse, *Pseudolabrus sieboldi* (Ohta *et al*., 2008). Female *P. sieboldi* are known for their yellow anal fin. As transition progresses the yellow colouration diffuses, and a red colouration becomes prominent.

This was the first study to quantitatively analyse the colour changes during sexual transitioning. However, the study of colour change in fish is not new. Colour changes in fish and other animals can occur rapidly or slowly. Rapid colour changes are associated with physiological colour changes, attributed to rapid motile responses of pigmented cells (chromatophores). The slowly process of colour change is linked to morphological colour changes, which may take weeks to a few months and usually a response to the changes in the density of chromatophores in specific areas of the body (Fujii, 1993; Fujii, 2000; Sugimoto, 2002; Leclercq *et al.*, 2010). Anal fin colour changes, during sexual transitioning, were slow, with some individuals taken approximately 4 months to complete the colour transition.

This study was also able to correlate the colour changes to the gonadal rearrangement, via sexual transition stages. As the colour changes began to occur during ET stage, inside the gonads, the degradation of PVOs by atresia becomes evident. Studies analysing the sex steroids have found that the degradation of oocytes coincides to the decrease of the female steroid, E_2 and the proliferation of spermatogonial germ cells is stimulated by 11-KT (Nakamura *et al.*, 2003; Bhandari *et al.*, 2003; Ohta *et al.*, 2008; Muncaster *et al.*, 2013). Although, sex steroids were not quantified in the study, they are a key physiological regulator of gonadal structure. The decrease in oocyte number and increasing presence of spermatogonia in the gonad is likely to reflect the switch of endocrine environment from estrogen to androgen as seen in other protogynous fish (Kroon & Liley, 2000; Bhandari *et al.*, 2003; Frisch, 2004; Todd *et al.*, 2016). Ohta *et al.* (2008) was able to find a significant correlation between the anal fin hue value and sex steroids. As sex change progresses to MT, PVOs continues to become scarce, and spermatogonia begins to proliferate in the centre of the lamellae. However, spermatogonia tends to be around the periphery of the lamellae (Muncaster *et al.*, 2013). At LT, the degeneration of PVOs and proliferation of spermatogonia are concomitantly observed with the increase of the male sex steroid 11-KT (Bhandari *et al.*, 2003; Muncaster *et al.*, 2013). Testis is almost completed and functional. At the same time, the anal fin colour changes are also close to their completion. Spermatogonia has now proliferated from the periphery of the gonad into the centre part of the lamellae, as well as different stages of spermatogenesis can be observed. This proliferation of the spermatogonia from the periphery to the centre of the lamellae, have also been observed in others protogynous species (Nakamura *et al.*, 1989; Bhandari *et al.*, 2003; Muncaster *et al.*, 2013). Finally, at TP, the individual has completed the entire process and now it has become a fully functional male. In this study, all males had no more signs of female gonad characteristics. Spermatogenesis is happening at various stages of spermatozoid development occurring at the same time. This has also been consistent with others protogynous wrasse species, such as the Ballan wrasse (Muncaster *et al*., 2013), and *Thalassoma duperrey* (Nakamura *et al*., 1989).

In summary, colour changes and gonadal restructuring are a vital part of sex change. This study demonstrates that the anal fin colour changes progressively throughout gonadal transition. Quantitatively the hue changes from a mean of 95° to 136° as the ovary becomes a functional testis. This is observed as a progressive loss of yellow pigmentation and dispersal of the dark spots to yield a largely clear fin with a darkened horizontal midline stripe. It is yet to be confirmed as to whether this corresponds to sex steroid concentrations. Finally, *N. celidotus*, has made a useful model to investigate two vital part of sex change in protogynous species.

Chapter 4

General discussion

The study attempted to develop an accurate methodology to assist with the colour analysis of a reliable external landmark prior, during and after sex change in the temperate wrasse *N. celidotus*. Once achieved, this methodology was then used in a manipulative study, with the objective to quantify the anal fin colouration as individuals transitioned from female to male. Histological examination was also analysed and classified according to the transitional phase of every fish.

4.1 Colour analysis methodology

The study was able to develop a methodology for measuring colour changes during sex change. It was found that the mean angular hue (*H*°ab) from the anal fin of the female *N. celidotus* had a reading of 95°, which correlates to a yellow hue (*refer to* Figure 2.3). This yellow colour matches the description of Choat (1965). The findings in the present study that the male anal fin has a mean *H*°ab of 141° or faded green, also supports the white-green description of Choat (1965). It was also determined that the anal fin presented the most significant colour variation compared with other parts of the body. This is similar to the tropical wrasse *Pseudolabrus sieboldi*, where the anal fin also presented the most colour change between genders (Ohta *et al*., 2008). Presently, very few sex change studies have included external colour change into the investigation. However, this study indicates that colour change is a significant event associated with sex change and should not be overlooked, due to its potential role as a visual stimulus. Ross *et al*. (1983) claimed that visual stimulus was crucial to the sex change process in the wrasse *Thalassoma duperrey*. Overall, it was possible to quantify the differences in the anal fin colouration between female and male. The same technique has made it possible to analyse the full process of the anal fin colour change during sexual transition in this species.

4.2 Colour changes and histological analysis of sex change

Using the developed technique for colour measurement, this study was able to identify key changes in the anal fin during sex change. The transition of the colour yellow to a pale white green colour was not rapid, but most of the yellow hue was faded by MT phase. Another observed feature in the anal fin was the presence of two dark spots. Both spots eventually become diffuse and slowly disappear during MT. These are replaced by a horizontal stripe that forms across the midline of the fin, close to the TP stage. These findings support the visual description made by Choat (1965), however, they provide greater detail in the changes during sex change. The slow change in colours suggested that the colour change is morphological. This transition takes weeks until fully completed. The slow colour change was also found in *P. sieboldi* (Ohta *et al*., 2008). Morphological skin colour change has been extensively discussed (Fujii, 1993; Sugimoto, 1993; Sugimoto, 2002; Leclercq *et al*., 2010). Using the developed technique, the study was also able to quantify the colours as transition progresses. Using circular statistics, as recommended in the literature it was possible to verify the angular hue during sexual transition. No significant variation in hue was observed between non-breeding females (NBF) and ET. However, at MT and LT the yellow hue had already faded significantly. Yellow hue was also observed to fade substantially in *P. sieboldi* at the LT phase. However, by ET phase, the yellow hue angle of the anal fin had already decreased to a red hue value (Ohta *et al*., 2008).

An interesting aspect of the current study was that all the female fish, including those in the control tanks, had a decrease in the yellow colouration during the study despite not changing sex. This corresponded as a more translucent yellow. One of the reasons for that occurrence may have been the fact that the background of the tank was plain blue, and not the wild habitat. Therefore, the fish may have been adapting to the colour to the background. Studies in zebrafish (*Danio rerio*) have illustrated that the colour of tank background can have an effect in the skin colouration. For example, zebrafish placed into a dark background tank will adjust its colour skin to a dark pigmentation. The dark colouration occurs naturally with the increase in melanophores, a dark pigmented cell, density. Whereas, using the same experiment, but in a white tank, will result in the fish becoming lighter, due to the natural decrease in melanophore density (Sugimoto, 2002; Logan *et al.*, 2006). Finally, the technique developed was successful in the sense of quantifying the fin colouration, as well as linking with the stages of sex change.

For the second part of this experiment, histological examination of the gonads was also made. Gonadal examination has been used in many studies (Bhandari *et al.*, 2003; Nakamura *et al.*, 2003; Kokokiris *et al.*, 2006; Ohta *et al.*, 2008; Muncaster *et al.*, 2013). By examining the gonads histologically, it is possible to determine the gender of the individual and the relevant stage of sex change. The histological findings correlated with that of other protogynous wrasse species (Ohta *et al.*, 2008; Muncaster *et al.*, 2013). Overall, the histological analysis of the gonads in the *N. celidotus*, contributed to the study demonstrating the internal changes as it moves from each sexual stage.

4.3 Study limitations

One of the major factors during the manipulative experiment was the water temperature. The facility where the fish were allocated had a refrigeration system in place. However, the extreme heat during the summer months made the water temperature increase from 17ºC to over 30ºC on the hottest days. Temperatures above 26ºC have been suggested to cause heat stress in the *N. celidotus* (Iftikar *et al.*, 2015). Another limitation that may have contributed to increased stress was the time in captivity. In total, the animals spent 4 months in the tanks. The length of exposure time to a stressor may influence its impact. In this study, the fish were subjected not only to extreme temperature changes, but also handling, captivity, and change in diet. Chronic stress can reduce immunocompetence, alter levels and patterns of sex steroids, and affect growth (Barton & Iwama, 1991). Attempts were made in the experimental design to minimise stress. For example, social structure was arranged to simulate natural social groups. Housings were added in each tank, in order to provide a cryptic shelter for the fish. However, in view of the other unavoidable significant stresses that the fish endured, it cannot be excluded that the stress may have influenced the number of fishes that changed sex during the study.

4.4 Future studies and recommendations

Through the development of a method to quantify external colour in relation to sexual state, this work creates opportunities for future studies. Investigations of sex change in this species can now be conducted without the need for invasive or destructive sampling to identify sex changing individuals. Further to this, individuals that are at different stages of sexual transition may be identified and selected for sampling by their external colour. A natural progression from this study would be to better understand the mechanisms influencing pigmentation in the anal fin. Based on the work by Ohta *et al.* (2008) androgen measurement, such as 11KT, may be worth further investigation. Studies on the composition of the pigments would also be interesting to understand if they are deposited or neuronally controlled. It would also be interesting to test the hypothesis that tank colour may influence fin hue. In a broader context, this study adds to the current understanding of sex change in temperate protogynous species. This is relevant as most literature reports on tropical species. With this in mind, it would be interesting to compare the time taken to complete sex change in both temperate and tropical species. One major recommendation is to reduce the length of time of the experiment to try and limit chronic stress or alternatively, study the fish in the natural habitat.

4.5 Conclusion

In conclusion, this study was able to quantify the external colouration of *N. celidotus* before, during and after sex change. It was also able to track and allocate the anal fin changes into distinct stages. Visually, these changes are recognisable, but this study aimed to quantify them in a statistically robust manner. The study was also able to analyse the external anal fin changes in association with the restructure of the gonads during sex change. This work adds further tools for the use of *N. celidotus* as a temperate model species to investigate the mechanisms of vertebrate sex change.

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