

Department of Environment and Agriculture

**Sex reversal and early larval biology of farmed silver perch,
Bidyanus bidyanus (Mitchell, 1838) in Western Australia**

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This thesis is presented for the Degree of

**Doctor of Philosophy
of
Curtin University**

October 2017

DECLARATION

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ACKNOWLEDGEMENTS

I am highly indebted to Prof. Ravi Fotedar, my supervisor who painstakingly guided me through the conduct of this research project. I really appreciate his patience and readiness to help any time I needed him. I also express my sincere thanks to Simon Longbottom and Anne Barnes who helped in diverse ways from behind the scene. I also thank Lidia who taught me histology. She did not hesitate to answer my questions even if the time was inappropriate.

I am thankful to my lovely wife Najmiah and my beautiful daughters Ummu Aiman and Haura Ainun and to my sons Muh. Fathan and Muhammad Naufal for their encouragement and support during my stay in Perth. They all allowed me to stay late in the laboratory and permitted me to work during weekends. I am extending my special gratitude to my Father Marhali who passed away during my study period and to my mother and my relatives who wholeheartedly prayed for my success.

I also thank my friends Irfan Ambas, Ilham, Ky, Ha, Tui and Ardiansyah for making my stay in Perth worthwhile. I really appreciate their friendly care. I also thank the pemprov scholarship group Ferdi, Dedi, Santi, Nurwati, Dino for their prayers and support. I deeply appreciate Pemrov for giving me this opportunity by way of the scholarship to study this course in Australia.

Above all, I am thankful to almighty Allah for giving me all the energy, strength, and patience needed to complete the dissertation. I thank the Almighty God who made everything, in its finest detail possible.

Dedication

This body of work is dedicated to my family Najmiah, Ummu Aiman Sulaeman, Muh. Fathan Sulaeman, Haura Ainun Sulaeman and Muhammad Naufal Sulaeman for their guidance and support. I would also like to dedicate this work in the memory of my Father Marhali who passed away during my study.

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LIST OF ABBREVIATIONS

AEC	Animal ethics committee
AN	Anus
ANOVA	analysis of variance
CARL	Curtin Aquatic Research Laboratory
Ctrl	Control
CV	Coefficient of variation
DO	Dissolve Oxygen
dph	days post hatching
E	Eye
E	East
E2	Estradiol-17 β
ED	Eye diameter
F1	The first filial generation
F2	The second filial generation
FAO	Food and Agriculture Organization
FCR	Feed Conversion Ratio
FOM	Final oocyte maturation
h	Hour
hCG	Human Chorionic Gonadotropin
HD	Head depth
HG	Hindgut
hph	Hour post hatching
hps	Hours post spawning
ind.	Individual
IU	International Unit
L	Litre
L0	Initial length
MG	Midgut
Min	Minute
MO	Mouth opening

MS	Microsoft
MT	Methyl testosterone
n	Number
NSW	New South Wales
OG	Oil globule
OgD	Oil globule diameter
OgV	Oil globule volume
pH	Potential of hydrogen
PNR	Point of No return
ppt	Parts per thousand
S	South
SB	Swimming bladder
SD	Standard deviation
SE	Standard error
SGR	Specific growth rate
SPSS	Statistical package for the social science
SPSS	statistical product service solution
T	Average temperature
tf	Time from hatching to first feeding
TL	Total length
ty	Time from hatching to yolk absorption
ty-PNR	Time from yolk absorption to PNR
W	Watt
WA	Western Australia
YS	Yolk sac
YsH	Yolk sac height
YsL	Yolk sac length
YsV	Yolk sac volume

LIST OF SPECIES

Artemia, *Artemia spp*

Asian sea bass, *Lates calcarifer*

Asp, *Aspius aspius*

Atlantic cod, *Gadus morhua*

Atlantic halibut, *Hippoglossus hippoglossus*

Atlantic salmon, *Salmo salar*

Atlantic sturgeon, *Acipenser oxyrinchus*

Australian bass, *Macquaria novemaculeata*

Ballan wrasse, *Labrus bergylta*

Betta fish, *Betta splendens*

Blue tilapia, *Tilapia aurea*

Brook trout, *Salvelinus fontinalis*

Brown trout, *Salmo trutta*

Brown trout, *Salmo trutta fario*

Burbot, *Lota lota*

Carp, *Aspidoparia morar*

Channel catfish, *Ictalurus punctatus*

Chinese sturgeon, *Acipenser sinensis*

Chinook salmon, *Oncorhynchus tshawytscha*

Chum salmon, *Oncorhynchus keta*

Clownfish, *Amphiprion sebae*

Coho salmon, *Onchorhyncus kisutch*

Common carp, *Cyprinus carpio*

Common pandora, *Pagellus erythrinus*

Cryfish, *Cherax albidus*

Dove Sole, *Microstomus pacificus*

Electric blue hap, *Sciaenochromis ahli*

Eurasian perch, *Perca fluviatilis*

European eel, *Anguilla anguilla*

European sea bass, *Dicentrarchus labrax*

Gilthead seabream, *Sparus aurata*

Golden barb, *Puntius gelius*

Goldfish, *Cyprinus auratus*

Grouper, *Epinephelus coioides*

Herring, *Clupea harengus*

Honmoroko, *Gnathopogon caurulescens*

Ide, *Leuciscus idus*

Japanese flounder, *Paralichthys olivaceus*

Koi, *Cyprinus carpio koi*

Largemouth Bass, *Micropterus salmoides*

Lined sole, *Achirus lineatus*

Loach, *Misgurnus anguillicaudatus*

Macquarie perch, *Macquaria australasica*

Mangrove red snapper, *Lutjanus argentimaculatus*

Medaka, *Oryzias latipes*

Mirror carp, *Cyprinus carpio* L

Mud loach, *Misgurnus mizolepis*

Murray cod, *Maccullochella peelii peelii*

Nile tilapia, *Oreochromis niloticus*

Pikeperch, *Stizostedion lucioperca*

Rabbitfish, *Siganus guttatus*

Rainbow trout, *Oncorhynchus mykiss*

Rainbow trout, *Salmo gairdneri*

Red sea bream, *Pagrus major*

Roach, *Rutilus rutilus*

Rock bream, *Oplegnathus fasciatus*

Rohu, *Labeo rohita*

Rotifer *Brachionus calyciflorus*

Sea bream, *Archosargus rhomboidalis*

Sharp snout sea bream, *Diplodus puntazzo*

Siberian sturgeon, *Acipenser baeri*

Silver perch, *Bidyanus bidyanus*

Singhi, *Heteropneustes fossilis*

Summer flounder, *Paralichthys dentatus*

Thai pangas, *Pangasius sutchi*

Tilapia, *Oreochromis spilurus*

Tilapiine, *Tilapia zillii*

Trout, *Oncorhynchus mykiss*

Turbot, *Scophthalmus maximus*

Walleye, *Stizostedion vitreum*

Yellow catfish, *Pelteobagrus fulvidraco*

Yellow perch, *Perca flavescens*

PREAMBLE

Silver perch, *Bidyanus bidyanus* (Mitchell, 1838), a protandrous hermaphrodite species, is an economically viable aquaculture species in Western Australia, however, the farm production is relatively low and thus requires improvement in culture technique and productivity. The purpose of this research is to assess the potential for monosex culture of silver perch through studying the reproductive biology, early life of larvae culture and the sex reversal. The research relevant to this area is described in eight chapters of this thesis. Out of eight chapters, five chapters, chapters 3 to 7, are direct result of five independent experiments that are or will be published in the form of five independent research articles. All these research based chapters are focused on meeting the aim and objectives of this research, highlighted in chapter 1.

Chapter 1 briefly introduces the role of aquaculture in meeting the demand of aquatic protein in the fast growing world population. It also discusses the present status of silver perch culture in Western Australia, constraints in improving aquaculture productivity and potential application of monosex culture based upon the experiences of the other species. The aim and objectives of the current research are also presented in this chapter.

Chapter 2 presents a taxonomic classification and biology of silver perch. This chapter also reviews sexual differentiation in fish and potential for application of sex reversal techniques to achieve monosex population of silver perch. The chapter attempts to critically review the existing literature related to silver perch and similar species.

Chapter 3 explores the reproductive performance of a domesticated broodstock and the relationship between oil globule fragmentation and the egg quality of silver perch. This chapter in the form of research article has been accepted for publication in Indonesian Aquaculture Journal Vol. 12, No. 2 (2017).

Chapter 4 delivers early life-history data on the silver perch larvae, obtained from the domesticated broodfish. The research investigates and details the biological

information during the early larval stages with respect to the yolk absorption rates, the onset of feeding, and the mortality response of the unfed larvae. This chapter is published in the journal of International Aquatic Research 9:107-116. DOI 10.1007/s40071-017-0160-7.

Chapter 5 determine the 'Point of No Return' (PNR) and investigates the larval growth and survival of silver perch larvae after progressive starvation initiated by the delayed initial feeding under laboratory conditions. This chapter has been submitted for publication to the journal of Aquaculture International (2017).

Chapter 6 describes the efficacy of artificial diets supplemented with estradiol-17 β to produce a predominantly female population of silver perch and is published in the Journal of the World Aquaculture Society 48 (3):444-452. doi: 10.1111/jwas.12352.

Chapter 7 assess the potential of two concentrations of dietary supplementation of 17 α -MT to produce an all-male population of silver perch as a step to produce XX genotype males which later can be used to produce an all-female population. This chapter is published in Egyptian Journal of Aquatic Research 43:109-116. <http://dx.doi.org/10.1016/j.ejar.2016.10.002>

Chapter 8 summarises and discusses the broodstock performance and their egg quality, larval development, and sex reversal of silver perch which leads to a series of conclusions, followed by the recommendations for future research.

ABSTRACT

Aquaculture of silver perch, *Bidyanus bidyanus* has been ‘works in progress’ in Western Australia as the industry has been established in Queensland and Victoria. This leads to the expectation that the development of silver perch industry should be easier in Western Australia. However, the scarcity of broodstock from natural resources, a farmer in Western Australia needs to develop its own domesticated broodfish. Another constrain in the development of industry in Western Australia is the lack of information related to larval biology which is important for intensive larviculture.

The objectives of the experiments were to assess the potential for monosex culture of protandrous hermaphrodite silver perch while studying the reproductive biology, early life of larvae and the sex reversal. The result of a series of five laboratory scale experiments performed showed that the tank-reared domesticated broodstock of 6 years old, were able to support the viable larvae production with high performance. Even though eggs and larvae produced from the domesticated broodstock were inferior than the wild broodstock, their larger oil globule could last longer during the weaning periods and hence showed higher viability. The research also showed that the eggs categorization based on the oil globule fragmentation could be a useful tool for the quality evaluation of silver perch eggs. The larvae resulted from the domesticated broodstock indicated that the exogenous feed should be available at 5 days post hatch (dph), well after the yolk sac is completely depleted at 4 dph. The survival decreased exponentially when the initial feeding was started beyond the point of no return (PNR) of between 8 and 9 dph. The results also suggested that the supplementation of estradiol-17 β (E2) at 60–120 mg/kg or MT at 9–18 mg/kg diet for 30 days from 31-60 dph could be applied to establish all-female or all-male populations respectively.

In conclusion, from the domesticated broodstock, it is possible to produced viable larvae that can be reared in intensive rearing system in hatchery to produce monosex population through sex reversal techniques.

CHAPTER 1: Introduction

1.1. Background

The global human population is expected to increase from 7.5 billion in 2017 to an estimated 8.5 billion by 2030 (Worldometers 2017). This will led to an increased demand for high quality food by future generations. Aquaculture, as the fastest-growing sector for food production (FAO 2014), has contributed about 44% to global fish production (FAO 2016), which is projected to increase to about 62% in 2030 (FAO 2014) to meet growing demand for seafood.

The silver perch, *Bidyanus bidyanus* is an Australian endemic, superior freshwater fish for aquaculture. The industry has been intensively developed across Australia, with total production in 2015 reached 314 tonnes, Western Australia contributed about 5% (Savage and Hobsbawn 2015). Even though its contribution to inland water aquaculture production in Western Australia was still low, it has been considered as the focus for future development. This species has also been cultivated in Israel since 1997 (Moiseeva 2001) and in Taiwan since the early 1990s (Yang et al. 2006; Yang et al. 2011).The species reaches market size (0.5-1 kg) in 16-24 months (Rowland 1995). Further, over-wintering of fingerlings in ponds which is a routine step in the production system, faced some problems on the reduction of feeding activity, growth rate, and immune response (Rowland 1995; 2009).

The development of tank-based recirculating aquaculture systems (Foley et al. 2010), and cage culture (Rowland 2004b) have been confirmed to effectively reduce bird predation and health problems both for fingerlings and harvest sized fish (Allan and Rowland 2002; NSW 2006). However, poor survival and growth rate at low water temperatures is remain problems in this system. The use of tank-based hatchery for larvae production and grow-out systems could overcome mortality and growth problems, especially during winter (Foley et al. 2010), but some biological and technical aspects still need to be elucidated.

Hatchery production systems of silver perch have mainly been developed based on the use of wild broodstock, with one dose of human chorionic gonadotrophin (hCG) injection, spawning, and hatching taking place in tanks, and larval rearing being performed in ponds (Thurstan and Rowland 1994; Rowland 2004a; 2009). As wild broodfish became scarce, domesticated broodfish have become more important. Pond domesticated broodfish introduced by Rowland (2004a) is promising where first time spawning was successful with good performance. However, different aspects of domesticated silver perch broodfish still need to be investigated especially related to their performance after a few years in captivity (Rowland 2004a). This will become more important once dealing with broodfish quality improvement through a breeding program (Guy 2009).

At the larval rearing stage, an extensive rearing system is well developed, which produces fingerlings to meet aquaculture, stock enhancement and conservation needs (Thurstan 1992). However, intensive larval rearing needs to be elaborate to meet the demand that will develop when silver perch aquaculture becomes more intensified. In addition, it needs to anticipate the development of a full tank-based aquaculture system, where the all cycle of production system takes place in tanks. Even though some research has been initiated (Phipps 1999) to support intensive larvae rearing, some basic information is still required with regard to broodfish performance, egg quality assessment, and larval rearing. Broodfish domesticated in ponds, feeding on natural food may have a different performance to broodfish cultivated in tanks supplied with artificial feed (Papanikos et al. 2003).

Egg quality is an important parameter when talking about broodfish performance (Papanikos et al. 2003; Mylonas et al. 2010; Jakobsen et al. 2016) and is still not fully understood (Kjørsvik et al. 1990; Brooks et al. 1997). The fatty acid composition of eggs is suspected to be a key factor in improving reproductive performance, including better egg quality and the survival of fry (Komen and Thorgaard 2007; Bobe and Labbé 2010). The quality of the eggs produced by silver perch have been identified as essential in controlling reproduction. Variability in

the size of newly hatched larvae has also been identified (Moiseeva 2001; Rowland 2004b; 2009), which is likely due to the high variability of oocyte quality.

Another important aspect related to intensive larval rearing system is to understand early larval development especially during ‘critical period’ or ‘mixed-feeding period’ (the period between the used of endogenous nutrition and the used of external food). The identification of the point of no return (PNR) and the effect of the first feeding delay on mortality rate and growth rate are vital (Dou et al. 2005; Pena et al. 2014; Jakobsen et al. 2016). This is mainly because silver perch larvae are similar to many other species, being small and poorly developed at first feed (Blaxter and Ehrlich 1974; Pitcher and Hart 1982).

Silver perch displays sexual dimorphism of growth, as females can reach a larger maximum size than males. In this species, the sex determination mechanism is not yet clear. Sex steroid treatment is commonly used to control sex differentiation and investigate sex determination systems in fish (Rougeot et al. 2002). The optimisation of steroid use in sex reversal is the first step to be completed in order to develop new methods to control sex differentiation or the sterilisation of silver perch. This is of value for aquaculture and gives rise to new choices in terms of management options. The production of an all-female fish would allow for quick propagation, as in many cases the milt from a male fish is capable of fertilising eggs from several numbers of females (Goetz et al. 1979). Furthermore, the occurrence of precocious males in silver perch is substantial, resulting in early small sexually mature males. These males do not reach their maximum growth capacity and are of lower market value than normal males.

Several methods have been tested in order to increase silver perch aquaculture production, but stunted growth and precocious maturation have halted anticipated productivity (Gordon 1995). Silver perch exhibit sexual dimorphism, where males are smaller than females (Mallen-Cooper 2003). Most silver perch males induce maturation at two years of age and thus divert their energy resources into gonadal development. On the other hand, females use resources for somatic growth and

mature at three years, when they are just approaching marketable size, at 1 kg (Rowland 2004a). Hence, females have one additional year compared to males in which they can divert their energy resources into somatic growth. Consequently, since females do not mature before harvest size and grow bigger than males, to obtain an all-female population is an economical choice for silver perch aquaculture.

Monosex cultivation has been proven efficient in the production of fish and can improve growth of biomass in many fish species (Phillay and Kutty 2005). The growth performance of single-sex and mixed-sex populations has also been compared for many cultured aquatic species (Goudie et al. 1994; Pongthana et al. 1999; Sheehan et al. 1999; Triño et al. 1999; Dan and Little 2000; Lawrence et al. 2000; Nam et al. 2001). In Nile tilapia, *Oreochromis niloticus* L., the biomass of monosex fish at harvest was 30–50% higher than that of a mixed culture (Mair et al. 1995). Similarly, the biomass of monosex fish was approximately 30% higher in mirror carp, *Cyprinus carpio* L., 6–8% higher in other common carp, *Cyprinus carpio* (Kocour et al. 2005), and 70% higher in cryfish, *Cherax albidus* (Lawrence et al. 2000). Production of higher-yielding monosex populations has also been achieved in Japanese flounder, *Paralichthys olivaceus*, and walleye, *Stizostedion vitreum* (Malison 1998). However, there are no reports regarding the monosex culture of silver perch.

1.2. Aim

The aim of the present research is to assess the potential for monosex culture of protandrous hermaphrodite silver perch through studying the reproductive biology, their larvae culture and sex reversal.

1.3. Objectives

The aim of the research will be achieved by meeting the following objectives:

1. To evaluate the performance of domesticated broodstock of silver perch and to determine the quality of silver perch eggs in relation to the fragmentation of oil droplets in ovulated oocytes.
2. To investigate the utilization of yolk sacs and oil globules by silver perch larvae produced from domesticated broodfish.
3. To determine the occurrence of the point of PNR, and investigate larval growth and the survival performance of silver perch larvae in laboratory conditions after progressive starvation by initial feeding delay.
4. To evaluate the efficacy of artificial diets supplemented with estradiol-17 β to produce a predominantly female population of silver perch.
5. To evaluate the possible use of a dietary supplementation of 17 α -methyl testosterone (MT), and to produce an all-male population of silver perch, as a step forward in producing neomales, which can later be used to produce an all-female population.

The research relevant to the above objectives is described in eight chapters of this thesis. Out of eight chapters, five chapters, chapters 3 to 7, are direct result of five independent experiments that are or will be published in the form of five independent research articles. All these research based chapters are focused on meeting the aim and objectives of this study.

CHAPTER 2: Literature review

2.1. Background

The final objective of directing reproduction and sex-reversal in aquaculture is to increase the cost-efficiency of fish production processes (Afonso et al. 2001; Senior et al. 2012). Since different fish species have a wide variances in their ability to tolerate an aquatic environment, an aquaculture technique established for a certain species is not always suitable for other fish (Quiñones-Arreola et al. 2015). However, some basic information and technologies established for a model fish species that answer basic questions can be expected to be adapted for other species (Tokumoto 2014). Therefore, efforts to control the reproduction of numbers of farmed fish species have become a routine procedure and will continue to grow (Pandian 2010).

The northern and western rivers and upper ranges of the Murray-Darling river system in New South Wales, Australia is the natural occupation area of silver perch, *Bidyanus bidyanus* (Rowland 1995). As a freshwater fish, it has also been known to survive and grow well in farm dams (Barlow and Bock 1984) and is known to have high potential for aquaculture (Rowland 2009). In Western Australia, silver perch farming has been the focus for aquaculture development over the last decade (Doupe et al. 1999); however, from 2011 to 2014 annual production was still very low (13-14 tonnes) compared to national production (256-306 tonnes) (Savage and Hobsbawn 2015). Even though its contribution to inland water aquaculture production was still very low, the Government of Western Australia (2015) consistently included silver perch as a priority for future aquaculture development.

2.2. Silver perch Taxonomy

The scientific name for silver perch is taken from an aboriginal name for the species *Bidyan*, as introduced by Major Mitchell after his expedition held in 1832. It is also

called bidyan, black bream, silver bream or grunter (Merrick and Schmida 1984). The taxonomic classification of silver perch is as follows:

Kingdom: Animalia

Phylum: Chordata

Subphylum: Vertebrata

Class: Actinopterygii

Order: Perciformes

Family: Terapontidae

Genus: *Bidyanus*

Species: *B. bidyanus*

2.3. Silver perch Biology

Silver perch is a freshwater fish with a small head and small scales with a moderately elongated body shape (Merrick and Schmida 1984). The colour can vary depending on water conditions, but it is silver-grey with darker scale margins often found in adult fish. The ventral part and the pelvic fins are white, while the median fins are grey. The oldest fish recorded was 27 years old, 440 mm in length and 1.2 kg in weight; it was collected from Cataract Dam (Mallen-Cooper 2003). The growing period from hatchery, to fingerling and grow-out stages is about 16-24 months (Rowland 1995, 2009). The initial phases of silver perch growth can be divided into four classifications: (1) the egg stage from fertilization up to hatching; (2) the prolarva period, where the yolksac grows; (3) the postlarva period, where yolksac absorption and the completion of fin formation takes place; and (4) the young period, from fin formation to the formation of an adult fish. However, the used of the term larva may refer to either prolarva or postlarva (Lake 1967b).

In aquaculture, knowledge about fish biology is crucial in order to provide optimum environmental requirements and suitable cultivation techniques. Success in controlling reproduction and growth in a controlled or semi-controlled environment largely depends on providing optimal environmental requirements (Mylonas and Zohar 2001; Fuiman and Cowan 2003; Jalabert 2005; Selvaraj et al. 2016). Silver

perch is known to have a wide range of temperature adaptations (2–38°C) (NSW 2006). Even if the optimal temperature for growth of silver perch has never been determined (Rowland 2009), the temperature range of 23-28°C (Rowland 1994; Anonymous 1999) or 20-30°C has been shown to support good performance in a pond culture.

2.3.1. Reproductive performance

As in higher vertebrates, fish reproduction is affected by environmental conditions that, in particular can affect fecundity and age at maturity (Jakobsen et al. 2016). In an aquaculture species, broodfish often face un-suitable conditions and fish are subjected to stress during periods of handling such as grading, transport, confinement, overcrowding, and water quality deterioration (Kumar et al. 2015). However, little is known about the effects of such treatments on the reproductive physiology of fish (Pickering et al. 1987). Fish reproduction is a physiological process that is sensitive to the effect of disruption (Schreck et al. 2001). Several events are involved in the final maturation process, including the integration of yolk proteins and lipids into oocytes (Devlin and Nagahama 2002). Commonly in the process of domestication, the wild animals customised the new, captive environmental condition and they may adjust their behavior, physiology, and morphology (Krejszefz 2009). When chronic stress occurs during the final stages of maturation it not only interrupts the reproductive endocrinology, it can also result in reduced egg quality (Campbell et al. 1994).

Age is another factor that can affect the reproductive performance of broodstock fish (Izquierdo et al. 2001). Different ages or sizes of females may produce differences in the viability of eggs (Jakobsen et al. 2016), where larger females may produce larger eggs with higher energetic reserves. Various reproductive parameters such as egg quantity, egg quality, spawning success, fertilization success, egg hatchability, and larval quality can be used to evaluate broodstock performance (Izquierdo et al. 2001; Watanabe and Vassallo-Agius 2003).

2.3.2. Egg quality

Success in the mass production of fish fry in aquaculture is often affected by unpredictable egg quality (Kjørsvik et al. 1990). Egg quality may also have an impact on the size and growth rate of progeny (Reglero et al. 2014), as observed in commercial species such as common carp (Kocour et al. 2005), chum salmon (*Oncorhynchus keta*) and coho salmon (*Oncorhynchus kisutch*) (Beacham et al. 1985), including silver perch (Barki et al. 2000; Moiseeva 2001). The term ‘egg quality’ has been used in various ways by different authors. However, due to our limited knowledge of this field, it has been difficult to find a valid quality criteria. Kjørsvik et al. (1990) and Grant et al. (2016) defined egg quality as an egg's potential to produce viable larvae. Eggs quality may be affected by egg size and their developmental process (Kjørsvik et al. 1990; Kjørsvik et al. 2003; Kamler 2005; Grant et al. 2016). High egg quality may increase the chance that newly hatched larvae have of surviving (Kjørsvik et al. 2003; Jakobsen et al. 2016). A number of physical, genetic, and chemical parameters, as well as the initial physiological processes occurring in the egg, have been identified to affect egg quality and have been used to determine egg quality (Bobe and Labbé 2010). On the other hands, egg development may fail at a certain stage if essential factors are lacking during development (Gisbert et al. 2000). Therefore, egg quality evaluation should be performed after spawn and the fertilization process are completed (Kjørsvik et al. 1990). This definition is, however, of very little practical value, since fry viability obviously cannot be determined before the fry is produced (Jakobsen et al. 2016). A simple criterion for egg quality determination should be that it is both possible to categorize and be easy to implement.

The fatty acid composition contained in eggs is suspected to play an important role in improving reproduction success, including better egg quality and the survival of fry (Komen and Thorgaard 2007; Bobe and Labbé 2010). Moreover, the oil globule in fish eggs is an important component of larval development as a material for energy resources or as physical constituents in membrane biogenesis (Sargent 1995). In relation to it physical performance, the distribution of oil globules in

oocyte has been identified as an indicator of egg quality for European perch, *Perca fluviatilis* (Żarski et al. 2011b) and brown trout, *Salmo trutta fario* (Mansour et al. 2007). On the other hand, the ability of eggs to influence water turbidity has also been reported as an indicator for egg quality (Wojtczak et al. 2004). Nevertheless, there was no relationship between oil globule distribution in the oocyte and the quality of the eggs especially with respect to the reproductive performances of domesticated rainbow trout (Ciereszko et al. 2009).

2.3.3. Larval food and feeding

Silver perch is a schooling, omnivorous fish that feeds naturally on zooplankton, insects, crustaceans, molluscs, small fish and some aquatic plants, in particular, filamentous algae (Allan and Rowland 2002). Silver perch are classified as dietary generalists in term of feeding behaviour, but on an individual level they exhibit highly specialised consumption patterns (Warburton et al. 1998). Given information on prey densities in the environment, these patterns can be predicted. The newly hatched silver perch larvae that from wild broodfish are weak and exhibit slow moving behavior. Therefore, it is necessary to provide enough aeration to avoid larvae from settling down at the bottom of the tank (Thurstan and Rowland 1994), which may give uneven distribution. As in many other species, silver perch larvae are light sensitive (Gehrke 1994); thus light intensity needs to be adjusted in order that they might avoid stress (Thurstan and Rowland 1994).

In order to maintain a high survival during larval development in captivity, rotifers are a suitable choice of food (Phipps 1999; Kailasam et al. 2015). The rotifer, *Brachionus plicatilis*, belongs to the phylum rotaria and is an euryhaline species (Watanabe et al. 1983), which is the most common first feed in larviculture (Watanabe et al. 1983; Baskerville-Bridges and Kling 2000; Preston et al. 2000; Curnow et al. 2006; Khan and Malik 2012; Mahjoub et al. 2013; Varghese et al. 2013; Kailasam et al. 2015). This is because rotifers are of ideal size for young larvae. They have the ability to reproduce rapidly and can be fed various unicellular algae such as *Pavlova lutheri*, *Isochrysis* spp., *Dunaliella tertiolecta*, *Tetraselmis*

chuii, *Chaetoceros gracilis* and *Phaeodactylum tricornutum* (Watanabe et al. 1983; Kailasam et al. 2015). After a certain stage, silver larvae can be fed with artemia (Phipps 1999). *Artemia* spp., a crustacean, is a live feed that is also commonly used in larval rearing (Watanabe et al. 1983; Lavens and Sorgeloos 2000; Sorgeloos et al. 2001; Callan et al. 2003; Curnow et al. 2006; Mahjoub et al. 2013; Royan 2015).

2.3.4. Onset of first feeding and the PNR

The initial life phases of fish are among the most essential stages in fish development (Palińska-Żarska et al. 2014). The changes happening during this period can directly affect the rest of their life. The transition process from the endogenous (yolk sac) to external food is the period where tiny larvae are prone to environmental changes and they often experience mass mortality (Gisbert et al. 2000; Palińska-Żarska et al. 2014). Therefore, the moment of exogenous food supply in larviculture is critical. If larvae are fed exogenous food too early, it can lead to a variety of fish sizes that can ultimately trigger cannibalism (Baras et al. 2003). Conversely, if the exogenous food is given too late, irreversible changes in the digestive system may have occurred and the larvae will not be able to digest the ingested food (Blaxter and Ehrlich 1974).

The start of feeding can also be defined as the period where food ingestion is possible until the larval is grow, or the irreversible starvation has been achieved (Blaxter and Ehrlich 1974). The most important thing here is the availability of energy sources which are essential to endure larval development and support them to change over from yolk reserves to exogenous feeding (Popova and Sytina 1977; Peña and Dumas 2005; Peterka and Mateřna 2011). This behavioral shift can only be successfully passed if all the required organs and related structures to food capture, digestion, and food assimilation have been ready in due time, and that suitable food is made available (Yin and Blaxter 1987; Dou et al. 2002). Such information is still relatively scarce and patchy in comparison to the growing larval stages of silver perch.

The time of first feeding is a crucial time in fish larvae growth. This period is claimed to be a major cause of deaths in hatcheries (Pandian 2013). Food deprivation or inappropriate food sizes, feeding time, food quality and feeding practices in larval rearing can increase mortality during the first few days (Miller and Kendall 2009). Commonly, larvae survival during the first feeding period is varied according to the species, quality of spawning, age and rearing techniques (Yin and Blaxter 1987; Yúfera and Darias 2007). At this stage, larval death can also be triggered by environmental conditions, especially when new diets are developed or new species are investigated (Cahu and Infante 2001; Yúfera et al. 2005).

Before feeding, yolk (yolk sac and oil globule) is used for larvae growth at a specific rate for each species (Yúfera et al. 2000). The temperature at hatching and the yolk size are two main factors influencing the absorption rate of the yolk sac and their assimilation into larvae tissues (Polo et al. 1991; Hardy and Litvak 2004). All these aspects control the duration of a mixed-feeding period and the ability of larva to survive under starvation.

The readiness of the right food at the right time is important because food shortage can cause mass mortality (Dou et al. 2002). It has also been stated that low larval survival commonly occurs during initial feeding periods (Bisbal and Bengtson 1995; Yang 2007), which is mostly affected by a delayed in initial feeding (Dou et al. 2000; Gisbert et al. 2004; Peña and Dumas 2005). The initial feeding in Chinook salmon, *Oncorhynchus tshawytscha* (Twibell et al. 2009) and Atlantic salmon, *Salmo salar* (Solberg et al. 2014) are due as swim-up stage. In relation to this critical part of aquaculture, the idea of the PNR has been applied to study the effect of starvation on fish larvae mortality (Peña and Dumas 2005; Kailasam et al. 2007; Shan et al. 2008; Jinbo et al. 2013). PNR is interpreted as the stage where the effects of food shortage become irremediable. Hence, about 50% of starving larvae remain alive but are powerless to feed even if food is available, thus they cannot successfully pass the ontogenetic stage. Hence, a relatively short starvation period may cause abnormalities, as well as feeding and digestive complications that will

hinder the larvae's ability to survive past the PNR (Gwak and Tanaka 2001; Dou et al. 2002).

Obviously, failure to provide proper nutrition can cause adverse effects at every stage of larval growth, but resistance to hunger will increase with the increasing of age and size (Gisbert et al. 2004). Therefore, the occurrence of PNR is the deadline to start feeding (Gisbert and Williot 1997; Gisbert et al. 2004). Even then, it does not mean that fish larvae that start feeding on the stage will be alive until fingerling (Koss and Bromage 1990; Gisbert and Williot 1997; Kailasam et al. 2007). Inversely, the delay of initial feeding for a short period of time generally does not result in physical deformity and a decrease in growth (Peña and Dumas 2005). The duration between yolk sac exhaustion and PNR is specific depending on the fish species (Shan et al. 2008); however, there is currently no information available for silver perch.

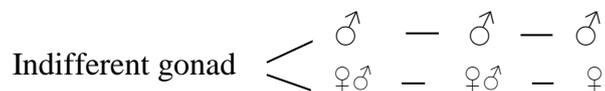
2.3.5. Hermaphroditism

The physiology of sex is depends on the type of primary sex organ known as gonadal sex (Degani and Kushnirov 1992). Gonadal sex can then be grouped into gonochorism where either testes or ovaries only present in the fish and hermaphroditism where both testicular and ovarian tissues are found in the same fish (Devlin and Nagahama 2002). The gonadal form of teleosts varies considerably from the general one in which individuals develop directly into females with ovaries and males with testes at sexual maturation to synchronous hermaphroditic species that can have functional female and male gonad at the same time (Degani and Kushnirov 1992; Devlin and Nagahama 2002).

Hermaphroditism can also be defined as the appearance of both a male and female reproductive function in a single individual either simultaneously or sequentially (De-Mitcheson and Liu 2008). In fish, simultaneous hermaphroditism, protandry, and protogyny are three basic forms of hermaphroditism (Pandian 2010). In simultaneous hermaphroditism, each individual fish functions both as a male and female concurrently. In protandry, individual fish first function as a male before

become a permanent female during their life. In marine fishes, hermaphroditism is considered as a normal type of reproduction such as commonly found in groupers (Bruslé-Sicard et al. 1992). However, in freshwater fish, this phenomenon is not commonly known and only few species have been identified (Devlin and Nagahama 2002; Pandian 2012; Pandian 2013) including the silver perch (Moiseeva 2001).

Moiseeva (2001) suggested that silver perch is a protandrous hermaphrodite, where indifferent gonad grows to all male gonad before male gonads grow in a direct way while female gonads develop in an indirect way. Therefore, according to Devlin and Nagahama (2002) sex differentiation in this species can be presented as follows:



2.3.6. Sex reversal

Some fish species reverse their sex naturally at different developmental stages (Chen et al. 2013). However, sex reversal can also be done using a certain technique to obtain a monosex male or female offspring. Furthermore, feminization or masculinization through hormones can be done in several ways such as by injection (Mirza and Shelton 1988), immersion (Kim et al. 1997) or orally (through food) (Goudie et al. 1983; Devlin and Nagahama 2002; Haffray et al. 2009). The hormone is commonly applied before the fish undergo sex differentiation, which usually begins to occur well before the eggs hatch (Baker et al. 1988; Kato et al. 2001; Haugen et al. 2011), after the eggs hatch and before or after the fish started to eat (Yamazaki 1983). Oral administration can be done by mixing a hormone with artificial feed or with natural feed through bio-encapsulation. Nowadays, hormone treatment through oral administration is common but it is only limited to fish that can accept artificial feed and require a longer time. In addition, the used of a steroid hormone is likely to undergo leaching during treatments (Tan-Fermin et al. 1994).

On the other hand, hormone treatment through bio-encapsulation requires fewer hormones, however from a technical perspective this method is less practical because live food has to be immersed in a hormone solution before being given to fish larvae. Hormone application is considered to be more simple and efficient with the immersion method. The immersion of eyed-eggs in a hormone solution have been successfully applied on the eggs of Chinook salmon (Baker et al. 1988). Donaldson and Hunter (1983) stated that the success of genital alteration is influenced by several factors, including the type and dose of hormones used, the length of treatment, the species of fish, the age of fish at the commencement of treatment and the water temperature at the time of treatment.

In contrast to hermaphroditic species (Beardmore et al. 2001), sex reversal has been achieved in many gonochoristic species (Yamazaki 1983; Domagala et al. 2015). The first step in developing a suitable method for producing monosex progeny is to identify the critical period in which the gonads are responsive to hormonal treatment (Pandian and Koteeswaran 1999; Blázquez et al. 2001; Haugen et al. 2011). During this period, the gonads are labile and sensitive to different environmental change including hormonal treatment, therefore it is also called as labile period (Pandian 2013). After this period is determined, monosex or sterile populations can be produced by administering hormones at a known dose (Hunter et al. 1986). However, few information are available on the timing and histological processes of protandrous hermaphroditic fish, especially silver perch, except for a brief description by Moiseeva (2001). Sex reversal can be more complicated in hermaphroditic species because the sex genes may remain active until the later stages of gonadal development (Yamazaki 1983). In silver perch, the gonads are anatomically formed in 30-day fry but cytological differentiation occurs only in 2-month-old fry (Moiseeva 2001).

2.4. Monosex Culture

Silver perch show sexual dimorphism, where, at a certain stage, male fish are smaller compared to females (Merrick and Schmida 1984). The males of silver

perch reach the maturity stage at one to two years earlier compared to females, and grow to a smaller size as an alteration of the larger gamete energy requirements (Mallen-Cooper 2003). The same phenomenon is also displayed in other Australian freshwater terapontids, where females often complete maturation at a larger size compared to males (Merrick and Schmida 1984). Sexual dimorphism also happens in other Australian percichthyids, such as Australian bass, *Macquaria novemaculeata* (Harris 1987), Murray cod, *Maccullochella peelii peelii* (Gooley et al. 1995), and in Macquarie perch *Macquaria australasica* (Appleford et al. 1998).

Separation between male from female sexes manually does not require high technology and has been applied successfully in several places for monosex culture. However, this technique is hard to apply because fingerlings need to be grown to a large size in order to determine sex before manual separation and culling of the slower-growing sex. The procedure itself is a stressful practice for the fish, liable to mistakes, and in several cases results in relatively high mortality and a low success rate compared to other methods (Beardmore et al. 2001; Cnaani and Levavi-Sivan 2009).

Many fish species are receptive to hormonal induction of sexual development and determination (Pandian and Sheela 1995). Furthermore, more detail examination in many cultured species revealed that sex is genetically established at fertilization. However, phenotypic sex determination may occur later in development, and the timing of sex determination varies among species (Pandian 2010). Hormonal treatment on sexually undifferentiated fry has been successfully to reverse sex in a wide range of fish species. However, production of monosex populations by direct hormonal treatment requires knowledge about the labile period and the time point of sexual differentiation during which the fish are susceptible to hormonal masculinization or feminization (Devlin and Nagahama 2002). Hormonal treatments are not only common to apply in gonochoristic species but have also been successfully administrated in some hermaphroditic species in order to provide broodstocks (Pandian and Sheela 1995; Navarro-Martín et al. 2009).

Hormones are usually administrated via dietary supplementation by dissolving the steroid in alcohol prior to mixing with the diet (Pandian 2012). Immersion techniques have also been applied successfully, and in some species, they appear to be more efficient than dietary administration. The labile period for sex reversal is variable among species, depending on the sexual differentiation pathway (Piferrer 1994; Pandian and Sheela 1995). Generally, hormone treatment for sex reversal are applied early during embryo stages in Salmonidae (Nagy et al. 1981; Piferrer and Donaldson 1991; Piferrer and Donaldson 1992; Piferrer 1994; 2001), but in Cichlidae and Cyprinidae is more effective in post-hatching stages. Hormone application through injections and implants are used limited to some hermaphrodite and fish species with late sexual maturation, with less successful result (Edmunds et al. 2000; Beardmore et al. 2001).

The critical period for hormonal treatment can be identified through the application of hormones at different periods, as has been applied to different species such as European sea bass, *Dicentrarchus labrax* (Blázquez et al. 2001); rainbow trout (Cousin-Gerber et al. 1989; Kato et al. 2001); honmoroko, *Gnathopogon caurulescens* (Fujioka 1993); and red sea bream, *Pagrus major* (Kato et al. 2001) or by identifying early gonadal development through microscopic examination, as used in silver perch (Moiseeva 2001).

2.4.1. Feminization

As presented in Table 2.1, all-female germ cells of many cultured species can be produced by hormonal manipulation (Yamazaki 1983; Domagala et al. 2015). Methods of estrogen administration that have been commonly applied include injection (Yamazaki 1983; Devlin and Nagahama 2002), immersion, and oral administration through food (Hunter et al. 1986; Purwati et al. 2004; Putra 2011; Lin et al. 2012). Hormone treatment is applied prior to sex differentiation at stages including pre-hatching (Beardmore et al. 2001), post-hatching, and before or after exogenous feeding (Yamazaki 1983). Despite the usefulness of steroids for fish sex reversal, its application in fish for consumption purposes has been questioned by

the scientific community on their relation to environmental hazards and health safety for human being. However, the level of contamination of steroids and their metabolites have been shown to be negligible, as steroid-treated fish is reported to have tendency for high clearance from blood plasma. Steroid can be rapidly metabolized in the liver and kidney, and are cleared from blood plasma with a short half-life (Mlalila et al. 2015). Therefore, the application of steroids at low dosage and short period of time should not be hazardous for human consumption.

Table 2. 1. Reported complete feminization of different species treated at different doses of Estradiol-17 β (E2)

Species	E2 doses (mg/kg diet)	References
<i>Anguilla Anguilla</i>	60	Degani, Kushnirov (1992)
<i>Acipenser brevirostrum</i>	50-100	Flynn, Benfey (2007)
<i>Micropterus salmoides</i>	50-100	Garrett (1989)
<i>Micropterus salmoides</i>	200	Arslan et al. (2009)
<i>Oncorhynchus kisutch</i>	10	Goetz et al. (1979)
<i>Salmo gairdneri</i>	20	Johnstone et al. (1978)
<i>Oncorhynchus mykiss</i>	20	Johnstone et al. (1978)
<i>Sciaenochromis ahli</i>	20-60	Karsli et al. (2016)
<i>Gadus morhua</i>	80-120	Lin et al. (2012)
<i>Perca flavescens</i>	15-120	Malison et al. (1986)
<i>Salmo salar</i>	20	Johnstone et al. (1978)
<i>Salvelinus fontinalis</i>	20	Johnstone et al. (1979)

2.4.2. Masculinization

Lots of different steroids have been applied for sex manipulation in fish. The most commonly used androgen to induce masculinization is 17 α -Methyltestosterone (MT) (Pandian and Sheela 1995; Pandian and Koteeswaran 1999; Piferrer 2001; Devlin and Nagahama 2002; El-Sayed et al. 2012; Megbowon and Mojekwu 2014), which has been tested successfully in selected representatives fish species (Table 2.2). On the other hand, the nonsteroidal aromatase inhibitor has also been successfully used to completely masculinize Nile Tilapia (*Oreochromis niloticus*) (Afonso et al. 2001; Didik et al. 2010), Atlantic halibut (*Hippoglossus hippoglossus* L.) (Babiak et al. 2012) and Japanese flounder (*Paralichthys olivaceus*) (Kitano et al. 2000).

Table 2. 2. Reported complete masculinization of different species treated at different doses of 17 α -Methyltestosterone (MT)

Species	MT doses (mg/kg diet)	References
<i>Salmo gairdneri</i>	1-9	Solar et al. (1984);
<i>Dicentrarchus labrax</i>	10	Blázquez et al. (1995)
<i>Oreochromis niloticus</i>	60	El-Greisy, El-Gamal (2012)
<i>Tilapia aurea</i>	60	Guerrero (1975)
<i>Perca fluviatilis</i>	40	Rougeot et al. (2002)
<i>Oreochromis spilurus</i>	70	Ridha, Lone (1990)
<i>Salvelinus fontinalis</i>	3	Johnstone et al. (1979)
<i>Salmo salar</i>	3	Johnstone, Youngson (1984)

CHAPTER 3: Reproductive performance of a domesticated broodstock and the relationship between oil globule fragmentation and the egg quality of silver perch

The article has been published in Indonesian Aquaculture Journal, 12 (2017): 43-51. DOAJ: 10.15578/iaj.12.2.2017.43-51

3.1. Introduction

Silver perch, *Bidyanus bidyanus* (Mitchell, 1838) which has long been known as a potential species for aquaculture (Allan and Rowland 2002); Rowland (2004a) is a native Australian fish, inhabiting inland waters around the Murray Darling River system. Although silver perch has been cultivated for decades and is expected to become the largest freshwater aquaculture industry in Australia, as declared by Rowland (2009), this has not materialized so far. A number of bottlenecks related to the larval production techniques including the understanding of broodstock performance and egg quality still need to be overcome.

Mastery of the techniques of fish reproduction is an important step as it is directly related to aquaculture productivity (Żarski et al. 2011a; Żarski et al. 2011b). Silver perch hatchery has been based on the hypophysation technique to spawn the broodstocks and pond-based larval rearing techniques (Rowland 1984; 2009). In the hatchery environment, a broodstock can be captured from the natural environment or can be domesticated using farming techniques (Rowland 2004a). However, Rowland (2009) stated that the performance of the wild-caught broodstock is reduced after five years of continuous spawning.

As the silver perch culture matures and farmers are more familiar with modern farming techniques of the species, some farmers are now able to produce their own larvae (McCormack 2017). However, various bottlenecks in pond-based larval culture are still challenging farmers as larval quality in pond-based rearing systems cannot be controlled and/or predicted (Mosig 2005). When domesticated

broodstock is used for spawning, the use of hormone and formulated diets, and handling procedures can considerably affect the egg quality and survival of larvae (Fernández-Palacios et al. 1997; Almansa et al. 1999; Izquierdo et al. 2001; Watanabe and Vassallo-Agius 2003; Targońska et al. 2010). Eggs that do not develop normally, die after a while, triggering protozoan and fungal growth that can infect surrounding embryos during incubation (Ciereszko et al. 2009) and then it intensifies during the egg hatching period resulting in mass mortalities due to water degradation (Rowland 2009).

Egg quality is a complex area to quantify and is still not well understood (Jakobsen et al. 2016). The fatty acid composition of eggs plays an important role in improving reproduction success, including egg quality and the survival of fry (Komen and Thorgaard 2007; Bobe and Labbé 2010). Egg quality of silver perch has been identified as a predominant indicator in assessing reproductive success and a key factor causing the high variation in the size of newly hatched larvae (Anonymous 1999; Moiseeva 2001; Rowland 2004b; 2009).

It is widely accepted that egg quality affects both egg and larvae viability which in turn is reflected in several characteristics of larvae including physical deformity, growth rate, mortality rate, and size variability (Żarski et al. 2011b). The difference in individual performance can lead to high cannibalism which, at the end, will directly determine production effectiveness (Rowland 2009). In silver perch, egg quality is commonly evaluated based on the viability of larvae after hatching (Rowland 1984; 2009) while earlier determination of egg viability could be a beneficial tool to eliminate low quality eggs leaving incubation of only good eggs quality as in Eurasian perch, *Perca fluviatilis* (Żarski et al. 2011b).

The distribution of oil globules has been used as an indicator of egg quality in European perch, (Żarski et al. 2011b) and brown trout, *Salmo trutta fario* (Mansour et al. 2007). There was no relationship between oil globule distribution in the oocyte and the quality of the eggs especially with respect to reproductive performance of the domesticated rainbow trout (Ciereszko et al. 2009). However, oil globule

fragmentation has never been used as a tool to evaluate the reproductive performance of silver perch. The present experiments were conducted to investigate any relationship between the degree of oil globule fragmentation and the reproductive performance and egg quality of the domesticated broodstock of the silver perch.

3.2. Material and Methods

The experiments and the procedure in this study were approved by the Animal Ethics Committee of Curtin University with the approval number of AEC_2011_70. The Australian Code of Practice for the care and the use of animals for scientific purposes also followed.

3.2.1. Broodstock source and preparation

The experiments were conducted during the summer season which coincided with silver perch maturation time after they were exposed to a low temperature in the winter months in Western Australia. Three pairs of broodstock aged approximately six years with an individual weight and TL range of 1.2–3.1 kg and 37–47 cm for males and 2.5–3.7 kg and 42–53 cm for females were obtained from Curtin Aquaculture Research Laboratory, Curtin University, Bentley (31°59'38.26"S 115°53'18.09"E). The broodstock were maintained in a semi-closed water recirculating system and fed commercial floating feed at a rate of 2% body weight/day containing 35% crude protein.

The mature females were marked by the soft and swollen stomach and pink-red genital papilla whereas mature males were marked by the release of the milt when gentle pressure was applied to the abdomen; these were selected for this experiment. The selected mature broodstock were then moved into a 200 litre fiberglass tank and anesthetize with AQUI-S at 100–120 ppm solutions before hormonal injections. All broodstock were injected intramuscularly with hCG hormone at 200 IU/kg body weight using a disposable syringe and a 21G x 1.25 needle to initiate spawning. Each pair of induced broodstock were placed in a 2-ton fiberglass tank until

spawning (Rowland 2004a). The water was vigorously aerated and the water temperature was maintained at 23°C. The tanks were inspected periodically to observe the spawning. As the spawning was completed, the post spawning broodstock were anesthetized and weighed before they were returned to the rearing tank. The female weight was used for relative fecundity calculations.

3.2.2. Egg quality classification

Under the stereo microscope, the oil globule was clearly visible, located in the middle of the yolk sac of newly fertilized eggs (diameter range 2.1–2.41 mm). The eggs of silver perch were classified based on the oil globule fragmentation following the category stated by Żarski et al. (2011b) with minor modifications. However, only three out of four categories were observed in this research. These categories were: category-1, a clearly visible, single droplet of oil globule, category-2, several small droplets of the oil globule along with a large one, and category-3, a highly-fragmented oil globule in the form of several droplets (Figure 3.1).

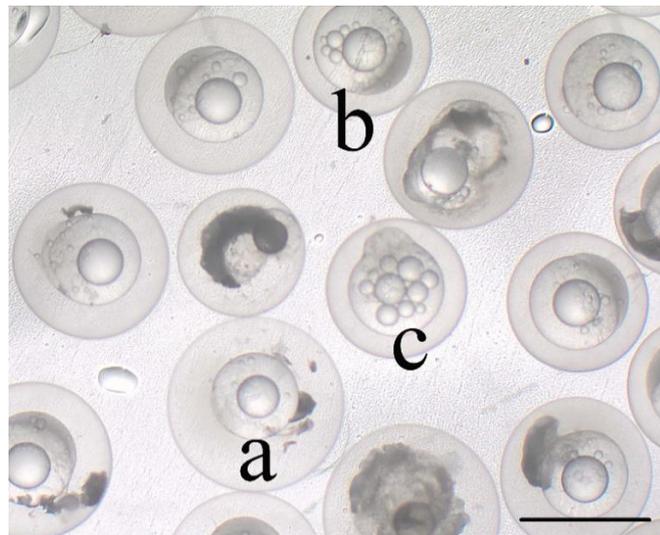


Figure 3. 1. Different categories of ovulated eggs of silver perch, *Bidyanus bidyanus* with different oil globule fragmentation: category-1 (a), category-2 (b), and category-3 (c). Bar = 2 mm.

3.2.3. Reproductive performance of the female broodstock (experiment-1)

The reproductive performance of the broodstock (n=3) was evaluated based on the fecundity, fertilization rate, hatching rate, and egg categories. The relative fecundity was defined as the number of recently spawned eggs divided by the female body weight (Hunter et al. 1992; Cerdá et al. 1994; Coward and Bromage 1999). Fecundity and fertilization percentage were estimated according to established procedure (Panini et al. (2001) with some modifications.

For fecundity determination, the eggs produced by each individual female were initially placed in a 10 litre bucket equipped with aeration from the bottom to provide gentle agitation of the eggs. A subsampling of 10 ml was taken and the number of eggs were counted before conversion to the bucket volume. The relative fecundity was then calculated and presented as a number of eggs/kg female body weight after spawning. At the same time, the fertilization rate was also estimated by counting the number of fertilised eggs divided by the total number of eggs in 10 ml subsamples.

To determine the hatching rate, eggs from the 10 litre bucket were taken in a 250- μ m mesh filter and were rinsed with filtered freshwater (Aqua-pure Model-AP12S, 5 microns) and placed in a 2 litre beaker. A petri dish was used to scoop 100–200 eggs from the beaker which was then placed under a stereoscope. Only fertilised eggs were taken one by one with a pipette and transferred to the wells of the microtiter plates at a rate of one egg per well. The microtiter plates were then covered with a plastic lid, and placed in a controlled-temperature incubator set at 23°C until hatching. Using a stereoscope, the number of larvae were counted and the hatching rate was calculated as the number of larvae divided by the number of fertilised eggs initially loaded in the microtiter plates.

To classify the eggs, another sample of 10 ml in volume were taken from the 10 litre bucket and placed them into five petri dishes (50 mm diameter and 12 mm height). The eggs in the petri dishes were photographed under a stereo microscope

using an Olympus SC30 camera with an image acquisition software, getIt, from Olympus Soft Imaging Solutions (Papanikos et al. 2003). The eggs were then classified based on its oil globule fragmentation as explained earlier. From the images, the number of eggs in each egg category of each broodstock was counted and the percentage of egg category was calculated.

3.2.4. The relationship between the degrees of oil globule fragmentation and egg quality (experiment-2)

3. 2.4.1. Embryo and larval performance at different egg categories

The eggs from a female were sampled from the 10 litre bucket, as in fecundity determination, using a 250- μ m mesh filter, rinsed with filtered freshwater, and placed in a 2 litre beaker (Panini et al. 2001). A petri dish was used to scoop 100–200 eggs from the beaker and was placed under a stereoscope and only fertilised eggs were taken one by one with a pipette and transferred into microtiter plates at a rate of one egg per well. Each egg category was placed in three separate microtiter plates as triplicates. The microtiter plates were then covered with a plastic lid, placed in a controlled-temperature incubator where the temperature was maintained at 23°C. Using a stereoscope, the survival rate of the embryo from each egg category was calculated at 10 and 20 hour post hatching (hps) and the hatching rate was calculated following the formula: the number of larvae/number of eggs in the microtiter plates \times 100. The deformity rate was calculated as the number of larvae with physical deformity/the number larvae in the microtiter plate.

3.2.4.2. Effect of egg quality on growth and survival rate

As soon as the entire batch of eggs were hatched, the larvae from each egg category (section 2.4.) were pooled and placed in 5 litre cylindrical glassware as stock for a growth and mortality experiment. The glassware was placed in an incubator tank where the temperature was set at 23°C as in the incubation temperature.

Two-day-old larvae were randomly taken from each holding glassware (categorized eggs) and placed in 1 litre beakers at 20 larvae/litre stocking density in triplicates. Larvae were fed rotifers at a density of 10 ind./ml starting at 4 days post hatching (dph). The density of rotifers was maintained throughout the experiment by daily adjustments whereas water temperature was set to 23°C. The faeces and debris, as well as dead fish, were removed from the glass beaker every day before feeding and the mortality was recorded. At the end of the experiment at 12 dph, the survival rate was calculated while larval growth was determined by measuring the TL (mm) of ten larvae from each egg category.

3.2.5. Data analysis

The IBM SPSS 24 and MS Excel 2007 for Windows software were used for the analysis of data and statistical analysis. The data was presented as mean±S.E where the mean value is derived from duplicate groups. The data expressed in percent was subjected to arcsine transformation before the statistical analysis was performed. The one way analysis of variance (ANOVA) followed by the Tukey's post hoc test was at the significant level less than 5% ($P<0.05$) when the analysis of variance revealed statistically significant differences.

3.3. Results

3.3.1. Reproductive performance of the female broodstock (experiment-1)

The reproductive performance of domesticated silver perch is shown in Table 3.1. The relative fecundity was high, ranging from 120,000 to 134,200/body weight. The fertilization rate and the hatching rate were high between 91.95 %–96.38% and 87.94±1.23% respectively (Table 3.1). The mean percentage of eggs in category-1 of 78.11% was significantly higher ($P<0.05$) than category-2 and category-3 at 21.26% and 0.40% respectively.

3.3.2. The relationship between the degrees of oil globule fragmentation and egg quality (experiment-2)

The survival rate of embryos in all egg categories was high during the incubation period. No mortality was observed until ten hours post spawning (hps) and only a few embryos were observed to die at 20 hps. A significant difference in embryo survival from different egg categories was detected at 20 hps (Table 3.2), whereas no significant difference in the deformity rate among the egg categories was found.

At 8 dph, the fragmentation of oil globules was still visible (Figure 3.2). The growth of silver perch larvae was clearly affected by the egg's categories. The TL of larvae in category-1 and category-2 (8.4 ± 0.2 and 8.3 ± 0.2 mm respectively) did not differ significantly ($P>0.05$), but they were much higher than category-3 (7.0 ± 0.1 mm) at 12 dph (Figure 3.3).

Table 3. 1. Reproductive performance and egg quality composition of silver perch, *Bidyanus bidyanus* broodstock (n=3) domesticated in a semi-closed recirculation system.

Broodstock (female)	Fecundity (eggs/kg)	Fertilization rate (%)	Eggs quality			Hatching rate (%)
			Category-1	Category-2	Category-3	
1	145,200	96.38	78.16	21.31	0.50	90.38
2	132,000	94.47	73.87	25.48	0.00	86.48
3	120,000	91.95	82.30	16.98	0.71	86.96
Average	132,400±7,218	94.27±1.28	78.11±2.44 ^c	21.26±2.45 ^b	0.40±0.21 ^a	87.94±1.23

Note: The eggs quality data labelled with different superscript in the same row of egg quality were statistically different ($P<0.05$).

Table 3. 2. The percentage of survivors of silver perch, *Bidyanus bidyanus* embryos at different periods of incubation, hatching rate, and the rate of deformity of newly hatched larvae at different categories of egg quality.

Egg Category	Mean survival rate (%)		Hatching rate (%)	Deformities at hatching (%)
	10 hph	20 hph		
1	100.00+0.00 ^a	95.33+0.00 ^b	93.33+0.00 ^b	0.00+0.00 ^a
2	100.00+0.00 ^a	90.00+0.00 ^b	85.00+0.00 ^b	0.33+0.003 ^a
3	100.00+0.00 ^a	72.33+1.76 ^a	60.33+0.00 ^a	0.50+0.003 ^a

Note: The data labelled with different superscript in the same column were statistically different ($P<0.05$).

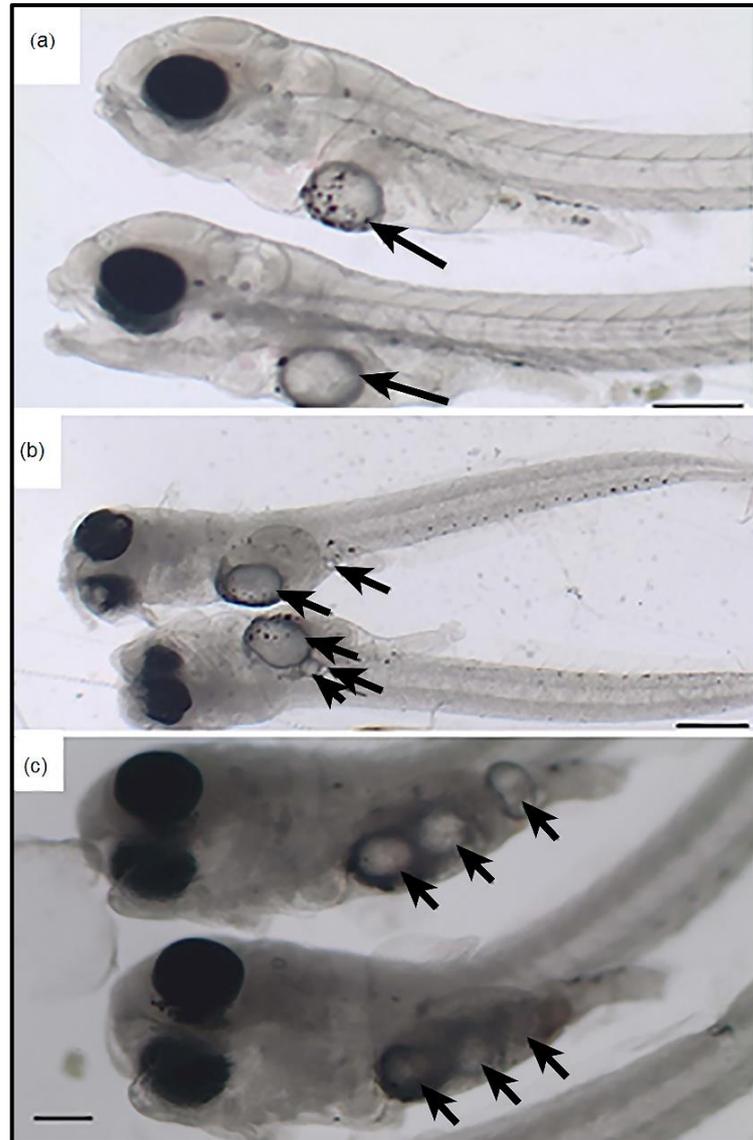


Figure 3. 2. The appearance of oil globule fragmentation of silver perch, *Bidyanus bidyanus* larvae originating from category I (a), category II (b), and category III (c). Bar (a)=500 μ m, (b)=500 μ m, and (c)=200 μ m.

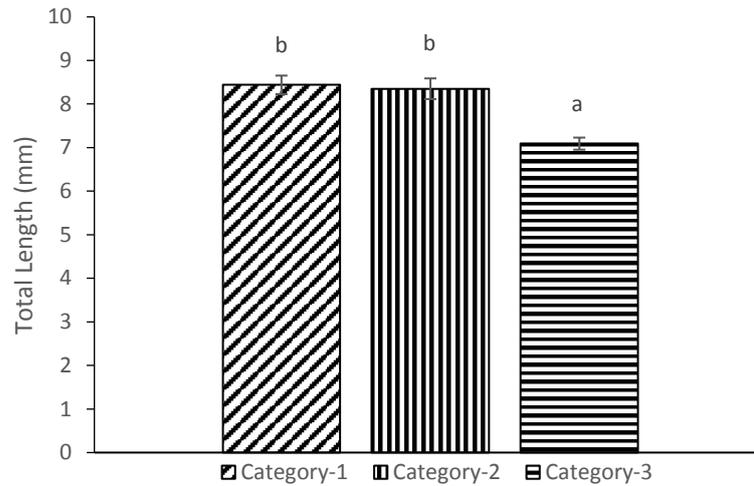


Figure 3. 3. The total length (TL) of silver perch, *Bidyanus bidyanus* larvae resulting from different egg quality at 12 dph. The same letter over the bars indicates no significant difference ($P>0.05$).

After 12 days of the rearing period, the lowest survival rate of $61.3\pm 0.3\%$ was noted in egg category-3, which was significantly ($P<0.05$) lower than the survival of category-1 and category-2 at $71.3\pm 0.9\%$ and $66.7\pm 0.9\%$, respectively. The survival rate of category-1 did not show any significant difference ($P>0.05$) when compared to category-2.

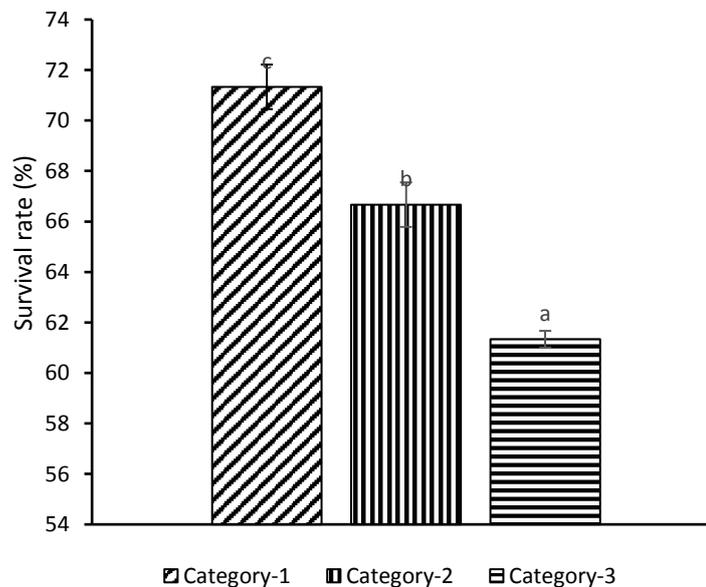


Figure 3. 4. Survival rate (%) of silver perch, *Bidyanus bidyanus* larvae from different egg categories at 12 dph. Different labels over bars designates significant difference ($P<0.05$).

3.4. Discussion

The reproductive performance of the 6-year-old silver perch broodstock was found to be high as displayed by all studied parameters. The relative fecundity per kg of female ($139,286 \pm 11,405$) 3-year-old domesticated silver perch broodstock, as reported by Rowland (2004a), is slightly higher than the fecundity ($132,400 \pm 7,218$) of the 6-year-old broodstock used in the present study. However, the fertilization and hatching rates of $94.27 \pm 1.28\%$ and $87.94 \pm 1.23\%$ were much higher than reported by Rowland (2004a) at $84.5 \pm 3.7\%$ and $76.8 \pm 2.8\%$ respectively. These different results may be due to the different age and level of domestication of the broodstock population used. Rowland (2004a) used a 3-year-old F1 broodstock at first maturation, while in this study, 6-years-old F2 broodstock was used. According to Izquierdo et al. (2001), the age of the fish is an aspect that affects reproductive performance, wherein the first maturation commonly results in poor performance than the older fish (Emata 2003).

The eggs produced by these 6-year-old broodstock were classified as good quality eggs since most (99%) eggs produced were category-1 and category-2. Other than the egg quality, the results also confirmed that the reproductive performance of domesticated broodstock reared in captivity for about 6 years was still high as indicated by the high viability of larvae. These findings confront the previous study by Thurstan, Rowland (1994) who proposed that the fecundity of silver perch broodstock may be reduced after 5 years reared in captivity.

As in most other cultured fish, silver perch showed segregation in oil globules and this phenomenon is related to the process of oocyte maturation and spawning. The process of oocyte maturation is known to incorporate different components such as yolk protein and oil globule (Bromage et al. 1992). Another study showed that the process of maturation can be disturbed by different activities in controlling reproduction such as through hormonal stimulation or the manipulation of environmental conditions (Żarski et al. 2011b). As a result, the segregation of oil droplets during oocyte maturation as has been reported for Eurasian perch (Żarski

et al. 2011b), the European eel, *Anguilla anguilla* (Palstra et al., 2005), burbot, *Lota lota* (Palińska-Żarska et al. 2014), *Salmo trutta fario* (Mansour et al. 2007), and rainbow trout, *Oncorhynchus mykiss* (Ciereszko et al. 2009). The segregation of oil globules in a different category of fragmentation has been identified as affecting egg quality and regarded as an indicator of egg quality (Żarski et al. 2011b).

The egg quality not only affects embryonic development and their success in hatching but also affects larvae growth and mortality in the beginning of the developmental stages (Houde 1974). The survival rate of eggs in category-1 and 2 showed higher viability than category-3 eggs as indicated by their high survival rate of the embryo during incubation, especially at 20 hps. Further evidence was shown in the hatching rate where only about 60% of eggs in category-3, while in category-1 and 2 the hatching rate were higher at 93% and 85% respectively. Even if the embryo survival and hatching rate differed according to egg category, the frequency of developmental abnormalities was low in each egg category (0.0–0.5%) and there was no difference between egg categories. However, the indication of higher deformation at higher oil globule fragmentation, as in category-3, may indicate that these eggs were of poorer quality than those in category-1 and 2. Żarski et al. (2011b) reported that viability of Eurasian perch eggs at category-3 was lower compared to category-1 and 2. On the other hand, Ciereszko et al. (2009) reported the inconsistency of the relationship between oil fragmentation and egg quality of rainbow trout where the high fragmentation of the oil globule did not always result in lower egg quality.

The mechanism on how oil globule fragmentation affects survival rates during incubation of silver perch eggs, particularly in category-3, is unclear. However, according to Abi-ayad et al. (2000), the oil content of eggs is constant during embryogenesis and is not used until the larvae's metamorphosis stage is reached. This means that oil globule fragmentation may not directly affect the survival rate of the embryo during incubation. Therefore, another mechanism may act as a stressor resulting in higher mortality during the incubation phase and lower the hatching rate of eggs in category-3. One possible explanation is the process of

ovulation is too early and some eggs do not complete the normal final maturation of the oocytes as in category-1 and 2. This explanation is also reported by Żarski et al. (2011b) where early ovulation could have disturbed the final maturation of the oocyte in Eurasian perch which resulted in a low quality of eggs in category-3 and 4 as indicated by the low survival rate during incubation, low hatching rate, low growth rate, and high mortality of the new larvae.

The visibility of oil globule fragmentation during larval development as noted at 8 dph (Figure 3.2), may suggest that the fraction is permanent until all oil is absorbed completely. This is different from Eurasian perch where the fraction of the oil globule may be merged, partly or totally, immediately after eggs were ovulated and get in contact with water at spawning (Żarski et al. 2011b) and in medaka, *Oryzias latipes* (Iwamatsu et al. 2008). The oil fragmentation may contribute to the growth and survival rate differences of silver perch larvae from different egg categories. Related to mortality, Li, Mathias (1982) pointed out that the high mortality of Walleye larvae, *Stizostedion vitreum*, occurred during yolk sac consumption and complete absorption of oil globules.

The egg quality also affected the TL of the larvae as detected at 12 dph. The more fragmented the oil globule, the lower the TL achieved. The same was true with the survival rate where the lowest survival was observed at category-3. It has been reported that the oil globule contained vitamin A and nutrient ingredients of high caloric value (Iwamatsu et al. 2008), which is crucial for larvae development during the transition from endogenous to exogenous feeding (Iwamatsu et al. 2008). The process of assimilation of fragmented oil globules into the larval body may be obstructed and reflected in small TL and low survival rates at 12 dph. This has also been the reason for low viability and high growth variability in Eurasian perch (Żarski et al. 2011b).

In conclusion, our results suggest that domesticated (F2) silver perch broodstock reared in captivity for about six years are still viable with acceptable performance

of high quality of eggs. In addition, an oil globule fragmentation test is recommended as a valuable tool for evaluating the egg quality in silver perch.

CHAPTER 4: Yolk utilization and growth during the early larval life of the silver perch

The article has been published in *Journal of International Aquatic Research*, 9 (2017):107-116. DOI 10.1007/s40071-017-0160-7

4.1. Introduction

The yolk stages (yolk-sac and oil globule) of most of the teleost with early-life planktonic stages and lack of parental care, are characterized by reduced capacities in swimming, vision, and feeding ability (Jug-Dujaković et al. 1995). The knowledge about the role of early fish ontogeny is particularly essential for better understanding of larval dynamics and mechanisms of adapting to variable environmental conditions. The essential events in larviculture are not only the period of these susceptible ontogenetic stages, but also a diversity of vital physical appearances such as the hatching size, onset of feeding, and the efficacy of yolk utilization (Polo et al. 1991; Koumoundouros et al. 2001). Some fish species are more highly prone to mass mortalities during the early feeding time than during the advanced development phase (Paperna 1978; Gisbert and Williot 1997; Qin et al. 1997; Mookerji and Ramakrishna 1999; Dou et al. 2005; Kailasam et al. 2007). Fish larvae are also highly vulnerable during the period of transition from consuming endogenous reserves of food to exogenous nutrient consumption. Failure to perform first feeding response immediately when the mouth has opened often causes morphological deformities, abnormal moving behavior, and an inability to swim and feed (Kjørsvik et al. 1991; Gwak and Tanaka 2001), leading to high mortality (Houde 1974; Dou et al. 2002).

Generally, the rate of survival of fish larvae is poor during the early life stages (Bisbal and Bengtson 1995; Yang 2007). The availability of food at the right time is crucial because starvation can lead to high larval mortality (Bisbal and Bengtson 1995; Kohno et al. 1997; Gisbert et al. 2004; Peña and Dumas 2005). Some other factors such as larval size, yolk and oil quantity and absorption rate, feeding

behavior, and the time of initial feeding may also affect mortality (Dou et al. 2000; Blaxter 1974). In Chinook salmon and rainbow trout, *Salmo gairdneri*, initial feeding starts after the swim-up stage (Twongo and MacCrimmon 1976; Heming et al. 1982) while in burbot, *Lota lota*, exogenous food needs to be given at 9-10 dph (Palińska-Żarska et al. 2014). The elapsed time from yolk sac depletion to irreversible starvation also differs between fish species (Bisbal and Bengtson 1995; Shan et al. 2008).

Silver perch, *Bidyanus bidyanus* (Mitchell, 1838), endemic to the Murray-Darling river system in New South Wales, is a native Australia freshwater fish, belonging to the Theraponidae family (Guo et al. 1993). Also known as freshwater bream, silver bream and grunter, the silver perch occupies the northern and western rivers and upper reaches of the Murray-Darling river system (Rowland 1995). It is an important freshwater aquaculture species which has been intensively cultured in Australia and producing around 500 metric tons per year (Rowland 2009). It has also been cultured in China, Taiwan, and Israel (Allan and Rowland 2002). Limited information on its morphogenesis and larval ontogeny originates from wild broodfish has been reported by Lake (1967b), but no detailed information has been published about domesticated broodfish. Since the life cycle of the silver perch can be closed in captivity (Rowland 2004a), it is important to assess the ontogeny and morphogenesis of larvae from the domesticated broodfish and compare it with the larvae from the wild broodfish.

This paper delivers early life-history data on the silver perch larvae, obtained from the domesticated broodfish. The research details the information found during the early larval stages with respect to the yolk absorption rates, the onset of feeding, and the mortality response of the unfed larvae.

4.2. Material and Methods

Each experiment was accepted by the Animal Ethics Committee of Curtin University with the approval number of AEC_2011_70. The Australian Code of Practice for the care and use of animals for scientific purposes was also followed.

4.2.1. Fish and experimental setup

The second generation of domesticated silver perch broodfish (6 years old), reared in a farmer's pond at Gingin, Western Australia (31°20'0.53" S, 115°46'20.51" E), were used in this experiment. Eggs were obtained after human chorionic gonadotropin (hCG) hormone was injected into both male and female broodfish at a dosage of 200 IU/kg (Rowland 1984; Levavi-Sivan 2004; Rowland 2009). Following hormone injection, both males and females were kept together in a 3-tonne fiberglass tank filled with two tons of freshwater until they spawned. The spawning tank was placed in a hatchery hall at a room temperature of 20 to 25°C, equipped with gentle aeration that was placed in the mid-bottom of the tank.

After spawning, the eggs were transported from Gingin to the Curtin Aquaculture Research Laboratory (CARL) for incubation and for the production of the fry used in the experiment. On arrival, about 5,000 eggs were placed into two 200-L, conical-shaped, fiberglass incubator tanks filled with freshwater, with a flow-through system that had a flow rate of 500 ml/min. With this system, egg shells were retained in the incubation tank while larvae from the two incubation tanks were moved into the sump tank. Larvae from the sump tank were then transferred to a holding tank (200-L volume) at a density of 50 larvae/L. The holding tank was filled with 6 ppt saline water and gently aerated. The water temperature in the holding tank was preserved at 20±0.5°C by a thermostatically controlled heater (Sonpar, Model-Ha200). The newly hatched larvae were then haphazardly taken to perform morphometric measurements, age at first feeding, and mortality experiments. The rest of the larvae were kept unfed in this tank until the end of the experiment. The swimming behavior of the larvae in this tank were observed simultaneously to identify the initial time of starvation signalling by slow-moving swimming, hanging from the head down into the water column, and almost no reactions to gentle probing.

4.2.2. Morphometric measurements

The hatching time is appointed at which 90% of the viable eggs were hatching (Shan et al. 2008). Meanwhile, morphometric measurement was performed at 6 hour intervals, from hatching until 10 dph, using 7 to 53 larvae (average=25) per sample. Yolk utilization (yolk sac and oil globule) was dignified based on the reduction in the volume of the yolk-sac and oil globule over time. The depletion time of each yolk sac and oil globule is employed at which more than 90% larvae have depleted their yolk sac or oil globule. The yolk utilization of the unfed larvae was studied using a stereoscopic microscope (Olympus-SZH) while morphometric characteristics were monitored using photographs taken with a digital camera (Olympus-SC30) attached to the microscope. On the photographs, five morphometric characteristics (TL: total length; YsH: yolk sac height; YsL: yolk sac length; OgD: oil globule diameter) were quantified to the nearest 0.001 mm (using Photoshop image software), while yolk sac volume (YsV) and oil globule volume (OgV) were estimated. The YsV was estimated using the formula introduced by Bagarinao (1986) for a prolate spheroid, $V = \pi/6 l h^2$, where l is the length of yolk sac and h is the height of yolk sac. The volume of the oil globule in the larvae was computed from the formula $V = \pi/6 d^3$, where d is the oil globule diameter. The specific growth rate (SGR) of the TL at different periods of their larval stage was calculated by the formula: $SGR = (e^g - 1) \times 100\%$ (Hopkins 1992; Árnason et al. 2009), where $g = [\ln(l_2) - \ln(l_1)] / (t_2 - t_1)$, and l_2 and l_1 are the mean TL on day t_2 and t_1 , respectively. All measurements were performed parallel to the body-length axis, and all depths and diameters were measured perpendicular to this axis (Figure 4. 1). The measurements for the diameter of ova, water-hardened egg, yolk sac, and oil globule, and the TL of each larva at hatching from domesticated broodfish in this study were then compared to the corresponding characteristic for larval performance from wild broodfish as reported by Lake (1967b). The data presented by Lake (1967b) were based on the wild broodfish of silver perch stocked in a pond and spawned naturally before eggs were collected for examination in accordance with the egg and larvae parameters.

4.2.3. Age at first feeding

To determine the age at first feeding, 10 one-day-old larvae were introduced into a beaker glass containing 1 L of water with rotifers (*Brachionus calyciflorus*) at a density of 10 ind./ml. Beakers were placed in the incubator tank, each equipped with a thermostat (Thermomix, B. Braun Biotech International) in order to maintain the water temperature at approximately $20\pm 0.1^{\circ}\text{C}$. To facilitate prey contrast, the trough was covered with black plastic on the sides and bottom, and illuminated from above by a 36W fluorescent light. The larvae were netted out after 45 min, lightly anaesthetized with AQUI-S (0.025 mg/L) and preserved in 8% buffered formalin for subsequent gut content observation. The incidence of feeding was expressed as the percentage of test fish (n=10) with food (at least one food item) in their gut. The feeding trials were repeated every 24 h with unfed silver perch until they were 144 h or 6 days old.



Figure 4. 1. Newly hatched silver perch, *Bidyanus bidyanus* larvae (eyes, jaws, and gut not formed) showing morphometric measurements of total length (TL), yolk sac length (YsL), yolk sac height (YsH), and oil globule diameter (OgD), (bar= 250 μm).

4.2.4. Mortality

The experiment was conducted using three 2-L, conical-shaped glass containers placed in an incubator tank to determine the mortality of unfed larvae during their yolk stage. Fifty 1-dph larvae from the holding tank were transferred to the experimental glass container. Mild aeration was provided, along with illumination

using 40W fluorescent light regulated at about 200 lux. Dead fish were counted and removed along with bottom sediments before changing the water about 30% from the rearing volume every 24 h. Cumulative mortality was then calculated daily. Water temperature was maintained at 20°C.

4.2.5. Data analysis

Statistical differences in egg and larva parameters (the TL, diameter of ova, diameter of water hardened egg, Ysh, and the Ogd) between domesticated and wild broodfish were analyzed by one sample t-test. The comparison between TL and SGR at different stages were analyzed by an independent-sample t-test between two periods of development i.e. from 0-96 hph and from 96-240 hph. Mortality rates at a different age of larvae were analyzed by one-way analysis of variance (ANOVA) followed by a post hoc test (Tukey's) with the level of significance below 5% ($P < 0.05$). The relationship between the mortality rate and the time after hatching was studied using regression analysis. Statistical analysis was performed using IBM SPSS Statistic-22 and regression analysis using Microsoft Excel 2013.

4.3. Results

4.3.1. Larval size, yolk reserves, and early growth

The eggs obtained from the pond-reared broodfish hatched about 32 h after fertilization, at $20 \pm 1.0^\circ\text{C}$. The newly-hatched silver perch larvae were transparent with a large yolk sac prolonging from the tip of the snout to about half of the ventrolateral of the body (Figure 4.1) with an initial mean length of 1.35 ± 0.03 mm and a volume of 0.71 ± 0.07 mm³. An oil globule (OG) of 0.64 ± 0.00 mm in diameter and 0.02 ± 0.00 mm³ mean volume was present at the caudal tip of the yolk sac. The frontal part of the body was bent about the yolk sac and the border of finfold formed surrounded the body from the dorsal and ventral part of the body to the caudal part. The intestine was not fully formed, with anus and mouth sealed. Eye pigmentation was observed 12 hours post-hatching (hph) and completed after 3 dph. The

intestinal canal started formation at 2 dph, and a simple, straight, tubular gut was observed. Further development of the alimentary system was the formation of the intestinal loop at 3 dph and at the same time, the lower jaw was completely formed.

At hatching, the silver perch larvae each measured 2.52 ± 0.05 mm in total length (TL). They grew to 4.12 ± 0.13 mm TL in about 96 h, by which time there was no more yolk sac left and about 45% of the oil globule remained (Figure 4.2). After this stage, the TL growth subsequently slowed down and was significantly ($P < 0.05$) lower than the first 96-hour period.

4.3.2. Yolk utilization rate

The yolk-sac utilization rates were much higher than the oil-globule exhaustion rate during the early life history of the silver perch larvae. Nearly 34% and 0.5% of the initial yolk sac and oil globule reserves, respectively, were utilized within 6 h. More than 50% of the silver perch larvae exhausted their yolk sac completely by the fourth day at 96 hph, whereas the oil globule traces (<1%) could still be seen up to 240 hph (Figure 4.2). At the same time, the TL increased significantly ($P < 0.05$) as the SGR reached approximately 12% per day during the first 96 h, followed by a slow growth rate of 4.8% only from 96 hph to 240 hph.

4.3.3. Age at first feeding

Even before the yolk exhausted completely, the larvae of the silver perch have demonstrated a sign of food searching behavior, comprising prey search, pursuit, and attempts to capture prey. The initial timing of first feeding was noticed at 5 dph. However, only about 5% of larvae could successfully initiate feeding instantly after the yolk sac was totally absorbed. The incidence of 100% of larvae feeding was observed at 6 dph; that is, one day after the yolk sac was fully absorbed. When silver perch larvae initiated first feeding, they already had functional mouths and active swimming behavior, which indicated that most of the larvae needed some time to establish active feeding before and during the yolk exhaustion period. The symptoms of starvation of unfed larvae were observed at 5 dph.

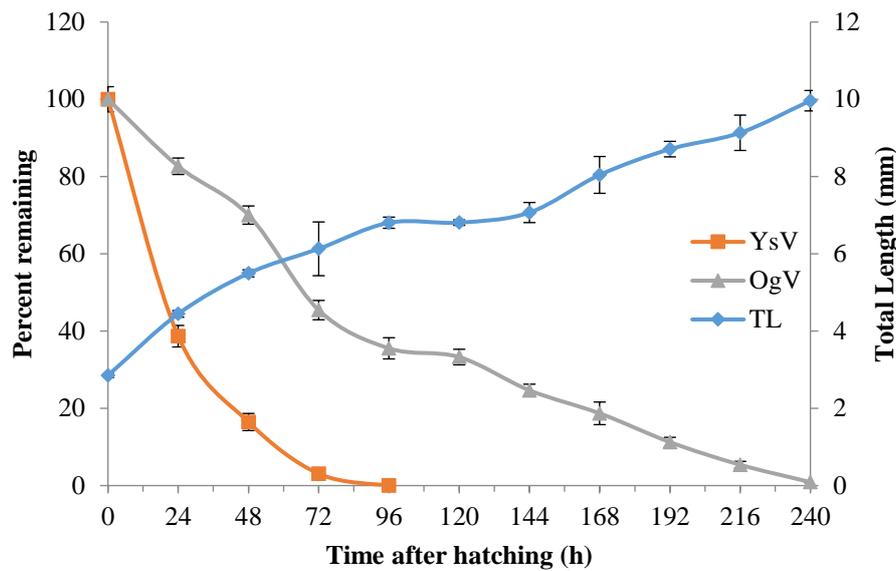


Figure 4. 2. Yolk sac volume (YsV) and oil globule volume (OgV) utilization and the total length (TL) development in relation to the time after hatching in hours (h) of the unfed silver perch, *Bidyanus bidyanus*, larvae. Data points are mean \pm S.E. of 7-20 larvae per sample.

4.3.4. Mortality

All silver perch larvae died no later than 240 hph. The daily cumulative mortality during the early life history of silver perch larvae increased with age (Figure 4.3). The daily mortality rate underwent a rapid increase ($P < 0.05$) after the exhaustion of the yolk sac at 96 hph onward and 50% mortality was reached at 175 hph (Figure 4.3). The correlation analysis between mortality and yolk sac-volume showed that mortality rate was significantly ($P < 0.05$) affected by the availability of the yolk sac.

4.4. Discussion

Earlier research has shown that the silver perch would not spawn at a temperature below 24°C in the natural environment and at a temperature below 23.3°C under captive pond conditions (Lake 1967a). The domesticated broodfish of the silver perch does not spawn naturally under the Gingin (Western Australia) pond conditions in temperatures ranging between 11-34 °C.

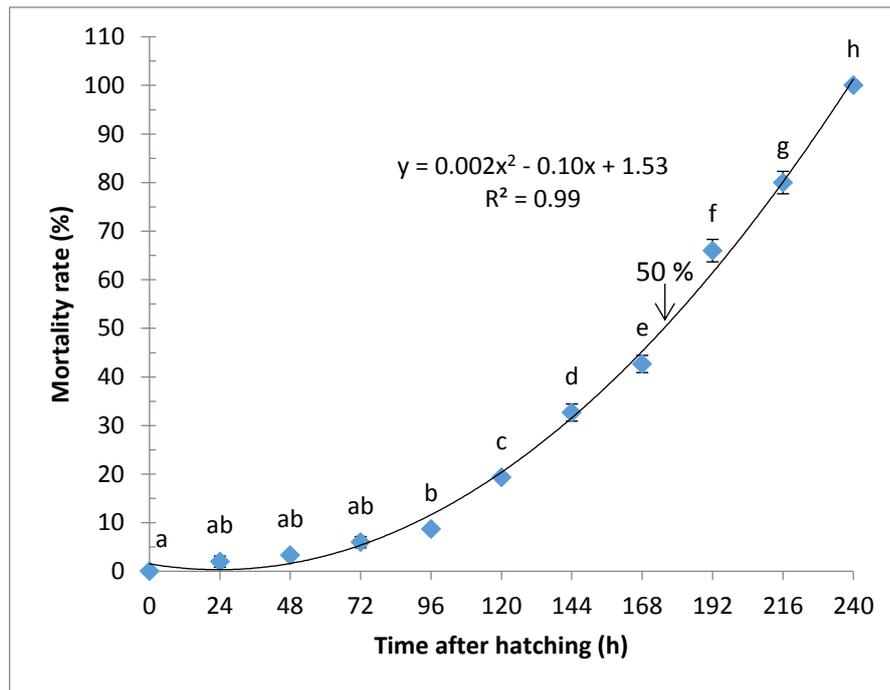


Figure 4. 3. The effect of starvation on mortality in silver perch, *Bidyanus bidyanus* larvae. Data points are mean \pm S.E. of three replication. The marker with the same label indicate not significantly different ($P < 0.05$)

However, after a single injection of hCG, the domesticated broodfish in this experiment spawned at 21°C. This is in the range of temperatures (21-24 °C) within which silver perch spawned in Israel (Levavi-Sivan 2004), which are both lower than the spawning temperature of 25°C as reported by Rowland (1984). Hence, no evidence of spawning deterioration was observed in this experiment. This may indicated that the domesticated silver perch could have adapted to spawn under different ranges of temperature after long time acclimation in pond conditions. This phenomena has also been stated by Lam (1983) for the goldfish, *Cyprinus auratus* where spawning temperature changed after acclimation to the new environmental condition. Another interesting information here is the relationship between the incubation temperature and the hatching time (Table 4.1). The difference in incubation temperature did not make much different in hatching time. This may possible that the incubation period does not only depending on the temperature but also depending on the broodstock and eggs quality (Mylonas et al. 2010). Therefore, the internal factor related to the eggs development stage at hatching may also affect the hatching time. On the other hands, the hCG injection before spawning may also

contribute to influence the period of incubation. However, no detail information to support this argument yet.

Table 4. 1. Egg and larval development of silver perch, *Bidyanus bidyanus* from wild and domesticated broodfish

Factor	After Lake (1967b)	This experiment (mean±S.E.)
Broodfish source	wild	domesticated
Broodfish size (kg)	0.7-3.2	1.5-3.5
Temperature at spawning (°C)	23.3	21
Nature of egg	Pelagic	Pelagic
The diameter of ova (mm) – averages in parentheses	0.7-1.3 (1.2) ^b	1.08±0.01 ^a (n=600)
The diameter of water-hardened egg (mm) – averages in parentheses	2.75-2.80 (2.78) ^b	2.17±0.01 ^a (n=600)
The diameter of yolk (mm) – averages in parentheses	1.00-1.25 (1.14) ^b	0.81±0.01 ^a (n=70)
Yolk length at hatch (mm)	-	1.35±0.03(n=70)
Yolk volume at hatch (mm ³)	-	0.58±0.06(n=70)
The diameter of oil globule (mm) – averages in parentheses	0.45-0.59 (0.54) ^a	0.62±0.01 ^b (n=70)
Oil globule volume at hatch (mm ³)	-	0.14±0.01(n=70)
Number of oil globules and position in yolk sac (minor ones neglected)	1 Posterior	1 Posterior
Egg color	Colorless	Colorless
Hatching time (hr) and temperature range (°C) in parentheses	30-31 (26-27)	30-32 (21-22)(n=3)
TL of larvae at hatching (mm) – averages in parenthesis	3.5-3.7 (3.6) ^b	2.85±0.06 ^a (n=70)
Maximal larval TL attained on the yolk sac reserves (mm)	-	11.97
Maximal larval TL attained on the oil globule reserves (mm)	-	14.31
Time from hatching to attainment of maximal TL on yolk sac (h)	-	96
Time from hatching to attainment of maximal TL on oil globule (d)	-	10
Age at first feeding (days)	6	5
Age at free swimming (days)	5	4

Note: Data in the same row having the same superscript letters (a,b) indicate not significantly different at α level of 0.05.

The measured TL of the newly-hatched larvae, egg's parameters and larvae morphometric of the domesticated silver perch were significantly lower than for the

parameters derived from wild broodfish as reported by Lake (1967b), except for the OgD (Table 4. 1). The reason may be due to differences in parent nutrition resulting in different biochemical composition of eggs. Eggs obtained from naturally foraging broodfish should contain a biochemical composition that is suitable for producing larger eggs and larvae with higher viability of offspring (Kjørsvik et al. 1989). On the other hand, the biochemical composition of eggs resulting from domesticated broodfish can be altered through changes in diet (Kjørsvik et al. 1990; Sargent 1995; Bromage 1998). However, until now, the quality of eggs produced from domesticated silver perch have not been compared to those wild broodfish. Regardless of the comparison with larvae resulting from wild broodfish, the observed TL in the current study (2.85 ± 0.06 mm) is still comparable to the Japanese flounder, *Paralichthys olivaceus* (2.62 ± 0.03 mm) (Dou et al. 2002). However, it is smaller than the Thai pangas, *Pangasius sutchi*, which has a TL of 2.98–3.10 mm (Islam 2005), the rohu, *Labeo rohita*, which has a TL of 4.58 ± 0.04 mm, and singhi, *Heteropneustes fossilis*, which has a TL of 3.32 ± 0.054 mm (Mookerji and Ramakrishna 1999). Even though the initial size of a silver perch larva is smaller, its initial yolk sac volume is relatively larger in terms of the YsL-TL ratio (0.54) than for Thai pangas (0.42) (Islam 2005), illustrating that the larvae is better equipped, in terms of its endogenous reserve as an energy source, to challenge the possibility of delayed exogenous feeding. It also demonstrates that the increase in total length is a biologically important aspect of the yolk-sac larval stages (Peterson et al. 1996) as the TL is directly related to the mouth opening and the size of prey that the larvae are able to ingest (Klimogianni et al. 2011).

During the first 96 hph the TL increased significantly ($P < 0.05$) compared to the next 144 hours, whereas at the same time, the yolk sac volume were reduced significantly ($P < 0.05$) due to their absorption. Conversely, the volume of the oil globule was not intensely absorbed. The oil globule consumption during the yolk-sac stage of the silver perch seems lower than the marine species common pandora, *Pagellus erythrinus* reared at comparable temperature at 18-21 °C (Klimogianni et al. 2004). A probable cause is a delay in oil globule adherence to the larval body (Klimogianni et al. 2011), which occurred mostly at 120-240 hph (Figure 4. 2). This

is much later compared to another marine species, the sharp snout sea bream, *Diplodus puntazzo*, which occurred at 4-6 hph. This could be advantageous to silver perch larvae as they get the opportunity to utilize both endogenous yolk and exogenous food (termed as the 'mixed feeding period') (Chai et al. 2011) for a longer time (6 days). This is much longer than the Chinese sturgeon, *Acipenser sinensis*, whose mixed feeding period only lasts for 2 days (Chai et al. 2011). Larvae have to start their capability to feed on an exogenous diet during the time when both endogenous and exogenous sources of diet are available; if not, they will hurt from continuous food shortage (Blaxter and Hempel 1963). An extended period of the mixed-feeding time is advantageous for larvae to gather enough feeding skills to develop their exogenous feeding capability, to escape from food shortages, and hence increase their probability of survival rates.

Since the onset of feeding occurred at 5 dph and the yolk-sac stage of the silver perch larvae lasted after 10 dph, it indicates that the mixed feeding period occurs for 6 days (from 5 to 10 dph). Consequently, the existence of appropriate prey organisms at this stage is of particularly significance for their endurance and the recruitment achievement of the silver perch. The same pattern is demonstrated by the Chinese sturgeon, where exogenous food deprivation can be maintained for 7 days (Chai et al. 2011).

The yolk-utilization rate of the silver perch during the first 24 h was about 61%, which is faster compared to 40% for singhi, another freshwater fish with a nearly equal activity pattern, illustrated by the intermittent bursts of rapid movement followed by relatively longer periods of rest during the yolk-sac stage (Mookerji and Ramakrishna 1999). However silver perch have a longer period of mixed feeding (6 days) compared to only two days for singhi.

Only about 5% larvae initiated feeding at 5 dph, when <5% of the initial yolk reserves (yolk-sac+oil globule) were remained, lower than for singhi where nearly 40% of the larvae initiate feeding when about 20% of the yolk sac reserves remained at about 26°C. Furthermore, the rate of oil-globule utilization was slightly lower

and about 33% still remained when the yolk sac totally disappeared at 5 dph. These trends were also observed by Mookerji, Ramakrishna (1999) in rohu and singhi. Exogenous feeding generally commences before the yolk is fully absorbed (Mookerji and Ramakrishna 1999; Gisbert et al. 2000; Gisbert et al. 2004; Islam 2005; Peña and Dumas 2005; Kailasam et al. 2007; Yang 2007; Shan et al. 2008; Ghelichi et al. 2010; Wang 2010; Klimogianni et al. 2011; Haga et al. 2014), as was also observed in the present study.

The size at first feeding of silver perch must be taken into account for successful rearing of larvae, with the use of suitable live feed or formulated diet. The temperature and level of activity are two important factors influencing the rate of depletion of yolk reserves in newly hatched larvae (Quantz 1985; Wang et al. 1987; Rana 1990). Since, in the current study, the temperature was set at a constant temperature, internal factors related to the natural activity of the silver perch larvae is the only main cause of yolk sac absorption. In an aquaculture context, however, the effect of temperature may be sufficiently relevant to regulate it under controlled nursery conditions to allow larval yolk reserves to last longer by setting a lower rearing temperature (Bagarinao 1986).

In general, nearly all freshwater fish species are demersal spawners, providing their eggs and larvae with a relatively more hospitable and predictable environment. Consequently, relative to marine fishes, freshwater fishes are generally less fecund and produce larvae which, at hatching, are larger and suffer less pre-metamorphosis mortality (Houde 1974; Mookerji and Ramakrishna 1999). The mortality of unfed silver perch during the early life history of larvae was significantly affected by the availability of the yolk sac. After the exhaustion of the yolk sac, the larvae may suffer from a nutrient deficiency due to delayed first feeding, and result in significantly increased mortality at 5 dph onward, reaching 50% at 7 dph. This suggests that the availability of the oil globule alone cannot maintain a high survival rate until the end of the yolk-sac stage, but supports fish during the 'mixed feeding period'. At this phase, exogenous feedstuff would help to satisfy the nutritive necessities for larval growth.

In conclusion, at hatching, silver perch larvae from the captive broodfish measured around 2.5 mm in TL and had a large yolk sac which is completely resorbed at 90 hph, whereas the oil globule remained until 240 hph. Even if the onset of feeding occurred at 5 dph, 100% feeding occurred later, at 6 dph. This indicates that silver perch larvae have about 5 days (from 6 to 10 dph) to perform exogenous feeding before the endogenous deposits are totally absorbed. The unfed larvae experienced low mortality (<10%) during the first 96 hph before it increased significantly (more than double) in the next 24 hours. No larvae survived when initial feeding was delayed beyond 240 hph. In addition, although most of eggs and larval performance from domesticated broodfish were inferior compared to the wild one, it has larger oil globule that could make longer of its mixed feeding period and therefore could have better in viability.

CHAPTER 5: The effects of delayed initial feeding on the growth and survival performance of silver perch larvae

The article has been published in AACL Bioflux Journal, 11 (2018):1025-1037

5.1. Introduction

Some fish species have been reported as being more prone to mass mortality in their early life phase than in the advanced developing phase (Dou et al. 2005). Their larval growth and survival are highly dependent on the early-life-history stage, which is extremely critical, especially during the period of transition from the use of endogenous nutritional reserves to exogenous food consumption. The delay in initial feeding after endogenous reserves are exhausted often causes morphological deformities, abnormal swimming behavior, and an inability to catch prey (Gisbert and Williot 1997; Qin et al. 1997; Mookerji and Ramakrishna 1999), leading to high mortality rates (Dou et al. 2005; Kailasam et al. 2007).

The availability of the right food at the right time is crucial as starvation can lead to high mortality (Dou et al. 2002). It has also been described that low larval survival commonly occurred during the initial feeding periods (Bisbal and Bengtson 1995; Yang 2007), which is most affected by a delay in the initial feeding (Dou et al. 2000; Gisbert et al. 2004; Peña and Dumas 2005). In Chinook salmon, (Twibell et al. 2009) and Atlantic salmon (Solberg et al. 2014) suggested that the initial feeding should have started after the swim-up stage. Related to this critical part of aquaculture, the idea of the point of no return (PNR) has been applied to study the effect of starvation on fish larvae mortality (Peña and Dumas 2005; Kailasam et al. 2007; Shan et al. 2008; Jinbo et al. 2013). The PNR is interpreted as the stage where the effects of food shortage become irremediable. Hence, about 50% of starving larvae remain alive, but are powerless to feed even if food is available, thus they cannot pass the ontogenetic stage successfully. The duration between yolk sac exhaustion and the PNR is specific depending on the fish species (Shan et al. 2008), but there is currently no information available for silver perch, *Bidyanus bidyanus*.

Silver perch is a freshwater fish endemic to the Murray-Darling river system in New South Wales, Australia, and is also known as freshwater bream, silver bream, and grunter. Silver perch belong to the Terapontidae family, and occupy the northern and western rivers and upper reaches of the Murray-Darling river system (Rowland 1995). Silver perch have also been introduced in different parts of the world, for example, in Israel in 1997 (Moiseeva 2001), and in Taiwan in the early 1990s (Yang et al. 2006; Yang et al. 2011). The newly hatched silver perch larvae are fragile and weak swimmers, especially during the first three days (Thurstan and Rowland 1994). At this stage, the larvae are light sensitive (Gehrke 1994) and thus light intensity should be kept to a minimum (Thurstan and Rowland 1994). Not only is the availability of high-quality feed vital, but feeding technique is also important for the species being cultured (Twibell et al. 2009). Failure to provide the initial feed results in a lowered survival rate (Foley et al. 2010). Due to the lack of knowledge regarding the silver perch larvae, feeding techniques have generally been developed using and error (Phipps 1999).

The use of earthen ponds for larval production is popular due to their practicality, reliability, and commercial viability (Ogburn et al. 1994). Considerable problems have been identified, however, such as intensive labor, bird predation (Barlow 1994), and vulnerabilities related to exposure to disease organisms, such as the frequently encountered ectoparasitic protozoan, *Trichodina* sp. (Thurstan and Rowland 1994), which causes trichodiniasis (Callinan and Rowland 1994). Due to the disadvantages associated with pond rearing of silver perch larvae, aquaculturists are looking to develop hatchery rearing techniques. Hatchery production of silver perch larvae is possible, but feed and feeding techniques need to be improved (Phipps 1999).

The description of how an initial feeding delay affects the fish larvae conditions under specific culture systems can be a useful tool for assessing the capability of the hatchery settings, and could aid the discovery of ways to prevent death triggered by hunger. The aim of this research was to determine the occurrence of the PNR,

and investigate the larval growth and survival performance of silver perch larvae in laboratory conditions after progressive starvation by an initial feeding delay.

5.2. Material and Methods

The experiments and the procedure in this study were approved by the Animal Ethics Committee of Curtin University (approval number AEC_2011_70) and the Australian Code of Practice for the care and use of animals for scientific purposes.

5.2.1. Experimental larvae

The fish larvae used in this experiment were produced from the domesticated silver perch broodstock that has been reared at the Curtin Aquaculture Research Laboratory (CARL) for around five years. The human chorionic gonadotropin (hCG) hormone was injected into both female (weight 3.5 kg) and male (weight 1.6 kg) broodstock at a dosage of 200 IU/kg of fish to obtain eggs (Rowland 1984; Levavi-Sivan 2004; Rowland 2009). After the hormone injections, paired broodstock were kept in the hatchery within the same tank at a room temperature of 20–26°C until they spawned. After spawning, around 20,000 eggs were transferred to two 200 L conical shape fiberglass incubator tanks filled with filtered fresh water equipped with an aeration from the bottom. The time at which 90% of the viable eggs were hatched was defined as the hatching time (Shan et al. 2008), with a hatching rate of 86.5%. The larval density in the incubation tanks was then estimated with a volumetric system by taking triplicate 10 ml sub-samples (Jensen et al. 2013), which were then stocked into a 200 L cylindrical holding tank at a density of 100 ind./L. The water salinity in the holding tank was increased to 6 ppt at 16 hours post-hatching in order to optimize the survival rates of the larvae (Mather and Capra 1993). The holding tank was equipped with gentle aeration and the temperature was maintained at 23°C. The larvae in this tank were kept unfed until the end of the experiment as a stock larvae for PNR determination, growth experimentation, and survival examination.

5.2.2. Experimental setup

Three set of experiments were presented in this study. The first experiment, PNR determination consisted of eight initial feeding treatments, each with three replicated. The experimental chambers used for this experiment were 1-L volume of glass beakers. The second experiment, the effect of delayed initial feeding on larval development was performed using 20-L plastic tanks. Five treatments of delayed initial feeding of 1, 2, 3, 4, and 5 d, respectively and the control was tested. Three replication for each treatment were applied. In the third experiment, the influence of delayed initial feeding on larval survival was investigated in five treatment (started at 4, 5, 6, 7, and 8 dph) and a control (unfed larvae) each of triplicated. The same container as in the second experiment were used in experiment. Rotifers, *Brachionus calyciflorus* was used as a live food during larval rearing. Aeration was provided to encourage a homogeneous distribution of live food, and also to maintain the dissolved oxygen levels. Prey density was checked daily before feeding to maintain them at the desired level. The water quality parameters, including temperature, pH, and ammonia, were measured and recorded daily. Temperature was recorded by a temperature data logger (Onset HOBO Data Loggers), pH was measured with a pH meter (Cyberscan pH 300, Eutech Instruments, Singapore), and total ammonia was measured with chemical test kits (Aquarium pharmaceutical Inc., Chalfont, PA, USA). More details of experimental setup of each experiment was provided below.

5.2.3. PNR determination

From 4 to 11 dph at 24 h intervals, 20 larvae were randomly taken from the holding tank and moved into three 1 L glass beakers representing triplicated treatment. The beakers were placed in an incubator tank equipped with thermostatic control (Thermomix, B. Braun Biotech International) to maintain the water temperature at around 23°C. A temperature data logger (Onset HOBO Data Loggers) was placed in the incubator tank to record the temperature, and 40W fluorescent light regulated at around 200 lux was used to accommodate the visual feeding behavior of silver

perch (Thurstan and Rowland 1994; Phipps 1999). In addition, every beaker was provided with individual mild aeration. The swimming and feeding behavior of the larvae was then visually observed, and the time when the larvae indicated signs starvation, such as sluggish swimming behaviors and hanging their heads down in the water column, were monitored during 4 h of feeding time.

In order to recognize the presence of prey in their guts, larvae were first placed in the 1-L contained rotifers at a density of 10 ind./ml. After the larvae were exposed to rotifers for 4 h (Dou et al. 2005), all larvae were observed under a stereo microscope to count the number of rotifers in the gut (Wang 2010). This was used to calculate the feeding rate and feeding intensity. The feeding rate, as defined by Dou et al. (2005), is the proportion of larvae that are able to catch food after progressive food shortages imposed on the total number of stocked larvae. The feeding intensity is the number of prey in the gut at dissection. The time when the feeding rate of progressively starved larvae is lower than 50% of the maximum feeding rate when food was supplied is defined as the PNR (Dou et al. 2005). The experiment was continued until all larvae died, or the starving larvae could no longer initiate feeding.

5.2.4. Effect of delayed initial feeding on larval development and growth

Six treatments were applied in triplicate in this experiment. The delayed initial feeding treatment of 1, 2, 3, 4, and 5 d, respectively, was assigned to the first five treatments, while the sixth treatment involved unfed larvae assigned as a control group. At 2 dph, the larvae were randomly taken out from the holding tank and were placed in 20 L experimental tanks at 20 larvae/L of stocking density. The larvae were fed on rotifers at a density of 10 ind./ml. The density of rotifers was maintained throughout the experiment via daily adjustments. From the beginning of the experiment, 10 larvae were periodically and randomly sampled from each experimental unit for larval development and growth examination. The sample was taken every four days at 12:00 during the experiment and anesthetized with AQUIS at 1.0 ppm before being preserved in a 10% formalin buffer and stored at 4°C.

The control treatment lasted up until 11 dph as all larvae died within this period. The other treatments terminated at 20 dph, however.

Four body measurements were used to identify the effect of food deficiency on the general body morphology, i.e total length (TL), eye diameter (ED), head depth (HD), and mouth opening (MO) (Figure 5.1). All measurements are related to larval quality and are commonly used to differentiate larval performance between treatments (Koumoundouros et al. 1995; Gisbert et al. 2002). They were measured vertically or horizontally lateral to the body axis (Gisbert et al. 2004; Kailasam et al. 2007). Measurements were performed to the accuracy of 0.1 mm using a stereoscopic microscope equipped with an Olympus SC30 camera that had image acquisition software getIT from Olympus Soft Imaging Solutions GmbH.

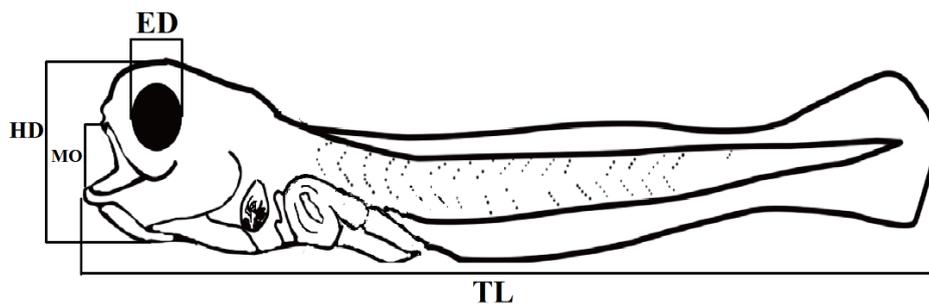


Figure 5. 1. Morphometric measurements of silver perch, *Bidyanus bidyanus* larvae. ED, eye diameter; HD, head depth; TL, total length, and MO, mouth opening.

The morphometric data were processed to examine the effects of late initial feeding on larval growth rate. The specific growth rate (SGR) of the TL at different treatments was calculated by the formula: $SGR = (e^g - 1) \times 100\%$ (Hopkins 1992; Árnason et al. 2009), where $g = [\ln(l_2) - \ln(l_1)] / (t_2 - t_1)$, and l_2 and l_1 are the mean TL on days t_2 and t_1 , respectively. The initial size differences between treatments were minimized so that the average could represent the initial length (L_0) of the individuals at 4 dph. A sample of 10 larvae was taken from the holding tank for the L_0 measurement just before the initial food was offered. The coefficients of variation (CV) of TL were calculated within each treatment with the formula

$CV=100 \times SD / \text{mean TL}$ (SD=standard deviation of mean TL) to find out the effects of delayed initial feeding on the individual growth variation of the larva.

5.2.5. The influence of delayed initial feeding on larval survival

The third experiment was planned to investigate the changes in the survival rate of silver perch larvae exposed to different initial feeding treatments. Silver perch larvae were reared in 20 L plastic tanks at a stocking density of 20 larvae/L and different initial feeding treatments started at 4, 5, 6, 7, and 8 dph. The feeding practice in Experiment 2 was also applied to this experiment. The experiment was conducted in triplicates and lasted after 20 dph. The experimental tanks were siphoned once every day, just before rotifers' density was adjusted to the desired density (10 ind./ml). At the same time, the dead larvae were removed from the tank through siphoning. Based on the daily count of dead larvae, the survival was calculated as the percentage of the alive fish in the tank.

5.2.6. Statistical analysis

The data analysis was performed using the IBM SPSS-24 software and a significance level of $P < 0.05$ was used. One-way ANOVAs, followed by Tukey tests when appropriate, were used to evaluate the differences in the means of the morphometric variables, including TL, HD, ED, MO, and survival rate at different delayed initial feeding times. The data in percentage were arcsine transformed prior to statistical analysis. Results are shown as mean \pm standard error of the mean (SE) where the mean is derived from duplicate groups.

5.3. Results

5.3.1. PNR determination

The eggs were hatched after 35 h of incubation at $21 \pm 1^\circ\text{C}$. The newly hatched larvae had a slim and elongated shape with a large yolk sac ($0.32 \pm 0.02 \text{ mm}^3$) containing an oil globule on the posterior tip, which had drifted frontally as the yolk

sac reduced. Pigmentation appeared as small dots scattered around the body and yolk sac. No eye and mouth openings were observed for the 2.65 mm TL of newly hatched larvae (Figure 5.2a). At 2 dph, the yolk sac reduced extensively and the pigmentation of the eyes was noticed (Figure 5.2b).

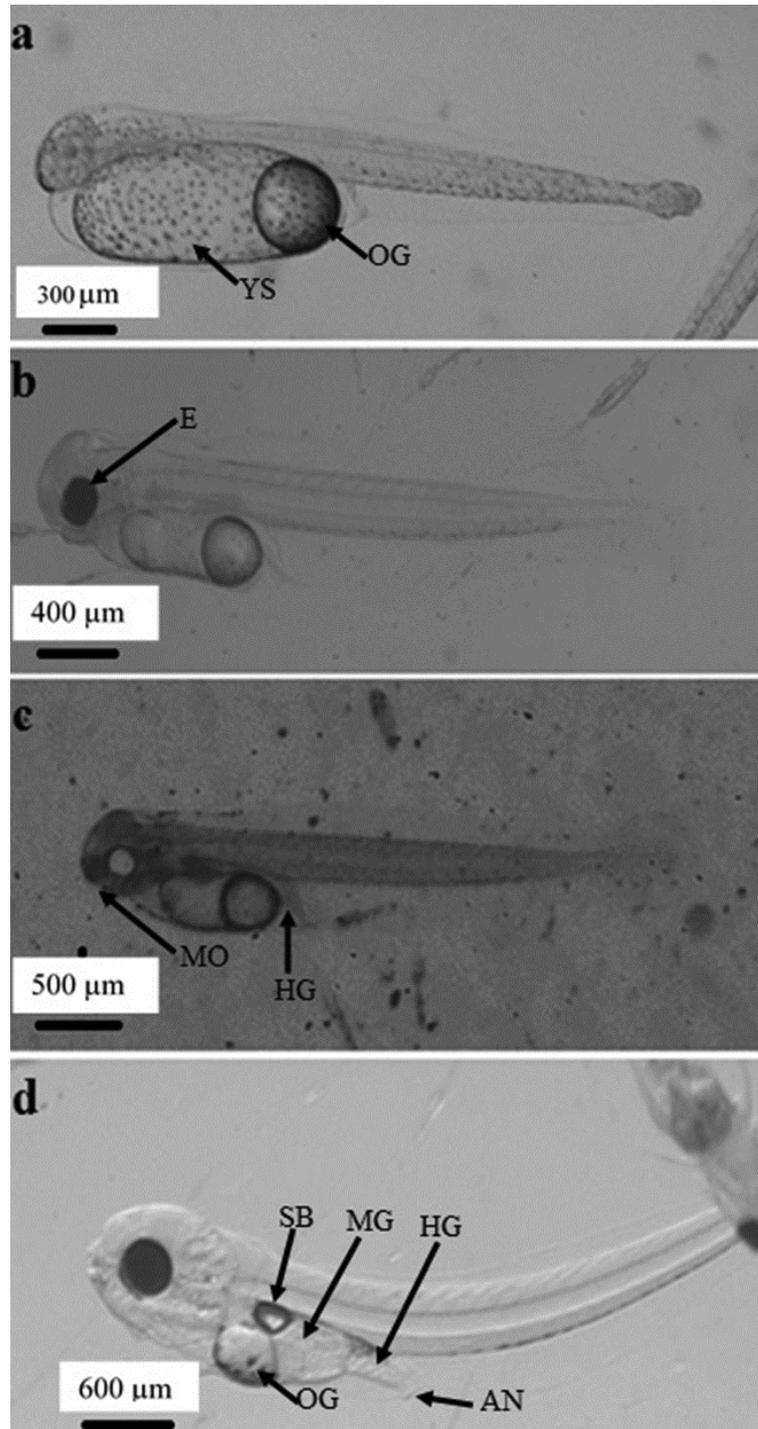


Figure 5. 2. Larval development of silver perch (*Bidyanus bidyanus*): the newly hatched larvae (a); larvae at two dph (b); larvae at 3 dph (c); and larvae at 4 dph (d). OG= oil globule; YS=yolk sac; E= eye, MO= mouth opening; SB= swim bladder; HG= hindgut, MG= midgut; AN= anus.

The distal region of the digestive tract was visible as a tube behind the oil globule. At 3 dph, the yolk reserves were intensively utilized, and the oil globule moved forward. The eye pigmentation appeared to have higher intensity and the mouths started to develop (Figure 5.2c, inverted in color). Yolk sac exhaustion started at 4 dph, and the remaining oil globule was visible.

At this time, the larvae had developed most of their organs, such as eyes, jaws, mouths, swimming bladders, and digestive systems (Figure 5.2d). The onset of the first feed was noticed at 5 dph, however, the maximum feeding rate and feeding intensity occurred at 6 dph. Around $28.3 \pm 1.7\%$ of larvae were able to feed at 5 dph (Figure 5.3), which increased significantly ($P < 0.05$) to $80.0 \pm 0.3\%$ at 6 dph when the maximum feeding rate occurred (Figure 5.3). Beyond 6 dph, the feeding rates dropped down to 53.3 ± 0.1 (higher than 50% of the maximum feeding rate) at 8 dph and $33.0 \pm 0.1\%$ (less than 50% of the maximum feeding rate) at 9 dph. Therefore, the PNR of silver perch was between 8 and 9 dph. This point is shown in Figure 5.3 where the dash line (50% of the highest feeding rate) is intercepted by the line graph of the feeding rate. The feeding rates at 6 and 7 dph showed no significant differences ($P > 0.05$). The symptoms of starvation were noticed at 8 dph and most larvae were starved irreversibly at 9 dph. The feeding rate decreased to only 10% at 10 dph, at which time most larvae were dead. The pH, dissolved oxygen, and ammonia were found to be within the accepted ranges of 7.51 ± 0.01 , 7.45 ± 0.01 mg/L, and 0.00 ± 0.00 mg/L, respectively, over the experimental period (Frances et al. 2000; Foley et al. 2010).

5.3.2. Effect of delayed feeding on larvae growth rate

The growth rate of silver perch larvae was strongly affected by delayed initial feeding. Starved larvae could survive until 10 dph, but the different initial feeding treatments lingered until the end of the experiment at 20 dph. Therefore, the

relationship between delayed initial feeding and larval growth rate at a period of 4–12 dph and 12–20 dph were investigated at 12 and 20 dph respectively (Table 5. 1). The TL and the SGR showed significant differences ($P<0.05$) at both 12 dph and 20 dph for different initial feeding times. The growth rates of 5 and 6 dph initial feeding larvae (SGR=5.19±0.63, TL=6.45±0.25 mm, and SGR=4.85±0.53, TL=6.27±0.29 mm, respectively) were not statistically different ($P>0.05$), but they were significantly higher ($P<0.05$) than unfed larvae (SGR=1.23±0.36, TL=5.11±0.39 mm) at 12 dph. The growth rate of 6 dph initial feeding larvae was also significantly higher than the 8 dph initial feeding larvae (SGR=1.12±0.37, TL=4.99±0.38 mm) at 12 dph (Table 5.1).

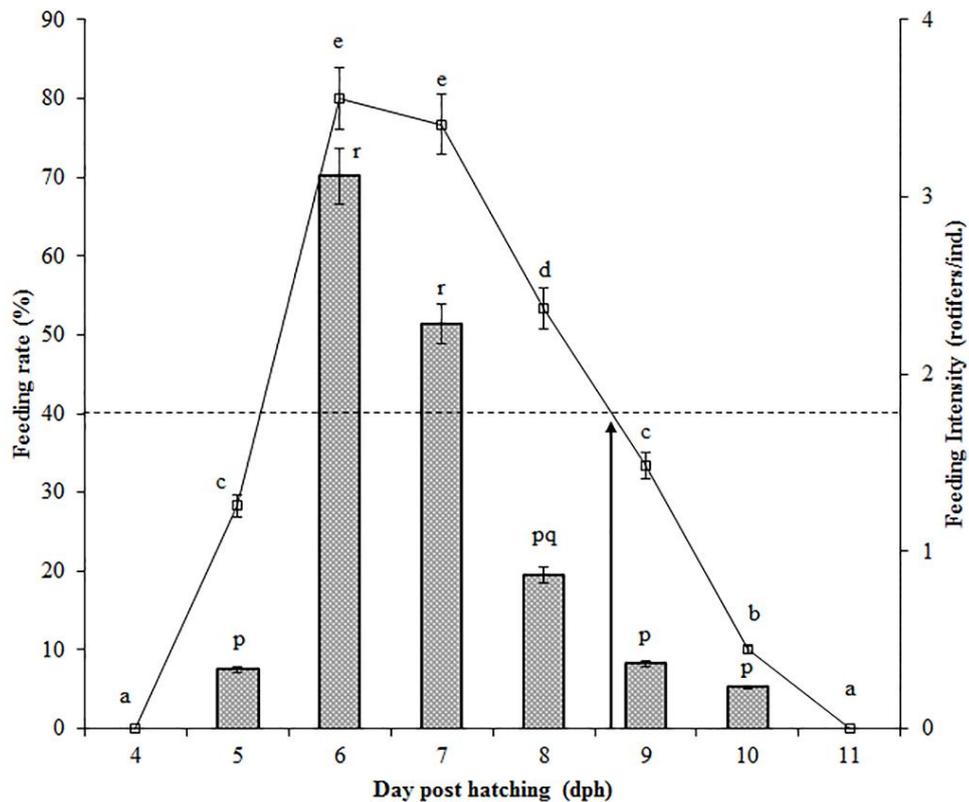


Figure 5. 3. Changes in the initial feeding rate (line graph) and feeding intensity (bar graph) of starved silver perch (*Bidyanus bidyanus*) larvae when firstly presented with food from 4 to 11 dph, the intersection of feeding rate curve and broken line signals the PNR (↑). Points sharing different lower case letters in the same dph for each graph indicated significant differences between treatments ($P<0.05$)

At 20 dph, the growth rate did not show any significant differences ($P>0.05$) between the 4, 5, and 6 dph initial feeding larvae (SGR=5.39±0.24, TL=9.94±0.41 mm; SGR=5.72±0.09, TL=10.45±0.41 mm; and SGR=5.56±0.02, TL=10.19±0.2 mm, respectively), but they were all significantly higher ($P<0.05$) than the 7 and 8 dph initial feeding larvae (SGR=4.59±0.14, TL=8.24±0.37 mm and SGR=3.00±0.18, TL=6.05±0.19 mm, respectively) (Table 5. 1). Whether at 12 dph or at 20 dph, size variation tended to increase with a delay of initial feeding (Table 5.1).

Table 5. 1. Final Total Length (TL, Mean ± SE), Coefficient of variation of Total length and Specific growth rate of total length (SGR, Mean ± SE) for silver perch (*Bidyanus bidyanus*) larvae at a different time of initial feeding.

Initial Feeding	TL (Mean ± SE)	CV	SGR (Mean ± SE)
<i>12 dph</i>			
D4	5.76±0.20a	5.3	4.67±0.32b
D5	6.45±0.25a	6.2	5.19±0.63b
D6	6.27±0.29a	7.9	4.85±0.53b
D7	5.57±0.26a	8.1	2.97±0.66ab
D8	4.99±0.38a	13.3	1.12±0.37a
UF	5.11±0.39a	13.3	1.23±0.36a
<i>20 dph</i>			
D4	9.94±0.41c	3.16	5.39±0.24c
D5	10.45±0.2c	3.32	5.72±0.09c
D6	10.19±0.03c	3.57	5.56±0.02c
D7	8.24±0.37b	7.87	4.59±0.14b
D8	6.05±0.19a	5.71	3.00±0.18a

Note: Different lower case superscript letters in the same column at the same dph indicated significant differences between treatments ($P<0.05$).

The effects of delayed initial feeding time on the measured morphometric of silver perch larvae are presented in Figure 5.4. Significant differences ($P<0.05$) for the

morphometric parameters were noticed in the initial feeding larvae of 4, 5, 6, 7 and 8 dph, which represented 0, 1, 2, 3, and 4 days after yolk sac exhaustion, respectively. No significant differences in TL were identified, however, from 4 to 20 dph between the 4, 5 and 6 dph initial feeding larvae. On the other hand, when the larvae were starved for 3 and 4 days (7 and 8 dph initial feeding), the TL was significantly smaller ($P<0.05$) than the 4, 5 and 6 dph initial feeding larvae at 12, 16, and 20 dph (Figure 5.4a). The TL of the 4, 5, 6 and 7 dph initial feeding larvae gradually grew between 4 and 16 dph ($P>0.0$) before rapidly growing at 20 dph ($P<0.05$). The 8 dph initial feeding treatment showed fluctuating growth patterns between 4 and 20 dph. Changes in HD, ED, and MG all followed a similar pattern to the TL development with the 4, 5, and 6 dph initial feeding larvae being significantly higher in this measurement ($P<0.05$) than the 7 and 8 dph initial feeding larvae at 12, 16, and 20 dph (Figures 5.4b, 5.4c, and 5.4d, respectively). The pH, dissolved oxygen, and ammonia were found to be within the accepted ranges of 7.51 ± 0.01 , 7.45 ± 0.01 mg/L, and 0.00 ± 0.00 mg/L, respectively, over the experimental period (Frances et al. 2000; Foley et al. 2010).

5.3.3. Effect of initial feeding on survival rate

The survival rate of silver perch was affected by different delays in initial feeding (Figure 5.5). After 20 days of the rearing period, the highest survival rate of 50 ± 2.3 % was noted for the 5 dph initial feeding larvae, which was not significantly different ($P>0.05$) to the survival rate of the 4 dph initial feeding larvae. A lower final survival rate was observed when the larvae were exposed to an initial feeding delay of 6, 7, and 8 dph (39%, 20%, and 8%, respectively). Whole mortality of unfed larvae was noted on 10 dph. The survival rate decreased significantly ($P<0.05$) from 84% in the 5 dph initial feeding larvae, to 57% in the unfed larvae at 8 dph (Figure 5.5). The 3 and 4 days delayed initial feeding and unfed larvae had the same survival trends, dropping down rapidly from 5 dph to 8 dph (Figure 5.5). The water quality in all experimental units, including pH, dissolved oxygen, and ammonia, was within the range for optimum growth of silver perch (7.50 ± 0.01 ,

7.44±0.01 mg/L, and 0.00±0.00 mg/L, respectively) over the experimental period (Frances et al. 2000; Foley et al. 2010).

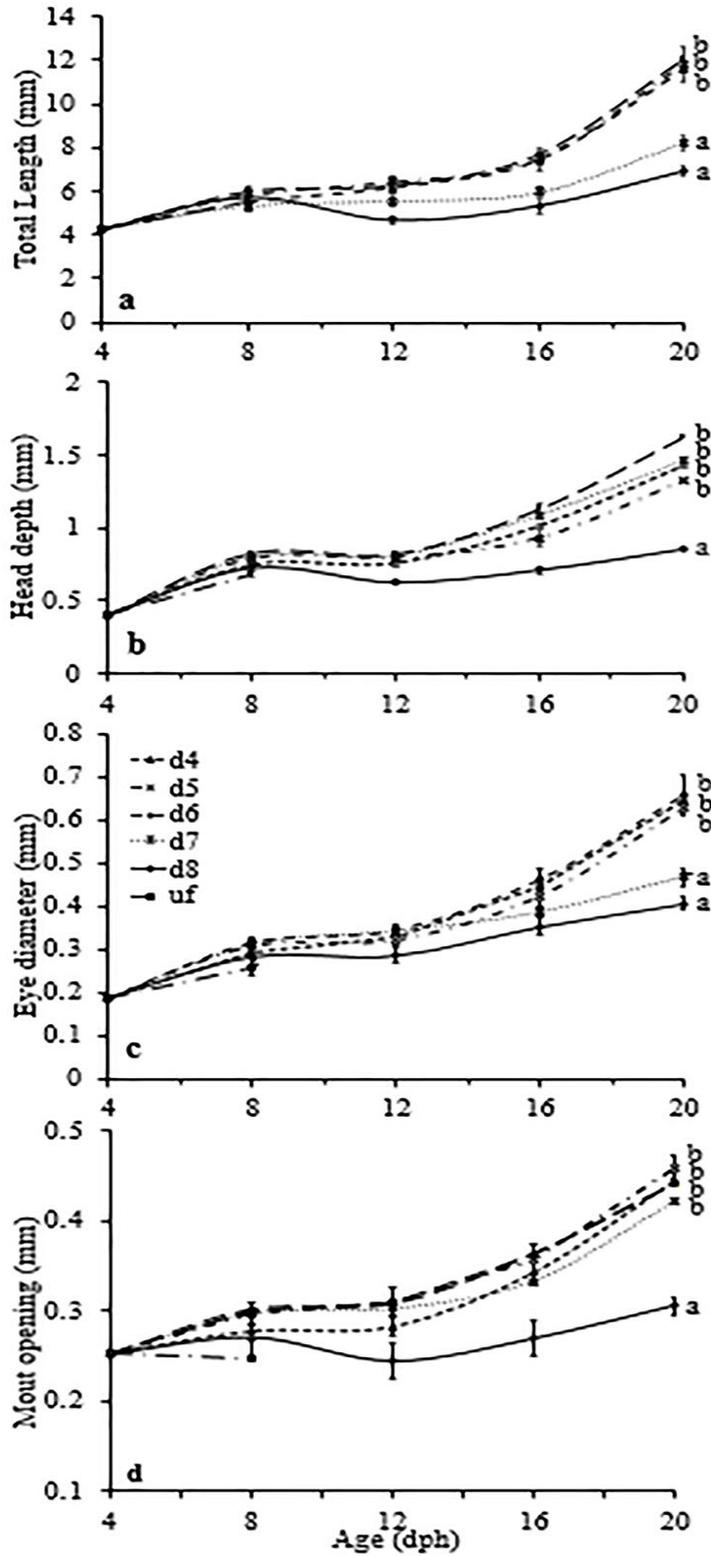


Figure 5. 4. Morphometric changes of silver perch (*Bidyanus bidyanus*) larvae at different initial feeding time of 4, 5, 6, 7 and 8 dph. Points sharing different lowercase letters 20 dph indicated significant differences between treatments ($P<0.05$)

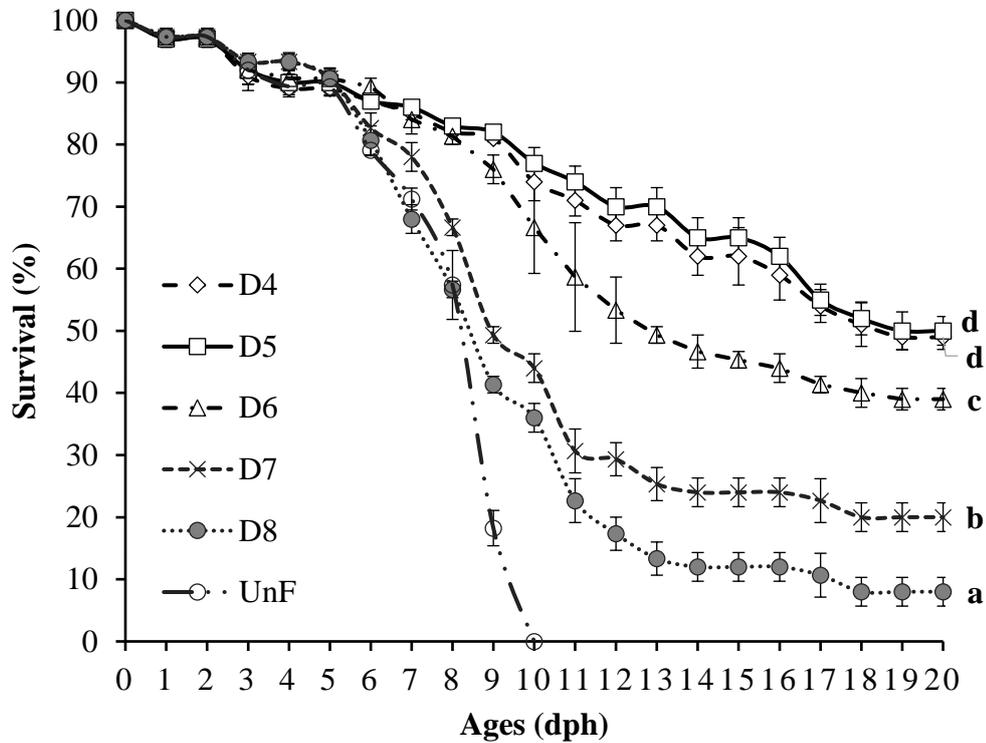


Figure 5. 5. Survival rate of silver perch (*Bidyanus bidyanus*) larvae from 0 to 20 dph at different delayed initial feeding. Different lower case letters at 20 dph indicated significant differences between treatments ($P<0.05$).

5.4. Discussion

The commencement of the initial feeding of fish larvae varies depending on the species (Dou et al. 2005; Yúfera and Darias 2007). Silver perch larvae initiating feeding exogenously at 5 dph was consistent with our previous experiment (unpublished data). The onset of feeding on rotifers was not related to MO, as larvae opened their buccopharynx two days before the first feeding was noticed (3 dph). In the current study, the initial feeding of silver perch corresponded with the change of the larva's behavior from passive to actively swimming, and from sinking to the bottom to maintaining their position in the water column. According to Kamler

(1992), these kinds of changes in fish activity are commonly associated with the depletion of endogenous food and the onset of exogenous feeding. Hence, alterations in the dispersal activities of fish larvae in rearing tanks are often used as visual criteria to start feeding (Shan et al. 2008). Compared to other fresh water fish larvae, silver perch larvae start feeding at a moderate-time (5 dph). Some species start earlier (2-4 dph), while others start later (6-9 dph) as summarized in (Table 5.2).

The duration from yolk depletion to PNR is different from one species to another. In this research, the PNR of silver perch was detected between 8 and 9 dph, or about 4.7 days after the yolk-sac was depleted. This period in other fish species varies from less than one day in sea bream, *Archosargus rhomboidalis*, and lined sole *Achirus lineatus* (Houde 1974), to 6 weeks in Atlantic salmon (Koss and Bromage 1990; Peterson and Martin-Robichaud 1995). Hence, the ability of fish to survive under food shortage conditions is also dependent on the eggs and yolk sac sizes (Gisbert et al. 2000). Large eggs are likely to carry more nutrients, resulting in a bigger yolk sac, which allows a longer time before initial feeding, and lengthens the time required to reach the PNR. This means that the PNR of larger eggs will be relatively longer than smaller eggs. Compared to species with a large egg size such as Siberian sturgeon, *Acipenser baeri* (2.8–4.1 mm), and with a strong starvation resistance (Gisbert et al. 2000), the silver perch larvae in this study had a lower egg size (averaging 2.2 mm).

The initial feeding of fish larvae tends to relate to the development and functionality of different body parts, such as sensory, buccal, and swimming organs. The late development of these organs often causes initial feeding rates in most fish larvae at lower a range of 10–50% (Shan et al. 2008). Based on the onset of feeding and the ability to survive during periods of food shortage, silver perch larvae belong to type ‘A’, where the feeding rate at the initial feeding is low, before sharply improving, then continuing to rapidly decline (Shan et al. 2008). At the beginning, the feeding rate was 28%, which then increased to the highest feeding rate of 80% at 6 dph, and decreased to 35% at 9 dph. The initial feeding time occurred when the swimming capability of the silver perch larvae was still poor, and some structures connected

to food digestion had not yet become fully functional. This was in agreement with the findings regarding other fish species (Dou et al. 2005). On the other hand, the number of larvae involved in feeding activities increased with starvation until 6 dph, indicating that the desire for food enhanced to recompense food constraint during starvation. After 7 dph onward, however, both feeding intensity and feeding rate experienced a significant decrease ($P<0.05$). This may indicate that the digestive system of silver perch can rapidly decline when exposed to starvation. The empty guts of the larvae showed incomplete development with weak pigmentation and were thus transparent in color. The same phenomenon was observed by Shan et al. (2008) in sea bream, where the development of their digestive tracts stops and the intestine becomes pale after starvation.

Table 5. 2. Average temperature (T), time from hatching to first feeding (tf), time from hatching to yolk absorption (yolk sac + oil globule) (ty), time from hatching to PNR (PNR), and time from yolk absorption to PNR (ty-PNR) of some fresh water fish.

Species	T (°C)	tf (d)	ty (d)	PNR (d)	Ty-PNR (d)	Sources
Tilapia, <i>Oreochromis mossambicus</i>	28.0	6.0–7.0	15.0	16.0–19.5	1.0–4.5	(Rana 1985)
Singhi, <i>Heteropneustes fossilis</i>	26	3.0	6.0	8.0	2.0	(Mookerji and Ramakrishna 1999)
Rohu, <i>Labeo rohita</i>	26.0	2.0	9.0	14.0	5.0	(Mookerji and Ramakrishna 1999)
Carp, <i>Aspidoparia morar</i>	14.0	4	9.0	14.0	5.0	(Malhotra and Munshi 1985)
Siberian sturgeon, <i>Acipenser baeri</i>	18	9	10	-	-	(Gisbert and Williot 1997)
Chinese sturgeon, <i>Acipenser sinensis</i>	22-24	8	10	15	5	(Chai et al. 2011)
Loach, <i>Misgurnus anguillicaudatus</i>	23	3	6	9-10	3-4	(Wang 2010)
<i>Aspidoparia morar</i>	13-15	7	9	14	5	(Malhotra and Munshi 1985)
<i>Burbot, Lota lota</i>	12	5	14	10	5	(Palińska-Żarska et al. 2014)

Silver perch, <i>Bidyanus bidyanus</i>	21	5	10	8-9	4-5	This study
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The increase in the length of silver perch larvae was significantly affected by the delayed initial feeding. Even if the mean size of the larvae displayed an increase at a shorter delay of initial feeding, it caused negative growth at a longer delay of initial feeding. The negative effect of delayed initial feeding was clearly shown in TL and SGR. When the initial feeding was delayed for 4 days, the TL and SGR of the silver perch larvae were significantly lower than at 0 and 1 day initial feeding delays at 12, 16, and 20 dph. Hence, negative growth was most likely related to the fact that larvae in this treatment could not procure exogenous feeding. On the other hand, the TL and SGR did not differ significantly between un-delayed initial feeding larvae and 1day delayed initial feeding larvae. The negative effects on larval growth after the delayed initial feeding (the time threshold) was observed at 2 days before the PNR at 23°C. The delayed initial feeding of the silver perch larvae also lead to an increase of TL variation. The longer the delay of initial feeding, the larger the CV values observed. The same condition has also been observed in other fish, such as Chinese sturgeon, *Acipenser sinensis* (Chai et al. 2011), summer flounder, *Paralichthys dentatus* (Bisbal and Bengtson 1995), and rock bream, *Oplegnathus fasciatus* (Shan et al. 2008). The size variation here is probably promoted by the differences in individual feeding ability and the behavior of the larvae after being exposed to different initial feeding delays. The energy needed to maintain life and the allocated energy for growth as well as for feeding activity could vary greatly, resulting in high individual variability in size. The longer the initial feeding delay, the more energy spent on the life maintenance of the starving larvae, and the higher the individual variation.

The delayed initial feeding was not only important in influencing the growth performance of the larvae but also contributed significantly to the survival rate (Houde 1974). The best survival rate at the end of this research was observed for un-delayed initial feeding, and at initial feeding delayed by 1 day, which was higher compared to the survival rates at 2, 3, and 4 days. All larvae were dead at 6 days of delayed feeding at 10 dph. This was comparable to another freshwater fish, the

loach, *Misgurnus anguillicaudatus*, where the survival rate at 3 dph initial feeding was higher than at initial feeding of 4, 5, and 6 dph (Wang 2010). The delayed initial feeding approaching the PNR may alter the feeding ability more seriously, which is generally accompanied by high mortality as a result of decreased growth (Abi-ayad et al. 2000). Hence, the reduction in larval sizes is conclusive evidence that starved survivors have done so at the expense of tissue formation (Dou et al. 2005). Even if whole larval death was noticed at 10 dph in this study, the growth and survival rates were very poor for the 7 and 8 dph initial feeding larvae. Based on the survival and growth rate performance of the silver perch larvae, it is suggested that a delay of initial feeding can be tolerated for 2 days, or as late as 6 dph.

In conclusion, the beginning of exogenous feeding in the silver perch larvae occurred at 5 dph, one day later than the yolk sac exhaustion. The elapsed time from the beginning of initial feeding to the PNR was around 3 days, placing it between 8 and 9 dph. Larval growth and their survival rate were directly associated with the time of initial feeding. Findings indicated that 5 dph initial feeding gives the best growth and survival rate performance. Therefore, an initial feeding of 5 dph is suggested for the hatchery production of silver perch larvae.

CHAPTER 6. Effect of dietary estradiol-17 β on the feminization of silver perch

The article has been published in Journal of the World Aquaculture Society 48 (3):444-452. doi: 10.1111/jwas.12352

6.1. Introduction

Techniques for governing sex differentiation in the silver perch, *Bidyanus bidyanus*, are expected to provide new choices in aquaculture. The occurrence of precocious male gonadal development in silver perch (Gordon 1995) as in many other cultured species (Longalong et al. 1999; McClure et al. 2007; Shewmon et al. 2007) results in males reaching sexual maturity when they are of small size and lower economic value than normal males (Johnstone et al. 1978; Beardmore et al. 2001). Silver perch also have significant size variability (Gordon 1995; Moiseeva 2001), which poses management problems during their culture (Barki et al. 2000). Monosex culture can resolve these issues as for many other aquaculture species (Dan and Little 2000; Beardmore et al. 2001; Devlin and Nagahama 2002; Cnaani and Levavi-Sivan 2009). Other benefits of monosex cultivation include the ability for controlled reproduction, improved growth, reduced variation in harvest size, and reduced environmental risk of escapees that could breed in the wild (Beardmore et al. 2001; Phillay and Kutty 2005).

The growth performance of all-female population in many cultured aquatic species have been proved more viable compared to all-male or mixed-sex culture (Goudie et al. 1994; Pongthana et al. 1999; Sheehan et al. 1999; Nam et al. 2001). The biomass of all-female population of mirror carp, *Cyprinus carpio* and common carp, *Cyprinus carpio* were approximately 30% and 8% higher compared to mixed-sex population respectively (Kocour et al. 2005). Production of higher-yielding all-female population has also been suggested for Eurasian perch (Rougeot et al. 2004). However, there are no research regarding monosex culture of silver perch.

All-male or all-female germ cells of many cultured species can be produced by hormonal manipulation (Yamazaki 1983; Domagala et al. 2015). Methods of estrogen administration include injection (Yamazaki 1983; Devlin and Nagahama 2002), immersion, and oral administration through food (Hunter et al. 1986; Purwati et al. 2004; Putra 2011; Ansai et al. 2012) has been commonly applied. Hormone treatment is applied prior to sex differentiation at stages including pre-hatching (Beardmore et al. 2001), post-hatching, and before or after exogenous feeding (Yamazaki 1983). Despite the usefulness of steroids for fish sex reversal, its application in fish for consumption purposes have been questioned by the scientific community on their relation to environmental hazards and human safety. However, the degree of contamination of steroids and their metabolites have been reported to be minimal as steroid-treated fish is reported tendency for high clearance from blood plasma. Steroid can be rapidly metabolized in the liver and kidney and cleared from blood plasma with a short half-life (Mlalila et al. 2015). Therefore the application of steroid at low dosage and short period of time should not be expected to leave any hazard for human consumption.

Sex reversal has been achieved in many gonochoristic species (Yamazaki 1983; Domagala et al. 2015), in contrast to hermaphroditic species (Beardmore et al. 2001). The first step in developing a suitable method for producing monosex progeny is to identify the critical period in which the gonads are sensitive to hormonal treatment (Blázquez et al. 2001; Devlin and Nagahama 2002; Haugen et al. 2011). After this period is determined, monosex or sterile populations can be produced by administering hormones at a known dose (Hunter et al. 1986). However, few data are available on the timing and histological processes of protandrous hermaphroditic fish, especially silver perch, except for a brief description by Moiseeva (2001). Sex reversal can be more complicated in hermaphroditic species because sex genes may remain active until later stages of gonadal development (Yamazaki 1983). In silver perch, the gonads are anatomically formed in 30-day fry but cytological differentiation occurs only in 2-month-old fry (Moiseeva 2001). Therefore, we introduced hormonal treatment for sex reversal before 60 dph. The aim of this study was to evaluate the efficacy of

artificial diets supplemented with estradiol-17 β to produce a predominantly female population of silver perch.

6.2. Material and Methods

This experiment was approved by the Animal Ethics Committee of Curtin University and performed according to the Australian Code of Practice for the care and use of animals for scientific purposes.

6.2.1. Preparation of test diets

The supplementation levels (0, 60, and 120 mg/kg diet) of estradiol-17 β (E2; Sigma no. E-1024) used in this experiment were based on levels used in other cultured species (Pandian and Sheela 1995; Strüssmann et al. 1996; Ansai et al. 2012; Hamdoon 2013). The alcohol saturation (Hendry et al. 2003) and evaporation method (Rougeot et al. 2002; Hendry et al. 2003; Ansai et al. 2012) was used to incorporate E2 into manufactured feed. First, the E2 was dissolved in 95% ethanol (1 mg/L) to prepare a stock solution. Commercial feed for fish larvae (20 mg; spectrum micron diets, NRD[®] 0.4–0.6 mm; protein: 55%, crude fat: 9%, fiber: 1.9%; INVE-Thailand) was then placed evenly on Petri dishes and saturated with 50 mL of E2–ethanol solution. The final concentrations of 60 and 120 mg E2/kg diet required 1.2 and 2.4 mL of 1 g/L E2, respectively. The control (Ctrl) feed was saturated with ethanol only. The prepared diets were placed in a fume hood overnight to dry. All diets were stored at 4°C until further use.

6.2.2. Experimental fish and setup

Silver perch broodstock maintained in a 10-ton fiberglass tank for approximately six years at the Curtin Aquaculture Research Laboratory (CARL), Western Australia, were used to produce fry. Eggs were collected after human chorionic gonadotropin (hCG) hormone was injected into male and female broodstock at a dose of 200 IU/kg fish (Rowland 1984; Levavi-Sivan 2004; Rowland 2009).

Following hormone injection, broodstock were maintained in the same tank at room temperature (20–26°C) until they spawned.

After spawning, approximately 10,000 eggs were transferred into a 200-L conical incubator tank filled with fresh water with a flow-through rate of 500 mL/min. Salinity in the incubator tank was increased to 6 g/L at 16 hours post-hatching to optimize larval survival (Mather and Capra 1993) and to enable the use of rotifers and *Artemia* as live food. After newly hatched larvae separated from their eggshells, they were transferred from the incubator to nursery tanks.

Three 300-L circular tanks, each with 1000 larvae, were used as nurseries. The saline water (6 g/L) was gently aerated, and the temperature was maintained at 20 \pm 1.0°C. During the first two weeks, all larvae were fed exclusively with live feed (marine rotifers and *Artemia* nauplii). From 15 dph, the live feed was replaced by a dried formulated diet (NRD 2/4, 200–400 μ m). However, the larvae were occasionally supplemented with *Artemia* nauplii until 25 dph.

At 26 dph, haphazardly chosen larvae were acclimated to nine 10-L experimental glass aquaria containing filtered fresh water (Aqua-pure Model-AP12S, 5 microns) at a stocking rate of 50 larvae per aquarium. All aquaria were placed in an incubator bath equipped with a heater (stirred thermostatic circulator, Model-GD120) to maintain the water temperature at approximately 23°C. Each aquarium was wrapped with black plastic sheeting to minimize light penetration and equipped with one air stone placed in the middle. Uneaten food and faeces were siphoned out before feeding and water exchange at a rate of 30% per day. A temperature data logger (Onset HOBO) was placed in the incubator tub to record the temperature during the experiment. During the four-day acclimation period, dead larvae were replaced with new larvae obtained from the same broodstock. From 26 to 30 dph, only NRD 2/4 feed was provided.

At 31 dph, the three E2-supplemented diets were assigned in silver perch larvae using nine prepared aquaria, where each treatment had three replications. The diets

were delivered manually until satiation three times per day during daylight hours. The treatments were applied for 30 days, from 31 to 60 dph, after which all fish were fed a normal diet.

At 75 dph, all fish from each group was transferred to a separate 200-L tank to keep treatments and replicates separated. The tanks were designed as a flow-through system (water flow rate of 0.2 L/min) and equipped with one heater (Sonpar, Model-Ha200) and an air stone. The fish were maintained on a normal artificial diet (NRD G8, 0.8 mm) until termination of the experiment (225 dph). Temperature and oxygen concentration were controlled daily and maintained at $23 \pm 2^\circ\text{C}$ and 7 ± 1 mg/L, respectively. The experimental fish were reared beyond the termination of the oral E2 treatment to confirm the permanent effects of hormone treatment on sexual differentiation and to facilitate sex identification.

6.2.3. Sampling and parameter measurement/calculation

Fish body weight (BW, precision 0.01 g) was measured on day 1 and at 75, 105, 135, 165, 195, and 225 dph; total length (TL, precision 1 mm) was measured at the beginning and end of the experiment only. At each sampling, 10 fish from each tank were anesthetized using AQUI-S (0.025 mg/L) before weighing, and total length was individually measured. At the end of the experiment, all fish were euthanized with AQUI-S (100 mg/L) before the gonads were excised and visually identified as ovaries or testes. Gonad weight (GW, precision 0.0001 g) and length (GL, precision 0.01 mm) were measured, and percent survival was calculated. At the same time, visual identification of fish sex was confirmed using the standard gonadal squash technique (Guerrero III and Shelton 1974; Conover and Fleisher 1986). The gonadosomatic index (GSI) was determined according to the formula suggested by Razak et al. (1999): $\text{GSI} = (\text{GW}/\text{BW}) \times 100$, where GW and BW are in grams. Specific growth rate was calculated using the formula: $\text{SGR} = (e^g - 1) \times 100\%$ (Hopkins 1992; Árnason et al. 2009), where $g = [\ln(w_2) - \ln(w_1)] / (t_2 - t_1)$, and w_2 and w_1 are the mean weights on day t_2 and t_1 , respectively.

6.2.4. Histology

The gonads were sliced and fixed in 40% buffered formalin solution (BFS) (Luna 1968) for histological examination. After washing in running water, the samples were dehydrated in an ascending series of ethanol (70%, 80%, 90% and absolute ethanol) and clarified with xylene. The gonads of each fish embedded in paraffin blocks were trimmed and 7 μ m thick sections taken were stained with Eirlich hematoxylin and eosin. Slides were photographed under a microscope using an Olympus SC30 camera and Olympus GetIt software. Early stages of gonadal development were compared to descriptions published by Beams (1973), Morrison (1990), Ansai et al. (2012), Maack, Segner (2003), Almeida et al. (2008), and Lubzens et al. (2010).

6.2.5. Statistical analyzes

The effects of different E2 treatments on the sex ratio, survival rate, weight growth, gonad weight, gonad length, GSI, and SGR were analyzed using one-way ANOVA. Data expressed as percentages were arcsine-transformed before the analysis of variance. If the ANOVA revealed significant differences, Tukey's post-hoc test was performed (significance level, $\alpha = 0.05$). The data analyzes were performed in IBM SPSS Statistics 22; data were expressed as the mean \pm SE where the mean is derived from duplicate groups.

6.3. Results

Survival in all treatment groups at the end of the experiment ranged from 38% to 46% (Table 6.1); most mortality occurred during the early feeding period. The survival of the silver perch was independent of dietary E2 dosage.

Morphological examination of gonads, wet-squash and histological methods yielded similar results for sex identification. Visual differences between ovaries and testes are illustrated in Figure 6.1. Gonads in females with a pair of ovaries and in

males with a pair of testes had similar transparency or whiteness. Ovaries were rounded, and testes were triangular in cross-sections. The differences were clearer in gonadal squashes (Figure 6.1B and E). Ovaries showed the presence of oocytes with lightly stained nuclei surrounded by darker cytoplasm (Figure 6.1B). Testicular tissue and their stages was more difficult to identify, but male gametes were usually seen in the lobule tissue (Figure 6.1E).

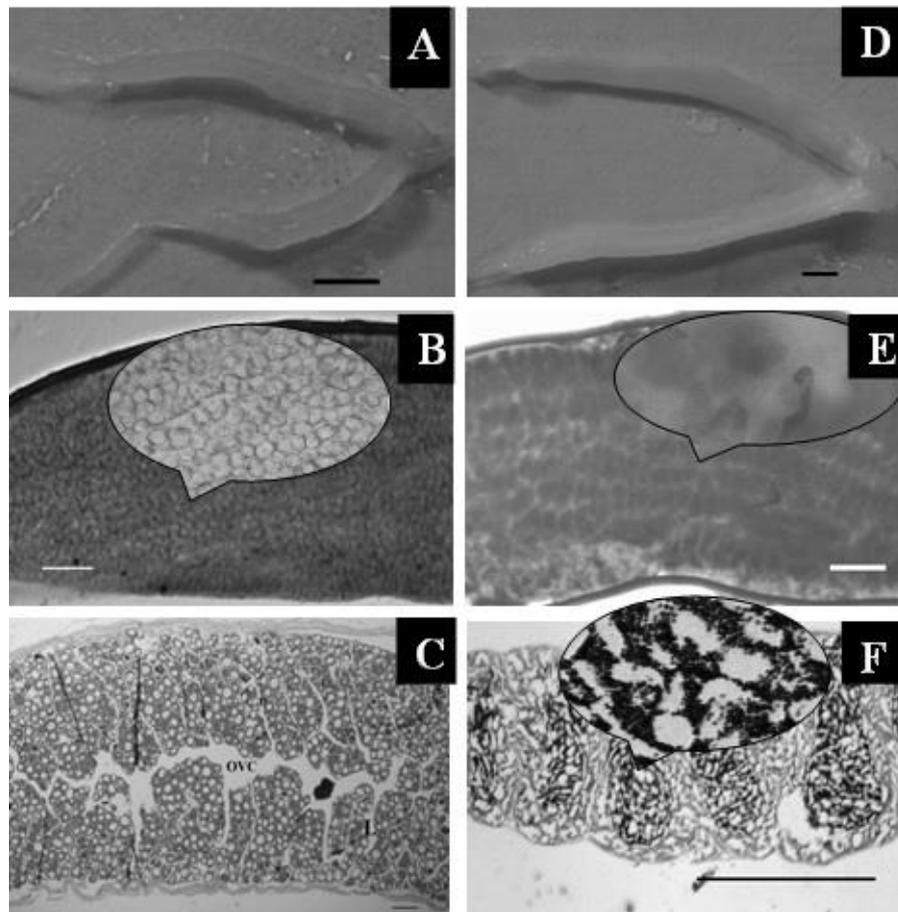


Figure 6. 1. Sex identification in juvenile silver perch (*Bidyanus bidyanus*) at 225 dph. (A) Photomacrograph of an ovary showing pale white color and rounded cross-section; scale bar = 10 mm. (B) Wet-squash preparations of an ovary with previtellogenic oocytes; scale bar = 200 μ m. (C) Histological section of an ovary with oocytes located in the ovarian lamellae; scale bar = 100 μ m. (D) Photomacrograph of testis showing color similar to that of the ovary but with a triangular cross-section. (E) Wet-squash of testis showing typical testicular tubules; scale bar = 200 μ m (F) Histological section of testis showing spermatogonia in the testicular tubules; scale bar = 20 μ m.

Histological analysis of all treatments showed that females had immature ovaries containing oocytes in the perinucleolar stage (Figure 6.1C). The testes (Figure 6.1F) consisted of testicular lobules with spermatogonia, spermatocytes, and spermatids. All ovaries showed the ovarian lamellae projecting into the lumen, whereas testes showed cyst formation in the testicular tubules. The ovaries of all female fish in E2-fed treatment groups were well developed, and their developmental stages and structures were similar to those of the Ctrl groups. However, fish fed the 60 mg E2/kg diet developed smaller gonads than with the control diet. Gonad weights were similar, but the gonad length of fish fed the 60 mg E2/kg diet was shorter than that of the control groups.

The mean percentage of females that resulted from the 60 mg E2/kg diet was not significantly different ($P > 0.05$) from that of the 120 mg E2/kg diet, and the percentage of females with both diets was significantly higher ($P < 0.05$) than that of females in the Ctrl populations (Figure 6.2).

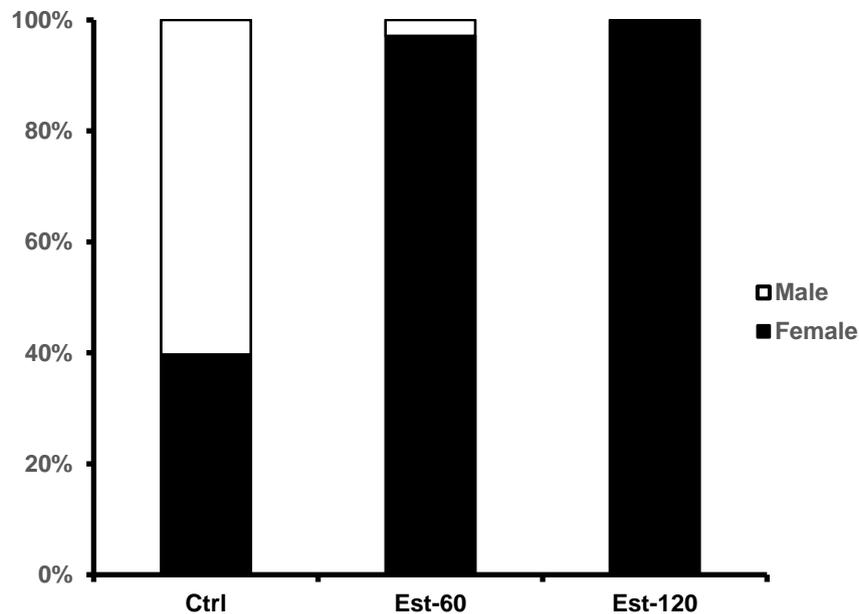


Figure 6. 2. Proportion of males and females of silver perch (*Bidyanus bidyanus*) at the end of the experiment. Ctrl, provided feed saturated with ethanol only; E2-60, estradiol-17 β fed at 60 mg/kg diet; E2-120, estradiol-17 β fed at 120 mg/kg diet.

Fish weight increased steadily after slow growth during the first two and a half months (Figure 6. 3), but there were no significant differences in mean weight ($P > 0.05$) at 75 dph or two weeks after hormone treatment ended. However, at the end of the experiment (225 dph), the total weight gain of fish fed 120 mg E2/kg feed was significantly ($P < 0.05$) higher than that of fish fed 60 mg E2/kg feed and that of the control groups (Figure 6.3).

The daily increases in body weight with all treatments are given in Table 6.1. No significant differences ($P > 0.05$) in SGR were found among the groups at 75 dph; however, at 225 dph (165 days after fish were fed 120 mg E2/kg feed for 30 days), fish grew faster than in the Ctrl groups (Figure 6.3). Gonadosomatic index did not differ significantly ($P > 0.05$) among the feeding groups.

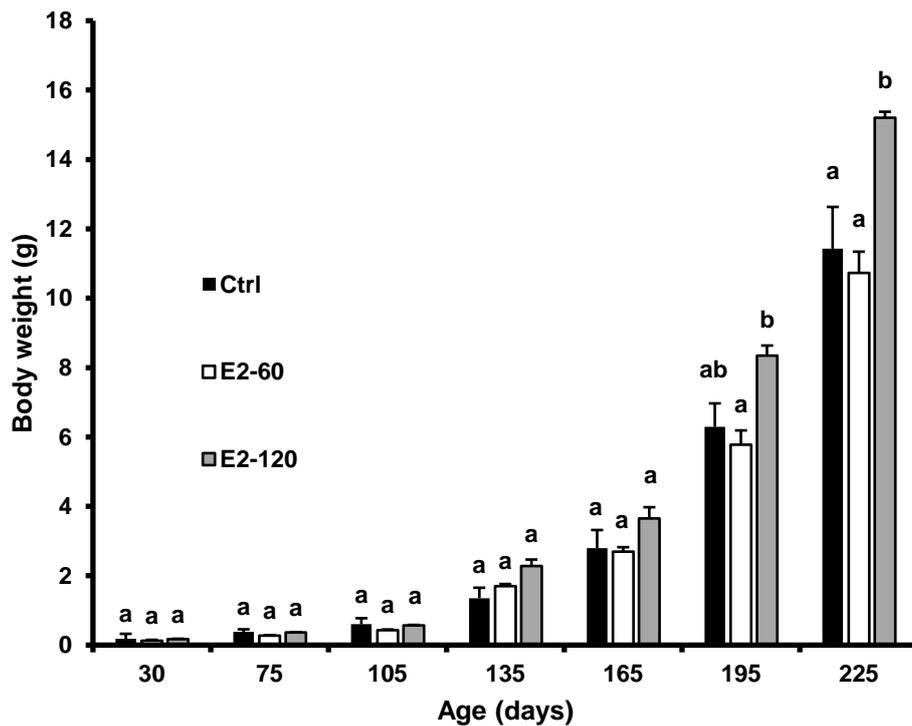


Figure 6. 3. Increase in body weight of juvenile silver perch (*Bidyanus bidyanus*) treated with estradiol. Bars indicate SE. E2-60, estradiol-17 β fed at 60 mg/kg diet; E2-120, estradiol-17 β fed at mg/kg diet; Ctrl, provided feed saturated with ethanol only. Sample sizes were $n = 7-12$ for each replicate, and different letters for each age indicated that the means are significantly different at the $P < 0.05$ level.

6.4. Discussion

The survival range observed here is in the range of other species in which E2 was used. For example, survival rates of 17–76% for European perch, *Perca fluviatilis* (Rougeot et al. 2002), 4–50% for Atlantic halibut, *Hippoglossus hippoglossus* (Hendry et al. 2003), and 17–50% for rainbow trout, *Salmo gairdneri* (Johnstone et al. 1978; Herman and Kincaid 1988) have been reported. Survival was not affected by E2 supplementation in any of these studies.

Table 6. 1. Effects of hormonal (E2) treatments on survival, growth, and gonadal development in juvenile silver perch, *Bidyanus bidyanus*.

Parameter	Treatment		
	Ctrl	60 mg/kg diet	120 mg/kg diet
Initial weight (g)	0.16	0.16	0.16
Survival rate (%)	38.33 \pm 3.33 ^a	44.29 \pm 2.97 ^a	45.95 \pm 2.12 ^a
Weight at 75 dph (g)	1.94 \pm 0.39 ^a	1.24 \pm 0.11 ^a	1.87 \pm 0.64 ^a
Weight at 225 dph (g)	11.18 \pm 1.20 ^a	11.34 \pm 0.62 ^a	15.20 \pm 0.17 ^b
Gonad weight (g)	0.02 \pm 0.02 ^a	0.028 \pm 0.00 ^a	0.02 \pm 0.00 ^a
Gonad length (mm)	18.55 \pm 2.52 ^b	10.11 \pm 0.43 ^a	12.60 \pm 1.56 ^{ab}
GSI (%)	0.26 \pm 0.10 ^a	0.12 \pm 0.02 ^a	0.14 \pm 0.03 ^a
SGR at 75 dph (%)	1.94 \pm 0.39 ^a	1.24 \pm 0.11 ^a	1.87 \pm 0.64 ^a
SGR at 225 dph (%)	2.20 \pm 0.06 ^a	2.22 \pm 0.03 ^{ab}	2.37 \pm 0.01 ^b
Female portion (%)	39.69 \pm 2.77 ^a	97.22 \pm 3.17 ^b	100.00 \pm 0.00 ^b

Note: Means with different superscripts in the same row are significantly different at $P < 0.05$ (Tukey test). GSI, gonadosomatic index; SGR, specific growth rate (n=3).

In *H. hippoglossus* (Hendry et al., 2003), mortality occurred at the beginning of the hormonal treatment, similar to our findings. Similarly, no correlation was found between E2 treatment and mortality in rainbow trout (Johnstone et al. 1978). Navarro-Martín et al. (2009), also reported that the use of E2 for feminization did not influence the survival of the European sea bass, *Dicentrarchus labrax*. Even though all survival rates in this study were not significantly different among treatments, the lower survival rate in the controls means that these tanks must have had a lower stocking biomass per tank than the E2 groups. However, the low

stocking density applied in this study (20-30 fish juvenile/200 L) would not affect the growth data at the end of experiment.

The suppression of reproduction or production of mono-sex species in fish is one method for channeling additional energy into growth. The present study demonstrated that male silver perch could easily be converted into females. The application of E2 at 120 mg/kg diet for 30 days from 31 to 60 dph produced 100% females showing that sex differentiation in silver perch is highly responsive to dietary supplementation of exogenous sex steroids during this period. When the lower dosage of 60 mg E2/kg diet was used, the proportion of females was still high (97%, more than double that of the control groups) and did not differ significantly from that of fish fed the 120 mg E2/kg diet. This result is in agreement with findings for other fish species in which complete feminization was observed after feeding of E2 (Gimeno et al. 1998; Blázquez et al. 2001). However, the average gonadal length of fish fed the 60 mg E2/kg diet was shorter than that of the control group. This deviation may have been a result of the low E2 dosage or of the relatively shorter treatment duration that led to incomplete gonadal development. Because the gonad weight remained similar, the reduced gonad length may not affect functionality. This was also in agreement with the GSI data, which did not differ between E2-fed fish and controls. Morphological and histological differences in sex-reversed gonads have been reported in rainbow trout (Krisfalasi and Cloud 1996) and Atlantic cod, *Gadus morhua* (Haugen et al. 2011). Such differences included smaller ovaries with fewer oocytes, gonads exhibiting both sexual characteristics, and gonadal shapes that differed from those of the controls (Krisfalasi and Cloud 1996). In contrast, Yamazaki (1983) showed no abnormalities in feminization of medaka that received dietary estrogen for eight months.

There were no histological abnormalities in female ovaries of control and E2-fed fish at 225 dph, suggesting that sex-reversed genotypic males (neo-females) may be able to produce viable ova. Similarly, functionally female medaka reversed from genotypic males were previously shown to produce fertilizable and viable eggs (Yamamoto 1953).

Previous studies of other species showed suppressed growth after feeding of E2, in contrast to this study where E2 did not lead to changes in SGR at 75 dph or after 15 days of feeding. Feeding of E2 has been shown to result in slower growth in the Atlantic halibut (Hendry et al. 2003), rainbow trout, and Atlantic salmon (Johnstone et al. 1979). However, at 225 dph, silver perch fed 120 mg E2/kg feed grew faster than those fed the 60 mg E2/kg diet. The reasons for the enhanced growth performance remain unclear but may have resulted from the direct feeding of E2 or could have been intrinsic, as sexual growth dimorphism has been reported in different species (Craig 2000). In the yellow perch, *Perca flavescens*, for instance, sexually related dimorphic growth appears during early life stages (110-mm length) and is correlated with the onset of vitellogenesis and spermatogenesis (Malison et al. 1986). In contrast, growth advantages of dietary E2 have also been reported in teleosts and were attributed to increased appetite and enhanced food utilization and protein synthesis (Kocour et al. 2005). Thus, the response to a given steroid hormone is species-specific.

Estradiol-17 β works well to produce all-female populations of silver perch when administered to larvae before sex differentiation at two months (Moiseeva 2001). Gonadal sensitivity to dietary estrogens in 31–60 dph fish larvae corresponds to a body weight between 0.15 and 0.34 g and a total length between 21 and 27 mm. Because this critical period occurs immediately after early weaning onto artificial dry feeds (Phipps 1999), E2 can easily be applied by mixing with the diet. Sex steroids used at the critical time can influence gonad development and overrule genetic sex determination (Devlin and Nagahama 2002). The suggested critical period derived from our results is within the range of critical periods reported for other species. For example, the critical period in Nile tilapia, *Tilapia niloticus* occurs in the early larval stage at 7–14 dph (Kwon et al. 2000), whereas in the European sea bass (Blázquez et al. 2001; Navarro-Martín et al. 2009), the critical period occurs during the late juvenile stage, at 96–126 days post fertilization.

Based on the results presented here, we concluded that dietary supplementation of E2 at 60–120 mg/kg diet could be used to establish all-female populations of silver perch.

CHAPTER 7. Masculinization of silver perch by dietary supplementation of 17 α -methyltestosterone

The article has been published in Egyptian Journal of Aquatic Research
43 (2017): 109–116. <http://dx.doi.org/10.1016/j.ejar.2016.10.002>

7.1. Introduction

The protandrous hermaphrodite silver perch (*Bidyanus bidyanus*, Mitchell 1838) is recognized as an important cultivated species in Australia. Faster growth is an important factor for profitable aquaculture. Several ways have been tried to increase silver perch aquaculture production but stunted growth and precocious maturation has halted the anticipated productivity (Gordon 1995). Silver perch exhibits sexual dimorphism, where male smaller than female (Mallen-Cooper 2003). Most silver perch males induce maturation at two years of age and thus divert their energy resources into gonadal development. On the other hand, females still use the resources for somatic growth and mature at three years, when they are just approaching marketable size, at 1 kg (Rowland 2004a). Hence, females have one additional year than males in which they can divert their energy resources into somatic growth. Consequently, since females do not mature before harvest size and grow bigger than males, to obtain an all-female population is an economical choice for silver perch aquaculture.

Hormonal sex control has been successfully applied for the direct feminization or masculinization in a substantial amount of fish species (Pandian and Sheela 1995; 2013). However, no published paper related to sex reversal on silver perch has been reported. Our previous study (unpublished data) suggested that direct feminization was successfully induced all-female population by the used of estradiol 17- β hormone. It is also well known that all-female populations can be achieved by indirect feminization through mating functionally sex-reversed females (neomales) with regular females (Donaldson 1996; Pongthana et al. 1999; Devlin and Nagahama 2002). The induced sex reversal of XX male sex genotypes in the latter instance is a means of producing monosex all-female population of fish under XY sex-determining system, as expected for silver perch. Even though more time and

labour consuming than the direct feminization, the indirect method has an advantage where harvested fish have never been treated with steroids hormone. Therefore, its application would avoid a clash with the restriction of using hormones in fishes produced for human eating purpose. Furthermore, the indirect application of hormone is also recognized as beneficial tools in recognizing the homogametic sex (Blázquez et al. 1995; Liu et al. 2013).

The masculinization technique using hormone intended to produce XX male fish has been applied in numerous fish species, for example yellow fin perch, *Perca flavescens* (Malison et al. 1986); rainbow trout, *O. mykiss* (Cousin-Gerber et al. 1989); Nile tilapia, *Oreochromis niloticus* (Mair et al. 1991), common carp, (Gomelsky et al. 1994); and redbfin perch, *Perca fluviatilis* (Rougeot et al. 2002). However, an inconsistent increase in percentage of male resulted from hormonally treated fish has also been mentioned by Blázquez et al. (2001) in several fish species such as channel catfish, *Ictalurus punctatus*; rainbow trout; coho salmon and Chinook salmon, *Oncorhynchus tshawytscha*. As the influence of sex androgen on gonadal differentiation is species specific (Blázquez et al. 2001), the timing and optimum dosage of hormonal treatment for targeted cultivated species, including silver perch, need to be investigated.

An important step in establishing an effective regime of hormonal usage to masculinize fish is the identification of the 'labile period' i.e. the period where gonad has highly sensitive to the exogenous factors, including treatment of steroids (Blázquez et al. 2001). In silver perch, the gonads are anatomically formed in 30-day fry but cytological differentiation occurs only after the 60-day old larvae stage (Moiseeva 2001). Therefore, in this study, we introduced androgen treatment for masculinization at 31–60 dph.

The present research aims to assess the potential of two concentrations of dietary supplementation of 17 α -MT to produce an all-male population of silver perch as a step of producing XX genotype males which later can be used to produce an all-female population.

7.2. Material and Methods

This experiment has been approved by the Animal Ethics Committee of Curtin University (approval number AEC_2011_70). Besides, the Australian Code of Practice for the care and use of animals for scientific study was also followed.

7.2.1. Preparation of the MT-containing diets and handling

The MT powder (Sigma, M-7252) was reconstituted in 95% ethanol (1 mg/L) to prepare a stock solution before being incorporated into manufactured feed using alcohol saturation methods (Hendry et al. 2003) and evaporation methods (Rougeot et al. 2002; Lin et al. 2012). Commercial feed (spectrum micron diets, NRD[®] 2/4 200–400 µm; protein: 55%, crude fat: 9%, fiber: 1.9%; INVE-Thailand) was saturated with 50 mL MT-ethanol solution in petri dishes (each of 20 mg feed). The concentrations of 9 and 18 mg MT/kg diet need 0.18 and 0.36 mL of 1 g/L MT, respectively. The control feed was saturated with ethanol only. The diets were dried overnight under a fume hood before being kept at 4°C until further use.

The MT-supplemented and control diets were fed in triplicate to silver perch juveniles in the nine prepared glass aquaria. The diets were given manually to 31 to 60 dph silver perch, until satiation, three times per day during daylight hours. The post 60 dph fish were then fed an untreated artificial diet (NRD G8, 0.8 mm) until termination of the experiment at 225 dph.

7.2.2. Broodstock handling

The domesticated silver perch broodstock, which had been maintained in a 10-ton fibreglass tank for approximately six years at Curtin Aquaculture Research Laboratory (CARL), Western Australia, was used to produce fry. Male and female broodstock were introduced with human chorionic gonadotropin (HCG) hormone at a dose of 200 IU/kg fish (Levavi-Sivan et al. 2004; Rowland 2009) to promote spawning. After hormonal injection, two sets of broodstock were maintained in a

two-tonne cylindrical fiberglass tank at room temperature (20–26°C) until they spawned.

7.2.3. Experimental fish preparation and maintenance

About 10,000 hardened eggs were transferred into incubator tanks an hour after the spawning. The incubator tanks were designed as a flow-through system equipped with a sump tank to hold newly hatched larvae, which were then transferred to a 300-L circular tank (as a holding tank) at a stocking density of 50 larvae/L. Water salinity in the holding tank was increased to 6 ppt to optimize larval survival. The holding tank was gently aerated and equipped with a heater (Sonpar, Model-Ha200) to maintain the temperature at $20 \pm 1.0^\circ\text{C}$. Initially, fish larvae were fed live rotifers and *Artemia* nauplii that were then replaced by a dried formulated diet (NRD 2/4) at 15 dph and occasionally supplemented with *Artemia* nauplii until 25 dph.

Haphazardly chosen larvae at 26 dph, at a density of 50 larvae per aquarium, were placed in nine 10-L glass aquaria filled with filtered fresh water (Aqua-pure Model-AP12S, 5 microns). The aquaria were arranged in an incubator bath equipped with a heater (stirred thermostatic circulator, Model-GD120) to keep the water temperature stable at 23°C . In order to minimize light penetration into the water column in the aquaria, each aquarium was wrapped with a black plastic sheet. An air stone was installed in the middle of each tank for DO diffusion and food dispersion. During the acclimation period, the dead larvae were replaced with new larvae obtained from the holding tank. From 26 to 30 dph, only NRD 2/4 feed was provided. Two weeks after the completion of hormonal treatment, all fish from each experimental unit were transferred to a separate 200-L plastic container to keep treatments and replicates separated. The containers were designed in a flow-through system where temperature and oxygen concentration were maintained at $23 \pm 2^\circ\text{C}$ and 7 ± 1 mg/L, respectively.

7.2.4. Data collection and sex determination

Fish body weight (BW, precision 0.01 g) was regularly monitored at 1, 75, 105, 135, 165, 195, and 225 dph, but total length (TL, precision 1 mm) was only measured at the commencement and at the termination of the experiment. Fish were anaesthetized using AQUI-S (0.025 mg/L) prior to weighing and measuring TL individually during sampling. At the end of the experiment, all fish were killed with an overdose of AQUI-S (100 mg/L). Gonads were removed and visually identified as ovaries or testes. Gonad weight (GW, precision 0.0001 g) and length (GL, precision 0.01 mm) were also measured. Survival was calculated by comparing the number of live fish at 225 dph with the initial number of fish, and the survival percentage was calculated. At the same time, fish sex was confirmed by the standard gonadal squash technique (Guerrero III and Shelton 1974; Conover and Fleisher 1986; Protocol M- 2009).

At the end of the experiment, the gonads of all fish were fixed in formalin buffer solution (FBS), embedded in paraffin wax, sectioned at a thickness of 7 μm , and stained with hematoxylin and eosin (Luna 1968). Slides were photographed under the microscope using an Olympus SC30 camera and GetIt software Olympus. The early stages of gonadal development were compared to published descriptions by Coward, Bromage (1998), Lin et al. (2012), Maack, Segner (2003), Almeida et al. (2008), and Lubzens et al. (2010).

The gonadosomatic index (GSI) was determined according to the formula suggested by Razak et al. (1999): $GSI = (GW/BW) \times 100$, where GW and BW are in grams. Specific growth rate was calculated using the formula: $SGR = (e^g - 1) \times 100\%$ (Hopkins 1992; Árnason et al. 2009), where $g = [\ln(w_2) - \ln(w_1)] / (t_2 - t_1)$, where w_2 and w_1 are the mean weights on day t_2 and t_1 , respectively. Condition factor (K) was calculated as: $K = BW \times TL^{-3} \times 100$ (Jamet and Desmolles 1994).

7.2.5. Statistical analyzes

Mean and standard errors (SE) were considered for BW and TL at each sampling times, and also for GW, GL, GSI, and SGR at 225 dph for each dietary treatment. The mean is derived from duplicate groups and the data expressed in percentages were arcsine transformed to ensure normality prior to further analyses. The significant differences between treatments in each parameter determined by one-way ANOVA followed by Tukey's post-hoc (multiple comparisons) tests. SPSS 22.0 package was used for all statistical analysis. Differences were accepted as significant when $P < 0.05$. Only male fish were included in the control group.

7.3. Results

7.3.1. Sex differentiation and masculinization

There were no visual deviations, such as lordosis or superficial blackening of the skin, in fish from any treatment groups. Sex identification through the examination of gonad morphology enabled the separation of male from the female fish. There was no discrepancy, between morphological identification of gonads, wet-squash and the histological examinations of gonads in the identification of sexes. Morphological differences between ovaries and testes were clear. A pair of ovaries in females and a pair of testes in males had similar transparency or whiteness in color but had different shapes, in that the ovaries were rounded and the testes were triangular under cross-section examination (Figure 7.1A and 7.1D). Female gonads from the control groups were arranged in lamellae containing oocytes with lightly stained nuclei, surrounded by darker cytoplasm (Figure 7.1B). Testicular tissue from all treatments with early beds of developing spermatocytes was present in the lobule tissue (Figure 7.1E).

In addition, histological analysis of females from the control groups had immature ovaries containing oocytes and oogonia (Figure 7.1C). The testes from MT-treated and control groups (Figure 7.1F) however, consisted of testicular lobules with the

different stage of male gametes such as spermatogonia, spermatocytes, and spermatid. All female gonads showed the ovarian lamellae projecting into the lumen, whereas male gonads showed the formation of the cyst in the testicular tubules.

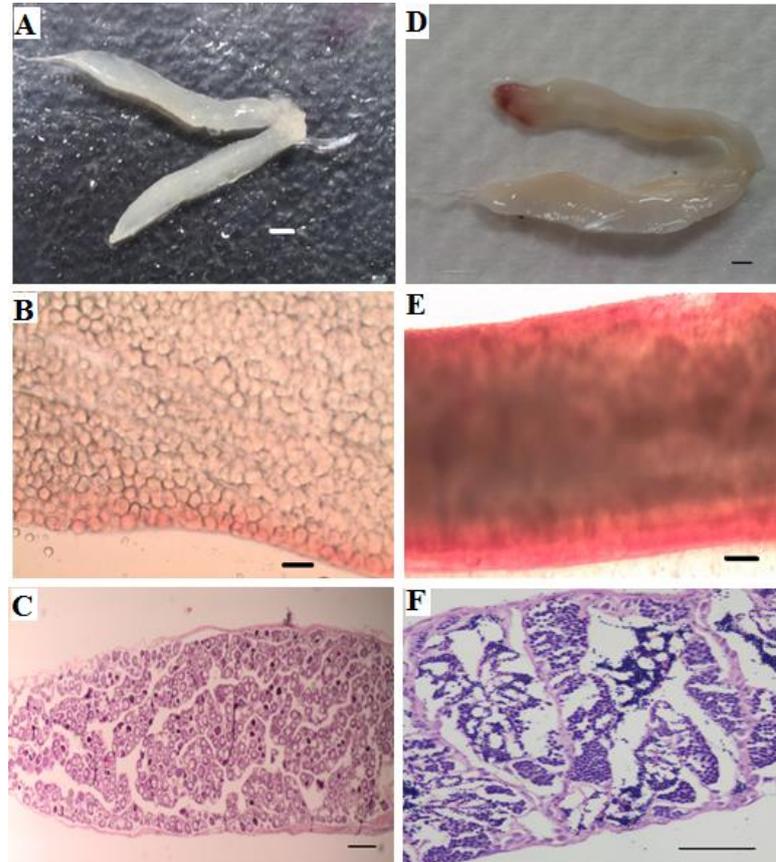


Figure 7. 1. Gonad visualization in silver perch, *Bidyanus bidyanus* juvenile at 225 dph. (a) Photomicrograph of an ovary showing pale white color and rounded cross-section; scale bar = 10 mm. (b) Wet-squash preparations of an ovary with previtellogenesis oocytes; scale bar = 100 μm . (c) Histological section of an ovary with oocytes located in the ovarian lamellae; scale bar = 100 μm . (d) Photomicrograph of testis showing color similar to that of ovary but triangular in cross-section; scale bar = 10 mm (e) Wet-squash preparations of testis showing typical testicular tubules; scale bar = 100 μm (f) Histological section of testis showing spermatogonia in the testicular tubules; scale bar = 50 μm .

The testes of all male fish fed MT were well developed, similar to those of the control groups. No indication of abnormalities such as gonadal retardation, intensification of connective tissue, lack of germinal tissue, and lack of testicular tubules, as commonly found in sterile gonads, were observed. There was no sign of

ovarian degradation, which showed that no males had ever gone through female stages in their life histories. Contrary to this, the females from the control population showed visible clusters of degraded male cells, demonstrating that they first went through male stages before changing into females (protandrous hermaphrodite), as described by Moiseeva (2001). Fish fed the MT-diet developed similar gonads, in terms of weight and length, to the fish fed control diet.

The lack of females, as opposed to 40% of females in the control group were induced by dietary MT supplementation. MT supplementation either at 9 mg/kg or 18 mg MT/kg diet resulted in the induction of a significantly ($P < 0.05$) higher percentage of males compared to control, which shows the complete masculinization of the silver perch (Figure 7.2).

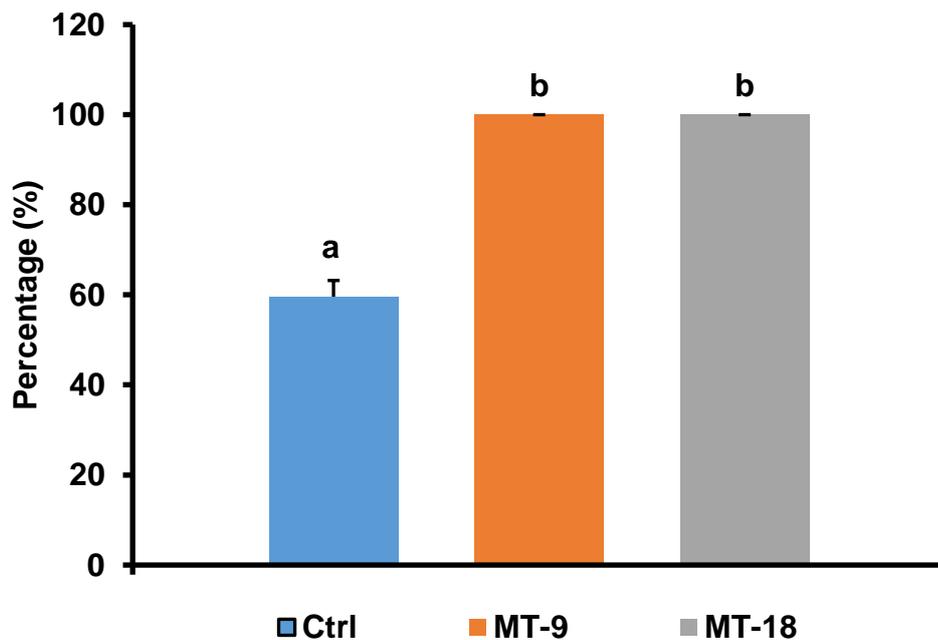


Figure 7. 2. Proportion of males of silver perch, *Bidyanus bidyanus* at the end of the experiment. Ctrl, provided feed saturated with ethanol only; MT-9, MT fed at 9 mg/kg diet; MT-18, MT fed at mg/kg diet. Different letter for each treatment indicate that the means are significantly different at the 0.05 level.

7.3.2. Growth

Fish fed MT had significantly ($P < 0.05$), higher weight gains but showed no significant ($P > 0.05$) differences in GSI (Table 7.1). Fish weight increased steadily after slow growth during the first two and a half months (Figure 7. 3). There were no significant ($P > 0.05$) differences) in mean weight at 75 dph or at two weeks after the withdrawal of dietary supplementation. However, at the end of the experiment (225 dph), the total weight of fish fed MT was significantly ($P < 0.05$) higher than that of the control groups (Figure 7.3). Food efficiency (weight gain/food intake) was significantly higher in MT-fed fish than in control groups (Table 7.1).

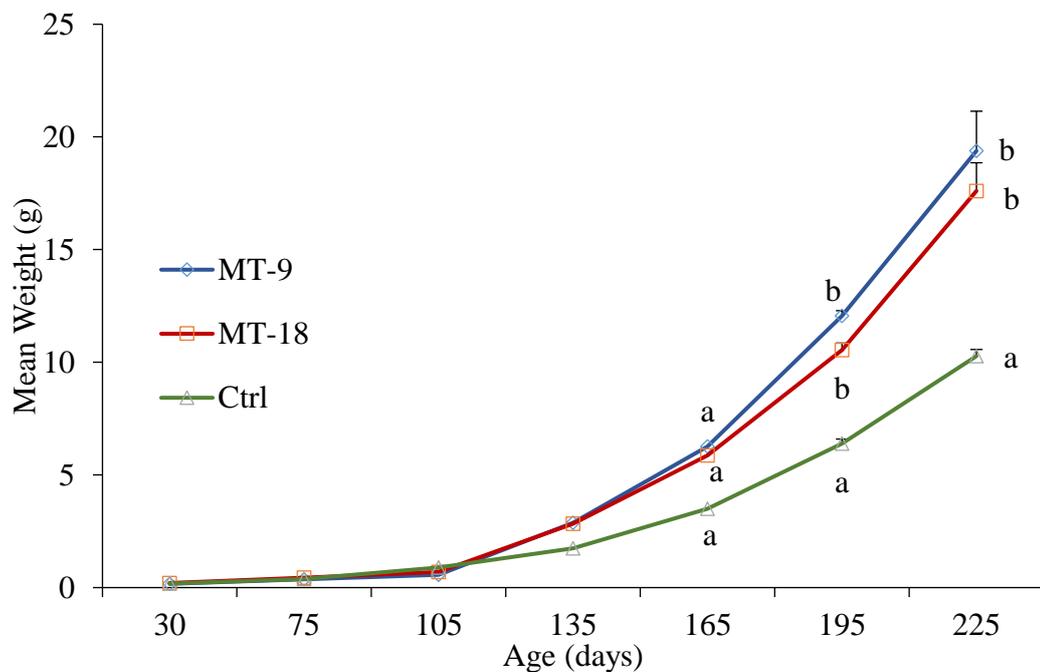


Figure 7. 3. The increase in body weight of silver perch, *Bidyanus bidyanus* juvenile fed MT. Vertical bars indicate SE. MT-9, MT fed at 9 mg/kg diet; MT-18, MT fed at 18 mg/kg diet; Ctrl, provided feed saturated with ethanol only. Sample sizes of 7–12 were used for each replicate, and different letters for each age indicate that the means are significantly different at the 0.05 level.

Table 7. 1. Effects of hormonal (MT) treatments on survival rate, specific growth rate, and food conversion efficiency in silver perch, *Bidyanus bidyanus*.

Parameters	Treatment		
	Control	9 mg/kg diet	18 mg/kg diet
Initial weight (g)	0.18 ± 0.03 ^a	0.17 ± 0.03 ^a	0.20 ± 0.03 ^a
Survival rate (%)	39.33 ± 3.33 ^a	51.33 ± 1.33 ^{ab}	44.42 ± 2.40 ^b
Final Weight (g)	11.52 ± 0.98 ^a	19.62 ± 1.49 ^b	17.60 ± 0.81 ^b
Gonad weight (g)	0.06 ± 0.02 ^a	0.07 ± 0.02 ^a	0.06 ± 0.03 ^a
Gonad length (mm)	18.79 ± 3.22 ^b	21.07 ± 1.21 ^a	22.60 ± 1.90 ^a
GSI (%)	0.37 ± 0.10 ^a	0.33 ± 0.12 ^a	0.33 ± 0.15 ^a
SGR (%)	3.3 ± 0.04 ^a	3.56 ± 0.03 ^a	3.51 ± 0.02 ^a
FE	29.78 ± 1.00 ^a	34.76 ± 0.47 ^b	33.18 ± 0.89 ^b

Note: Means with different superscripts in the same row are significantly different at $P < 0.05$ (Tukey test). GSI, gonadosomatic index.

7.3.2. Mortality

At the end of experiment, survival in all treatment ranged from 39% to 51% (Table 7.1), with most of the mortalities arising in the beginning period of the experiment: that is at the commencement of the feeding on formulated diet. The survival of silver perch was independent of the dietary MT dosage.

7.4. Discussion

The doses (0, 9, and 18 mg/kg diet) of MT hormone (Sigma, M-7252) used in this experiment were based on the supplementation level applied to the other species such as rainbow trout, *Salmo gairdneri* (Solar et al. 1984); European sea bass, *Dicentrarchus labrax* (Blázquez et al. 2001); and golden barb, *Puntius gelius* (Montajami 2012). The production of all-male or all-female populations through sex reversal in aquaculture operation is one way of directing feed-derived energy into growth. There are three main variables involved in the success of sex reversal:

(i) the timing of hormonal treatment in relation to gonadal improvement; (ii) the duration of hormone treatment; and (iii) the dose and type of sex steroids (Blázquez et al. 2001). The best time for hormonal exposure, termed as a critical period here, is the period where gonads reveal a high response to exogenous sex steroids.

The critical period for hormonal treatment can be identified through the application of hormone at different periods, as has been applied to different species such as European sea bass (Blázquez et al. 2001); rainbow trout (Cousin-Gerber et al. 1989; Kato et al. 2001); honmoroko, *Gnathopogon caurulescens* (Fujioka 1993); and red sea bream, *Pagrus major* (Kato et al. 2001) or by identifying early gonadal development through microscopic examination, as used in this experiment.

The present study demonstrated that female silver perch can easily be converted into males. The dietary supplementation of MT from 31 to 60 dph showed high sensitivity to exogenous androgen and resulted in complete masculinization at relatively low dosages and over a short period of time. The sex undifferentiated period for silver perch that is identified before two months of age (Moiseeva 2001) showed its sensitivity to hormonal treatment. This period is shorter than another hermaphrodite fish, European sea bass, whose equivalent period lasts 11 months, during which the gonads remain sexually undifferentiated (Blázquez et al. 2001; Saillant et al. 2003; Navarro-Martín et al. 2009). However, the silver perch is still comparable to the other short sexually undifferentiated periods of fish such as the 7–14 days post-hatch of Nile tilapia (Kwon et al. 2000), the 30 mm fork length and 45 days onwards for Atlantic halibut, *Hippoglossus hippoglossus* (Hendry et al. 2002); the 19 mm total length (TL) for Atlantic cod (Chiasson et al. 2009; Haugen et al. 2011), and approximately 15 mm and 30 days for golden rabbitfish, *Siganus guttatus* (Komatsu et al. 2006).

The dietary dosage of 9 and 18 mg MT/kg diet resulted in a high percentage of males (Figure 7.2) which is comparable to results from experiments involving rainbow trout and European sea bass, although a relatively higher dosage was applied to Nile tilapia, blue tilapia (*Tilapia aurea*) and redfin perch (Table 7. 2). In

contrast, a relatively lower dosage has also been reported to completely masculinize black tilapia, *Tilapia mosambica* and rainbow trout (Table 7. 2).

Table 7. 2. Reported complete masculinization of different species treated at different doses of 17 α -Methyltestosterone (MT)

Species	MT doses (mg/kg diet)	References
Rainbow trout, <i>Salmo gairdneri</i>	1-9	Solar et al. (1984);
European sea bass, <i>Dicentrarchus labrax</i>	10	Blázquez et al. (1995)
Nile tilapia, <i>Oreochromis niloticus</i>	60	El-Greisy, El-Gamal (2012)
Blue tilapia, <i>Tilapia aurea</i>	60	Guerrero (1975)
Redfin perch, <i>Perca fluviatilis</i>	40	Rougeot et al. (2002)
Silver perch, <i>Bidyanus bidyanus</i>	9-18	This study

While a small MT dosage can result in incomplete masculinization, an overdose can, potentially, lead to infertility and even inconsistent feminization resulting aromatization of androgens to estrogens (Goudie et al. 1983). Since the 9 and the 18 mg MT/kg diet resulted in the same percentage of males, 9 mg MT/kg diet is therefore preferred to avoid the adverse effect of the higher dosage of MT. Reducing MT concentration below 9 mg/kg diet or increasing above 18 mg/kg diet may reduce the male percentage and promote the appearance of intersex fish. This phenomenon has been reported in Nile tilapia where the percentage of intersex fish was greatest at a lower dosage of 3.75 mg/kg, decreased rapidly at a higher dosage of 30 mg/kg, and then increased again by further increasing the dosage to 120 mg/kg (Phelps and Okoko 2011).

The duration of hormonal exposure for 30 days in this study started when the fish were ready to accept the formulated diet, at 31 dph, to the time of sex differentiation, at 60 dph. The result showed that the duration is sufficient to completely masculinize silver perch juveniles. The same length of MT exposure has been reported by Rougeot et al. (2002) for redfin perch, but at higher MT concentration

(40 mg/kg diet). Nearly the same duration is also reported for blue tilapia, which were fed for 28 days, at a concentration of 30 mg MT/kg diet (Tayamen and Shelton 1978). The period before sex differentiation identified histologically by Moiseeva (2001) coincides with the critical period for MT treatment in silver perch.

The average gonadal weight, length, and GSI of fish did not show any significant ($P>0.05$) differences due to any dietary supplementation of MT. Morphological differences in sex-reversed gonads have been reported in European sea bass (Blázquez et al. 2001) where androgen treatment encouraged a robust obstruction of the gonadal development, producing in reduced testes sizes with a great content of fibrous-connective tissues. However, in our research, there were no histological abnormalities in the male gonad of control fish or MT-fed fish at 225 dph, suggesting that sex-reversed genotypic males (neo-females) may be able to produce viable sperms. Similarly, male-functional blue tilapia (Guerrero 1975), yellow catfish, *Pelteobagrus fulvidraco* (Liu et al. 2013), and rainbow trout (Cousin-Gerber et al. 1989) reversed from genotypic females, were able to produce viable sperms.

Histological analysis indicated no degraded female cells in the male gonads of MT-fed fish, indicating that MT induces the direct development of males, which is different from the normal sequence of sexual change from male to female (protandrous hermaphrodite) resulting in visibly degraded male cells (Moiseeva 2001). The female gonads of control groups showed their normal development where different stages of the oocyte were visualized with the sporadic presence of degraded male cells. This indicates that natural females have to go through male stages prior to transforming into the female. When MT was applied to the fish larvae before the onset of sex differentiation, genetically female fish were sexually reversed to male before their female gonads were developed.

In the present study, the potential of different doses of MT to promote growth rate was also investigated. Monosex silver perch did not show significant difference in their TL after being fed MT. However, feeding MT appears to have a higher

anabolic effect given that the weight gained by the fish treated with MT was higher than the control groups, after 195 and 225 dph. Hence, the dietary MT not only influences the sex ratio in favour of males but can also promote growth, as in Nile tilapia (Little et al. 2003; El-Greisy and El-Gamal 2012). Dietary MT has also proven to be an effective growth promoter in common carp (Nagy et al. 1981); coho salmon (Shelbourn et al. 1992); European sea bass (Navarro-Martín et al. 2009); rainbow trout (Cousin-Gerber et al. 1989); red sea bream, *Chrysophrys major* (Woo et al. 1993); and golden barb, *P. gelius* (Montajami 2012).

The reasons for the enhanced growth performance in this study might have been a result of the direct feeding of MT which led to increased appetite, resulting in higher SGR. Other studies in Nile tilapia, have also stated that the enhancement of food conversion efficiency on sex-reversed fry could have attributed to the higher mean weights (Chakraborty et al. 2011; El-Greisy and El-Gamal 2012).

While dietary MT induces increased appetite and food consumption in coho salmon (Fagerlund et al. 1979), it depressed the appetite and feeding rate in rainbow trout (Yamazaki 1976). It is unclear whether dietary MT improved the growth directly or by MT's ability to masculinize the sex, as, for example, it is established that the male tilapia, under normal conditions, grows better than females. Hence, it can be stated that the response to a given steroid hormone is species specific (Kuwaye et al. 1993) which could promote higher growth rate, by means of increased food conversion, activate the formation of other androgenic anabolic hormones, and as the direct effect of MT on the gene expression in the muscle cells. In the present study, the increased feeding rate was observed in hormonal-fed fish, leading to higher food ingestion in silver perch.

Complete masculinization without abnormality can only be achieved if the right dose of hormones is used at the correct time. Gonadal sensitivity to dietary MT in 31–60 dph in fish larvae corresponds to body weight between 0.15 and 0.34 g and to total length between 21 and 27 mm. Since this critical period occurs just after early weaning onto artificial dry feeds (Phipps 1999), sex androgen applied at the

critical time can influence the gonadal development and overrule genetic sex determination (Devlin and Nagahama 2002). The suggested critical period, derived from our results, is within the range of critical periods reported for other species. For example, the critical period in Nile tilapia occurs in the early larval stage, at 7–14 dph (Kwon et al. 2000); and in the European sea bass (Blázquez et al. 2001; Navarro-Martín et al. 2009) during the late juvenile stage, at 96–126 days post fertilization.

In conclusion, the application of MT at 9–18 mg MT/kg feed from 31 to 60 dph is effective in inducing masculinization in silver perch.

CHAPTER 8. General discussion, conclusions, and recommendations

In this study, the sexual manipulation procedure used to obtain a monosex population, by the feminization or masculinization of silver perch (*Bidyanus bidyanus* Michell, 1838), involved feeding larvae with hormone-supplemented feed for 30 days. Prior to the feminization and masculinization experiments, a series of experiments was conducted to evaluate the reproductive performance of domesticated silver perch and their larval biology. The study began with an evaluation of silver perch broodstock performance and egg quality in relation to the oil globule fragmentation, using the classification established by Źarski et al. (2011b). An experiment was also conducted to evaluate the ontogenic performance of silver perch larvae resulting from domesticated broodstock in comparison to larvae resulting from wild broodstock. The experiment then continued to elucidate larval performance during exposure to the different delayed of initial feeding, including their growth rate and mortality. Finally, the experiment on sex manipulation was conducted to assess the potential for feminization and masculinization as a way to produce a monosex stock. The silver perch was chosen in this study as it is a major native aquaculture industry in this country and there is no previous comprehensive research on the egg quality, larval development, and sex manipulation of silver perch.

8.1. Broodstock Performance

In many aquaculture species, especially newly domesticated fish, reproductive ability is an important limiting factor for the successful mass production of fry. For some fish species proficient knowledge in gonad maturation and spawning induction techniques and procedures is required to successfully obtain high-quality fertilised eggs (Mylonas et al. 2010). Spawning induction for silver perch has been described in detail (Rowland 1983; 1984; 2009), with hCG found to be a potent agent for final oocyte maturation, ovulation, and spawning. The optimum hCG dosage of 200 IU/kg for both male and female broodfish (Rowland 1984) has been routinely applied in research and commercial hatcheries (Rowland 2009). It is also common for farmers to cultivate broodfish in farm dams and move them to the

hatchery during the breeding season (November-March) (Rowland 2009). While information about the gonad maturation of silver perch is limited, fingerlings grown in a commercial pond and given a formulated diet have been found in a mature gonad stage at 3 years old (Rowland 2004a).

To date, information about the nutritional requirements of broodstock fish is limited to a few species. Research on the nutritional requirements of silver perch for grow-out purposes has commenced, focusing on phosphorus and lysine (Yang et al. 2006; Yang et al. 2011). A preliminary study on practical diet for silver perch has also been initiated by primarily using fishmeal and soymeal with a protein content of 35% and lipid content of about 5-10% (Allan 1992; Allan and Rowland 2002). Although the use of a commercial grower diet with 35% protein, as in this study, supports silver perch reared in tanks to reach maturity with a good quality of eggs and larvae, further research on broodstock diet is needed.

The comparison of tank-domesticated broodstock used in this experiment (Chapter 3) with pond-domesticated silver perch and wild broodstock is presented in Table 8.1. Pond-domesticated broodstock demonstrated the highest fecundity but had the lowest hatching rate. In contrast, the wild broodstock demonstrated the lowest fecundity but had the highest hatching rate. According to fecundity and hatching rate, the tank-domesticated broodstock had a superior performance compared to the other sources of broodstock. Aside from these attributes, however, the water-hardened eggs and the TL of newly hatched larvae of wild broodstock were significantly larger than the domesticated ones, as explained in Chapter 4. However, the yolk sac of larvae from the domesticated broodstock was larger than that of the wild broodstock and therefore considered to have better viability. This result differs to other species, where the wild broodstock shows a better reproductive performance compared to the domesticated broodstock (Emata 2003; Mazorra et al. 2003). The age and size range (6 years and 2.5-3.7kg respectively) of the tank-reared broodstock may not be the only reason for better performance in this study, as the commercial feed given routinely to both tank- and pond-domesticated broodstock may also have contributed to their performance. According to Jams, Sampath (2002) and Izquierdo et al. (2001), food limitation can severely inhibit

gonadal maturation in several fish species, including the ornamental fish, *Betta splendens*, the European seabass, and the male Atlantic salmon (Thorpe et al. 1990; Herbinger and Friars 1992). Furthermore, the importance of nutrients, especially dietary lipid levels, on fecundity and hatching rate has been described by several researchers (Watanabe et al. 1984a; Watanabe et al. 1984b; Watanabe et al. 1985a; Watanabe et al. 1985b; Duray et al. 1994; Fernández-Palacios et al. 1995; Izquierdo et al. 2001; Mazorra et al. 2003).

In brief, the domesticated (F2) female silver perch broodstock reared in tanks (Chapter 3) for about 6 years are viable, with acceptable performance and high-quality eggs.

Table 8. 1. Reproductive performance of domesticated and wild silver perch (*Bidyanus bidyanus*) broodstock,

Source	Domesticated in tank	Domesticated in pond	Wild
Age (years)	6	3	-
Weight (kg)	2.5-3.7	0.55±0.27	0.4-2.3
Fecundity (eggs/kg)	132,000±7218	139,286 ±11,405	125,000
Hatching rate (%)	87.94±1.23	76.8±2.8	88.8±2.8
References	This study	(Rowland 2004a)	(Lake 1967a; Thurstan and Rowland 1994)

8.2. Egg Quality Indicators

The distribution of oil globules is an egg quality indicator that can be employed for fish (Kjørsvik et al. 1989; Brooks et al. 1997). The relationship between the level of oil globule fragmentation and egg quality has been demonstrated in Eurasian perch (Żarski et al. 2011b). In the present study, the relationship between oil globule fragmentation and embryo and larval survival parameters was examined, in order to assess its potential as a marker for egg quality in silver perch.

High variation in the size of silver perch larvae and fingerlings has been reported (Anonymous 1999; Moiseeva 2001; Rowland 2004b; 2009) and is thought to be caused by low egg quality. The fatty acid composition of eggs has been identified as a key factor influencing egg quality and therefore is often enhanced to ensure improved egg quality (Watanabe et al. 1984b; Fernández-Palacios et al. 1995; Fernández-Palacios et al. 1997; Fernández-Palacios et al. 1998; Henrotte et al. 2010). Evaluation of eggs based on the viability of larvae after hatching as commonly applied for silver perch (Rowland 1984; 2009) and other cultured species (Migaud et al. 2006; Targońska et al. 2010; Źarski et al. 2011b) was considered to be too late. Earlier determination of egg quality for instance immediately after spawning is beneficial as it allows for the elimination of low-quality eggs from incubation and further processing to fingerling stage, as has been applied in European perch (Źarski et al. 2011b).

The oil globule fragmentation observed in silver perch eggs in this study (Chapter 3) meets the criteria suggested by Źarski et al. (2011b) for European perch, except category-4, which was not found in the silver perch eggs. Category-1 and 2 eggs demonstrated better performance in all parameters including embryo survival, hatching rate, larger TL of larvae, and higher survival rate of larvae, compared to category-3 eggs. This is different to European perch (Źarski et al. 2011b) and brown trout (Mansour et al. 2007), where the oil globule fragmentation was subjected to change from dispersion to coalescent during incubation, as the oil globule fragmentation in silver perch eggs is still visible in newly hatched larvae until 8 dph. Therefore, oil globule fragmentation as an indicator of egg quality in silver perch may be an evaluation method with high validity. Since no data is yet available regarding the application of this evaluation method in commercial hatcheries, further research may be needed before recommendations can be issued.

Some researchers agree that oil globule fragmentation can be caused by the disturbance of FOM during controlled reproduction, including hormonal and environmental manipulation (Migaud 2003; Targońska et al. 2010; Źarski et al. 2011b) and stress caused by handling (Schreck 2010). Furthermore, Campbell et al. (1994) has found that chronic confinement stress during the final stages of

reproduction could reduce egg size, result in lower progeny survival, and disrupt the endocrinology of brown trout and rainbow trout (Bromage et al. 1992). While this possibility still needs to be researched for silver perch, stress-avoiding activity during handling and hormone injection of silver perch broodstock as a standard procedure in hatcheries (Rowland 1984) is therefore important to maintain good egg quality.

8.3. Yolk-sac Absorption and the PNR

The time of initial feeding, food availability, and the capacity for food ingestion are crucial for the survival of newly hatched larvae (Sanderson and Kupferberg 1999). These factors are strongly related to yolk-sac absorption and the ability of larvae to maintain good performance until they can develop exogenous feeding (Blaxter and Ehrlich 1974). Better understanding of yolk-sac absorption and the PNR of silver perch is expected to provide accurate information to establish feeding procedure on larval rearing in hatcheries.

In the present study, yolk-sac absorption in silver perch larvae (Chapter 4) occurred intensively during the first few hours post-hatching, while the oil globule remained available until 240 hph. The yolk-sac absorption was linearly correlated with the SGR of larvae and its exhaustion was one day before the onset of initial feeding at 5 dph. Interestingly, the reduction rate of the oil globule was slower than the yolk-sac usage, and this is considered beneficial to support larvae during the mix feeding period. Only about 0.5% of the oil globule volume was utilised within 6 hours when the yolk sac had already reduced to 34%. The initial feeding then commenced at 5 dph after the yolk sac depleted completely at 4 dph (Figure 8.1).

The appearance of a gap between the yolk-sac exhaustion and the initial feeding may not be critical for larvae as no serious mortality occurred at this period (Chapter 5). The oil globule, which is still available at about 35% (Chapter 4) may support nutritional requirements during this period until oil globule exhaustion at 10 dph. In some other species, the initial feeding begins far sooner or just as the yolk sac is

depleted, as demonstrated in rohu and singhi (Mookerji and Ramakrishna 1999) and in Mozambique tilapia (Rana 1985).

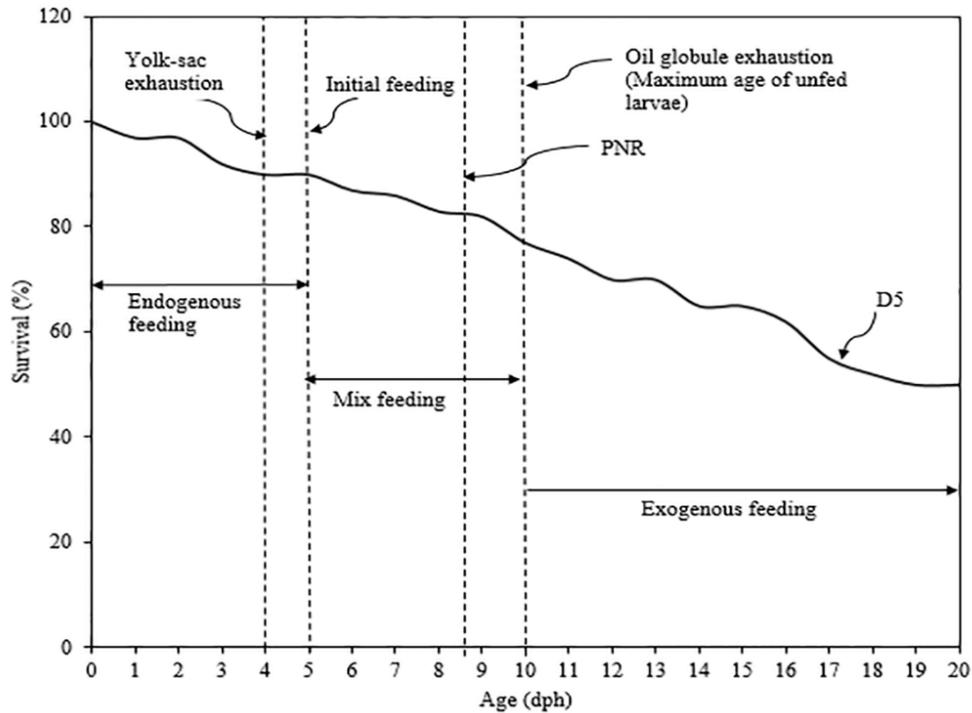


Figure 8. 1. Survival of silver perch (*Bidyanus bidyanus*) larvae at 5 dph initial feeding (graph) and larval development in relation to the yolk sac absorption and initial feeding.

8.4. Sex Reversal

Sexual development is an important element of the continued improvement of cultured fish stocks (Frisch 2004; Cnaani and Levavi-Sivan 2009). Several traits of high economical value have been observed that relate to sexual development (Lutz 2001; Frisch 2004; Chen et al. 2013; Mylonas et al. 2013). Economically important sexual dimorphisms in some edible fish species are observed in growth rate, time and age of maturation, and body shape. Monosex stock culture may also be advantageous to avoid unwanted or uncontrolled reproduction (Lutz 2001; Frisch 2004; Cnaani and Levavi-Sivan 2009; Liu et al. 2013; Pandian 2013).

Silver perch display sexual growth dimorphism, where males grow more slowly than females (Mallen-Cooper 2003). This sex-related dimorphic growth is correlated with gonad maturity level and appears before marketable size at about 1

kg in weight (Rowland 2004a). Therefore, the development of a procedure to produce all-female silver perch would increase the productivity of this species in commercial aquaculture (Frisch 2004).

Monosex female stock can be obtained by direct feminization through hormone sex reversal (Pandian and Sheela 1995; 2013) or by indirect feminization through mating functionally sex-reversed females (neomales) with normal females (Donaldson 1996; Pongthana et al. 1999; Devlin and Nagahama 2002). However, no published papers relating to sex reversal in silver perch have been found. The success or failure of hormonal sex reversal treatments depends on several major factors that are species-specific, such as (1) labile period, (2) chemical inducer, and (3) the methods of administration (Malison et al. 1986; Pandian and Varadaraj 1990; Pandian and Sheela 1995; Rougeot et al. 2002; Pandian 2013). The time of application is important as hormones applied after the onset of sexual distinction frequently have reduced or no effect. However, even if the hormone is applied at the right time, low doses and/or short duration may produce incomplete or no sex reversal, whereas excessively high doses and/or treatment duration produce sterile fish or individuals that develop no gonads (Bye and Lincoln 1986; Chatain et al. 1999; Demska-Zakes and Zakes 1999).

Silver perch is a sequential protandrous hermaphrodite fish according to the criteria proposed by Pandian (2010), in which sex change from male to female occurs naturally during the juvenile stage (Moiseeva 2001) as illustrated in Figure 8.2. Male gonads are first detected in one-month-old larvae. In an all-male population (1 to 3 months old), some change to female while others stay male for their entire lifecycle. The sex change occurs at the threshold size of 3 cm TL and 1 g in weight, between the age of 3 and 6 months, as reported in the histological study of Moiseeva (2001). According to Pandian (2013), the sex change can be induced only during the labile embryonic period. Based on the gonad developmental stage of silver perch, the labile period may be assumed to occur between hatching and 6 months of age.

The present study suggests that the application of E2 and MT hormone at 30-60 dph significantly increased the mean percentage of females or males respectively (Chapters 6 and 7). This indicates that 30-60 dph is part of the labile period for silver perch, which coincides with the period of male genital cell formation (Figure 8.2). According to Pandian (2013), the labile period commonly covers the period of the undifferentiated or during differentiating gonads stage. As such, the labile period of silver perch can be seen to have occurred from hatching to 180 dph, where dimorphic sex differentiation commenced after the period of sex change (Figure 8.2). However, 30 dph is the earliest time in which silver perch are ready to accept formulated food and are suitable for hormone applications through diet. Although further research is needed to confirm the labile period for silver perch, hormone application of MT or E2 at a 30 to 60 dph was shown to produce an all-male or all-female population respectively.

In most cases, the labile period is detected by applying the same hormone inducer and dose to individuals at different durations. The period where a high percentage of the treated individuals have undergone the desired sex reversal is defined as the labile period (Hunter and Donadson, 1983; Devlin and Nagahama, 2002; Piferrer, 2001). However, this study showed that part of the labile period of silver perch can be accurately detected with the assistance of histological data on gonad development. This finding is of value to the future study of different species.

Another important finding is that the use of E2 hormone resulted in 100 % feminization of silver perch at the relatively low dosage of 60 mg/kg diet (Chapter 6). This dosage is about a half of the dosage application of E2 required to achieve 100% feminization of bluegill sunfish, *Lepomis macrochirus*, at 150 mg E2/kg fed to post-larvae for 60 days (Wang et al. 2008). In addition, no intersex individuals were detected in this study, while 5% were found to be intersex in the bluegill sunfish. Similar findings were demonstrated with MT treatment, as the dosage of MT supplementation at 9 mg/kg diet resulted in complete masculinization of the silver perch (Chapter 7). This dosage is comparable to the minimum effective dose for Nile tilapia at 14 mg/kg diet (Phelps and Okoko 2011) or 15 mg/kg diet for *Betta*

splendens (Kavumpurath and Pandian 1994). This demonstrates that silver perch are responsive to MT and E2 hormones.

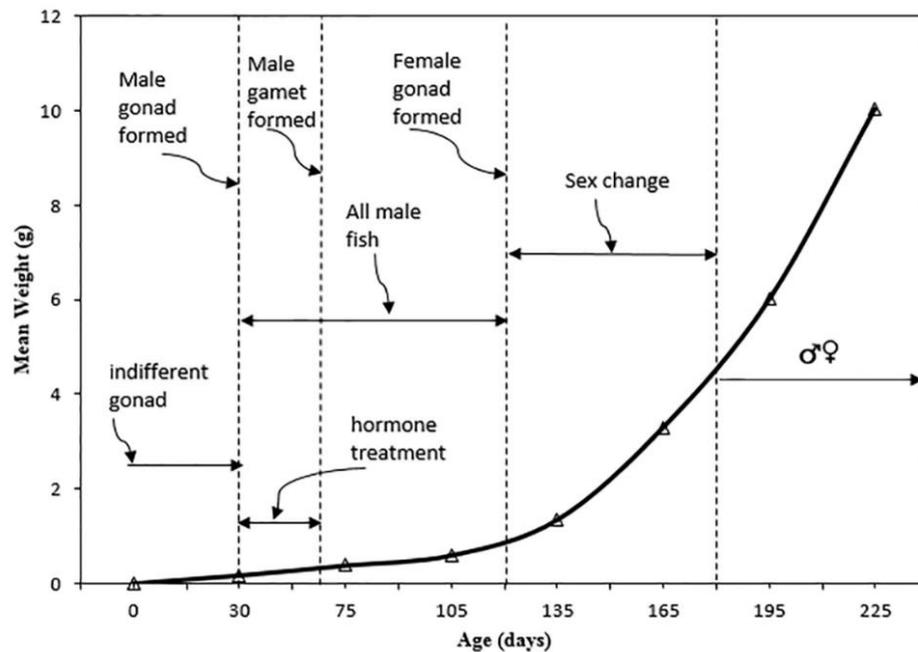


Figure 8. 2. The mean weight (graph) of silver perch (*Bidyanus bidyanus*) larvae reared in laboratory and gonad development according to Moiseeva (2001) in relation to the period of hormone treatments.

There appears to be a tendency for steroid hormones to cause growth disorders in some aquaculture species, such as the Atlantic halibut, *Hippoglossus hippoglossus* (Hendry et al. 2003) and brook trout (Johnstone et al. 1979) treated with E2 and a reduced appetite and feeding rate in rainbow trout (Yamazaki 1976) after MT treatment. However, this study demonstrated no adverse effects on silver perch growth from hormone treatment, as the use of E2 and MT treatments indicated improvement in growth compared to control groups. This enhanced growth performance may have resulted from increased appetite and enhanced food utilization and protein synthesis (Kocour et al. 2005). MT has also been shown to promote growth in Nile tilapia (Little et al. 2003; El-Greisy and El-Gamal 2012), common carp (Nagy et al. 1981); coho salmon (Shelbourn et al. 1992), European sea bass, *Dicentrarchus labrax* (Navarro-Martín et al. 2009), rainbow trout (Cousin-Gerber et al. 1989), red sea bream, *Chrysophrys major* (Woo et al. 1993), and golden barb, *Puntius gelius* (Montajami 2012).

8.5. Conclusion and Recommendation

A summary of conclusions drawn from this research are listed below.

1. The female domesticated silver perch broodstock could maintain a good performance after 6 years old (objective 1).
2. The oil globule fragmentation is a possible tool for evaluating egg quality in silver perch (objective 1).
3. The yolk sac is completely resorbed at 90 hph, whereas the oil globule remains until 240 hph at $20\pm 0.5^{\circ}\text{C}$ (objective 2).
4. The PNR was detected 3 days after onset of feeding (at 5 dph), between 8 and 9 dph (objective 3).
5. Initial feeding at 5 dph gives the best growth and survival rate performance for silver perch larvae (objective 3).
6. Supplementation of E2 at 60–120 mg/kg diet could be applied for direct feminization to establish all-female populations of silver perch (objective 4).
7. The dietary supplementation of MT at 9–18 mg/kg diet, from 31 to 60 dph, is effective in inducing masculinization in silver perch (objective 5).

Based on the findings in this study, further research and recommendations are suggested. These recommendation are as follows:

1. To support silver perch tank-domestication, further research on broodstock diet is required.
2. Further research on the use of oil globule fragmentation as an indicator of egg quality for silver perch in hatcheries is needed before recommendation can be issued.
3. The initial feeding at 5 dph is suggested for the hatchery production of silver perch larvae.
4. Further research is required to evaluate the growth and reproductive performance of adult silver perch after feminization with the E2 hormone.
5. Based on the optimised sex reversal using steroids in this study, further research on the genetic sex-determining mechanism in silver perch can be

performed. This is important for attempts to obtain all-female populations by crossing hormonally sex-reversed males with original females.

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APPENDIX

List of Publications

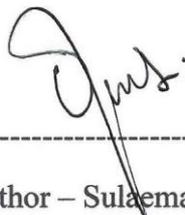
- Sulaeman, R. Fotedar. 2017. Reproductive performance of a domesticated broodstock and the relationship between oil globule fragmentation and the egg quality of silver perch, *Bidyanus bidyanus* Mitchell 1838. Indonesian Aquaculture Journal 12 (2017): 43-51. DOAJ: 10.15578/iaj.12.2.2017.43-51 (Chapter 3)
- Sulaeman, R. Fotedar. 2017. Yolk utilization and growth during the early larval life of the silver perch, *Bidyanus bidyanus* (Mitchell, 1838). International Aquatic Research 9 (2017):107-116. DOI 10.1007/s40071-017-0160-7. (Chapter 4)
- Sulaeman, R. Fotedar. 2018. Effects of delayed initial feeding on growth and survival performance of silver perch, *Bidyanus bidyanus* (Mitchell, 1838) larvae. AACL Bioflux AACL Bioflux 11 (4):1025-1037. (Chapter 5)
- Sulaeman, R. Fotedar. 2017. Effect of dietary estradiol-17 β on the feminization of silver perch, *Bidyanus bidyanus* (Mitchell, 1838). Journal of the World Aquaculture Society 48 (3):444-452. doi: 10.1111/jwas.12352 (Chapter 6)
- Sulaeman, R. Fotedar. 2017. Masculinization of silver perch (*Bidyanus bidyanus* Mitchell 1838) by dietary supplementation of 17 α -methyltestosterone. Egyptian Journal of Aquatic Research 43:109-116. <http://dx.doi.org/10.1016/j.ejar.2016.10.002> (Chapter 7)

Statement of Contribution

I, Sulaeman, as the first author of the publications entitled:

1. Sulaeman, R. Fotedar. 2017. Reproductive performance of a domesticated broodstock and the relationship between oil globule fragmentation and the egg quality of silver perch, *Bidyanus bidyanus* Mitchell 1838. Indonesian Aquaculture Journal Vol. 12, No. 2 (2017). (accepted for publication).
2. Sulaeman, R. Fotedar. 2017. Yolk utilization and growth during the early larval life of the silver perch, *Bidyanus bidyanus* (Mitchell, 1838). International Aquatic Research 9 (2017):107-116. DOI 10.1007/s40071-017-0160-7.
3. Sulaeman, R. Fotedar. 2018. Effects of delayed initial feeding on growth and survival performance of silver perch, *Bidyanus bidyanus* (Mitchell, 1838) larvae. AACL Bioflux AACL Bioflux 11 (4):1025-1037.
4. Sulaeman, R. Fotedar. 2017. Effect of dietary estradiol-17 β on the feminization of silver perch, *Bidyanus bidyanus* (Mitchell, 1838). Journal of the World Aquaculture Society 48 (3):444-452. doi: 10.1111/jwas.12352.
5. Sulaeman, R. Fotedar. 2017. Masculinization of silver perch (*Bidyanus bidyanus* Mitchell 1838) by dietary supplementation of 17 α -methyltestosterone. Egyptian Journal of Aquatic Research 43:109-116. <http://dx.doi.org/10.1016/j.ejar.2016.10.002>.

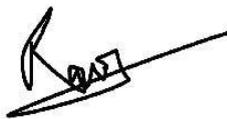
declare that the above works were primarily designed, experimentally executed, interpreted and written by the first author.



First Author – Sulaeman

Date 1-11-2018

I, Ravi Fotedar, as a Co-author, endorse that this level of contribution by the first author indicated above is appropriate.



Co-author, Ravi Fotedar

Date 2-11-2018