Technique development for marking of Yarra Pygmy Perch (*Nannoperca obscura*) with calcein, including field validation.

Simon Westergaard

Supervisors Dr James Harris and Dr Qifeng Ye

Presented for the Bachelor of Science Honours (Aquaculture)

School of Biology

Flinders University, South Australia

Title page image: Left to right top row, Female *Nannoperca Obscura*, Calcein stained *Nannoperca Obscura*, second row, Calcein staining, Male *Nannoperca Obscura*. Images S. Westergaard and M. Hammer.

Declaration

This work contains no material which has been accepted for the award of any degree or diploma in any university or other tertiary institution to S. Westergaard, and to the best of my knowledge and belief, contains no material previously published or written by any other person, except where due reference has been made in the text.

I give consent to this copy of my thesis when deposited in the university library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within each thesis (as listed on page) resides the copyright of that work.

_____
___/___/_____

Contents

List of Tables

List of figures

Abstract

Drought conditions across South Australia, during the period 2001-2009, led to significant reductions in the availability and quality of aquatic habitat. The Yarra Pygmy Perch (*Nannoperca obscura*) is a small-bodied Australian native freshwater fish. Yarra Pygmy Perch from the Lower Lakes, the sole location of the species in the Murray-Darling Basin were impacted severely. In early 2008 the Department of Environment Water and Natural Resources (DEWNR) rescued stocks of Yarra Pygmy Perch. With no Yarra Pygmy Perch seen in the MDB since February 2008, the species has likely been extirpated from the system. Conditions in the Lower Lakes improved markedly in 2010-2013. Combined efforts to raise numbers of fish in captivity for release have been successful. Increased water levels and much improved water quality and habitat show much promise for re-establish the species in its natural habitat. Methods for monitoring and evaluating the success of re-introductions are important for restocking, to inform management agencies and maximise conservation out-comes. Methods for quick cost effective mass marking of fish with calcein were trialled, adjusted from those developed by previous authors. Retention trials investigated survival, growth, effect of sunlight radiation and field use with mark identification accuracy determined at 89% at 8 months, via external examination on live fish. Sunlight radiation was established as the main driver of stain fading and the potential for complete stain fade by 4 months under highlight conditions was established. Mark identification accuracy via internal examination of otoliths was determined to be 76.9% at 8 months. The addition of new post-staining protocol was trialled and showed delaying early exposure to sunlight radiation by 2 weeks post-treatment increased mark retention by a factor of 3.

Introduction

The Yarra Pygmy Perch (*Nannoperca obscura*) is a small-bodied native freshwater fish, attaining a total length of 75mm. The species is nationally listed as vulnerable under the Environmental Protection and Biodiversity Conservation Act 1999 (EPBC), critically endangered at a state level (Hammer *et al.* 2009), vulnerable on the IUCN Red List of Threatened Animals (IUCN 2003) and listed on the Australian Society for Fish Biology threatened species list (ASFB 2001). In South Australia the species is protected under the *Fisheries Act* (1982).

Drought conditions across South Australia and in the Murray-Darling Basin during the period 2001-2009, led to significant reductions in the availability and quality of aquatic habitat (Hammer 2007; Hammer 2009; Bice *et al.* 2011) for Yarra Pygmy Perch. Drought conditions impacted severely on Yarra Pygmy Perch populations from the Lower Lakes, the sole location of the species in the MDB (Smith and Hammer 2006) which is considered of high conservation value (Saddlier and Hammer 2010). Populations here showed a marked decline in abundance in the period 2005-2007 (Bice and Ye 2007) and 2007-2008 (Bice *et al.* 2008). Subsequently for conservation of the Yarra Pygmy Perch in accordance with the National Recovery Plan for the Yarra Pygmy Perch (Saddlier and Hammer 2010), the situation required the removal of fish from natural habitat where where severe impacts that threatened the species' survival had occurred. Therefore in early 2008 before sites dried, a number of fish were rescued and transferred to a captive location funded by Department of Environment, Water and Natural Resources (DEWNR), South Australia.

Yarra Pygmy Perch have <u>not</u> been seen in the MDB since February 2008, despite a number of targeted monitoring events (Wedderburn *et al.* 2010; Bice *et al.* 2011);

Bice, Hammer et al 2012) . It is thought likely the species has been extirpated from the system (Bice *et al.* 2012; Wedderburn *et al.* 2012) and that the only remaining individuals were now contained in captivity.

During 2008-2009 numbers of fish were released into two surrogate dams, where good numbers have become established. Also in 2010/2011 new facilities were constructed at Flinders University for the breeding, rearing and production of Yarra Pygmy Perch with genetic analysis informing breeding strategies to maximise preservation of the genetic diversity remaining in the small population (Beheregaray 2011).

Conditions in the Lower Lakes of the MDB have improved markedly in recent times, with increased water levels and much improved water quality and habitat. Restocking with the aim to re-establish wild-stocks of this threatened native fish population is occurring, but it is important to have methods for monitoring and evaluating the success of re-introductions, i.e. mark recapture methods. Successful mark recapture methods enable insight into population dynamics for the Yarra Pygmy Perch, and clues to environmental factors conducive to either, persistence, recruitment or demise, which can thereby inform management agencies of strategies to maximise the success of release programs.

There are several established methods of marking fish. Methods include the use of genetic marks, and physical or chemical marks which may be either intrinsic or artificial. The different methods each have unique advantages and disadvantages. Genetic marks are highly accurate but like intrinsic physical marks such as scale and otolith annuli analysis require specialised staff and costly laboratory equipment. Artificial physical marks such as coded wire tags show variable and sometimes poor

retention and high mortality. Intrinsic chemical marks such as analysis techniques using Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LAICPMS) also require specialised staff and costly laboratory equipment. Of several chemical marking methods, calcein so far is the only one to have established field detection methods and is also relatively cheap to implement. Calcein marking potentially offers a marking method with the least stress and damage to the fish with the added benefit of being cost effective.

The chemical fluoro-chrome dye calcein has been widely trialled for marking a number of species with variable results. Calcein binds with calcium phosphate in bony structures, and when it complexes with alkaline earth salts a fluorescence emission results (Bart *et al.* 2001; Honeyfield 2006). This produces a greenish colour seen with either the aid of filter glasses and a UV black-light, fluorescence microscopy, or detected with an electronic detector (fluorometer). A variety of techniques to mark fish with this chemical have been trialled including immersion, injection, dietary uptake and 'osmotic induction'.

Immersion techniques showed some promising results (Wilson *et al.* 1987; Brooks *et al.* 1994; Mohler 1997) but can take up to 48 h, with subsequent issues in relation to volume, dye cost and water quality concerns. These increase rapidly as fish numbers increase and are likely to present a management problem. The application of calcein via injection is time-consuming(Monaghan 1993), a critical point when large numbers of marked fish may be desired, also dosage rates via injection are critical with 80% mortality rate observed in some injection treatments (Gelsleichter *et al.* 1997).

Investigations into dietary intake of calcein showed complications upon food palatability by the inclusion of calcein, hampering the use of the technique with some

species (Honeyfield 2006). The extra processes required to produce feeds that contain calcein are considered a disadvantage of the technique.

A technique using hypo-osmotic water to dehydrate the fish followed by immersion in a calcein solution to induce uptake of the chemical has been termed 'osmotic induction' (Alcobendas *et al.* 1991; Mohler 2003; Crook and O'Mahony 2009a). Osmotic induction allows good stain quality to be obtained in around 15 mins with little or no mortalities (Mohler 2003), and has been successfully used on Golden Perch, Murray cod and Silver Perch (Negus and Tureson 2004; Crook and O'Mahony 2009a; Crook *et al.* 2009b). Thus for the production of calcein marks, the advantages seem to be with the osmotic induction technique.

Calcein trial methodology was based on osmotic induction protocol developed by Crook (2009a) for Golden Perch.

Sunlight has been suggested as a factor affecting mark retention and fading with some good supporting evidence (Leips *et al.* 2001; Bashey 2004; Honeyfield *et al.* 2008), suggesting this factor needs to be taken into account where investigating the technique with new species. Longer retention of internally detectable calcein marks compared to external marks has been shown, which is important knowledge for use of the technique (Elle *et al.* 2010).

When reintroductions occur, important information can be gained from monitoring efforts utilising calcein marking as an effective population study tool. Development of calcein marking techniques for the species will enable mark recapture studies of population dynamics post release. Knowledge of population persistence, recruitment, population growth rates and population number estimates will inform management agencies of the best locations for releases there-by maximising outcomes

from restocking and ultimately contribute to conservation of the species. As such the technique refined by this project for this species specifically should provide knowledge to guide management agencies (DEWNR) to save Yarra Pygmy Perch from extinction in the MDB.

The aim of the current work was to develop protocol for the non-lethal marking of Yarra Pygmy Perch with calcein via osmotic induction, determine whether the developed method has any effects upon growth or survival for this species and investigate and quantify mark retention/fading in the developed method under natural lighting compared to reduced light exposure, under natural temperature conditions for the species, to trial and assess accuracy, capabilities and limitations of the technique in the field, and to maximise capabilities of the developed methods where-ever possible.

Materials and Methods

General

Fish used in Experiment 1 A & B were collected from Crouch dam, a DEWNR refuge site for the species (Bice *et al.* 2012). At initiation of Experiment 1 A fish were of mean length 34.2 mm (SD 3.4), and mean weight 0.56g (SD 0.19) (n=180), for Experiment 1 B fish were of mean length 43.8 mm(SD 3.8), and mean weight 1.14 g(SD 0.58) (n=48). Fish were fed equal quantities per tank of either, frozen brine-shrimp, live black-worm or live *Daphnia* sp. on Mondays, Wednesdays and Fridays, with all tanks receiving the same food type on any given day. Any mortalities were recorded on a daily basis, being bagged, labelled and frozen. All analyses for this project were performed in SPSS 20.0 with graphing performed in SigmaPlot 12.0.

Experiment 1A: Laboratory trial of Calcein, the effect of sunlight and growth on mark retention.

Experimental set-up consisted of a total of 6 treatment and control groups that were replicated three times, with 10 fish per replicate, using a total of 18 Aquaria, and 180 fish in total. Aquaria were housed outdoors in a shaded position (shade-cloth roof) to replicate ambient conditions of temperature and natural light conditions representative of those likely to be experienced by released fish. 'Dark' treatment tanks were additionally covered with black plastic to reduce light exposure; 'Light' treatments were not. All tanks were supplied recirculated water, however different treatments were run from separate 40L biofilter systems to limit the possibility of cross-

contamination of dissolved calcein between treatments. Biofilter systems were aerated and filled with Kaldnes K1 moving bed™ bio-filter media, Developed by Professor Halvard Odergard at Trondheim University of Science & Technology, Norway.

Calcein trial methodology was based on osmotic induction protocol developed by Crook (2009a) for Golden Perch, who suggested a 5 min salt bath in 50 g/L followed by 10min bath in 5 g/L of calcein. Crook (2009a) investigated exposure time and found 5mins in salt solution the optimum, and that exposure time was adopted here. However because of species specific physiological differences, evidenced by comparatively reduced tolerance to salinity in Yarra Pygmy Perch when compared to Golden Perch (Kefford *et al.* 2004; McNeil *et al.* 2010b; Mahon 2012), osmotic induction protocol required modifications. As such 25 g/L and 15 g/L salt balts were trialled.

Calcein solutions were buffered with sodium bicarbonate to a pH of 7.0 ($\pm$0.2) prior to use. To minimise stress to the fish all salt and calcein bath temperatures were brought in line with that of the husbandry tanks by floating sealed containers in the sump until temperatures were $\pm$0.1ºC. A kitchen strainer and bowls were used to transfer the fish between treatments minimising fish handling. In line with methods developed by (Crook and O'Mahony 2009a), the fish were placed into the salt solution for 5 mins, momentarily dipped into freshwater and drained, then placed into the calcein solution for 10 mins, momentarily dipped into freshwater and placed into the appropriate husbandry tank. Rinsing and draining protocol was designed to prevent contamination of the calcein solution with salt, and likewise the husbandry tanks with calcein. The calcein solution was thereby conserved for future use, and the effect of excessive amounts of free calcein in the laboratory trial was minimised.

Individual treatments are stipulated below in Table 1:

Table 1: Experimental design for experiment 1A, 10 fish per replicate tank, all treatments except controls received a 10min bath in 5 g/L of calcein.

Staining pre-treatment salinity concentration (5 minute bath)	light exposure	
	Light	Dark
25 g/l (High)	3 replicate tanks	3 replicate tanks
15 g/l (Low)	3 replicate tanks	3 replicate tanks
control (no salt)	3 replicate tanks	3 replicate tanks

All tanks were initially covered with a tarpaulin for 48 h to reduce stress.

Sample Fluorescence Ratio

Fluoro-meter readings ranging 0-1400 Sample fluorescence ratio (SFR) were taken from fish with an Opti-sciences® GFP-meter before marking and after marking then monthly (month1-month 8) to give quantified data of mark intensity over time. Three recordings of SFR were taken from each fish, with the highest value used for analysis, methods suggested by (Crook *et al.* 2009b). The highest value is used because false negative values are more likely than false positives, due mostly to an often patchiness in mark distribution seen about the fish. SFR recordings were taken from the gill pre-operculum alternating between each side of the fish in-between recordings, with the fish under a black sheet to reduce any possible effects of ambient light. Initially also an additional single recording was taken from the caudal peduncle, however these were later dropped when quicker fading was noted than from gill pre-operculum. The same calibration was used for all of the sampling events, with 10 data points recorded

from the calibration standards (control and reference cards from Opti-sciences®) at each sampling event to give an estimation of background variance. For the purposes of data analysis where signals were above the fluoro-meter measurement limit (signal overload=above 1400), these were entered as 1400.

Light exposure

Light levels were recorded with an Extech® light probemeter at the water surface three times from each tank in Lux, on three days at each time period: Initiation; Month 3; Month 6; and on completion at Month 8. The light probemeter had an upper measurement limit of 2000 lux.

Growth

Fish were weighed (to nearest 0.01g) and measured (Total Length (TL) to nearest mm) before and after the trial. Fish were measured on a measuring board then picked up with the use of a plastic card and dropped into a container of water on-top the scales that had been zeroed before and between fish. Specific Growth Rate Weight (SGRW) and Specific Growth Rate Length (SGRL) were calculated (Cook *et al.* 2000).

Water Quality

Salinity (ppt), DO (ppm), temperature (°C), pH, ammonia (ppm), nitrite (ppm), nitrate (ppm), general hardness (ppm) and carbonate hardness (ppm) were recorded weekly for the first six weeks, and from then on weekly, with the exception of ammonia, nitrite, nitrate, general hardness and carbonate hardness being performed monthly. Salinity, temperature and pH were measured with a YSI® 63/10 FT, DO with a Handy Polaris by OxyGuard®, while ammonia, nitrite, nitrate, general hardness and carbonate

hardness determined by Aquarium Pharmaceuticals® test strips. The YSI® and OxyGuard® meters were calibrated monthly.

Data analysis

Sample Fluorescence Ratio, visual assessment of QQ-plots investigated for normality, and homogeneity of variance assumptions were tested by Brown-Forsythe. A one-way repeated measures analysis of variance (RM ANOVA) was performed to investigate the effect of light exposure and salinity pre-treatment level through month 1 to 8, with month used as the within subject factor, and 25 g/L Light, 15 g/L Light and control Light treatments as the between subject factors, note Dark treatments were not included in this analysis as these were considered comparative controls only, and are not statistically relevant to the realistic use of the technique. A Kruskal-Wallis test across the three different light exposed treatments was performed at month 8 (normality assumptions for ANOVA were violated) to determine where significant differences were no longer evident between light exposed treatments and controls, thereby suggesting when marked and unmarked fish can no longer be separated on the basis of fluorometer readings. A cut-off value to denote marked/unmarked fish was set at of 50 SFR, being control mean SFR +2 Standard Deviations (mean 9.55, SD 16.5) rounded up to the nearest factor of 10 SFR, and assessed for accuracy(%) at determining marked/unmarked fish by month.

Light Exposure, Light and dark treatments were examined separately in terms of Lux with visual assessment of QQ-plots investigated for normality, and homogeneity of variance assumptions proven using Brown-Forsythe. One way between groups analysis of variance (ANOVA) was performed to explore light levels between the treatment groups.

Growth, one way between groups ANOVA was performed to determine any potential effects of the calcein staining protocol, associated handling and light exposure on growth or potential growth differences between treatments that could theoretically contribute to calcein mark fade. Visual assessment of QQ-plots were investigated for normality, and homogeneity of variance assumptions by Brown-Forsythe.

Water Quality, visual assessment of QQ-plots investigated for normality in temperature, pH and TDS (ppt) across treatments. Homogeneity of variance assumptions across treatments was investigated using the Brown-Forsythe test. One way between groups ANOVA was used to investigate for differences across treatments within temperature, pH, TDS.

Post trial otolith examination (using samples from experiment 1A).

5 fish per treatment were randomly selected at the completion of experiment 1A, euthanized with 20 ml/L of AQUI-S, then stored in ethanol for a week prior to otolith removal. 12 fish per treatment from an earlier pilot trial were also included to test for possible effects of ethanol on auto-fluorescence. Sagittae (otolith) were removed under dissection microscope and examined under fluorescence microscopy using a BX-51 Olympus® microscope 4x magnification and filter number 3 (U-MW1B2 Exc. 460-495DM, 505 Em: 510 LP). Visual assessment of stain was trialled, allocating a score to relative ease of mark visualisation (0=no mark, 1=low visibility, 2=medium visibility, or 3=high visibility). An image of each otolith was recorded and analysed for mean intensity and area of fluorescence with the image analysis software package FIJI®. All Fluorescence measurements are reported as mean $\pm$ SE.

Experiment 1B: Calcein mark optimisation, binding in dark conditions pre-sunlight exposure.

To test if delaying exposure to sunlight would allow more binding of the calcein with calcium in bony structures, increase stain quality, and subsequent stain retention when exposed to sunlight radiation, an experiment was set-up consisting of 2 treatments. Treatments were replicated three times, with 8 fish per replicate, using a total of 6 Aquaria and 48 fish. As per experiment 1A, aquaria were housed outdoors in a shaded position. All tanks were supplied re-circulated water with different treatments run from separate sump-bio-filter systems. Osmotic induction protocol as per Experiment 1A (25 g/l salt solution) was followed. The two post marking treatments were as follows:

- No dark.
- 2 weeks dark.

Osmotic induction of calcein was staggered so as all treatments were first exposed to light on the same day, however the same calcein solution was used for all treatments. The experiment ran for 4 months. Protocol for the recording and analysing of SFR, light, growth and water quality were the same as per experiment 1A.

Experiment 2: Field validation of Calcein marking

Yarra Pygmy Perch for this trial were obtained from DEWNR captive held stock. A total of 288 Yarra Pygmy Perch were calcein marked using 25 g/L salt solution as per Experment 1A osmotic induction methods above. Following marking, these fish were held in captivity for three days in dark plastic tubs prior to release. Prior to release

Total length (mm) was measured from a sample (n=57) of fish. The marked fish were then released into Tupelogrove Dam on the 29/04/2011, a DEWNR refuge site for the species (Figure 1).

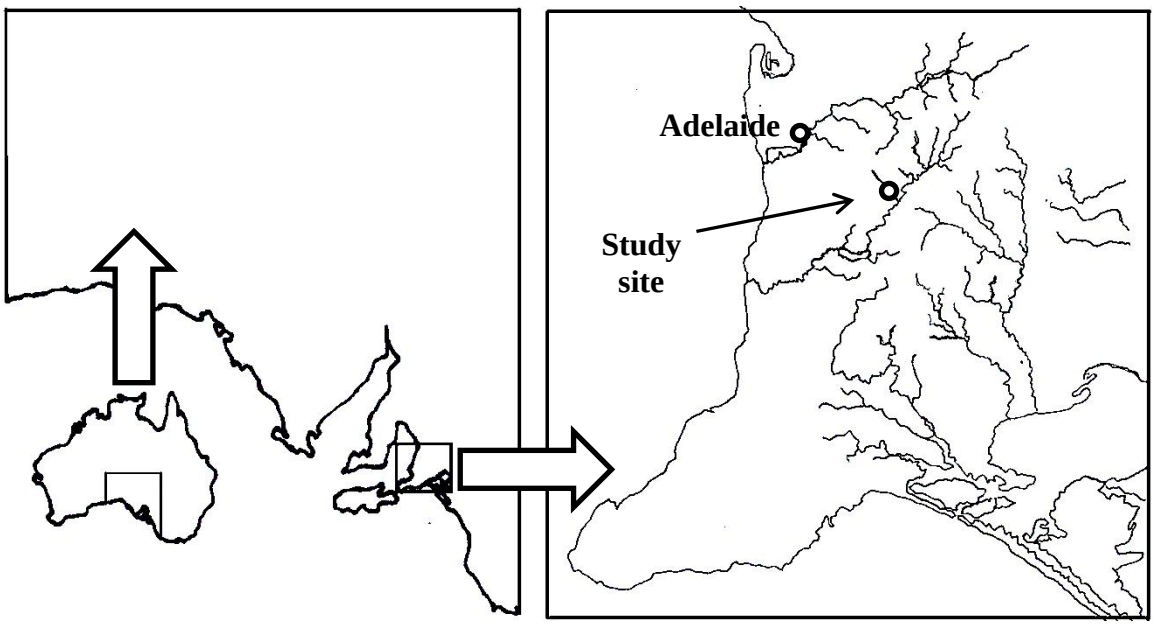


Figure 1 : Location of Tupelogrove field trial site (experiment 2).

Tupelogrove dam has abundant macrophyte cover (figure 2 in appendix 2) similar to the species' original habitats in the Murray-Darling Lower Lakes. DEWNR pre-release surveys of Tupelogrove dam showed an absence of any fish species. It is a closed system with no connection to other aquatic habitats. These facts make it an ideal field trial site to establish accuracy of calcein marking in the field.

Sampling in this dam occurred at 8 months and 12 months post-release. Seven fyke nets were set over-night and retrieved the following morning. Upon net retrieval all fish were transferred to holding tubs, 40L black plastic tubs of water, supplied with aeration, used to temporarily hold fish in the field until measured/counted.

SFR were taken with an Opti-sciences® GFP-meter with equipment and calibration as per Experiment 1A. A subsampling protocol was employed, where a

random sample of 600 fish maximum were measured, with all fish being measured if numbers caught were below this, and any numbers of fish above 600 were simply counted. SFR were recorded from each fish as per procedure described in Experiment 1A. Corresponding data on TL (to nearest mm) was also recorded from each SFR measured fish. All fish were released back into the dam.

Data analysis

Data analysis aimed to determine the origin of fish (original release verses new recruitment by co-hort) and estimate stain retention rate and accuracy of mark detection via comparative analysis of length and fluoro-meter reading. Population estimate at 8 and 12 months post release assumed a mortality rate of zero, and an equal recapture rate between released fish and new recruits, and was calculated by

$$Total\ population\ estimate = \left(\left(\frac{no.of\ released\ fish}{known\ mark\ recaptures}\right) \times (no.of\ known\ unmarked\ fish)\right) + no.of\ released\ fish$$

Recapture rate was calculated by

$$Recapture\ rate = \frac{no.of\ released\ fish}{known\ mark\ recaptures}$$

Light exposure

Light intensity (lux) was recorded by habitat type (Open water, Typha, Myriophylum) and depth (0, 0.25, 0.5, 0.75 and 1.0m) under broken cloud conditions in November. Light intensity was measured with an Extech® light probemeter, which had an upper measurement limit of 2000 lux. The probe on the unit was sealed inside a plastic container to enable sub-surface readings, cord length prevented measurements below 1m.

Water Quality

Water Quality parameters of Salinity, DO, Temperature, pH, ammonia, nitrite, nitrate, general hardness and carbonate hardness were recorded at initial release and at 8 and 12 months with equipment as per Experiment 1A.

Results

Experiment 1A: Laboratory trial of Calcein, the effect of sunlight and growth on mark retention.

Sample Fluorescence Ratio

SFR recordings taken from all test fish prior to any treatment showed low background SFR (mean 1.0 SD, 4.5, max 64). Fish recovered quickly from all treatments and the associated handling. Fish fed well in all tanks within 6 hours post treatment.

SFR recorded post treatment showed high initial recordings from both staining protocols tested, with 100% of fish registering a signal overload (above 1400 SFR). Post-treatment SFR recordings from both control groups showed no evidence of contamination with calcein, and indeed for the complete duration of the experiment low SFR recordings were seen in both Control/Light (mean 3.7 SFR, SD, 9.0) and Control/Dark (mean 0.9 SFR, SD, 2.9). SFR from both staining protocols in dark conditions remained high for the duration of the trial, where 25 g/L Dark was (mean 1369.6 SFR, SD, 120.0), and 15g/L Dark was (mean 1400.0 SFR, SD, 0.0), minimum recording from a stained fish in a Dark treatment was 631 SFR, from ‘High’ protocol.

SFRs in both salinity pre-treatment protocols in light conditions showed initially high recordings, where both 25 g/L Light and 15g/L Light were signal overloads (mean 1400.0 SFR, SD, 0.0). However values observed in both 25 g/L Light and 15g/L Light treatments reduced through-out the experiment (Figure 2). SFR in all calcein stained Light treatments underwent the largest reductions during month one.

One-way repeated measures analysis which showed a significant effect for time, Greenhouse Geisser =0.157, $F(1.2, 35) = 218$, $p = 0.0005$, multivariate partial eta squared = 0.97, and Treatment, Greenhouse Geisser =0.157, $F(2.5, 35) = 54.5$, $p = 0.0005$, multivariate partial eta squared = 0.94. Pairwise comparisons suggested 25 g/L light and 15 g/L light were not significantly different ($p>0.05$), where control Light treatments were significantly different ($p=0.005$). Assessment of 25 g/L light, 15 g/L Light and control Light at 8 months showed violation of homogeneity of variance assumptions by Levene's, welch and Brown forsythe. As such the non-parametric Kruskal-Wallis test was used. A Kruskal-Wallis test at month 8 showed a statistically significant difference in SFR across the three different light exposed treatments; 25 g/L Light (n=22) and 15 g/L Light (n=30), Control Light treatments (n=22) X^2 (2 n=74) =31.30, p=0.000. 15g/L Light recorded the highest mean rank (52.73) followed by 25 g/L Light (35.73) and Control Light (18.93) treatments respectively. Although significant differences between these treatments are evident at 8 months the use of 50 SFR and above as a cut-off value to denote marked/unmarked fish could not separate these with sufficient accuracy (Table 2). The use of 50 SFR and above as a cut-off value to denote marked/unmarked fish showed decreasing accuracy with time in light exposed treatments, but no changing trend was seen in control or Dark treatments (Table 2).

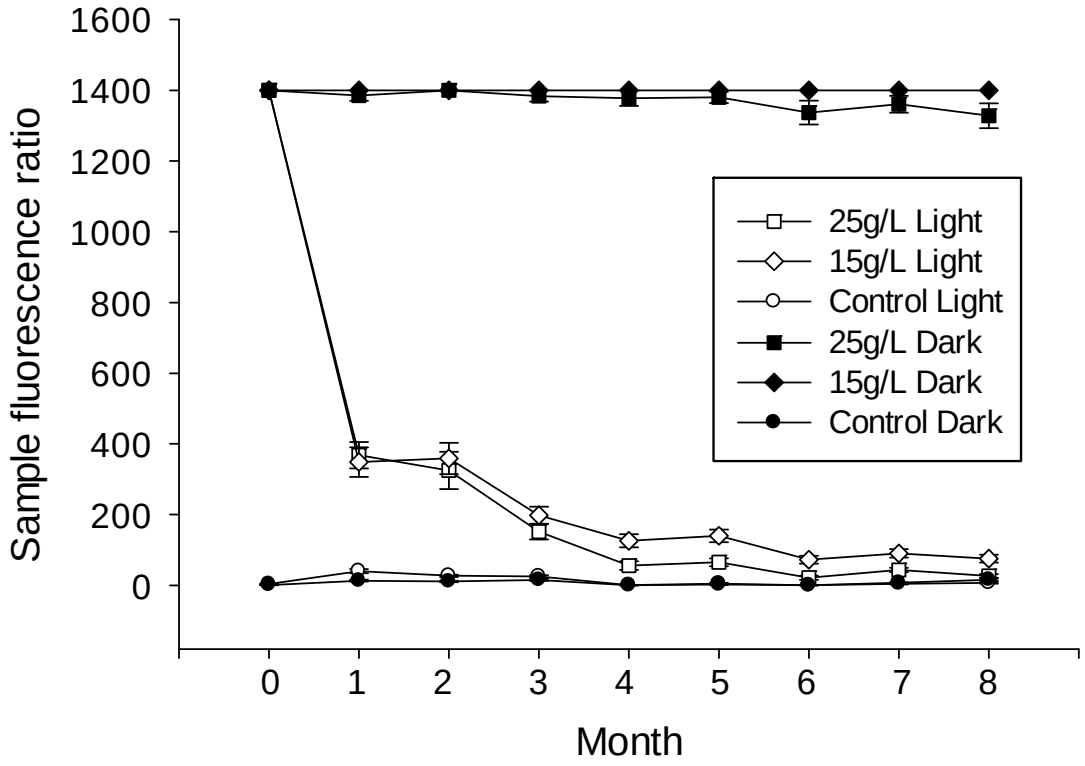


Figure 2: Experiment 1A, SFR (mean of maximum values ± SE) by treatment with time (n/treatment=30).

Table 2: Experiment 1A, Accuracy (%) of mark recognition, testing the validity of 50 SFR and above, as a cut-off value to denote mark.

	Treatment					
month	25 g/L Light	15 g/L Light	Control Light	25 g/L Dark	15 g/L Dark	Control Dark
0	100	100	100	100	100	100
1	100	100	73.3	100	100	93.3
2	100	100	89.3	100	100	100
3	100	86.2	87.5	100	100	100
4	34.8	69	100	100	100	100
5	54.5	70	100	100	100	100
6	18.2	60	100	100	100	100
7	21.7	66.7	100	100	100	100

8	13.6	56.7	100	100	100	96

Light exposure

Light levels in terms of Lux within light exposed treatments were similar, as were those in dark exposed treatments, but mean differences are obvious between Light and Dark treatments (Table 3). Light and Dark treatment were examined separately in terms of Lux and showed normality, and homogeneity of variance assumptions shown at the $p < 0.05$ level by Brown-Forsythe for light ($p = 0.953$) and Dark ($p = 0.812$). One way between groups ANOVA showed no significant differences at the $p < 0.05$ level within light treatments: $F(2,131) = 0.048$, $p = 0.953$, and within dark treatments: $F(2,108) = 0.208$, $p = 0.812$.

Table 3: Light (Lux, mean ± SD)) by treatment in experiment 1A.

Treatment	Light exposure (Lux)
25g/l Light	754 ± 564
15g/l Light	725 ± 526
control Light	721 ± 570
25g/l Dark	6 ± 5
15g/l Dark	7 ± 9
control Dark	7 ± 7

Growth

Growth rates in terms of both weight (g) and Length (mm) across treatments were equal. Normality was seen in SGRW and SGRL, homogeneity of variance assumptions across treatments were shown at the $p < 0.05$ level by Brown-Forsythe for SGRW ($p = 0.199$) and SGRL ($p = 0.117$). One way between groups ANOVA showed no significant differences at the $p < 0.05$ level in SGRW between treatments: $F(5,12) = 2.4$, $p = 0.102$, and in SGRL between treatments: $F(5,12) = 3.05$, $p = 0.053$. As such it can

be said the calcein staining protocol, associated handling and light exposure levels had no lasting effects on growth.

Table 4: Specific growth rates (SGR, mean ± SD) for Weight (g)/day and Total Length (mm)/day by treatment from Experiment 1A.

Treatment	SGRW	SGRL
25g/l Light	0.10 ± 0.01	0.30 ± 0.04
15g/l Light	0.07 ± 0.01	0.12 ± 0.05
control Light	0.10 ± 0.01	0.20 ± 0.03
25g/l Dark	0.08 ± 0.04	0.16 ± 0.12
15g/l Dark	0.08 ± 0.02	0.21 ± 0.03
control Dark	0.10 ± 0.02	0.25 ± 0.07

Water Quality

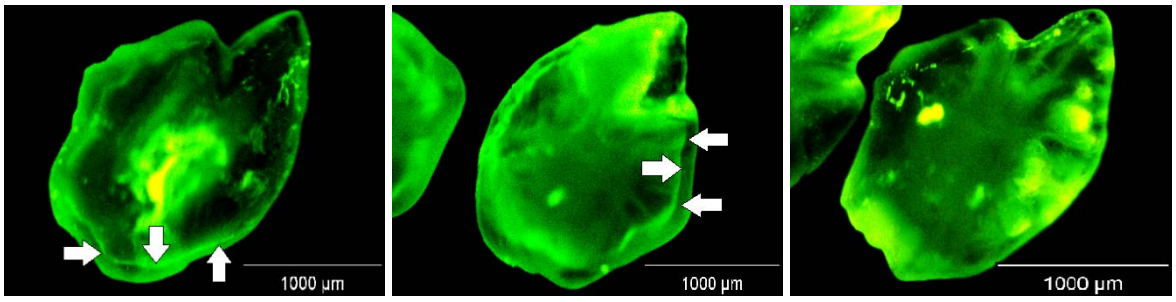
Normality was seen in temperature, pH and TDS across treatments. Homogeneity of variance assumptions across treatments were shown at the $p > 0.05$ level by Brown-Forsythe for temperature ($p = 0.995$), pH ($p = 0.681$) and TDS ($p = 0.995$). One way between groups ANOVA showed no significant differences across treatments at the $p < 0.05$ level in temperatures: $F(2,138) = 0.005$, $p = 0.995$, pH : $F(2,123) = 0.385$, $p = 0.681$ and TDS: $F(2,137) = 0.412$, $p = 0.663$ (Table 5). Temperature varied through time during the experiment with large fluctuations (see figure 10 in appendix 3). General hardness and carbonate hardness remained steady at 120 ppm for the duration of the experiment. Ammonia, nitrite and nitrate remained below detectable concentrations for the duration of the experiment.

Table 5: Water quality parameter during Experiment 1A (mean ± SD).

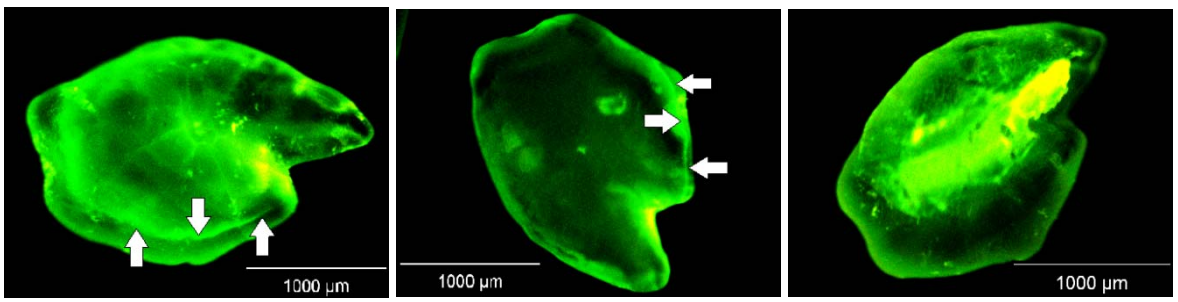
Treatment	Temperature (°C)	pH	Salinity (ppt)	DO
25g/l Light and Dark	17.9 ± 0.6	7.9 ± 0.1	0.3 ± 0.0	104.9 ± 2.7
15g/l Light and Dark	17.9 ± 0.7	7.9 ± 0.0	0.3 ± 0.0	105.1 ± 2.9
control Light and Dark	17.9 ± 0.8	7.8 ± 0.0	0.4 ± 0.0	104.2 ± 4.1

Post trial otolith examination (using samples from experiment 1A).

Visual assessment of whole otolith (sagittae) under fluorescence microscopy from fish 8 months after staining at the completion of experiment 1A showed variable mark retention, with mark loss due to light exposure (Figure 3). Accuracy at determining mark presence/absence on otoliths visually was 100% on all Dark treatment samples, and all Control samples, independent of light/dark exposure, alcohol/no alcohol exposure (Table 6). As such there is no evidence here for auto-fluorescence due to alcohol exposure. Conversely mark fade on light exposed treatments made mark recognition difficult, accuracy at determining mark on 25 g/L Light was 76.9% where 15 g/L Light treatments was lower at 64.3%. The use of the image analysis software FIJI was unsuccessful at determining mark due to high background auto-fluorescence.



25 g/L Dark **15 g/L Dark** **Control Dark**



25 g/L Light **15 g/L Light** **cControl Light**

Figure 3: Calcein marks identified visually on whole Sagittae under fluorescence microscopy (arrows indicate mark location, note: no marks seen on controls), samples from experiment 1A.

Table 6: Mean score, SD, N and % accuracy at mark recognition by Treatment from examination of sagittae, samples from experiment 1A (* indicates samples from and earlier pilot trial used)

Treatment	Score	n	% accuracy at mark recognition	% error at mark recognition
25g Light	1.1 ± 0.2	13	76.9	23.1
15g Light	0.9 ± 0.2	14	64.3	35.7
control Light	0.0 ± 1.0	8	100	0
25g Dark	2.7 ± 0.2	9	100	0
15g Dark	2.7 ± 0.2	8	100	0
control Dark	0.0 ± 1.0	9	100	0
25g no alcohol *	2.3 ± 0.2	12	100	0
Control no alcohol *	0.0 ± 1.0	11	100	0

Experiment 1B: Calcein mark optimisation, binding in dark conditions pre-sunlight exposure.

Sample Fluorescenece Ratio

Delaying of exposure to sunlight increases retention of the stain when exposed to sunlight radiation, but only slightly (Figure 4). Although SFR initially faded slightly more in the first month in the 2 week dark post treatment fish, they remained consistently higher than control for the remainder of the trial. Normality justified one-way repeated measures analysis which showed no significant effect for time, Greenhouse Geisser $=0.302$, $F\ (1.2, 4.8) = 218$, $p= 0.096$, multivariate partial eta

squared = 0.51. However a Kruskal-Wallis test at month 4 showed a statistically significant difference in SFR between 2 week Dark treatment (n=21) and control (n=25); X^2 (2 n=46) =6.44, p<0.05. The 2 week dark post treatment group recorded the highest mean rank (28.95) followed by control (18.92). Mean difference between the two treatments at month four shows the 2 week dark treatment (at 1095 SFR) some 364.4 SFR above the control (at 844 SFR).

The use of 50 SFR and above to denote marked fish was 100% accurate for both treatments for the duration of Experiment 1B. Overall rate of stain loss (excluding first month as mean here is above measurement limit) for the 2 week dark post-treatment group is 49.6 SFR/month, where control group was 148.9 SFR/month. Delaying early exposure to sunlight radiation by 2 weeks post-treatment increased mark retention, there-by without this treatment significant decreases in mark retention occurred.

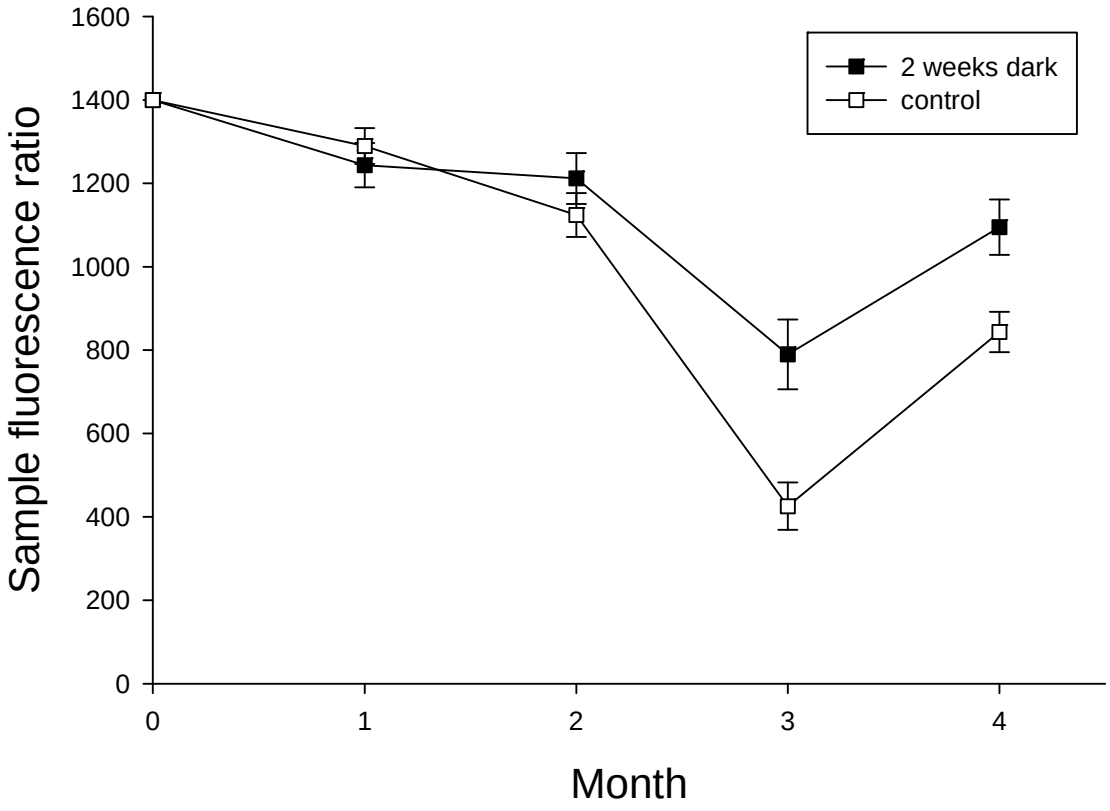


Figure 4: Experiment 1B, SFR (mean of maximum values ± SE) by treatment with time (n/treatment=27).

Light exposure

Light levels in terms of Lux between 2 weeks Dark and control were similar (Table 3). Light and Dark treatment were examined in terms of Lux with normality observed, and homogeneity of variance assumptions shown at the $p > 0.05$ level by Levene's. Independent samples t-test showed no significant differences between 2 weeks Dark and control groups: $t(52) = 0.505$, $p = 0.616$, two-tailed.

Table 7: Experiment 1B, Light (Lux, mean ± SD) by treatment (n/treatment=27).

Treatment	Light exposure (Lux)
2 weeks Dark	317.5 ± 176.6
Control	292.3 ± 189.7

Growth

Growth rates in terms of both weight (g) and length (mm) across treatments were similar. Normality was observed in SGRW and SGRL, homogeneity of variance assumptions across treatments were shown at the $p > 0.05$ level by Levene's for and SGRL ($p = 0.650$) but not for SGRW ($p = 0.03$). Independent samples t-test showed no significant differences between 2 weeks Dark treatment and control groups for SGRW ($t(2.0) = 1.801$, $p = 0.211$, two-tailed), or SGRL ($t(4) = -0.249$, $p = 0.67$, two-tailed).

Table 8: Experiment 1B, Specific growth rates (SGR, mean ± SD) for Weight (g)/day and Length (mm)/day by treatment (n/treatment=27).

Table 9: Experiment 1B, Specific growth rates (SGR, mean ± SD) for Weight (g)/day and Total Length (mm)/day by treatment (n/treatment=27).

Treatment	SGRW	SGRL
2 weeks Dark	0.01 ± 0.02	-0.01 ± 0.01
Control	-0.19 ± 0.19	0.00 ± 0.01

Water Quality

Normality was observed in temperature, pH and TDS across treatments. Homogeneity of variance assumptions across treatments were shown at the $p < 0.05$ level by Levene's for temperature ($p = 0.985$), pH ($p = 0.756$) and TDS ($p > 0.05$). Independent samples t-test showed no significant differences across treatments at the $p < 0.05$ level in temperatures: ($t(34) = -0.116$, $p = 0.909$, two-tailed), pH : ($t(34) = 0.155$, $p = 0.877$, two-tailed) and TDS: ($t(34) = -0.517$, $p = 0.608$, two-tailed) (Table 5). General hardness and carbonate hardness remained steady at 120 ppm for the duration of the experiment. Ammonia, nitrite and nitrate remained below detectable concentrations for the duration of the experiment.

Table 10: Experiment 1B, water quality parameters (mean ± SD).

Treatment	Temperature (°C)	pH	Salinity (ppt)
2 weeks Dark	19.8 ± 2.9	8.0 ± 0.3	0.3 ± 0.1
Control	19.9 ± 2.9	8.0 ± 0.3	0.3 ± 0.2

Experiment 2: Field validation of Calcein marking

8 months post release

A total of 288 fish, with a mean total length of 31.7mm (SD 7.1), were released into the site. At 8 months post release a total of 119 fish were captured. Length data clearly shows two distinct groups of fish: larger fish released into the site (35-60mm) and new recruits (14-23mm). Large variation in SFR was seen within the group of larger size fish

(Figure 5), which are undoubtedly the released fish, while the group of smaller size fish all showed low-no recordings of SFR, clearly indicating them as new recruits. In terms of length there is no overlap between the two size groups. Thereby we can make the assumption that the two cohort sizes are distinct, group A: released fish (35-60mm), and group B: new recruits (14-23mm), and are without overlap. The large variation in SFR seen within the group of larger size fish suggests variation in calcein retention, with much fade of mark evident in some individual fish but others no measurable fade.

The use of the highest observed value and 500 SFR as a cut-off point to signify marked fish from non-marked fish applied here resulted in mark identification accuracy rate for positive identification of a marked fish at 8 months of 61%. Using 50 SFR as a cut-off value, the resultant accuracy rate is 88.3%, at 8 months post release. This suggests a recapture rate of 20.8%.

No data are available on mortality rate in the released fish, there-by a mortality rate of zero was assumed, and an equal recapture rate between released fish and new recruits. Calculations estimated 283 new recruits, and a total population of 571 Yarra Pygmy Perch in the dam at 8 months post-release.

Temperature at the site at 8 months post release was 20.1°C, pH was 7.1, TDS was 0.1ppt, Dissolved oxygen was 102.4%, general hardness and carbonate hardness were both 120ppm.

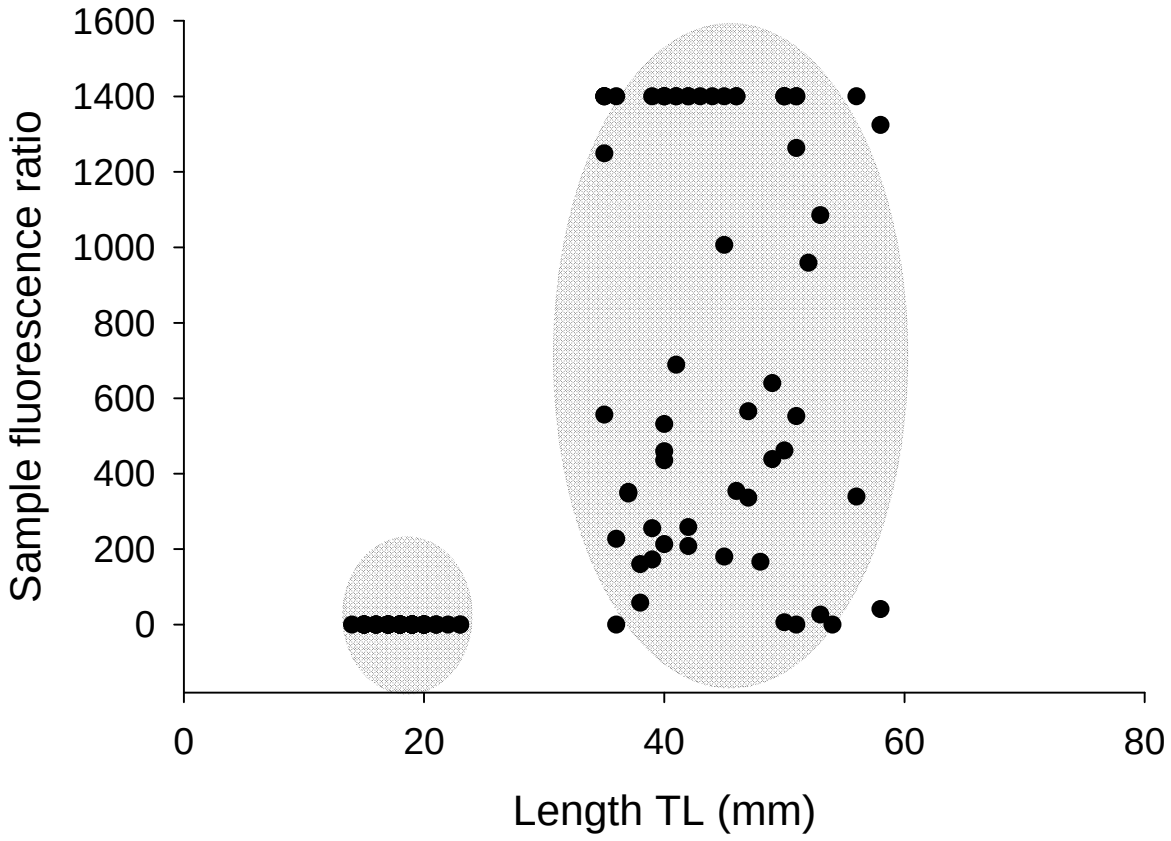


Figure 5: Experiment two, SFR (max value) by length in Yarra Pygmy Perch at 8 months post release (n=119), shading represents separate co-horts, being released fish and new recruits.

12 months post release

At 12 months post release, 1028 fish were captured. SFR recordings taken from the 600 sampled fish showed large variation (Figure 6). Two groups of fish are evident, the larger fish (released fish) are (40-70mm) and the new recruits (25-46mm). More overlap of length between the two groups is seen than at 8 months. The use of the highest value and 50 SFR as a cut-off suggests an estimated a recapture rate of 14.9%. Because of the overlap of size ranges accuracy of the method at 12 months is undetermined. Assuming 88.3% accuracy as found at 8 months, a mortality rate of zero,

and an equal recapture rate between released fish and new recruits, there are an estimated 3730 new recruits, and a total population of 4018 Yarra Pygmy Perch in the dam, 12 months post-release. That represents a population increase by a factor of 14 in 12 months.

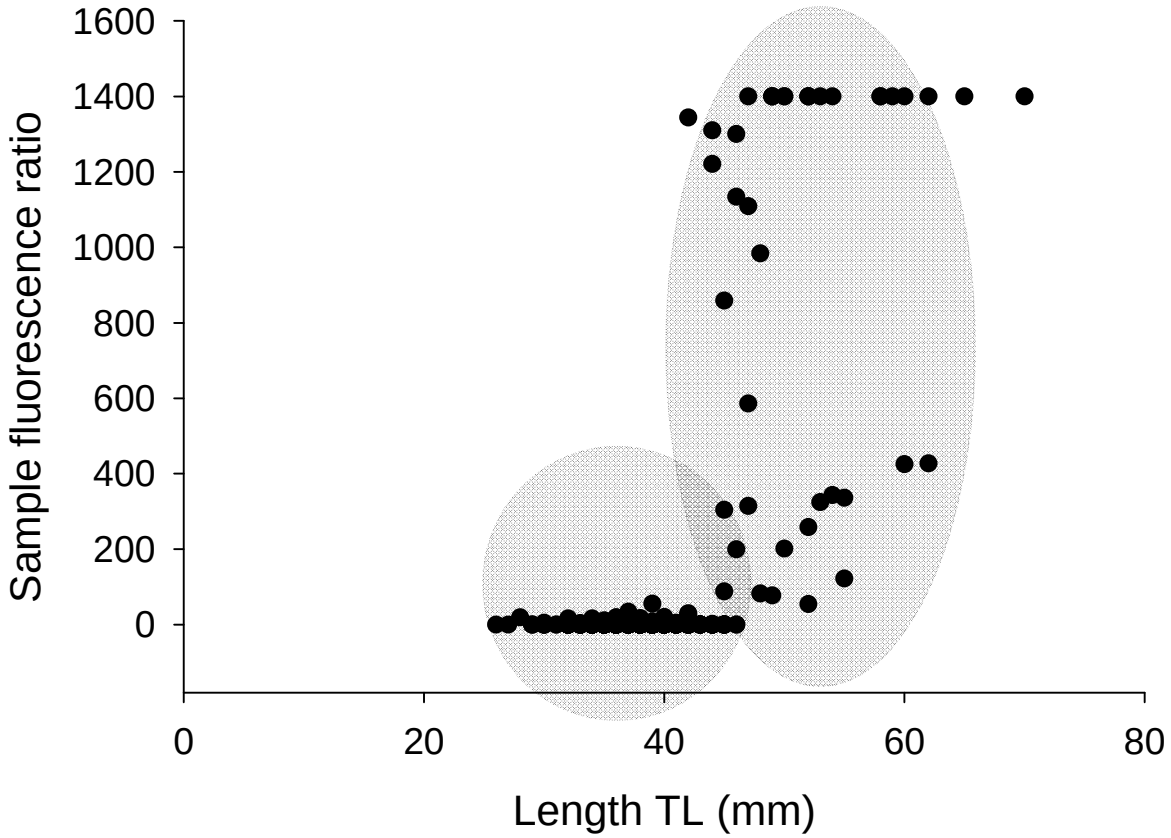


Figure 6: Experiment two, SFR (max value) by length in Yarra Pygmy Perch at 12 months post release (n=600) , shading represents co-horts, being released fish and new recruits.

Light levels (Lux) at Tupelogrove showed variation with vegetation type and reduction with depth from the water surface (Figure 7). Maximum depth at the site was 1.6m.

Temperature at the site at 12 months post release sampling was 17.4°C, pH was 7.2, TDS was 0.1ppt, Dissolved oxygen was 101.4%, general hardness and carbonate hardness were both 120ppm.

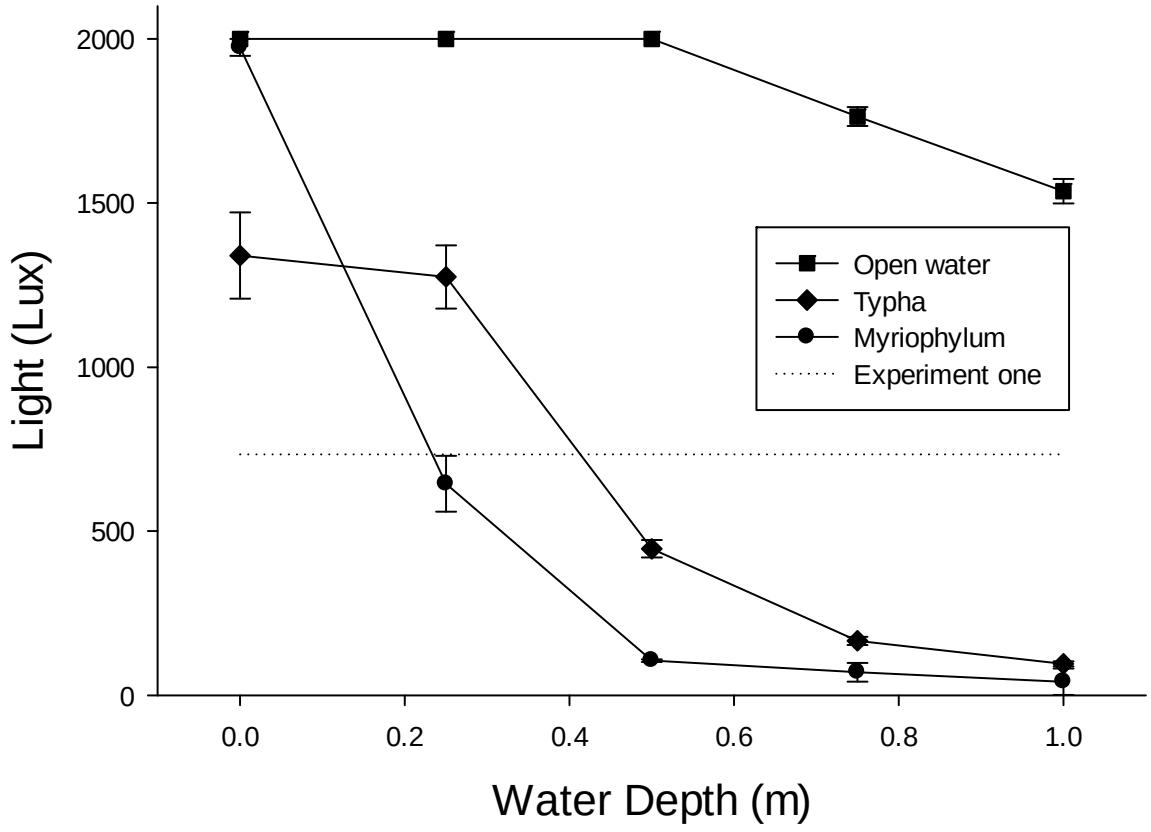


Figure 7: Experiment two, Light (Lux, mean ± SE) at Tupelogrove by vegetation/habitat type (Experiment 1A mean plotted for comparison only).

Discussion

Calcein marking as a non-lethally detectable marking method

Pilot trials established that Yarra Pygmy Perch are less tolerant to increased salt than Golden Perch, thus for osmotic induction purposes require, either, reduced salt concentrations or exposure times. Salinity tolerance reported for the species concurs with this observation, with very short survival times at high salinities (McNeil *et al.* 2010b). The choice of reducing salinity concentrations rather than exposure times for osmotic induction purposes was made with the logic that if 50 g/L causes mortality at 10 min exposure it is likely to cause undesirable cellular damage to the gills at shorter exposure times therefore increasing the risk of secondary infections. Additionally a thorough understanding of the effect of time upon dehydration is lacking, therefore it is considered a safer choice to stay within exposure times similar to those already successfully demonstrated for Golden Perch (Crook and O'Mahony 2009a). The use of 25 g/L NaCl solution pre-treatment for osmotic induction was chosen after close examination of recent salinity tolerance data for the species (McNeil *et al.* 2010b), and indeed has proven to produce strong marks with 100% survival, and little apparent stress.

Experiment 1A suggests that the main cause of calcein mark loss for Yarra Pygmy Perch is radiation from the sun, which corroborates findings for other species (Bashey 2004; Honeyfield *et al.* 2008; Elle *et al.* 2010). Photo-bleaching of calcein in the presence of free-radicals has been suggested as a factor by (Leips *et al.* 2001). UVA radiation causes the release of free iron in human cells (Pourzand *et al.* 1999; Reelfs *et al.* 2004),

which degrades calcein (Hasinoff 2003), and may be the mechanism behind the observed loss of mark in the current study. Though UVa is likely the cause, we cannot be certain from the current study and literature, but the use of light measured in Lux as in the current study seems a useful proxy for whichever wavelength in sunlight radiation is causing fade.

No evidence for an association between growth and mark loss was found in the current study. This does not however rule out growth as a factor worthy of consideration for mark trials. The Opti-sciences® GFP-meter used in the current study measures to a maximum of 1400 units with all detections at this and above registered as 'signal overload', so considerable calcein fade could have occurred but still remained above the measurement limit and therefore be undetectable. This measurement limitation means there could have been undetected mark fade in dark treatments in experiment 1A attributable to growth. However the small adult size of Yarra Pygmy Perch infers relatively small growth potential compared to other species upon which this technique may be employed. As such growth is not likely to be an issue for mark fade in Yarra Pygmy Perch. Calcein mark loss associated with growth has been reported (Negus and Tureson 2004), and there-fore it is suggested for other species especially larger bodied fishes, that growth should be considered as a potential factor influencing mark loss.

The use of the highest observed value and 500 SFR as a cut-off point to signify marked fish from non-marked fish has been suggested (Crook *et al.* 2009). The highest value from a known non-marked fish in the entire current study was 124 SFR, with a mean of 9.55 SFR (SD, 16.5). There-fore 500 SFR as a cut-off value is considered high and unjustified, with 50 SFR suggested more applicable. The use of 50 units and above

as a cut off to denote marked fish showed good accuracy with field trial of the method showing marked verses non-marked recognition accuracy of 88.3% at 8 months. Adequate stain retention was seen in the field and was comparatively better than that seen in the light exposed laboratory trials, though stain retention was more variable in the field. Comparatively, light levels in experiment 1A were within the range observed at Tupelogrove, though fish inhabiting vegetated areas below 0.25-0.45 are likely to receive less exposure to sunlight radiation than those fish in experiment 1A. There-by sunlight radiation expose differences between, experiment 1A and in the field (experiment 2), explain retention differences seen between these, furthermore, variation in habitat use by individual fishes likely explain the larger variability in retention seen in the field. As such higher stain retention observed in the field suggests fish are being exposed to less sun-light than in experiment 1A, which infers habitat use for the large part remains below and amongst vegetation or at maximum pool depth (1.5-1.6).

Although adequate stain retention has been seen here for Yarra Pygmy Perch, and recently good stain retention in the field shown for Southern Pygmy Perch (*Nannoperca australis*), poor stain retention has been reported for Southern Purple Spotted Gudgeon (*Mogurnda adspersa*), all within the MDB (Bice *et al.* 2011; Bice *et al.* 2012). I have personally observed captive juvenile Southern Purple Spotted Gudgeon in outdoor tanks actively rising to the surface and basking in sunlight, this may have been a behaviour in response to cool water with fish seeking slightly warmer water at the surface or because of lower dissolved oxygen (DO) with depth (McNeil and Closs 2007), although fish were not showing obvious signs of hypoxic stress (ie.rapid opercula movement). Any such surfacing behaviour is likely to drastically reduce stain retention.

Sites where Southern Purple Spotted Gudgeon showed poor stain retention were shallow and relatively open (Bice *et al.* 2012). Evidence for the effects of sunlight on mark retention from the current work and these observations suggest this as a risky habitat type for successful calcein use. Relative growth is likely greater in Southern Purple Spotted Gudgeon, than in Yarra Pygmy Perch, and is therefore a factor worthy of future investigation.

Shipping fish out for release 24 h post calcein staining has been a suggested protocol (Crook *et al.* 2009b). Experiment 3 shows the 2 weeks' dark treatment protocol increasing SFR 364.4 units above control at four months, with mark retention improved by a factor of 3 As fading continues this will increase mark identification accuracy for an appreciable and useful duration. In light of the current data, a two week period of dark or low light exposure post staining is recommended for fish headed for low turbidity environments and especially those headed for such habitats with little cover such as aquatic vegetation. Indeed sunlight exposure should be limited whereever practical to maximise calcein stain retention but especially so for post staining early exposure to sun-light. Protocol for calcein marking using external detection methods should also include sampling wild unmarked fish prior to release of any marked fish whereever possible, to give data on site and species-specific background SFR ranges.

Calcein marking as an internally detectable marking method

Examination of otolith samples post trial aimed to inform internal mark longevity. Internal mark longevity is thought likely to persist longer than external marks (Elle 2010) and there-by the investigation of this technique may justify data collection via internal methods where fish sacrifice is applicable. If internal mark retention is

longer than external mark retention this would increase and widened the scope for the use calcein marking methodology and the understanding of such data is relevant to the wider use of calcein with other species.

Externally detectable calcein marks have been shown to fade to undetectable levels by 200 days post stain in outdoor hatchery conditions, however internally detectable marks could still be found on the otolith (Elle *et al.* 2010). In the current work for light exposed fish at 8 months, external detection methods showed mark identification accuracy at 13.6-56.7%, where internal examination of the otolith showed higher mark identification accuracy at 64.3-76.9%. This corroborates with previous findings, that internally detectable marks last longer than external marks, but shows that marks on otoliths are not completely resistant to the effects of sun-light radiation.

Conclusion and Implications for conservation management of Yarra Pygmy Perch

The evidence points to the successful use of calcein in marking studies to be dependent on specific environmental conditions. Sunlight exposure and high growth rates appear to be the strongest factors affecting calcein mark retention. The effect of these factors upon the success of calcein marking studies will be different for each species, and situation. The specific duration of data required for each study will need to be considered and measurement of survival and retention rates should be inbuilt into the study whereever possible. This study suggests that for the Yarra Pygmy Perch, in the typically thick vegetated habitat type where they are encountered, calcein marking via osmotic induction using 25 g/L salt solutions is a successful technique for the study of population dynamics and reintroduction success. It is also apparent the population in

Tupelogrove dam is persisting and recruiting well which is obviously a great outcome for conservation.

Acknowledgements

Thanks go to Biology Society of S.A. for a field research grant which facilitated purchase of fyke nets enabling the collection of field data. Thanks go to Adam Watt and Arkellah Hall (DEWNR), Dr Luciano Beheregaray (FUSA) for allowing the project to link in with a current ARC project and access to fish, and to Hilary Mahon, Keith Westergaard, Jessica Buss, Mel Tucker, and James Lean for assistance during data collection. Thanks also go to Chris Bice (SARDI), SARDI and Adelaide Microscopy for access to equipment and technical advice.

References

Alcobendas m., Lecomte f., Castanet j., Meunier f., j., Maire p., Holl m. (1991) Technique de marquage en masse de civelles (Anguilla anguilla L.) par balnéation rapide dans le fluorochrome. Application au marquage à la tétracycline de 500 Kg de civelles. *Bull. Fr. Pêche Piscic.*(321),43-54.

ASFB (2001) *Conservation Status of Australian Fishes*. In. ')

Bart A. N., Kindschi G. A., Ahmed H., Clark J., Young J., Zohar Y. (2001) Enhanced transport of calcein into rainbow trout, Oncorhynchus mykiss, larvae using cavitation level ultrasound. *Aquaculture* **196**(1–2),189-197. (doi:http://dx.doi.org/10.1016/S0044-8486(00)00579-2).

Bashey F. (2004) A comparison of the suitability of Alizarin Red S and calcein for inducing a nonlethally detectable mark in juvenile guppies. *Transactions of the American Fisheries Society* **133**(6),1516-1523. (doi:10.1577/T03-073.1).

Beheregaray L. (2011) *ARC - Restoration genetics of five threatened freshwater fishes.* In. ' Adelaide)

Bice C., ., Whiterod N., ., Wilson P., Zampatti B. P., Hammer M. (2012) *The critical fish habitat project: Reintroductions of threatened fish species in the coorong, lower lakes and murray mouth region in 2011/2012.* . 43pp. SARDI Publication No. F2012/000348-1. SARDI Research Report Series No. 646.43pp.

Bice C., Hammer M., Wilson P., Zampatti B. (2011) *Fish Monitoring for the Drought Action Plan for the South Australian Murray-Darling Basin threatened freshwater fish populations Summary for 2010/2011.* South Australian Research and Development Institute (Aquatic Sciences), Adelaide. 214pp SARDI publication number F2010/000647-2. SARDI Research Report series no. 564.

Bice C. M., Wilson P., Ye Q. (2008) *Threatened Fish Populations in the Lower Lakes of the River Murray in spring 2007 and summer 2008.* South Australian Research and Development Institute (Aquatic Sciences), Adelaide. 32 pp.

Bice C. M., Ye Q. (2007) *Monitoring threatened fish communities on Hindmarsh Island, in the Lower Lakes of the River Murray, South Australia, in the summers of 2006 and 2007 with reference to baseline data from 2005.* South Australian Research and Developement Institute (Aquatic Sciences), Adelaide, 47 pp.

Brooks R. C., Heidinger R. C., Kohler C. C. (1994) Mass-marking otoliths of larval and juvenile walleyes by immersion in Oxytetracycline, Calcein, or Calcein Blue. *North American Journal of Fisheries Management* **14**(1),143-150. (doi:10.1577/1548-8675(1994)014<0143:MMOOLA>2.3.CO;2).

Cook J. T., McNiven M. A., Richardson G. F., Sutterlin A. M. (2000) Growth rate, body composition and feed digestibility/conversion of growth-enhanced transgenic Atlantic salmon (Salmo salar). *Aquaculture* **188**(1–2),15-32. (doi:10.1016/S0044-8486(00)00331-8).

Crook D. A., Munro A. R., Gillanders B. M., Sanger A. C., O'Mahony D. J., Thurstan S., Woodcock S., Baumgartner L. (2009) *Chemical marking of hatchery fish: Techniques and applications*. In. ' (June 2009 workshop CD-ROM. Murray-Darling Basin authority: Canberra, ACT, Australia.)

Crook D. A., Munro A. R., Gillanders B. M., Sanger A. C., O'Mahony D. J., Thurstan S., Woodcock S., Buumgartner L. (2009b) '*Chemical marking of hatchery fish: techniques and applications*.'. In June 2009 workshop CD-ROM. (Murray Darling Basin Authority.: Canberra, ACT, Australia.)

Crook D. A., O'Mahony D. J. (2009a) Development and evaluation of methods for osmotic induction marking of Golden Perch Macquaria ambigua with Calcein and Alizarin Red S. *North American Journal of Fisheries Management* **29**,279-287.

Elle F. S., Koenig M. K., Meyer K. A. (2010) Evaluation of Calcein as a mass mark for Rainbow Trout raised in outdoor hatchery raceways. *North American Journal of Fisheries Management* **30**(6),1408-1412. (doi:10.1577/M10-006.1).

Gelsleichter J., Cortés E., Manire C. A., Hueter R. E., Musick J. A. (1997) Use of Calcein as a fluorescent marker for elasmobranch vertebral cartilage. *Transactions of the American Fisheries Society* **126**(5),862-865. (doi:10.1577/1548-8659(1997)126<0862:UOCAAF>2.3.CO;2).

Hammer M. (2007) *Status report on South Australian threatened freshwater fish populations during 2007 drought conditions*. In. ' Ed. R. t. D. f. E. a. H.-S. A. Government))

Hammer M. (2009) Status assessment for nationally listed freshwater fishes of south east South Australia during extreme drought, spring 2008. *Report to Department for Environment and Heritage, South Australian Government. Aquasave Consultants, Adelaide.*

Hammer M. P., Wedderburn S., Van Weenen J., Australia N. F., Australia S. (2009) '*Action plan for South Australian freshwater fishes*.' (Native Fish Australia (SA) Incorporated)

Hasinoff B. B. (2003) The intracellular iron sensor calcein is catalytically oxidatively degraded by iron(II) in a hydrogen peroxide-dependent reaction. *Journal of Inorganic Biochemistry* **95**(2–3),157-164. (doi:10.1016/s0162-0134(03)00099-0).

Honeyfield D. C. (2006) Dietary Calcein marking of Brook Trout, Atlantic Salmon, Yellow Perch, and Coho Salmon scales. *North American Journal of Fisheries Management* **26**,431-437.

Honeyfield D. C., Kehler T., Fletcher J. W., Mohler J. W. (2008) Effect of Artificial Sunlight on the Retention of External Calcein Marks on Lake Trout. *North American Journal of Fisheries Management* **28**,1243-1248.

IUCN (2003) Red List Categories Version 3.1. Prepared by the IUCN Species Survival Commission. *IUCN, Gland, Switzerland.*

Kefford B. J., Papas P. J., Metzeling L., Nugegoda D. (2004) Do laboratory salinity tolerances of freshwater animals correspond with their field salinity? *Environmental Pollution* **129**,355-362.

Leips J., Baril T. C., Rodd F. H., Resnick D. N., Bashey F., Visser G. J., Travis J. (2001) The suitablility of Calcein to mark Poeciliid fish and a new method of detection. *Transactions of the American Fisheries Soociety* **130**,501-507.

Mahon H. C. (2012) *Effect of temperature and salinity on growth of juvenile Yarra pygmy perch (Nannoperca obscura: Percichthydae)*. Flinders University of South Australia.

McNeil D. G., Closs G. P. (2007) Behavioural responses of a south-east Australian floodplain fish community to gradual hypoxia. *Freshwater Biology* **52**, 412–420.

McNeil D. G., Westergaard S., Ye Q. (2010b) Chapter 3. Larval salinity tolerance of nine native fish species from the Lower River Murray, South Australia. In Influences of salinity, water quality and hydrology on early life stages of fishes in the Lower River Murray, South Australia. . In '*Influences of salinity, water quality and hydrology on early life stages of fishes in the Lower River Murray, South Australia.* .' (Eds Q. Ye, K. J. Cheshire and D. G. McNeil) pp. 73-102. (South Australian Research and Development Institute (SARDI), Aquatic Sciences: Adelaide, Australia)

Mohler J. W. (1997) Immersion of larval Atlantic Salmon in Calcein solutions to induce a non-lethally detectable mark. *North American Journal of Fisheries Management* **17**,751-756.

Mohler J. W. (2003) Producing fluorescent marks on Atlantic Salmon fin rays and scales with Calcein via osmotic induction. *North American Journal of Fisheries Management* **23**(4),1108-1113. (doi:10.1577/M02-143).

Monaghan J. P. (1993) Notes: comparison of Calcein and Tetracycline as chemical markers in summer flounder. *Transactions of the American Fisheries Society* **122**(2),298-301. (doi:10.1577/1548-8659(1993)122<0298:NCOCAT>2.3.CO;2).

Negus M. T., Tureson F. T. (2004) Retention and nonlethal external detection of Calcein marks in Rainbow Trout and Chinook Salmon. *North American Journal of Fisheries Management* **24**(2),741-747. (doi:10.1577/M03-119.1).

Pourzand C., Watkin R. D., Brown J. E., Tyrrell R. M. (1999) Ultraviolet A radiation induces immediate release of iron in human primary skin fibroblasts: The role of ferritin. *Proceedings of the National Academy of Sciences* **96**(12),6751-6756. (doi:10.1073/pnas.96.12.6751).

Reelfs O., Tyrrell R. M., Pourzand C. (2004) Ultraviolet A Radiation-Induced Immediate Iron Release Is a Key Modulator of the Activation of NF-[kappa]B in Human Skin Fibroblasts. *J Investig Dermatol* **122**(6),1440-1447.

Saddlier S., Hammer M. (2010) *National Recovery Plan for the Yarra Pygmy Perch Nannoperca obscura.* . Department of Sustainability and Environment, Melbourne.

Smith B., B., Hammer M. (2006) *Mapping and current distribution of native and exotic freshwater fish within the South Australian Murray-Darling Basin.* Final report to PIRSA Rural Solutions, prepared by the South Australian Research and Development Institute (Aquatic Sciences), Adelaide. 60 pp.

Wedderburn S., Hammer M., Bice C. (2012) Shifts in small-bodied fish assemblages resulting from drought-induced water level recession in terminating lakes of the Murray-Darling Basin, Australia. *Hydrobiologia* **691**(1),35-46. (doi:10.1007/s10750-011-0993-9).

Wedderburn S., Shiel R., Hillyard K., Brookes J. (2010) *Zooplankton response to watering of an off-channel site at the lower lakes and implications for Murray Hardyhead recruitment*. Report to the Murray Darling Basin Authority and the South Australian Murray-Darling Basin Natural Resources Management Board.

Wilson C. A., Beckman D. W., Dean J. M. (1987) Calcein as a fluorescent marker of Otoliths of larval and juvenile fish. *Transactions of the American Fisheries Society* **116**(4),668-670. (doi:10.1577/1548-8659(1987)116<668:CAAFMO>2.0.CO;2).

Appendix

Target Journal- Guide to Authors