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The influence of saline concentration on the success of Calcein marking techniques for hatchery-produced Murray cod (*Maccullochella peelii*)

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Abstract

Chemical marking is a useful technique to determine natal origin of fish and is increasingly used to determine the success of fish stocking programs. This study sought to optimise an osmotic-induction batch marking technique, using the calcium-binding chemical, Calcein, to enable future identification of hatchery-marked Murray cod (*Maccullochella peelii*). It was hypothesised that higher saline concentrations would create a more reliable bone mark but it was unknown whether saline exposure would influence fish survival. A laboratory trial was undertaken to determine the optimum saline concentration required to maximise survival of Murray cod and marking of bony body parts. Fish were exposed to a no salt control, a no Calcein control or one of three different saline concentration treatments then housed in either 60 L aquarium tanks or hatchery ponds and monitored for 43 days post marking. There was no significant difference in mortality rates among the three treatments under controlled aquarium conditions or among marked fish released into hatchery ponds. Whilst saline concentration did not influence fish survival, marking using concentrations less than seawater produced a detectable mark and reduced stress on Murray cod fingerlings. Mark intensity, however, was greater when fish were exposed to higher saline concentrations.

Introduction

Fish stocking is used globally as the most common management tool to increase recreational fisheries following decline or overharvest (Cowx, 1994; Halverson, 2008). Restocking activities aim to either create new recreational opportunities or improve existing fisheries but few studies are undertaken pre or post-stocking to determine overall effectiveness (Verspoor and De Garcia Leániz, 1997; Pearsons and Hopley, 1999). Discrimination between hatchery-reared and wild fish would substantially assist assessment of stocking success (Crook et al., 2012) but validated techniques are largely unavailable for many recreational species (Crook et al., 2007). Techniques relying on chemical marking agents, such as calcein (2,4-bis-[N,N'–

di(carbomethyl)- aminomethyl]fluorescein) and oxytetracycline, that mark otoliths and other calcified tissues have been developed (Crook et al., 2012) and are routinely used as a means of monitoring fish stocking programs (Johnson, 2005). There is concern about the retention rates of calcein in different species (Crook et al., 2012). There is a subsequent need to verify appropriate marking techniques for each new species that is considered.

Murray cod (*Maccullochella peelii*) is an iconic species within the Murray-Darling River system (Australia) that has experienced substantial declines in recent times (Allen *et al.*, 2009). Historical populations supported a large-scale commercial fishery but declining catches led to an eventual fishery closure (Rowland, 2005). Efforts to recover the species focus largely on restrictive harvest controls for recreational fishers (Allen *et al.*, 2009) or restocking activities (Rowland, 1988). Over nine million Murray cod fingerlings have been stocked into the Murray-Darling Basin since commercial-scale hatchery production of Murray cod commenced (Rourke et al., 2011). There has been no structured effort to quantify stocking program success and no suitable technique developed to distinguish wild and stocked fish. The lack of an effective marking technique is presently limiting efforts to determine stocking success throughout the natural range of this species.

Several techniques to discriminate hatchery and wild fish exist but the most successful, and widely accepted, is chemical marking (Coghlan *et al.*, 2007; Hill and Quesada, 2010). The main benefits of chemical marking are a reduced requirement to handle and transport fish and that many fish can be marked over a very short time period (Nielsen, 1992). Most chemical marking techniques focus on staining the otolith and therefore require fish to be sacrificed in order to determine natal origin (Crook et al., 2007). Non-lethal identification techniques are therefore preferred, and in recent years osmotic induction has been widely used to apply a reliable long-term mark (Smith et al., 2010).

Osmotic induction requires fish to be immersed in a hyperosmotic solution to effectively 'dehydrate' cells (Smith et al., 2010). Fish are then placed within a chemical marking bath, usually containing Calcein, and all bony structures within the fish retain a permanent chemical signature (Guy *et al.*, 1996; Mohler, 2003). Calcein binds with alkaline earth metals and causes calcified parts of organisms (e.g., otoliths, fin spines and scales) to fluoresce when examined under an ultraviolet light source (Wilson et al., 1987). Subsequent capture and identification of live Calcein marked individuals has determined excellent retention rates for salmonid species up to twelve months (Frenkel *et al.*, 2002; Mohler, 2003; Negus and Tureson, 2004; Crook *et al.*, 2007). Similarly, external marks have been demonstrated in live percichthyids for up to 100 days (Crook et al., 2009), and salmonids for up to 19 months (Game and Wildlife Trust, Unpublished Data).

Immersion into a saline bath for extended periods could have substantial physiological impacts on fish and exert stress which could influence post-marking survival and it is generally accepted that many freshwater taxa have critical levels of salinity tolerances (Hart et al., 1991). Saline impacts are classified as either lethal, where fish may die following contact, or sub-lethal, where fish exhibit adverse physiological responses but eventually recover (Chessman and Williams, 1974; Kefford *et al.*, 2004). Sub-lethal

effects are relevant to batch marking studies. Fish may exhibit stress during the marking process and make an apparent recovery but could have sustained physiological damage during saline immersion. Murray cod exposed to sub-optimal water quality are known to exhibit epithelial cell degeneration and mucous membrane sloughing (Schultz et al., 2011). Recovery from these conditions can be slow. If exposure to a saline bath elicits a similar physiological response, it may influence post marking survival and exclude chemical marking as a useful technique for this species.

This study sought to identify impacts of saline exposure to osmotically-induce Calcein marks in Murray cod fingerlings. It was expected that higher concentrations of salinity could influence fish welfare through increased osmotic stress which may influence post-release survival. Fish were marked using a range of salinity concentrations to facilitate osmotic induction of Calcein. Post release survival of fingerlings was monitored for up to 57 days under pond and aquarium conditions. Physiological responses were also monitored through regular random sampling to determine potential immune system repression resulting from saline bath exposure. The overall aim of this project was to identify an optimal saline concentration that could maximise success of Calcein batch marking programs, as indicated by mark intensity and survival rates for Murray cod fingerlings.

Methods

Study location

The study was carried out at the Narrandera Fisheries Centre (NFC) which is located four kilometres south east of Narrandera, New South Wales in south-eastern Australia. Juvenile Murray cod were sourced from a commercial hatchery (Uarah Fisheries) and were transferred to the experimental facility and allowed to acclimate for five days prior to commencement of experimental procedures.

Laboratory trials

Fish were Calcein marked using an osmotic induction method modified from Crook *et al,* 2009. Fish were first placed in a saline treatment bath for at least three minutes, then briefly rinsed in freshwater before being transferred to a 0.5 % Calcein solution for a further three minutes (Figure 1). Saline baths for an experimental control (Group A – 0 % salt) and three different treatment regimes (Group B – 1 % salt; Group C – 3 % salt and Group D – 5 % salt) were prepared by dissolving coarse natural salt (Lake Charm Salt Co.) into 10 L of hatchery water. Group C sought to replicate a salinity close to that of seawater (Doroudi *et al.*, 2006) whilst Group D represented higher salinity than seawater (1.5 times) which was consistent with salinity levels commonly used to osmotically induce chemical marks in other species (Mohler, 2003; Crook *et al.*, 2007; Smith *et al.*, 2010). A 0.5 % Calcein solution was prepared by adding 50 g of Calcein powder (Sigma Aldrich) to 10 L of hatchery water. Previous work on another percichthyid species, Golden perch (*Macquaria ambigua*) determined that altering Calcein concentration had no effect on mark intensity (Crook et al., 2009). On this basis it was subsequently decided to maintain a consistent Calcein concentration (0.5 %) for each experimental treatment.

Fish were immersed into control and treatment saline baths for a total of three minutes prior to Calcein marking for all

except treatment group B. These fish were held in the 1 % salt solution for one hour (60 min) prior to marking. This treatment was included to replicate standard hatchery preventative practice to reduce the likelihood of fungal or bacterial infection with Murray cod fingerlings. If standard preventative practice saline concentrations elicited a strong mark on Murray cod fingerlings, then batch marking could be simplified and incorporated into normal hatchery practices without requiring additional handling.

A total of 1,120 Murray cod were marked in four treatment groups of 280 fish (3 months old, average weight 1.35 ± 0.2 g. Following marking, fish were transferred to a large freshwater tank (2000 L) to rinse excess Calcein, and to recover for two hours post-marking (Figure 1). Following recovery, fish from each treatment were divided into 28 sub-groups of 10 fish. Each sub-group was placed in one of 28 glass aquaria (60 L) which had been partitioned into four separate, equally sized zones using 3 mm black polyethylene mesh (Figure 1). Aquariums were individually numbered and each zone randomly designated as A, B, C or D according to experimental treatment. A standard sized house brick with 14 holes was placed into each zone to provide habitat for the fish. Two additional aquaria were stocked with 10 Murray cod fingerings that were not exposed to either saline conditions or immersed into Calcein. These fish were untreated controls and used to compare against Group A for any potential effect from Calcein immersion.

Aquaria were maintained in a temperature controlled room (22.8 ^{o}C) with fish fed Skrettings Gemma Diamond 1.5 mm dry diet (57 % protein, 15 % oil) three times per week. Fish were inspected daily with any dead fish removed, recorded and stored in 10 % buffered formalin. Each tank was cleaned and had a 25 % water exchange once a week during the trials. After approximately 11 days in the tanks an outbreak of white spot disease (caused by the ectoparasite *Ichthyopthirius multifilus*) was observed. To control this disease aquarium water was maintained at 0.5 % salt solution for the remainder of the experiment.

Uncontrolled pond trials

Assessment of fish survival under controlled laboratory conditions may not be directly applicable to standard stocking operations. It was determined that wild-released fish would be subjected to water quality changes which may be influenced by the marking technique. To replicate a more-natural release scenario, hatchery fry ponds were used to house a further 600 Murray cod (3 months old, average weight 1.40 ± 0.2 g) which were Calcein marked according to methods described for Treatment C (3 % saline solution and three minute immersion).

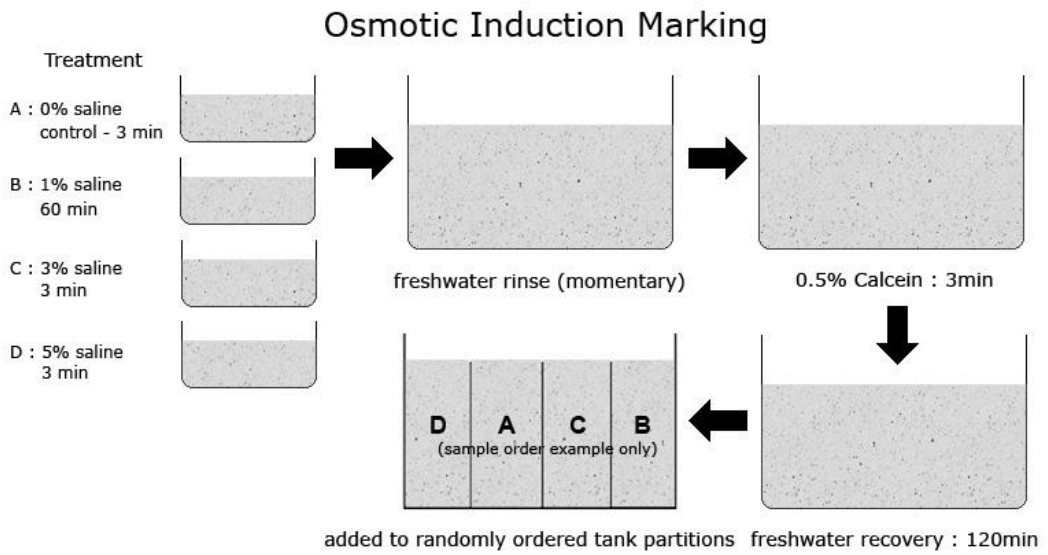

Figure 1: Schematic representation of the experimental approach. Fish from each replicate were allocated to a salinity concentration treatment, momentarily rinsed in freshwater, and then placed into a Calcein bath.

A further 600 Murray cod were exposed to conditions consistent with Treatment C but not exposed to Calcein. This sought to control for any potential effects arising from Calcein immersion. Insufficient fish were available to replicate all four experimental groups and treatment group C was selected after a preliminary analysis of aquaria data. Fish were counted into groups of 30 marked and 30 unmarked fish and then placed into one of twenty cages (0.91 m x 0.91 m x 0.71 m) situated in two fry rearing ponds. Each cage received approximately 10 g of blood worm (distributing organisation Aqua-One) every second day to supplement any natural food that may have entered the cages from the pond.

After 57 days fish were removed from the cages, anaesthetised with Benzocaine (100 gL^{-1}; Pharmaq Ltd), and examined under UV light to determine mark retention. The number of marked and unmarked fish returned from each cage was recorded.

Mark detection strength

Rather than just report whether marking was achieved or not, we used mark intensity as a surrogate for longevity of the mark following Negus and Tureson (2004) who found marks of greater intensity persisted longer in Chinook salmon. Intensity was quantified using a battery-operated general purpose modulated probe fluorometer (GFP meter; Opti-Sciences Inc., Hudson, New Hampshire). After calibration using standard compounds with known ppm fluorescein, the meter reports fluorescence intensity in 'tics' in the range from zero to 1,800 (maximum detectable limit). Any values exceeding the maximum detectable limit returned as a system overload, at which point the maximum value of 1,800 was assigned for statistical analyses.

Mark intensity was assessed following the completion of controlled aquaria trials. Five

fish were removed from each control and treatment group. One intensity reading (ppb fluorescein) was taken from the inner operculum, anal spine and jaw of each fish and recorded for later analysis.

Data analysis

All data analyses were performed using the SAS software package and statistical tests were considered significant at $P < 0.05$.

Laboratory trials

A generalised linear model using a probit link function for a binomial distribution was used to compare fish survival between the four different laboratory salinity treatments (A, B, C, D). The proportion of survivors in each treatment at the conclusion of the laboratory trials (after 43 days) was compared among treatments after the effect of tanks was partitioned. Wald confidence intervals were calculated for these four adjusted survival rates.

Pond trials

A generalised linear model using a probit link function for a binomial distribution was fitted to assess the survival rate of Calcein marked individuals with non-marked fish in hatchery ponds (after 57 days). In this analysis, the proportion of marked and unmarked fish were compared after the partitioning out the effects of each pond and the cages which were treated as randomised blocks nested within the ponds.

Mark detection strength

A mixed linear model was used to compare the fluorescence tics returned from different body parts in the different treatments 43 days post-tagging. Treatment (A, B, C or D) were treated as fixed effects and fish were considered experimental subjects. Fluorometer readings from each body part (inner operculum, anal spine and jaw) were treated as repeated measures taken from each fish. The fluorescence measurements were log (base10) transformed and the assumptions of normality and homoscedastic variances confirmed by inspection of the residuals. Significant effects were followed up by comparing the least squares means in each treatment group against the control (Group A) for each body part using Dunnett's adjustment for multiple comparisons to maintain the family-wise error rate at 0.05.

Results

Immediate response

Fish exposed to low salinity concentrations (Control group A or Treatment group B and C) exhibited few signs of distress, maintained equilibria and demonstrated excellent flight response during saline bath exposure. Fish exposed to relatively high saline concentrations (Treatment group D) exhibited signs of distress, including increased opercular beat rate and some degree of equilibrium loss during saline immersion. Fish visibly recovered within one hour of immersion and no fish died during the marking process or in the immediate post-marking period during transportation to ponds or aquaria.

Laboratory trials

There was a significant tank effect in the experiment ($\chi^2 = 120.1$, df = 18, $p < 0.0001$) After removing the tank effect, the average survival rate varied between 72 % (Control group A), 66 % (Treatment group B), 58 % (Treatment group C), and 74 % (Treatment group D), however survival rates were not significantly different between the four treatments ($\chi^2 = 1.24$, df = 3, $p = 0.75$) (Table 1).

Treatment	Lower 95% CL	Average survival rate	Upper 95% CL
A (0% control)	0.49	0.72	0.95
B (1% saline)	0.45	0.66	0.88
C (3% saline)	0.37	0.58	0.79
D (5% saline)	0.52	0.74	0.96

Table 1. Average survival rate (and 95% confidence limits) of Murray cod fingerlings exposed to four treatment groups A (0% saline control), B (1% saline solution for 60 minutes), C (3% saline solution for 3 minutes) and D (5% saline solution for 3 minutes).

A white spot outbreak was detected in several aquaria after 11 days. Fish from all treatments were infected, including the Calcein-immersion controls (n = 3 fish). Five aquaria were removed from the experiments because of increased mortality rates from white spot. The experimental groups experienced similar mortality over the seven day period whilst the infestation was contained (Group A, n = 13 from 9 tanks; Treatment B, n = 19 from 11 tanks; Treatment C, n = 24 from 12 tanks; Treatment D, n = 17 from 11 tanks).

Uncontrolled pond trials

There was no significant difference in the survival rate between the two ponds (χ^2 = 0.05, df = 1, p = 0.50) or between fish that were Calcein marked or not (χ^2 = 1.63, df = 1, p = 0.20). After removing the pond effect, 92.3 % of Calcein marked fingerlings survived compared to 90.2 % of unmarked fingerlings.

Mark detection strength

Strong mark detections were recorded from all three body parts (Figure 2). There was a significant difference in the mean tics returned from each experimental group which was dependent on which body part was being measured (F = 13.7, df = 6, 24, p < 0.001). Follow up comparisons indicated that experimental groups A and B did not differ but both had significantly lower tics than treatments C and D from jaw and anal spine readings (Figure 3). Measurements taken from the inner operculum did not differ among any of the saline treatment groups.

Discussion

Changing salinity concentration had little impact on Murray cod fingerling mortality. No significant differences were observed under pond or controlled aquarium conditions during the post-marking observation period suggesting that salinity concentration had no effect on survival of Murray cod fingerlings. A major expectation from fisheries managers is that chemical marking could become a reliable tool to determine stocking success (Mohler, 1997). Further research is therefore required to ensure that chemical marking does not influence long-term survival, and that the chemical mark is reliable and detectable over longer timescales.

Most mortality observed in the post-marking period was attributed to a white-spot outbreak. A prophylactic salt treatment was subsequently applied and infected fish recovered rapidly so the trial continued. It was originally hypothesised that a potential effect of saline immersion could be epithelial cell destruction or mucous membrane degradation which may influence immune system efficiency. There are four pieces of evidence which suggest the experimental trials did not contribute to the disease outbreak.

Firstly, the disease outbreak infected saline immersion controls and treatment groups which suggest that infection was not correlated with exposure to a saline bath.

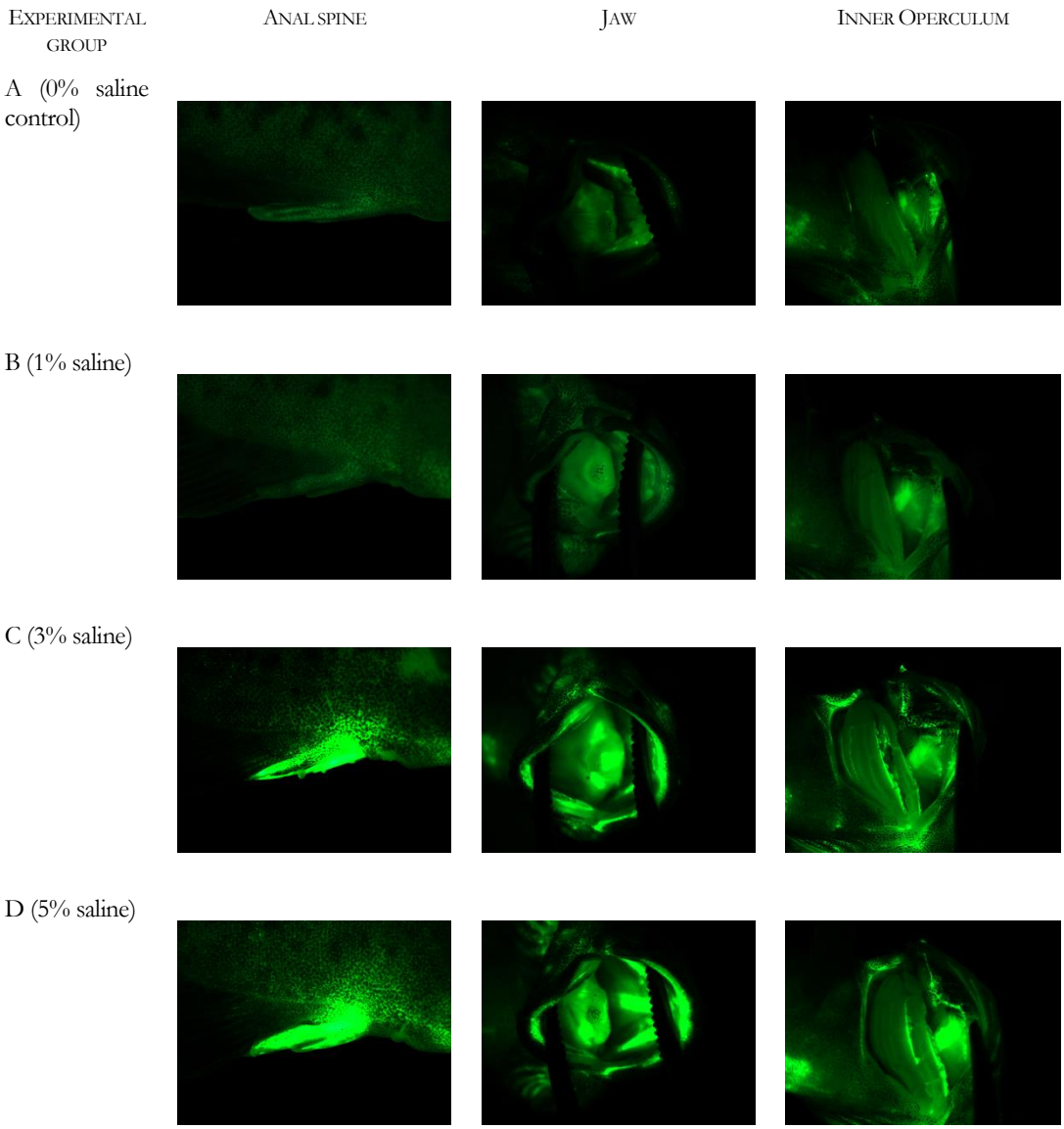

Figure 2. Stereo microscope images of Calcein-marked Murray cod. Images show the interaction between experimental group (and hence salinity concentration) and the strength of fluorescence from various body parts of juvenile Murray cod. (Images converted to greyscale and examined with a Leica M165FC with GFP3 UV filter).

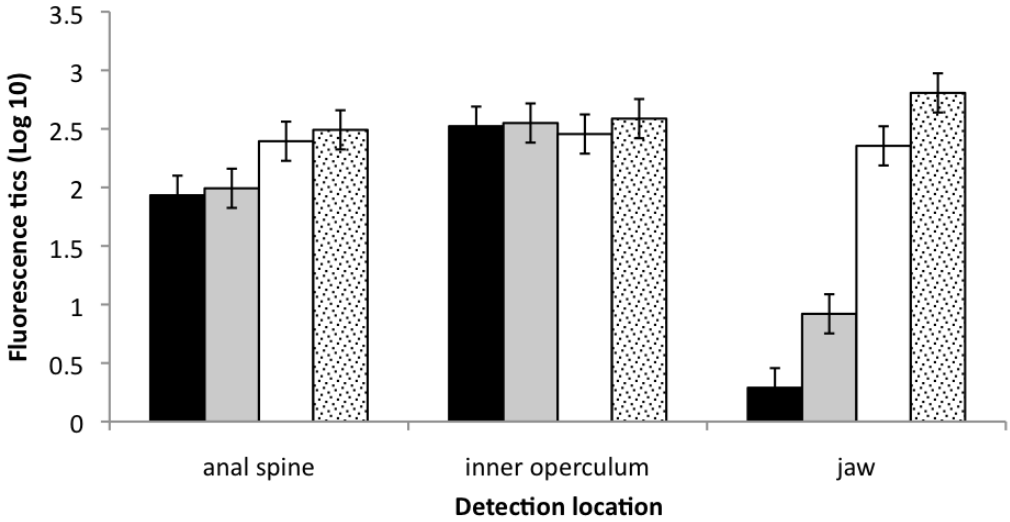

Figure 3. Comparison of fluorometer readings returned from different body parts of fish from each experimental group. Mean fluorescence tics were log (base 10) transformed for subsequent analysis. Treatment groups are defined as A (0% saline control - black), B (1% saline solution for 60 minutes - grey), C (3% saline solution for 3 minutes - white) and D (5% saline solution for 3 minutes - mottled).

Secondly, fish within the Calcein controls also succumbed to the disease suggesting that immersion in the marking chemical was also unlikely to have facilitated the outbreak. Third, low mortality was recorded from both marked and unmarked fish from the hatchery pond trials where fish were not held under controlled conditions. Finally, juvenile Murray cod are aggressive, territorial and are known to be susceptible to white spot disease if stressed when held under relatively high densities (Rowland and Ingram, 1991). The observed levels of mortality likely resulted from these behavioural responses rather than experimental effects. This justified the application of a prophylactic treatment to continue the experiment.

Australian biota are known to possess some tolerance to hypersaline conditions but most lethal effects are not observed until concentrations are many times that of sea water (Hart *et al.*, 1991; Doroudi *et al.*, 2006). For example, the LD50 of Murray cod in saline water is shown to be 15,700mg/L. (James et al 2003). Juvenile Murray cod exposed to saline levels equal to or lower than sea water were relatively unaffected by the saline immersion technique. Treatment groups exposed to salinity levels greater than seawater experienced substantial stress responses during saline immersion. These observations suggest juvenile Murray cod have an inherent intolerance to highly saline conditions. The implications of this are important for osmotic induction techniques. Firstly, this means that saline bath concentration needs to be carefully prepared and that salinity levels should be assessed prior to immersion. This would prevent the possibility of accidental mortality if techniques are not precisely followed.

Secondly, our study did not examine the influence of saline immersion longer than three minutes. Precision in immersion timing could be critical to ensuring the welfare and long-term survival of batch marked fish. To minimise stress, saline concentrations lower than sea water should be considered for future osmotic induction work on juvenile Murray cod. Maintaining accurate saline concentrations and immersion times are likely to be critical to ensure successful marking of juvenile Murray cod with minimal stress.

Mark intensity varied from different body parts suggesting that calcified structures absorbed different levels of fluorescein. The anal spine and jaw consistently reported lower intensity under reduced osmotic pressure during the marking process. Increased mark intensity from higher saline concentrations are observed in salmonid marking programs (Negus and Tureson, 2004). Some chemical marking techniques are known to induce autofluorescence which can increase the probability of false positives (Crook et al., 2009). Chemical marks are also more readily incorporated into structures containing large amounts of calcium carbonate (Lochet et al., 2011). The operculum is the largest single bony structure within teleost fish and could therefore be expected to absorb high amounts of Calcein during the marking process. Increased intensity under fluorescence could be expected from such regions, irrespective of saline concentration, if localised Calcein uptake was high. Lower readings from both the jaw and anal spine justify the use of osmotic induction to maximise uptake (See Figure 2).

The main objective of chemical marking programs is to provide a non-lethal mechanism to identify natal origin which persists indefinitely. Ongoing fish growth results in deposition of additional layers on calcified structures and deposition of opaque tissue suggests that external detectability of Calcein fluorescence may decrease with age (Crook *et al.*, 2009; Lochet *et al.*, 2011). Calcein was detected from all bony structures post marking but long-term non-lethal detection using external analysis methodologies requires further investigation.

It is possible to induce a Calcein mark into fish without osmotic induction (Smith *et al.*, 2010). Indeed our study validated this because all marked fish, including no salt controls, returned a positive reading in the short assessment period. There is, however, much evidence to suggest that mark intensity degrades in response to prolonged exposure to ultra-violet light (Honeyfield *et al.*, 2008; Smith *et al.*, 2010) and becomes less detectable as fish grow (Game and Wildlife Trust, Unpublished Data). Fish stocked into rivers and impoundments, where prolonged ultraviolet exposure could be expected, may therefore have a limited time period where Calcein marks can be reliably detected using non-lethal techniques. Using osmotic induction to increase the uptake of Calcein seeks to prolong the external detection of Calcein marks over the long-term. Internal structures, like otoliths or vertebrae, should retain a permanent mark and Calcein fluorescence should be detectable via examination of thin sections well beyond the external detection period (Crook et al., 2009). External detection should therefore be considered in conjunction with other detection techniques when planning stocking success studies relying on chemical marking (Frenkel *et al.*, 2002; Crook *et al.*, 2012).

Improved intensity can also be achieved by increasing Calcein concentration in some species (Mohler, 1997; Smith *et al.*, 2010) but was not considered practical for Murray cod juveniles. Compared to other commercially-

available marking chemicals, Calcein is substantially more expensive and has limited potential for re-use on multiple batches of fish (Crook *et al.*, 2007). High levels of Calcein are also known to be toxic under some circumstances (Bumguardner and King, 1996) or result in no discernable intensity increase in others (Crook *et al.*, 2009). This variability in response to altered concentration suggested that increasing uptake of Calcein by manipulating osmotic pressure could reduce potential toxic effects whilst also minimising cost. These are both important considerations if chemical marking is to be applied on a large spatial scale by commercial operators.

Conclusion

Murray cod fingerlings were successfully marked using Calcein under a range of saline bath concentrations. Potential welfare issues under higher salinity suggest that moderate to low saline concentrations should be considered when facilitating osmotic induction. Under these conditions fish exhibited low mortality and relatively high mark retention rates. Further research into the length of time Calcein marks are externally detectable would be useful when considering non-lethal methods of hatchery fish discrimination. Further research is also required to ensure that chemical marking does not influence long-term survival. Over the long term, combining non-lethal external techniques with lethal validation using thin sectioning of saggital otoliths would provide a useful mechanism to report on success of Murray cod stocking programs.

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