

Article

Multiple Lines of Evidence Indicate Limited Natural Recruitment of Golden Perch (*Macquaria ambigua*) in the Highly Regulated Lachlan River

Foyez Shams ^{1,*}, Fiona Dyer ^{1,2}, Ross Thompson ^{1,2}, Richard P. Duncan ¹, Jason D. Thiem ³, T. Gabriel Enge ⁴ and Tariq Ezaz ^{1,*}

¹ Institute for Applied Ecology, Faculty of Science and Technology, University of Canberra, Canberra, ACT 2617, Australia; fiona.dyer@canberra.edu.au (F.D.); Ross.Thompson@canberra.edu.au (R.T.); Richard.Duncan@canberra.edu.au (R.P.D.)

² Centre for Applied Water Science, Faculty of Science and Technology, University of Canberra, Canberra, ACT 2617, Australia

³ Department of Primary Industries, Narrandera Fisheries Centre, Narrandera, NSW 2700, Australia; Jason.Thiem@dpi.nsw.gov.au

⁴ Research School of Earth Sciences, Australian National University, Canberra, ACT 2601, Australia; Gabriel.Engel@anu.edu.au

* Correspondence: Foyez.shams@canberra.edu.au (F.S.); Tariq.ezaz@canberra.edu.au (T.E.)

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Abstract: Freshwater ecosystems and their associated biota have been negatively impacted by the human development of water resources. Fundamental to restoration activities for target species is an understanding of the factors affecting population decline or recovery. Within Australia's Murray–Darling Basin, recovery efforts to address the population decline of native freshwater fish include stock enhancement, habitat restoration, and the delivery of environmental water. Essential to guiding future management actions is information to assess the efficacy of these efforts. We undertook a study to investigate whether natural spawning and recruitment, stock enhancement, or a combination of the two is contributing to sustaining populations of golden perch (*Macquaria ambigua*) in the highly regulated Lachlan River, Australia. Otolith microchemistry and genetic analyses were used as complementary tools to determine the source (hatchery origin or wild-spawned) of existing populations in the catchment. We identified that natural spawning and recruitment was contributing to riverine populations in some years but that populations were heavily reliant on stocking. It was not possible to distinguish hatchery and wild-born fish using genetic tools, highlighting the value of using multiple lines of evidence to establish causal mechanisms contributing to population recovery.

Keywords: otolith; fisheries management; conservation ecology; freshwater fish; stocking; Murray–Darling Basin

1. Introduction

Recruitment is a fundamental requirement for sustaining fish populations [1,2]. In freshwaters, fish and other biota have undergone global population decline, primarily as a result of anthropogenic disturbances such as climate change, river regulation, over-exploitation, pollution, and pests and diseases [3–6]. Often, these disturbances directly impact a species' ability to complete life-history requirements such as spawning, migration, and recruitment into adult populations [7,8]. Consequently, stock enhancement is often used as a remediation tool to subsidize a vulnerable fish population with no or limited natural recruitment, with varying degrees of success [9–11]. In many cases, stocked fish have been shown to have poor post-stocking survival and reproductive success [12–14]. In catchments

where the survival rate of stocked fish is high, stocked fish have been found to be replacing the existing wild population [15]. In other situations, stocking has effectively supported wild populations through increased abundance, natural spawning, and increased effective population sizes [16–18].

A significant challenge in fisheries management has been identifying the source of individuals in populations receiving stocked fish to determine the contribution of stocked individuals to population viability. Analysis of the chemical composition of fish ear stones (otoliths) is an effective method to determine where fish have spent time, as the layers of the otoliths retain a geochemical signature of the feeding habitat during the fish's life stages [19–22]. Otolith analysis has been used to identify hatchery-bred individuals in multiple marine and anadromous fishes [23,24] because otoliths can contain a signature of the hatchery conditions under which the fish were raised, allowing hatchery-bred individuals to be distinguished from wild-bred individuals. However, the use of such natural tags is still an emerging field in ecology [25].

One disadvantage of otolith analysis is that it requires killing fish to obtain samples. The molecular analysis of tissue samples also has the potential to determine fish origins and evaluate stock enhancement programs but without the need to kill fish, allowing the non-lethal sampling of a larger number of individuals. This approach has been applied to both freshwater and marine fishes [12,26–29]. Molecular studies have shown the effects of stocking on population genetic structure and genetic diversity but have yet to be applied to identifying hatchery-bred stocked and wild-born individuals.

Stock enhancement is a major conservation practice for many Australian native freshwater fishes [30,31]. Australian freshwater drainages, particularly the rivers of the Murray–Darling Basin (MDB), have been severely impacted by anthropogenic disturbances, primarily water resource development, which has changed flow regimes and introduced in-stream barriers. This has resulted in a large decline in native fish abundance since European settlement [32,33]. Despite an initial aim to boost recreational fisheries with salmonid fishes, the re-establishment of native fish populations in many rivers of the MDB through stock enhancement has been a major priority since late-last century [31]. To date, golden perch (*Macquaria ambigua*) (Percichthyidae) has been the most stocked species in the MDB to boost native fish recovery as well as recreational fisheries [30,31].

In the present study, we aimed to investigate the status of the *M. ambigua* population in the Lachlan River catchment (MDB) with respect to the role of stocking and natural recruitment. To achieve this aim, we used otolith microchemistry analysis and genetic markers to identify wild-born and hatchery-bred *M. ambigua*. The results of this study address a current knowledge gap regarding the level of the natural recruitment of golden perch and the effect of stock enhancement on populations of this species in a highly regulated river.

2. Materials and Methods

2.1. Study Species and the Catchment

Macquaria ambigua (golden perch) (Richardson 1845; Percichthyidae) is a medium-sized (commonly 30–40 cm, 1–1.5 kg), long-lived (20+ years) freshwater fish endemic to three major river basins (MDB, Lake Eyre Basin, and the Fitzroy Basin) of central and eastern Australia [34]. The spawning of *M. ambigua* is strongly associated with environmental cues such as water temperature and high-flow flooding events [35,36], which trigger upstream movement to spawn, although fish will also breed in isolated waterhole habitats [37–40].

The Lachlan River is a major drainage within the MDB, flowing from upland areas in inland New South Wales (NSW) to a distributary system and terminal wetland that infrequently connects with the Murrumbidgee River during high natural flow events. The system is characterized by a dynamic hydrologic regime and habitat fragmentation through water extraction [41–43]. The Lachlan River underwent a long period of disconnection from the remainder of the Murray system during the Millennium Drought (2001–2009) [44]. Monitoring over the period 2014–19 found no evidence of *M. ambigua* spawning [45].

The Lachlan River has been extensively stocked with hatchery-bred *M. ambigua* since 1973. On average, more than twenty thousand juvenile *M. ambigua* are stocked annually along the river between Wyangala Dam and Hillston (Figure 1). The NSW government hatchery (Narrandera Fisheries Centre, NSW Department of Primary Industries (DPI) Fisheries) and two private hatcheries (Murray Darling Fisheries and Uarah Fish Hatchery) are the sources of juvenile golden perch being used for stock enhancement. In these hatcheries, the breeding of golden perch occurs in captive conditions using either water from a different catchment (the Murrumbidgee River) or groundwater. Broodstock for hatchery production are collected from the Lachlan River according to NSW DPI guidelines [46].

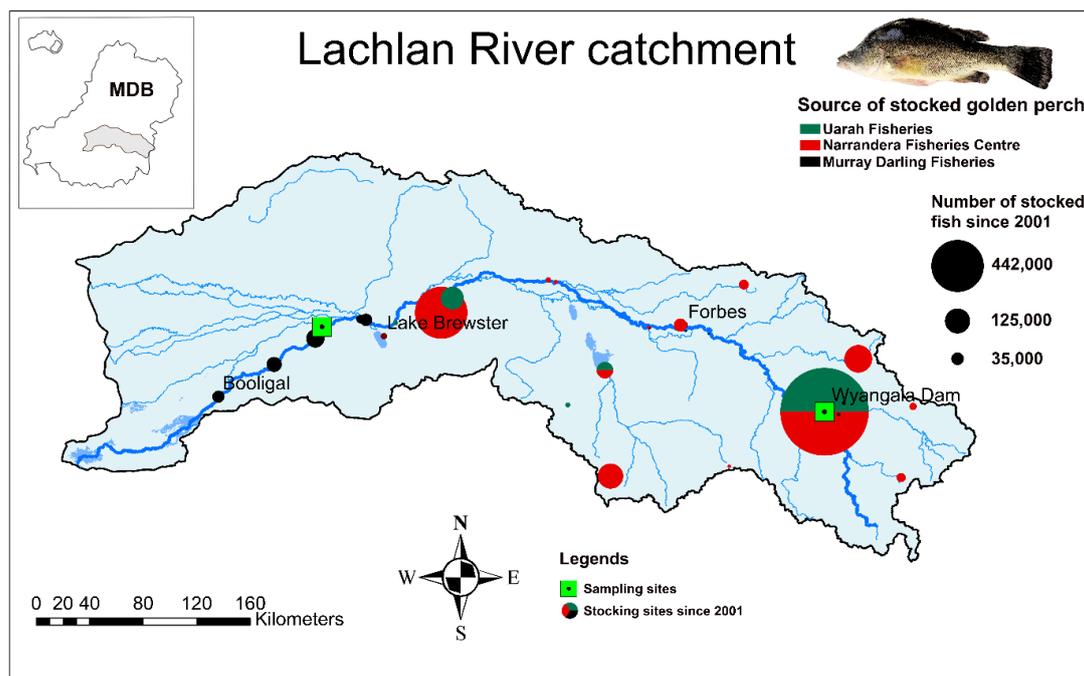


Figure 1. Cumulative number and hatchery-source of *Macquaria ambigua* (golden perch) stocked into the Lachlan River since 2001 (Unpublished data from NSW DPI 2019). Sampling sites cover the Upper Lachlan (above Wyangala dam) and Lower Lachlan (Downstream Lake Brewster). Sampling sites represent the locations from where fish for this study were collected.

Under this long-term isolation scenario and the hatchery practice regime, we hypothesized that the source of *M. ambigua* recruitment in the Lachlan River in the last twenty years will be either natural recruitment or stocking from the hatcheries. The otolith core will have developed at the site of the natal origin of a fish, while the otolith edge will have developed in the habitat from which the fish was sampled. Consequently, we assumed a fish born in the Lachlan River would have the same chemical signature in both the core and the edge of the otolith, while a stocked fish would have a different chemical signature in the otolith core, reflecting the hatchery conditions, relative to the edge, reflecting conditions in the Lachlan River.

2.2. Otolith Sampling

We collected otoliths from a total of 44 *M. ambigua* sourced from two sites on the Lachlan River (upper Lachlan and lower Lachlan) (Figure 1). An additional 19 young of year (YOY) juvenile *M. ambigua* were collected from a commercial hatchery (Murray Darling Fisheries) as a control group to provide a hatchery reference collection. (Table 1). Fish were humanely euthanized following the animal ethics protocol approved by the University of Canberra Animal Ethics Committee, AEC 17–18 and Fisheries NSW Animal Care and Ethics permit 14/10. The otoliths were separated from the head using sterile forceps (washed with 100% EtOH followed by a wash with distilled water), and otoliths

were washed in Milli Q (Millipore, Merck) water. Air-dried otoliths were stored in sterile 5 mL tubes in dry conditions until further use. All collections were performed following appropriate ethics approval and permits (University of Canberra Animal ethics Committee, AEC 17–18; Fisheries NSW Animal Care and Ethics permit 14/10 and Scientific Collection Permit P01/0059(A)-2.0).

Table 1. Sources of sampled fish in the Lachlan River. Upper Lachlan—upstream of Wyangala dam; Lower Lachlan—downstream of Lake Brewster; Hatchery—Murray Darling Fisheries (located in the Murrumbidgee catchment). The spawning year was calculated using the annual rings in the otoliths.

Locality	Sampling Year	N (Sample Size)	Spawning Year Range
Lower Lachlan	2015	24	2001–2014
Upper Lachlan	2017	20	2003–2012
Hatchery (YOY)	2017	19	2016

2.3. Otolith Processing and Laser Ablation ICP-MS Analysis

The core of each otolith was externally marked with a permanent marker after examining the surface and placed on a mounting tray with the marked side upward. The otoliths were then embedded in a 2-part epoxy resin containing 5:1 resin (ChemAlert) to hardener (methyl ethyl ketone peroxide catalyst (Nuplex)) and hardened at 45 °C for 4 h. Resin blocks were then sectioned using a low-speed diamond saw (TechCut 4™, ALLIED). To expose the core of the otoliths, each block was sectioned into 3 slices (200–300 µm each) with the marked position of the otolith in the middle slice (Figure S1). The sections were washed with Milli-Q water and examined for the shape of the sulcus groove in each slice. The slice containing the core (based on the shape of the sulcus groove) was selected for further analysis. The selected otolith sections were polished on a 3M lapping film (50-30130, ALLIED) to expose the core as well as to remove saw marks. The polished otolith sections were mounted on a glass slide using a mounting adhesive (Crystal Bond 509, ProSciTech).

Laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) was performed on the samples at the Research School of Earth Science, Australian National University using a 193 nm ArF beam (LPF 200, Lambda Physik) and an iCap RQ (ThermoFisher) quadrupole-ICP-MS. Samples were pre-ablated to remove surface contaminants; the otoliths were then analyzed for a suite of trace element concentrations based on previous work [22,47]. The analyzed elements included ²⁴Mg, ⁸⁶Sr, ¹³⁷Ba, ⁵⁵Mn, ⁵⁷Fe, ⁵⁹Co, ⁶⁰Ni, ⁶³Cu, and ⁶⁶Zn. The analyses were bracketed by analyses of NIST 612 (National Institute of Standards and Technology) and the in-house Davies Reef Coral standard [48] before and after each sample batch. Samples were normalized to Ca and then evaluated against the coral standard for Mg, Sr, and Ba and against the NIST 612 for all other elements.

Spot analyses were carried out by ablating square 50 µm spots with a repetition rate of 10 Hz and an ablation time of 60 s per spot at the core of the otoliths, as well as at the outer edge. These two locations on the otolith were chosen because it would be expected that hatchery-sourced fish would have a different signature at the edge compared to the core of the otolith, while wild-bred fish should have the same signature at the edge and core. This assumption is based on the geographic distance and differences in source water between the commercial hatchery (source of the hatchery group) and the Lachlan River.

2.4. Genetic Data

In this study, we used a subset of samples used in [49] (Shams et al. submitted manuscript 2020) for genetic analysis. We followed the tissue sample collection procedure as described in Shams et al. (submitted manuscript 2020). Briefly, fin clips and/or muscle tissue from the same individuals used for the otolith analysis (44 *M. ambigua* from Lachlan river and 19 hatchery YOY) (Table 1) were collected using sterile scissors and washed with 100% EtOH to avoid genetic contamination. Tissue samples were stored at 4 °C in 95% EtOH until further use.

Genomic DNA extraction was performed from fin clip and muscle tissue using protocols developed by Diversity Array Technology Pty Ltd (DArTseq™) [50]. The quality of genomic DNA was evaluated by running agarose gel electrophoresis (1.2% agarose). Genotyping by sequencing was performed by DArTseq™ using a combination of DArT complexity reduction methods and next-generation sequencing following protocols described in [50–55]. Markers with a high call ratio (threshold, 0.25) were filtered based on their polymorphic information content (>0.025) using a software package developed by DArT PL (DArTsoft14). Here, a high call ratio represents the least proportion of missing values (to call both alleles of a marker) caused by sequencing error or low-quality genomic DNA.

2.5. Statistical Analysis

2.5.1. Otolith Microchemistry

For each element, we tested for a difference between the otolith core and edge of wild-caught fish using Student's *t*-test with the statistical computing software R (version 3.3.1) [56].

To determine the structure of the *M. ambigua* population in the Lachlan River and to identify hatchery and wild-born fish, we performed non-metric multidimensional scaling (NMDS) [57] and a PERMANOVA test [58] based on the Bray–Curtis distance using otolith trace element composition (Table S1). The analysis was carried out using the function metaMDS in the R package 'vegan' (version 2.5-3) [59]. The settings included a Wisconsin double standardization, a maximum number of 100 runs to find the best solution, and 1000 permutations for calculating the coefficient of determination (r^2) and significance (*p*-value) [57]. The automatic rotation of the ordination space displays the value's main gradient in the first axis (NMDS1). In addition to the NMDS, we performed a hierarchical cluster analysis using individual dissimilarities based on Euclidean distance to identify groupings of fish based on otolith trace element composition. The cluster analysis was performed using the Ward.D2 implementation of the clustering method (minimum variance method) described by Ward [60,61]. All analyses were carried out with the statistical computing software R (version 3.3.1) [56].

2.5.2. Single Nucleotide Polymorphism (SNP) and SilicoDArT Data Analysis

Data processing, quality assessment, and filtration to reduce potential errors from low-quality genomic DNA and sequencing errors were performed using dartR (version 1.1.11) [62], a package developed for the statistical computing software R. To differentiate hatchery-bred stocked fish from wild-born fish, Principal Component Analysis (PCA) using the packages "factoextra" [63] and "factoMineR" [64] in R was performed on the Single Nucleotide Polymorphism (SNP) and SilicoDArT data.

3. Results

3.1. Otolith Micro-Chemical Composition

Four of the nine elements analyzed showed a significant difference in concentration ($\mu\text{g}\cdot\text{g}^{-1}$) between the otolith core and edge for wild-caught fish from the Lachlan River (^{24}Mg , ^{86}Sr , ^{55}Mn , and ^{66}Zn ; Student's *t*-test *p*-values all < 0.01) (Figure 2, panel LL_edge vs. LL_core and UL_edge vs. UL_core).

The NMDS clearly separated the chemical signatures from the edges of otoliths from fish from the Lachlan River and those of the hatchery (YOY) samples (stress: 0.07) (Figure 3a). A PERMANOVA test with 999 bootstrapping using the Bray–Curtis dissimilarity index found a significant difference between the clusters (*p*-value = 0.001, *F* model = 76.35, ANOVA for homogeneity of multivariate dispersion *p* = 0.39), indicating a unique chemical signature for each locality. The analyses were then repeated on the lower Lachlan and upper Lachlan edge and core samples separately (Figure 3b,c). For the lower Lachlan, this revealed two distinct groups comprising core and edge samples (Figure 3b). The signature from the hatchery reference samples was more similar to the lower Lachlan core chemical signature than

the edge signature of the lower Lachlan River individuals. In addition, a small group of individuals displayed similar core and edge signatures (Figure 3b), suggesting wild-born individuals from the lower Lachlan. Cluster analysis using elements significantly contributing to the core and edge variation (^{24}Mg , ^{86}Sr , ^{55}Mn , and ^{66}Zn) identified three primary groups (clusters) representing predominantly edge, core, and hatchery signatures. This analysis clearly identified six *M. ambigua* individuals for which core signatures were clustered with the edge signature indicating natural recruitment in the lower Lachlan catchment. Within the core and hatchery (YOY) groups, four individuals displayed core signatures that clustered with the hatchery reference group while the remaining individuals displayed core signatures that grouped separately (Figure 3d).

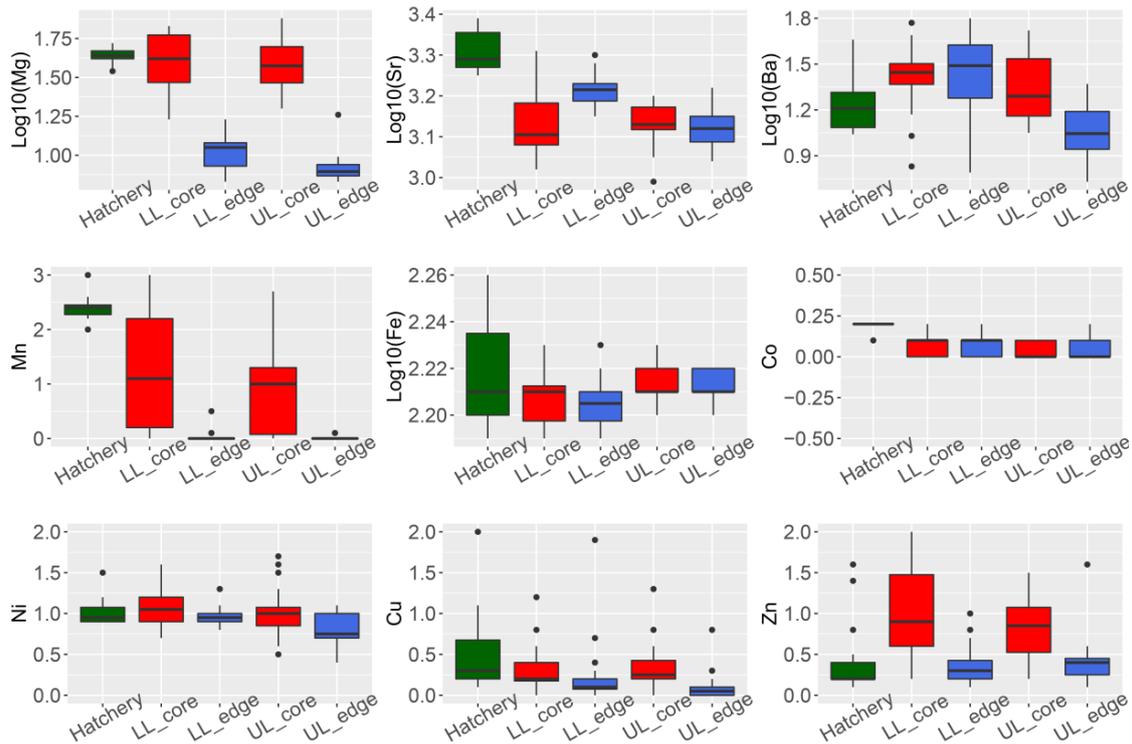


Figure 2. Summary of trace element composition ($\mu\text{g}\cdot\text{g}^{-1}$) in otoliths of *Macquaria ambigua* (golden perch) from three localities. Hatchery = hatchery sourced YOY samples ($n = 19$). LL = samples from the lower Lachlan (downstream Lake Brewster) ($n = 24$); UL = samples from the upper Lachlan (upstream Wyangala Dam) ($n = 20$); core = measurements from the core of the otolith; edge = measurements from the edge of the otolith.

In the upper Lachlan River samples, the NMDS revealed three distinct groups comprising core, edge, and hatchery reference samples, but the cluster analysis using only those elements significantly contributing to the core and edge variation suggests more complex groupings within the data set (Figure 3e). The cluster analysis identified two primary clusters separating the core and edge measurements of the Lachlan River samples from the hatchery reference collections. Five tentative sub-clusters belonging to two major groups of Lachlan River samples and hatchery samples were evident; however, the groupings were less distinct within the clusters (Figure 3e). The results suggest none of the upper Lachlan samples were wild-born, while five individuals displayed a core signature that clustered with the hatchery reference group.

Our analysis revealed the presence of both hatchery-bred stocked and wild-born *M. ambigua* from the Lachlan River. Among the 44 fish collected from Lachlan River, a total of six *M. ambigua* were identified as wild-born fish (14%), while the rest of them were either hatchery-bred stocked fish or from unknown sources (Figure 4). The observation of annual rings in the otolith revealed that all six wild-born *M. ambigua*, sampled from the lower Lachlan, were spawned in 2002, 2007, 2009, 2014

(one fish each year), and 2010 (two fish). A comparison between the daily discharge data from the lower Lachlan (Figure 4) revealed no clear association between high flow events and the spawning year of the six wild-born fish.

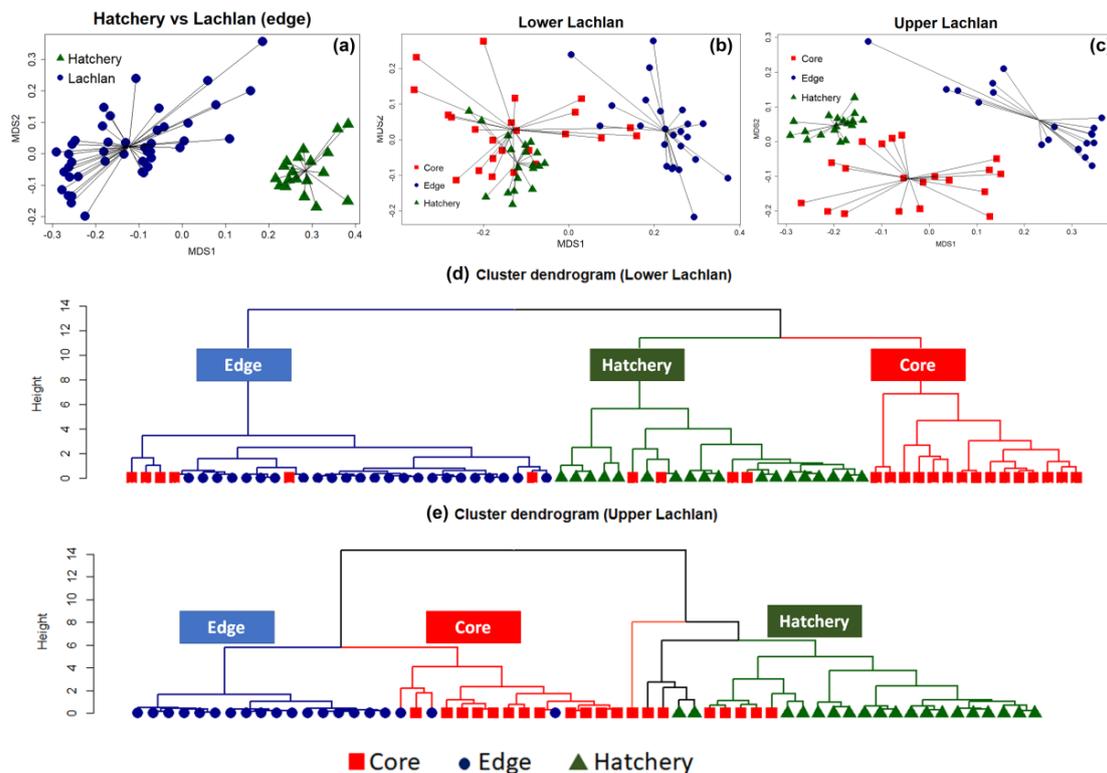


Figure 3. Non-metric multidimensional scaling (NMDS) and cluster analysis showing the presence of both wild-born and hatchery-bred stocked *M. ambigua* from the Lachlan River. (a) NMDS spider plot between the edge of the Lachlan river sample and the hatchery reference individuals (Hatchery YOY); (b) NMDS spider plot among the edge and core of the lower Lachlan river sample with the presence of the hatchery reference; (c) NMDS spider plot among the edge and core of the upper Lachlan river sample with the presence of the control group (Hatchery YOY); (d) Cluster dendrogram of the lower Lachlan samples and the hatchery reference based on a Euclidean distance matrix representing the presence of natural recruitment in the lower Lachlan catchment; (e) Cluster dendrogram of the upper Lachlan samples and hatchery reference group based on a Euclidean distance matrix representing no natural recruitment with two distinct clusters between the Lachlan River samples and hatchery reference group.

3.2. Genetic Data Analysis

The Diversity Arrays Technology (DArT) pipeline scored 5207 single nucleotide polymorphisms (SNPs) and 6956 SilicoDArT (presence-absence) markers after filtering based on polymorphic information content (>0.025). For the present study, we filtered out 2124 SNP markers based on the call ratio (0.90) and reproducibility average (0.90), resulting in a total of 3083 SNPs (null allele = 2.1%). Further filtering based on the Hardy–Weinberg equilibrium (HWE) was performed to remove markers under selection. Finally, 1481 SNPs were used for the present study. The same criteria (call ratio and polymorphic information content) were also used to filter out low-quality SilicoDArT markers. After filtering, 2902 (nNull allele = 5.7%) SilicoDArT markers were used for further analysis. Filtering for SilicoDArT loci under selection was not performed, as the allelic form of the loci is unknown (presence and absence of restriction fragment).

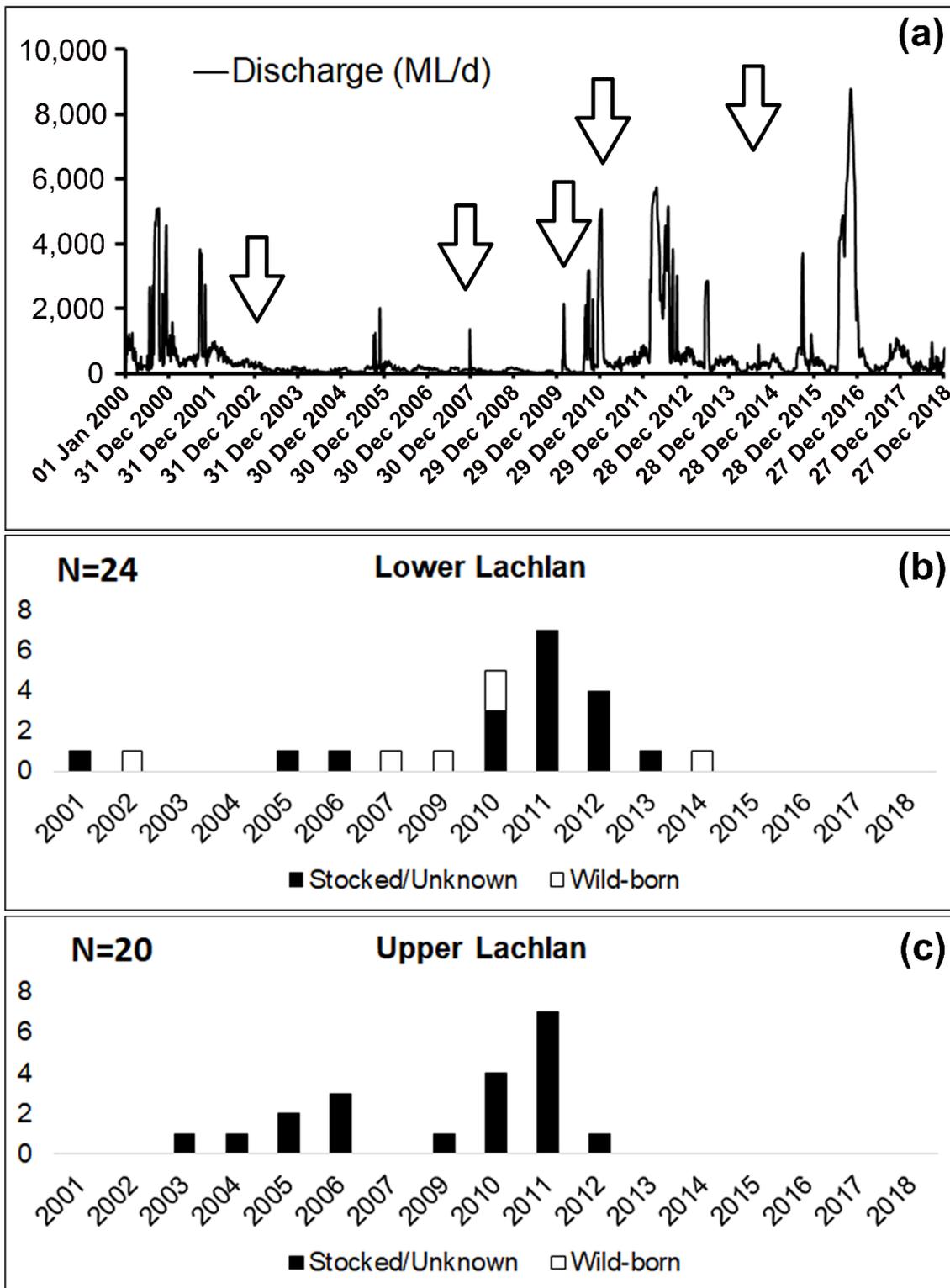


Figure 4. Daily discharge from the lower Lachlan since 2000 (Source: WaterNSW, <https://realtime.data.watnsw.com.au/>), and the number of stocked/unknown and wild-born fish from the Lachlan River and their spawning year. (a) Daily discharge (ML/day) at Hillston Weir (lat: -33.4873 lon:145.504) from 1 January 2000 to 31 December 2018; (b) Samples from the lower Lachlan (N = 24); (c) Samples from the upper Lachlan (N = 20). White bars represent the number of wild-born fish, and the black bars represent the number of fish either stocked or from unknown sources.

Despite a small cluster of hatchery reference individuals (hatchery YOY) (Figure 5a), the principal component analysis (PCA) suggests no specific clustering using SNP markers. High similarity among the Lachlan River samples and hatchery YOY and a lack of genetic structure means that the SNP markers could not conclusively distinguish the wild-born fish from the hatchery-bred stocked or unknown-source fish. We also performed a principal component analysis using only Lachlan River samples testing for differences between wild-born fish identified a priori and the rest from the otolith data (Figure 5b) and could find no genetic differences. The principal component analysis using SilicoDArT markers found a similar result, although it did indicate differences between the lower Lachlan fish (widely spread across PC1/Dim1 in the PCA plot) and upper Lachlan fish (a single cluster, which included the hatchery-bred YOY fish) (Figure 5c,d).

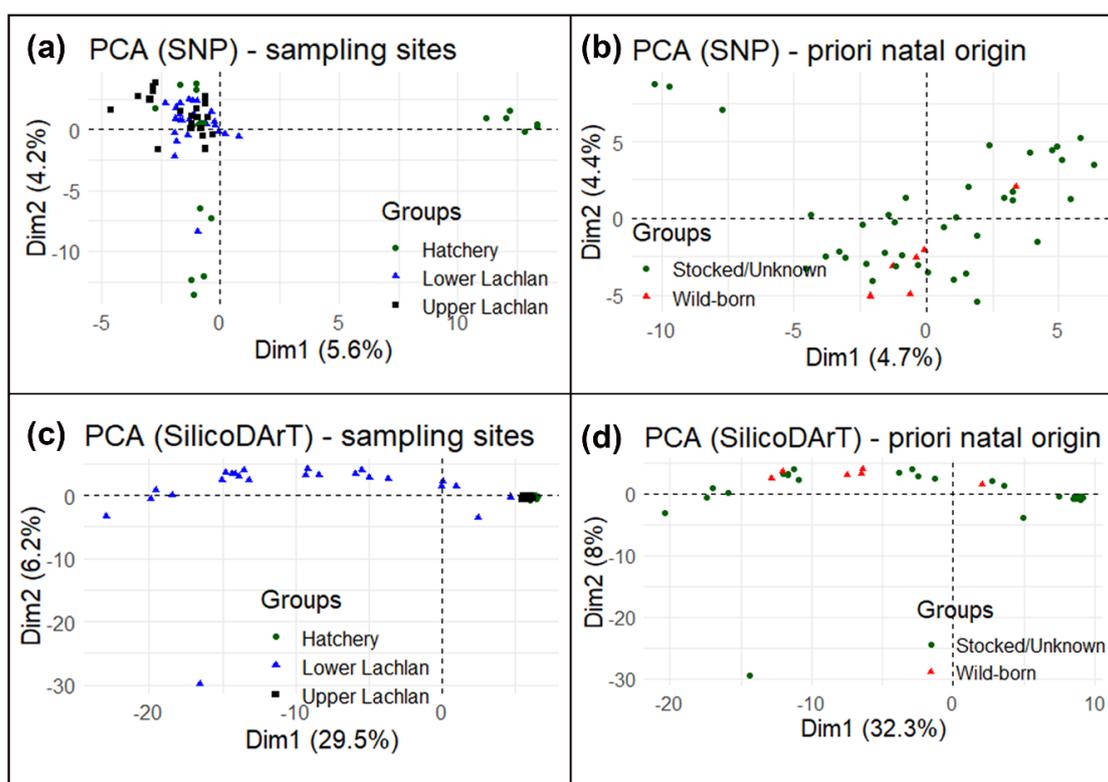


Figure 5. Principal component analysis (PCA) of *M. ambigua*; (a) PCA plot using Single Nucleotide Polymorphism markers—plotting based on geographic locations such as lower Lachlan, Upper Lachlan, and Hatchery; (b) PCA plot of only Lachlan samples using Single Nucleotide Polymorphism markers—plotting based on natal origins such as wild-born fish and hatchery-bred stocked/unknown-source fish; (c) PCA plot using SilicoDArT markers—plotting based on geographic locations such as Lower Lachlan, Upper Lachlan, and Hatchery; (d) PCA plot of only Lachlan samples using SilicoDArT markers—plotting based on natal origins such as wild-born fish and hatchery-bred stocked/unknown-source fish.

4. Discussion

The aim of this study was to investigate whether there was evidence of the natural recruitment of the existing *M. ambigua* population at the Lachlan River, or whether all the fish were derived from hatchery stocking. In doing so, we tested the effectiveness of genetic and otolith data as comparative tools. The analysis of otolith microchemistry identified limited natural recruitment along with the co-existence of wild-born and hatchery-bred stocked fish in the lower reach of the Lachlan River. While the data set was less clear about the origin of the upper Lachlan individuals, it appears that no natural recruitment was evident in our samples. Genetic data were not able to clearly distinguish hatchery-bred stocked and wild-born fish. Understanding the effects of stocking on population

composition is essential to evaluate the effectiveness of stocking to inform effective management and population recovery [31]. To date, the mark-recapture of chemically or physically tagged fish is the predominant method used to identify hatchery-bred stocked fish in Australia [17,65–67]. Otolith trace element composition has been widely applied in identifying the natal origin of fish [68–73]. This method has also been used to identify hatchery-bred stocked fish in wild salmonid populations [24,74,75].

We performed an otolith spot measurement to compare the chemical composition of the core (indicating natal origin) and edge (indicating current habitat). We observed that ^{24}Mg , ^{86}Sr , ^{137}Ba , and ^{55}Mn were the elements significantly contributing to the variation between core and edge composition. This is consistent with previous observations of otolith trace element composition in carp (*Cyprinus carpio*) from the Lachlan River [22].

In the upper Lachlan samples, no natural recruitment was evident using otolith microchemistry data. However, the clustering of the otolith edge, core, and hatchery reference groups was more complex than for the samples from the lower Lachlan. A different chemical signature was displayed by the core of otoliths compared to the edge as well as the YOY hatchery samples, indicating that the natal origin of these fish was neither the Lachlan River nor the Murray Darling Fisheries. Although our data could not conclusively identify the natal origin of these fish, plausible explanations for such results are (i) that they are fish stocked from hatcheries other than the Murray–Darling Fisheries such as DPI Fisheries (Figure 1) and (ii) that they are caused by interannual variability in the multi-element composition in the otolith, or a combination of both.

The fact that no association between high-flow events in the lower Lachlan and a priori recruitment events was evident in the present study suggests a flow-independent recruitment pattern in this species, which has also been reported in previous studies [40,76]. However, this could be an artifact of there being very few individuals from each spawning year. Larger sample sizes from each cohort will be required to provide conclusive evidence.

M. ambigua is a highly migratory, long-lived species and widely distributed in the MDB [34]. Within this region, *M. ambigua* is considered as a single panmictic population with high gene flow across connected catchments, resulting in high genetic similarity among individuals from different drainages of the MDB [77,78]. Our approach to identify natal origin using SNP and SilicoDArT markers was unsuccessful. Such an outcome would be expected if (i) either one or both parents of the river-born fish were previously stocked hatchery-bred fish, which would indicate the natural recruitment of stocked golden perch in the Lachlan River; or (ii) the broodstock being used in the hatchery were previously stocked fish from a different cohort. The longevity of the species, the intensity of *M. ambigua* stocking in Lachlan River, and the hatchery quality assurance program (the frequent replacement of old broodstock with wild-caught fish from the Lachlan River), coupled with abundant stocked fish in the river (the results from this study), suggests the second assumption is more likely to be correct. However, the natural recruitment in stocked fish is not infeasible considering a similar result in other species [16].

One possible limitation of this study that resulted in the inability of the genetic data to identify natal origin could be the low genomic representation of the hatchery samples. We sampled only a single cohort for the hatchery reference sample for the present study. Given the high genetic similarity of the species within the MDB as well as in Lachlan River, we recommend further analysis using multiple hatchery cohorts to demonstrate the effectiveness of genetic markers to identify hatchery-bred stocked and wild-born fish. The necessity of multiple-cohort sampling was also reflected in the otolith microchemistry analysis. We recommend further otolith study using multiple cohorts of hatchery reference as well as covering all the hatcheries contributing to the Lachlan River stocking. This will also reduce the probable error caused by physiological influences on otolith microchemistry, which was not considered in the present study [79,80].

There are increasing numbers of examples of studies that combine otolith microchemistry and genetic analyses [24,81–84]. As a combined approach, most studies have used otolith data to perform the fine-scale identification of spawning grounds and genetic data to investigate gene flow and connectivity

among populations. Focusing on stock enhancement, we identified only one study where a similar research question to ours was addressed using a combination of genetic and otolith data. Perrier, Daverat, Evanno, Pécheyrans, Bagliniere, and Roussel [24] used this multi-marker approach to infer information about the breeding activity of stocked *Salmo salar* (Atlantic Salmon) in wild conditions. However, the utility of a multi-marker approach is dependent on the presence of fine-scale genetic differentiation between hatchery and wild fish. For genetically homogenized populations, otolith trace elements are the more useful approach to identify sub-populations from different localities (e.g., Svedäng, André, Jonsson, Elfman, and Limburg [84]).

In conclusion, the present study has found evidence of limited natural recruitment in the lower Lachlan River, while no natural recruitment was evident in the upper Lachlan. The study successfully demonstrates the utility of otolith microchemistry analysis to identify stocked and wild-born *M. ambigua*, particularly where a lack of genetic structure rules out other approaches. Along with the use of otoliths, we propose that parent–offspring assignment approaches with genetic data from the hatchery broodstock could be effective in terms of the genetic differentiation of stocked and wild-born fish.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4441/12/6/1636/s1>, Figure S1: A schematic diagram of otolith processing. The best section containing core of the otolith was selected to mount on glass slide. 1 and 3: 200–300 μm sections from each side of the core. 2: the otolith section with the core; Table S1: Trace element compositions ($\mu\text{g}\cdot\text{g}^{-1}$) in core and edge of Lachlan River samples and core of the hatchery YOY samples. H = Hatchery, LL = Lower Lachlan and UL = Upper Lachlan.

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