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Otolith microchemistry identifies shallow, intertidal-dominated estuaries as important nurseries for sand flounder in New Zealand

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ABSTRACT: Estuaries provide critical habitat, food, and refuge for juvenile fish. Elemental concentrations in otoliths can retrospectively identify estuarine nursery habitats that contribute disproportionately to adult populations, providing valuable information for fisheries and coastal ecosystems. The present study aimed to (1) compare elemental signatures of juvenile (age 0+) sand flounder *Rhombosolea plebeia* otoliths collected from 9 estuaries in east Otago, New Zealand, and (2) assess the application of otolith microchemistry for identifying the nursery habitats contributing to adult (age 1+) sand flounder from the coastal shelf population. Otoliths from juvenile flounder were collected in November 2020, and adults of the same cohort were collected in the winter/spring of 2022. Laser ablation inductively coupled plasma mass spectrometry quantified 12 element concentrations within the post-settlement region of juvenile and adult otoliths using depth-profiling and ablation transects, respectively. Significant differences in post-settlement elemental concentrations were detected among estuaries. Juvenile and adult sand flounder were classified to their nursery origin using canonical analysis of principal coordinates. Grouping estuaries by the New Zealand Estuary Trophic Index classification scheme improved the overall juvenile classification accuracy from 56.7% (51/90) to 82.2% (74/90). Adult sand flounder (n = 78) were classified to have originated from shallow intertidal dominated estuaries (62%), followed by Otago Harbour (32%) and shallow, short residence time river and tidal river with adjoining lagoon estuaries (6%). The results highlight how otolith microchemistry can provide information on the use of critical estuarine nursery habitats for a commercially important species.

KEY WORDS: *Rhombosolea plebeia* · Otolith microchemistry · Laser ablation inductively coupled plasma mass spectrometry · LA-ICP-MS · Estuarine nursery · Flounder · Ontogenetic migration

1. INTRODUCTION

Estuaries and inshore coastal waters support many economically and ecologically important fish and invertebrate species, notably during their juvenile life history phase (Beck et al. 2001). The shallow depths, diverse habitat types, and high productivity of inshore coastal waters provide a safe environment rich in resources for vulnerable juveniles through provision of habitat, food, refuge, and/or favourable hydrodynamic and eco-physiological conditions (Litvin et al. 2018). Migration between estuarine nurseries and adult spawning populations is a critical linkage in the life cycle of many coastal fishes and has a major influence on the dynamics, productivity, and resilience of their populations (Cowen & Sponaugle 2009). However, the role of coastal marine and estuarine ecosystems as nursery and feeding grounds is under enormous pressure from anthropogenic degradation worldwide, threatening up to 80% of commercially

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and recreationally harvested species in some areas (Litvin et al. 2018, Lefcheck et al. 2019). As estuaries continue to be degraded and exploited, it becomes increasingly important to maintain connectivity between spatially segregated juvenile and adult populations by identifying those estuaries that are most essential to completion of the life cycle (Beck et al. 2001, Gillanders 2005).

Ontogenetic fish movement and connectivity can be quantified by a variety of approaches ranging from morphometric variations to utilising artificial and natural tagging methods (Reis-Santos et al. 2015). Natural tagging methods use chemical information in hard structures present in fish, for example otoliths, to reconstruct their life and environmental histories and determine population connectivity (Sturrock et al. 2012). As all individual fish in the population are already tagged since birth, the bias, cost, and logistical constraints associated with artificially applied tags tracking larval/juvenile movement are eliminated (Gillanders 2002a, Elsdon et al. 2008). Otoliths are paired, metabolically inert calcium carbonate ($CaCO_3$) concretions forming the auditory system of teleost fishes that are necessary for spatial orientation and hearing (Hüssy et al. 2021). The microchemistry of otoliths has been extensively used as a natural tag to reflect local physico-chemical conditions of the environment, albeit with significant physiological regulation (Campana 2005, Walther 2019, Reis-Santos et al. 2023). Variation in otolith elemental signatures and their use as natural tags has been applied to fish in rivers (e.g. Hicks et al. 2021), estuaries (e.g. Schilling et al. 2018), seagrass beds (e.g. Dorval et al. 2007), the coastal ocean (e.g. Marriott et al. 2016), and pelagic regions (e.g. Artetxe-Arrate et al. 2019).

Beam-based assays that target and quantify multiple elemental concentrations simultaneously in a particular region of otoliths can provide environmentally induced tags related to specific life stages of fish (Campana 2005, Beer et al. 2011, Reis-Santos et al. 2023). Provided that elemental signatures in the otoliths of juveniles vary spatially and can be resolved in the corresponding region of adults, critical juvenile habitats can be identified for a specific cohort of fish (Yamashita et al. 2000, Gillanders 2002a, Brown 2006, Ley & Rolls 2018, Artetxe-Arrate et al. 2019). For example, the otolith microchemistry of juvenile snapper from estuaries in New South Wales, Australia, were distinguished in adult fish, and the findings suggested that 89% of the fishery in the Sydney region originated from local estuaries (Gillanders 2002a). Nursery habitats vary in their ability to support juvenile fish growth, survival, and recruitment, subsequently resulting in varying contributions to recipient adult populations (Mohan et al. 2012). Identifying and protecting important inshore habitats, especially those that contribute disproportionately to adult populations (nursery habitats), is crucial for maintaining the survival and persistence of many marine species through potentially supporting the design of effective coastal management strategies (Beck et al. 2001, Brown 2006).

Elemental incorporation in otoliths is driven by a complex interaction between physiology and the environment (Martino et al. 2021). Strongly discriminatory elements among environments are often those that are influenced primarily by the ambient water physico-chemical conditions (Marriott et al. 2016). Conservative elements, including lithium (Li), magnesium (Mg), strontium (Sr), and barium (Ba), as well as some bioactive elements, including manganese (Mn) and zinc (Zn), have commonly been utilised to reconstruct the environmental histories of fishes (Brown 2006, Arai et al. 2007, Mohan et al. 2012, Marriott et al. 2016, McMullin et al. 2017). For example, conservative elements such as Sr and Ba are correlated with salinity and have been extensively used to study diadromous movements of fish (Tabouret et al. 2010, Doubleday et al. 2014, Izzo et al. 2018). Despite environmental influence, many conservative and bioactive elements can also be physiologically regulated by ontogenetic (Martin & Thorrold 2005, Brown 2006), genetic (Clarke et al. 2011), and trophic processes (Campana et al. 2000, Buckel et al. 2004). Elements less widely applied as markers can include those under stronger physiological influence such as sodium (Na), phosphorus (P), and potassium (K), and highly scavenged elements such as copper (Cu) and lead (Pb) (Hüssy et al. 2021). Regardless of the incorporation mechanisms, elemental concentrations in otoliths can serve as useful markers and environmental indicators provided that concentrations differ among the groups being considered (Campana et al. 2000).

As the large number of New Zealand estuaries (~443) range in size, tidal exchange, and physico-chemical characteristics (Francis et al. 2011, Berthelsen et al. 2020), their role as nursery habitats is expected to vary. Sand flounder (pātiki) *Rhombosolea plebeia* (Richardson, 1843) are important components of inshore soft sediment ecosystems in New Zealand and rely on estuaries as nursery and feeding grounds (Roper & Jillett 1981). Along the coastline of New Zealand's South Island, Banks Peninsula and Otago Peninsula have extensive areas of estuarine habitat that support juvenile flounder, providing a recruitment source for commercially, culturally, recreationally, and ecologically important adult flounder populations on the outer coast (Roper & Jillett 1981). Previous studies have focussed on the movement and feeding habits of adult sand flounder populations (Mundy 1968, Kilner 1974, Colman 1978); however, very few studies have provided the direct links between juvenile and adult distributions that are important for understanding population dynamics (Roper & Jillett 1981). Sand flounder are the most common flounder species in the Otago region and are prevalent in local inshore and offshore soft-sediment habitats (Roper & Jillett 1981, Currie et al. 2024). Adults are often found in spawning condition in Otago's waters and are believed to spawn offshore within the region during winter—spring months, with larvae transported to inshore soft-sediment coastal habitats where juveniles settle (Colman 1978, Francis et al. 2011). Age 0+ sand flounder are known to remain in the inner parts of estuaries (Kilner 1974), where they become abundant, followed by the individuals leaving the estuary to offshore waters at 1+ or 2+ years of age to spawn (Mundy 1968). As flounder experience a wide range in temperature, salinity, and water chemistry during the completion of their life cycle, otolith microchemistry provides an effective tool for identifying habitat use through the lifetime of individual fish (Elsdon et al. 2008, Earl et al. 2014, Walther 2019).

Here we investigated the role of specific estuary types as nursery habitats for supporting the adult population of sand flounder in east Otago, New Zealand. The aims of the present study were to (1) assess variation in the otolith elemental signature in the edge (post-settlement) region of otoliths from juvenile (age 0+) sand flounder collected from 9 different estuaries in east Otago, and (2) determine the recruitment estuary of sand flounder (age 1+) collected from the coastal fishery in east Otago by analysing the otolith region that corresponds to the juvenile phase. The post-settlement region of the flounder otoliths can be retrospectively identified and represents the estuarine life history stage.

2. MATERIALS AND METHODS

2.1. Study region

The east Otago coastline in south-eastern New Zealand is punctuated by an array of estuaries (Fig. 1) that are used as nursery habitats by juvenile fishes, including sand flounder (Roper & Jillett 1981, Francis et al. 2011, Currie et al. 2024). Estuarine sites were chosen based on published and anecdotal reports of the presence of juvenile flounder (Roper & Jillett 1981, Setyono 1996, Taddese 2019), and each of the sites was given a broad-scale classification based on the New Zealand (NZ) Estuary Trophic Index (ETI) classification scheme (Fig. 1; Robertson et al. 2015, Plew et al. 2018). Five of the sampled estuaries were classified as shallow, intertidal-dominated estuaries (SIDEs), Otago Harbour was classified as a deep, subtidal-dominated, longer residence time estuary (DSDE), and Kaikorai Lagoon was characterised as a shallow, short residence time river and tidal river with adjoining lagoon estuary (SSRTRE) (Fig. 1). Two larger areas (Otago Harbour and Waitati Inlet) had distinct intra-site habitat differences. Both Otago Harbour and Outer Waitati were characterised by seagrass patches and sand, while substrate at Andersons Bay Inlet and Inner Waitati had a greater mud content (Goerlitz et al. 2013). As Andersons Bay Inlet and Inner Waitati are nested within Otago Harbour and Waitati Inlet, respectively, they had no published assignment or measured environmental characteristics. The environmental conditions within Andersons Bay Inlet and Inner Waitati supported their classification as a SIDE and SSRTRE, respectively, in the present study (Fig. 1).

2.2. Sample collection

Juvenile (age 0+) sand flounder were collected during November 2020 from 9 sites along a 65 km portion of the east Otago coast (Fig. 1). Juvenile flounder were collected using a seine drag net (L × H = 14 × 1 m; 10 mm mesh size) during low tide at 3 or 4 replicate areas within each site. A total of 60 juvenile flounder were collected from each of the Kaikorai Lagoon, Hoopers Inlet, Papanui Inlet, Purakaunui Inlet, and the Waikouaiti River estuary sites (Fig. 1), while 30 flounder were collected from each of Andersons Bay Inlet, Inner Waitati, Otago Harbour, and Outer Waitati. Juvenile flounder were humanely euthanised with an overdose of AQUI-S following the University of Otago ethics protocol AUP-18-183 and immediately frozen. Adult sand flounder (age 1+, n = 81) were acquired in August and October 2022 from the commercial fisheries in Blueskin Bay (n = 62) and Wickliffe Bay (n = 19) within the east Otago area (Fig. 1).

At the Portobello Marine Laboratory, University of Otago, juvenile and adult sand flounder were completely defrosted and their morphometrics, including total length, were measured using electronic callipers for juveniles and a fish measuring board for adults. Dorsal and anal fin ray counts and the snout to first dorsal fin ray distance measurements were used to

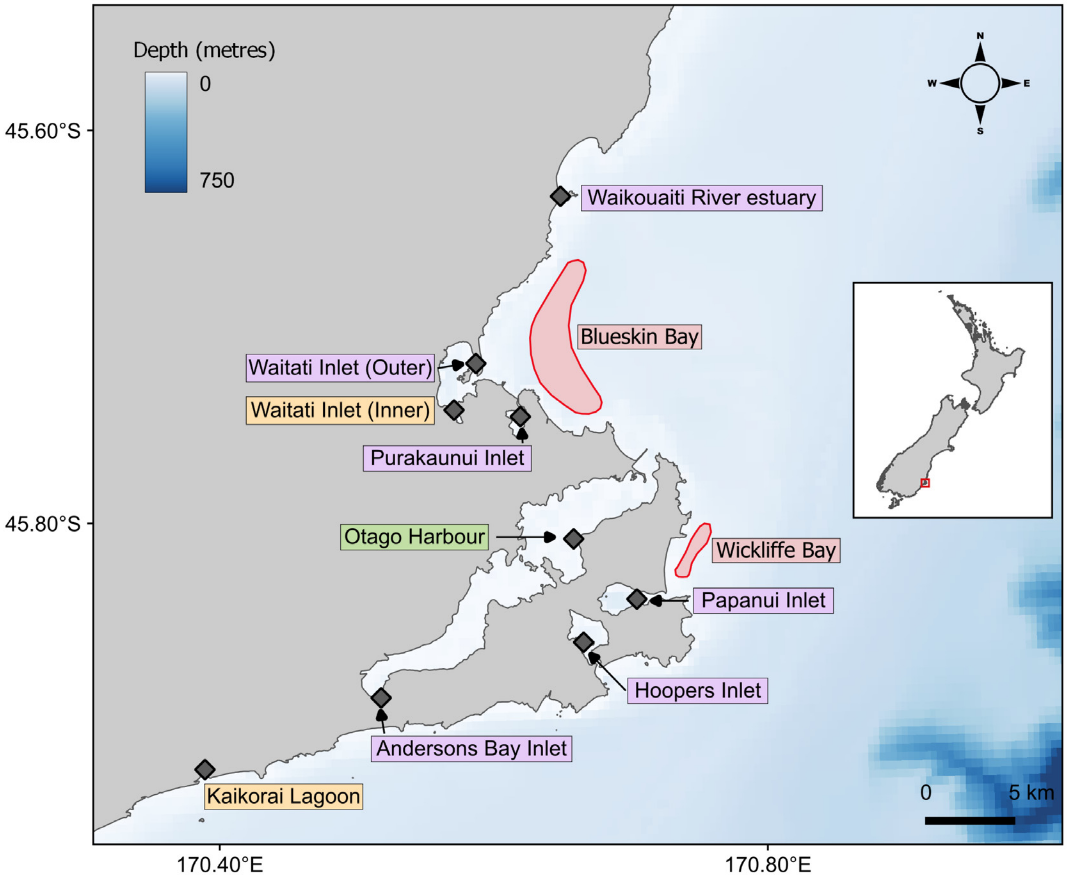

Fig. 1. Nine study sites in east Otago, New Zealand, where juvenile sand flounder were collected for the present study. The label colour relates to the classification of estuaries as shallow intertidal dominated estuaries (SIDEs, purple); shallow, short residence time river and tidal river with adjoining lagoon estuaries (SSRTREs, orange); or deep, subtidal dominated, longer residence time estuaries (DSDE, green). The sites and polygons in red represent the areas where commercial fishers caught adult sand flounder (Blueskin Bay, n = 62; Wickliffe Bay, n = 19)

confirm that juvenile and adult individuals were sand flounder (Eldon & Smith 1986, Roberts et al. 2015, Currie et al. 2024). The juvenile sand flounder collected ranged in size from 18.17 to 126.32 mm (mean ± SD, 46.99 ± 16.46 mm), while the adults collected ranged in size from 213 to 306 mm (271.4 ± 23.8 mm). When possible, both otoliths were extracted using a scalpel and fine-tip tweezers, cleaned of adhering tissue, washed with ultrapure Milli-Q water, and air-dried before storage in individual Eppendorf tubes.

As otolith microchemistry is known to differ among years, there is potential to confound temporal differences with spatial differences (Gillanders 2002a, Buckel et al. 2004). Consequently, only the age 1+ cohort of adult fish collected in 2022, which were recruited from the summer of 2020/21, were included in the present study. Adult otoliths were aged by examining annual opaque bands under a compound microscope (Olympus BX51) with transmitted light (Colman 1974a), with the edge of the first opaque zone delineated as the individual's first spring (~1 yr old) (Mundy 1968, Setyono 1996, Coulson et al. 2021). Although many of the adults were closer to age 2, the term age 1+ is used to accommodate the inherent ambiguity in the otolith markings, as the second winter band was forming but not consistently clear across all individuals.

2.3. Otolith preparation

Ten juvenile sand flounder between 20 and 70 mm total length were randomly selected from those collected from each of the sites (Table 1). The size range of 20–70 mm was chosen to ensure that the time period occupied within the estuary was sufficient for otolith elements to be detected accurately, that the otolith was adequately sized for distinguishing regions, and that ontogenetic effects on otolith microchemistry were minimised (Gillanders 2002b, Elsdon &

Table 1. Mean ± SD of the total length (mm) of juvenile sand flounder collected from 9 estuaries in east Otago, New Zealand, and mean ± SD concentrations ($\mu g\ g^{-1}$) of 12 elements within their otoliths. n = 10 for all sites

Site	Total length	Li	Na	Mg	P	K	Mn
Andersons Bay Inlet	39.40 ± 6.65	0.70 ± 0.06	3872.07 ± 256.82	51.84 ± 17.47	107.65 ± 60.08	516.68 ± 25.28	2.25 ± 0.55
Hoopers Inlet	41.06 ± 6.56	0.73 ± 0.05	3956.38 ± 160.90	26.96 ± 5.33	75.85 ± 17.35	793.20 ± 98.10	1.46 ± 0.51
Inner Waitati	43.51 ± 10.13	0.49 ± 0.12	3966.32 ± 170.76	49.54 ± 22.95	210.14 ± 125.29	706.16 ± 84.87	17.16 ± 8.91
Kaikorai Lagoon	44.68 ± 8.48	0.30 ± 0.06	3762.78 ± 188.79	30.33 ± 5.99	87.83 ± 34.97	699.38 ± 140.76	7.69 ± 3.31
Otago Harbour	43.64 ± 10.25	0.77 ± 0.09	4191.73 ± 297.43	34.07 ± 5.40	110.59 ± 47.20	839.07 ± 131.23	1.69 ± 0.39
Outer Waitati	43.81 ± 7.11	0.68 ± 0.08	4063.01 ± 171.81	55.30 ± 36.40	135.97 ± 119.51	578.78 ± 93.70	1.67 ± 0.83
Papanui Inlet	39.11 ± 7.57	0.75 ± 0.06	3924.23 ± 272.04	24.95 ± 8.81	106.76 ± 75.46	889.23 ± 118.41	1.21 ± 0.65
Purakaunui Inlet	48.51 ± 9.02	0.70 ± 0.08	4065.75 ± 211.85	34.92 ± 6.87	126.37 ± 60.46	764.91 ± 86.47	1.70 ± 0.73
Waikouaiti River estuary	43.97 ± 8.11	0.64 ± 0.09	4072.93 ± 265.48	42.23 ± 14.26	190.28 ± 121.69	885.19 ± 159.67	3.54 ± 2.26
Site	**Total length**	**Cu**	**Zn**	**Rb**	**Sr**	**Ba**	**Pb**
Andersons Bay Inlet	39.40 ± 6.65	0.17 ± 0.03	0.59 ± 0.18	0.05 ± 0.01	2010.92 ± 204.70	0.84 ± 0.30	0.01 ± 0.00
Hoopers Inlet	41.06 ± 6.56	0.18 ± 0.04	0.50 ± 0.08	0.08 ± 0.01	2157.85 ± 149.87	1.14 ± 0.25	0.01 ± 0.00
Inner Waitati	43.51 ± 10.13	0.18 ± 0.04	0.78 ± 0.20	0.08 ± 0.02	1809.48 ± 272.22	3.18 ± 2.73	0.01 ± 0.00
Kaikorai Lagoon	44.68 ± 8.48	0.15 ± 0.04	0.64 ± 0.15	0.16 ± 0.07	1975.06 ± 321.94	4.78 ± 1.52	0.01 ± 0.00
Otago Harbour	43.64 ± 10.25	0.19 ± 0.05	0.76 ± 0.45	0.10 ± 0.04	1954.56 ± 211.30	1.33 ± 0.50	0.01 ± 0.00
Outer Waitati	43.81 ± 7.11	0.17 ± 0.05	0.55 ± 0.09	0.05 ± 0.01	2114.96 ± 315.33	1.87 ± 1.73	0.00 ± 0.00
Papanui Inlet	39.11 ± 7.57	0.17 ± 0.04	0.50 ± 0.11	0.09 ± 0.02	2299.68 ± 207.62	1.54 ± 0.50	0.01 ± 0.00
Purakaunui Inlet	48.51 ± 9.02	0.18 ± 0.07	0.48 ± 0.16	0.08 ± 0.02	1929.91 ± 175.32	1.11 ± 0.46	0.01 ± 0.00
Waikouaiti River estuary	43.97 ± 8.11	0.16 ± 0.04	0.66 ± 0.25	0.10 ± 0.03	1903.83 ± 110.11	1.80 ± 1.12	0.01 ± 0.00

Gillanders 2006). From the randomly chosen juvenile individuals that ranged in total length from 27.12 to 62.60 mm (43.08 ± 8.39 mm), one of the paired sagittal otoliths was randomly chosen and mounted on a gridded glass microscope slide with double-sided adhesive tape. The forceps used to transfer otoliths to the glass microscope slide were washed with ultrapure Milli-Q water and dried with lint-free wipes between each otolith.

The left (blind-side) otolith from each age 1+ individual was selected for analysis (for more information, see Text S1 in the Supplement at www.int-res.com/articles/suppl/m742p113_supp.pdf). Each otolith was embedded in Crystalbond$^{\mathrm{TM}}$ 509 thermoplastic and sanded down from both sides of the longer axis by sequentially using a series of grade P400, P800, and P1500 sandpaper followed by finer 12 and 3 µm lapping films (3 M$^{®}$) lubricated with ultrapure Milli-Q water. The transverse section (~400 µm) was at the centre of the otolith core (Fig. 2C) and was cleaned with Milli-Q water, air dried, and mounted on a glass slide in preparation for otolith laser ablation.

2.4. Laser ablation

Concentrations of 13 elements commonly found and analysed in otoliths (^{7}Li, ^{23}Na, ^{25}Mg, ^{31}P, ^{39}K, ^{43}Ca, ^{55}Mn, ^{63}Cu, ^{66}Zn, ^{85}Rb, ^{88}Sr, ^{138}Ba, and ^{208}Pb) were measured in the otoliths of juvenile and adult sand flounder using a laser ablation inductively coupled plasma mass spectrometer (LA-ICP-MS). Data were collected on an Agilent 7900 quadrupole ICP-MS coupled to an Applied Spectra (AppliedSpectra.com) LA system with a Coherent ArF excimer laser (193 nm) and a Laurin Technique M50 cell located in the Centre for Trace Element Analysis at the University of Otago, Dunedin, New Zealand.

Otolith mounts were placed in the sampling cell and visually located via a 400× magnification video imaging system. For juvenile otoliths, the laser was fired at the estimated location of the primordial core (Fig. 2A) using the depth-profiling technique (Warburton et al. 2017) with a spot size of 75 µm and an approximate ablation time of 200 s, or until the laser travelled through the whole juvenile otolith. For adult otoliths, the transects ran from one side of the estimated central primordium core, passed through the core, and continued across the growth rings until the dorsal edge of the otolith transect was reached (Fig. 2D). The laser was fired on individual transects with a pre-ablation spot size of 75 µm to remove contaminants on the otolith surface, and a final spot size of 50 µm and scan speed of 10 µm s^{-1}. Across juvenile and adult otoliths, a washout time of 20 s between samples was applied and on-sample laser fluence was 2.6 J cm^{-2} at a repetition rate of 10 Hz. All ablations occurred under software-controlled helium flow (300 ml min^{-1}) mixed with an ICP-MS controlled argon stream (1 l min^{-1}) before the ICP torch. The helium atmosphere minimises re-condensation of ablated materials and elemental fractionation (Eggins et al. 1998). Nitrogen gas was added (~3.0 ml min^{-1}) to enhance sensitivity.

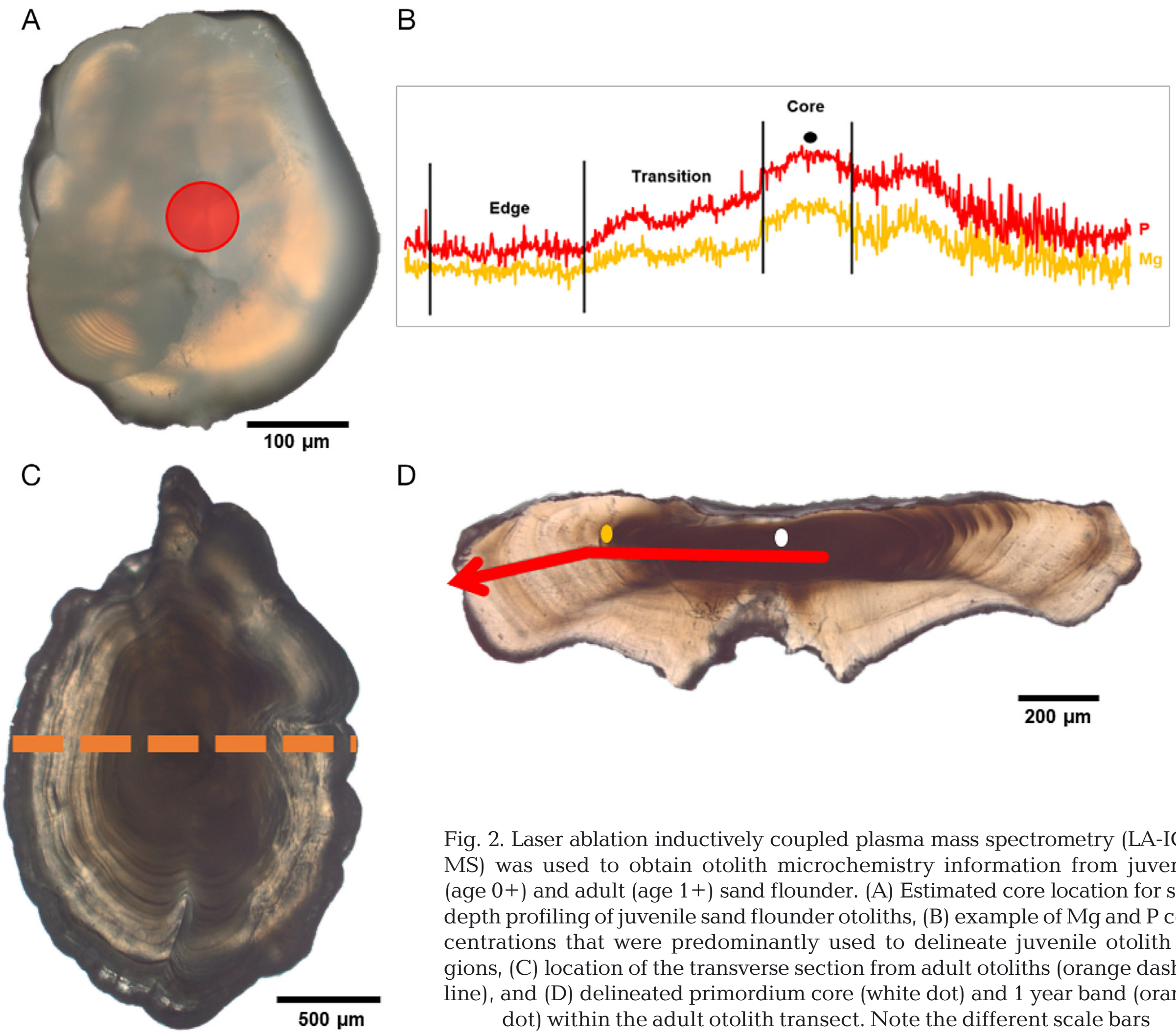

Fig. 2. Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) was used to obtain otolith microchemistry information from juvenile (age 0+) and adult (age 1+) sand flounder. (A) Estimated core location for spot depth profiling of juvenile sand flounder otoliths, (B) example of Mg and P concentrations that were predominantly used to delineate juvenile otolith regions, (C) location of the transverse section from adult otoliths (orange dashed line), and (D) delineated primordium core (white dot) and 1 year band (orange dot) within the adult otolith transect. Note the different scale bars

External standards, including National Institute of Standards and Technology (NIST, USA) Standard Reference Material (SRM) 610, NIST SRM 612, and United States Geological Survey Geochemical Reference Materials and Certificates (MACS)-3, were analysed at the start and end of each session as well as after every 6–8 samples to correct for mass bias and instrument drift. Background signals were obtained for 20 s between samples to establish a baseline and account for possible machine drift.

2.5. Data processing

Data refining and reduction were performed using the Trace_Elements_IS data reduction scheme in IOLITE version 3.71 (Paton et al. 2011) run on Igor Pro 7, with ^{43}Ca set as the internal standard. The calibration standard was NIST 610, while the calibration verification and matrix-matched reference materials were NIST 612 (Jochum et al. 2011) and MACS-3 (Jochum et al. 2019), respectively. The results of the analysed elements were within the published values of NIST 610 and NIST 612 (Jochum et al. 2011), and MACS-3 (Jochum et al. 2019) (see Table S1), while all measurements were found to be above the instrument's limits of detection (see Table S2).

Elemental concentrations were normalised using ^{43}Ca in a time-series graph (i.e. plotted versus laser distance) and are presented as micrograms per gram ($\mu g\ g^{-1}$). Elemental concentrations of ^{25}Mg have been observed to share a relationship with the life-stages of fish (Hüssy et al. 2021). Concentrations of ^{25}Mg, which often co-varies with ^{31}P, were higher in the central primordium core than on the edges of juvenile sand flounder otoliths, with a transition zone being identified (Fig. 2B). Each juvenile otolith was separated into 3 regions (pre-settlement, transition, and post-settlement), with the post-settlement (edge) region representing the elemental signature in otoliths corresponding to the natal estuary.

Following LA-ICP-MS, each adult otolith was photographed under transmitted light using a compound microscope (Olympus BX51) with an attached digital camera (Olympus XC50). Each image was imported into ImageJ 1.53v, and the length of the entire ablation transect was measured, including individual distance measurements between the start of the transect, outer edge of estimated primordium core, edge of first opaque zone, and the end of the transect. The distance between the outer edge of the estimated primordium core and the outer edge of the first opaque zone covered by the transect was measured to identify the distance of the transect that corresponds to the first year of growth. The distance measures were converted into time periods (distance/speed, where speed was 10 $\mu m\ s^{-1}$) that were incorporated and aligned onto the elemental time series graph to assist the delineation of otolith regions. The adult otolith time series were also separated into 3 regions (pre-settlement, post-settlement, and adulthood) based on their changes in elemental concentrations, particularly Mg, across the time series and distance measures. The average of each element's concentration within the post-settlement region of adult otoliths was calculated to use as a standardised, comparable value for analysis with the juvenile post-settlement regions. Two standard deviations were also calculated within the post-settlement regions (see Table S3). The resulting data standardised to time period ensured that data from transects and vertical profiling could be compared.

2.6. Data analysis

2.6.1. Pre-analysis

As non-normality was present for most elements, all average elemental concentrations ($\mu g\ g^{-1}$) in the post-settlement region of juvenile and adult sand flounder otoliths were $\ln(x + 1)$ transformed in R Studio (version 4.2.0) (R Core Team 2020) to improve the distribution and range of values. The significance level used for all statistical tests was $\alpha = 0.05$.

2.6.2. Relationship of juvenile total length with elements and sites

Identifying correlations between fish total length and otolith elemental concentrations can reduce confounding factors and/or indicate elemental incorporation patterns (Gillanders 2002a, Comyns et al. 2008). Therefore, the pattern of each element's relationship with fish total length (which is correlated with otolith weight/size) was visually and statistically assessed (linear regression analysis) (Gillanders 2002a, Comyns et al. 2008). An analysis comparing the total length of the selected juveniles between sites was also performed with a 1-way ANOVA or a Kruskal-Wallis rank sum test if assumptions of normality were not met. If fish total length significantly differed among sites, the standardised residuals of elements that had significant relationships with fish total length would be used in univariate analyses to eliminate possible spatial bias due to fish size.

2.6.3. Variation in juvenile otolith microchemistry between estuaries

A linear mixed effects model, with otolith region (pre- and post-settlement) as a fixed effect and site as a random effect, was run for each element to determine whether elemental concentrations differed between the pre- and post-settlement regions.

Both univariate and multivariate statistical analyses were performed to investigate spatial differences in single- and multi-elemental concentrations in the post-settlement region of juvenile sand flounder otoliths collected from different estuaries. As parametric assumptions of ANOVA were not met, univariate differences between the concentration of each element between sites (fixed effect) for each species were tested using 1-way Kruskal-Wallis rank sum tests, with pairwise Dunn's post hoc tests being performed if a significant overall result occurred to identify what locations significantly differed from one another. The adjusted p-values based on the Holm method in the Dunn's post hoc test were used to control for the type-1 error rate across the large set of comparisons (n = 36). All univariate analyses were performed in R Studio (version 4.2.0) (R Core Team 2020).

Multi-elemental differences in the post-settlement region were assessed between sites by conducting permutational multivariate ANOVA (PERMANOVA) and permutational homogeneity of variance (PERMdisp) tests in Primer v.6 with the PERMANOVA + v.1 add-on (PRIMER-E) (Clarke & Gorley 2006, Anderson et al. 2008). The non-parametric methods available in PRIMER are robust to unbalanced designs and correlations among response variables, and they do not rely on normality and homogeneity assumptions, as p-values are calculated under permutation (Anderson et al. 2008). Prior to multivariate analysis, element concentrations were normalised to ensure that they were all the same magnitude (all had a mean of 0 and standard deviation of 1) by using *Z*-scores (the difference between the observation and overall mean, divided by the overall standard deviation). All PERMANOVA and PERMdisp analyses were performed on the Euclidean distance matrices, which were constructed from each of the normalised data sets, and analyses had 9999 unrestricted permutations with pairwise tests.

Lastly, canonical analysis of principal coordinates (CAP) with leave-one-out cross validation was conducted to classify juvenile samples to one of the estuaries sampled (*a priori* groups) based on their multielemental otolith signature (Anderson et al. 2008, Chittaro et al. 2009, Ley & Rolls 2018). The CAP estimates the number of samples from each estuary that could be correctly predicted to have been taken from that estuary. Linear and canonical discriminant analysis (LDA and CDA, respectively) have been frequently used as methods of classification in otolith microchemistry studies (Comyns et al. 2008, Leakey et al. 2008). However, CAP was utilised in the present study as the assumptions of LDA and CDA could not be met. The centroid for each CAP group represents the centrepoint of all samples from each group (equivalent to multivariate mean in MANOVA) in multidimensional space, and an associated permutation test with 9999 permutations was performed to test for significant differences among group centroids (Anderson et al. 2008). To assess how elements contributed to the discrimination of groups, Spearman rank correlations of individual elements with the CAP axes were also interpreted. Two CAP models were performed: a model with a fine-scale (individual estuaries) grouping and a model with a broad-scale (groups of similar estuaries) grouping based on the NZ ETI classification scheme (Robertson et al. 2015, Plew et al. 2018).

2.6.4. Assigning the nursery origin of adult fish

Adult sand flounder (age 1+, n = 81, unknown nursery origin) were assigned to an estuary and NZ ETI estuary type in east Otago by comparing the multielemental signature in their post-settlement otolith region to the signature in the post-settlement otolith region from juvenile sand flounder of known origin. Prior to adult classification, the multi-elemental signatures of the juvenile stage of adult otoliths were compared with juvenile sand flounder otoliths (collected in summer 2020) using PCA on non-normalised values (Chittaro et al. 2009, Reis-Santos et al. 2013). To reduce the potential bias of uncharacterised sources, the adult individuals that were outside the 95% confidence interval ellipse of all juvenile elemental signatures in the PCA were classified as originating from a nursery habitat outside of the east Otago region and were removed from subsequent analyses.

For each of the remaining adults (n = 78), the average concentration of each individual element in the juvenile portion of the adult otoliths was normalised with the juvenile samples to ensure equal weighting of elements in the CAP, converted into a Euclidean distance matrix, and incorporated onto the canonical axes of the existing CAP model with juvenile sand flounder otoliths as the baseline values to classify each of the adult samples into one of the existing groups. Each adult individual was allocated to the estuarine group whose centroid was closest to it in canonical space (Anderson et al. 2008). A CAP model was constructed for assigning adult origin to individual estuaries and to the broad-scale NZ ETI classification. The assignments of adults were then used to assess the proportion of individuals that likely recruited from each of the estuaries.

3. RESULTS

3.1. Relationship of juvenile total length with elements and sites

Linear regression analyses detected 3 (out of 12) significant correlations between elements in the post-settlement region of sand flounder otoliths with their total length. Significant positive correlations were found between K ($r^2 = 0.094$, $F_{1,88} = 9.44$, $p = 0.0028$) and Rb ($r^2 = 0.12$, $F_{1,88} = 12.23$, $p = 0.001$) with fish total length, while Mg was negatively correlated with fish total length ($r^2 = 0.052$, $F_{1,88} = 4.75$, $p = 0.032$). However, both K and Mg had trivial effect sizes ($r^2 < 0.1$), while Rb had a weak effect size ($0.1 < r^2 < 0.3$) with the total length of juvenile sand flounder. Although significant correlations were detected, the influence on the results and requirement for the removal of fish size was considered limited, as the total length of juvenile sand flounder analysed did not significantly differ among sites (one-way ANOVA, $F_{8,81} = 1.22$, $p = 0.30$).

3.2. Spatial variability in the edge otolith elemental signature of juveniles

The pre- and post-settlement otolith regions of juvenile sand flounder had significantly different elemental concentrations, with only Ba being not significantly different between the 2 otolith regions (linear mixed effects model, $F_{1,152} = 0.41$, $p = 0.52$).

Nine elements (out of 12 analysed) in the post-settlement regions of sand flounder otoliths differed significantly between sample sites (Kruskal-Wallis, $p < 0.05$, Table 2). The 3 elements not significantly different between the 9 sites were P, Cu, and Pb, and were subsequently excluded from the multivariate analyses. Based on the significant p-values, significant post hoc comparisons, and visual observation, Li, K, Mn, Rb, and Ba were the elements with the greatest variability among estuarine sites (Tables 1 & 2).

Significant differences in multi-elemental signatures (with 9 elements) were also detected between estuaries (PERMANOVA, pseudo-$F_{8,81} = 8.29$, $p_{(perm)} = 0.0001$). Although 1/20 pairwise comparisons would be expected by chance, most of the sites were found to significantly differ in their multi-elemental signature (33/36 pairwise tests with $p_{(perm)} < 0.05$). Non-significant multi-elemental pairwise comparisons (n = 3/36) were detected between Andersons Bay Inlet and Outer Waitati, Papanui Inlet and Hoopers Inlet, and Otago Harbour and Waikouaiti River estuary. Although significant differences were detected by the PERMANOVA, a significant overall dispersion effect (PERMdisp, $F_{8,81} = 3.89$, $p_{(perm)} = 0.0068$) and associated significant pairwise tests (16/36 pairwise tests with $p_{(perm)} < 0.05$) removed the ability to determine whether the significant differentiation was due to the separation and/or dispersion of groups in multivariate space.

The CAP analysis performed with the 9 elements that significantly differed among sites produced the highest overall classification success of 56.67% (51/90) on an individual estuary scale, ranging from 40% (Hoopers Inlet, Outer Waitati, Waikouaiti River estuary) to 90% (Kaikorai Lagoon) (see Table S4). Overall, 6 of the 9 estuaries had ⩾50% of their juvenile sand flounder correctly classified, supporting the conclusion that the 9-estuaries model accounted for a considerable portion of the variation in the data. The sizes of the canonical correlations with the first 2 CAP axes were relatively strong ($\delta1 = 0.89$ and $\delta2 = 0.74$), and the CAP trace statistic was also significant ($p = 0.0001$). Misclassifications were shared between overlapping sites in multivariate space (Fig. 3), which included both adjacent sites (for example, Hoopers Inlet samples misclassified to Papanui Inlet) and those with expected similar physico-chemical conditions (for example, Andersons Bay Inlet and Outer Waitati Inlet). The broad-scale classification, which grouped estuaries by their NZ ETI classification (Fig. 1), increased the overall classification success to 82.2% (74/90) (Table 3). Both models' classification successes of 56.7 and 82.2% were more than would be expected by chance alone (11.1 and 33.3%, respectively). The vector overlay in the CAP plot (Fig. 3) conveys the direction and discriminatory strength (length of rays) of the elements measured in the otoliths. The vector representing Li was associated with samples from SIDEs/DSDE and is well correlated with CAP1 (Spearman's $r = -0.92$). Both Na and Sr were less influential (indicated by shorter vector lengths) but were also aligned in the same direction across CAP1 (Na, $r = -0.66$; , $r = -0.37$), being at higher concentrations in otoliths from SIDEs/DSDE. Three elements (Ba, Mn, Zn) were positively correlated with CAP1 and at higher concentrations for SSRTREs, with Ba ($r = 0.45$) and Mn ($r = 0.60$) being the most influential. Correlations with CAP2 included K ($r = -0.84$), Rb ($r = -0.83$), and Mg ($r = 0.68$).

3.3. Nursery origin of adult sand flounder

Only 3 adults, located outside the 95% confidence interval ellipse in the PCA plot, were considered outliers and subsequently removed from the analysis (Fig. 4). Two individuals had high concentrations of Zn and Rb, while one individual was noted as having abnormal otolith calcification and had comparatively

Table 2. Univariate analysis comparing individual element concentrations in the post-settlement region of otoliths from juvenile (age 0+) sand flounder collected from 9 estuaries in east Otago, New Zealand. **Bolded** elements are those that varied significantly ($p < 0.05$) between sites, with means and standard deviations across all sites combined. Kruskal-Wallis rank-sum tests were used to determine overall significance, while the post hoc pairs are the number of significant pairwise tests (out of 36) that were revealed by the Dunn's post hoc test

Element	Mean ($\mu g\ g^{-1}$)	SD ($\mu g\ g^{-1}$)	Kruskal-Wallis χ^2	p	Post hoc pairs
Li	0.64	0.16	52.00	< 0.0001	9
Na	3986.13	248.06	20.16	0.010	1
Mg	38.91	19.35	36.98	< 0.0001	5
P	127.94	89.66	14.33	0.074	0
K	741.40	162.32	52.86	< 0.0001	8
Mn	4.26	5.88	58.86	< 0.0001	11
Cu	0.17	0.05	3.41	0.906	0
Zn	0.60	0.23	20.39	0.009	2
Rb	0.09	0.04	53.25	< 0.0001	10
Sr	2017.36	260.39	29.30	< 0.0001	5
Ba	1.95	1.71	44.60	< 0.0001	8
Pb	0.01	0.00	11.59	0.170	1

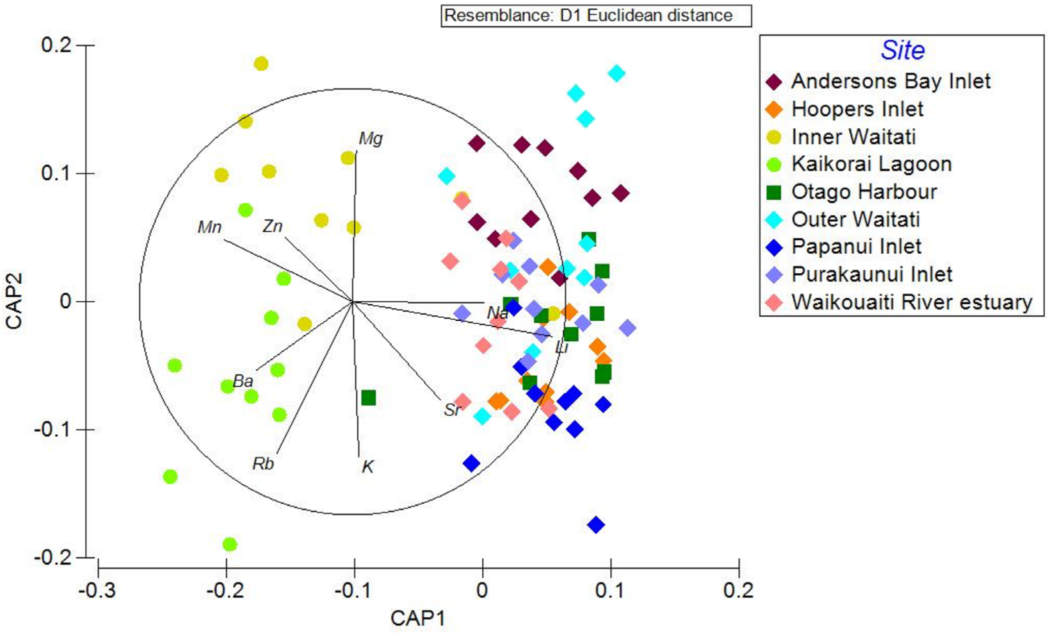

Fig. 3. Canonical analysis of principal components (CAP) plot, displaying variation in the multi-elemental (Li, Na, Mg, Mn, K, P, Zn, Rb, Sr, and Ba) signature in the post-settlement region of juvenile (age 0+) sand flounder collected from 9 estuaries in east Otago, New Zealand. Each symbol represents an individual otolith in multivariate space, with 10 individuals/otoliths analysed per site. The symbol shapes are representative of the New Zealand Estuary Trophic Index (NZ ETI) classification scheme: SIDE (diamond), SSRTRE (circle), DSDE (square) (see Fig. 1 for group definitions). The vector overlay (based on Spearman rank correlations) shows the direction and weight of individual elements in discriminating sites

higher Li, Mg, and Mn, and lower Na, K, and Sr compared to other individuals. The first 2 axes in the PCA plot explained 48.4% of the total variation, indicating that less than half of the variation was contained in the first 2 eigenvalues.

Each of the 78 adult (age 1+) individuals was assigned to a juvenile habitat (east Otago estuary) whose centroid was closest in multivariate space via the CAP routine (Table 4). The highest number of adult sand flounder were assigned to Hoopers Inlet (n = 25), representing 32% of the adult samples. However, as many estuaries overlapped in multivariate space, the reliability of the classifications at the fine-scale (individual estuaries) resolution was low. Consequently, adult classifications at a broad-scale (grouped estuaries) resolution were considered more reliable and revealed that most adult sand flounder of the spring 2020 cohort were allocated to SIDEs (n = 48, 62%), followed by DSDE (n = 25, 32%) and SSRTREs (n = 5, 6%) (Table 4).

Table 3. Canonical analysis of principal component (CAP) classification matrix constructed using otolith multi-elemental signatures (Li, Na, Mg, Mn, K, Zn, Rb, Sr, Ba) from juvenile (age 0+) sand flounder in east Otago, New Zealand, to classify juveniles to estuary groupings based off the New Zealand Estuary Trophic Index (NZ ETI) classification scheme (broad-scale resolution). The 3 estuary groupings are shallow intertidal dominated estuaries (SIDEs, includes Andersons Bay Inlet, Hoopers Inlet, Outer Waitati, Papanui Inlet, Purakaunui Inlet, Waikouaiti River estuary), shallow, short residence time river and tidal river with adjoining lagoon estuaries (SSRTREs, includes Inner Waitati and Kaikorai Lagoon), and deep, subtidal dominated, longer residence time estuaries (DSDE, includes Otago Harbour)

Collection locality	— Predicted grouping — SIDEs	SSRTREs	DSDE	No. correct	No. analysed	% correct
SIDEs	50	1	9	50	60	83.3
SSRTREs	2	18	0	18	20	90
DSDE	4	0	6	6	10	60
Overall	56	19	15	74	90	82.2

% chance per site = 33.3%
Permutation test trace statistic = 1.04, p = 0.0001

4. DISCUSSION

4.1. Otolith microchemistry characterisation of juvenile habitats

The first step in effectively using elemental concentrations in otoliths as a natural tag for identifying the nursery origin of adult fish is determining whether fish residing in different estuaries can be discriminated (Forrester & Swearer 2002, Campana 2005, Gillanders 2005, Brown 2006). Signifi-

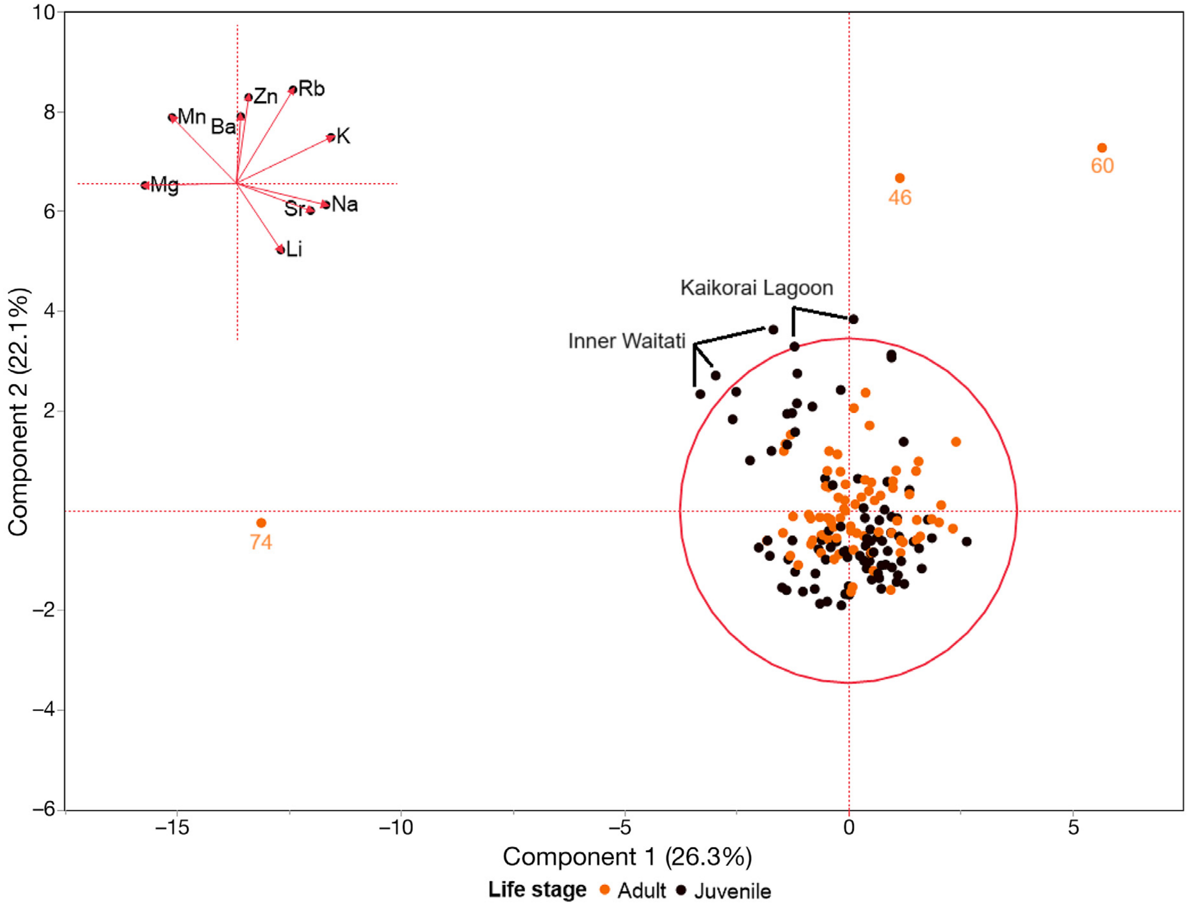

Fig. 4. Ordination plot of principal component analysis (PCA) comparing the multi-elemental signatures of the post-settlement region of otoliths from adult (age 1+) sand flounder (orange) of unknown estuarine origin and juvenile (age 0+) sand flounder of known estuarine origin (black). A 95% confidence interval ellipse surrounds the juvenile data, with 3 adults (individuals 46, 60, 74) and 5 juveniles from Inner Waitati (n = 3) and Kaikorai Lagoon (n = 2) falling outside the ellipse. The biplot (top left) displays the strength and direction of elements in discriminating among individuals

cant differences in individual and multi-elemental signatures within the post-settlement region of juvenile sand flounder otoliths were detected among the 9 estuaries in east Otago, indicating that multi-elemental signatures could be used as natural tags for the nursery grounds of sand flounder. To our knowledge, the present study was the first to investigate the fine- and broad-scale spatial variability of a natural tag in sand flounder in New Zealand, providing insights for understanding population connectivity, specifically, important links between nursery habitats in estuaries and adult populations on the outer coast and shelf.

Otolith microchemistry studies on flatfish have been successful in assigning individuals to geographic origins over a vast range of spatial scales (for example, 10s to 100s of km) (see Marriott et al. 2016 for review). Most of these studies had overall classification successes ranging from 58 to 92%; however, they were conducted on a scale of hundreds of kilometres compared to the present study, which was conducted on a scale of tens of kilometres (~65 km between most northern and southern sites). The overall classification success of 50.7% for juvenile sand flounder is greater than what would be expected by chance (11.1%) (Anderson et al. 2008). Variability in otolith elemental signatures between estuaries can be associated with differences in environmental, catchment input, and physico-chemical conditions, while levels of variation within estuaries is often attributable to habitat heterogeneity and temporal fluctuations of environmental conditions (Campana 1999, Gillanders 2002b).

Otolith elemental signatures from Inner Waitati and Kaikorai Lagoon were notably different from all other estuaries in east Otago as they had higher reclassification successes of juveniles and less overlap in multivariate space, which could be attributable to more freshwater influence. A relatively unexpected result was the similarity between the Waikouaiti River estuary and estuaries classified as SIDEs, as the Wai-

Table 4. Allocations and relative proportions of adult (age 1+) sand flounder (n = 78) that were classified to their potential nursery areas in east Otago, New Zealand, based on the multi-elemental signatures in the post-settlement region of their otoliths. Allocations were performed by a canonical analysis of principal components (CAP) with multi-elemental signatures of juvenile (age 0+) sand flounder otoliths from the 9 different sites (n = 10 per site) as the baseline data. Two separate CAPs were performed for (1) the individual sites and (2) the sites grouped by the New Zealand Estuary Trophic Index (NZ ETI) characterisation with group abbreviations as in Table 3 (also see Fig. 1). The surface area at spring high tide and shoreline perimeter length of each site were sourced from Hume et al. (2016), except for Andersons Bay Inlet and Inner Waitati, which were calculated in QGIS 3.28.1 and subtracted from the Otago Harbour and Outer Waitati values

Site	Number allocated by CAP	Proportion allocated by site/group	Surface area (km^2)	Shoreline length (km)
Andersons Bay Inlet	3	0.04	0.13	1.64
Hoopers Inlet	25	0.32	3.75	12.21
Inner Waitati	3	0.04	0.17	3.37
Kaikorai Lagoon	4	0.05	0.64	10.56
Otago Harbour	14	0.18	47.79	78.98
Outer Waitati	5	0.06	6.06	17.03
Papanui Inlet	12	0.15	3.63	10.76
Purakaunui Inlet	7	0.09	1.13	7.05
Waikouaiti River estuary	5	0.06	1.27	19.78
Sites (by ETI)				
SIDEs	48	0.62	15.97	68.46
SSRTREs	5	0.06	0.82	13.94
DSDE	25	0.32	47.79	78.98

kouaiti River estuary has a freshwater fraction of 35%, which is higher than the freshwater fraction of Kaikorai Lagoon (21%) and much higher than remaining SIDEs (mean ± SD, ~4 ± 3.6%) (Plew et al. 2018). Caruso (2001) concluded that the Waikouaiti River regularly has extreme low flows and durations, which in combination with the low water residence time (<1 d) and sampling flounder in the middle/lower reaches of the estuary may explain why relative elemental concentrations did not indicate an estuary with more freshwater influence.

Other studies assigning fish to locations based on otolith microchemistry found that incorrect classifications often occur between neighbouring or adjacent sites, as they likely have the closest similarities in environmental conditions (Comyns et al. 2008, Ley & Rolls 2018). In the present study, the increased misclassifications and greater overlap in multivariate space among the 7 remaining estuaries (SIDEs and Otago Harbour) was likely indicative of similar physico-chemical conditions (Campana 1999, Fairclough et al. 2011). The possibility of misclassifications between sites has also been attributed to potential movement between habitats by individuals (Leakey et al. 2008). However, low numbers of misclassifications and the observed distinct otolith microchemistry between connected sites supports existing knowledge on the high site fidelity of juvenile flounder (Reis-Santos et al. 2015, Marriott et al. 2016). In future studies, classification accuracy may potentially be increased by higher sample sizes (Mohan et al. 2012, Norrie et al. 2016, Ley & Rolls 2018) and/or additional methods, including stable isotopes of otoliths (δ^{13}C and δ^{18}O) and eye lenses (δ^{13}C, δ^{15}N, δ^{34}S) (Comyns et al. 2008, Clarke et al. 2009, Stounberg et al. 2022).

The results of the present study suggest that otolith microchemistry may be used more reliably as a natural marker for sand flounder at a broader spatial scale by grouping estuaries with similar physico-chemical conditions together. For example, after reviewing 30 different design components across 14 publications on inter-estuary comparisons using otolith microchemistry, Ley & Rolls (2018) concluded that the number of groups compared is negatively correlated with the overall cross-validation success. The use of otolith microchemistry as fine-scale tags has been consistently challenging in previous studies (Stransky et al. 2005, Lara et al. 2008, Gerard & Muhling 2010, Liu et al. 2010, Martinho et al. 2020), with many lowering the fine-scale resolution by pooling source sites (Gillanders & Kingsford 1996, Forrester & Swearer 2002, Gillanders 2002a, Fodrie & Levin 2008, Clarke et al. 2009). For example, Fodrie & Levin (2008) grouped 14 locations into exposed coasts, bays, lagoons, and estuaries, improving the overall classification success from 33% (14 sites) to 66% (4 groups). As expected, when the 9 estuaries in the present study were classified into 3 groups (SIDEs, DSDE, and SSRTREs) based on the similarities in their physical characteristics underpinning the NZ ETI classification, the overall classification accuracy for juvenile sand flounder improved from 56.67% to 82.2%. Characterising estuaries into groups to ensure that microchemical signatures become more distinct results in a greater cross-validation success rate and improved accuracy when assigning nursery origin to individuals of unknown origin (Mercier et al. 2011), thereby potentially informing management actions.

While some studies have reduced the elemental set to improve the overall classification success of individuals to their respective estuaries, others have suggested to include all significantly different elements (Vasconcelos et al. 2007, Marriott et al. 2016). The present study found that the largest amount of variation in the data was explained by including all 9 significantly different elements in the model, a number that has been used in previous stock discrimination studies (Norrie et al. 2016). As all elements interact differently with the environment and/or physiology, they can all provide a level of discrimination among groups, which is especially important for studies over smaller geographic ranges (<500 km) (Marriott et al. 2016). However, certain conservative and bioactive elements previously utilised as environmental tracers and markers among coastal and estuarine habitats had stronger discriminatory strength for juvenile sand flounder collected from SIDEs and Otago Harbour than for those collected from SSRTREs (Gillanders 2002b, Swearer et al. 2003, Brown 2006, Mohan et al. 2012, Doubleday et al. 2014, Marriott et al. 2016). In general, elements associated with SSRTREs were Mn, Zn, Rb, and Ba, while elements at higher concentrations associated with SIDEs and Otago Harbour were Li, Na, and Sr.

The reduced salinity in SSRTREs likely resulted in higher concentrations of the conservative elements that have greater bioavailability in freshwater (Rb and Ba) and lower concentrations of conservative elements that have greater bioavailability in saltwater (Li and Sr) compared to SIDEs and Otago Harbour (Dorval et al. 2007, Brown & Severin 2009, Walther & Limburg 2012). However, many other factors are known to influence the incorporation of these elements, including temperature (Tanner et al. 2013), chlorophyll *a* (Grammer et al. 2017), and physiological processes (Brown 2006), all of which could have contributed to discrimination among groups. Bioactive elements, including Mn and Zn, were also elevated in juvenile flounder from SSRTREs, likely associated with exposure to highly enriched anthropogenic nutrient sources, the added physiological stress of variable salinity, sediment re-suspension, and/or low-oxygen conditions (Geffen et al. 2003, Leakey et al. 2008, Tabouret et al. 2010, Limburg et al. 2015). Despite being considered under strong physiological control (Hüssy et al. 2020), the concentrations of Na, Mg, and K in juvenile flounder otoliths also contributed to discrimination among estuaries (Gillanders 2002a, Clarke et al. 2009). As many of the estuaries in east Otago share similar environmental and physicochemical conditions, physiologically influenced elements likely provide an additional tool to distinguish between juveniles from these similar estuaries (Martino et al. 2021).

4.2. Application of otolith microchemistry as a natural tag in sand flounder

Identifying important juvenile nursery grounds for sand flounder in Otago is crucial for understanding the recruitment dynamics of the local population, potentially contributing to enhanced management strategies, including links to estuarine environments to support a more sustainable sand flounder fishery (Vasconcelos et al. 2011, Marriott et al. 2016, Tanner et al. 2016). Only 3 (out of 81) adult sand flounder analysed were excluded from analysis due to a lack of similarity to multi-elemental signatures of juvenile sand flounder otoliths. The inability to assign certain individuals to a location can be attributable to a range of factors, including otolith accretion and elemental incorporation abnormalities of individuals, ageing error, incorrectly characterising otolith regions, and/or other potential source locations (nursery areas) (Gillanders 2002a, Campana 2005). For example, as sand flounder along the east coast of the South Island have been recorded to move over 200 km (Colman 1978) and are considered to be one stock (Hannan et al. 2016), immigrations of adults from regions outside of east Otago are possible, although the extent is unknown. As only 3 individuals were excluded from analysis, the estuaries sampled in east Otago likely encompassed most, if not all, of the likely source habitats of sand flounder in Otago (Colman 1978). It is possible that individuals originated from uncharacterised estuaries in other regions that have similar otolith elemental signatures to those in east Otago (Gillanders 2002a, Fodrie & Levin 2008). However, the potential influence of adults originating from uncharacterised source habitats outside the east Otago region was minimised through elucidating the nursery origin of age 1+ fish that had only recently emigrated from their nursery habitat to join adult populations on the outer coast and shelf (Colman 1974b, 1978). It would be interesting to assess whether the number of adults not classified increases in later year classes as flounders may begin to migrate greater distances as they increase with age. An assumption not tested in the present study was the presence of temporal variation within the recruitment period of juveniles in late spring and early summer of 2020. If large temporal variation was present in the recruitment period of the young-of-year cohort, classifications of adults to their natal nursery area may be confounded (Elsdon & Gillanders 2006).

The results of both the fine-scale (all 9 individual estuaries) and broad-scale (DSDE, SIDEs, SSRTREs) resolution models indicate that all juvenile habitat types in east Otago likely contributed to the adult population of sand flounder in east Otago for the 2020 year class. As precision and accuracy limitations restrict reliable conclusions from the fine-scale model, overall results suggest that most adult sand flounder (62%) from the 2020 year class were reared in SIDEs in east Otago. A disproportionate contribution of SIDEs to the adult population may be associated with those habitats supporting higher densities, higher growth rates, lower mortality, and/or greater habitat connectivity and transport mechanisms with the adult population (Beck et al. 2001). The size and shape of estuaries often contribute to their value as nurseries, with larger estuaries and wider openings to the ocean believed to facilitate greater recruitment through the ability of larvae to enter and remain in the area (Beck et al. 2001, Martinho et al. 2009). Although Otago Harbour (DSDE) has the greatest area compared to all other estuaries, the amount of suitable habitat for juvenile flounder will encompass a much smaller portion of the total area as the intertidal areas of DSDEs are typically less than 5% (Plew et al. 2018). Comparatively, a higher proportion of suitable habitat may occur within shallow, intertidal dominated estuaries as their intertidal areas are typically greater than 40% (Plew et al. 2018) and their higher frequency along the east Otago coastline may also facilitate more settlement and residence of juvenile flounder. For instance, as 6 out of the 9 estuaries in the present study were classified as SIDEs, a higher contribution of SIDEs to the adult population of sand flounder would be expected. Over 150 out of around 440 estuaries in New Zealand are classified as SIDEs (Robertson & Savage 2021), so their importance for juvenile flounder is likely retained throughout New Zealand. Many theories can be postulated as to why Inner Waitati and Kaikorai Lagoon had comparatively lower contributions of recruits, including reduced habitat areas, increased stress, and restricted immigration and emigration. Although increased freshwater flow has been shown to improve recruitment of greenback flounder in southern Australia (Earl 2014) and flounder species worldwide (Amorim et al. 2016), this hypothesis cannot be supported with the current results for sand flounder in east Otago, New Zealand.

4.3. Limitations

The present study employed 2 distinct analytical techniques for obtaining the elemental concentrations within juvenile and adult sand flounder otoliths. The decision to use depth profiling for juvenile otoliths stemmed from feasibility concerns and the risk of sample loss. The small size of juvenile otoliths, represented by maximal lengths as low as 0.4 mm, presented challenges with otolith embedding and sectioning; this is consistent with Easey & Millner (2008), who suggested that otolith sections of less than 0.3 mm have a high risk of fracturing. The decision to use horizontal transects for adults emanated from the depth of laser ablation that would be required to sample the post-settlement region, thereby limiting the accuracy of elemental detection and precise life history zone identification.

Sampling the proximal–distal plane of juvenile otoliths and anterior–posterior plane of adult otoliths introduces potential limitations to our ability to reliably compare concentrations in juvenile and adult otoliths, primarily attributable to differences in otolith accretion rates across different otolith planes. To mitigate the limitations associated with the analytical sampling approaches, the post-settlement region of juvenile and adult otoliths was detected and focussed on average elemental concentrations across the region rather than fine-scale changes. Additionally, grouping estuaries based on their NZ ETI classification supported the reliability of the comparative analysis through reducing the reliance on fine-scale differences in concentrations associated with classifying adults to all 9 sampled estuaries. Lastly, the distribution of sample means and standard deviations of juvenile and adult samples for the significant elements were similar and overlapping for the elements that were compared (see Figs. S1 & S2). Despite these mitigations, using the same analytical technique for both juvenile and adult otoliths might have yielded different results.

4.4. Future studies

Recruitment and connectivity patterns of fish are influenced by both biotic and abiotic factors (Kraus & Secor 2005). Despite the results of the present study suggesting that SIDEs in east Otago contribute disproportionately to the adult population of sand flounder, this may not be stable through time as both environmental and ecological conditions are temporally variable. For example, Yamashita et al. (2000) suggested that 65 and 32% of adult stone flounder

Platichthys bicoloratus originated from estuarine habitats across 2 years (1994, 1995), highlighting that estuarine contributions are likely variable among year classes, a pattern which has been found in many other nursery contribution studies (Chittaro et al. 2009, Clarke et al. 2009, Reis-Santos et al. 2013, Tanner et al. 2013). As year-to-year variation in commercial landings of flounder species in New Zealand has been noted as a potential result of recruitment variation (Fisheries New Zealand 2022), a longer-term study across different year classes and through time is required to resolve interannual patterns in recruitment and otolith microchemistry. Additionally, investigating recruitment through time should also monitor and incorporate environmental variables that vary on different time scales and influence recruitment (Ley & Rolls 2018). Following and researching year classes, otolith microchemistry, and environmental and biotic variables in estuaries through time would provide an extraordinary wealth of information on the dynamics and connectivity patterns of sand flounder populations as increasing environmental variability from marine heatwaves and eutrophic events may affect viability of some nursery habitats.

4.5. Conclusion

Natural tags successfully identified the nursery origin of age 1+ sand flounder collected from the coastal fishery in east Otago in 2022. Although the mechanisms behind spatial differences between estuaries in otolith microchemistry are not completely understood, they were still able to provide a broad-scale natural tag for the nursery origin of sand flounder. The findings of the present study highlighted that many estuarine habitats in east Otago are important nursery habitats for juvenile flounders, especially those estuaries characterised as SIDEs. Conserving these estuarine nursery habitats will be an important component in maintaining high levels of recruitment from estuaries to the adult population(s), although further research is required on more cohorts to assess the reliability and variability of results through time. The present research demonstrates the ability of otolith microchemistry to quantify the ontogenetic connectivity of a vital taonga (treasured), commercial, recreational, and mahinga kai (customary fishery) species.

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