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Otolith development in wild populations of stickleback: Jones & Hynes method does not apply to most populations

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This paper critiques Jones & Hynes (1950) findings by analysing sequential samples of otoliths from three wild populations of *Gasterosteus aculeatus* from North Uist, Scotland and Nottingham, England. Contrary to Jones & Hynes (1950), but coincident with the finding of later researchers, our results showed that no true translucent ring formed in the otolith of *G. aculeatus* during their first summer. The first translucent ring was probably starting to be formed by the end of summer and was completed by the end of their first winter. There was no second opaque ring in the otoliths of *G. aculeatus* before they passed their first winter. The second opaque ring was just starting to appear by early April of the second year in the southern population *i.e.* Nottingham, but later, by May, in the northern populations *i.e.* North Uist. Formation of the opaque ring in *G. aculeatus* mostly occurs in spring and summer, with younger fish starting earlier. In contrast, the formation of translucent rings is mostly during autumn and winter, but can be more widespread through the year, possibly as a result of reproductive investment.

KEYWORDS

ageing, *Gasterosteus aculeatus*, otolith, seasonal ring, three-spined stickleback

1 | INTRODUCTION

The determination of age in fishes by counting of annual rings on otoliths has been well established among researchers and fisheries managers for decades (Campana, 2005; Jones & Hynes, 1950). The method is still widely used (Koeda *et al*., 2016; Robertson *et al*., 2016; Uriarte *et al*., 2016) though more recent approaches for otolith analysis, such as daily increment quantification, microchemistry, carbon dating, plasma mass spectrometry and three dimensional x-ray scanning (Hippel *et al*., 2013; Jones & Chen, 2003; Mapp *et al*., 2016; Thorrold & Shuttleworth, 2000) are more powerful for age estimation. Reading of otolith annual rings, if validated, is still useful in ecological studies because it is cheap and more easily applied to large samples. Otolith reading is also generally considered more accurate as a method of age estimation than similar reading of other calcified structures (Buckmeier *et al*., 2002; Campana, 2001; Erickson, 1983).

The method of reading annual rings of otoliths to obtain age estimates relies on contrasting growth patterns during good *v.* poor growing conditions and is probably related to temperature (Hüssy *et al*., 2004) and food availability. Rich food conditions, that usually occur in summer, correlate with faster growth and growing otoliths; while poor food conditions, that usually occur in winter, are related to slower somatic growth and little or no growth of otoliths (Beckman & Wilson, 1995; Chilton & Beamish, 1982; Williams & Bedford, 1974), but see Jones and Hynes (1950). In the great majority of fishes, faster growth of otoliths results in formation of an opaque ring that consists mainly of organic material, while slow growth produces a translucent ring (for a definition see Kalish *et al*., 1995) which is dominated by inorganic materials (Beckman & Wilson, 1995; Chilton & Beamish, 1982; Hüssy *et al*., 2004; Williams & Bedford, 1974). This opaque-translucent pattern repeats over the years, following the growth of the fish and makes it possible to use the resulting rings for age estimation. Although the term otolith generally refers to three kinds of ear stone in the labyrinth system of the fish, the largest otolith, *i.e.* sagittal, is the most studied and used for age determination among researchers. In keeping with the wider literature, we use the term otolith to refer to the sagitta.

Significant problems with reading rings in calcified structures, which can lead to inaccurate age determination, are the presence of smear or fake ring checks and inconsistent patterns from year to year, place to place or between fish species (Williams & Bedford, 1974). Checks probably arise from short-term alterations in growth as a result of brief decreases (or increases) in otherwise good (or poor) conditions (Campana & Neilson, 1985; Uriarte *et al*., 2016). These genuine problems of interpretation have sometimes been compounded by lax methodology, such as failing to read otoliths blind to other information about them, using small samples, not accounting for different patterns between age groups and failing to adequately validate findings (Campana, 2001).

Here we review the use of otoliths to age three-spined stickleback *Gasterosteus aculeatus* L. 1758, a small circumboreal fish of no significance to commercial fisheries, but which has increasingly become a model organism for the study of behaviour, ecology, parasitology, ecotoxicology, evolution and recently, and most prominently, evolutionary molecular genetics (Bell & Foster, 1994; Colosimo *et al*., 2005; Jones *et al*., 2012; Robertson *et al*., 2016). The increasing interest in this fish demands a careful approach if reliable ages of wild-caught individuals are to be inferred, since many patterns of interest to researchers are age-related, including growth, senescence and other aspects of life history, as well as population dynamics, epidemiology and natural selection. To date, the main description of otolith reading in this species is Jones and Hynes (1950), but while still cited for ageing of *G. aculeatus* (Defaveri & Merila, 2013; MacColl *et al*., 2013; Robertson *et al*., 2016; Zeller *et al*., 2012; Zimmerman, 2007), their main conclusions suggest a pattern for the deposition of opaque and translucent rings that contradicts that for most fishes and has substantial consequences for age estimation in this short-lived species.

We summarize these contradictions into three main points. Firstly, the presence of a first translucent ring in the first summer of life (*i.e.* June or early July) in Jones and Hynes's (1950) was not found in a later study by Borland (1986), but see Blouw and Hagen (1981). Secondly, the development of an opaque ring in the first autumn suggested by Jones and Hynes (1950) contradicts Allen and Wootton (1982; Borland, 1986; Defaveri & Merila, 2013). Thirdly, Jones and Hynes (1950) finding that translucent rings in *G. aculeatus* are formed mostly in summer (*i.e.* June to September), while opaque rings are formed in other months contradicts the general pattern for other fishes (Uriarte *et al*., 2016; Williams & Bedford, 1974), but see Swan and Gordon (2001). These differences in interpretation could result in 6 months to 1 year discrepancy in age estimation of *G. aculeatus* (*e.g.* a fish aged as 6 months by Jones & Hynes's method could really be 12–18 months old). For a fish with a life expectancy of 1–2 years (Baker, 1994; Katsiadaki *et al*., 2007; Gambling & Reimchen, 2012), this would represent a substantial error.

In this study, we re-examined Jones & Hynes (1950) method by using consecutive sampling through the year in three geographically and ecologically contrasting populations of wild *G. aculeatus*, from Nottingham in central England and North Uist in the Scottish Western Isles, U.K. Our overall aim was to provide a method for the interpretation of *G. aculeatus* otoliths in order to age them reliably. Our objectives were, first, to describe the pattern of ring formation and validate its relation to age using edge analysis, to the extent that we would be able to assign *G. aculeatus* reliably to year classes. Second, to further corroborate our interpretation by examining correlations between age and size. We hypothesized that otolith growth in *G. aculeatus* will follow either the Jones and Hynes (1950), Greenbank and Nelson (1959), and Blouw and Hagen (1981) model or the opposite one from Borland (1986), Williams and Bedford (1974) and Uriarte *et al*. (2016) (Table 1). We recorded the reproductive status of a sub-sample of fish, because reproduction is known to inhibit the formation of otolith rings in some species.

2 | MATERIALS AND METHODS

2.1 | Populations and sampling

Five hundred and twenty-two *G. aculeatus* from three different populations were sampled in three sampling periods during 2014–2015 (Table 2) in North Uist (57° 36′ N; 7° 20′ W) and Nottingham (52° 93 N′; 1° 20′ W). Sampling took place only from the early spring until the late summer since the models of both Jones and Hynes (1950) and Borland (1986) proposed little accretion of seasonal rings during autumn–winter. In addition, growth during autumn and winter can be tracked as it will be continuously opaque according to Jones and Hynes (1950) or translucent according to Borland (1986). Due to easier access, sampling in Nottingham was more regular than in North Uist.

The two populations, Reiv and Bhar, from North Uist, were chosen because of substantial differences in water chemistry of the lakes and morphology of the *G. aculeatus* (MacColl *et al*., 2013). Reiv *G. aculeatus* are large and the lake they live in is shallow and resource rich, while Bhar *G. aculeatus* are very small and live in a deep, resource-poor lake (MacColl *et al*., 2013; Rahman, 2017). The third population, Tottle Brook in Nottingham was sampled because of its different latitude (800 km south of North Uist) and associated differences in seasonality. Tottle Brook is resource rich and *G. aculeatus* are relatively large and long-lived.

TABLE 1 Comparison of otolith growth in *Gasterosteus aculeatus* between Jones and Hynes' (1950) and Borland's (1986) models

Otolith rings	Growth–formation	Jones and Hynes (1950)	Borland (1986)
1	The first transparent ring	June and July of the year the fish were born	In the first autumn of the year fish were born and throughout their first winter
2	The second opaque ring	Directly after the growth of first transparent ring until the following June	Starting in the spring of the following year until the end of summer
3	Growth of the otolith through the year	Transparent growth is formed in June to September, while opaque growth in other months	Transparent ring is mostly formed during the coldest part of the year i.e. autumn and winter, while opaque growth occurs in other months

TABLE 2 Indices of samples, and the pattern of otolith edges and types among three populations of *Gasterosteus aculeatus* from North Uist and Nottingham, Great Britain

			Sample size (*n*)				Edge (%)		Otolith type					
Location	Populations	Sampling date	Total	Unreadable	Doubt	Clear	Opaque	Transparent	S−	S	S+	2S	2+	3S
North Uist	Bhar	April 15, 2014	42	10	6	25	0	100		24		1		
	Bhar	May 25, 2014	45		2	43	28	72		27	12	4		
	Bhar	October 13, 2014	26			26	0	100		21		5		
	Reiv	April16, 2014	41	8		33	0	100		20		13		
	Reiv	May 21, 2014	32	4		28	60	40		7	16	4	1	
	Reiv	October 11, 2014	21	2		19	5	95	1	15		3		
Nottingham	Tottle Brook	April 2, 2015	110	3	7	97	15	85		68	13	14	2	
	Tottle Brook	June 16, 2015	102	2	9	89	42	58		24	23	22	14	6
	Tottle Brook	August 28, 2015	103	1	9	91	65	35	45	16	13	11	1	5

Note. Data are actual number of samples, except for edges are in percentages. See text for details of otolith types. S−, Otolith with an opaque ring only; otolith with one opaque, one translucent; S+, otolith with two opaque rings flanking a translucent ring; 2S, otolith with two sets of seasonal rings; 2S+, otolith with three opaque rings flanking two translucent rings; 3S, otolith with three sets of seasonal rings.

Gasterosteus aculeatus were caught using unbaited minnow traps (Gee's traps, Dynamic Aqua; www.dynamicaqua.com) that were set overnight from the shore in water 0.3–3 m deep. The fish caught were sorted to ensure no other species of fish were sampled. Samples of the remaining fish were then haphazardly selected for analysis and killed immediately by overdose of MS-222, followed by brain destruction (U.K. Home Office licence held by A.C.D.M.; U.K Gov, 1986). We recorded individual standard length (L_S, mm) of all selected fish. We also recorded the female reproductive status of the *G. aculeatus* from Tottle Brook. Collection of reproductive status data was restricted to females because of the difficulty of unambiguously assigning reproductive status to males. Female reproductive status was confirmed by dissection of the body cavity and observation of the ovaries. If the ovules were all small and the same size, then fish were recorded as immature. If any ovules were of larger size then the fish was recorded as maturing–mature. Fish were then preserved in 70% ethanol until their otoliths were extracted. To avoid the fragility of otoliths identified by Borland (1986), all of the otoliths were processed no later than 6 months after fish were preserved.

2.2 | Otolith processing and reading

Under a ×10 dissecting microscope, otoliths were extracted only from the left side of the fish because both Jones and Hynes (1950) and Borland (1986) found more than 90% consistency in ring patterns between left and right otoliths of *G. aculeatus*. If the left otolith could not be found, the right one was taken out instead. The extracted otoliths were put into a dish containing several drops of water and were cleaned of the remnants of their sacculus. Cleaned otoliths were stored (maximum 2 months) in an individual tube with 70% ethanol until they were mounted on microscope slides using Sigma Aldrich DPX (www.sigma-aldrich.com).

Each mounted otolith was examined twice (by A.R.S.) using two different methods: direct reading under a microscope and reading of a photograph, blind to information about fish size. Microscope reading was conducted under a compound microscope at ×40 magnification using reflected light and dark backgrounds. To corroborate the microscope reading, a photograph reading was carried out, blind to the result from the microscope reading, by analysing the pictures of each otolith which were obtained with a Panasonic Lumix L10 camera (www.panasonic.com) under reflected light and with a dark background. Any results that differed between microscope and photograph reading were clarified by re-analysing the photographs and, or the slide under the microscope. Based on the results of the two readings, otoliths were categorized as unreadable, in doubt or clear.

Otoliths were labelled as unreadable when no clear image could be captured due to rupture, lack of clarity or otoliths having been mounted perpendicular to the slides. The in-doubt category contained otoliths with vibrant images, but with rings that could not be determined due to indistinct borders of seasonal rings or presence of checks. Unreadable otoliths were excluded directly, while in-doubt otoliths were re-checked at least twice by each reading method before being excluded from the analysis if they proved uninterpretable.

Edge pattern, number and type of seasonal rings, and the presence and type of checks on each otolith were recorded. Edge pattern and type of seasonal rings were categorized as either opaque or translucent, while the pattern of rings was coded following Jones and Hynes (1950): S− for an otolith with an opaque ring only, S for two seasonal rings (one opaque, one translucent), S+ for two opaque rings flanking a translucent ring, 2S for two sets of seasonal rings and so on. Identification of checks was done by referring to Williams and Bedford (1974), Swan and Gordon (2001), Waldron and Kerstan (2001) and Uriarte *et al*. (2016). The difference between true translucent rings and checks was mainly based on the wholeness and the clarity of the rings. Most of the checks were incomplete–interrupted, obviously narrower or less clear in comparison with true rings. Otoliths with complete and clear translucent rings, but with unusually close spacing, were also categorized as having checks and were excluded from the analysis (Williams & Bedford, 1974).

For those asymmetrical otoliths that could be interpreted differently depending on which sector was examined (Blouw & Hagen, 1981), the reading was made from the larger side. Where otolith materials are distributed unevenly during growth, there is usually more information in the longer axis (Williams & Bedford, 1974). The following measurements (μm) were recorded from otoliths using ImageJ (Schindelin *et al*., 2012): maximum radius of each otolith (the longest axis of each individual otolith), the width of opaque and translucent rings of 1 year old *G. aculeatus* from April sampling and the width of translucent rings of young-of-the-year (YOY) from August sampling.

2.3 | Data analysis

Edge patterns, growth of otoliths through the year and types of checks were analysed descriptively. Number of individuals as well as number of opaque or translucent edges in each age class were summarized as the percentage of total number of samples for each sampling period for each population. Generalized linear models (GLM) with normal error distributions and identity link functions were used to model morphological measurements. In this way we tested the difference in translucent ring width of S-type otoliths between August and April sampling in Tottle Brook and the difference in ring width of 1 year olds during April sampling among populations. We also used GLMs to analyse the relationships between L_S and otolith radius (R_O), age of fish and R_O, and L_S and age of the fish. In comparison of edge patterns between different reproductive statuses, L_S and age, edge was coded as a binomial variable (translucent or opaque) and the data were analysed by GLM with a binomial error distribution and a logit link function.

3 | RESULTS

Of 522 samples, 484 (93%) were successfully analysed, while 30 were unreadable and eight were lost during processing. Of the 484, 451 (93%) were categorized as clear, with the remainder as in doubt. The highest percentage in doubt was found in Bhar (14% of the Bhar samples or 3.2% of the total sample), while the lowest was in Reiv (4%, <1% of the total sample). The two different reading methods, microscope and photograph, gave coherent results with more than 92% similarity indicating that both methods are appropriate for otolith reading.

3.1 | The pattern of otolith edges through the year

Most of the otoliths (90%) from fish sampled in April were translucent at the edge and this was also true (98%) of fish sampled late in the year (October) on North Uist (Table 2), consistent with translucent rings being laid down during the colder part of the year (autumn to spring on North Uist, where the summer season is short). The only exceptions to this pattern were 15 fish (10%) from Tottle Brook sampled in April, when it is likely that growth had already begun at this latitude and one fish from Reiv (2%) sampled in October. The proportion of opaque edges increased in all populations during sampling in May–June, with up to 60% in the Reiv population, but only 28% in Bhar and 42% in Tottle Brook. This percentage climbed to 65% in Tottle Brook in August sampling (Bhar and Reiv were not sampled at this time). The opaque edges were then almost completely replaced by translucent edges in autumn sampling in Bhar and Reiv with only one opaque edge in the Reiv population. When sampling period was controlled in Tottle Brook fish, translucent edges of otoliths were significantly more common in larger (Wald $F_{1,271} = 20.36$, $p < 0.001$) and mature (Wald $F_{1,183} = 4.01$, $p < 0.05$) fish.

3.2 | Growth patterns of otoliths through the year

The simplest otolith pattern (S–; opaque with no clear translucent ring; Figures 1(a) and 2(a)) was dominant (49%) in the August sample from Tottle Brook, suggesting that these fish are YOY. Size of fish with this type of otolith in Tottle Brook ranged from 28.90 to 57.10 mm (mean ± S.D. $L_S = 38.53 \pm 0.14$ mm). The next most complex pattern of otolith is S type with an opaque centre surrounded by a translucent ring at the edge (Figures 1–2(b) and 3(a), (b)). These fish were significantly larger than their counterparts with opaque edges (S–) (Wald $F_{1,60} = 13.29$, $p < 0.001$, mean ± S.D. $L_S = 47$ mm ± 0.21 *v.* 38.53 ± 0.14 mm) potentially indicating that they had faster growth, or had hatched earlier in the season.

In the October sample from North Uist, there was only one S– fish, caught in Reiv (Table 2). This is consistent with the later date of sampling on North Uist, where 80% of fish from Bhar and Reiv had S type otoliths (Table 2). S type otoliths were also common (18%) in Tottle Brook in August samples, consistent with the first development of the translucent edge towards the end of the growing season in late summer or in autumn. Possession of an S type otolith during August sampling was significantly more prevalent in matured fish (Wald $F_{1,23} = 6.85$, $p < 0.01$), but was unrelated to the number of their checks (Wald $F_{1,59} = 0.86$, $p > 0.05$).

April sampling showed that the majority of fish (72%) in all populations still have otoliths of the S type. As this is before the start of, or very early in, the breeding season and none of the fish sampled were < 20 mm, it seems that these fish with S type otoliths are approximately 1 year old (0+ age class). The only difference from August–October samples is that the translucent ring becomes larger (Wald $F_{1,53} = 10.72$, $p < 0.01$; mean ± S.D. $R_O = 37.46$ μm ± 0.16 *v.* 52.29 ± 0.15 μm) and more contrasting (Figures 1 and 3(a), (b)) suggesting the rings from August–October had not completed formation. Thus the evidence suggests that the translucent ring is laid down in the autumn and winter (non-growing season). Correspondingly within a single population, Tottle Brook, there was no difference in size of S–otolith *G. aculeatus* in April in comparison with those with S– or S otoliths in August (Wald $F_{1,127} = 0.02$, $p > 0.05$, mean ± S.D. $R_O = 40.75 \pm 0.04$ *v.* 40.97 ± 0.05 μm).

April samples showed that new opaque ring had begun to grow on the otoliths of some fish from Tottle Brook (for both 1S+ (Figure 1 (b)) or 2S+ (Figure 1(e))), but not in Bhar and Reiv, where the spring comes later. By May, new opaque growth had begun to occur in Reiv (Figures 1 and 2(c)) and Bhar (Figures 1 and 3(c)). The presence of a new opaque edge in all populations during May sampling suggests that new opaque growth had just been started. In Tottle Brook, the second ring of opaque growth continues to be formed until at least August when it still appeared in 14% of the total sample (Table 2). The average size of S+ fish in August was 43% larger (55.22 ± 0.21 *v.* 38.53 ± 0.06 mm) than the average of S– fish indicating it was unlikely that these two opaque zones could have been grown in the same year (*i.e.* implying that S+ fish are a year older than S– fish). At the same time, the otoliths of some *G. aculeatus* were starting translucent growth (Figure 1(d)) and this became more evident during autumn sampling (Figures 1–2(d) and 3(d)). The fact that 95% of the

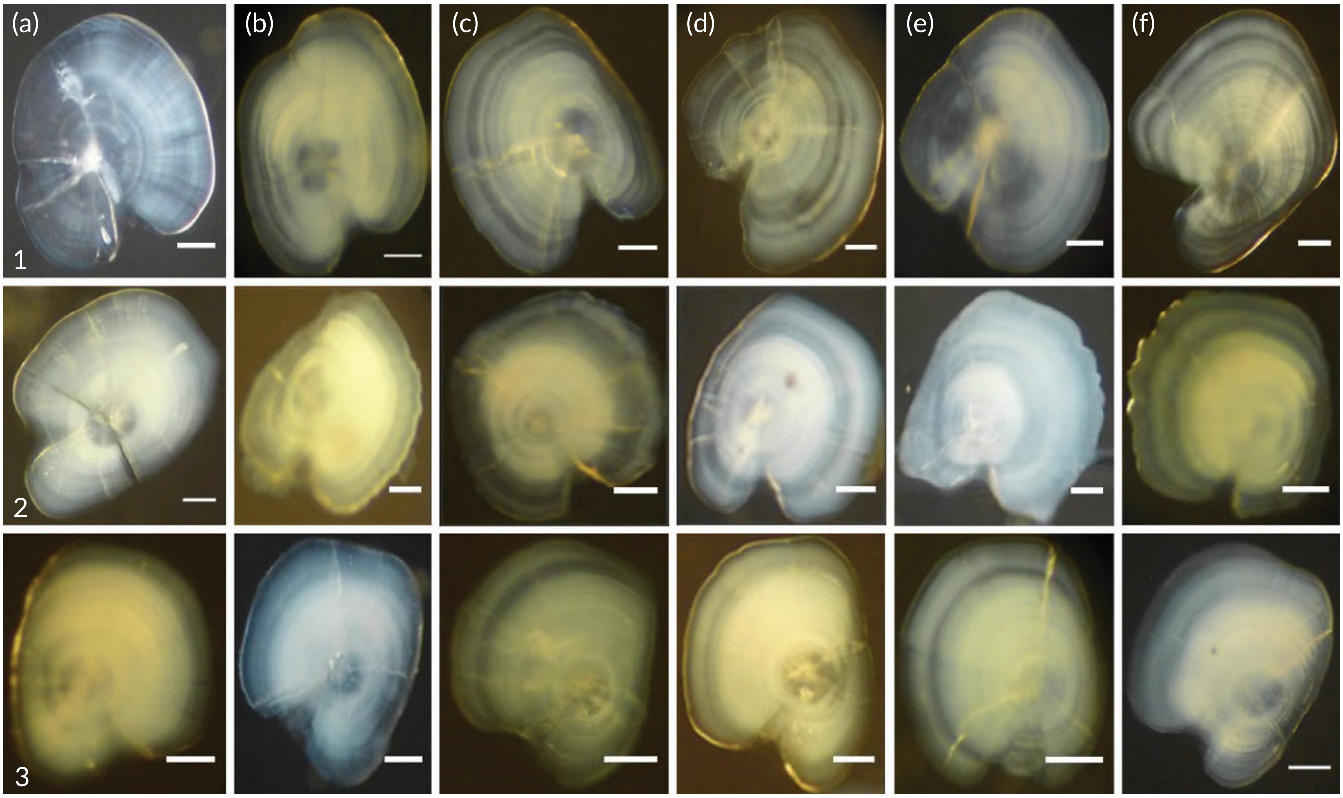

FIGURE 1 Contrasting otolith patterns of otolith growth by months in the same year in three populations of *Gassterosteus aculeatus*: Row 1, Tottle Brook, Nottingham, (a) August, (b) April, (c) June, (d) August, (e) April, (f) June;); row 2, Reiv and row 3, Bhar, North Uist, (a) October, (b) April, (c) May, (d) October, (e)–(f) May. Otoliths were pictured under reflected light with a dark background that results in opaque rings appearing cloudy white colour, while transparent rings are black. Scale bar = 100 μm

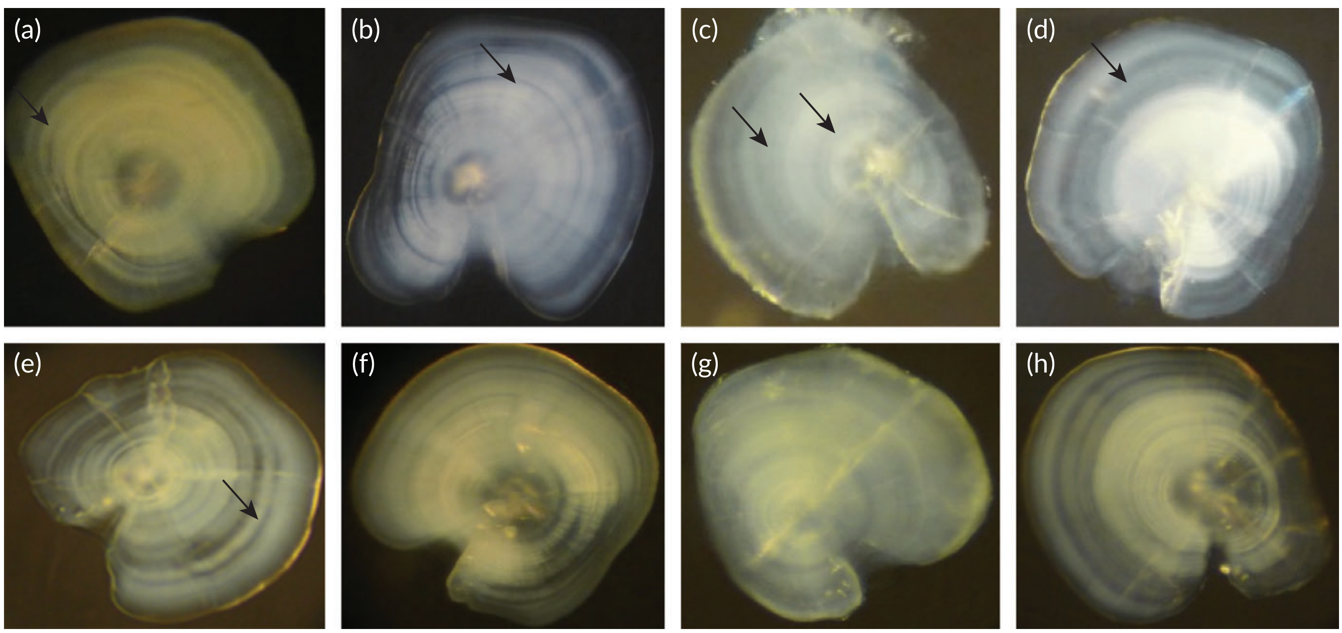

FIGURE 2 Types of common checks (⟶) in the otoliths of *Gasterosteus aculeatus*. Otoliths were pictured under reflected light with a dark background that results in opaque rings appearing white and transparent ones as black

autumn samples had translucent edges, either 1S or 2S (Table 2), provides a clear sign that the translucent ring was starting to be formed by autumn at the latest.

The otolith growth pattern (opaque rings in summer, clear in winter) seems consistent from year to year as some samples from Tottle Brook, where spring (and growth) begins earlier, also had an outer opaque ring (S+) in April sampling (Figure 1(e)), which was absent in Reiv (Figures 1 and 2(e)) and Bhar (Figures 1 and 3(e)). The most complex of all the otoliths from Tottle Brook showed three opaque (growth) rings (3S) (Figure 1(f)), while in Reiv the most complex otolith

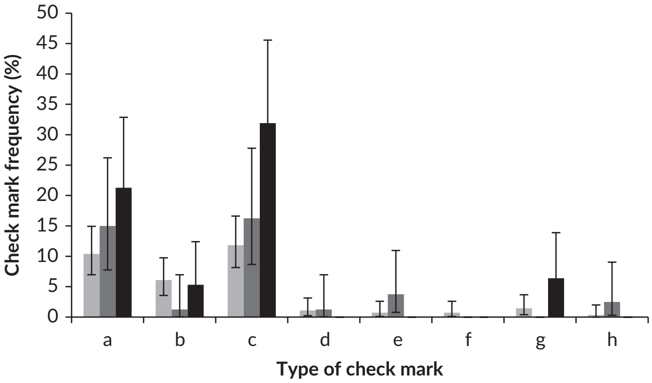

FIGURE 3 Frequency distribution (mean ± 95% c.i.) of *Gasterosteus aculeatus* with the occurrence of each type of otolith check mark shown in Figure 2 among three populations of three-spined stickleback from North Uist and. ■, Tottle Brook, Nottingham; ■, Reiv, North Uist; ■, Bhar, North Uist

had two sets of growth rings plus an opaque edge (*i.e.* 2S+, Figures 1 and 2(f)). A fish with a 2S+ otolith was also found in Bhar (Figure 1 and 3(f)), but its opaque edge was not as clear as the fish from Reiv (Figure 1 and 2(f)), suggesting that it had not begun to grow in the year it was caught. Comparison of 1 year old *G. aculeatus* from Tottle Brook showed the width of otoliths had increased following the sampling periods (average mean ± R_O = 319.24 ± 3.5 μm, in June R_O = 337.29 ± 2.5 μm and in August R_O = 376.01 ± 7.38 μm).

3.3 | Checks and doubts

We detected seven different kinds of irregular rings or checks in our samples. The commonest check was the presence of a fake translucent ring before the first true translucent ring. These fake rings can be either in the form of an incomplete smear ring that is easy to detect (Figure 2(a)), or three forms of complete (but often narrow) clear rings (Figure 2(b), (c)) that were assumed to be fake. These had either thinner size (Figure 2(b)), smaller radius or lower clarity (Figure 2(c)) than true translucent rings. These four types of check (Figures 2(a)–(c)) were more common in the Bhar population (Figure 3) and may be related to poor resource conditions. Another type of check that was found is where a narrow opaque ring splits the translucent ring (Figure 2(d)) or vice versa (Figure 2(e)). A sixth type is when otoliths lack clear translucent or opaque rings (Figures 2(f), (g)). The seventh type, that quite often led to doubt, is the persistence of nice regular, but very closely spaced rings (Figure 2(h)).

3.4 | Age structure of the populations

Based on the pattern of the development of edges and the growth of *G. aculeatus* described above, age-class determinations were made from the number of rings, but in a season-dependent way. Briefly, fish with opaque otoliths (caught in summer; S–) were counted as YOY while those with an opaque centre and translucent ring (S) were assumed to be YOY when caught in the autumn and 1 year old when caught in the spring. Any samples with two opaque rings flanking a translucent ring (S+) were also described as yearlings. Samples with two pairs of rings (2S) were assumed to be *c.* 1.5 years old when caught in the autumn and 2 years old when caught in the spring. Fish with two pairs of rings plus an opaque edge (2S+) were counted as 2 years old and so on.

We found that more than half of the samples (59%) across all sampling periods belonged to 1 year old fish, while 22% were YOY, 18% were 2 years old and only six individuals (1%) were in their third year. All 3 year olds were from the Tottle Brook population. Fish that were 1 year old were dominant in spring (April–May–June) sampling (70%), while YOY were dominant in late summer–autumn (August–October–November) sampling (71%) (Figure 4).

When population and sampling period were controlled for, older age of *G. aculeatus* was significantly correlated with larger size (Wald $F_{1,446}$ = 67.30, p < 0.001; Figure 5) and larger otolith size (Wald $F_{1,446}$ = 61.56, p < 0.001 for age) and therefore, larger *G. aculeatus* also had larger otoliths (Wald $F_{1,446}$ = 559.95, p < 0.001). As older fish were larger and there was a positive relationship between fish size and the presence of translucent edges on their otoliths, younger fish were more likely to have otoliths with opaque edges (Wald $F_{1,447}$ = 18.77, p < 0.001; Figure 6). A higher percentage of opaque edges in younger age classes suggests either that they begin feeding earlier in spring or their otoliths grow faster or there is a tighter relationship (less lag time) between otolith and somatic growth for

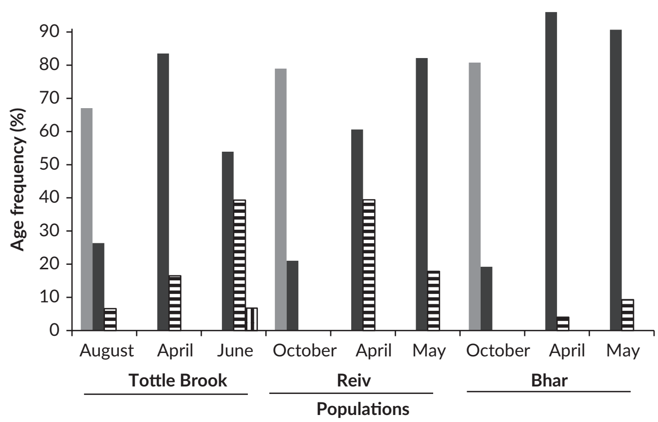

FIGURE 4 Frequency distribution of *Gasterosteus aculeatus* age groups by months from Tottle Brook, Reiv and Bhar. ■, young-of-the-year; ■, I year old; ▤, 2 years old; ▥, 3 years old

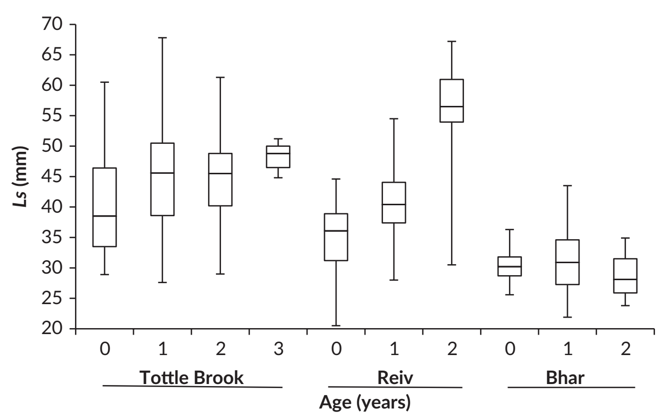

FIGURE 5 Box plots (–, median; box, 25th and 75th percentile; |, range) of i *Gasterosteus aculeatus* standard length (L_S) at age for populations from Tottle brook, Reiv and Bhar

younger fish. The negative relationship between L_S and age of *G. aculeatus* in the Bhar population (Figure 5) at first seems unusual and may indicate that there was an overestimation of *G. aculeatus* age in this population. However, in a resource-poor population like Bhar, this pattern is quite consistent with expectations from life-history theory in suggesting small 2 year old fish are the most slowly growing or latest hatched that have delayed maturity until 2 years of age.

Among 1 year old *G. aculeatus* that were sampled during April, the width of opaque rings differed significantly between populations (Wald $F_{2,54}$ = 26.25, p < 0.001) even between the two North Uist populations (Wald $F_{1,37}$ = 26.59, p < 0.001; Figure 7). However, the translucent ring only differed between regions, (Nottingham *v.* North Uist, Wald $F_{1,55}$ = 51.16, p < 0.001) and not between Bhar and Reiv (Wald $F_{1,37}$ = 3.36, p > 0.05). This is consistent with longer growing seasons and shorter winters in more southerly populations (Tottle Brook) resulting in narrower winter rings and better resource conditions in Reiv, in comparison to Bhar, resulting in wider summer rings.

4 | DISCUSSION

Our results on the otolith development pattern of *G. aculeatus* are more compatible with Borland (1986) and studies of many other fishes (Williams & Bedford, 1974; Jones, 1992; Uriarte *et al.*, 2016), than with Jones and Hynes (1950) or Greenbank and Nelson (1959). In keeping with Borland (1986), our study shows that it is more likely that the first translucent ring is formed after juvenile *G. aculeatus* stop growing at the end of their first summer, rather than during that first summer, *i.e.* in June or early July. Samples from the end of August show that most YOY from Nottingham still have opaque edges, suggesting continued growth, while the presence of translucent edges in the remainder are probably an indication that growth had ceased and the formation of the translucent ring was just starting. The appearance of these translucent rings, the majority of which are thinner in size and lighter in colour than those from April, supported this assumption. Moreover, the great majority of *G. aculeatus* sampled in

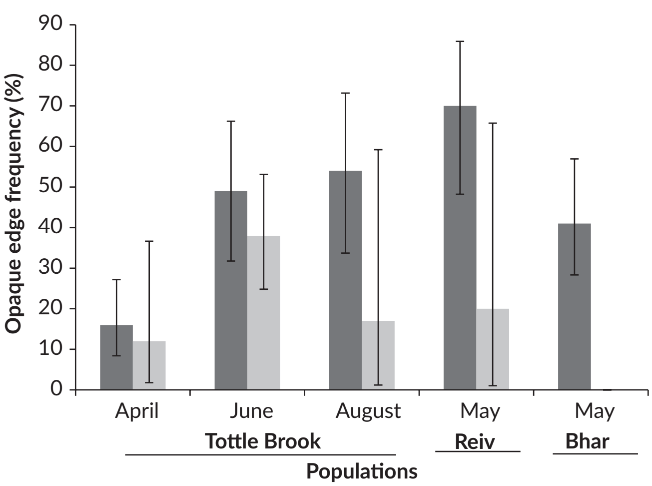

FIGURE 6 Frequency distribution (mean ± 95% C.I.) of *Gasterosteus aculeatus* otoliths with opaque edges from Tottle Brook, Reiv and Bhar. ■, I year old; ■, 2 years old. There were no opaque edges in other months sampled

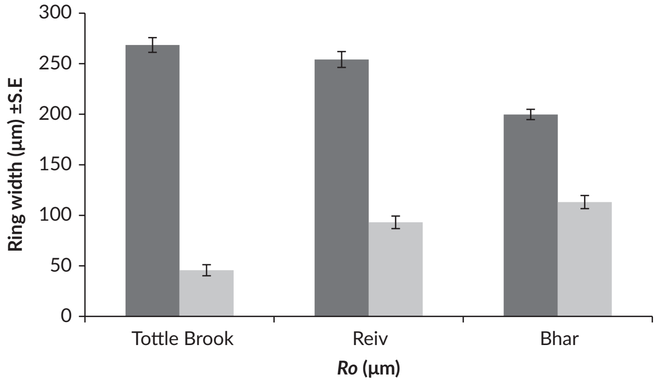

FIGURE 7 The width (mean ± S.E.) of opaque (■) and transparent (■) otolith rings (R_O) among 1 year old *Gasterosteus aculeatus* in Tottle Brook, Reiv and Bhar

April had translucent edges, suggesting that the formation of translucent growth was still continuing.

Our findings also disagree with Jones and Hynes's (1950) claim that a second period of opaque growth in the otolith of *G. aculeatus* starts by July of the year fish were born and lasts until the following June. Sampling in late August in Tottle Brook showed only 14% of the samples in their second period of opaque growth (S+), which matches with the results of Jones and Hynes (1950). However, as the mean L_S of this 14% S+ is substantially larger than those with one opaque growth ring (S− category), it is very unlikely these two groups were born in the same year. This 14% of S+ fish is more likely to be yearlings, not YOY. They were probably growing their second opaque edge since April, not July. Autumn and April sampling also supported our interpretation over Jones and Hynes's (1950), as there were almost no otoliths with opaque edges in autumn and only 10% in April. The fact that all of these 10% were from Tottle Brook, suggests the earlier onset of growth in this southerly population and the formation of these edges after, not before, the winter. Williams and Bedford (1974) claimed that, in the northern hemisphere, the growth of opaque otolith rings usually started earlier in more southerly populations. These patterns provide clear evidence that *G. aculeatus* do not develop a second opaque ring before their first winter.

The growth pattern of *G. aculeatus* otoliths through the year clearly shows that the opaque zone is formed during spring and through the summer, while most translucent zones are formed during autumn and winter. There was no indication of opaque growth during autumn or winter, while the appearance of translucent edges in the June and August samples is probably a result of reproductive investment, as it is more common in mature and older *G. aculeatus*. This result differs from Jones and Hynes (1950) and Greenbank and Nelson (1959) but is consistent with other studies either in *G. aculeatus* (Allen & Wootton, 1982; Borland, 1986) or other fishes (Jones, 1992; Uriarte *et al.*, 2016).

In comparison with our interpretation, Jones and Hynes's (1950) method could lead to a large bias, in that *G. aculeatus* ages will often be underestimated by 1 year for those sampled in June onwards to the end of the year. This is because they state that the first translucent ring (S type otolith) will be prominent in the *G. aculeatus* otolith by June or early July of the year the *G. aculeatus* was born; therefore, this type of otolith belongs to YOY. In fact, our results demonstrate that, as in other fishes (Borland, 1986; Jones, 1992; Uriarte *et al*., 2016; Williams & Bedford, 1974), *G. aculeatus* with S type otoliths during these months are the yearlings that were born in the previous year. Secondly, by following Jones and Hynes (1950), fish with S+ otoliths that are found after July until the end of the year would also be counted as YOY, because of their assertion that the second opaque ring starts to grow by July. Again, our results showed a different pattern; S+ otoliths only appear by the following spring and persist until of the end of August in more than a half of the yearling samples. Therefore, those S+ fish should be counted as yearlings, not YOY. This 1 year underestimation will probably not apply for those sampled in other months, *i.e.* beginning of the year to early June, as long as S and S+ fish are grouped as the same age class.

We believe that the inaccuracy of Jones and Hynes's (1950) method could have affected some previous studies (Pennycuick, 1971a, 1971b, 1971c; Zeller *et al*., 2012; Zimmerman 2007) as their sampling was conducted in June onwards and the age estimation of their samples was based purely on Jones and Hynes' (1950) method. If true, this inaccuracy might have resulted in overestimation of growth rate, underestimation of life span and misjudgement of the degree of infection and selection. The failure to detect an effect of predation on body size in Zimmerman's (2007) study could be due to underestimation of yearling age. If they misinterpreted 2 year old stickleback as 1 year old, the effect of predation would appear less as the fish might already have passed predators' gape limits. In Zeller *et al*.'s (2012) study, body size and growth rate of YOY were outliers (and possibly overestimated) in comparison with analogous studies (Allen & Wootton, 1982; MacColl *et al*., 2013; Zimmerman, 2007). Zeller *et al*.'s (2012) YOY group might really have been yearlings.

Other previous studies of *G. aculeatus* (Allen & Wootton, 1982; Borland, 1986; Defaveri & Merila, 2013) have displayed an awareness of the inaccuracy of Jones & Hynes 91,950). Allen and Wootton (1982) for example identified that the first translucent ring of *G. aculeatus* will become evident only by the end of the first autumn, while the second opaque ring begins to be formed in spring. A detailed analysis by Borland (1986) found similar results to those of Allen and Wootton (1982). Defaveri and Merila (2013) also seem to have differed in their approach from Jones and Hynes (1950). They did not clearly describe their method for ageing, but they stated that fish with a couple of seasonal rings were counted as 1 year old. If they followed Jones and Hynes (1950) strictly, a couple of seasonal rings should be counted as YOY.

In keeping with most previous studies (Beckman & Wilson, 1995; Chilton & Beamish, 1982; Williams & Bedford, 1974), we found that the formation of opaque rings occurred under conditions with ample resources, *i.e.* spring–summer and longer days. The translucent rings were mostly laid down during periods of slower or no growth, which usually correlate with low resource conditions, *i.e.* autumn and winter. Therefore, researchers applying ageing methods which rely on counting seasonal rings of the otolith should be aware of the effects of growth patterns in their samples and locations.

Consistent with previous studies, we found that otoliths having a translucent edge in late summer are more common in older fish and those that were reproducing (Campana, 2001; Williams & Bedford, 1974). This gives rise to a potential error of a year in ageing these fish, because it is hard to tell whether these edges grew in the previous winter, or were forming at the time of sample collection, but as *G. aculeatus* are short-lived, this should affect few individuals.

Idiosyncratic differences in otolith growth between sampling locations was probably the main reason for the difference between our results and Jones and Hynes (1950). According to Hynes (1950), the Birket river, where Jones and Hynes' (1950) study was conducted, was choked by vegetation and had reduced in flow in summer. In addition, there was a conspicuous shortage of large prey items in *G. aculeatus* stomachs during June to September (Table 8 in Hynes, 1950). It therefore seems likely that for *G. aculeatus*, growth conditions in the Birket were at their poorest in the summer and improved in the winter. The pattern of resource conditions in the Birket also probably explains why the otolith growth pattern of *G. aculeatus* in this river was reversed in comparison with other locations in most of the northern hemisphere.

All types of checks that were found in this study have been reported by previous researchers (Uriarte *et al*., 2016; Waldron & Kerstan, 2001; Williams & Bedford, 1974). The most common check found was one before the first translucent ring that can appear as a complete but narrow ring or an incomplete smear. The former can be identified as a check either by their thinness or their low clarity. This type of check was reported by Waldron and Kerstan (2001) in horse mackerel *Trachurus trachurus* (L. 1758) otoliths and by Uriarte *et al*. (2016) in European anchovy *Engraulis encrasicolus* (L. 1758). Uriarte *et al*. (2016) claimed that this type of check occurred in juvenile *E. encrasicolus* because of changing temperatures and, or poor feeding conditions. The fact that this type of check is more prevalent in the Bhar population is consistent with their explanation, since it is a lower quality environment compared to Reiv (MacColl *et al*., 2013).

The presence of abrupt opaque growth in the middle of a translucent ring or vice versa is probably due to rapidly changing environmental conditions. This type of check occurs commonly in the first translucent ring of *E. encrasicolus* when slower growth is interrupted by higher temperatures or better food conditions (Uriarte *et al*., 2016). An interruption of an opaque zone could be due to spawning (Uriarte *et al*., 2016). A complete and clear ring within a short distance of a previous one could be a true ring in a slow growing fish, but also could be a check splitting a single translucent ring (Williams & Bedford, 1974). It is probably most appropriate to exclude this type of sample when analysing *G. aculeatus* otoliths.

We also observed artefacts in the form of translucent or opaque rings at the outermost edge of otoliths, due to the thinner edge structure and the effect of light refraction (Jones and Hynes, 1950). A thinner outermost edge can appear as a false translucent ring when an edge is really opaque. On the other hand, an edge can appear as a heavy or very dark opaque zone because of refraction or incorrect focus. Both of these artefacts are less likely to have affected our results because with experience they are not difficult to distinguish. Fake translucent edges were mainly found in the otoliths of YOY and tended to reduce in older age class as edges became thicker. Fake translucent edges were usually narrower, cloudier and had an indistinct border with the previous ring. Fake opaque edges usually looked heavier (darker) than true rings and can be avoided by maintaining good focus.

Ability to classify edges as opaque or translucent is important, because we relied on it to validate our ageing method (a form of "edge analysis"; Campana, 2001). This has many recognized problems of application and interpretation (Campana, 2001). We believe these problems can be avoided in *G. aculeatus* through experience, by reading otoliths blind to other information about them, and because *G. aculeatus* are short lived, since the most serious problems with using edge analysis arise from applying to older fish validation based on young fish.

The size of the *G. aculeatus* in this study was tightly correlated with their inferred age, and both age and L_S correlated with larger otolith size, supporting our inferences about the relationship between otolith development and age. The positive relationships of age, L_S and otolith size in fishes have been reported widely by previous researchers (Harvey *et al*., 2000; Koeda *et al*., 2016; Williams & Bedford, 1974). A test by Harvey *et al*. (2000) on 63 species of fish found that 45 of them exhibited a linear relationship between body size and otolith size. Since size of fish also correlates positively with their age (Koeda *et al*., 2016), it is reasonable to expect a linear correlation between age and otolith size. However, we found there was an apparent inconsistency in the relationship between age and L_S in the Bhar population, where 2 year old fish were smaller than yearlings. This could be an indication that the age of those fish was overestimated. As *G. aculeatus* in this population are annual and mostly matured in their second spring (Rahman, 2017), the presence of a second translucent ring during May might suggest a maturation ring, not a seasonal ring. Alternatively, we prefer the explanation that those 2 year olds were late-hatched or slow-growing fish that have delayed maturation. Established models (Reznick *et al*., 1990; Stearns & Koella, 1986) predict that smaller individuals often delay maturation.

In conclusion, our results clearly differ from Jones and Hynes (1950) in three main ways. Firstly, there is no development of a first translucent ring among juvenile *G. aculeatus* during their first summer, *i.e.* June–July. The formation time of translucent rings does not differ greatly between juvenile and adult fish. They are formed during late summer and through the winter. Second, there is no formation of a second opaque growth band in the otoliths of juvenile *G. aculeatus* until they pass their first winter. Thirdly, formation of the opaque ring in *G. aculeatus* mostly occurs in spring and summer, with younger fish starting earlier. In contrast, the formation of translucent rings is mostly during autumn and winter, but can be more widespread since it was detected in late August and can apparently still occur until the middle of June for some individuals. This conclusion is exactly the same as Borland's (1986).

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REFERENCES

Allen, J. R. M., & Wootton, R. J. (1982). Age, growth and rate of food consumption in an upland population of the three-spined stickleback, *Gasterosteus aculeatus* L. *Journal of Fish Biology*, *21*, 95–105.

Baker, J. A. (1994). Life history variation in female threespine stickleback. In M. Bell & S. Foster (Eds.), *The evolutionary biology of the threespine stickleback* (pp. 144–187). New York, NY: Oxford University Press.

Beckman, D., & Wilson, C. (1995). Seasonal timing of opaque zone formation in fish otoliths. In D. Secor, J. Dean, & S. Campana (Eds.), *Recent developments in fish otolith research* (pp. 27–43). Columbia, SC: University of South Carolina Press.

Bell, M., & Foster, S. (1994). *The evolutionary biology of the Threespine stickleback*. Oxford, England: Oxford University Press.

Blouw, D. M., & Hagen, D. W. (1981). Ecology of the fourspine stickleback, *Apeltes quadracus*, with respect to a polymorphism for dorsal spine number. *Canadian Journal of Zoology*, *59*, 1677–1692.

Borland, M. (1986). *Size-assortative mating in threespine sticklebacks from two sites on the Salmon River, British Columbia*. Vancouver, BC: The University of British Columbia.

Buckmeier, D. L., Irwin, E. R., Betsill, R. K., & Prentice, J. A. (2002). Validity of otoliths and pectoral spines for estimating ages of channel catfish. *North American Journal of Fisheries Management*, *22*, 934–942.

Campana, S. E. (2001). Accuracy, precision and quality control in age determination, including a review of the use and abuse of age validation methods. *Journal of Fish Biology*, *59*, 197–242.

Campana, S. E. (2005). Otolith science entering the 21st century. *Marine and Freshwater Research*, *56*, 485–495.

Campana, S. E., & Neilson, J. D. (1985). Microstructure of fish otoliths. *Canadian Journal of Fisheries Aquatic Sciences*, *42*, 1014–1032.

Chilton, D. E. & Beamish, R. J. (1982) Age determination methods for fishes studied by the Groundfish program at the Pacific Biological Station. *Canadian Special Publication of Fisheries and Aquatic Sciences* 60. Ottawa, Canada: Department of Fisheries and Ocean. Retrieved from publications.gc.ca/collections/collection_2016/mpo-dfo/Fs41-31-60-eng.pdf

Colosimo, P. F., Hosemann, K. E., Balabhadra, S., Villarreal, G., Dickson, M., Grimwood, J., … Kingsley, D. M. (2005). Widespread parallel evolution in sticklebacks by repeated fixation of Ectodysplasin alleles. *Science*, *307*, 1928–1933.

Defaveri, J., & Merila, J. (2013). Variation in age and size in Fennoscandian three-Spined sticklebacks (*Gasterosteus aculeatus*). *PLoS One*, *8*, 8–13.

Erickson, C. M. (1983). Age determination of Manitoban walleyes using otoliths, dorsal spines and scales. *North American Journal of Fisheries Management*, *3*, 176–181.

Greenbank, J., & Nelson, P. (1959). Life history of the threespine stickleback *Gasterosteus aculeatus* Linnaeus in Karluk lake and bare lake, Kodiak Island, Alaska. *Fish B-NOAA*, *59*, 537–559.

Gambling, S. J., & Reimchen, T. E. (2012). Prolonged life span among endemic Gasterosteus populations. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, *90*, 284–290.

Harvey, J. T., Loughlin, T. R., Perez, M. C., & Oxman, D. S. (2000). Relationship between fish size and otolith length for 63 species of fishes from the eastern North Pacific Ocean. *NOAA Technical Report NMFS*, *150*, 2–7. Retrieved from www.spo.nmfs.noaa.gov/Technical%20Report/tr150.pdf

Hüssy, K., Mosegaard, H., & Jessen, F. (2004). Effect of age and temperature on amino acid composition and the content of different protein types of juvenile Atlantic cod (*Gadus morhua*) otoliths. *Canadian Journal of Fisheries and Aquatic Sciences*, *61*, 1012–1020.

Hynes, H. B. N. (1950). The food of fresh-water sticklebacks (*Gasterosteus aculeatus* and *Pygosteus pungitius*), with a review of methods used in studies of the food of fishes. *Journal of Animal Ecology*, *19*, 36–58.

Jones, C. C. M. (1992). Development and application of the Otolith increment technique. In D. K. Stevenson & S. E. Campana (Eds.), *Otolith microstructure examination and analysis Canadian special publication of fisheries and aquatic sciences* (Vol. 117, pp. 1–11). Ottawa, Canada: Department of Fisheries and Oceans. Retrieved from www.dfo-mpo.gc.ca/Library/141734.pdf

Jones, C. M., & Chen, Z. (2003). New techniques for sampling larval and juvenile fish otoliths for trace-element analysis with plasma mass spectrometry (SF-ICP-MS). In H. I. Browman & A. B. Skiftesvik (Eds.), *The big fish bang: Proceedings of the 26th annual larval fish conference* (pp. 431–443). Bergen, Norway: Institute of Marine Research. Retrieved from www.fishlarvae.org/wp-content/uploads/2014/01/BigFishBangBook.pdf

Jones, F. C., Grabherr, M. G., Chan, Y. F., Russell, P., Mauceli, E., Johnson, J., … Kingsley, D. M. (2012). The genomic basis of adaptive evolution in Threespine sticklebacks. *Nature*, *484*, 55–61.

Jones, J. W., & Hynes, H. B. N. (1950). The age and growth of *Gasterosteus aculeatus*, *Pygosteus pungitius* and *Spinachia vulgaris*, as shown by their otoliths. *Journal of Animal Ecology*, *19*, 59–73.

Kalish, J. M., Beamish, R. J., Brothers, E. B., Casselman, J. M., Francis, R. I. C. C., Mosegaard, H., … Wright, P. J. (1995). Glossary for otolith studies. In D. Secor, J. Dean, & S. Campana (Eds.), *Recent developments in fish otolith research* (pp. 723–729). Columbia, SC: University of South Carolina Press.

Katsiadaki, I., Sanders, M., Sebire, M., Nagae, M., Soyano, K., & Scott, A. P. (2007). Three-spined stickleback: An emerging model in environmental endocrine disruption. *Environmental Sciences*, *14*, 263–283.

Koeda, K., Ishihara, T., Fukagawa, T., & Tachihara, K. (2016). Life cycle differences between two species of genus *Pempheris* based on age determination around Okinawa-Jima Island. *Ichthyological Research*, *63*, 519–528.

MacColl, A. D. C., El Nagar, A., & De Roij, J. (2013). The evolutionary ecology of dwarfism in three-Spined sticklebacks. *Journal of Animal Ecology*, *82*, 642–652.

Mapp, J. J. I., Fisher, M. H., Atwood, R. C., Bell, G. D., Greco, M. K., Songer, S., & Hunter, E. (2016). Three-dimensional rendering of otolith growth using phase contrast synchrotron tomography. *Journal of Fish Biology*, *44*, 1–6.

Pennycuick, L. (1971a). Differences in the parasite infections in three-spined sticklebacks (*Gasterosteus aculeatus* L.) of different sex, age and size. *Parasitology*, *63*, 407–418.

Pennycuick, L. (1971b). Quantitative effects of three species of parasites on a population of three-Spined sticklebacks, *Gasterosteus aculeatus*. *Journal of Zoology (London)*, *165*, 143–162.

Pennycuick, L. (1971c). Seasonal variations in the parasite infections in a population of three-Spined sticklebacks, *Gasterosteus aculeatus* L. *Parasitology*, *63*, 373–388.

Rahman, A. (2017). Life history evolution in three-spined stickleback. PhD thesis, University of Nottingham.

Reznick, D. A., Bryga, H., & Endler, J. A. (1990). Experimentally induced life-history evolution. *Nature*, *346*, 183–187.

Robertson, S., Bradley, J. E., & Maccoll, A. D. C. (2016). Measuring the immune system of the three-Spined stickleback – Investigating natural variation by quantifying immune expression in the laboratory and the wild. *Molecular Ecology Resources*, *16*, 701–713.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., … Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis. *Nature Methods*, *9*, 676–682.

Stearns, S. C., & Koella, J. C. (1986). The evolution of phenotypic plasticity in life-history traits: Predictions of reaction norms for age and size at maturity. *Evolution*, *40*, 893–913.

Swan, S. C., & Gordon, J. D. M. (2001). A review of age estimation in Macrourid fishes with new data on age validation of juveniles. *Fisheries Research*, *51*, 177–195.

Thorrold, S. R., & Shuttleworth, S. (2000). *In Situ* analysis of trace elements and isotope ratios in fish otoliths using laser ablation sector field inductively coupled plasma mass spectrometry. *Canadian Journal of Fisheries and Aquatic Sciences*, *57*, 1232–1242.

U.K. Gov. (1986). *Animals (Scientific Procedures) Act 1986*. Retrieved from www.legislation.gov.uk/ukpga/1986/14/contents

Uriarte, A., Rico, I., Villamor, B., Duhamel, E., Duenas, C., Aldanondo, N., & Cotano, U. (2016). Validation of age determination using otoliths of the European anchovy (*Engraulis encrasicolus* L) in the Bay of Biscay. *Marine and Freshwater Research*, *67*, 951–966.

Von Hippel, F. A., Smayda, L. C., Zimmerman, C. E., & Bell, M. A. (2013). Validation of daily growth increments in otoliths to age Threespine stickleback (*Gasterosteus aculeatus*). *Evolutionary Ecology Research*, *15*, 947–957.

Waldron, M. E., & Kerstan, M. (2001). Age validation in horse mackerel (*Trachurus trachurus*) otoliths. *ICES Journal of Marine Science – ICES Journal of Marine Science*, *58*, 806–813.

Williams, T., & Bedford, B. (1974). The use of otoliths for age determination. In T. Bagenal (Ed.), *Ageing of fish* (pp. 114–123). Woking, England: Unwin Brothers Ltd..

Zeller, M., Lucek, K., Haesler, M. P., Seehausen, O., & Sivasundar, A. (2012). Signals of predation-induced directional and disruptive selection in the Threespine stickleback. *Evolutionary Ecology Research*, *14*, 193–205.

Zimmerman, M. S. (2007). A field study of brook stickleback morphology: Multiple predators and multiple traits. *Canadian Journal of Zoology*, *85*, 250–260.

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