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Supplementary material

Validation of age determination using otoliths of the European anchovy (*Engraulis encrasicolus* L.) in the Bay of Biscay

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Otoliths of European anchovy in the Bay of Biscay: seasonal characterisation by age classes, as seen by incident light on whole mounted otoliths over black slides

The Supplementary material provides a tabular description of the development and characterisation of otoliths throughout the year by age classes, along with a set of pictures of otoliths by ages in different months of the year (Fig. S1). In addition it provides a table with the monthly monitoring of the occurrence of edge types by age classes from the collection of otoliths between 1984 and 1993 (Table S2).

The otolith edge characterisation basically followed the nomenclature for the edge types in otoliths adopted by ICSEAF for hake (1983 – which originated from Jensen 1965) by naming HN, HW, ON and OW to the narrow (N) and wide (W) Hyaline-Translucent (H) or Opaque (O) edges respectively. To increase our precision we then established a border for the opaque edge between narrow and wide types in 33% of the expected opaque growth zone of the otolith by age, as measured with a micrometer. The expected size of the annual mean opaque growth zone for ages 1, 2 and 3 were measured in a subset of otoliths resulting in 479 µm for age-1 (n = 121, CV = 7.3%), 124 µm for age-2 (n = 62, CV = 7.5%) and 56 µm for age-3 (n = 14, CV = 15.7%). Additional edge types, OH and HO were defined for the transition edges from opaque to hyaline and from hyaline to opaque respectively; these correspond to an opaque or hyaline edge starting to be formed which is not yet visible all around the margin of the otolith respectively. By OH2, HN2 and HW2, we mean new, partially, completely or intensely formed hyaline edges after having resumed (or completed) the annual marginal opaque growth.

Checks have been typified and named with a C plus two digits according to the age of the fishes when formed (first digit) and to the approximate relative position over the expected annual growth of the otolith at that age (second digit). For instance, the most typical checks formed during the first year of life (age-0) are named C05 or C08 because they are formed approximately at 50 or 80% of the expected annual growth of the otolith at age-0. Other typical checks are C12, C15 or C18, which correspond to checks formed at age-1 at ~20, 50 or 80% of the expected annual growth of the otolith for that age. Checks C12/15 refer to checks C12 and C15 together due to the difficulty of discerning one from the other when annual opaque growth is not complete and because they define a continuous range of relative positions.

Table S1. Tabular seasonal description of otoliths by age classes (see also Table S2)

Quarters	Main features occasional deviations
Age-0	Main features of age-0 otoliths and occasional deviations
Q1	Not present
Q2	Not present
Q3	Opaque – entirely opaque otoliths up to the edge or occasionally faint hyaline edges (OH) at the end of summer. The small size of anchovies and their otoliths, usually sampled from schools of pure juvenile fishes, make samples very homogeneous, being evident that those fishes are juveniles 0 group.
Q4	Opaque – entirely opaque otoliths up to the edge or occasionally faint hyaline edges (OH) of increasing occurrence as the autumn progresses. Occasional deviations: sometimes the outer opaque zone shows up in a white of lesser intensity than inner zones and occasionally there can be some inner checks of easy recognition due to their faint nature (C05 or C08).
Age-1	
Quarters	Main features of age-1 otoliths and occasional deviations
Q1	In January some anchovies still show entirely opaque otoliths but a majority of them show hyaline edges either OH (in formation) or HN due to the winter growth detention. In February the majority are HN and by March about half of them have already laid down a narrow opaque edge (ON) (Fig. S1.a1), laying behind the first winter hyaline zone. Occasional deviations: in some cases the winter zone is a composite of successive hyaline zones, typically two or three alternated by one or two narrow opaque zones, resulting in a double or triple hyaline rings conforming the first Winter mark (split rings, see Fig. 1h and 1i of the paper and Fig. S1.a1 age 1 in March).
Q2	By April almost all 1-year-old anchovies will show a narrow opaque edge (ON) after the first winter hyaline zone. During the spring the edge will be growing continuously, becoming a wide opaque edge (OW) by May or during June (varying between years) (Fig. S1.b1 age-1 May ON). By the end of June most of them will already achieve an OW edge, surpassing 1/3 of their annual expected growth. Occasional deviations: In June some 1-year-olds may show a partially hyaline edge (OH, after an initial substantial opaque growth, called OH2), corresponding to the formation of a spawning check. Not all fishes lay down this check and its incidence changes between years. The type of opaque edge achieved by the 1-year-old anchovies in spring, or occasionally the faint hyalinity in June (OH2) in case of laying the spawning check, make them clearly distinguishable from the 2-year-old, which in turn show hyaline (HN or HW edges) throughout spring, without starting a neat opaque edge deposition of the current year (except some in June).
Q3	Intense growth of the opaque edge (OW) happens during summer months July (Fig. S1.c1 age-1 July OW) and August, so that by September ~80–90% of the total expected annual otolith growth for age-1 is already achieved (Fig. S1.d1), corresponding with a very wide opaque edge (OW). Occasional deviations: there are always some 1-year-olds with semi-hyaline edges (OH2) not entirely visible all around the otolith. If the anchovy laid down a spawning check this will usually be seen from July. The distinction between spawning check and true winter hyaline zone should be made on the following basis a) judging if the distance to the former winter hyaline zone matches with the expected annual opaque growth of 1-year-old anchovies or is less than expected and b) judging the intensity of this hyaline mark (as faint), though if well marked then the reader should base its judgment only on the *a* criteria. Both are a bit subjective criteria and this is probably the time of major difficulties for age determination of the 1-year-old fishes. Besides this around the time of the spawning check, or later on, the intensity of the white colour of the opaque growth band may occasionally weaken (becoming a bit more grey as if there were two different growth colour bands in the year) (see example of this check on older fish in Fig. 1 of the paper and in Fig. S1 in several age 2 fish (c2 & d2) and for an age-3 fish in c3) (red stars) (case of change in colour in Fig. S1.d3).

Quarters	Main features occasional deviations
Q4	The growth band of the 1-year-old has been completed, with opaque edges (OW) at the beginning of the period and more hyaline (usually OH2 or HN2) at the end of the period (Fig. S1.e1 showing C12). Occasional deviations: Same comments as for Q3 and it should be noticed that occasionally there can be some interruptions and resuming of growth resulting in some usually faint checks at the end of the 1-year-old otolith opaque growth (C18). (Fig. S1 age-2 in winter (a2) and in Aug–Sep as well as age-4 Nov (e4) showing C18).
Age-2 Quarters	Main features of age-2 otoliths and occasional deviations
Q1	Otoliths with the two first annuli formed having its second hyaline zone just at the edge either in formation OH (Fig. S1.a2 age-2 in winter) or as hyaline narrow zones HN. Occasional deviations: In few cases the second hyaline zone is not yet formed having an edge entirely opaque. And in some cases within the opaque growth zone of the previous year checks C12/15 or more rarely C18 can be seen (see Fig. 1 for several examples and the checks C18 in Fig. S1.a2 age-2 in winter – very faint)
Q2	Otoliths with the two first annuli formed having its second hyaline zone just at the edge either as narrow hyaline zones (HN), mainly in April, or as wide hyaline zones with increasing occurrence from April to June (Fig. S1.b2 age-2 in April–May HN). By the end of May and in June some otoliths will start the deposition of the opaque growth zone showing either HO or ON edge types. Occasional deviations: the starting date of appearance of the opaque edge may change from year to year ranging, the earliest about mid-May and usually not later than end of June.
Q3	In July most of the otolith edges are already opaque, either narrow or wide zones (ON or OW) even though there might still be some hyaline wide edges (HW) (Fig. S1.c2 age-2 –July OW). By the end of July and in August edges will show up as wide opaque growth bands (OW) and by September most of the expected annual growth of the otoliths will be achieved (Fig. S1.d2 age-2 Aug–Sept OW). Occasional deviations: By September a few age-2 otoliths may show partially new hyaline edges (OH2).In addition see examples of checks C15 and C18 (very faint) in Fig. S1.d2.
Q4	The expected annual growth of the otoliths for age-2 will be achieved. Edges will show up either as wide opaque (OW) partially hyalines (OH2) or a few as narrow hyaline edges (HN2). (Fig. S1.e2 age-2 Nov OW).
Age-3 (and older) Quarters	Main features of age-3+ otoliths and occasional deviations
Q1	Otoliths with the three first annuli formed having its third hyaline zone just at the edge either in formation OH or as hyaline narrow zones HN (Figs. S1a3 and a.4 both with HN). Occasional deviations: in a few cases the third hyaline zone may not yet be, formed having an edge entirely opaque. And in some cases within the opaque growth zone of its second year of life (of the age 1) the previous year checks C12 / 15 (or more rarely C18)
Q2	Otoliths show its third hyaline zone just at the edge either as narrow hyaline zones (HN), mainly in April–May, or as wide hyaline zones (HW) with increasing occurrence from April to June (Fig. S1.b3 age-3 with HW and age-4 b.4-HN).By June some otoliths will start the deposition of the opaque growth zone (showing HO or ON edge types). Occasional deviations: the starting date of appearance of the opaque edge may change from year to year, but less than at younger ages, usually between June or July.

Quarters	Main features occasional deviations
Q3	The otolith opaque growth zone of the year is being formed: In July some HW edges coexists with ON edges (Fig. S1c3 age-3 July ON). In August edges will show up mostly as wide opaque growth bands (OW) and by September most of the expected annual otolith growth will be achieved (Figs S1.d3 and d.4 age-3 and age-4 in Aug–Sept with OW). Occasional deviations: by September a few age-3+ otoliths may show partially hyaline edges (OH2).
Q4	The expected annual growth of the otoliths for age-3+ will be achieved. Edges will show up either as wide opaque (OW) partially hyalines (OH2) (Fig. S1.e3 age-3 and e.4 age-4 in Nov).

Table S2. Percentages of occurrence of the different otolith edge types by age throughout the year, for the whole set of samples between 1984 and 1992 (Sub-tables for ages 0, 1, 2 and 3+)

Total Otol. refers to the total amount of otoliths examined per month. Edge types: OO refers to the otoliths entirely opaque until the edge, without bearing any hyaline zone inside them. Edge types HN, HW, ON and OW refer to the narrow (N) and wide (W) Hyaline (H) or Opaque (O) edges respectively. Edges OH and HO refer to the transition forms from opaque to hyaline and from hyaline to opaque respectively (i.e. not entirely visible all around the margin of the otolith). By OH2, HN2 and HW2, we mean new, partially, completely or intensely formed hyaline edges after having resumed (or completed) the annual marginal opaque growth.

	Month											
Edge type	1	2	3	4	5	6	7	8	9	10	11	12
Age 0												
OO								100	99	100	100	87
OH									1			10
HN												
Total Otol.	0	0	0	0	0	0	0	1	95	259	327	78
Age 1												
OO	36		1	0								
OH	55		7	1	0							
HN			29	1	0	1						
HW	2		14	1	0	1		1				
HO			1	0	0							
ON	7		47	95	61	27	2	1				
OW				1	24	51	68	83	73	33	69	17
OH2					13	20	30	16	21	50	31	
HN2									6	17		67
HW2									0			17
Total Otol.	87	0	435	541	1048	908	308	177	242	6	16	6
Age 2												
OO												
OH	33		13	2	1	0						
HN	67		67	41	33	12	2					
HW			18	54	39	33	9					
HO			0	2	8	13	10					
ON			1	1	17	29	26	18				
OW					2	12	54	80	52	20	64	
OH2								2	36	80	36	
HN2									8			
HW2									4			
Total Otol.	6	0	205	483	805	375	132	61	25	5	11	0
Age 3+												
OO												
OH			9	2	2		4					
HN			74	67	59	20	3					
HW			15	31	33	53	36					
HO					3	16	14					
ON			1	1	2	8	38		6			
OW						3	4	100	88		100	
OH2									6			
HN2												
HW2												
Total Otol.	0	0	149	271	266	116	69	1	17	0	2	0

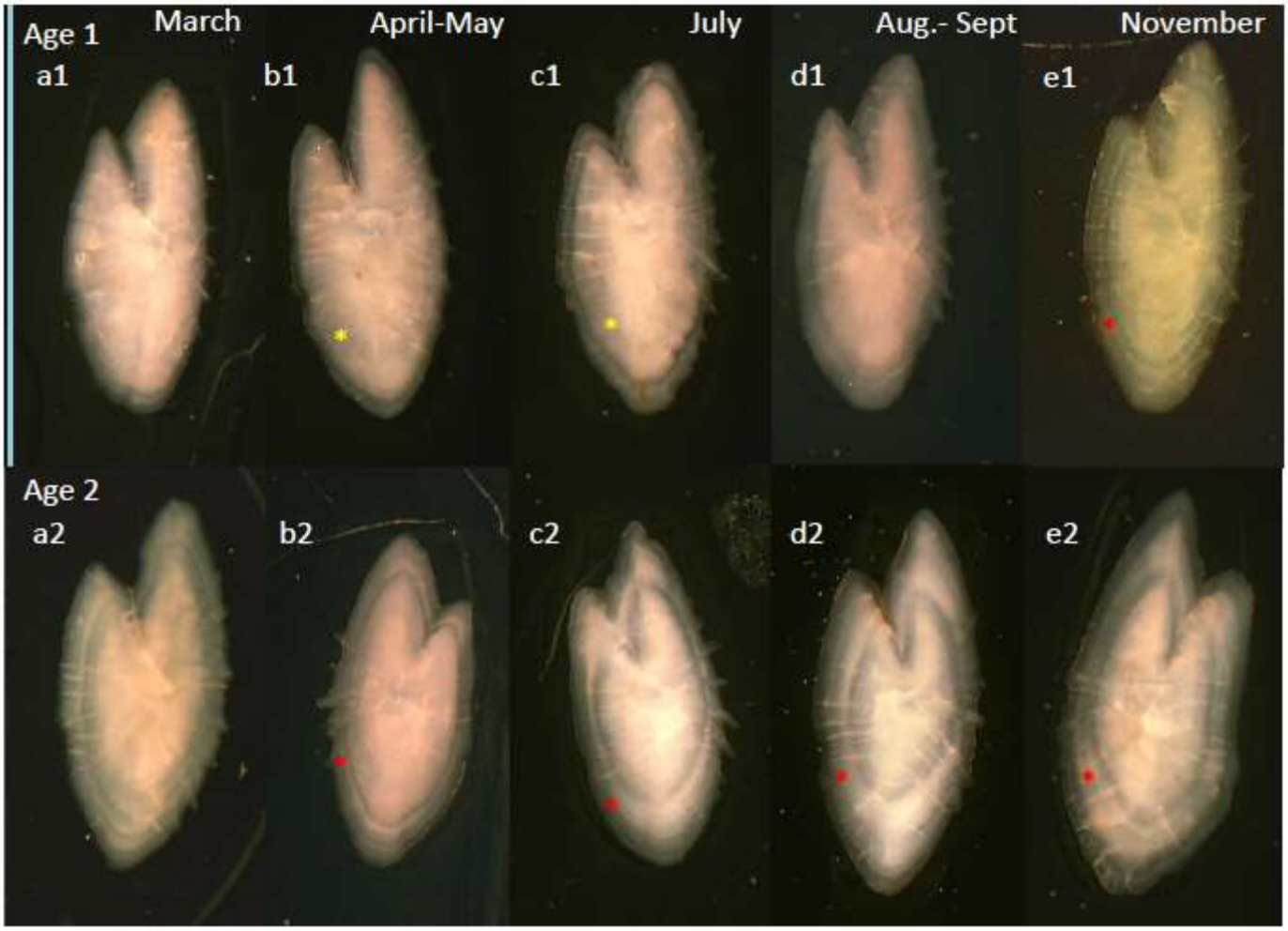


Fig. S1. Pictures of typical otoliths by age group (numbers) throughout the year (letters: a, March; b, April–May; c, July; d, August–September; e, November). Dates of the seasonal pictures by ages: Age-1: (a1) 07-Mar-1990, (b1) 18-May-1990, (c1) 18-Jul-1990, (d1) 26-Sep-1990, (e1) 14-Nov-1984; Age-2: (b1) 19-Mar-1984, (b2) 16-May-1991, (c2) 12-Jul-1989, (d2) 02-Aug-1989, (e2) 14-Nov-1984; Age-3: (a3) 18-Mar-1992, (b3) 22-May-1992, (c3) 26-Jul-1985, (d3) 04-Sep-1985, (e3) 14-Nov-1984; Age-4: (a4) 14-Mar-1986, (b4) 30-Apr-1986, (c4) not available, (d4) 27-Sep-2010, (e4) 18-Nov-2003. Scale: the longest side of otolith pictures (i.e. the blue line) measures 4354 μm. Some false checks are annotated: yellow stars, C08; red stars, C12/15.

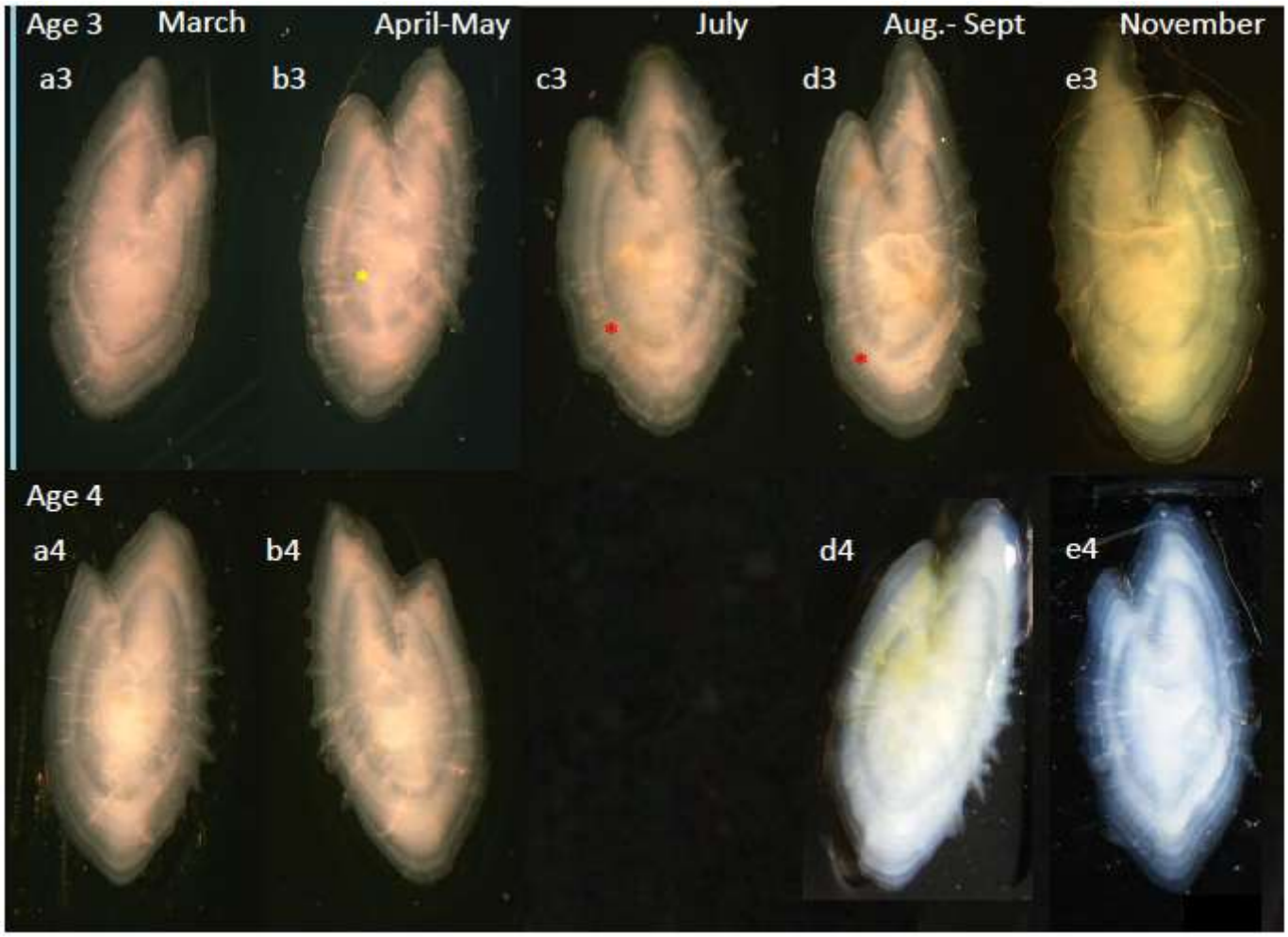


Fig. S1. (*Cont.*)

Otoliths of European anchovy in the Bay of Biscay: quantitative otolith growth analysis

The Supplementary material describes a quantitative analysis carried out on the size of the annual otolith increments by ages. For it, we analysed measurements of the annual otolith increments (between annual hyaline zones) carried out on a subset of the otoliths corresponding to samples in May from the years 2004–2009 in the context of a recent project (AFISA EU Project number 044132[A]) (Table S3). Measurements were taken on the radius from the core (or primordium) to successive annuli along the posterior axis of the otoliths (μm) using a light microscope (at 20× magnification) coupled with an image analyser (Visilog, TNPC Software, *v.*3.2, Ifremer, France).

Table S3. Number of otoliths measured by ages in AFISA project

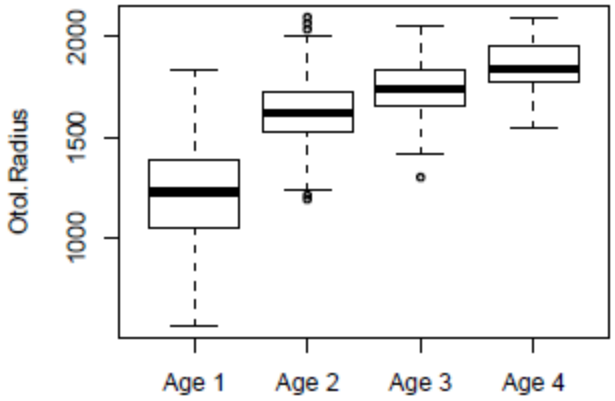
	Years						
Ages	2004	2005	2006	2007	2008	2009	Total
Age 1	166	129	232	250	148	198	1123
Age 2	23	164	131	203	187	152	860
Age 3	12	12	45	37	82	95	283
Age 4	7	1	0	0	3	24	35
Total	208	306	408	490	420	469	2301

It was found that that otolith growth is rather asymptotic due to the decreasing size of the annual increments (Fig. S2a–b): otolith growth during the first year of life (as age-0, until first winter translucent zone) is vast and usually supposes the major part of the otoliths (even for the oldest fishes). During the second year of life (age-1), the opaque growth is still substantial though reduces to ~50% (CV = 44%) compared to the growth achieved at age-0. And during the third year of life (age-2) the reduction of the opaque growth zone is very much pronounced, to ~29% (CV = 33%) of that achieved at age-1. In subsequent ages, opaque growth still diminishes but to a lesser extent, leading to a gradual (less intense) narrowing of subsequent increments. As such, at age-3 opaque growth is still ~48% (CV = 34%) of that produced at age-2, and at age-4 growth is probably larger than 50% of that achieved at the age of 3 (as deduced from the few ages 5 of the 1982 cohort – in Fig. 1 of the main text). In summary, the decreasing rate of annual growth with age (relative to the former age) is not constant but accelerates from ages 1 to 2 then slows down from age-3 onwards (Fig. S2c). Globally, the majority of otoliths correspond to the growth achieved during the first two years of life (ages 0 and 1) (furthermore the growth at age-0 alone usually accounts for the major part of the otolith).

Otolith increments during the second and third years of life (at ages 1 and 2) are inversely related to the growth achieved at age-0 (Fig. S3a–b), so that the greater the growth achieved until its first winter, the smaller will be the growth increments during ages 1 and 2, resulting in a positive relationship between the growth increments of the latter two ages (Fig. S3c). The increments of otoliths at age-3 were not significantly related to the growth at age of any previous ages. Much of the individual variability in the size of annual opaque growth increments by ages (CVs ~33%) comes therefore from the inverse relationship between the otolith growth increments at ages 1 & 2 *v.* that at age 0.

[A]AFISA PROJECT REPORT in http://www.ices.dk/explore-us/projects/EU-RFP/EU%20Repository/AFISA/FP6%20AFISA%20Final%20Activity%20Report.pdf

(*a*)



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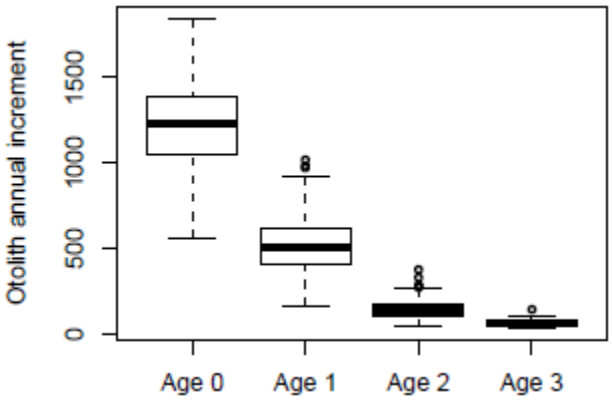

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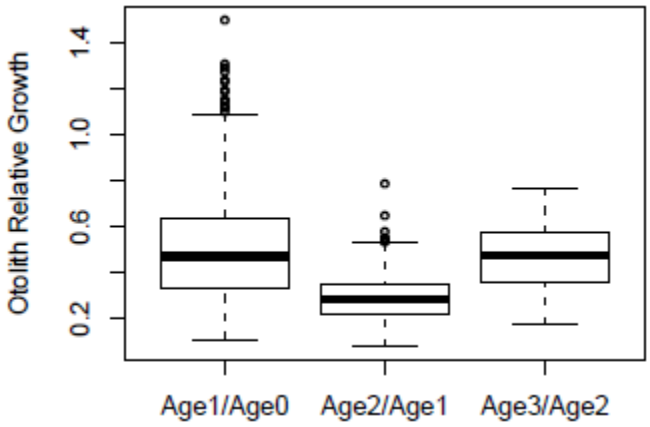
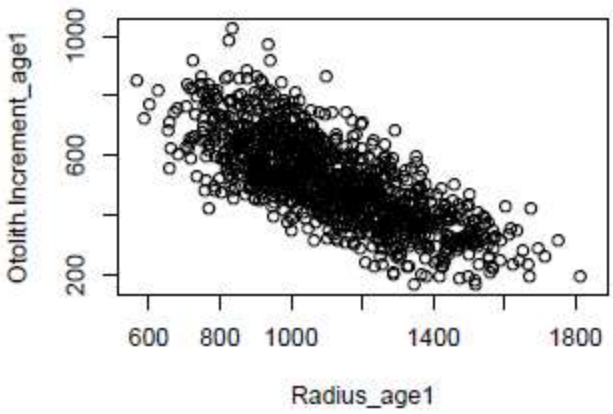


Fig. S2. Box-and-whisker plots of otolith growth by age in absolute (μm) and relative terms: (*a*) cumulative otolith growth along the post-rostrum axis; (*b*) absolute growth increment by age and (*c*) growth increment by age relative to former age growth increment.

(*a*)



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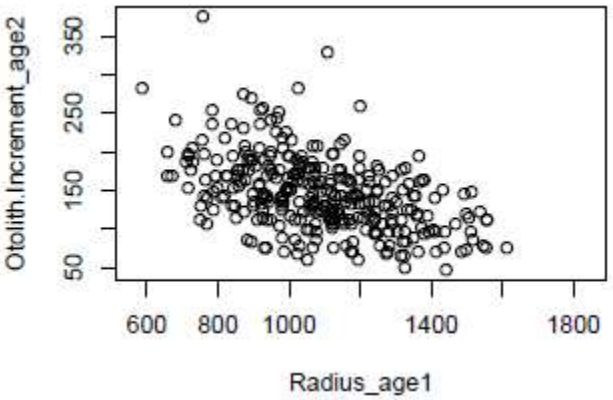

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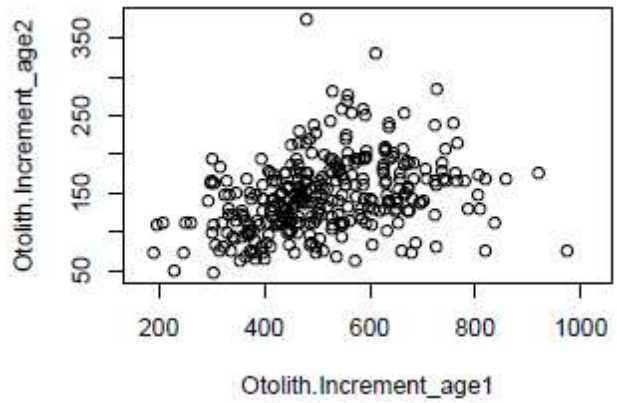

Fig. S3. Covariation of anchovy otolith growth along ages 0, 1 and 2: (*a*) Absolute otolith growth increment at age-1 *v*. growth achieved at age-0 (Radius.age_1); (*b*) absolute otolith growth at age-2 *v*. growth achieved at age-0 (Radius.age_1) and (*c*) absolute otolith growth increment at age-2 *v*. growth increment at age-1. Age growth measurements correspond to the distance between successive winter hyaline zones (for age-0 this applies to the radius from the primordium to the first hyaline winter zone).

Incidence of the most typical checks (C05, C08 and C12 or C15) in the collection of otoliths in May of the years 1984–1991

The Supplementary material contains the study of the incidence of the most typical checks C05, C08 and C12 or C15 as recorded by ages in May for the collection of otoliths of this month in years 1984–1991. For it, we analysed for a subset of otoliths by years and ages (1 and 2+) as shown in Table S4. Nomenclature of checks follows that described in the main text of this paper, as well as in the first section of this Supplementary material.

Table S4. Incidence (percentage) of checks C05, C08, C12/15 by ages 1 and 2+ in the May samples between 1984 and 1991

	Year								
Check	1984	1985	1986	1987	1988	1989	1990	1991	Mean
Age 1									
C05	18.2	0.0	4.7	12.7	19.4	5.1	4.9	12.1	9.6
C08	54.5	11.4	26.6	32.9	18.8	8.9	1.3	11.0	20.7
C12/15	0.0	8.6	8.3	2.5	2.6	0.0	0.0	0.0	2.8
Number of otoliths	11	35	84	158	191	79	390	91	129.9
Age 2+									
C05	0.0	3.0	0.7	8.8	0.0	0.5	2.9	2.8	2.3
C08	11.0	19.5	25.5	27.5	14.3	11.2	8.8	6.2	15.5
C12/15	49.0	51.2	54.5	35.7	33.3	25.7	20.6	42.8	39.1
Number of otoliths	100	162	145	182	105	187	34	145	132.5

It was found that that the incidence of Checks C05, C08 and C12/15 in otoliths sampled in May between 1984 and 1991 changes largely over these years (Table S4). Check C05 is the least frequent one with a yearly incidence ranging between 0 and ~20%, having an average incidence of ~9.6% on the 1 year-old fishes but being detected in a lesser extent at the age of 2 or older, probably due to some loosing of contrast as the otolith grows while aging. The incidence of Check C08 varies a lot across year classes, ranging between 0 and ~33%. On average, C08 is observed in ~20% of the otoliths. And Check C12/15, almost absent at age-1 in May, is the most frequent one at age-2 or older, ranging between 15 and 60% depending on year classes and ages, being detected on average in ~39% of the 2+ year-old fishes.