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THE OTOLITH AS STRESS INDICATOR OF PARASITISM ON EUROPEAN EEL

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EUROPEAN EEL
ANGUILLICOLA CRASSUS
OTOLTH SHAPE
ASYMMETRY

ABSTRACT. – The parasite *Anguillicola crassus* threatening European eel since the eighties is regarded as a factor of stress. It is a very successful colonizer, and can severely impair swim bladder function. Glass eels were collected from the coastal lagoon Salses-Leucate of the Languedoc-Roussillon Region and were reared in experimental basins in which they were contaminated by several parasites. Otolith shape was examined to investigate to what extent parasitism affects otolith shape. Univariate and multivariate analyses of variance conducted on the otolith parameters of eels were realized in order to test the hypothesis that the parasite has an effect on these parameters and can generate a change in the shape of the otoliths. In this experimental approach, the otolith parameters varied according to the abundance of the parasite. The mean values of the size parameters and the Fourier coefficients displayed significant differences between right and left otolith. The values of the coefficient of shape, the circularity and the ellipticity of the otoliths were sensitive to the abundance of the adult parasite.

INTRODUCTION

The European eel (*Anguilla anguilla* Linnaeus, 1758) is a species typical of European waters. All eel species have a complex and long life cycle, which makes them remarkable. Eels migrate over long distances across oceans and spawn in ocean waters (Lecomte-Finiger 2003). Today, renewed interest in the life history of the European eel is attributable to the management of its increasing fishing pressure and because eel is an endangered species (Bruslé 1994, Feunteun 2002, Dekker 2003). A synergetic effect of several causations (fisheries, habitat degradation, long term climatic change, parasitism…) is most likely.

In the Languedoc-Roussillon lagoons (Mediterranean, France), the average output of eels was estimated between 80 and 100 kg/ha/year in the seventies. Since 1990 the average output is in strong fall, between 40 and 50 kg/ha/year (Rulleau 2002). This fall could be related to two classes of factors: (1) biological factors, such as predation, bacterial infections or parasitism; and (2) anthropogenic factors, which result from human impacts on environment like fishing or pollution, preventing the migrations necessary to the development of eels. One of the most serious threats is due to a parasite, *Anguillicola crassus*, that was introduced in Europe from imports of Japanese eels in the 1980s (Blanc 1994). This endoparasitic worm (Nematode) lives in the swim bladder.

Fluctuating Asymmetry (FA) is the most used measurement of developmental instability. It is a macroscopic measure of the difference of development between left and right from perfect bilateral symmetry caused by environmental or biotic stresses (Van Valen 1962, Moller & Swaddle 1997). FA is a useful indicator of condition since it has been shown to be extremely sensitive to stress (Clarke 1992).

European eel parasites, in particular invasive species such as *A. crassus*, are suspected to have a strong influence on the population dynamics of their host. In a previous work, Fazio *et al*. (2005) studied the relationship between parasitic fauna of yellow eels caught in the Salses-Leucate lagoon (Mediterranean, France) and the FA of some functional bilateral traits: pectoral fins, eyes and otoliths. This study revealed different morphological characteristics between eels caught at different dates. In this study, only otoliths were found asymmetric.

Otoliths are crystalline structures composed of calcium carbonate. They are used for the maintenance of stability and the perception of depth pressure, gravity, angular movements and sound waves (Blacker 1974). Otoliths constitute some permanent recorders of the exposure to environment without being resorbed (Jones 1992). Otoliths are therefore an indirect mean for studying environment and organism relationships, through their crystal morph, chemical composition and shape (Campana & Casselman 1993, Campana 1999). The otolith shape may vary within each species according to fish size, geographical sites, depth and/or other environmental factors (Hoff & Fuiman 1993, Lombarte & Lleonart 1993, De Vries *et al*. 2002, Cardinale *et al*. 2004). Fluctuating asymmetry is assumed to reflect developmental instability caused by environmental stress.

We hypothesized that the shape of the otolith could be an indicator of a stress on the individual caused by the parasite *A. crassus*. Our study aimed to investigate to what extent the environmental stress factor *A. crassus* may induce an impact on the shape of the otolith and a bilateral asymmetry of the otolith of European eels reared in experimental conditions. Glass eels were collected when they enter the Mediterranean lagoon of Salses Leucate since at this transparent stage (1) the swim bladder is not functional, therefore not parasitized, and (2) otoliths are perfectly symmetric.

MATERIALS AND METHODS

Sampling

A total of 151 glass eels *A. anguilla* (stage VB, Elie *et al.* 1982) were collected on January-February 2004 from the coastal lagoon Salses-Leucate (Gulf of Lions, France, NW Mediterranean Sea). They were reared with the same life conditions (T° = 20 °C, food pellets…) in experimental tanks according to Da Silva's protocol (Da Silva 2005). Four groups of infested eels were prepared (respectively 25, 50, 75 and 100 parasites *A. crassus* at stage L_3). An intact group of eels was left as an animal control. After 3 months of rearing, individuals were grouped in six classes according to the abundance of parasites found in their swim bladder (respectively 0, 1, 2 to 5, 6 to 10, 11 to 30 and > 31).

Otolith data

Pairs of sagittae were extracted from each fish, cleaned with distilled water and stored dry in small tubes. Otoliths were placed on a microscope slide with the Sulcus acusticus oriented up to the observer. Each otolith was observed (magnification 18x) under a stereomicroscope (Leica Wild M8) coupled with a numerical video camera (XC-77 CE) connected to a computer. Acquisition and image analysis were achieved using image analysis software Visilog Noesis (v.5.1). Size parameters composed by the area of the otolith (A_o), its perimeter (P_o), its length (L_o), its width (l_o) and its eccentricity were calculated. Shape indices were obtained by combining the size parameters (Tuset *et al.* 2003).

The shape of each otolith can be described by the decomposition of its outline with the Fourier series. The elliptic Fourier method was used, as it is a powerful taxonomic descriptor (Rohlf & Archie 1984, Ferson *et al.* 1985, White 1988, Crampton 1995). The outline of the otolith is a periodic function based on a sum of trigonometric series of sinus and cosinus. These series are composed by several components, named harmonics. Each harmonic is characterized by 4 coefficients, issued from the projection of each point of the outline on axes (x) and (y). Higher is the number of harmonics, greater is the accuracy of outline description (Kuhl & Giardina 1982). All the Fourier coefficients were calculated by using the software Shape v.1.2 (Iwata & Ukai 2002), which made them invariant to the location, the size and the orientation of the otolith. In order to determine the necessary number of harmonics for the best construction of the otolith's outline (Crampton 1995), the Fourier Power spectrum (FP) was calculated. The FP of a harmonic is proportional to its amplitude and provides a measure of the amount of shape information described by this harmonic. For the n^{th} harmonic, Fourier Power (FP_n) is given by the expression:

$$PF_n = (A^2_n + B^2_n + C^2_n + D^2_n) / 2$$

where A_n, B_n, C_n and D_n are the Fourier coefficients of the n^{th} harmonic.

Then we calculated the cumulated power percentage (PF_c) defined by:

$$PF_c = \sum_1^n PF_n$$

To define the adequate number of harmonics to be considered in the analyses, the threshold of 99.9999 % of the mean cumulated Fourier's power was chosen. The cumulated power reached the fixed threshold at the 9 first harmonics. The Fourier analysis indicates that the otolith shape of *Anguilla anguilla* elvers could be summarized by 9 harmonics, i.e. 36 Fourier coefficients. The coefficients corresponding to the first harmonic were eliminated because this harmonic represents the starting point of the outlines and is not relevant to a shape analysis. So, 8 harmonics and 32 Fourier coefficients were used in the following data analysis.

Data analysis

Our first objective was to test whether or not the presence of

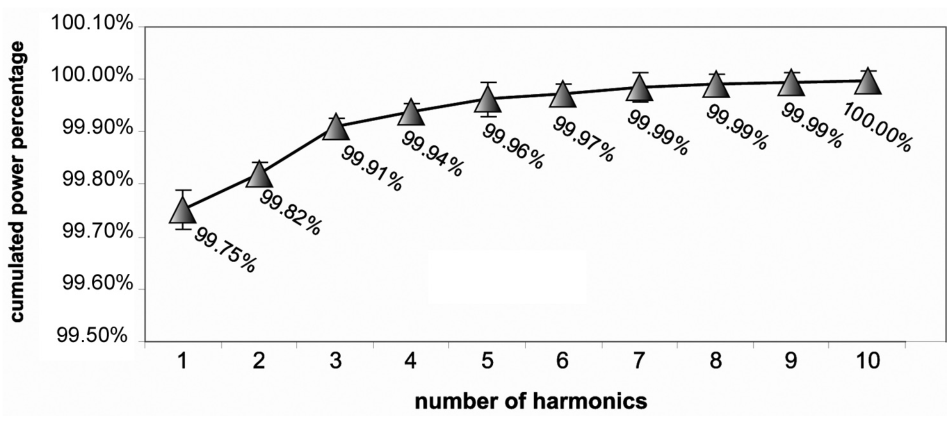

Fig. 1. – Number of harmonics calculated by Fourier power to reconstruct the otolith shape of *A. anguilla*.

A. crassus in the swim bladder of the young eels has an effect on the otolith's symmetry and parameters (i.e. size parameters, shape indices and Fourier coefficients). We modelled abundance as a function of *Parasite*, *Otolith* and *Reproduction*. All three factors are crossed and were treated as fixed. The factor *Parasite* corresponds to the six classes of abundance of *A. crassus* in the eels; the factor *Otolith*, with its two groups, denotes the left or right nature of the otolith; and the factor *Reproduction*, with its two groups, corresponds to the presence or absence of parasite eggs in the individuals. The linear algebric model was:

$$X_{ijkz} = \mu + P_i + O_j + R_k + PO_{ij} + PR_{ik} + OR_{jk} + POR_{ijk} + e_{z(ijk)}$$

where X_{ijkz} represents the set of abundances observed at the zth replicate of the kth level of the factor *Reproduction* (R) in the jth level of the factor *Otolith* (O) and in the ith level of the factor *Parasite* (P). μ represents the overall mean abundance vector. P_i represents the effect of the ith level of the factor *Parasite*; O_j denotes the effect of the factor *Otolith*; R_k represents the effect of the k^{th} level of the factor *Reproduction*. PO_{ij}, PR_{ik}, OR_{jk}, and POR_{ijk}, are the interaction effects; finally $e_{z(ijk)}$ represents the error term associated with each observation.

To test our hypotheses we used a semi-parametric approach, the permutational multivariate analysis of variance (PERMANOVA, Anderson 2001). This method analyses the variance of multivariate data explained by a set of explanatory factors on the basis of any distance measure choice. It also provides *P*-values by permutations, so that effects linked to each factor or interaction between factors may be tested. The permutation tests were realised using the DISTLM4 software (Anderson 2004). Each term in the model was tested through 4999 permutations to obtain *P*-values. The Euclidean distance was used and the significance level chosen was 0.05. When a significant interaction term was revealed, we conducted discriminant analyses with the Statgraphics Plus 5.0 software.

In addition to multivariate approaches, we also conducted univariate analyses on each variable of shape indices in order to test for the effect of the factor *Parasite* on the form of the otolith. Analyses were conducted using the same approach used for multivariate tests. When the effect of factor *Parasite* on the shape indices was significant, we conducted *a posteriori* pairwise comparison, also based on 4999 random permutations.

A second objective was to test for the effect of the abundance of the different developmental stages of the parasite found in the eels on the otolith's parameters. We used a semi-parametric multivariate multiple regression. This analysis was carried out with the DISTLM4 software with 4999 permutations. The four explanatory variables were respectively the abundance of parasites founded at stage L_3 (infested stage), L_4 (pre-adult stage), M (male adult stage) and F (female adult stage).

RESULTS

PERMANOVA on the otolith's parameters showed a significant effect of the factor *Parasite* (Table I). The mean values of the Size parameters and the Fourier coefficients displayed significant differences between right and left otoliths. The significant effect of the interaction between factors *Parasite* and *Otolith* showed that the val-

Table I. – Permutational multivariate analysis of variance conducted on the otolith's parameters.

Variables	Source of variation	df	SS	MS	*F*	*P*	
Size parameters (4 variables)	Parasitism (P)	5	3019609207.51	503268201.25	4.51150	0.00140	***
	Otolithes (O)	1	479163611.19	479163611.19	4.29541	0.03900	*
	Reproduction (R)	1	4467569594.30	4467569594.3	40.04908	0.00020	***
	P x O	5	1121363371.73	186893895.28	1.67539	0.13960	n.s
	P x R	5	723613307.508	120602217.91	1.08113	0.22220	n.s
	O x R	1	40969466.56	40969466.56	0.36727	0.54560	n.s
	P x O x R	5	93390395.13	15565065.85	0.13953	0.97880	n.s
	Residual	278	30565351632.4	111552378.22			
Shape indices (6 variables)	Parasitism (P)	5	94.45109	15.74185	2.45565	0.04740	*
	Otolithes (O)	1	11.37384	11.37384	1.77426	0.18780	n.s
	Reproduction (R)	1	0.67090	0.67090	0.10466	0.76360	n.s
	P x O	5	4.87480	0.81247	0.12674	0.97480	n.s
	P x R	5	5.95387	0.99231	0.15480	0.90300	n.s
	O x R	1	0.49497	0.49497	0.07721	0.78700	n.s
	P x O x R	5	14.66196	2.44366	0.38120	0.65940	n.s
	Residual	278	1756.46510	6.41046			
Fourier coefficients (32 variables)	Parasitism (P)	5	0.09602	0.01600	10.64541	0.00020	***
	Otolithes (O)	1	0.27314	0.27314	181.69811	0.00020	***
	Reproduction (R)	1	0.01702	0.01702	11.32377	0.00020	***
	P x O	5	0.01272	0.00212	1.40980	0.03100	*
	P x R	5	0.01030	0.00172	1.14186	0.03120	*
	O x R	1	0.00106	0.00106	0.70664	0.72120	n.s
	P x O x R	5	0.01218	0.00203	1.35006	0.01980	*
	Residual	278	0.41190	0.00150			

P-values: *** < 0.001; ** < 0.01; * < 0.05. n.s: non significant.

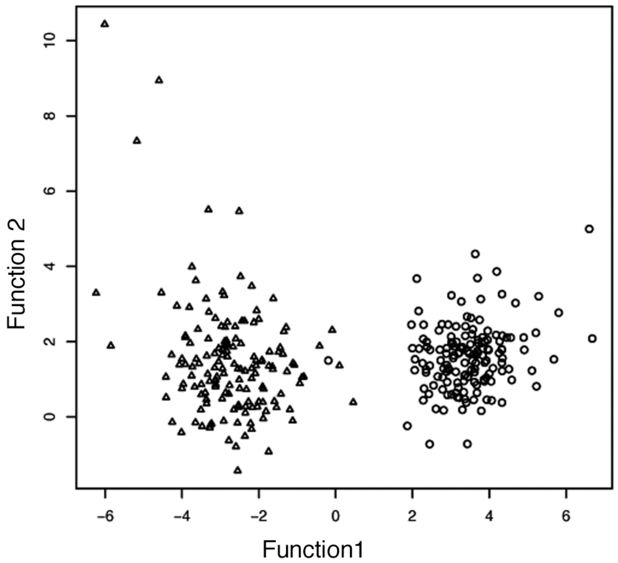

Fig. 2. – Discriminant analysis on Fourier coefficients according to the interaction *Parasite* x *Otolith* x *Reproduction*. The circles represent the group of right otolith and the triangles represent the group of left otolith.

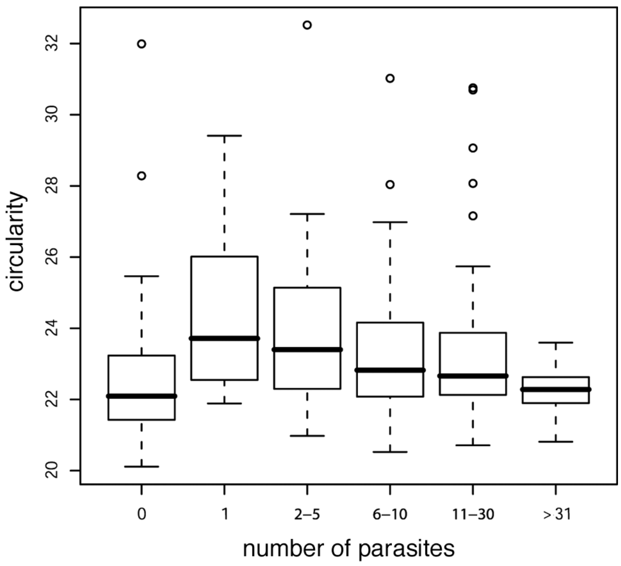

Fig. 4. – Boxplot of the circularity according to the number of parasites in the swim bladder of the eels.

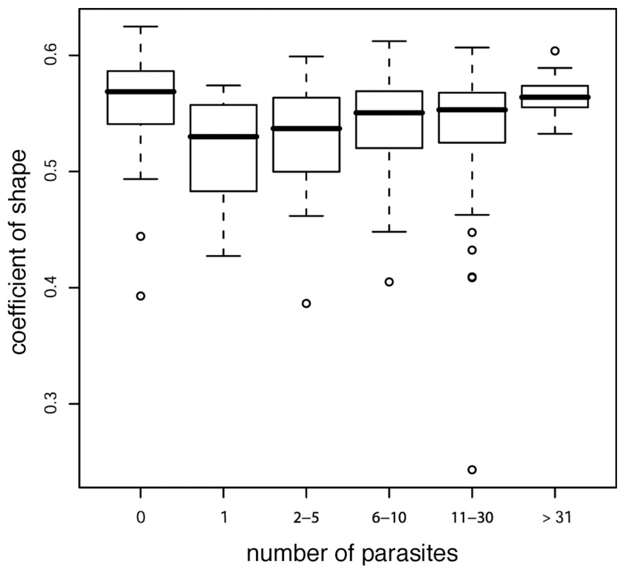

Fig. 3. – Boxplot of the coefficient of shape according to the number of parasites in the swim bladder of the eels.

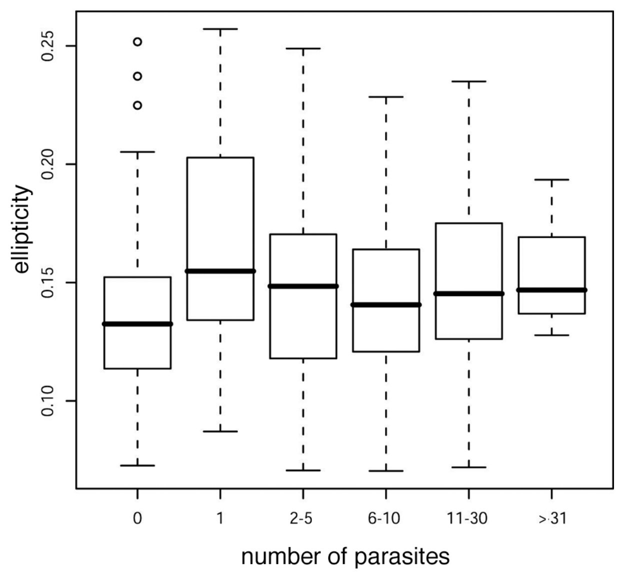

Fig. 5. – Boxplot of the ellipticity according to the number of parasites in the swim bladder of the eels.

ues of the harmonics between left and right otolith varied according to its parasite abundance (Table I). Discriminant analysis showed two distinct groups (Fig. 2) revealing a strong separation between the two groups of otolith in the individuals ($P < 0.05$). There is an asymmetry between otoliths right and left, whether the eels were highly infested or not, and whether the parasite is in a state of *Reproduction* or not. In the classification matrix, each case is placed in the group where its classification function value is largest.

Permutational univariate ANOVAs did not reveal a significant effect of the classes of parasite abundance on all shape indices (Table II). The abundance of parasite had a significant effect only on the coefficients of form, circularity and ellipticity. Pair-wise comparisons showed that the classes of abundance corresponding to the highest percentage of parasite L_3 (0 and > 31; Table III) had a significantly different coefficient of form from the other classes of abundance ($P < 0.05$). Their coefficient of form was higher than the other classes (Fig. 3). Otolith's circularity of the classes 0 and > 31 were significantly lower than the other classes ($P < 0.05$; Fig. 4). The variation of the coefficient of shape, the circularity and the ellipticity appeared in the otolith with the highest percentage of

Table II. – Permutational univariate analysis conducted on the shape indices of the otoliths.

Variables	Source of variation	df	SS	MS	*F*	*P*	
Coefficient of shape (1 variable)	Parasitism (P)	5	0.04511	0.00752	4.10713	0.00840	**
	Otolithes (O)	1	0.00948	0.00948	5.18077	0.02200	*
	Reproduction (R)	1	0.00197	0.00197	1.07442	0.30160	n.s
	P x O	5	0.00304	0.00051	0.27693	0.92320	n.s
	P x R	5	0.00263	0.00044	0.23899	0.87680	n.s
	O x R	1	0.00002	0.00002	0.01352	0.91000	n.s
	P x O x R	5	0.00553	0.00092	0.50333	0.67400	n.s
	Residual	278	0.50161	0.00183			
Roundness (1 variable)	Parasitism (P)	5	0.03780	0.00630	2.03668	0.06580	n.s
	Otolithes (O)	1	0.01579	0.01579	5.10606	0.02180	*
	Reproduction (R)	1	0.09350	0.09350	30.23037	0.00020	***
	P x O	5	0.00827	0.00138	0.44549	0.83380	n.s
	P x R	5	0.00924	0.00154	0.49814	0.64560	n.s
	O x R	1	0.00004	0.00004	0.01373	0.90960	n.s
	P x O x R	5	0.00133	0.00022	0.07167	0.99720	n.s
	Residual	279	0.84750	0.00309			
Circularity (1 variable)	Parasitism (P)	5	94.34978	15.72496	2.45564	0.04740	*
	Otolithes (O)	1	11.34241	11.34241	1.77125	0.18800	n.s
	Reproduction (R)	1	0.52053	0.52053	0.08129	0.79220	n.s
	P x O	5	4.85813	0.80969	0.12644	0.97480	n.s
	P x R	5	5.93419	0.98903	0.15445	0.90280	n.s
	O x R	1	4.85813	0.80969	0.12644	0.97480	n.s
	P x O x R	5	14.65124	2.44187	0.38133	0.65820	n.s
	Residual	278	1754.58760	6.40360			
Rectangularity (1 variable)	Parasitism (P)	5	0.00063	0.00010	0.18164	0.97700	n.s
	Otolithes (O)	1	0.00413	0.00413	7.18302	0.00840	**
	Reproduction (R)	1	0.00012	0.00012	0.21127	0.65440	n.s
	P x O	5	0.00029	0.00005	0.08368	0.99500	n.s
	P x R	5	0.00080	0.00013	0.23062	0.86480	n.s
	O x R	1	0.00008	0.00008	0.13466	0.71760	n.s
	P x O x R	5	0.00245	0.00041	0.71147	0.45300	n.s
	Residual	278	0.15748	0.00057			
Ellipticity (1 variable)	Parasitism (P)	5	0.01599	0.00266	2.20629	0.04320	*
	Otolithes (O)	1	0.00198	0.00198	1.63855	0.20580	n.s
	Reproduction (R)	1	0.04883	0.04883	40.43089	0.00020	***
	P x O	5	0.00475	0.00079	0.65553	0.67340	n.s
	P x R	5	0.00610	0.00102	0.84158	0.35140	n.s
	O x R	1	0.00018	0.00018	0.14749	0.70380	n.s
	P x O x R	5	0.00128	0.00021	0.17598	0.96980	n.s
	Residual	278	0.33089	0.00121			
Eccentricity (1 variable)	Parasitism (P)	5	0.00179	0.00030	2.04236	0.06060	n.s
	Otolithes (O)	1	0.00005	0.00005	0.32494	0.57060	n.s
	Reproduction (R)	1	0.00595	0.00595	40.74429	0.00020	***
	P x O	5	0.00033	0.00005	0.37250	0.88660	n.s
	P x R	5	0.00092	0.00015	1.04952	0.23280	n.s
	O x R	1	0.00003	0.00003	0.23840	0.62280	n.s
	P x O x R	5	0.00014	0.00002	0.15783	0.98100	n.s
	Residual	278	0.04001	0.00015			

P-values: *** < 0.001; ** < 0.01; * < 0.05. n.s: non significant.

adult parasites (class of abundance 1; Fig. 5).

Permutational multivariate regression analysis conducted on the otolith's parameters showed that 12% of the variability of the size parameters was explained by the adult male stage of the parasite ($P < 0.05$; Table IV). Two percent of the total variability was explained by the stage L_3 of the parasite, which led to 14% of the cumulative proportion. The four stages of parasite development had no significant effect on the shape indices and on the Fourier coefficients.

DISCUSSION

Stress is a state produced by any environmental or other factor which extends the adaptive responses of an animal beyond the normal range, or which disturbs the

Table III. – Percentages of the L_3 and the Adult stages of parasites according to their abundances in the swim bladder of the eels.

Number of parasites \ Parasite stages	L3	Adult
0	94 %	6 %
1	15 %	85 %
2 to 5	16 %	84 %
6 to 10	29 %	71 %
11 to 30	37 %	63 %
> 31	56 %	44 %

Table IV. – Permutational multivariate linear regression on otolith's parameters.

Variables	Stages	*P*	Proportion
Size parameters (4 variables)	Male	0.0002	0.1232
	L_3	0.0056	0.0235
	Female	0.3494	0.0025
	L_4	0.5020	0.0013
Shape indices (6 variables)	L_4	0.0352	0.0140
	L_3	0.1288	0.0071
	Female	0.4248	0.0019
	Male	0.3064	0.0030
Fourier coefficients (32 variables)	Female	0.6632	0.0016
	Male	0.9050	0.0006
	L_3	0.9836	0.0002
	L_4	0.9936	0.0001

normal functioning process to such an extent that, in either case, the chances for survival are significantly reduced (Brett 1958). Some authors (Esch *et al.* 1975) stated that stress could be identified as the product and not the cause of the change in homeostasis or environmental stability. It is expressed physiologically and/or behaviourally. Environmental factors may influence the shape of otoliths and induce an asymmetry in their morphology, being an indicator of growth disturbance. The Fluctuating Asymmetry (FA) arises as small deviations from perfect bilateral symmetry, which reflect "mistakes" in developmental processes resulting from the inability of the genotype to buffer itself effectively against environmental perturbations. FA is thought to be a sensitive indicator of both genetic (Somarakis *et al.* 1997) (e.g. inbreeding, hybridization) and environmental (Parsons 1990, Campana & Casselman 1993) (e.g. temperature extremes, pollutants, food shortage, parasite load, population density) stresses. However, Van Dongen *et al.* (2000) stated that, generally, studies failed to detect significant heritability for fluctuating asymmetry (FA). FA may index stress to a population accurately, but it may be a poor indicator of individual quality due to its random nature (Palmer 1994).

Besides environmental stressors, other factors, such as a stress by parasitism, can cause a bilateral asymmetry on fish species (Leary & Allendorf 1989). Parasites have been found to be associated with a deviance of symmetry in the development (Moller 1992, Polak 1993, Reimchen 1997, Thomas *et al.* 1998, Brown & Brown 2002). This may be explained by (1) a direct stress caused by the metabolic cost of the parasite on its host (Moller 1992, Polak 1993), (2) a variation of the abundance of the parasite related to stressful environmental conditions for hosts (Koskivaara & Valtonen 1992), or (3) a relationship between the genetic incapacity to interfere with developmental errors and the susceptibility to parasite (Moller 1996).

One of the objectives of this study was to examine the effect of *A. crassus* on otoliths morphology of eels reared in controlled experimental conditions. Significant differences in Fourier descriptors were found between right and left otoliths. Since eels were reared in tanks with the same environmental conditions, this asymmetry is due to a stress control caused by the parasitism. High density of parasites had also significant effects on the coefficient of form, circularity and ellipticity of the otoliths. The energetic cost due to *A. crassus* forced the eels to make compromises. The parasite disturbs specifically the growth of the otolith by interfering with its calcification. Barus *et al.* (1998, 1999) measured the concentration of several inorganic substances (among which the calcium) and amino acid in the muscles of uninfected and infected eels. They found that *A. crassus* generated significant differences that could be explained by the hematophagous feeding and pathogenicitiy of the parasite.

In natural environment, there are no significant relationship between parasitism and otolith bilateral asymmetry of yellow eels caught at Salses-Leucate lagoon (Fazio *et al*. 2005). At this adult stage, eels are more resistant to parasitism. Similar results were found in this lagoon on glass eels (Lecomte-Finiger unpubl data). At this entering stage, VB glass eels were not parasitized. Eels have to eat the parasite intermediate host to be infested, the latter being only present in the lagoon.

In our experimental approach, eels were captured at early stages. They were infested with a high number of parasites and thus were stressed during their metamorphosis. This can explain why a bilateral asymmetry of the otoliths was related to the density of the parasite found in the eel's swim bladder when infested at early stages. Moreover, the adult stage of *A. crassus* is the most aggressive form on its final host (Da Silva 2005). Once in the eel, it can reduce the development of the individual by drawing its energy and limiting its resources. Sagittae of eels infested with adult stages of *A. crassus* were the most affected.

The possible stress caused by suboptimal environmental conditions may force the fish to allocate energy to cope with extreme physical parameters otherwise available for development of a stable phenotype (Moller & Swaddle 1997). Even if the number of parasites had a significant effect on the shape of otoliths, infested eels did not show differences in their morphological parameters. The absence of effect of the parasite on the general condition of the eel could be an artefact due to the experimental conditions of our study. In experimental conditions infected eels remaining in fasting highlight a big loss in body mass (Boon *et al*. 1990). In our study, eels were fed ad libitum. We suppose that infected eels have compensated the energetic loss due to the parasite.

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