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An evaluation of indices of gross pathology associated with the nematode *Anguillicoloides crassus* in eels

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Abstract

This study compares two alternative indices for quantifying the gross pathology of the swimbladder of eels, *Anguilla anguilla* (L.), infected with the nematode *Anguillicoloides crassus*. Two observers recorded twice the scores obtained by the two indices on the same set of 71 wild caught eels (from elver to silver eels, French Mediterranean lagoons). The Length Ratio Index (LRI), performed better than the Swimbladder Degenerative Index (SDI), in three of four predefined criteria of decision. First, the LRI better correlated with an estimate of the swimbladder volume reduction, a functional consequence of the infection (representativeness). Also, the LRI was less prone to subjectivity (inter-observer variability) and more precise (intra-observer variability), although less easy to generate (time needed for measurement/assessment). Using a sub-sample of 32 unaffected eels (showing minor if any swimbladder damage and no living worms at autopsy), we ascertained a linear relationship between the swimbladder length and the total body length, a prerequisite of isometric growth, to definitively accept the new ratio index as a valid alternative to the SDI. Also, because the LRI can be recorded on live specimens with radio-imagery (non-invasive method), we recommend its use, and provide a graph of correspondence between the SDI scores, the LRI scores and the estimated proportion of gas loss in the swimbladder.

Keywords: Anguilla anguilla, anguillicolosis, helminthiasis, indexing, introduced species, swimbladder.

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Introduction

The need to assess the health state of the eel swimbladder has long been recognized by investigators concerned with anguillicolosis, a disease caused by nematodes of the genera *Anguillicoloides* and *Anguillicola*. The first published attempt to score the pathological signs in the infected organ was by Liewes & Schaminee-Main (1987), a few years after the first reports of the Asian parasite, *Anguillicoloides crassus* (Kuwahara, Niimi & Itagaki 1974), in the European eel, *Anguilla anguilla* (L.) (for review, see Kirk 2003 and Table 1). The combined action of both larval and adult parasites results in haemorrhages, inflammation and fibrosis of the swimbladder wall, which may alter the gas composition in the swimbladder, and eventually lead to impaired functioning of the organ (Molnár, Baska, Csaba, Glávits & Székely 1993; Haenen, Van Wijngaarden, Van der Heijden, Höglund, Cornelissen, Van Leengoed, Borgsteede & Van Muiswinkel 1996; Würtz, Taraschewski & Pelster 1996; Nimeth, Zwerger, Würtz, Salvenmoser & Pelster 2000). In the most severe cases, after multiple and repetitive infections, the swimbladder may turn into a compact ball of necrotic tissue, with

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Table 1 Severity grades of damage in the first five published attempts to score the swimbladder health of eels, *Anguilla anguilla*, as a result of infections by the nematode *Anguillicoloides crassus*

Liewes & Schaminee-Main 1987	Csaba *et al.* 1993	Hartmann 1994	Molnár *et al.* 1994	Beregi *et al.* 1998
Grade 1: normal swimbladder, no worm	**Grade 1:** normal swimbladder, no worm	**Grade 1:** swimbladder without histological changes. Wall transparent, 0.6 to about 1.0 mm thick (depending on the size of the individual)	**Grade 1:** swimbladder wall less than 1 mm, although exhibiting signs of past infections (smoke-like opacity, parasitic nodules, pigmentation, minor haemorrhages, etc.)	**Grade 1:** swimbladder transparent and thin-walled, with the wall thickness not exceeding 0.3 mm. Pneumatic duct not containing air. Swimbladder collapsing after opening
Grade 2: normal swimbladder, a few worms	**Grade 2:** normal swimbladder, containing a number of worms	**Grade 2:** weak histological changes in the swimbladder wall. Swimbladder with slightly thickened wall and little opacity	**Grade 2:** swimbladder with a 1–3 mm thick wall	**Grade 2:** swimbladder normally filled with air; wall slightly opacified, sometimes containing haemorrhages but not exceeding 1 mm in thickness. Lumen usually containing only a few worms of small size. In some cases, worms also in the lumen of the air-filled pneumatic duct
Grade 3: enlarged swimbladder, partly filled with red–brown fluid; wall can be inflated	**Grade 3:** enlarged swimbladder, partly filled with brownish liquid, wall with inflammatory processes	**Grade 3:** strong histological changes in the swimbladder. Wall opaque and less elastic. Swimbladder lumen reduced, but filled with gas.	**Grade 3:** swimbladder wall thickness exceeding 3 mm	**Grade 3:** dilated, air-filled lumen of the swim bladder containing a small amount of exudates and numerous worms (hollows in the wall). Dilated lumen of the pneumatic duct often containing worms. Wall opaque and slightly thickened (up to 2 mm but only near the two ends of the sac)
Grade 4: enlarged swimbladder, filled with red–brown fluid; actively moving nematode larvae can be noticed	**Grade 4:** greatly thickened wall, lumen filled with brownish liquid containing L2 larvae actively moving	**Grade 4:** histological changes and other pathological effects. Swimbladder as in grade 3, plus haemorrhage, inflammation, necrosis, and exudates. Lumen filled with strong pigment deposits, narrowed by the remains of nematode tissues. Swimbladder severely damaged, but still partly functional		**Grade 4:** one or both ends of the swimbladder atrophied, and sometimes one of its halves completely devoid of air. Lumen containing more or less exudates and few small dead worms. Wall markedly thickened: 2 or 3 mm in the middle third, and even 3 to 5 mm at the ends. Pneumatic duct looking like an airless bundle
Grade 5: rupture of the swim bladder wall or of the *ductus pneumaticus* which is highly irritated. Secondary infections of surrounding tissues externally visible as a swollen and inflamed abdomen	**Grade 5:** wall of the *ductus pneumaticus* burst, secondary infections arise	**Grade 5:** advanced histological changes in the swimbladder wall and loss of function. Wall up to 5 mm thick, lumen narrow, gasless and constricted		**Grade 5:** swimbladder markedly shrunken. Rigid wall containing neither air nor any other material (exudates, worms), uniformly and markedly thickened, reaching 2–5 mm. Pneumatic duct thin-walled, containing no air
Grade 6: swimbladder wall replaced by a thick layer of connective tissue; remains of the nematodes can be found	**Grade 6:** thick wall, remains of lumen worms			
Grade 7: swimbladder replaced by a hard brown–black mass in which remains of the nematodes can be found	**Grade 7:** hardened tissues, remains of worms, liver often of pale colour			

no lumen left (Molnár *et al.* 1993; Würtz & Taraschewski 2000). A similar pathogenic situation was later observed with the infection of the American eel, *A. rostrata* (Ooi, Wang, Chang, Wu, Lin & Hsieh 1996; Barse, McGuire, Vinores, Eierman & Weeder 2001; Sokolowski & Dove 2006), whereas no such pronounced effects have been documented for the native eel host, *A. japonica* (Egusa 1979; Nagasawa, Kim & Hirose 1994; Knopf & Mahnke 2004). It thus seems that severe damage only occurs in the case of recent allopatric interactions, which may explain the low immune response observed in the new hosts and the high parasite abundance (Taraschewski 2006; Kennedy 2007; Sasal, Taraschewski, Valade, Grondin, Wielgoss & Moravec 2008). This 'useless virulence' (Combes 2001) creates a situation of infection-state dependence, in which the tissue degradation caused by previous infections may limit and eventually prevent the establishment of new infections (feedback effect) (Van Banning & Haenen 1990; Molnár, Székely & Perényi, 1994; Lefebvre, Contournet & Crivelli 2002). In such a context, classical epidemiological parameters (i.e. intensity, abundance, prevalence) by themselves cannot capture the overall impact suffered by eels. Indeed, the absence of living parasites at the autopsy may correspond to two opposite situations: either a very low or extremely high parasite pressure.

Assessing the health state of the eel swimbladder is also important to investigate the fitness costs imposed by the infection since, for instance, the severity of the damage in the swimbladder was shown to be the best predictor of the eel mortality rate in two independent studies conducted in hypoxic conditions (Molnár 1993; Lefebvre, Contournet & Crivelli 2007). Moreover, one may reasonably question the chance of silver eels reaching their spawning site in the Sargasso Sea (6000 km transoceanic migration) with an impaired swimbladder (Nimeth *et al.* 2000; Lefebvre, Acou, Poizat & Crivelli 2003; Münderle, Sures & Taraschewski 2004). For instance, in a recent mark-recapture study in the Baltic Sea, the most severely affected eels appeared to cover less distance and to swim in shallower waters, lacking the typical vertical migrations of healthy silver eels (Sjöberg, Petersson, Wickström & Hansson 2009). The conclusion of the last international collaborative investigation of the reproductive status of the European eel (EELREP 2005) was explicit: 'in case of heavy swimbladder infection and/or damage… [silver eels]… will never reach the spawning grounds and cannot contribute to recruitment' (also see Palstra, Heppener, Van Ginneken, Székely & Van den Thillart 2007; Székely, Palstra, Molnár & Van den Thillart 2009). For all the above reasons, and also to compare the actual infection spread between sites, dates and eel species (cross-specific data being very informative concerning the origin and the evolution of the disease), it is essential to have suitable measures to assess the health state of the infected organ.

There have been six published attempts to score eel swimbladder damage (Liewes & Schaminee-Main 1987; Csaba, Láng, Sályi, Ramotsa, Glávits & Rátz 1993; Hartmann 1994; Molnár *et al.* 1994; Beregi, Molnár, Békési & Székely 1998; Lefebvre *et al.* 2002), all based upon the severity of the gross pathologies observed at autopsy (see Table 1). In 2005, Palstra and co-workers in the EELREP project introduced an alternative index based on the shortening of the swimbladder as a result of infection (thereafter LRI, for Length Ratio Index, also see Palstra *et al.* 2007). This metric is *de facto* promising and offers interesting new perspectives (especially when coupled with non-invasive radio-diagnostic methods), but its biological rationale needs to be validated and its scores compared to the commonly used index (i.e. SDI, Swimbladder Degenerative Index, see Lefebvre *et al.* 2002).

The primary objective of the present study was thus to compare and critically evaluate the two approaches using data from one set of fish. To do so, we first defined the expected attributes of a 'good' index, and then investigated the performance of the two indices on the following criteria: representativeness, objectivity, precision and easiness. In addition, the LRI prerequisite of isometric growth between the swimbladder length and the total body size was checked (in the absence of any infection sign). Based on the obtained results, the pros and cons of the two alternative indices are considered and consensual recommendations suggested.

Materials and methods

Detailed history of swimbladder damage assessments

Previous attempts to score swimbladder damage at autopsy (see Table 1) all used the thickness of the swimbladder wall as a key pathological sign. They also focussed on the progressive loss of the natural

yellowish transparency of the swimbladder wall into a smoke-like opacity in case of infection. It was argued that, besides the observed changes in the swimbladder wall, the amount of exudate and worm remains within the swimbladder lumen were also an important factor for evaluating the severity of past infections. Probably because of the difficulties in clearly distinguishing between the different grades (some mixing histopathological changes with the presence/absence of lumen worms), and also because of the restricted availability of some original publications, these early attempts never entered into common usage. Following on from previous workers, Lefebvre *et al.* (2002) proposed a codified metric (SDI, see below), based on the cumulated values in predefined criteria to be scored individually.

The Swimbladder Degenerative Index

The assessment was based on gross pathology of excised swimbladders. Three criteria were used, each one coded by 0, 1 or 2 (increasing degradation). The first criterion focussed on the opacity of the swimbladder wall. A value of 0 was assigned to normal-looking swimbladder (i.e. transparent-yellowish colour, see Clarke & Witcomb 1980). Total opacity (when no reading is possible through the swimbladder wall) was assigned a value of 2, and all intermediate cases a value of 1. The second criterion examined the presence of pigmentation on the swimbladder wall and exudate instead of gas in the swimbladder lumen (dead worms, erythrocytes, decaying swimbladder tissue, eggs and L2 stages of *A. crassus*). A value of 0 was given to swimbladders with no pigmentation and no exudate. A value of 2 was assigned to swimbladders that exhibited both pigmentation and exudate, and value 1 to those that showed either signs of pigmentation or exudate. The third criterion concerned the thickness of the swimbladder wall. The codification was adapted from an earlier proposition by Molnár *et al.* (1994): a value of 0 was assigned to thin walled-swimbladders (< 1 mm), a value of 2 to swimbladders with little if any lumen left (more than 3 mm thick wall), and value 1 to all other intermediate cases. Thus, individual criteria can be conveniently and quite safely scored applying extreme values (0 or 2) to normal and severely degraded swimbladders, respectively. In cases of doubt, and for all intermediate situations, a medium value of 1 is applied. The SDI is then computed by adding the scores obtained for the three separated criteria, and so may span over seven discrete values, ranging from 0 to 6.

The Length Ratio Index

In 2005, EELREP warned of possible subjectivity and mis-interpretations in using the SDI, and introduced a synthetic but easy metric for capturing the overall pathological damage (i.e. LRI, later discussed in Palstra *et al.* 2007). They based their index on the observation that swimbladders thicken and shorten as a result of multiple infections events (also see, for example, Würtz & Taraschewski 2000), so that the severity of the pathology could be tentatively encapsulated into a linear measure of the infected organ (in relation to body size, by dividing the swimbladder length by the total length). In other words, the higher the parasite pressure suffered by the eel, the shorter the relative size of the swimbladder, and so the smaller the resulting LRI score.

General considerations on indexing

The process of indexing is similar in many ways to the process of sampling, in the sense that the otherwise complete operation (measuring the true value through exhaustive enumeration or measurement) is either unsustainable or impracticable, and finally unnecessary if the very nature and properties of the estimators are properly considered. Expected attributes of a good index should thus closely match those expected for an adequate sample or subsample. There follows a proposed list of criteria of decision for evaluating any index metric (for similar considerations, see for example Bolger & Connolly 1989, in selecting suitable body condition indices):

Representativeness (or the capability to reflect the biological characteristics measured). A good index should provide useful and meaningful information, whilst being able to accurately discriminate between a continuum of possible biological values. With the two indices at hand, we aim to score the damage done to the swimbladder as a means to ultimately evaluate the functional impact of the infection on the eel hosts. The 'true' biological value to investigate is here tentatively assumed to relate to the loss of internal swimbladder volume. The eel swimbladder is an organ involved in many important functions such as buoyancy control, gas exchange, absorption and secretion (Zwerger, Nimeth, Würtz, Salvenmoser & Pelster 2002; Tesch 2003), so that any reduction in

the lumen gas volume is expected to severely impact on the general metabolism and physiology of the fish (Würtz *et al.* 1996; Palstra *et al.* 2007).

Precision (or the reproducibility between repeated measurements). A good index should not vary substantially in its score between separate measurements, and be closely reproducible under similar conditions. The swimbladder is a delicate, flexible and inflatable organ, and all related data (length or pathological signs) are expected to vary to some extent between repeated measurements. Here, the precision (or inversely the measurement error) of the indices could be investigated by recording twice the scores of the same observers (i.e. intra-observer variability).

Objectivity (or the concordance between multiple observers). A good index should be unequivocally understood and clearly defined to minimize inter-observer variability (subjectivity). The swimbladder is embedded in a conjunctive tissue at both poles so that its delimitation may differ from observer to observer. Also, when the criteria used to assess pathological damage do not correspond to objective metric measures (as for the SDI), it has *de facto* some subjectivity. Here, the objectivity of the indices could be tested by recording the scores of two independent observers (i.e. inter-observer variability).

Easiness (or the simplicity in the measurement, computation or interpretation). A good index should be cost- and time-effective enough to ensure a widespread application amongst possible end-users (i.e. managers, researchers), and to ultimately serve comparative purposes between individuals, or to track changes between years or between areas. In our case, both indices are logistically easy to generate (as compared to the 'true' value, herein approximated by the internal swimbladder volume), and to understand (the LRI decreases and the SDI increases with the severity of the observed damage). Here, the criterion is the time spent in recording the necessary data for computing each index, i.e. the histopathological aspect and the swimbladder length.

Detailed protocol for comparing the two indices

Eels originated from the brackish waters of the Vaccarès lagoon in the Rhône river delta (Camargue, southern France). Several fyke nets (6 mm mesh in the funnels) were set on eight consecutive days in the third week of June 2009. On the day of capture, eels were placed in a tank of 10–20 cm water depth, and killed by adding an overdose of anaesthetic (0.5 mL L^{-1} Eugenol).

Animals were examined fresh, closely following published protocols (i.e. Lefebvre *et al.* 2002 for the SDI; Palstra and co-workers in EELREP 2005 for the LRI). The total body length of the eel (L_T) was recorded to the nearest 1 mm (from the tip of the snout to the tip of the tail), and the vertical and horizontal diameters of both eyes (D_V and D_H) were measured to the nearest 0.1 mm with a Vernier calliper. Eels were then ventrally opened, sexed whenever possible (for individuals longer than 300 mm), and the swimbladder, together with the *ductus pneumaticus* (in functional connection with the swimbladder in physostome fish, see Pelster 1998), were carefully detached from the rest of the body. The naturally gas-filled organ was immersed in a water-filled graduated tube, and the volume of water displaced (to the nearest 0.1 cm^3) was used to approximate the total inflated volume of the swimbladder (including the *ductus pneumaticus*).

Swimbladders were then measured to the nearest 1 mm with a Vernier calliper (L_S, natural extended length) (see text in page 70 and Fig. 4, in EELREP 2005). Observed pathologies were recorded using the SDI by attributing individual scores (from 0 to 2, increasing damage) to the three criteria of opacity, presence of pigmentation/exudate, thickness of the swimbladder wall (see above). To quantify both objectivity and precision in the measurement/assessment, the L_S and the SDI scores were recorded twice by two different observers. Thus, for each eel, after a first set of measurements by observer 1, measurements were taken by observer 2, then repeated again by observer 1, and finally one more time by observer 2. Throughout, the duration of each of these operations (metric measurement and damage assessment) was recorded to the nearest second, so that the elapsed time could be used to estimate the ease of scoring the two indices.

At this stage, the swimbladder (and the *ductus pneumaticus*) was fully opened and the number of living lumen worms was recorded (preadults and adults). The volume of parasites (in cases of current infection) and the deflated volume of the swimbladder (after total removal of the internal gas, and subsequently exudate and other dead worm remains) were estimated using again the volume of water displaced after full immersion in a water-filled graduated tube.

Analyses and statistical treatments

The stage of maturation of the eels was evaluated using the Ocular Index (based on the relationship between the mean dimensions of both the right and left eyes and the total body size), according to Pankhurst's formula: $OI = ((D_V + D_H)/4)^2 \times (\pi/L_T) \times 100$. Only those eels that met the threshold value of $OI \geq 6.5$ were considered to be in the silvering process (i.e. onset of sexual maturation, see Pankhurst 1982). The internal volume of the swimbladder (V_S in cm^3, including the *ductus pneumaticus*) was calculated by subtracting the deflated volume (open swimbladder), and the volume of parasites (in case of infection), from the full inflated volume (as measured immediately after autopsy). Normality was checked with the Shapiro–Wilk (W) test because of its good power properties for small to medium sample sizes (StatSoft Inc. 2001). Central tendencies were expressed by arithmetic means ± standard deviations (SD), or by median values ± 25th percentiles (0.25–0.75 quartiles interval) in case of severe departure from normality. The amount of variability around the two indices (either between the two observations of the same observers or between the two observers for the same set of observations) was compared using the coefficient of variation (CV), which is the SD expressed as a percentage of the mean (and calculated from the average of each pairwise CV). It is an absolute measure of dispersion in the sense that it is independent of the unit employed, and of the magnitude of the mean (Sokal & Rohlf 1995). Apart from their use in estimating variability, the scores obtained in the four observations of the same swimbladder were pooled as arithmetic means (and approximated back to the closest integral in the case of the SDI), so that $n = 71$ for all variables unless otherwise mentioned. Non-parametric Spearman rank (Rs) correlations, Mann–Whitney U-tests and Wilcoxon matched-pair (T) tests were applied whenever conditions for parametric analyses were violated. All statistical treatments and graphics were performed using STATISTICA 6 (StatSoft Inc. 2001).

Results

For the 71 eels collected in the Vaccarès lagoon the L_T ranged from 188 to 761 mm, with a mean value of 428.07 ± 135.46 mm. The overall length frequency was normally distributed (Shapiro–Wilk test: $W = 0.98$, $P = 0.21$), and, with the exception of the youngest elver eels (too small to be caught by the 6 mm mesh of the nets), all size classes were represented in the sample. Nearly one-fourth of the eels were in the silvering process according to the criterion of Pankhurst ($IO \geq 6.5$, $n = 17$), and the sex-ratio was strongly female-biased (≈ 8% males). The number of adult and preadult *A. crassus* in the eel swimbladder (and *ductus pneumaticus*) ranged from 0 to 21, for an overall mean abundance of 2.17 ± 4.04 worms per host (median abundance: 0.00 ± 0.00–2.00; prevalence: 41%, $n = 29$; mean and median intensities: 5.31 ± 4.85 and 3.00 ± 1.00–8.00). The parasite distribution strongly deviated from normality (Shapiro–Wilk test: $W = 0.61$, $P < 0.001$), and best fitted a negative binomial function.

The two indices and the measure of swimbladder volume

SDI. The SDI scores extended over the seven possible values of the index (i.e. from 0 to 6). However, the frequency distribution of the SDI was skewed to the right (Fig. 1a), and significantly deviated from normality (Shapiro–Wilk test: $W = 0.90$, $P < 0.001$). More than half of the sample (43/71 = 61%) showed a $SDI \leq 2$ (slightly or not degraded swimbladders) for an overall median value of 2.00 ± 1.00–3.00 (Fig. 1a). Compared to previous years in the same area (see for instance Lefebvre *et al.* 2002), the swimbladders of the 71 investigated eels were considered quite healthy, with only a minor fraction (10/71 = 14%) showing SDI values ≥ 5 (severely degraded swimbladders). Multiple Spearman correlations revealed that the two SDI criteria of opacity and thickness were highly auto-correlated ($Rs = +0.84$, $P < 0.001$), whereas the presence of pigmentation/exudate was relatively independent of the other two (vs opacity: $Rs = +0.50$, $P < 0.001$; vs. thickness: $Rs = +0.63$, $P < 0.001$). Overall, thickness best fitted with the final SDI value (thickness vs. SDI: $Rs = +0.92$, $P < 0.001$; opacity vs. SDI: $Rs = +0.87$, $P < 0.001$; pigmentation/exudate vs. SDI: $Rs = +0.78$, $P < 0.001$). The SDI score increased with the size of the eel ($Rs = +0.31$, $P < 0.01$) and with the parasite abundance ($Rs = +0.30$, $P < 0.05$), but there was also a trend for longer eels to harbour more parasites (L_T vs. abundance: $Rs = +0.23$, $P = 0.06$). Overall, silver eels showed a significantly higher SDI score than the rest of the

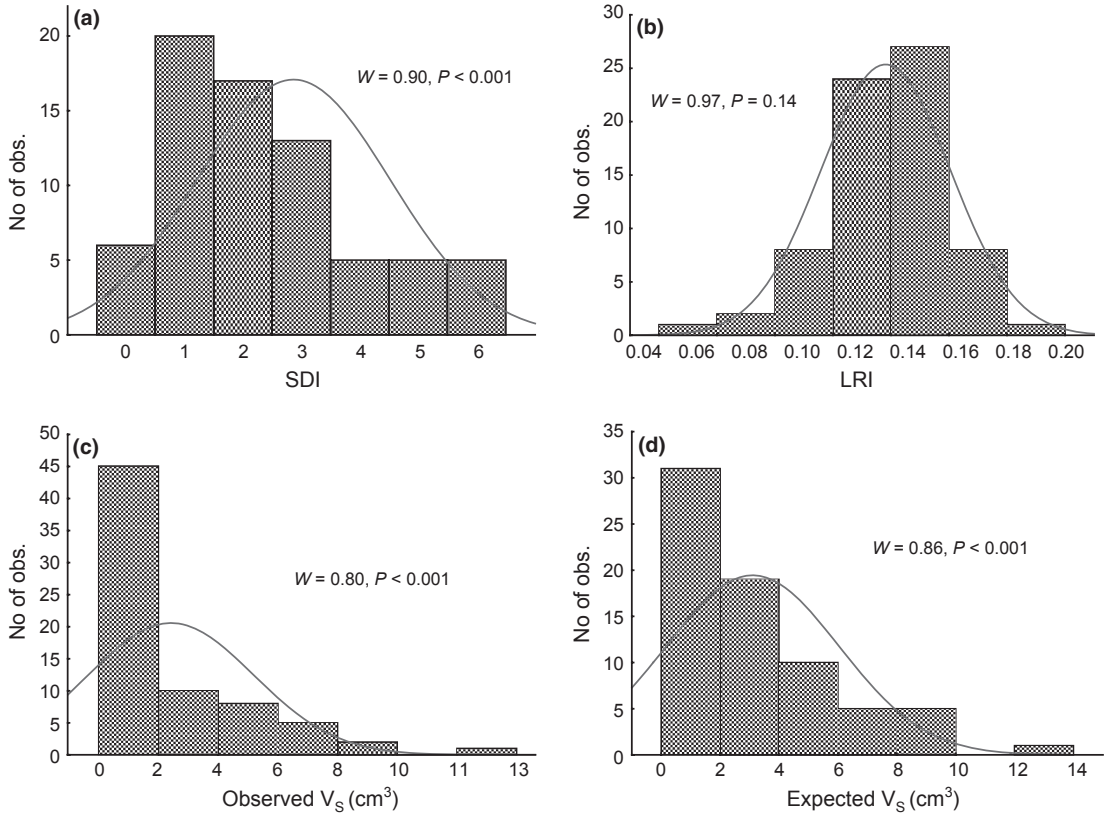

Figure 1 Frequency distribution of (a) the Swimbladder Degenerative Index, (b) the Length Ratio Index, (c) the observed internal swimbladder volume, (d) the expected internal swimbladder volume in the absence of any sign of infection, for the 71 investigated eels. The curve shows a normal distribution. The Shapiro–Wilk parameter W measures the closeness to normality (in which case its value approaches unity). Small W and P values indicate severe departure from a normal distribution.

sample (median values: 4.00 ± 2.00–5.00 vs. 2.00 ± 1.00–3.00; Mann–Whitney test: U = 206.50, $P < 0.001$).

LRI. The LRI scores ranged from 0.04 for the most severely degraded swimbladders to 0.20 for healthy ones (Fig. 1b). The frequency distribution did not deviate from normality (Shapiro–Wilk test: $W = 0.97$, $P = 0.14$) with a mean value of 0.13 ± 0.02. For comparison purposes, in Palstra *et al.* (2007), the LRI values of silver eels ranged from 0.03 to 0.19 with a mean of 0.11 ± 0.03. Thus, based on the two available studies, it seems that the swimbladder length can vary from 1/5 (or 20%) of the total body length (upper relative size in normal condition) to 1/33 (or 3%) when severely damaged. Within the sub-sample of healthy swimbladders (harbouring no parasites and having a SDI value ≤ 2, $n = 32$), the swimbladder length L_S (numerator of the LRI) was almost normally distributed (Shapiro–Wilk test: $W = 0.93$, $P = 0.03$; Kolmogorov–Smirnov test: $D = 0.13$, $P > 0.20$; mean: 51.15 ± 15.75 mm), as was the total length of the eel L_T (Shapiro–Wilk test: $W = 0.95$, $P = 0.18$; mean: 386.09 ± 108.52 mm). For these healthy eels, the relationship between the two components of the LRI significantly fitted a straight line (Fig. 2a; $R = +0.83$, $P < 0.001$). Attempts to transform one or both of the variables did improve the R value (i.e. natural logarithm of both variables: $R = +0.86$; inverse function of both variables: $R = +0.88$), whilst the same transformations did not work on the whole sample ($R = +0.79$ on natural scaled values; $R = +0.81$ on log transformed values; $R = +0.80$ on inverse transformed values). In other words, in the absence of any sign of infection, eel body length may account for more than 75% of the swimbladder length variability ($R^2 = 0.77$ for the inverse function, $n = 32$), and so the prerequisite of isometric growth between the two variables of the ratio index was confirmed. For the total sample, there was a trend for the LRI to decrease with L_T

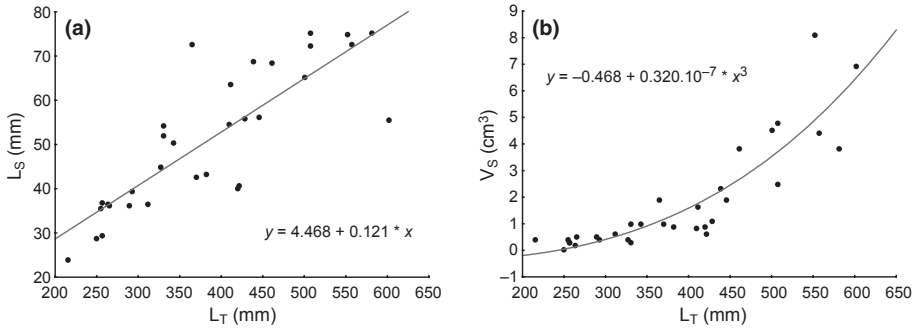

Figure 2 Relationships between (a) eel length and swimbladder length, (b) eel length and the internal swimbladder volume, in the absence of parasites and degradation of the swimbladder (sub-sample $n = 32$).

($Rs = -0.21$, $P = 0.08$) and to increase with parasite abundance at autopsy ($Rs = +0.21$, $P = 0.08$), but there was also a trend for longer eels to harbour more parasites (L_T vs. abundance: $Rs = +0.23$, $P = 0.06$). Overall, silver eels showed a significantly lower LRI score than the rest of the sample (median values: 0.12 ± 0.11–0.13 vs. 0.14 ± 0.12–0.15; Mann–Whitney test: $U = 281.00$, $P < 0.05$).

Swimbladder volume. The internal swimbladder volume, V_S, ranged from 0 to 13.4 cm^3. The frequency distribution strongly deviated from normality (Shapiro–Wilk test: $W = 0.80$, $P < 0.001$) and was highly right-skewed (best fit by a log-normal distribution) with a median value of 1.00 ± 0.40–3.80 cm^3 (Fig. 1c). For the sub-sample of healthy swimbladders (harbouring no parasites and having SDI values ≤ 2, $n = 32$), V_S positively and significantly correlated with L_T ($Rs = +0.91$, $P < 0.001$). Multiple transformations were applied to best relate the two variables (including polynomial, power, square root, logarithmic functions). The relationship between L_T and V_S was found to best fit a power 3 function: $V_S = -0.468 + 0.320 \times 10^{-7} \times {L_T}^3$ ($R = +0.89$, $P < 0.001$, see Fig. 2b). Then, using the above formula, an expected V_S (i.e. swimbladder volume in the absence of degradation and parasites) can be inferred for all eels (Fig. 1d), so that it became possible to estimate the gas loss due to the infection [% gas loss = $(V_{exp} - V_{obs})/V_{obs} \times 100$].

Comparison between the two indices

There was a significant negative relationship between the SDI and the LRI ($Rs = -0.38$, $n = 71$, $P < 0.005$). In other words, the higher the SDI score, the smaller the LRI value, and the more severe the corresponding damage in the swimbladder (Figs 3 and 4).

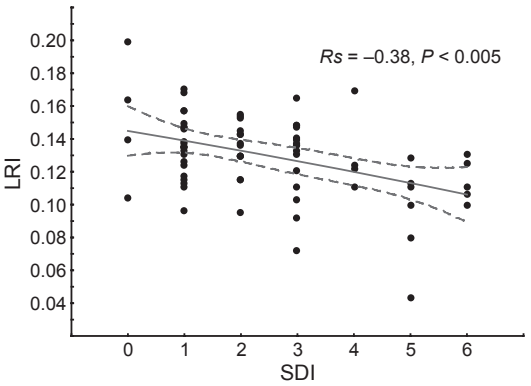

Figure 3 Relationship between the Swimbladder Degenerative Index and the Length Ratio Index, for the 71 investigated eels (±0.95 confidence limits).

Representativeness. There was a significant positive relationship between the estimated percentage of gas loss and the SDI ($Rs = +0.27$, $P = 0.03$), so that the higher the SDI score the smaller the relative remaining swimbladder volume (Fig. 4). In contrast, there was a significant but negative relationship between the LRI and the estimated percentage of gas loss ($Rs = -0.33$, $P < 0.01$). Thus, the higher the swimbladder volume reduction, the smaller the LRI value, and the higher the SDI score (Fig. 4). Assuming that the percentage of gas loss was properly estimated and closely represents a functional measure of parasite impact (relative swimbladder volume reduction), then the LRI performed slightly better (although still poorly) in term of biological significance.

Precision. The intra-observer variability between the two sets of repeated measures on each of the 71

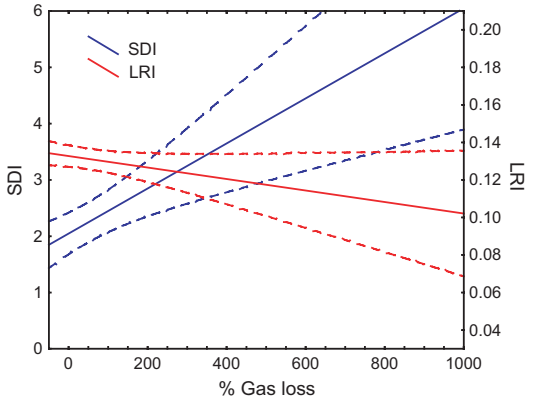

Figure 4 Relationship between the estimated percentage of gas loss (based on the relationship between the V_S and L_T in the absence of infection sign, *x*-axis) and the corresponding scores in the Swimbladder Degenerative Index (left axis, blue line ±0.95 confidence limits), and in the Length Ratio Index (right axis, red line ±0.95 confidence limits).

investigated eels is given in Table 2 for the SDI and the L_S. The SDI showed a much higher coefficient of variation than the L_S (23.97% vs. 5.08%, combined data for observers 1 and 2, $n = 142$) which indicated that the swimbladder length was measured with higher repeatability (and so better precision). However, it must be emphasized that the LRI may suffer additional measurement errors from measuring the total length of the eel (i.e. L_T, the denominator of the index). From simple calculation, setting a CV on L_T of similar amplitude as for L_S (which actually corresponds to a relatively large measurement error of $5.08 \times 428.07/100 = 22$ mm) and applying the approximating formula $CV(X/Y) = [CV(X)^2 + CV(Y)^2]^{1/2}$ (see for instance Oyejola & Mead 1989), the overall coefficient of variation for the LRI would be about 7%, which is still much smaller than the 24% obtained for the SDI. The intra-observer variability in scoring the SDI slightly co-varied with the index value, whether measured by the SDI score (mean vs. SD: $Rs = +0.18$, $n = 142$, $P = 0.04$) or by the LRI (mean vs. SD: $Rs = -0.12$, $n = 142$, $P = 0.17$), whereas the intra-observer variability in measuring the L_S strongly co-varied with the index value (mean vs. SD for LRI: $Rs = -0.22$, $n = 142$, $P < 0.01$; mean vs. SD for SDI: +0.20, $n = 142$, $P = 0.02$). Since the two indices inversely varied in scoring the observed damage (see above), this would indicate that the intra-observer error of measure on both indices

Table 2 Intra-observer variability in measurements of swimbladder length (L_S) and Swimbladder Degenerative Index (SDI), for the 71 investigated eels

	Observer 1 (FL)		Observer 2 (GF)		Observers 1 and 2	
	L_S (mm)	SDI	L_S (mm)	SDI	L_S (mm)	SDI
Mean ± SD[a]	54.67 ± 18.37	2.39 ± 1.65	55.06 ± 18.46	2.29 ± 1.74	54.87 ± 18.38	2.34 ± 1.69
Median ± 25th percentiles[b]	55.00 ± 39.00–69.00	2.00 ± 1.00–3.00	54.00 ± 39.00–71.00	2.00 ± 1.00–3.00	55.00 ± 39.00–69.00	2.00 ± 1.00–3.00
Average pairwise mean[c]	54.67	2.39	55.06	2.29	54.87	2.34
Average pairwise SD[d]	±2.52	±0.51	±2.76	±0.33	±2.64	±0.42
Average pairwise CV[e]	4.86%	28.82%	5.31%	19.12%	5.08%	23.97%

[a] Mean and measure of dispersion over the two sets of repeated measures ($n = 142$ observations for observer 1, $n = 142$ for observer 2, $n = 284$ for observers 1 and 2).
[b] Median and measure of dispersion over the two sets of repeated measures ($n = 142$ observations for observer 1, $n = 142$ for observer 2, $n = 284$ for observers 1 and 2).
[c] Average of the within-observer means, i.e. between the two repeated measures per eel ($n = 71$ pairs of observations for observer 1, $n = 71$ for observer 2, $n = 142$ for observers 1 and 2).
[d] Average of the within-observer standard deviations, i.e. between the two repeated measures per eel ($n = 71$ pairs of observations for observer 1, $n = 71$ for observer 2, $n = 142$ for observers 1 and 2).
[e] Average of the within-observer coefficients of variations, i.e. between the two repeated measures per eel ($n = 71$ pairs of observations for observer 1, $n = 71$ for observer 2, $n = 142$ for observers 1 and 2).

tended to increase for severely degraded swimbladders.

Objectivity. The inter-observer variability over the two sets of independent measures on each of the 71 eels is given in Table 3 for the SDI and the L_S. The SDI showed a much higher coefficient of variation between the two observers (33.38% vs. 4.23%, combined data for the two sets of observations, $n = 142$) which may reflect a higher subjectivity in scoring the observed pathological signs. Again, if setting a CV on L_T of similar amplitude as for L_S (i.e. 4.23%) and applying the approximating formula $CV(X/Y) = [CV(X)^2 + CV(Y)^2]^{1/2}$, then the overall CV for the LRI would be about 6%, still very much smaller than the 33% obtained for the SDI. For the linear measure of the swimbladder (L_S), the parameter of variability significantly co-varied with the index value, whether measured by the LRI ($Rs = -0.20$, $n = 142$, $P = 0.02$) or by the SDI ($Rs = +0.33$, $n = 142$, $P < 0.001$), which would indicate that the subjectivity over the swimbladder limit was higher for severely degraded swimbladders. In contrast, the inter-observer variabilities in the SDI appeared relatively independent of the observed pathologies (mean vs. SD for SDI: $Rs = -0.03$, $n = 142$, $P = 0.73$; for LRI: $Rs = -0.1$, $n = 142$, $P = 0.94$). In detail, however, the subjectivity was maximal for intermediate SDI values and minimal for extreme scores (i.e. better objectivity for slightly and severely degraded swimbladders).

Easiness. The individual time necessary for assessing the SDI ranged from 6.17 to 37.46 s for a mean value of 15.49 ± 5.68 s ($n = 284$, median: 14.30 ± 11.48–18.10 s). The necessary time for taking the length of the swimbladder (L_S) ranged from 7.40 to 71.74 s for a mean value of 22.67 ± 7.70 s ($n = 284$, median: 21.48 ± 18.12–26.70 s). Both measures of time strongly deviated from normality (Shapiro–Wilk tests: $W < 0.94$, $P < 0.001$). No significant difference was observed between the duration of the first and the second measurements when measuring L_S (Wilcoxon matched-pair tests: $T = 1120.00$, $n = 71$, $P = 0.37$, for observer 1; for observer 2: $T = 1124.50$, $n = 71$, $P = 0.38$). When considering the SDI, a significant difference was revealed for one of the observers (observer 1: $T = 674.00$, $n = 71$, $P < 0.001$; observer 2: $T = 1086.00$, $n = 71$, $P = 0.27$), but not in taking a shorter

Table 3 Inter-observer variability in measurements of swimbladder length (L_S) and Swimbladder Degenerative Index (SDI), for the 71 investigated eels

	Observation 1		Observation 2		Observations 1 and 2	
	L_S (mm)	SDI	L_S (mm)	SDI	L_S (mm)	SDI
Mean ± SD[a]	55.34 ± 18.59	2.27 ± 1.68	54.39 ± 18.23	2.40 ± 1.71	54.87 ± 18.38	2.33 ± 1.69
Median ± 25th percentiles[b]	56.00 ± 40.00–70.00	2.00 ± 1.00–3.00	54.00 ± 39.00–68.00	2.00 ± 1.00–3.00	55.00 ± 39.00–69.00	2.00 ± 1.00–3.00
Average pairwise mean[c]	55.34	2.27	54.39	2.40	54.87	2.33
Average pairwise SD[d]	±2.19	±0.55	±2.19	±0.47	±2.19	±0.51
Average pairwise CV[e]	4.39%	32.04%	4.07%	34.72%	4.23%	33.38%

[a] Mean and measure of dispersion over the two observers ($n = 142$ observations for the first set of measures, $n = 142$ for the second set, $n = 284$ for the two sets of measures).
[b] Median and measure of dispersion over the two observers ($n = 142$ observations for the first set of measures, $n = 142$ for the second set, $n = 284$ for the two sets of measures).
[c] Average of the between-observer means, i.e. between the two independent measures per eel ($n = 71$ pairs of observations for the first set of measures, $n = 71$ for the second set, $n = 142$ for the two sets of measures).
[d] Average of the between-observer standard deviations, i.e. between the two independent measures per eel ($n = 71$ pairs of observations for the first set of measures, $n = 71$ for the second set, $n = 142$ for the two sets of measures).
[e] Average of the between-observer coefficients of variations, i.e. between the two independent measures per eel ($n = 71$ pairs of observations for the first set of measures, $n = 71$ for the second set, $n = 142$ for the two sets of measures).

time for the second measurement (median time: 16.04 ± 11.89–18.35 s for observation 1 vs. 18.64 ± 13.66–23.35 s for observation 2) which could have indicated a non-independence between the two sets of measurements (e.g. imprinting of the first score). No significant difference was observed between the time needed by the two observers when measuring L_S (observation 1: $T = 1078.50$, $n = 71$, $P = 0.25$; observation 2: $T = 993.50$, $n = 71$, $P = 0.10$). When scoring the SDI, a significant difference in time was consistently revealed between the two observers (observation 1: $T = 869.00$, $n = 71$, $P < 0.05$; observation 2: $T = 286.00$, $n = 71$, $P < 0.001$), but not because of a shorter time for the observer (FL) who was the most familiar with that index (median time over the two set of observations: 16.56 ± 12.95–21.97 s for observer 1 vs. 12.90 ± 10.78–15.44 s for observer 2). Overall, when comparing the time needed for each of the indices (average of the four individual measures per eel, $n = 71$), a Wilcoxon matched-pair test revealed that the SDI was significantly quicker to compute (median time: 15.51 ± 13.64–17.36 s for the SDI vs. 22.02 ± 18.78–25.24 s for L_S; $T = 60.00$, $P < 0.001$). Since the computation of the LRI also requires a measure of the total length of the eel (L_T), the SDI is obviously faster to generate. There was no significant correlation between the necessary time for assessing the SDI and the severity of the corresponding damage (SDI time vs. SDI score: $Rs = -0.20$, $n = 71$, $P = 0.09$; SDI time vs. LRI value: $Rs = +0.09$, $n = 71$, $P = 0.46$). Similarly, no significant correlation was revealed between the time needed for taking the swimbladder length (L_S) and the severity of the pathological damage, whether assessed by the LRI or the SDI (L_S time vs. LRI value: $Rs = -0.21$, $n = 71$, $P = 0.08$; L_S time vs. SDI score: $Rs = +0.05$, $n = 71$, $P = 0.66$). Although none of the above correlations were statistically significant they tend to indicate that the relationship between the scoring time and the swimbladder degradation differed for the two indices; for the SDI the more severe the degradation the shorter the time needed to attribute an index value, whereas for the L_S the more severe the pathologies the longer the time needed to measure the organ.

Discussion

This work aimed to compare the performance of two alternative approaches for measuring eel swimbladder degradation caused by the nematode *A. crassus*. Since the present study was co-written by the main authors of the two prevailing indices (FL and AJC on one side, APP and CS on the other), it represents a joint collaborative effort to critically evaluate the tools at hand, and to produce consensual but clear-cut recommendations for end-users.

Scoring the SDI requires having a visual imprinting of the appearance of a healthy organ on the one hand, and of an extremely damaged swimbladder on the other (that would score, respectively, 0 and 2 in each histopathological criteria). For that purpose, illustrations of normal-looking and degraded swimbladders are provided in Fig. 5, or can be found on the internet, for instance, at the following URL: http://cbetm.univ-perp.fr/pages/francais/biblio/publis/THESE-Fazio.pdf Fazio, 2007, fig. 23, p. 50). Assessing the SDI may also require previous training in order to assess the different severity grades it may involve. In the present investigation, however, one of the observers (GF) was not familiar with the SDI scoring (in comparison to FL) but reported very similar SDI values (overall mean: 2.29 vs. 2.39; median value of 2 in both cases), in similar (or even shorter) times. In fact, the difficulties inherent in any categorization process were here partly overcome by the use of three clearly defined criteria, each scored separately with only three possible values. Moreover, because the auto-correlation between the three criteria proved to be strong but not total ($+0.50 \leq Rs \leq +0.84$, $P < 0.001$), the use of multiple criteria encapsulates additive information whilst partial redundancy tends to inflate extreme values, and so the discrimination power of the index. As designed, the SDI is quite meaningful as a degradation index because it is directly based on the severity of observed pathologies, and easy to work out because it is bounded within constant, predefined, upper and lower limits.

Nonetheless, the computation of the SDI involves making categories instead of reporting observed metrics of underlying continuous variables, and so is intuitively more prone to subjectivity. Subjectivity (or inter-observer variability) was one the main criticisms of the SDI (EELREP 2005). We have shown here that the subjectivity in scoring the SDI was indeed much higher than the subjectivity in properly defining the limit of the swimbladder for the LRI (coefficient of variation: 33% vs. 4%, respectively).

Also, categorization can affect the magnitude and the outcome of statistical tests (by changing for

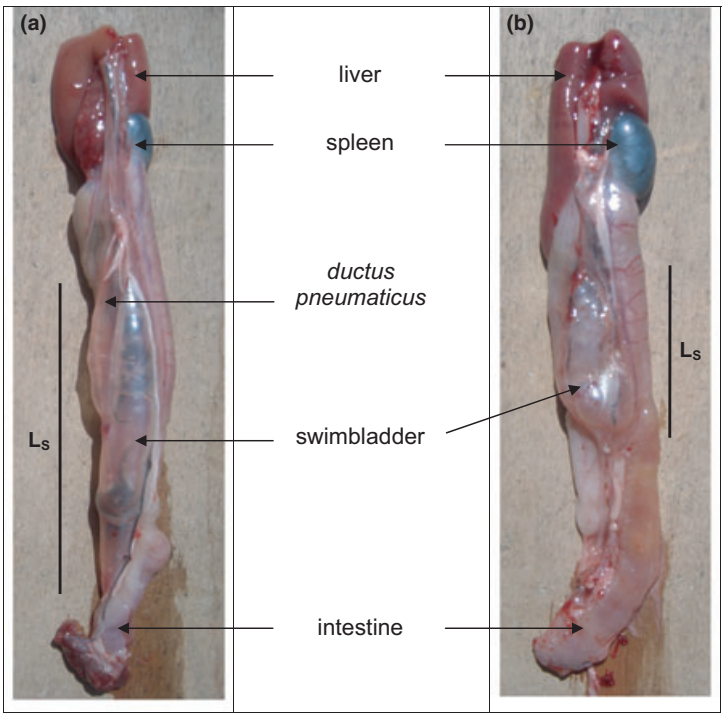

Figure 5 Ventral views of excised swimbladders. (a) Healthy organ with nine parasites (390 mm long male: SDI = 1, L_s = 58 mm, LRI = 0.15). (b) Degraded organ with no parasites (406 mm long female: SDI = 6, L_s = 40 mm, LRI = 0.10). In (b), because of multiple infection events in the past, the swimbladder has become a compact ball of necrotic tissues, with no visible *ductus pneumaticus*, thus offering little space for further parasite establishment.

instance the response distribution, see Bollen & Barb 1981; Lemon 2009), and the cruder the categories, the greater the discrepancy between the obtained score and the true value. As the SDI is entirely based on the pathological status of the swimbladder (and not a weighted-based index as for the LRI), it comes with inherent statistical problems such as the non-normality of the response distribution (e.g. asymmetry with regard to small and large values). For example, in a sample of eels all intensively affected by the parasite, then the distribution of the SDI scores would tend to cluster around the upper limit value of 6, which may require the use of specific but limited (i.e. non-parametric) tests to study the SDI variable in relation to other host or environmental parameters.

We have shown here that swimbladder length increased almost linearly with body length, indicating that the swimbladder follows an isometric growth with the rest of the body. This is a crucial verification in the absence of which the LRI values could not be comparable between eel size classes. Under this assumption, a small LRI value indicates a shortened swimbladder (as a result of infection) relative to, but independent, of the size of the eel.

The LRI is intuitively a meaningful and easy measure because it is based on the ratio of two linear, metric dimensions (swimbladder length divided by total eel length). In practice, the LRI performed better in comparison to the SDI with regard to the predefined criteria of representativeness, objectivity and precision. In contrast, and quite surprisingly, the LRI proved to take longer to generate than the SDI. This is mainly due to the difficulties in properly extending the swimbladder for the linear measurement and assessing the physical limit of the organ (embedded in connective tissues), especially for severely degraded swimbladders. In our experience, this can be partly overcome by placing the organ on absorbant paper so that the swimbladder sticks to the support and remains extended for the time of the measurement.

The main advantage and most interesting property of the LRI lies in the fact it can be computed using radio-diagnostic images so that the histopathological status of the swimbladder (and eventually the presence of swimbladder worms) can be assessed without causing damage to the fish (a non-invasive method). Indeed, Székely and co-workers repeatedly demonstrated that the diagnosis obtained by radiography on live-anaesthetized-eels showed good agreement with naked-eye examinations of the excised organ (Beregi *et al.* 1998; Székely, Molnár, Müller, Szabó, Romvári, Hancz & Bercsényi 2004; Székely, Molnár & Rácz 2005).

As with the SDI, the computation of the LRI (a weighted ratio of two correlated variables) comes with statistical and practical problems. It mainly revolves around the complex response distribution of a ratio (Creasy 1954), and the inherent difficulties in calculating a standard error or other measures of dispersion (Oyejola & Mead 1989). This was illustrated here by the need for Taylor's approximations to be able to generate a coefficient of variation around the measures of the LRI.

Another, more biologically orientated possible pitfall of the LRI, could concern the yet unclear interaction between the nematode infection and the growth of the eel. Indeed, in the event of an interaction with body growth, what would then be the rationale of using the eel length as a scaling factor for estimating the swimbladder degradation? So far, most studies on this aspect have not detected any significant impact, but some recent results tend to indicate that infected eels could actually grow faster (Fazio, Moné, Lecomte-Finiger & Sasal 2008), whilst others suggest that the infection may decrease fish growth (Liewes & Schaminee-Main 1987; Van Banning & Haenen 1990; Gollock, Kennedy, Quabius & Brown 2004). Obviously, the actual impact of the infection on the host life history traits remains to be better explored, but for now, in the absence of clearly demonstrated (and published) effect, it is possible to adhere to the position that the eel length is not, at least severely, affected by the nematode infection.

The two indices have both either actual or potential intrinsic limitations. Based on the results, however, we recommend the use of the LRI. For comparative purposes at least, we suggest using the historic index (SDI) with dead eels, but otherwise, and for obvious conservative reasons, to adopt the LRI in combination with radiographic methods. Indeed, the SDI cannot be estimated without dissecting the eel since the pathological criteria of opacity and thickness cannot be properly scored using radiography. The LRI is thus a new alternative tool in those situations where non-invasive methods are *de facto* needed (e.g. over-years monitoring). Also, a crucial question can be investigated using the LRI: can a damaged swimbladder recover in the absence of re-infection? Using radio-diagnostic methods, Székely *et al.* (2005) did not remark on any significant improvement after 3 months in the absence of re-infection, but longer term monitoring is needed.

Based on the obtained data, and to facilitate the use of the LRI, we provide a graph of correspondence between the scores of the two indices and the estimated loss in swimbladder volume (see Fig. 4). Thus, it is now possible to state that, for instance, a SDI score of 3 approximately corresponds to a LRI value of 0.12, and that significant gas loss starts to occur at about SDI = 2 or LRI = 0.13.

For both indices, we noted that the elapsed time between autopsy and swimbladder assessment tended to overestimate the actual damage (increased opacity, thickness and overall shrinkage of the organ). Thus, with dead eels, we strongly recommend measuring the swimbladder length, or alternatively to assess the SDI, immediately after the autopsy of the fish (storage in open air, in closed tubes or in tap water was inefficient).

More works would be welcome to precisely assess the factors that may interact with the swimbladder measures. For instance, the silvering process induces morphological, physiological and histological changes in the swimbladder (Durif, Van Ginneken, Dufour, Müller & Elie 2009; see also Kleckner 1980 for *A. rostrata* and Yamada, Zhang, Okamura, Tanara, Horie, Mikawa, Utoh & Oka 2001 for *A. japonica*), but what is the effect on the SDI and LRI measurements? In our sample, silver eels had swimbladders significantly more degraded than the rest of the sample, and the difference was particularly marked in the SDI scores, but more work is clearly needed (especially taking into account the eel size effect). It would be valuable if research on silver eels could test the effect of silvering on the two indices.

In conclusion, there are now two alternative and workable tools to record gross infection-induced pathologies, and so to accurately monitor (in conjunction with classical epidemiological parasite counts), the parasite pressure suffered by individual eels. We hope this will ultimately help estimate the quality of future spawners and the net losses due to anguillicolosis.

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