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Evaluating the histological-based condition of wild collected larval fish: A synthetic approach applied to common sole (Solea solea)

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Abstract :

In the present study, the histological-based condition of common sole (Solea solea) larvae in the English Channel was evaluated to assess the relevance of experimentally-calibrated indices for wild collected specimens. Based on topographical observation of foregut, midgut, hindgut, liver and pancreas, each of the 202 larvae analysed was attributed one of the six grades of a condition index that was developed for sole in the nineties. In parallel, a synthetic table of 20 criteria historically used as condition indicators was created from an extensive review of the literature. These criteria were related to tissular integrity, cells height, nuclei, presence of prey and vacuolisation of the same five target organs. For each larva, each criterion was scored on a scale from one to three. Multiple correspondence analyses coupled to clustering and indicator values were used to explore the relationship between the condition index and criteria as well as to highlight which complementary information the latter could provide to the former. Results showed that some adjacent grades were related to the same set of scored criteria, suggesting that these grades are not as distinguishable in the field as they can be in controlled conditions. Some criteria like vacuoles in the enterocytes, size of the hepatocytes, amount of zymogen, stain of acini, presence of prey in the digestive tract and goblet cells in the foregut, were independent of the condition index and depicted more recent food intake than a true condition status. Conversely, criteria related to tissular integrity as well as presence of energetics reserves like vacuoles in the liver appeared more relevant to evaluate the condition status of fish larvae since their increasing scores were associated to different grades. It is concluded that the grading system obtained from experimental observations may not be adapted to reflect the different larval condition states that could be encountered in the field. A simplified grading system, based on three grades and a reduced set of relevant criteria is then proposed.

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Highlights

► Experimental condition index is not adapted to wild specimens. ► Criteria of tissular integrity were the most discriminant. ► Criteria related to recent feeding were not linked to condition. ► Sole larvae can be grouped into three grades of condition.

Keywords : Condition index, eastern English Channel, fish larvae, histology, Solea solea, starvation

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1. Introduction

Many hypotheses rely on the potential of survival during early life history of fish to explain recruitment variability (Houde, 2008; Somarakis et al., 2019). Fish larvae survival can be assessed using condition indices (Ferron & Leggett, 1994; Gisbert et al., 2008). Among the wide range of available techniques including nucleic acid- and lipid-based-indicators, histological-based measurements have been recognised as the most appropriate to provide a reliable index of the larval nutritional status (including starvation) and point-of-no-return (Ferron & Leggett, 1994; Suthers, 1998; Gisbert et al., 2008). This approach gives a visual indication of direct effects of starvation whereas others indices are considered as proxies of the condition. Although some factors such as diseases or parasitism can also lead to a bad condition (Houde, 1987), starvation remains the main cause of tissue degeneration in larvae (Ferron & Leggett, 1994). Hence, since food deprivation leads to an abnormal development and degeneration at the cellular and tissular scales (Gisbert et al., 2008), starvation level of various organs was used to evaluate the larval condition (Koubbi et al., 2007; Diaz et al., 2013), based on the examination and scoring of target tissues and cells. Moreover, degradation patterns have been recognised as to be species, size, stage and temperature independent (O'Connell, 1976; Theilacker, 1978; Kashuba & Matthews, 1984; Theilacker, 1986; Oozeki et al., 1989; Yúfera et al., 1993; McFadzen et al., 1997; Sieg, 1998) which is one of the main advantages of histology-based indices.

Estimation of the larval condition for wild specimens requires the development of an experimentally-calibrated condition index that should be able to reflect starvation-induced degradation pattern at the organs scale (O’Connell, 1976). This kind of indices is rare (Boulhic, 1991; Catalán et al., 2006; Koubbi et al., 2007) and could rely on a multiplicity of target organs and biomarkers. Target organs may include those directly associated with the digestive tract (guts, liver and pancreas) which respond quickly (within a day) to food deprivation. Other organs such as muscles, nervous system, notochord and chondrocytes are also used and show histological changes under long-term starvation (Khashuba & Matthews, 1984; Theilacker, 1986; O’Connel 1976; Diaz et al., 2013). For each organ, many criteria (e.g cell sizes, intercellular spaces, cytoplasm color) are also available and their use vary widely between studies. Once calibrated, the condition index potentially needs to be adapted to reflect the different larval condition states observable in the field. Indeed, some degradation levels of larval condition described from experiments might not be observed in the field because of non-linear and unaccounted for processes in controlled conditions, e.g. stochastic variability of food availability or higher predation rate on poor condition individuals (Leggett & Deblois, 1994)

The main objective of the present methodological study was then to assess the relevance of experimentally designed condition indices to describe and evaluate the larval condition of wild collected fish. Common sole (*S. solea*) in the English Channel was used as the model species since a condition index was experimentally developed by Boulhic (1991). This index relies on a subjective and qualitative appraisal of the degradation status of nutrition-related organs. This could lead to a high rate of observer-induced bias if relevant criteria are not priorly standardised in the observation protocol (O'Connell, 1976; McFadzen et al., 1997; Koubbi et al., 2007). A synthetic scoring table was then created from an extensive review in the literature of the most commonly used criteria. Only criteria depicting short-term response of organs associated to nutrition (guts, liver and pancreas) were considered since they are the most pertinent for wild collected fish (Diaz et al., 2013). This scoring table was confronted to the Boulhic index using multivariate analysis in order to see which complementary information could be provided by a more objective and semi-quantitative evaluation of the larval condition. Based on these results, we finally proposed a simplified quotation system more adapted for wild collected fish larvae and based on a reduced set of criteria.

2. Materials and methods

2.1 Fish larvae sampling, fixation and identification

Sole larvae were collected during three surveys that were conducted in the EEC in March, April and May 2017 along the French coast from the Bay of Seine to the Belgium coast (Figure 1). Larvae were sampled using a bongo net (60 cm diameter, Smith & Richardson, 1977) fitted with two nets of 500 µm mesh size. The bongo was deployed through a double-oblique tow between the surface and five meters above the seabed during at least 10 minutes at a speed of 2 knots. A sub-sample from the second net was fixed in Bouin Holland solution during 48 hours and then preserved in ethanol 70°. Sole larvae were sorted, identified and staged under a

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stereomicroscope according to Russell (1976), Munk, (2005) and Ryland (1966). The planktonic phase of sole in the English Channel lasts for about 4-6 weeks depending on water temperature (Russell, 1976). Time for hatching is 5-8 days. Stage 1 larvae correspond to the vitelline stage and were not considered in the present study since they are badly sampled by the sampling device. Yolk absorption which corresponds to the start of stage 2 (pre-flexion of the notochord) happens at 4-6 days post hatching between 9 and 13°C. Stage 3 corresponds to the flexion of the notochord and stage 4 to the migration of the left eye which starts 22 days post hatching. Stage 5 corresponds to the complete migration of the left eye on the right face). In total, 202 larvae were analysed.

Figure 1: Location of the sampling stations of sole larvae

2.2 Histological preparation

Standard histological techniques were adapted from Martoja & Martoja-Pierson (1967). Sole larvae were dehydrated in successive and progressively more concentrated alcohol baths (70°, 95°, 100°) of 10 minutes each (replicated three times). Larvae where then cleared in three xylol baths of 15 minutes each and then immersed in two successive paraffin baths of 60°C during two hours each, before being embedded in paraffin blocks. Larvae were fully sectioned. Sagittal sections of 7 µm thick slices were mounted, dewaxed and rehydrated by successive baths (xylol, ethanol 100°, 95°, 70°, distilled water) of 5 minutes, replicated three times. Finally, slides were stained with Groat's hematoxylin (2 minutes) and picro-indigo-carmine (15 seconds), then flushed three times in absolute ethanol baths and covered with a microscope cover glass.

2.3 Larval condition scoring

Several histological sections of liver, pancreas and gastrointestinal tract (foregut, midgut and hindgut) were examined using optical microscopy and the best quality ones were used.

A value of larval condition was attributed to each larva using the Boulhic's grading system (Boulhic, 1991). It consists in six grades of starvation which are determined from a qualitative assessment of several criteria. This grading system was already applied to several wild collected larval fish species (Grioche, 1998 for sole, Koubbi et al., 2007 for *P. antarctica* and Harlay, 2001 for flounder) and grades are defined as follows:

Grade° 6: Larva is in good health related to a good nutritional state. Reserves of glycogen and lipids in the form of vacuoles are visible in the liver hepatocytes. The acini of the pancreas are well structured with a large amount of zymogen. Intestinal cells are high.

Grade° 5: Larva is in good health. Organs are in good condition but the liver has fewer vacuoles indicating lower levels of glycogen. Vacuoles are present in the intestine.

Grade° 4: The beginning of starvation is noted. An organ is altered, often the posterior intestine. Few vacuoles are visible in the intestine.

Grade° 3: Advanced starving or food recovery. The digestive tract and the liver are altered, intercellular spaces can be observed, but cells show some signs of absorption. In case of refeeding after a starving phase, goblet cells are present in the oesophagus.

Grade° 2: Severe starvation. Organs such as the liver, pancreas and digestive tract are altered. Cellular cohesion is very weak. Hepatocytes are small.

Grade° 1: Larva is at the point of no return. It is an irreversible state leading to death. All organs are altered. The pancreas is no longer structured, there is no more zymogen, nor glycogen in the liver. Some nuclei are pycnotic.

The Boulhic's grading system was completed by a semi-quantitative approach based on a standardised table created from an extensive review of the most commonly used criteria (Table 1). It was based on the work done by O'Connell (1976) on *Engraulis mordax*, Theilacker (1986) on *Trachurus symmetricus*, Boulhic (1991) on *Solea solea*, Margulies (1993) on three species (*Euthynnus lineatus*, *Auxis spp* and *Scomberomorus sierra)*, Yúfera et al. (1993) on *Sparus aurata,* McFadzen et al. (1997) on *Sardina pilchardus*, Sieg (1998) on *Engraulis anchoita*, Catalán (2003) and Catalán et al. (2006) on *Dicentrarchus labrax* and *Sardina pilchardus,* Koubbi et al., (2007) on *Pleuragramma antarctica* and Diaz et al. (2013) on *Pagrus pagrus*. In total, 20 criteria were retained (table 1). For each criterion (except one), three modalities (semi-quantitative scores), reflecting the level of degradation for the different criteria, have been resumed from the literature. Each larva was attributed a modality for each criterion.

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2.4 Statistical analyses

Analyses were applied under the R software (R Core Team, 2018) and the threshold of significance for all analyses was set to 5%. Criteria/modalities with less than 5 observations were not considered for the numerical analyses.

A Multiple Correspondence Analysis (MCA) was used to confront Boulhic's grades to criteria/modalities. It was performed on a complete disjunctive matrix with organs-criteria-modalities in columns and individuals in rows. Boulhic's grades were used as supplementary variables. Euclidean distance was calculated on the coordinates of the criteria on the first two axes of the MCA and a clustering using the aggregation criterion of Ward was performed to identify groups of criteria/modalities. The number of clusters was determined using the "gap statistic" (Tibshirani et al., 2001). Indicator values (IndVals, Dufrêne and Legendre, 1997) were calculated to identify links between groups of criteria/modalities and Boulhic's grades. The IndVal of a "x" Boulhic's grade, in relation to a "y" group of criteria and associated modalities, is defined within each cluster as follows:

$$IndVal.xy = Specificity.xy * Fidelity.xy * 100$$

where Specificity.xy is the proportion of criteria plus modality of the group "y" with the grade "x", and Fidelity.xy is the proportion of the number of individuals of grade "x" that are in a "y" group of criteria. This method allows identifying the best clustering levels in a dendrogram for each "y". IndVals varied from 0 to 1, in accordance with the level of association between "y" and "x". IndVals were tested by a permutation test (999 permutations) to identify significant associations between groups of criteria/modalities and grades.

A chi-squared (Chi²) test was then applied to test for the dependency between criteria, recent feeding and Boulhic's grades. From this test, criteria related to the presence of prey in the gut and/or independent of Boulhic's grades were removed. In order to identify groups of individuals with common criteria modalities, a new clustering was performed on coordinates of the individuals on the first two axes of the new MCA (without criteria removed from the first step). Six groups of individuals were defined and proportions of each Boulhic's grade within these groups were calculated in order to assess the relevance of the six grades for wild collected fish larvae. IndVals were also calculated (here "y" were groups and "x" were criteria plus modality).

3. Results

The synthetic table of criteria and modalities for the five organs associated to nutrition (liver, pancreas, foregut, midgut and hindgut) obtained from the literature review is given in Table 1. Foregut had one criterion related to goblet cells. Midgut and hindgut had both the same four criteria related to cells heights, microvilli density of brush border, amount of vacuoles and tissue organisation. The presence of prey in the gut was also considered. Liver was composed of six criteria focusing on cells (i.e hepatocytes size, cytoplasm texture, nuclei appearance and placement) and tissue (i.e integrity and amount of vacuoles). For pancreas, three criteria regarding the amount of zymogen, nuclei position and tissue organisation, were considered. Each criterion, except acini stain, had three modalities. For all criteria (except presence of prey), modality 3 corresponded to a healthy state, modality 2 corresponded to an intermediate one and modality 1 corresponded to a degraded one. This resulted in 59 combinations of criteria/modalities.

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Table 1: Summary table built from the literature review describing modalities of each criterion for the different organs. Modalities for presence of prey are also indicated.

<table>
<tr><th rowspan="2">Organs</th><th colspan="3" rowspan="2">Criteria</th><th colspan="3">Modality</th></tr>
<tr><th>1 - (Degraded)</th><th>2 - (Intermediate)</th><th>3 - (Healthy)</th></tr>
<tr><td>Foregut</td><td colspan="3">Goblet cells (FO_GC)</td><td>Absent</td><td>Present</td><td>Numerous</td></tr>
<tr><td rowspan="4">Midgut</td><td colspan="3">Epithelial cells height (MI_CH)</td><td>Reduced by more than 50% or cuboid</td><td>Reduced between 25% and 50% or cuboid</td><td>Large and convoluted</td></tr>
<tr><td colspan="3">Brush border (MI_BB)</td><td>Loss of most microvilli</td><td>Moderate microvilli, some degenerations</td><td>Many microvilli, intact</td></tr>
<tr><td colspan="3">Vacuoles (MI_VA)</td><td>Absent</td><td>Reduced</td><td>Developed</td></tr>
<tr><td colspan="3">Tissue organisation (MI_TO)</td><td>Large separations</td><td>Some detachments</td><td>Attached cells</td></tr>
<tr><td rowspan="4">Hindgut</td><td colspan="3">Epithelial cells height (HI_CH)</td><td>Reduced by more than 50% or cuboid</td><td>Reduced between 25% and 50% or cuboid</td><td>Large and convoluted</td></tr>
<tr><td colspan="3">Brush border (HI_BB)</td><td>Loss of most microvilli</td><td>Moderate microvilli, some degenerations</td><td>Many microvilli, intact</td></tr>
<tr><td colspan="3">Vacuoles (HI_VA)</td><td>Absent</td><td>Reduced</td><td>Developed</td></tr>
<tr><td colspan="3">Tissue organisation (HI_TO)</td><td>Large separations</td><td>Some detachments</td><td>Attached cells</td></tr>
<tr><td rowspan="6">Liver</td><td rowspan="5">Hepatocytes</td><td colspan="2">Hepatocytes size (LI_HS)</td><td>Small or atrophied</td><td>Medium and distinct</td><td>Large and distinct</td></tr>
<tr><td colspan="2">Cytoplasm (LI_CY)</td><td>Hyaline, lack of texture</td><td>Granular and homogeneous</td><td>Many textures</td></tr>
<tr><td rowspan="2">Nucleus</td><td>Size or placement (LI_NS)</td><td>Small and pycnotic</td><td>Centrals, extended or indistinct nucleoli</td><td>Laterals, reduced and distinct nucleoli</td></tr>
<tr><td>Stain (LI_ST)</td><td>Dark</td><td>Dark and grainy stains</td><td>Distinct nucleoli, slightly granular</td></tr>
<tr><td colspan="2">Tissue organisation (LI_TO)</td><td>Disjunct cells, loss of lamellar structure</td><td>Some detachments</td><td>Attached cells</td></tr>
<tr><td colspan="3">Vacuoles (LI_VA)</td><td>Absent</td><td>Rare and scattered</td><td>Numerous and wide</td></tr>
<tr><td rowspan="2">Pancreas</td><td colspan="3">Zymogen (PA_ZY)</td><td>Very little or no</td><td>Moderate (granular)</td><td>Abundant (homogeneous), filled sinuses</td></tr>
<tr><td>Acini</td><td colspan="2">Stain (PA_AS)</td><td>Dark</td><td></td><td>Pale</td></tr>
</table>

		Nucleus **(PA_NU)**	Indistinct or pycnotic nuclei	Many indistinct nuclei	Distinct nuclei in basal position
	Tissue organisation **(PA_TO)**		Large separations, acini indistinct and unsymmetrical	Some detachments, acini weakly distinct, of irregular shape, not symmetrical	Contiguous, well-defined acini, circular in shape, and symmetrical
Prey	Prey presence **(PR)**		Absent	Present	Numerous (>2)

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3.1 Relation between Boulhic's grades and criteria

Results of the MCA are presented in Figure 2. Two criteria/modalities (absence of goblet cells; FO_GC.1 and small and pycnotic hepatocyte nucleus; LI_NS.1) had less than 5 observations and were removed from the analyses. Both axes explained 33% of the variation. There was a clear trend in the distribution of modalities along the first axis with most of modalities 1 on the right, modalities 2 in the middle and modalities 3 on the left. On the second axis, the majority of modalities 1 was on the lower part and separated from the others which were on the upper part. Boulhic's grades were also distributed along the first axis with grade 1 on the right side, grades 2, 3 and 4 in the middle and grades 5-6 on the left. Again, grade 1 was separated from the others along the second axis.

Figure 2: MCA of criteria and their modalities (1-3). The factorial plan of the first two axes is represented. Boulhic's grades were used as supplementary variables and are plotted at their barycentric position. Colors depict groups (A-D) obtained from the clustering analysis and association of the grades with these groups. Abbreviations are given in table 1.

Four groups of criteria were obtained from the clustering analysis (Figure 3). Group A gathered a mix of different modalities from 1 to 3 for different criteria including presence of prey, vacuoles in hindgut and midgut and goblet cells in foregut. This group was clearly close to the origin of the MCA (Figure 3), traducing independence, and was not significantly linked to any particular Boulhic's grade. Group B gathered the majority of modalities 1 for most of the criteria and was close on the MCA and significantly (IndVal = 0.39) linked to Boulhic's grade 1. Group C included most of modalities 2 and some modalities 1 (vacuoles and nucleus stain in liver; LI_VA and LI_ST, and zymogen and acini stain in pancreas; PA_ZY and PA_AS) and was associated with Boulhic's grades 2, 3 and 4 (Indvals = 0.40, 0.45 and 0.37 respectively). Finally, group D included only modalities 3 for most criteria (except those that were in group A) and was significantly associated with Boulhic's grades 5-6 (Indvals = 0.47 and 0.68).

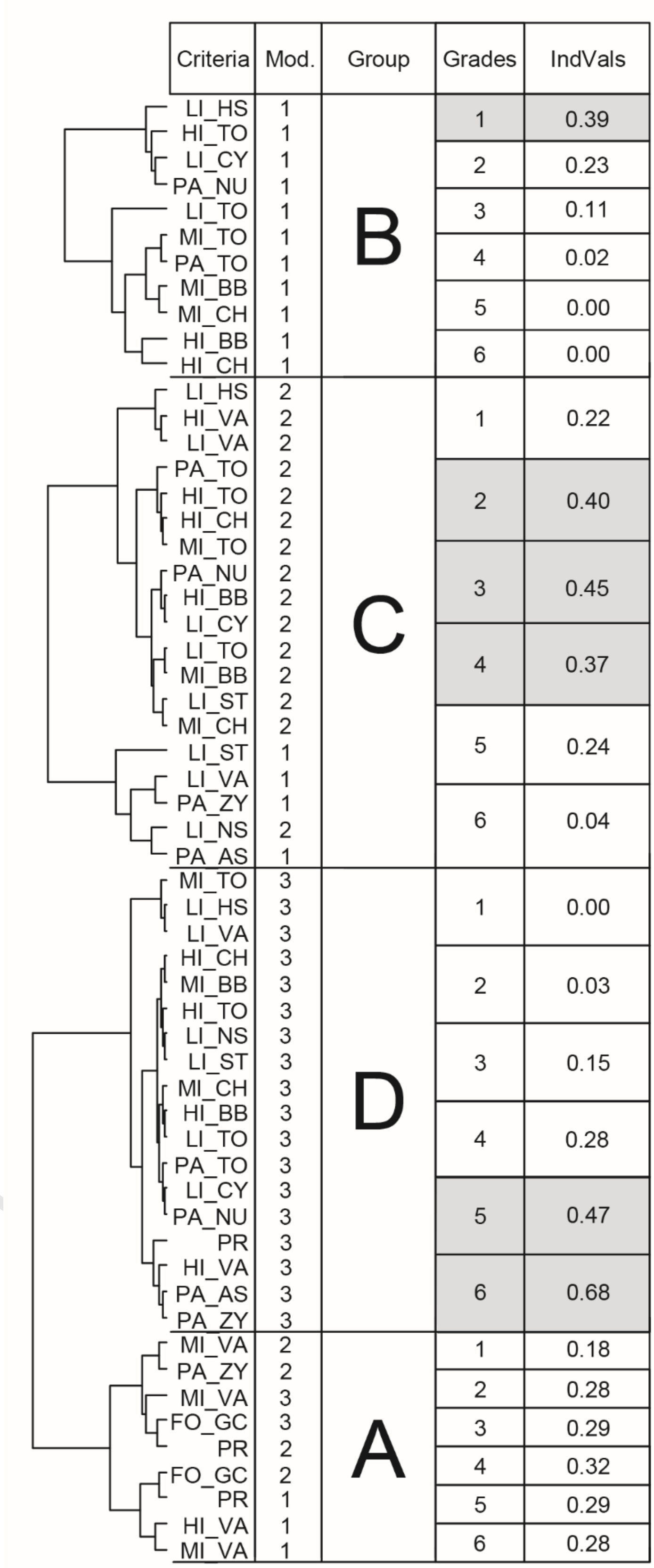

Figure 3: Groups of criteria with their modalities obtained from the clustering analysis. IndVals of the Boulhic's grades within each group are given and significant ones (p-value<0.05; 999 permutations) are indicated in grey cells.

Chi² test (Table 2) revealed that two criteria, namely goblet cells in the foregut (FO_GC) and vacuoles in the midgut (MI_VA) were independent from Boulhic's grades. On the contrary, there was dependence between the presence of prey (PR) and five criteria including vacuoles in the midgut and hindgut (MI_VA and HI_VA), hepatocytes size (LI_HS), amount of zymogen (PA_ZY) and stain of acinis (PA_AS). These six criteria were removed in the next analysis (see the discussion section).

Table 2: Chi² test of independence of criteria regarding Boulhic's grades and presence of prey. Grey cells indicate independence with grades and dependence with presence of prey.

Criteria	Chi² (p-value)	
	Grades	Prey presence
FO_GC	0.19	0.65
MI_CH	< 0.01	0.52
MI_BB	< 0.01	0.28
MI_VA	0.05	< 0.01
MI_TO	< 0.01	0.44
HI_CH	< 0.01	0.87
HI_BB	< 0.01	0.54
HI_VA	< 0.01	< 0.05
HI_TO	< 0.01	0.65
LI_HS	< 0.01	< 0.01
LI_CY	< 0.01	0.47
LI_NS	< 0.01	0.10
LI_ST	< 0.01	0.32
LI_TO	< 0.01	0.24
LI_VA	< 0.01	0.21
PA_ZY	< 0.01	< 0.05
PA_AS	< 0.01	< 0.01
PA_NU	< 0.01	0.89
PA_TO	< 0.01	0.30

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3.2 Individuals classification

Proportion of the six Boulhic's grades within each of the six groups (I - VI) obtained from the clustering of individuals based on their coordinates on the first two axes (28% and 13% of explained variation respectively) of the new MCA are represented in figure 4. Even if different grades were observed in the different groups, each of them was more represented in one or two groups. Group II gathered mainly grades 1 and 2 whereas group I was dominated by grade 3. Groups III and V were mostly dominated by grade 4, group IV by grade 5 and group VI by grade 6.

Figure 4: Proportions of the Boulhic's grades within the six groups of individuals obtained from the clustering.

Of the six groups obtained, only four (I, II, III and VI) were significantly associated with at least one criterion of the reduced set (Figure 5). Almost all criteria and modalities (except liver tissue organisation of modality 2; LI_TO.2 and hepatocytes vacuoles of modalities 1 and 2; LI_VA.1 and LI_VA.2) were significantly associated with a group. Group I was associated to only one criterion of modality 2 (size and placement of hepatocytes nucleus, LI_NS.2; IndVal = 0.22). Group II was characterised by all modalities 1 of eleven criteria (IndVals ranged from 0.3 to 0.8). Group III was significantly linked to modalities 2 of ten criteria (IndVals ranged from 0.25 to 0.33) and group VI to modalities 3 of 13 criteria (IndVals ranged from 0.35 to 0.48).

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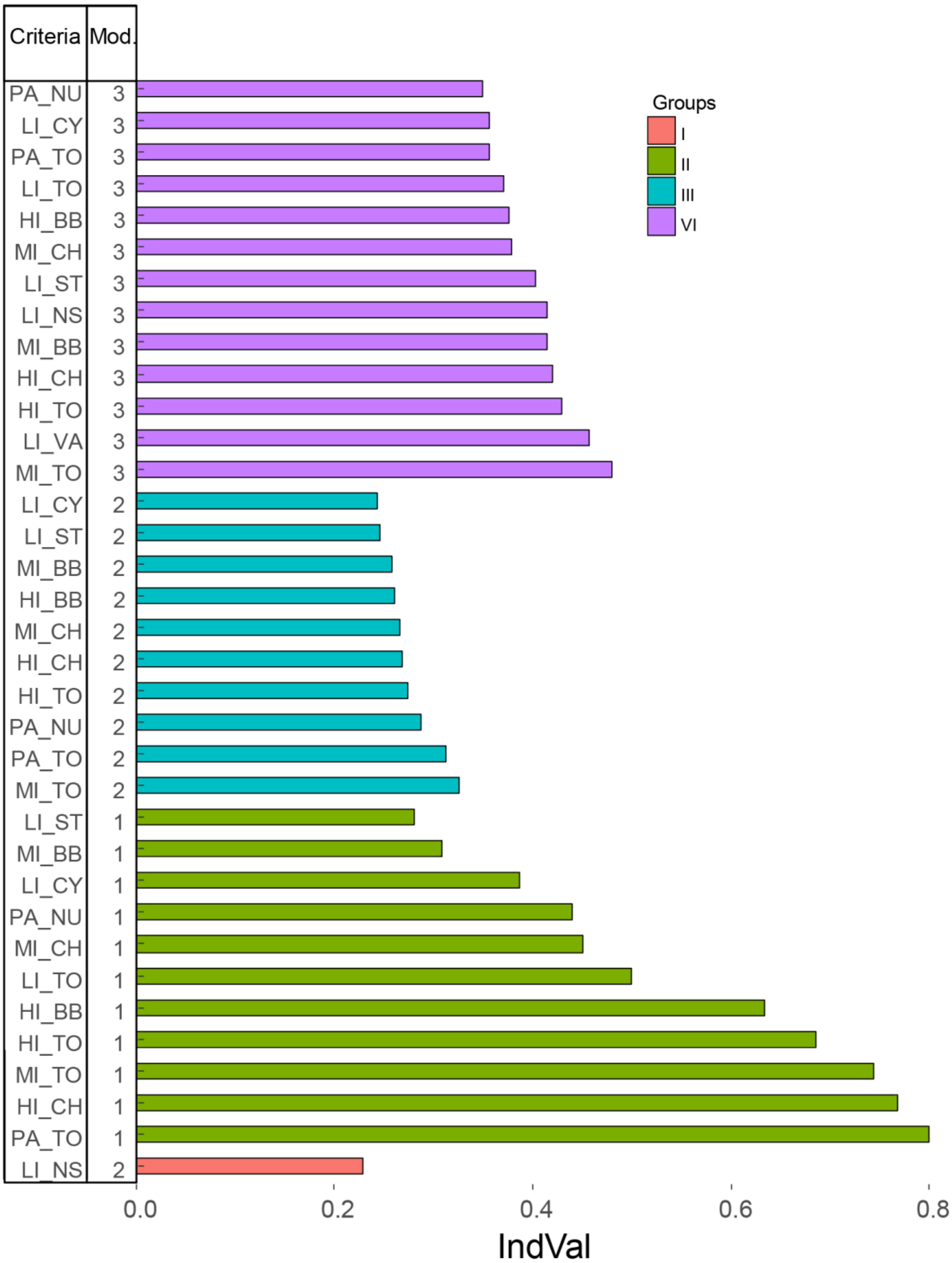

Figure 5: Association (only significant IndVals are represented, p<0.05, 999 permutations) between criteria and their modalities (Mod.) and the six groups of individuals obtained from the clustering. Groups IV and V were not associated with any particular criterion.

4. Discussion

Two approaches were tested to evaluate the nutritional condition of *in situ* caught sole larvae. The grading system developed by Boulhic (1991) from starvation experiments and used by Grioche (1998), Harlay (2001) and Koubbi et al. (2007), though coming from experimental studies, remains subjective and could lead to observer bias effect. As suggested by Koubbi et al. (2007), a semi-quantitative table of criteria was used to reduce this effect. The correspondence between Boulhic's grades and modalities highlights additional information that can be brought by this semi-quantitative approach. These aspects are discussed below.

4.1 Recent feeding and criteria of medium-term response

From our results, vacuoles in the enterocytes, size of the hepatocytes, amount of zymogen, color of acini, prey in the digestive tract and goblet cells in the foregut can be classified as very short-term condition criteria linked to recent feeding since many individuals with a low Boulhic's grade had a high modality for these criteria. On the contrary, criteria related to the tissular integrity and organisation as well as reserve indicators such as vacuoles in the liver appeared more relevant to assess the larval condition.

In the foregut, the amount of goblet cells was not related to presence of prey in the gut nor to the grade of starvation. It has been considered as a sign of recent feeding during starvation experiments (Boulhic, 1991) but Gisbert et al. (2004a) showed that the number of goblet cells is also size-dependent and increases during ontogeny.

In the midgut and hindgut, degradation pattern of enterocytes is well documented and was defined as some of the most accurate criteria (*e.g* Theilacker & Watanabe, 1989; Theilacker & Porter, 1995; Green & McCornick, 1999; Rodríguez et al., 2005). In the literature, vacuoles in the digestive tract were also considered as an indicator of good condition (Margulies, 1993 and references therein). In our results, the presence of vacuoles varied according to the gut region. In the midgut, it was not related to Boulhic's grades but to the presence of prey, whereas the number of vacuoles from the hindgut was related to the level of starvation and the presence of prey. Lipids are firstly absorbed during the digestive process mainly in the midgut in the form of lipid droplets whereas proteins are secondly absorbed in the hindgut in the form of acidophilic inclusions (Govoni et al., 1986; Deplano et al., 1991; Kjørsvik et al., 1991; Boulhic & Gabaudan, 1992; Yúfera et al., 1993; Sarasquete et al., 1995; Diaz et al., 1997; Olsen et al., 2000). The type and size of lipid inclusions in the midgut, also observed in the hindgut and rectal regions (García Hernández et al., 2001), are dietary-dependent as they increase with the fat content of food and the degree of unsaturation of ingested lipids (*i.e* prey quality, Gisbert et al., 2008). On the contrary, presence of vacuole can also indicate a poor condition since they can be used as a storage form of re-esterified fatty acids that cannot be metabolised by larvae (Kjørsvik et al., 1991) or they can reflect autophagy phenomenon (Segner et al., 1987). Moreover, hindgut enterocytes of starved larvae can display more abundant proteins inclusions than fed individuals (Catalán, 2003; Kjørsvik et al., 1991). Hence, the relevance of the amount of vacuoles in the gut to evaluate larval condition remains highly uncertain.

Almost all criteria related to liver (except hepatocytes size which was more related to recent feeding) were relevant to evaluate larval condition. Regarding vacuoles, their presence was used to discriminate healthy larvae with reserves (grade 6) from healthy larvae with no reserves (grade 5). Absence (modality 1) and scarcity (modality 2) appeared not relevant since their IndVal were not significant to discriminate groups of individuals (Figure 5). The liver is considered as being sensitive to starvation because of its role as energy reservoir. During digestion and assimilation, larvae store glycogen and neutral lipids in the form of vacuoles in the hepatocytes. This leads to an increase of hepatocytes size (Boulhic, 1991) and to a change in the position of the nucleus which becomes more peripheral. During starvation, reserves in the liver are the first ones to be mobilised (O'Connel, 1981; Segner et al., 1994) and smaller vacuoles lead to a large and central nucleus (Deplano et al., 1991). Proteins are used during more severe starvation once these reserves have been exhausted (Love, 1970). Vacuolation of modality 3 (numerous and wide) was found significant to discriminate individuals with reserves from those without reserves. Moreover, size and placement of nucleus (linked to vacuoles width) of modality 2 were the only significant criteria related to individuals of group I. These two criteria could be an indication of transition between larvae with no reserves and larvae starting to get starved. Indeed, a decreasing amount of vacuoles does not necessarily reflect a poor condition, but could be seen as a decrease in the potential of starvation without physiological consequences (*i.e* tissues degeneration).

Regarding pancreas, whereas tissular integrity was a good criterion to evaluate larval condition, zymogen amount and stain of acini were related both to starvation and recent feeding (i.e. poor amount of zymogen was found in larvae of poor condition or with numerous preys in the gut). Pancreas presents pyramidal acinar cells glands and acidophilic zymogen as pancreatic enzyme precursor. It has been recognised that starvation leads to degeneration of the exocrine pancreas (Crespo et al., 2001; Diaz et al., 2013) and variation in the amount of zymogen (Patt & Patt, 1969; Boulhic 1991). Several studies stated that under food deprivation, the enzymatic activity and amount of zymogen decrease (O'Connel, 1976; Boulhic, 1991) whereas others enunciated that accumulation of zymogen was observed in starved larvae (*e.g* Gisbert et al., 2004b; Margulies, 1993) due to a lack of feeding stimuli for its release (Pedersen et al., 1987; Yúfera et al., 1993). Stain of the acini was also a criterion related to recent feeding, probably due to the correlation with zymogen amount, i.e. a large amount of zymogen leads to a pale shade of acini. Hence, the amount of zymogen (and associated stain of the acini) should be considered more as an indicator of recent feeding than of the larval condition.

4.2 Condition assessment of wild collected larvae

Groups of individuals obtained from the organs-based scoring (criteria and modalities) were not homogeneous in terms of Boulhic's grades, i.e. a mix of different grades were found in each group. This suggests that the grading system of Boulhic might be too fine and not adapted for wild-collected larvae since it relies on observations from a continuous and/or linear kinetic of starvation. In the field, starvation is not linear because of stochastic larval feeding linked to spatio-temporal heterogeneity of food availability, i.e. starvation periods alternate with feeding periods. Observable response at the organ scale is hence more complex compared to what can be observed in controlled conditions. Also, fish larvae in poor condition might be rapidly caught by predators (Rosenthal & Alderdice,1976; Purcell et al., 1987; Leggett & Deblois, 1994; Hare & Cowen, 1997) because of lower buoyancy and mobility (Blaxter & Ehrlich, 1974; Sclafani et al., 2000). Moreover, larvae that do not feed sufficiently grow less rapidly (Garrido et al., 2015) which can increase their mortality as stated in the growth-mortality hypothesis by Anderson (1988) or by Miller et al. (1988) stating that "bigger is better". Faster growing individuals are less subject to predation since they have better swimming capacities and experience a shorter duration of the critical larval period (*e.g* Houde, 1997; Takasuka et al., 2007; Pepin, 2016). These phenomena result in some starvation grades, in particular those reflecting very poor condition, to be less observed in the wild compared to the others as the results of this study also indicate.

Very short-term response criteria linked to recent feeding can be a source of misinterpretation and should not be considered when one wants to attribute a condition index reflecting a more long-term response. Once these criteria were removed, three homogeneous groups of individuals characterised with the same level of modality were obtained. We then suggest that a quotation system based on three grades may be more appropriate to evaluate the larval condition of wild-collected specimens. The scoring table can then be simplified in order to keep only relevant criteria (as discussed above) like tissular integrity and organisation, height of the enterocytes and vacuoles in the liver. Criteria such as stain of cytoplasm or nucleus, even if significantly linked to condition in our results, remain more subject to observers' appreciation and resolution power of the observation devices. They can be observed secondarily as support for other criteria. The following quotation system is proposed and the simplified questionnaire (Figure 6) can be used to easily attribute a condition index. Correspondence of this grading system with this of Boulhic is shown in figure 7.

Grade 3: Fish larvae depict energetic reserves and the organs are well-structured. The key criterion is the presence of numerous vacuoles in the liver. Secondary criteria are linked to tissular integrity and organisation, i.e. enterocytes of hindgut and midgut are large and convoluted with good integrity and many microvilli, hepatocytes are attached and tissue organisation of the pancreas forms a cohesive whole, with well-defined acini (symmetrical circular shape). Majority of these criteria have a modality of 3. This grade encompasses majorly good condition grades 6 and 5 of Boulhic.

Grade 2: Fish larvae depict no sign of reserves (absence or few of vacuoles in the liver) and organs integrity has started to alter. Key criteria are related to height of enterocytes which are reduced in the hindgut and midgut with some detachments and moderate microvilli of the brush border. Pancreas can show some inter-cellular spaces with acini and nuclei weakly distinct. Majority of these criteria have a modality of 2 with a potential mix with modalities 1 or 3 for some of them. This grade encompasses majorly intermediate grades 3 and 4 of Boulhic.

Grade 1: Fish larvae are in a degraded state of severe starvation, all organs are deeply altered. Integrity of the epithelial cells of guts, brush border and mostly pancreas is strongly affected. Epithelial cells, especially for the hindgut, are very reduced or even cuboids. Acini's nuclei are indistinct and potentially pycnotic. Liver's cells are disjunctive leading to the loss of the lamellar structure. Majority of these criteria have a modality of 1 with a potential mix with modality 2 for some of them. This grade encompasses majorly poor condition grades 1 and 2 of Boulhic.

Figure 6: Simplified questionnaire used for the new grading system

The semi-quantitative character of this approach and the reduced set of criteria needed make it less subjective and more usable in a routine manner. For this purpose, it can be used to evaluate the larval condition of fish throughout ontogeny, but also in a spatial and multiannual context. Evaluation of the larval condition can be useful to identify critical periods during larval development (Di Pane et al., 2019). In a spatial context, the condition index could be mapped and related to environmental factors in order to identify suitable areas for larvae to develop and survive. In a multiannual context, larval condition might be used to study variations between years in the potential of larval survival. This is of particular interest to evaluate annual recruitment and year-class strength variations in fish populations from early-life stages (Hjort, 1914).

Figure 7: Correspondence (proportions of individuals) between the new grading system obtained from wild sole larvae and Boulhic's grades.

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Evaluating the histological-based condition of wild collected larval fish: a synthetic approach applied to common sole (*Solea solea*)

Di Pane, J.[1]; Gendrot, F.[1].; Giraldo, C.[1]; Marchal, P.[1]; Koubbi, P.[2], Loots, C.[1]

Highlights

- Experimental condition index is not adapted to wild specimens
- Criteria of tissular integrity were the most discriminant
- Criteria related to recent feeding were not linked to condition
- Sole larvae can be grouped into three grades of condition

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