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Modification of the feeding behaviour of sole (*Solea solea*) through the addition of a commercial flavour as an alternative to betaine

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Abstract

In aquaculture, a diet both nutritionally sound and organoleptically pleasing is essential to guarantee satisfactory intake. This has not been fully achieved with soleids (*Solea solea* and *Solea senegalensis*) because of their special palatability requirements, which are difficult to satisfy with conventional diets. Nevertheless, soleids have long been considered interesting species for aquaculture. To improve the palatability of diets for this family, various substances have been tried. Betaine has been the most effective substance by far, for all the species studied and especially for sole, reporting good results but at too high a cost. In other areas of animal production, the problems of palatability are solved with commercial flavours, which offer scope for specialisation to satisfy the most demanding organoleptic requirements. The aim of the present work is to evaluate the use of a bivalve commercial flavour as an alternative to betaine in sole diets. An ethological methodology, adapted to the feeding behaviour of sole, is developed to qualify and quantify behavioural changes when faced with experimental diets containing either this commercial flavour or betaine. Results show that the commercial flavour externally applied to feed particles performs efficiently as an *attractant* and as an *arrestant*, being able to attract the animal to the food source from some distance, provoke the ceasing of locomotion close to the food particle and promote initiation of feeding. In this step of the feeding sequence, it may be considered a viable alternative to betaine from the organoleptic point of view and economic cost. When the commercial flavour is applied internally to the feed, its performance as a *stimulant*, to encourage the continuation of feeding, is not distinguishable from betaine. Other factors such as time of feeding and size of fish, also assessed in the experiment, strongly influenced the level of acceptation of diets, even more than the composition of the diet itself. The distribution of feed at a time out of the endogenous rhythm of the species provoked a lower feed acceptation. Slight differences in the size of fish also affected the degree of acceptation, this being lower in the smaller fish.

Résumé

Modification du comportement alimentaire de la sole (*Solea solea*) par l'addition d'un arôme commercial en tant qu'alternative à la bétaïne. En aquaculture, il est essentiel qu'un aliment soit à la fois de bonne qualité nutritionnelle et agréable organoleptiquement pour garantir une alimentation satisfaisante. Cela n'a pas encore été réalisé avec les soléidés (*Solea solea* et *Solea senegalensis*) car leurs exigences particulières, en palatabilité, sont de satisfaire la plupart des exigences organoleptiques. Le but de ce travail est d'évaluer l'emploi d'arôme de bivalve provenant du commerce comme alternative à la bétaïne dans les aliments de la sole. Une méthodologie éthologique adaptée à son comportement alimentaire est difficile à satisfaire avec des aliments conventionnels. Néanmoins, les soléidés sont considérés comme des espèces intéressantes pour l'aquaculture. Pour améliorer la palatabilité des aliments destinés aux soles, différentes substances ont été testées. La bétaïne est de loin la substance la plus efficace pour toutes les espèces étudiées et pour la sole en particulier, donnant de bons résultats mais à un coût trop élevé. Dans d'autres sites d'aquaculture, les problèmes de palatabilité ont été résolus avec des arômes du commerce, qui offrent une gamme suffisante de spécialisation pour développer, pour évaluer de façon qualitative et quantitative les changements comportementaux des soles. Les résultats montrent que l'arôme commercial appliqué en surface des granules alimentaires provoque une attirance ainsi qu'une immobilisation des soles ; en effet, elles sont attirées par la source de nourriture à partir d'une certaine distance, cessent ensuite tout mouvement de locomotion près des particules alimentaires avant de s'alimenter. Lors de cette étape de l'alimentation, on peut considérer cet arôme comme une alternative viable à la bétaïne du point de vue organoleptique et économique. Lorsque l'arôme est incorporé à l'intérieur de

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l'aliment, il agit comme un stimulant et encourage la poursuite de la prise de nourriture, sans effet distinct de celui de la bétaïne. D'autres facteurs sont évalués, tels que l'heure de l'alimentation ou la taille du poisson, et influencent fortement le niveau d'acceptation des aliments et même davantage que la composition de l'aliment lui-même. La distribution de l'aliment lors d'une période hors du rythme endogène de l'espèce provoque une moindre acceptation de la nourriture. De légères différences dans la taille du poisson affectent aussi le degré d'acceptation qui est plus faible chez les poissons plus petits.

Keywords: Feeding behaviour; Commercial flavour; Betaine; Soleidae

1. Introduction

In aquaculture, as in any other animal production, a sufficient consumption of feed should be guaranteed to satisfy the objectives of production and profitability. More specifically, aquaculture systems need to maintain an optimum water quality, which can be achieved by reducing wasted feed. With this aim in mind, it is necessary to obtain a feed not only nutritionally sound and adapted to the species to be fed, but also pleasing for its organoleptic features, which will maximise its consumption. To design a sufficiently attractive feed, the study of the feeding behaviour of fish is a subject of major interest.

Soleids (*Solea solea* and *Solea senegalensis*), together with turbot and sea bass, have been considered apt for commercial aquaculture since the end of the 19th and the beginning of the 20th century (Person-Le-Ruyet, 1986). Although there still exist some constraints to full development, it remains a very interesting candidate to diversify the offer of cultured species (Howell, 1997; Flos et al., 1998; Dinis et al., 1999). Different commercial fish farms are growing sole from the juvenile stage up to the market size, but results can be improved. Today there are sole feeds available which ensure a relatively acceptable growth but at too high a cost. This is mainly due to the need to add expensive ingredients to satisfy the special requirements associated with sole feeding habits and behaviour.

The feeding behaviour of Soleids has been described in depth by various authors (Kruuk, 1963; de Groot, 1969, 1971; Mackie et al., 1980; Mackie, 1982; Appelbaum and Schemmel, 1983; Appelbaum et al., 1983; Batty and Hoytt, 1995) . Kruuk (1963) reported that in its natural habitat the sole remains inactive, buried in the sand the whole day and carries out all its activity, particularly feeding, during the night. To detect prey at night, the sole uses chemoreception (de Groot, 1969, 1971) but also possesses a well-developed mechanoreceptor system, rare even in other benthic species (Appelbaum et al., 1983; Appelbaum and Schemmel, 1983; Harvey, 1996).

Different authors agree in describing a trophic profile for *S. solea* mainly composed of polichaeta and molluscs (Reys, 1960; Braber and de Groot, 1973), as well as crustaceans (Ramos, 1981; Molinero and Flos, 1991, 1992). The trophic profile of *S. senegalensis* is very close to that of *S. solea* (García-Franquesa et al., 1996; García-Franquesa, 1996).

Due to the nocturnal habits and the feeding strategy of sole, it seems clear that its requirements will not be easily satisfied with the standard rearing methods. Moreover, fish is not present in the natural diet of sole, therefore it will be difficult to motivate it with conventional feeds formulated with fishmeal as a main ingredient.

The palatability problems of soles have been reported in several research papers. The addition of different substances, mainly betaine and L-aminoacids, improve the organoleptic features of feeds. Betaine has been, by far, the most effective substance for all the species studied, but especially for sole, either alone or in combination with other substances (Mackie et al., 1980; Cadena-Roa et al., 1982; Mackie, 1982; Cadena-Roa, 1983; Metailler et al., 1983). L-aminoacids are present in tissue of vertebrates and invertebrates (Mackie and Mitchell, 1985). While almost all marine invertebrates have a large amount of betaine, there is only a trace in teleost fish tissue (Love, 1980). This could explain the efficacy of betaine as a feeding stimulant for these species, considering their trophic profile. The best results in sole diets were reported with a dose of 4.4% of betaine, as part of a mixture of chemical feeding activators, which reach 7% of the diet (Cadena-Roa et al., 1982). Later on Metailler et al. (1983) reduced the economic cost of attractants by adding only betaine, glycine and inosine, with good results but still at too high a cost. When using betaine two facts should be considered, the first being the high price of the product. The second refers to the legislation concerning the use of additives because betaine is not included as such in the positive lists of additives, so it can only be incorporated into the fish feed under other names.

In other areas of animal production, there are also problems of palatability (medicated feeds, starting feeds), which are solved with commercial flavours. A *flavour* has been defined as a mixture of aromatic chemical substances, which is only used to give a particular odour or taste to any food, or to improve its odour or taste. This is an attainable solution, with a wide scope for specialisation, which can be tailored in order to satisfy the most demanding organoleptic requirements. This solution has been already tried in shrimp (Pittet et al., 1996) but there are no reports of its use in fish.

Thus, taking into account the advantages of the commercial flavours, i.e. price and suitability for adaptation to the requirements of different species, the aim of the present work is to evaluate the influence of a commercial flavour on the feeding behaviour of sole as an alternative to betaine. With this aim in mind, an ethological methodology specifically adapted to the feeding behaviour of sole is developed. This methodology should allow the evaluation and quantification of changes in the sole feeding behaviour when faced to different experimental feeds containing a commercial flavour or betaine and a feed with no organoleptic modification.

2. Materials and methods

2.1. Ethological methodology

The general criteria considered to develop an adequate ethological methodology for the aim of the trial were the following: (1) the type of behaviour (state and/or event) should be known and described, (2) the methodology should permit an independent evaluation of all phases of a behavioural sequence so as to monitor potential changes in motivation (Stradmeyer, 1989), (3) in order to compare and statistically analyse the results of an ethological methodology, the behaviour should be quantified by choosing a type of measurement; the most frequent measurements of states and events being total or partial frequency, rate and duration (Lehner, 1979), and (4) external and internal factors potentially affecting the results should be considered and controlled.

The method used was direct observation, which can be implemented inexpensively using a simple experimental layout. This technique has been previously used in diverse studies of feeding behaviour, both in fish (Stradmeyer, 1989; Brännäs and Alanärä, 1992), crustaceans (Pittet et al., 1996) and molluscs (Viana et al., 1994).

To describe the behavioural sequence of sole towards a feed, we used three different sources of information: First, the description of the feeding behaviour of sole as previously reported by different authors. Second, the classification of stimuli described by Mackie and Mitchell (1985), according to the sequence of chemoreception-mediated behaviour, after the definition of stimulus of Lindsted (1971) cited in Mackie et al. (1980) (Table 1). Third, our own information on the observation of captive sole during the weaning and maintenance period previous to the trials.

As a result of these considerations, a three-phase behavioural sequence was defined: (1) distant orientation (Do), (2) near orientation and initiation of intake (No), and (3) continuation of intake (Ci). In Table 2, positive (+), negative (–) and intermediate (o) reactions of individuals to a stimulus during each phase of the behavioural sequence were described. The main criteria to discriminate between a positive, negative or an intermediate response were the number of fish that developed a certain pattern of behaviour and the pace at which it is developed. The negative response includes both a nil response (no interest in the food) and aversive reactions.

The feeding rate was set in 2% and 1.9% of body weight per day for the smaller and the larger fish, respectively, according to water temperature and fish size. Feed was distributed in two meals, morning (08.30) and afternoon

Table 1
Classification of stimuli according to the response in chemoreception-mediated feeding behaviour (modified from Mackie and Mitchell, 1985)

Response	Stimulus	
	+	–
Orientation (distant)	Attractant (the animal responds orientating itself to the source of the stimulus, even at a certain distance)	Repellent (the animal responds leaving the area of the source of the stimulus)
Orientation (near)	Arrestant (provokes the cessation of the animal's locomotion close to the source of the stimulus)	Repellent (same as above)
Initiation of intake (biting and tasting)	Incitant (incites the animal to bite and taste the particles)	Suppressant (inhibits the initiation of intake)
Continuation of intake	Stimulant (promotes the continuation of intake for a certain period of time)	Deterrent (promotes the cessation of intake)

Table 2
Summary of the description of a positive (+), negative (–) and intermediate (o) response of the fish when facing a stimulus, in each phase of the behavioural sequence

Distant orientation (Do)	+	All the fish or most of them react when feed particles are thrown into the water. They react promptly.
	o	Some fish react, while the rest show no reaction towards the feed during the whole observation period (10 min).
	–	No fish or very few of them react. Only occasionally fear or escape reactions are observed.
Near orientation and initiation of intake (No)	+	All the fish or most of them try the feed and eat it. The reaction involves almost 100% of the population, and it is also rather fast.
	o	Most of the fish that have initially reacted try the feed and eat it but the behaviour is slower. Some of the fish, which were attracted from a distance, do not even try to taste the feed when they approach it, although they have the opportunity to do so. Usually those fish that did not react at the first phase, never tried to eat.
	–	None of the fish try to eat. In some cases, evident escape reactions are observed, showing clear displeasure.
Continuation of intake (Ci)	+	All the fish or most of them eat and keep doing so during the whole observation period. The feeding behaviour is evident and determined.
	o	Some fish—occasionally only a few—keep eating. There are frequent and prolonged interruptions in the feeding activity, which may be considered slow or very slow.
	–	None of the fish eat and if any try a feed particle clear rejections are observed including expelling of particles.

(16.00), following the feeding schedule stated after weaning. The time interval between the meals was sufficient to guarantee the emptying of the digestive tract. After the observation period, care was taken in order to detect and remove non-ingested particles of feed.

In the present experiment, 'normal' behaviour was assessed before fish were offered the experimental feeds. According to Macquart-Moulin et al. (1991) and Batty and Hoytt (1995), behaviour is 'normal' while soles are still at the bottom, concentrating in the darkest area of the tank. Two observers conducted evaluations, one of them acquainted with the feed type being administered, having prepared it beforehand, while the other had no previous knowledge of the feed. After the administration of the experimental diet, the two observers, working independently, closely watched the reaction of the fish for a 10 min period. Both recorded the reaction of fish for each phase and, still working separately, assigned a positive, negative or intermediate score according to the description given above (Table 2). This procedure was repeated for all experimental feeds. A careful schedule was set for the experimental period in order to obtain 50 repetitions for each of the six feeds, distributing them evenly between morning and afternoon meals. A commercial feed was given once a day to assess the 'normality' of the feeding behaviour of the sole.

At the end of the experimental period, for each step of the sequence (distant orientation, near orientation and initiation of intake, continuation of intake), results were expressed in terms of percentage of each type of response (positive, +, negative, –, intermediate, o). In this way, each feed obtained three values per phase and, therefore, a total of nine values in the whole feeding sequence.

2.2. Fish and facilities

A set of 120 soles (*Solea solea*) from a public hatchery was used. Even though they were coetaneous (200 d old), the size range among individuals was rather high. In order to reduce size heterogeneity, they were graded into two homogeneous groups taking the median weight (8.4 g) as the intermediate size, the larger group being ≥8.4 g, and the smaller <8.4 g.

Twenty fish were stocked in each of the six tanks, all small fish in three tanks (mean body weight in grams ± standard error were, respectively, 7.48 ± 0.12, 7.48 ± 0.13 and 7.64 ± 0.13 for tanks 1, 2 and 3) and all large fish in the other three (mean body weight in grams ± standard error were 9.35 ± 0.18, 9.19 ± 0.17 and 9.46 ± 0.22 for tanks 4, 5 and 6, respectively). The facilities used were in the Barcelona Zoo, with an open circuit of seawater at a temperature of ≅14 °C, with dissolved oxygen near saturation, salinity ≅36. The room was lightened with an intensity of 300–400 lux, using a natural autumn photoperiod (12 h).

2.3. Commercial flavour and application

A bivalve flavour was chosen for assessment in the present experiment. Using the legislation currently in force, this flavour may be defined as a mixture of natural-like chemical substances and is approved for human consumption.

According to the classification of stimulus by Mackie and Mitchell (1985), the mixture was required to perform as an attractant, an arrestant, an incitant and a stimulant: Attractant to guarantee a distant attraction of the animal to the food source. Arrestant to provoke the ceasing of locomotion close to the food particle. Incitant to promote the initiation of feeding. Finally, stimulant to encourage the continuation of feeding to satiation.

Two applications were developed to achieve this aim. One application was expected to behave as an attractant and arrestant, to be applied externally as a coating mixed with oil. The other application, expected to perform as an incitant and stimulant, was mixed with the constituents of the basal diet before pelleting. These two applications were obtained by changing the carrier of the flavour. Vegetable oil was used for the outer coating. Sole may reject fish oil due to its stronger organoleptic features.

2.4. Feeds

A basal diet containing 58% crude protein (N × 6.25, Kjeldahl method) and 18% fat (measured as ethereous extract by Soxhlet method) was formulated according to Cadena-Roa (1983) and Metailler (1990). It included fishmeal as the main ingredient, as well as soybean meal, wheat middlings and bran, brewers yeast, fish oil and a commercial turbot mineral–vitamin premix provided by Roche. Five feeds were obtained from this basal diet. One of them with betaine added and three with commercial flavour added, either outside and/or inside the feed particles as described in Table 3. In the nomenclature used, the first letter (upper case) refers to the internal treatment of the diet (A: flavour, B: betaine, O: none). The second letter (lower case) refers to the external treatment (a: flavour, o: none). Doses of commercial flavour, following the recommendation of the manufacturer, were 1.5% of the diet in the internal application and 0.5% in the external. There was 3% of betaine added to the basal diet to obtain one of the experimental feeds. This dose was chosen considering that Cadena-Roa (1983) reported 2.9% as the minimum dose that induces changes in the feeding behaviour of sole. The dosages applied also take into account the economic cost, which is an important factor for future utilisation at a production scale. The basal diet without any organoleptically active substance, i.e. betaine or flavour, was the fifth feed representing an 'internal' control. It was designated Oo, according to the above described nomenclature, and was used to test the efficacy of these diets regarding texture and composition.

The feeds were extruded at the University of Valencia to produce pellets of 1–2 mm diameter. Pellets were always coated with vegetable oil to equal the texture of all the

Table 3
Description of the experimental diets. The first letter, upper case, refers to the internal treatment and the second, lower case, to the external treatment or coating as follows: A/a flavour, B betaine and O/o none. C is the commercial diet or external control. Basal diet included fishmeal, soybean meal, wheat middlings and bran, brewers yeast, fish oil and a commercial turbot mineral–vitamin premix. Internal treatment is mixed with the consituents of the basal diet before pelleting. External treatment is mixed with vegetable oil and applied as coating after pelleting

Name of the diet	Type of diet	Internal treatment (identified with the first letter)	External treatment or coating (identified with the second letter)
Aa	Basal diet (58% crude protein, 18% ethereous extract)	Flavour (1.5%)	Flavour (0.5%)
Ao	Basal diet (58% crude protein, 18% ethereous extract)	Flavour (1.5%)	None
Oa	Basal diet (58% crude protein, 18% ethereous extract)	None	Flavour (0.5%)
Bo	Basal diet (58% crude protein, 18% ethereous extract)	Betaine (3%)	None
Oo	Basal diet (58% crude protein, 18% ethereous extract)	None	None
C	Commercial diet (55% crude protein, 14% ethereous extract)	Betaine + aminoacids (unknown proportions)	

experimental feeds and also to improve their stability in water.

Furthermore, a commercial feed for turbot containing 55% crude protein (Kjeldahl method) and 14% fat (Soxhlet method) was used as an 'external' control. This one was chosen to feed the fish after weaning and during the period previous to the trial. The fish accepted it and at the beginning of the trial, it was considered that soles were already adapted to it. Consequently, the reaction to this feed allowed evaluation of the 'normalcy' of the fish during the trial. It included betaine and aminoacids in unknown proportions and was designated C in the nomenclature of this work.

2.5. Analysis of data

The aim of the statistical analysis was to assess the reaction and the response of the fish to each diet, taking into account the influence of two factors, namely, daily time period of feed distribution and size of fish. The reaction observed in each phase was evaluated separately to assess the efficacy of the flavour as an attractant, an arrestant, an incitant and a stimulant.

With the nine values obtained for each diet, corresponding to the percentages of each type of response (+, –, o) in each of the three phases, two quantitative variables were defined. The Acceptation Index (AI) for each phase allows assessment of acceptation in each step of the feeding sequence. The Global Acceptation Index (GAI) is used to combine all those values and give a more comprehensive expression of the entire feeding response.

The AI for each phase was defined by assigning specific coefficients to each type of response, like weights, in the following way: $AI = [(a \times 1.0) + (b \times 0.5) + (c \times 0)] \times 100^{-1}$ where a is the percentage of positive responses, b that of intermediate responses and c that of negative or nil responses, obtaining a unique value per phase. In this way, a value of AI close to 1 means a very good response. The zero values are not excluded from the index because they are all of interest as they reveal the ethological meaning behind its formulation.

The GAI, obtained by adding up the three AIs corresponding to each phase, gives an overall evaluation of the response of the fish to each diet. Values close to three indicate very good responses.

An example is developed to show the procedure carried out to convert the observed responses into numerical values that will be statistically analysed. In the smaller fish group and in the morning meal, when the feed with flavour inside and flavour outside, i.e. Aa, was distributed, the following percentages of each type of response in each step were recorded:

Distant orientation			Near orientation and initiation of intake			Continuation of intake		
–	o	+	–	o	+	–	o	+
8	42	50	16	42	42	35	50	15

Using the definition of the variables AI and GAI, we have the following values:

Acceptation Index			Global Acceptation Index
Distant orientation	Near orientation and initiation of intake	Continuation of intake	
0.71	0.63	0.40	1.74

The values of AI for the three phases and GAI corresponding to each diet were statistically analysed ($\alpha = 0.05$) using the Statistics Analysis System software. The influence of the observers was assessed through a Homogeneity test applied to the set of data obtained by each observer. The Pearson product moment correlation coefficient was estimated in order to study the association between the responses registered according to phase. Normality tests were performed to assess the type of data distributions, as well as variance tests to perform hypothesis tests for equality or homogeneity of variances, in order to guarantee the use of parametric tests. None of these tests were significant, so General Linear Models were used to evaluate the influence of the different factors. Analysis of variance (ANOVA) tests were applied with time of feed distribution (morning and afternoon), size of fish (large and small) and diet (Bo, Aa, Oo, Ao, Oa, C) as main effects without interaction. When significant differences were observed a Tukey–Kramer test for multiple comparisons was performed (Montgomery, 1991).

3. Results

All results obtained, expressed as values of AI for each phase and GAI of the whole behavioural sequence, are shown in Table 4. The Homogeneity test applied to the values obtained by both observers do not find significant differences, so we consider that observers are not influencing the values of the recorded measurements. According to the test, both sets of data collected by the two observers will be analysed conjointly.

By means of the scatter plots used to represent data and with Pearson's correlation test, a high degree of interaction between the three phases is confirmed. The Pearson correlation coefficient obtained is 0.919 between distant (Do) and near orientation (No), and 0.795 between this (No) and continuation of intake (Ci). The positive correlations between steps suggest that what occurs in one step of the sequence influences the next one, i.e. a higher acceptation level in distant orientation will be associated to high acceptation levels in near orientation, and so on.

The mean and standard error of the AI obtained for both groups of fish separately (larger and smaller), in each of the phases of the feeding sequence (Do, No, Ci) and in both meals (morning and afternoon), without considering the feed factor, are shown in Fig. 1. The values of AI registered (Table 4) are always higher during the morning feeding, right after switching on the lights, than during the afternoon feeding, after 8 h of diurnal light. In the three steps of the feeding sequence, considering that one is the maximum score obtainable, the AI registered for the larger fish was as high as 0.96 (maximum value), while for the smaller fish, it was 0.75 (Table 4). Furthermore, the smaller fish are more affected by an adverse time of feeding and their responses at the afternoon meal are much poorer than those of the bigger fish (Fig. 1). For the GAI, without considering the feed factor, the values for the superior sized fish decrease about 11% between the morning and the afternoon feeding, while for the inferior sized fish the reduction between the two meals is around 29%.

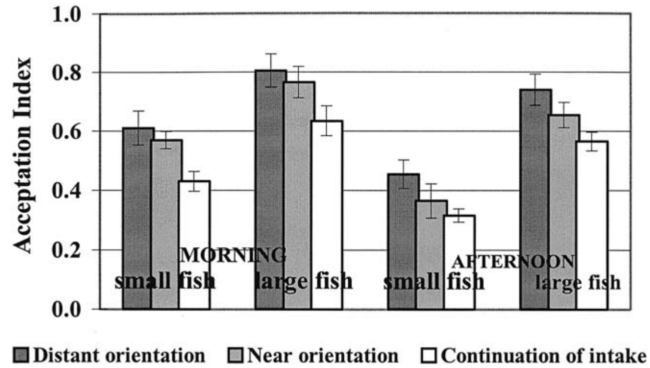

Fig. 1. AI expressed as mean and standard error obtained by different sizes of fish (large and small) and by the two administrations of feed (morning and afternoon) in each of the phases of the behavioural sequence (Do, No, Ci) considering all diets.

Fig. 2 shows the mean and standard error of the AI obtained for each of the feeds in the three steps of the feeding sequence. In this figure, some variations appear between the three phases, always maintaining the decreasing pattern in the acceptation level. For all the feeds, in the two first phases (distant orientation and near orientation and initiation of intake) the range of values is wider than in the continuation of intake. The GAI, which allows an overall evaluation of the reaction to a feed, is shown in Fig. 3 for each of the feeds in both groups of fish. This figure reveals the lower level of response of the small fish, regardless of the feed offered, in comparison to that of large fish. The maximum difference is observed in the feed with internal flavour only (Oa), in this case, the global level of acceptation (GAI) of larger fish is 67% higher than the GAI value of the smaller fish (Fig. 3).

Table 4
For each type of diet, values of AI and GAI are expressed, separating size of fish and time of feeding. The nomenclature of diets is Aa flavour inside and outside, Ao flavour inside only, Oa flavour outside only, Oo no flavour, no betaine, neither inside nor outside, and Bo betaine inside and C commercial diet

	Small fish (<8.4 g)				Large fish (≥8.4 g)			
	Acceptation Index			Global AI	Acceptation Index			Global AI
	Distant orientation	Near orientation	Continuation of intake		Distant orientation	Near orientation	Continuation of intake	
Morning (08.30)								
Aa	0.71	0.63	0.40	1.74	0.96	0.89	0.60	2.45
Ao	0.57	0.52	0.41	1.50	0.86	0.80	0.79	2.45
Oa	0.75	0.63	0.55	1.93	0.96	0.91	0.69	2.56
Oo	0.730.65	0.40	1.78	0.68	0.73	0.75	2.16	
Bo	0.44	0.50	0.50	1.44	0.74	0.69	0.51	1.94
C	0.45	0.50	0.32	1.27	0.65	0.57	0.49	1.71
Afternoon (16.00)								
Aa	0.51	0.52	0.36	1.39	0.86	0.79	0.59	2.24
Ao	0.63	0.51	0.28	1.42	0.76	0.62	0.54	1.92
Oa	0.49	0.22	0.28	0.99	0.91	0.75	0.67	2.33
Oo	0.43	0.31	1.19	0.72	0.59	0.58	1.89	
Bo	0.30	0.22	0.40	0.92	0.66	0.68	0.60	1.94
C	0.35	0.29	0.26	0.90	0.55	0.51	0.44	1.50

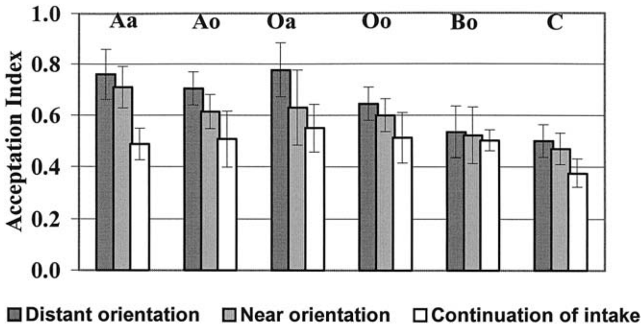

Fig. 2. AI expressed as mean and standard error, obtained by each diet in each of the phases of the behavioural sequence: Do being distant orientation, No near orientation and initiation of intake, and Ci continuation of intake. The first letter, in capitals, refers to to the internal treatment, while the second letter refers to the external treatment, A/a being commercial flavour, B betaine, O/o none and C commercial diet.

The ANOVA test used to evaluate the effect of the three factors indicates the existence of significant differences between both times of feeding and both sizes of fish, for the AI of each of the phases and for the GAI, at the level of $\alpha = 0.05$. The ANOVA test, used to assess only the effect of the diet, shows differences in the AI values for the two first phases of the sequence (Do and No) as well as in the global response (GAI), but not in the third phase or continuation of intake (Ci) with a *P*-value equal to 0.095.

The results of the Tukey–Kramer test, shown in Table 5, allow the comparison of the mean values of the AI for each diet in the three phases of the feeding sequence and in the whole sequence (GAI). When the differences in the test are significant, through the values achieved in each phase, the behaviour of each diet as an attractant, arrestant, incitant and stimulant is established. The feeds with flavour in the external coating (Oa, Aa) work more efficiently as an attractant than the feeds with betaine. The Aa feed (flavour inside and outside) is the most efficient as an arrestant and incitant, while no differences are found between all feeds when their efficiency as a stimulant is assessed.

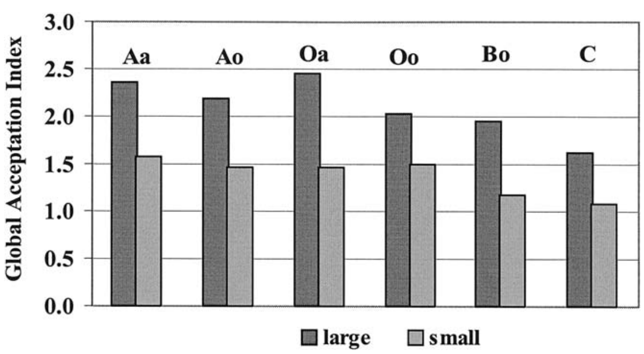

Fig. 3. GAI, expressed as means, obtained by each diet for each size of fish (small and large). Diet nomenclature: first letter, in capitals, is internal treatment, second letter external treatment, A/a being commercial flavour, B betaine, O/o none and C commercial diet.

4. Discussion

In the present trial, changes in the feeding behaviour of sole towards different organoleptic stimula have been assessed using a specifically designed ethological methodology, which has proved to be efficient to show differences in feeding behaviour. Moreover, the variables defined to measure those changes, i.e. AI and GAI, have been found useful to quantify the level of response and evaluate the changes statistically.

Although the time of feeding and the size of fish were not the main objectives of the study, they have shown a significant influence on the overall degree of response to various feeds, regardless of their organoleptic features. During the morning feeding, all feeds are better accepted than during the afternoon feeding. As care is taken to ensure that the digestive tract has been evacuated before the following meal, a worse acceptation of feeds after several hours of daylight could be more related to the nocturnal habits of sole. The magnitude of the difference between both feedings suggests that the nocturnal endogenous rhythm of the fish is still dominant over the artificial diurnal rhythm applied at the facility. These results are in agreement with those reported by Champalbert and Castelbon (1989) with juveniles and Kruuk (1963) with adults, which demonstrated only a slight alteration of the endogenous rhythm in sole exposed to an artificial photoperiod.

With reference to the fish size, it should be noted that the population is composed of coethaneous fish from the same spawn and with only a small difference in size. In spite of this slight difference, the smaller fish registered worse results than the bigger fish. It is not possible to infer from the present results whether the less active behaviour exhibited by the inferior sized fish is because they are smaller or they are smaller because they feed less actively and thus consume less feed. In any case, the application of a very precise classification routine would actually allow the optimisation of ingestion under culture conditions.

Table 5
Results of the Tukey–Kramer test are expressed for the mean values of AI for each phase and GAI, for each type of diet, identifying the internal and external treatment where A/a is flavour, B is betaine, O/o is none and C is commercial diet. In one row, there are no significant differences between values with the same letter in superscript $\alpha = 0.05$ level

Internal treatment	O	A	A	O	B	C
External treatment	a	a	o	o	o	
AI distant orientation (attractant)	0.777^{a}	0.760^{a}	$0.705^{a,b}$	$0.645^{a,b,c}$	$0.535^{b,c}$	0.500^{c}
AI near orientation and initiation of intake (arrestant and incitant)	$0.627^{a,b}$	0.707^{a}	$0.612^{a,b}$	$0.600^{a,b}$	$0.522^{a,b}$	0.467^{b}
AI continuation of intake (stimulant)	0.547^{a}	0.487^{a}	0.505^{a}	0.510^{a}	0.502^{a}	0.377^{a}
GAI	1.952^{a}	1.955^{a}	1.822^{a}	1.755^{a}	$1.560^{a,b}$	1.345^{b}

In relation to the three-phase feeding sequence defined for the present work, all the diets offered induced a similar decreasing pattern in the magnitude of the acceptation registered throughout the sequence: the AI registered in the first phase (distant orientation, Do) is always higher than that registered in the second phase (near orientation and initiation of intake, No). Following the same trend, the level of acceptation in the third phase (continuation of intake, Ci) is the lowest of the whole sequence (Figs. 1 and 2). This common tendency suggests that all the diets are more effective as an attractant, less as an arrestant and an incitant and even less as a stimulant. It could be a matter of texture, considering the low acceptability of dry diets by sole reported by various authors (Bromley, 1974; Cadena-Roa et al., 1982; Metailler et al., 1983). However, in general and according to Colgan (1986), fish feed more selectively and slowly as the meal proceeds. Therefore, the decreasing pattern observed in our trial in the AI throughout the sequence, could be in agreement with this idea. Further research focused on the initiation and continuation of intake in fish is needed to clarify this aspect.

Furthermore, a positive correlation between the three phases has been established through the experimental results. This fact is reasonable because the three phases have been defined as forming part of the same sequence. According to these results and the work of Colgan (1986), after an acceptance fish search more intensively in the immediate vicinity thus increasing the possibilities of showing a high level of acceptation in the next phase. For the feed manufacturer, this is an interesting connection because when a feed is efficient as an attractant, even though there is not a full guarantee of intake, the likelihood of complete feed consumption increases.

Looking at the results shown in Fig. 2, the effectiveness of each feed to maintain motivation throughout the behavioural sequence may be discussed. In the distant orientation phase (Do), the objective is to attract the fish from a certain distance. In this step, those feeds with external flavour are the most readily accepted, independently of the internal composition (Oa and Aa, where the lower case denotes external coating). Meanwhile, those including betaine are the least accepted, both the experimental feed formulated for this trial (Bo) and the commercial feed (C), which includes betaine in unknown proportions. The two feeds without external treatment (Ao, Oo) occupy an intermediate position, registering even better results than those with betaine. Therefore, the commercial flavour has shown to be a more efficient attractant than betaine.

In the phase of near orientation and initiation of intake (No), where the objective is to provoke the cessation of the fish locomotion close to the source of the stimulus and to incite it to bite and taste the particles, the differences are less marked. Only two diets show clear differences. The one with flavour both inside and outside (Aa) reports the best results, suggesting that the flavour works efficiently both as an arrestant and incitant. The commercial diet (C) registers the poorest level of acceptation.

In the continuation of intake phase (Ci), where the objective is to keep the fish eating, none of the diets offered stands out from the rest, which suggests that no particular one is a better stimulant than the others. The reason for that behaviour cannot be ascertained through the present results but, in any case, none of the chemical substances used is sufficiently stimulating to overcome the lack of motivation.

The information provided by the AI for each of the phases facilitates a study of the performance of flavour or betaine throughout the sequence, which will serve in the future to improve the presentation of the active substances (flavour, betaine) in feeds and thus optimise efficiency in the entire sequence. On the other hand, in order to fully answer the question posed by the initial objective, as to whether commercial flavour is a realistic alternative to betaine, an evaluation of the variable GAI is essential (Fig. 3). As already stated in Section 3, the GAI values obtained for the five diets specifically designed for this experiment (Oa, Aa, Ao, Oo, Bo) do not show a statistical difference between them. The commercial feed, which registered the worst results, is significantly different from the feed containing commercial flavour (Oa, Aa, Ao) and from the internal control (Oo), which does not include any organoleptically active substance. On the other hand, the commercial diet (C) does not show a significant difference from that with betaine (Bo). The feed Bo occupies an intermediate position in relation to the feeds with flavour and to the commercial feed containing betaine. This would suggest that both the dosage of betaine used and the way in which it is incorporated into the feed are important factors to be considered and require a more specific study. For instance, Mackie et al. (1980) and Metailler et al. (1983), incorporating very much higher doses than ours, obtained excellent results. The betaine doses used by those authors ranged between 4% and 7%, while the dose applied to our feed (Bo) was 3%. The economic cost of higher doses of betaine, which might not be affordable to a commercial fish farmer, should be taken into account.

Although the bivalve flavour is interesting to optimise the feed intake, the level of acceptation of the feed without any organoleptic modification (Oo) should be highlighted. Even assuming the need for improvement, this fact is encouraging for future sole production because it shows that this species can be fed on artificial commercial feeds mainly based on fishmeal, a fact that was doubted some years ago (Mackie et al., 1980).

The results obtained so far can be used as a reference for improvement of the flavour application. The external addition of flavour is revealed as an interesting instrument to improve the attraction of the feed (distant orientation, near orientation and initiation of intake). Considering that the dose applied externally is very low (0.5%), the economic cost is much less than that of betaine. Obviously the results here are limited to a bivalve flavour and to a specific dose. Further investigation into different dosages and types of flavours, considering their future application in production for diverse species and stages of lifecycle, could report more precise results.

In this work, the efficiency of the flavour to attract the fish to the feed, arrest them in the vicinity and incite them to taste it has been proved. But it would be interesting to find chemicals that result in rapid feed intake by inducing location and total consumption of feed, which is especially important to reduce leaching and feed losses. In order to guarantee a full intake of feed, further research is needed to formulate a flavour that would work better as a stimulant.

The results in the present work were obtained with sole, which has for long been considered a fastidious feeder on pelleted feeds including fish meal (Bromley, 1977; Mackie et al., 1980). Therefore, the use of flavours could be earnestly recommended for less exigent species that are interesting for aquaculture, in order to achieve higher feed profitability and environmentally sustainable production.

5. Conclusions

Various authors have chosen to use betaine to solve the palatability problems in sole, but the main drawback to this is its high economic cost as well as its legal status. In the present study, the replacement of betaine for a commercial flavour has shown to be a viable alternative from both organoleptic and economic points of view, which makes the future use of commercial flavours in sole feeds encouraging.

The ethological methodology and the variables (AI and GAI) developed for this study have proved to be efficient to assess the effectiveness of flavour in each step of the feeding sequence. Different combinations in the application of flavour (internal and external addition) have been tried to evaluate its efficacy when compared with betaine. The flavour, incorporated in the external coating of the particles, is a more efficient attractant than betaine for the feeding of sole and it is more economical. The internal addition of flavour (doses, way of incorporation, carrier) to stimulate feed intake could be improved, but, in general, the organoleptic modification applied to the feeds through the addition of a bivalve flavour improves their acceptation by sole.

The timing of feed administration is important since acceptation is better in all cases when feed is offered to the sole in early morning just when the day begins. In commercial production those schedules that adjust better to the endogenous cycle of the sole would be preferable. Taking into account the better results achieved by the larger fish, a strict classification routine would facilitate optimisation of feeding.

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