Aquat. Living Resour. 12 (2) (1999) 145-149 / © Ifremer/Cnrs/Inra/Ird/Cemagref/Elsevier, Paris

Note

Simple techniques for labelling prey and gut content analysis in short-term feeding experiments with fish larvae

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Received March 25, 1998; accepted February 24, 1999.

Abstract – Techniques were developed for labelling prey, and for gut content analysis in fish larvae. Labelling consisted of a short feeding period of the prey (rotifer and *Artemia* metanauplii) on black-drawing ink before supplying them to the larvae. Prey labelling was rapid and remained stable for at least 3 h when transferred into seawater. In addition, a method was tested to facilitate the observation of larval gut contents. The method, based on Dawson's technique, involved fixation, removal of lipids and clearance of turbot (*Psetta maxima*) larvae, which become transparent and ready for gut content analysis. This treatment did not interfere with the black ink label of the ingested prey. © Ifremer/Cnrs/Inra/Ird/Cemagref/Elsevier, Paris

Labelling technique / gut content / fish larvae / prey / turbot / *Psetta maxima*

1. INTRODUCTION

Gut content analysis is an important tool for feeding studies in fish larvae. Some studies involve the use of several types of prey that must be clearly distinguished at the time of gut content analysis [5, 10, 16, 18]. This is not a problem when prey are morphologically different (e.g. rotifers and copepods). However, some problems may arise when prey are only slightly different in size and morphology since digestion may affect both their size and shape.

In studies where the larvae are fed on a single prey type [12, 13, 19], it is convenient that the larvae taken for experimentation have already food in the gut, since it is known that feeding rate is affected by the degree of gut fullness [12, 15]. In this case, the observer must distinguish between prey ingested during and before the experiment. This may be achieved by labelling or staining the prey. In most cases, prey labelling is inconveniently long, e.g. 12–24 h [7, 11]. Microalgae [8, 15], radioactive compounds [17] or fluorescence techniques [2, 14] have also been used. Special optical equipment and/or complex analytical techniques are required for the latter. Labelling with microalgae and many colouring agents has the disadvantage that larvae must be analysed fresh since prey get discoloured after fixation or preserved larval tissues become opaque. Young larvae of some species are transparent and the identification of unlabelled prey in fresh or preserved larvae is feasible when digestion is not advanced [16]. However, tissues of older larvae are thicker and more pigmented and the direct observation of prey within the gut can be difficult or impossible.

This paper describes a rapid and simple technique for labelling prey with black drawing ink for short-term feeding experiments, and a clearance procedure for larval tissues in order to facilitate the observation of gut content in preserved turbot larvae with conventional optical equipment.

2. MATERIALS AND METHODS

2.1. Prey labelling

Rotifers (*Brachionus plicatilis*) and *Artemia* metanauplii were the prey used in the labelling experiments. Rotifers were fed on baker's yeast in mass culture tanks. *Artemia* cysts (Argentemia, grade I) were incubated and the newly hatched nauplii were maintained for 24 h at 28 °C under continuous lighting and strong aeration.

Black drawing ink (Rotring, Germany) was used for prey labelling. Two drops (100 µL approx.) of ink·L^{-1} of seawater were emulsified in 100 mL of aerated tap water and added to 5-L buckets gently aerated, containing 100 rotifers·mL^{-1} or 50 metanauplii·mL^{-1}. The following treatments were applied:

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For rotifers:

– Starvation and filtration (SF). The rotifers were taken from the culture tanks, filtered (53-μm mesh) to discard remaining food, transferred into clear seawater, starved for 6 h and labelled. Starvation allowed rotifers to empty their guts. Labelled rotifers were filtered again to discard the ink remaining in the water, washed and transferred to clear seawater.

– Filtration (F). The rotifers were treated as for SF treatment but not starved.

– Neither starvation nor filtration (NSF). The rotifers were taken from the culture tanks and put directly in buckets with culture tank water. After labelling, the rotifers were not filtered and directly taken with the remaining ink.

For *Artemia*:

– Filtration (F). The labelled metanauplii were filtered, washed, and transferred to clear seawater.

– No filtration (NF). The labelled metanauplii were not submitted to filtration and remained with the ink present in the water.

The number of labelled prey were determined and recorded in each bucket by observations through time under a stereoscopic microscope (Nikon SMZ-2C).

To determine the permanence of the ink in prey guts, samples were either directly taken from the vessels, or filtered to remove the remaining ink, and then transferred (5–10 prey·mL^{-1}) to 5-L buckets containing seawater without ink. The numbers of labelled prey through time were determined.

Preference of fish larvae for labelled or non-labelled prey was studied in turbot (*Psetta maxima*) larvae. The larvae were taken from rearing tanks at days 4, 6 and 8, transferred to 5-L buckets and fed simultaneously on ink-labelled (5 prey·mL^{-1}) and non-labelled (5 prey·mL^{-1}) rotifers. Samples of twenty larvae were taken 30 min later. The larvae were anaesthetised (MS-222) and the gut contents analysed under the microscope.

Experiments were performed at 18 °C in triplicate at different days.

2.2. Treatment of larvae for gut content analysis

Turbot larvae were reared up to day 11 post-hatching [6]. Larvae were transferred daily from the rearing tanks to three 10-L buckets (10 larvae·L^{-1}) at 18 °C and fed on labelled rotifers or *Artemia* metanauplii (10 prey·mL^{-1}). Samples of twenty larvae were taken 3 h after the addition of prey. The larvae were anaesthetised (MS-222) and preserved in Eppendorf tubes (6 % formaldehyde) for one week. Fixed larvae were treated with absolute ethanol (99–100 %) for 2 h to remove lipids and then cleared with 1 % KOH. The optimal clearing period was determined by direct observation of the larvae under the microscope. Gut contents were checked by microscopic observation.

3. RESULTS AND DISCUSSION

Two major problems may arise with the use of some staining methods. First, the time required for prey coloration is usually very long. Second, the resulting colour of the prey is not stable after larval fixation and the larvae must be analysed in vivo immediately after sampling. The labelling method proposed in this paper is efficient, rapid and flexible since larvae can be analysed fresh or fixed and analysed later without any negative effect on the stability of the prey label. Both rotifers and *Artemia* metanauplii were strongly labelled (*figures 1, 2*) after 10 and 30 min in drawing ink, respectively, with a labelling efficiency higher than 95 %. The ink inside the prey was observed as a big black spot (rotifers) or a large black rod (metanauplii) in the gut.

In treatments NSF and SF, more than 96 % of the rotifers remained labelled for at least 8 h (*figure 3a*). However, half of the F rotifers, which were filtered after labelling, lost their black label after 3 h. This period is considerably longer than in rotifers fed on microalgae. In fact, it has been reported that rotifers fed on microalgae emptied their gut in less than 40 min [9]. Furthermore, the loss of the ink-label can be largely delayed by adding small quantities of ink to the

Figure 1. Ink labelled rotifer (*Brachionus plicatilis*); a: spots of black ink.

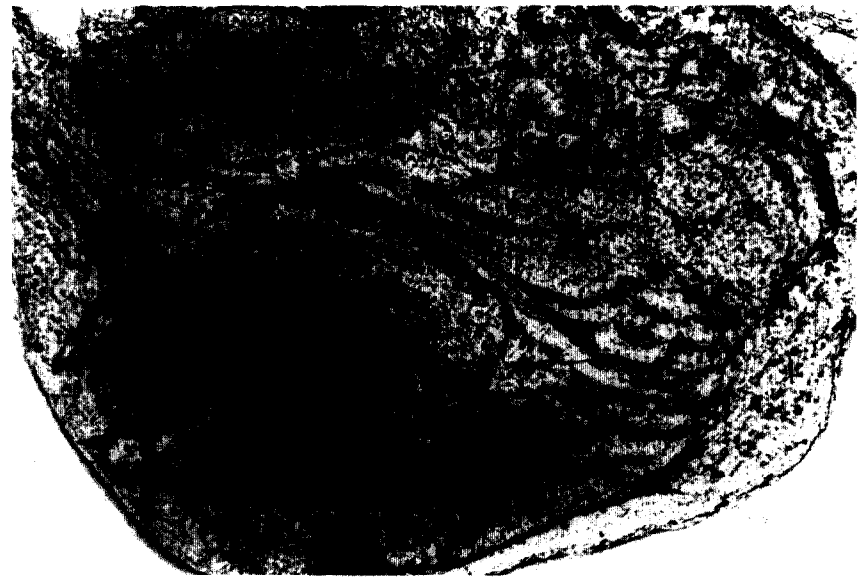

Figure 2. Labelled and non-labelled *Artemia* metanauplii (A) ingested by turbot larvae; a: ribbons of black ink.

experimental buckets. We found it to be non-toxic for turbot larvae for at least the following 48 h. In fact, larvae maintained in the buckets for 2 d in the presence of labelled prey displayed a normal behaviour and full guts. It is also interesting to note that the presence of both ink and baker's yeast in the gut of the rotifers (treatment F) accelerated the evacuation of ink. Therefore, treatments SF and, particularly NSF, were considered more adequate for labelling maintenance.

Artemia metanauplii remained coloured for at least 3 h when directly transferred (treatment NF) from the colouring tanks into seawater without ink (*figure 3b*). During this period the percentage of labelled prey was not significantly different from 100 % ($P < 0.05$). The labelling of the metanauplii that were filtered (F) declined after 2 h in seawater. In *Artemia*, it is very important to ascertain that all individuals are at the same developmental stage (stage III or later). The presence of less developed individuals would decrease the labelling efficiency, since the mouth is not open in early stages [1].

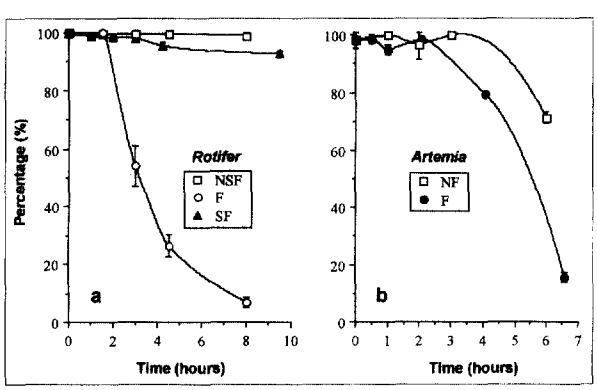

Figure 3. Change in the percentage of labelled prey: a, *Brachionus plicatilis*; b, *Artemia* metanauplii after transfer into seawater without ink. Data are mean ± SD of three to five samples.

Larval preference for prey colour should be considered in feeding experiments involving the use of dyed prey. Feeding of turbot larvae was not affected by the addition of ink labelled rotifers (*table I*). The same results were found in *Clarias gariepinus* larvae fed on coloured *Artemia* [11]. However, Dover sole (*Solea solea*) larvae improved their feeding when using stained *Artemia* [7]. The contradiction of these results could be explained by taking into account the light intensity and the colour of the tanks. These variables are of great importance in prey detection [3, 7] since they change the contrast existing between the prey and the environment. The advantage of using the proposed technique is that labelling affects only the gut and not the whole body of prey.

Another important topic in feeding studies is the simplicity and effectiveness of gut content analysis. Our method of larval clearing is based on Dawson's technique [4], which was modified and simplified for rapid observations. The clearing procedure with KOH was effective in turbot larvae aged from 3 to 11 d post-hatching (*table II, figure 4*). Larvae smaller than 5-mm standard length were directly mounted in groups of 10–20 on microscope slides and covered with large coverslips. Because of their larger volume, older larvae had to be dissected and the guts mounted separately. For these larvae, the optimal clearing time was about 8 d. With longer clearance times, these larvae became gelatinous making gut dissection difficult. Fat removal and clearing treatments did not affect the ink inside labelled prey.

Fixation in alcohol, used in Dawson's technique, is not recommended since it renders tissues much softer

Table I. Gut content (large rotifers·larva^{-1}) in turbot larvae fed on ink-labelled and non-labelled rotifers (*Brachionus plicatilis*) for 30 min. Data for each day are mean ± SD of two sets of twenty pooled larvae each.

	Day 4	Day 6	Day 8
Labelled rotifers	1.13 ± 0.62	1.37 ± 0.16	1.41 ± 0.05
Non-labelled rotifers	1.25 ± 0.25	1.20 ± 0.28	1.30 ± 0.28
Total rotifers	2.38	2.57	2.71
P-level (ANOVA)	0.700 n.s.	0.461 n.s.	0.792 n.s.

n.s., Non-significant.

Table II. Optimal clearance times for turbot larvae of different sizes.

Larvae		Days in 1 % KOH
Age (d)	Length (mm)	
3	3.8	5–7
5	4.3	8–10
7	4.8	10–11
9	5.3	8[1]
11	6.0	8[1]

[1] Guts were dissected and mounted separately for content analysis.

than when fixed in formalin, especially if the samples must be stored for a long time. The times given in the present paper as adequate for each chemical treatment

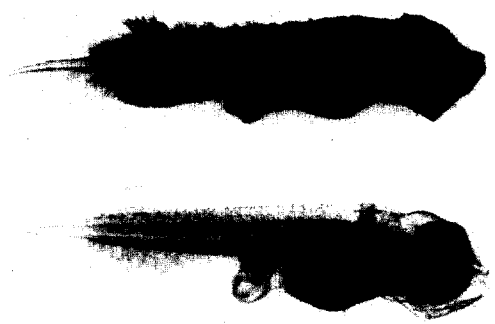

Figure 4. Six-d-old turbot larvae. Upper: larva fixed in formaldehyde. Lower: larva fixed and treated with ethanol and 1 % KOH.

with ethanol and KOH are only guidelines that should be modified for other species depending on size, thickness and other characteristics.

In conclusion, the procedures reported in this paper are very efficient tools for the study of short-term larval feeding since gut contents can be very easily observed both in fresh and preserved larvae.

Acknowledgements

This study was financially supported by the 'Comisión Interministerial de Ciencia y Tecnología' (CICYT, Spain) under Project AGF 185/92. I. Cunha was supported by a postgraduate scholarship from the JNICT (Portugal).

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