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Gut fluorescence technique to quantify pigment feeding in Downs herring larvae

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ABSTRACT: The ingestion of chlorophyll pigments (chlorophyll *a* and phaeopigments) by Downs herring *Clupea harengus* larvae (8–13 mm) collected in the English Channel and the North Sea during winter 2014 (International Bottom Trawl Survey) was quantitatively estimated via gut fluorescence analysis, a method classically used for copepods. Our results confirmed the consumption of chlorophyll pigments either directly, or indirectly through the consumption of herbivorous copepods. Higher mean pigment ingestion rates were observed for small larvae (8–11 mm, 52 ± 51 [SD] ng chl *a* eq. ind.$^{-1}$ d^{-1}), whereas lower mean rates (43 ± 48 ng chl *a* eq. ind.$^{-1}$ d^{-1}) were measured in larger larvae (12–13 mm). This decrease in the rate of pigment ingestion coincided with an ontogenetic shift in prey preference that occurred at 12–13 mm. Chlorophyll pigment ingestion covered on average up to 18% of the daily metabolic needs of 8–13 mm herring larvae and thus constitutes a non-negligible part of the larval diet mainly during the first feeding stages. Direct ingestion of autotrophic protist prey primarily involved small larvae (8–11 mm, 73 ± 38 to 84 ± 34%), as it can synergistically increase digestion efficiency of other prey items (e.g. copepods). The gut fluorescence method applied to fish larvae provides a global estimate of total ingested pigments and a snapshot of their diets. As a fast and easy method, it should be deployed in future surveys to compare food intake (ingestion) in different areas, and to quantitatively assess the nutritional status of fish larvae in the field.

KEY WORDS: *Clupea harengus* · Gut content analyses · Fish larval feeding · Chlorophyll pigment ingestion · Copepod · Daily metabolic needs

INTRODUCTION

Variability in fish recruitment can be explained by a combination of physical (e.g. ocean circulation) and biological processes (e.g. predation or poor feeding conditions; Nunn et al. 2012, Peck & Hufnagl 2012) acting on early life survival, and impacting year-class strength in many fish stocks (Houde & Schekter 1980, Pecuchet et al. 2015). During the 'critical period' (sensu Hjort 1914), when fish larvae shift from endogenous to exogenous nutrition, poor feeding conditions can lead to higher mortality rates due to reduced growth, i.e. increased larval stage duration, and higher vulnerability to predation (Houde 2008, Robert et al. 2014, Wilson et al. 2018). Survival of fish larvae therefore depends on their ability to acquire,

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ingest and assimilate sufficient amounts of appropriate food to avoid starvation and to ensure high growth rates (Blaxter 1965, Checkley 1982, Pryor & Epifanio 1993).

In the Eastern English Channel (EEC) and Southern Bight of the North Sea (SBNS), Downs herring constitutes a spawning component (sub-population) of North Sea herring *Clupea harengus* which reproduces in winter (Maucorps 1969, Corten 1986, 2013, Heath 1993). After hatching, released Downs herring larvae experience severe winter trophic conditions, with less food being available from the distant spring bloom period. This clear example of mismatch between plankton blooms and larval production (Cushing 1969) often leads to starvation and is worsened by trophic competition with other species (Corten 2013, Kellnreitner et al. 2013). The particular sensibility of Downs herring larvae to hydrology (Nash & Dickey-Collas 2005, Gröger et al. 2010, Hufnagl et al. 2015), their vulnerability to predation (Lynam et al. 2005, Torniainen & Lehtiniemi 2008) and disease/parasitism (Lusseau et al. 2014) are additional parameters to account for in recruitment failure.

Although fish larvae are thought to be exclusively carnivorous, feeding only on copepodites and naupliar stages of copepods (Munk & Kiørboe 1985), an increasing number of studies (Vallet et al. 2011, Arula et al. 2012, Denis et al. 2016) has underlined significant contributions of auto- and hetero/mixotrophic protists (diatoms, aloricate and loricate ciliates, and dinoflagellates) to larval feeding. However, many of these studies were qualitative and considered prey composition and occurrence from visual analyses of gut contents. The few quantitative estimates regarding feeding are based on dedicated predator/prey incubations (e.g. Lessard et al. 1996, Nagano et al. 2000, Friedenberg et al. 2012) or co-occurrence, combining field and modelling studies (Bils et al. 2017) and emphasize the need to quantify the contribution of total plankton (i.e. phyto- and zooplankton) to fish larval diets under natural environmental conditions.

Since the 1970s, zooplankton herbivory has been estimated by the gut fluorescence method (Mackas & Bohrer 1976). The methodology was a breakthrough compared to standard procedures (e.g. predator/prey incubation; Frost 1972, Roman & Rublee 1980), as it was fast and easy to set up and could be deployed *in situ,* thus being suitable for organisms distributed at depth. Although potential issues exist regarding gut pigment destruction (Conover et al. 1986, Durbin & Campbell 2007) and gut evacuation rate estimates (Perissinotto & Pakhomov 1996, Irigoien et al. 2008), it is still a widely used method adaptable to a great variety of planktonic (copepods, salps and krill; Pakhomov et al. 1996, Perissinotto & Pakhomov 1998, López et al. 2007) and benthic organisms (Díaz et al. 2012, Gaonkar & Anil 2012). However, to our knowledge the method has only been applied twice in feeding studies of fish larvae (Otake et al. 1990, Conway et al. 1996).

In a previous study, we demonstrated by a qualitative approach that autotrophic protists (i.e. phytoplankton) significantly contributed to Downs herring larval diet, particularly regarding the first feeding stages (Denis 2016, Denis et al. 2016). In the present study, the gut fluorescence method was used to quantify the contribution of chlorophyll pigments to Downs herring larval diet. Gut fluorescence contents were compared to those of dominant suspension feeders (potential prey of Downs herring larvae) in the area, i.e. copepods (*Calanus* spp., *Euterpina acutifrons*, *Oncaea* spp., *Paracalanus parvus*, *Pseudocalanus elongatus* and *Temora longicornis*) and are discussed with regard to the daily metabolic needs of herring larvae.

MATERIALS AND METHODS

Sampling

Hydrological parameters (seawater temperature and salinity, chlorophyll concentrations), mesozooplankton and fish larvae were collected at 14 sampling stations from mid-January to mid-February 2014, during the French part of the International Bottom Trawl Survey (IBTS), in the EEC and SBNS (Fig. 1). Sampling strategy and methods are detailed in Denis et al. (2016, 2017). Briefly, seawater temperature and salinity were continuously measured at 3–5 m depth using an SBE 21 SeaCAT thermosalinograph. Seawater samples for chlorophyll concentration estimations were collected in Niskin bottles deployed at 1 m depth. Mesozooplankton samples were collected using a double WP2 net (Tranter & Smith 1996) hauled obliquely through the water column at 0.75 m s^{-1}. Mesozooplankton from the first net was preserved in buffered formalin seawater solution (Mastail & Battaglia 1978, modified by Lelièvre et al. 2012) for later identification via ZooScan. The content of the second net was deposited on cellulose filters (Whatman Shark Skin) and frozen in liquid nitrogen for gut content analyses. Fish larvae were sampled at night using a mid-water ring net (ICES 2015). After sieving over 500 µm mesh net, herring

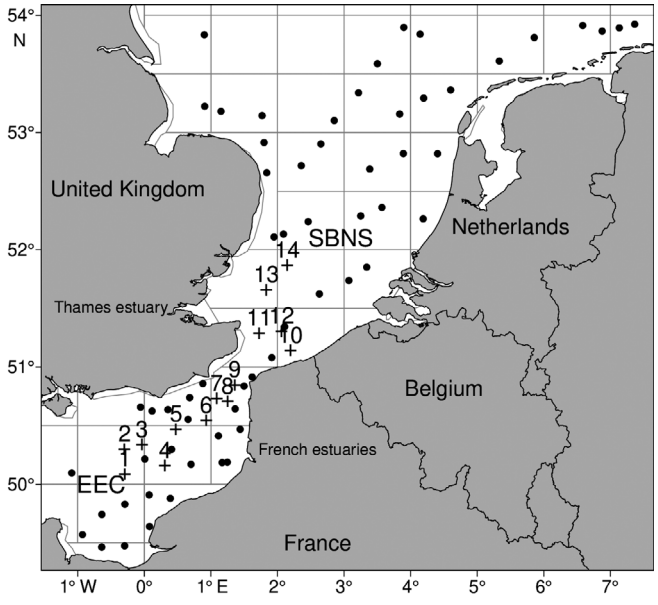

Fig. 1. Sampling locations (dots) of hydro-biological parameters, mesozooplankton and herring larvae during the International Bottom Trawl Survey (IBTS) in the Eastern English Channel (EEC) and the Southern Bight of the North Sea (SBNS) in winter 2014 (January–February). Crosses are stations where larval herring and copepod samples were collected for gut content analyses. ICES statistical rectangles are depicted

larvae were visually sorted, and only Downs herring larvae, i.e. those located south of 54° N (ICES 2015), were considered in this study. Sub-samples (1 per station) of 30 larvae were frozen in liquid nitrogen for gut fluorescence analyses, and the remaining samples were preserved in buffered formalin seawater solution for abundance estimates and visual analyses of gut content by stereomicroscopy.

Chlorophyll concentration, copepods and larval fish abundances

Detailed methodology for quantification of chlorophyll concentration, copepods and larval fish abundance can be found in Denis et al. (2016, 2017). Briefly, *in situ* chlorophyll *a* (chl *a*) and phaeopigment concentrations (T_{pig}, µg l^{-1}) were estimated from duplicate seawater samples (0.5 to 1 l), filtered onto glass-fibre filters (Whatman GF/C) and frozen at –20°C, using the spectrochromatic monochromatic method (Lorenzen 1967, Aminot & Kérouel 2004).

Mesozooplankton samples were processed using the ZooScan system (Grosjean et al. 2004, Gorsky et al. 2010) and plankton identifier software (Gasparini & Antajan 2013). Two size fractions (>500 µm and 200–500 µm) per sample were prepared and fractionated to reach 1000–2000 objects to be scanned (following Lelièvre et al. 2012). Results from each size fraction were summed to obtain total mesozooplankton abundance (ind. m^{-3}).

Herring larval abundance (ind. 5000 m^{-3}) was estimated from a fraction of the original sample using a Motoda splitter. At least 50 larvae per sub-sample were individually measured for standard length (SL, ±1 mm) and values were corrected for potential shrinkage due to preservation modes (i.e. either formalin or liquid nitrogen) using a linear model (ANOVA, $p < 0.05$, Fox 1996). To do so, freshly caught Downs herring larvae (n = 50) were measured before (i.e. SL) and after 15 d of preservation (Ls) in either formalin solution or liquid nitrogen. Corresponding corrections for shrinkage were SL = 1.2064 × Ls – 1.1224 and SL = 0.9588 × Ls + 0.892 for formalin and liquid nitrogen preservation, respectively. Counts and measurements carried out on each sub-sample were estimated for the total sample, and divided by the filtered volume.

Gut content analyses

Gut fluorescence content of 6 dominant copepod species (*Calanus* spp., *Euterpina acutifrons, Oncaea* spp., *Paracalanus parvus, Pseudocalanus elongatus* and *Temora longicornis*) was determined quantitatively by fluorometry (Mackas & Bohrer 1976). Depending on species abundance and size, 1–3 replicates of 1–2 individuals for *Calanus* spp. and 15–35 individuals for the other species per station were analysed (Fig. 1). Copepods were individually sorted on ice and picked from frozen shark-skin (Whatman filter) samples under a cool light stereomicroscope. As individuals must be quickly sorted, it was not possible to measure and stage them during this sorting phase. Individuals were rinsed in filtered 0.2 µm seawater to eliminate microplankton protist cells stuck to feeding appendages and were then transferred into 4 ml of 90% acetone. Individuals were ground and extraction was carried out in the dark at 4°C for 6 h. Fluorescence of the extract (chl *a* and phaeopigments) was measured before and after acidification with 10% HCl (Parsons et al. 1984) using a Trilogy Laboratory Fluorometer (Turner Designs EPA 445). Copepod gut fluorescence content (G_{cop}, ng chl *a* eq. $ind.^{-1}$) was obtained from the sum of chl *a* and phaeopigment concentrations. Values were not corrected for pigment degradation (as recommended by Durbin & Campbell 2007).

Gut contents of Downs herring larvae (n = 5 station^{-1}) were analysed by stereomicroscopy to estimate the number of copepod prey ingested as well as the number of empty guts. The number of larvae analysed was defined according to Denis et al. (2016), who demonstrated that using a higher number of larvae increases the number of rarely-ingested prey but does not change the overall picture of the larval diet. Each larva was placed in a petri dish filled with deionized water (Milli-Q). The larva was dissected, the gut removed and opened, and the contents were resuspended to facilitate optical examination (×10 magnification). Copepod prey were identified to the lowest taxonomic level depending upon their digestion state and were counted to estimate the number of individuals (N_{cop}) in each gut content. The proportion (%) of larvae without copepod prey (i.e. those with 'empty guts') was recorded according to stations and size classes.

Gut fluorescence content of fish larvae was measured following the same method as for copepods but contained specific adaptations to herring larvae during sample preparation and extraction. Several trials (not presented) were carried out to determine the optimal extraction time and the minimum number of larvae required for signal detection. Larval gut fluorescence was first measured every 2 h over a 12 h period. This allowed setting the optimal extraction time at 6 h, as no significant differences in pigment concentrations (Kruskal-Wallis test, $p > 0.05$) were observed thereafter. Fluorescence measurement on an increasing number (from 2 to 16) of larval guts allowed defining the optimal number of 10 larvae replicate^{-1} on a total of 1–3 replicates station^{-1}. To limit photodegradation of the gut fluorescence contents, sorting of larvae, dissecting and selection processes were carried out rapidly by working under a dissecting microscope equipped with dim cool light. Each larva was measured (±0.1 mm, Campana 1990) and grouped into 1 of 4 size classes: 8–9, 10, 11 and 12–13 mm, in order to have at least 5 individuals per size class. Frozen larvae were placed in petri dishes filled with milliQ water to allow soft thawing and examined on ice at 10× magnification. The gut was removed from each larva and transferred into a glass tube with 4 ml of 90% acetone. Pigment extraction was performed for 6 h at 4°C in the dark. 'Blank guts' (B_{gut}) were set at each station by emptying the guts of 10 randomly selected larvae with dissecting forceps. Larval gut fluorescence content (G_{fish}, ng chl *a* eq. ind.$^{-1}$) was estimated from the total amount of pigments (T_{pig}) recovered in the gut fluorescence content after subtracting B_{gut}. This gut fluorescence content (G_{fish}) comprises direct ingestion of autotrophic and heterotrophic/mixotrophic protist prey (i.e. phytoplankton) as well as indirect ingestion of herbivorous copepods. Although the contribution of microzooplankton to fish larvae gut content could not be separately estimated from phytoplankton ingestion, inferred from our study, we estimated the proportion of larvae for which gut fluorescence content was related to the ingestion of herbivorous copepods ($\%_{fishIC}$) as follows:

$$\%_{fishIC} = G_{cop} \times N_{cop} / G_{fish} \times 100 \quad (1)$$

where G_{cop} and N_{cop} are respectively the mean of the gut fluorescence content and numbers of copepod prey observed in the larval gut. This proportion was corrected by the number of empty guts recorded according to size class and by station.

Pigment ingestion rate (I_{fish}, ng chl *a* eq. ind.$^{-1}$ d^{-1}) was used as a proxy of larval ingestion (Denis et al. 2017) and was estimated from G_{fish} as follows:

$$I_{fish} = G_{fish} \times GER \quad (2)$$

where GER is the gut evacuation rate (d^{-1}). Given the difficulty in maintaining herring larvae onboard after collection, we did not directly measure GER. Instead, we used a GER value of 40 min^{-1} obtained from the conversion of hourly values of 0.667 h^{-1} (Fossum 1983) and 0.706 h^{-1} (Pedersen 1984) reported for herring larvae (8–40 mm) feeding continuously on zooplankton prey (e.g. copepod nauplii and copepodites) at temperature ranges matching those encountered during the winter sampling period (6–9°C).

The contributions of pigment ingestion to larval daily metabolic requirements (%DMR) were calculated from the daily ration (DR) to respiration rate (R_{fish}) ratios. Larval daily rations (DR, % body C d^{-1}) were estimated from the ratio of carbon ingestion to larval body carbon content. Pigment ingestion rates (I_{fish}) were converted to carbon (IC_{fish}, µg C ind.$^{-1}$ d^{-1}) using a C:chl *a* ratio of 50 (Banse 1977). Larval body carbon content (µg C ind.$^{-1}$) was estimated from the length–weight specific relationship (Hufnagl & Peck 2011) and converted to carbon assuming that carbon is 44.5% of dry weight (Arrhenius & Hansson 1996). Pigment ingestion rates were compared to estimates of basic respiratory requirements for fish based on the empirical relationship linking dry weight to respiration rates (De Silva & Tytler 1973). Oxygen values were converted into respiratory carbon (R_{fish}, µg C ind.$^{-1}$ d^{-1}) after calculating respiration rates (µl O_2 ind.$^{-1}$ d^{-1}) and assuming a respiratory quotient of 0.8 (Tytler & Calow 2012).

Mapping and data analyses

Copepods and larval distribution, pigment ingestion rate and contribution of copepod prey to the diet were mapped using the 'mapplots' package of the R software (R Development Core Team 2005).

Normality and homoscedasticity of these data were assessed using a Shapiro–Wilks test ($p < 0.05$) and a Levene's *F*-test ($p < 0.05$), respectively. Parametric tests (ANOVA and Tukey HSD) were then used to assess both spatial differences in larval distribution, empty guts and gut fluorescence contents, and size differences in the larval pigment ingestion rates and empty guts.

RESULTS

Hydrobiological parameters

In 2014, winter sea surface temperature ranged between 9.5°C (Stn 9) and 11°C (Stn 1), with slightly higher values in the EEC compared to the SBNS (Table 1). Variability in salinity values was essentially due to lower values (34.3–34.9) recorded at Stns 9, 10 and 12, whereas for most stations, values were 35.1–35.3. Total chlorophyll concentrations ranged between 0.70 and 0.92 µg l^{-1} in the SBNS, with slightly lower values (from 0.45–0.75 µg l^{-1}) in the EEC (Table 1).

Table 1. Sea surface temperature (°C), salinity, *in situ* chlorophyll *a* and phaeopigment concentrations (µg l^{-1}) and mesozooplankton abundance (ind. m^{-3}) during winter 2014 (January–February) in the Eastern English Channel (EEC) and the Southern Bight of the North Sea (SBNS). DL: detection limit (<0.025 µg l^{-1})

Stn	Temperature	Salinity	Chl *a*	Phaeopigment	Mesozooplankton
EEC					
1	11.0	35.3	0.48	DL	904
2	10.7	35.3	0.4	0.05	697
3	10.8	35.3	0.4	0.16	958
4	10.6	35.1	0.48	0.14	94
5	10.5	35.1	0.48	0.08	1277
6	10.6	35.2	0.67	DL	461
7	10.4	35.1	0.44	0.09	578
8	10.5	35.2	0.52	0.23	326
9	9.5	34.7	0.4	0.33	1786
SBNS					
10	9.6	34.3	0.64	0.28	817
11	10.6	35.1	0.48	0.44	1273
12	10.2	34.9	0.44	0.26	2980
13	10.2	35.1	0.4	0.36	2335
14	10.2	35.1	0.52	0.38	3634

Phaeopigment contribution to total pigments was high (23–48%) except for stations located in the centre of the EEC (Stns 1, 2 and 5 to 7: 0–17%).

Distribution of copepods and herring larvae

Mesozooplankton abundance ranged from <100 (EEC) up to >3600 ind. m^{-3} (SBNS; Table 1). In the EEC, abundance ranged between 600 and 900 ind. m^{-3}, although it could reach locally higher values (Stns 5 and 9: 1277 and 1786 ind. m^{-3}, respectively). The SBNS was characterized by generally higher mesozooplankton abundance, particularly at stations under the influence of the Thames estuary (Stns 11–14: 1273–3634 ind. m^{-3}). Copepod community composition in the EEC–SBNS was homogeneous during winter 2014 and comprised 6 dominant species (Fig. 2A,B). For all species, both copepodite (C4–C5) and adult (C6) stages dominated, with nauplii representing a negligible part of copepod abundance (<2%). *Pseudocalanus elongatus* and *Paracalanus parvus*, which together reached 79.8% of total abundance were dominant in the EEC–SBNS, as they were nearly the only species present in the SBNS (Stns 11–14). Copepod assemblages were also composed of *Temora longicornis* (up to 40.1% of total copepods), with a distribution restricted to coastal waters of the SBNS (Stn 10). *E. acutifrons*, *Calanus* spp. and *Oncaea* spp. represented >25% of total abundance in the southern part of the EEC and 10.2% in the SBNS.

Larval distribution showed a clear and significant southwestern–northeastern gradient coinciding with an increase in larval size and abundance (ANOVA, SS = 61 327 348, $F_{13,56} = 4.87$, $p < 0.001$; Fig. 2C,D). Smaller larvae (8–11 mm, 0–703 ind. 5000 m^{-3}) were mainly located in the EEC, while larger larvae (12–13 mm, 0–374 ind. 5000 m^{-3}) were recorded in the SBNS.

Copepod gut fluorescence content

Copepod gut fluorescence content varied from 0.09–9.02 ng chl *a* eq. $ind.^{-1}$, and the highest values were observed when the largest copepods were sampled (e.g. *Calanus* spp. and *P. elongatus* for Stns 2, 7, 9, 11, 13 and 14; Table 2). In contrast, lowest values (from 0.09 to 1.84 ng chl *a* eq. $ind.^{-1}$) were attributable to the smallest species, namely *Oncaea* sp. and *Euterpina acutifrons* (Stns 1, 4 and 12). Intermediate gut fluorescence content values ranged from 0.13–3.44 ng

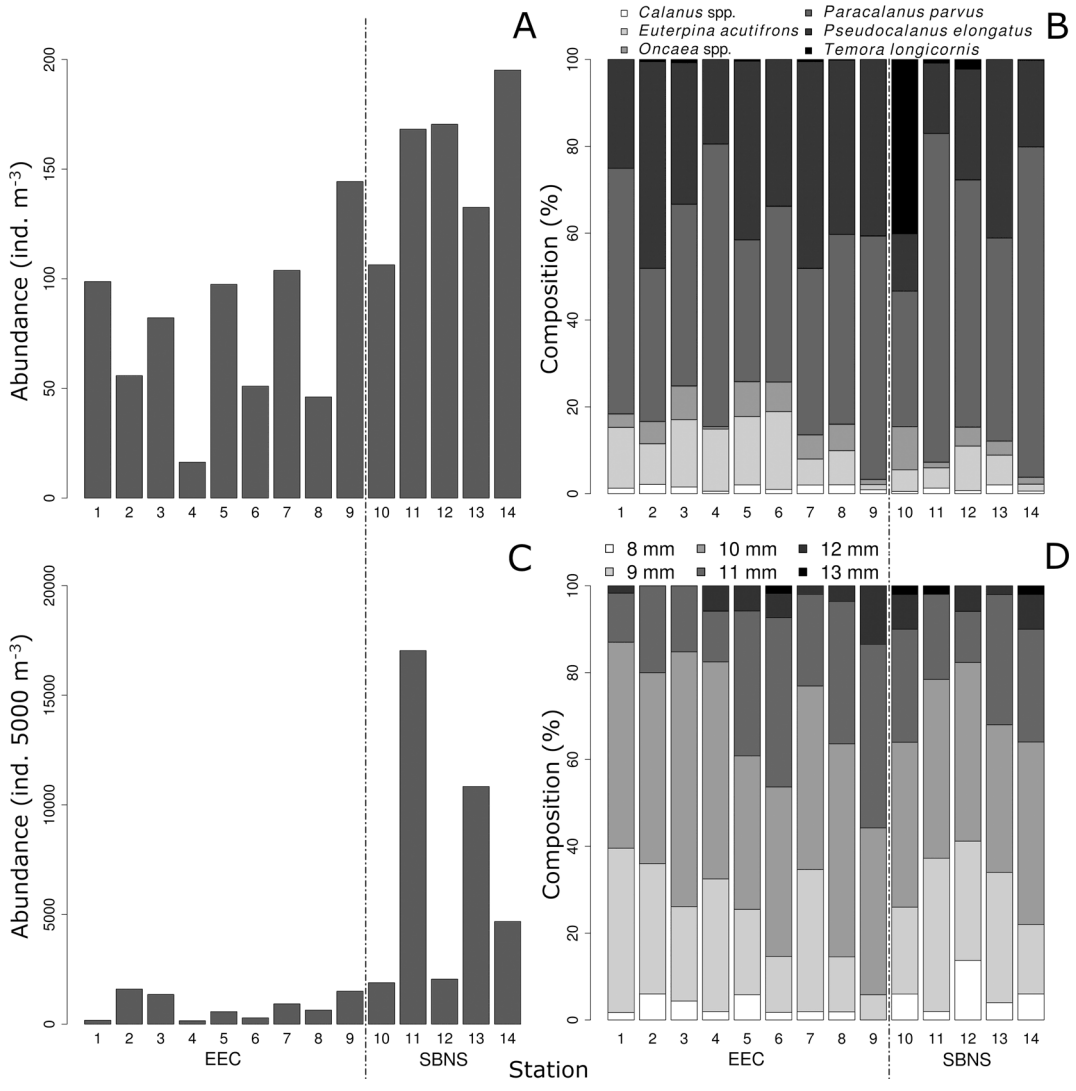

Fig. 2. Abundance distribution of (A,B) 6 dominant copepod species (ind. m^{-3}) and (C,D) Downs herring larvae (ind. 5000 m^{-3}) by size (mm) during winter 2014 (January–February) in the Eastern English Channel (EEC) and the Southern Bight of the North Sea (SBNS). Only stations where larvae were collected for gut analyses are shown

chl *a* eq. $ind.^{-1}$ and corresponded to the dominance of *P. parvus* (Stns 3, 5, 6 and 8) and *T. longicornis* (Stn 10). Phaeopigment contribution to gut fluorescence content was highly variable (5–100%), but most values were higher than those recorded for seawater (up to 48%).

Feeding by herring larvae

On average, 47% of the guts of herring larvae analysed were empty. The percentage of empty guts varied significantly (ANOVA, SS = 4.70, $F_{1,2}$ = 31.47, p = 0.03) according to larval size (Fig 3A), with the highest values (52%) observed for 8–9 mm larvae. For the other size classes, a decrease from 47% for 10 mm larvae down to 20% for the largest ones (12–13 mm) was recorded. The proportion of empty guts also varied significantly (ANOVA, SS = 2356, $F_{1,11}$ = 3.19, p = 0.10) in space (Fig. 3B), and the area with the lowest proportion (<40%) was identified in the middle of the EEC.

Larval diet comprised 5 of the 6 dominant copepod species (Fig. 3) sampled in the area (Fig. 2A,B) and no nauplii, suggesting that Downs herring larvae fed on the most available zooplankton prey. Larval diet composition matched copepod species distribution (Figs. 2B & 3B). Larvae of 8–9 mm consumed relatively few individuals (1–2 copepods) of *P. parvus* and *Oncaea* spp., whereas larvae of 10–13 mm in-

Table 2. Dominant copepods and Downs herring larvae fluorescence content (ng chl *a* eq. ind.$^{-1}$; range of min. and max. values) and contribution of phaeopigments (%) during winter 2014 (January–February) in the Eastern English Channel (EEC) and the Southern Bight of the North Sea (SBNS)

Stn	Copepods		Downs herring larvae	
	ng chl *a* eq. ind.$^{-1}$	% Phaeo-pigments	ng chl *a* eq. ind.$^{-1}$	% Phaeo-pigments
EEC				
1	0.09–0.7	34–39	0.35–0.78	70–74
2	0.12–4.24	50–97	1.08–7.30	66–83
3	0.51–3.44	38–68	0.49–2.51	75–81
4	0.16–0.62	47–72	0.74–2.81	67–72
5	0.51–4.7	50–91	1.14–2.89	72–79
6	0.13–2.49	34–98	0.45–3.36	66–72
7	0.21–4.16	51–100	0.35–0.69	68–73
8	0.51–2.79	19–48	0.43–3.58	67–70
9	0.31–8.9	5–96	0.30–0.91	73–85
SBNS				
10	0.08–2.12	21–94	0.15–0.70	65–77
11	0.31–5.55	46–85	0.12–0.43	65–68
12	0.17–1.84	39–63	0.55	74
13	0.40–4.11	52–79	0.71–0.90	84–88
14	0.34–9.02	38–74	0.79–1.67	66–69

gested higher numbers of copepods (>46), targeting mainly *P. elongatus*, *E. acutifrons* and *T. longicornis.*

Larval gut fluorescence contents were of the same order as values observed for copepods (Table 2). Values ranged between 0.12–0.43 (Stn 11) and 1.08–7.30 ng chl *a* eq. ind.$^{-1}$ (Stn 2) and were significantly higher in the EEC compared to the SBNS (ANOVA, SS = 37.3, $F_{2,346}$ = 12.18, p < 0.001). From 65 to 88% of pigments recorded in herring larvae gut fluorescence content were phaeopigments, a contribution located in the higher range of values observed for copepods in the study. Pigment ingestion by fish larvae was not significantly related to either prey standing stocks, i.e. *in situ* pigment concentrations (r = –0.09, p > 0.05), or to mesozooplankton abundance (r = –0.21, p > 0.05).

Larval pigment ingestion rates varied significantly with larval size (ANOVA, SS = 8.6, $F_{1,347}$ = 5.328, p = 0.022; Fig. 4A). Mean pigment ingestion rates increased from 24.6 ± 28.7 to 52.2 ± 50.9 ng chl *a* eq. ind.$^{-1}$ d^{-1} for 8–11 mm larvae, while mean values slightly decreased to 43.3 ± 48.2 ng chl *a* eq. ind.$^{-1}$ d^{-1} for 12–13 mm larvae. Spatial variability in mean pigment ingestion rates is depicted in Fig. 4B–E. For all size classes, a clear distinction could be made between rather high pigment ingestion rates of the EEC larvae (19.2–188.1 ng chl *a* eq. ind.$^{-1}$ d^{-1}) and lower values (8.8–83.5 ng chl *a* eq. ind.$^{-1}$ d^{-1}) recorded for larvae collected in the SBNS. For smaller larvae, pigment ingestion rates ranged

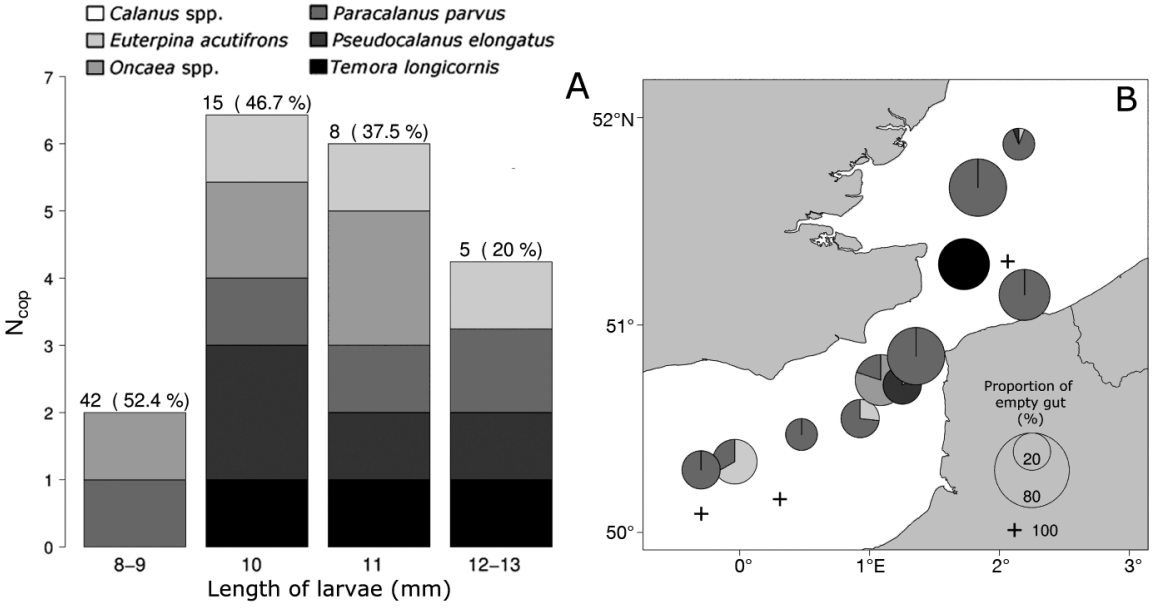

Fig. 3. Contribution of copepod prey to the diet of Downs herring larvae during winter 2014 (January–February) in the Eastern English Channel and the Southern Bight of the North Sea. (A) Number of copepods larva^{-1} (N_{cop}) by larval size classes (mm). Number of larvae analysed and proportion of empty guts (%; within brackets) are indicated. (B) Spatial distribution of larval gut content composition (copepod species) and proportion of empty guts (%; related to pie size)

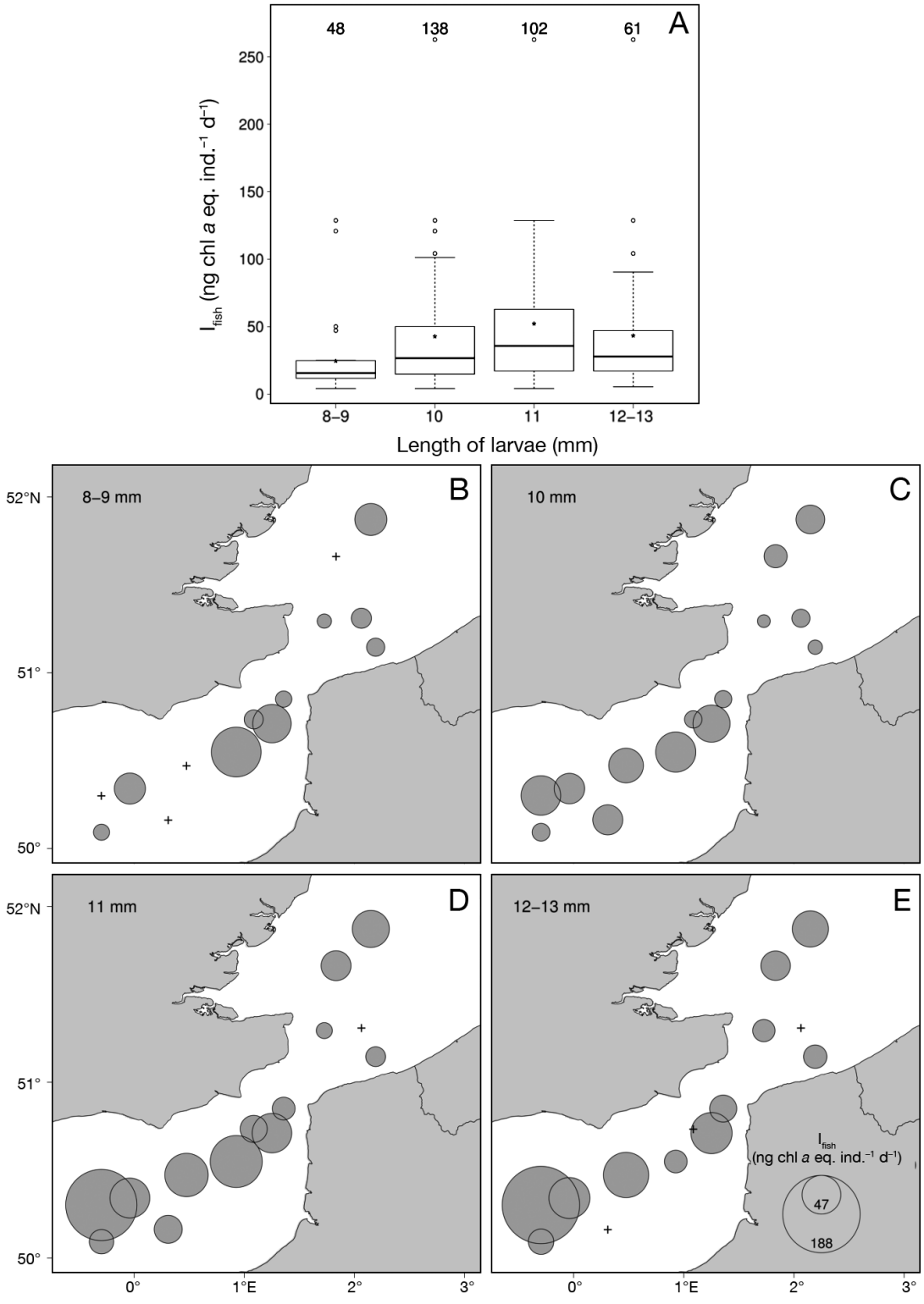

Fig. 4. Chlorophyll pigment ingestion by Downs herring larvae during winter 2014 (January–February) in the Eastern English Channel and the Southern Bight of the North Sea (SBNS). (A) Boxplots which represent the outliers, minimum, first quartile, median, third quartile, and maximum of the pigment ingestion rate (I_{fish}, ng chl *a* eq. ind.$^{-1}$ d^{-1}) by larval size. Stars represent mean values. The number of larvae analysed for each size class is indicated on the top of each bar. (B–E) Pigment ingestion rate at each station by larval size class (8–9, 10, 11 and 12–13 mm). Crosses on the map indicate the absence of larval size class for the station

Table 3. Downs herring larval parameters (mean ± SD) derived from allometric relationships (body carbon content; respiration rates, R_{fish}), gut content analyses (carbon ingestion rates, IC_{fish}; daily ration, DR) and the proportion of larval gut pigment ($\%_{fishIC}$) due to herbivorous copepod ingestion during winter 2014 (January–February) in the Eastern English Channel (EEC) and the Southern Bight of the North Sea (SBNS)

Size class mm	IC_{fish} µg C ind.$^{-1}$ d^{-1}	Body carbon content µg C ind.$^{-1}$	DR % body C d^{-1}	$\%_{fishIC}$	R_{fish} µg C ind.$^{-1}$ d^{-1}	R_{fish} % body C d^{-1}
8–9	1.1 ± 1.3	31.4 ± 9.9	2.8 ± 3.3	16.3 ± 33.5	7.6 ± 2	24.4 ± 1.4
10	2.3 ± 2.6	57.6 ± 0	3.9 ± 4.4	24.1 ± 39.3	12.6 ± 0	21.8 ± 0
11	2.3 ± 1.9	83.1 ± 0	2.7 ± 2.3	27.1 ± 38.2	17 ± 0	20.4 ± 0
12–13	2.6 ± 2.6	137 ± 29.6	2.2 ± 2.2	45.9 ± 40.6	25.5 ± 4.6	18.7 ± 0.7

between 8.8 and 173.2 ng chl *a* eq. ind.$^{-1}$ d^{-1}, while for larger ones, values ranged from 15.6–188.1 ng chl *a* eq. ind.$^{-1}$ d^{-1}.

Larval carbon ingestion increased with larval size and ranged between 1.1 ± 1.3 and 2.3 ± 1.9 µg C ind.$^{-1}$ d^{-1} for smaller larvae (8–11 mm) up to 2.6 ± 2.6 µg C ind.$^{-1}$ d^{-1} for larger larvae (12–13 mm; Table 3). Feeding exclusively on pigment would lead larvae to ingest 2.2 ± 2.2 to 3.9 ± 4.4 % of their body C every day (Table 3).

The proportion of larvae exhibiting indirect consumption of pigments via predation on copepods increased with larval size from 16 ± 34 % for smaller larvae (8–9 mm) up to 46 ± 41 % for the larger larvae (12–13 mm; Table 3).

Based on the relationship between body weight and respiration, herring larvae needed to ingest 18.7 ± 0.7 to 24.4 ± 1.4 % body C d^{-1} just to balance their respiratory needs, with smaller larvae (8–9 mm) having the highest daily respiratory requirements. Therefore, chlorophyll pigment ingestion covered 1 to 116 % (average ± SD, 15 ± 17 %) of the daily metabolic requirements of 8–13 mm herring larvae. About 5 % of these larvae covered more than half of their daily metabolic requirements when feeding exclusively on chlorophyll pigments, with values from 50–116 %. Larvae ranging in size from 8–9 to 11 mm covered on average 10 ± 14 to 18 ± 19 % of their daily metabolic needs when feeding on chlorophyll pigments, whereas intermediate values (15 ± 18 %) were observed for the largest larvae (12–13 mm).

DISCUSSION

One of the challenges in experimental ecology is to develop methods and techniques allowing fast, but reliable and repeatable, assessments of ecosystem variables such as diversity, productivity and trophic interactions. The present study combines the gut fluorescence technique and fish larval parameters (e.g. size class distribution and abundance) at the scale of the EEC-SBNS ecosystem to quantify pigment feeding by fish larvae. Measurements carried out simultaneously on dominant herbivorous suspension feeders (i.e. copepods) allowed a direct comparison of grazing intensities and interactions between pelagic grazers in the EEC-SBNS.

Shortcomings and advantages of the quantitative approach

The gut content method has proven its efficiency to quantify pigment ingestion in a wide range of zooplankton organisms such as copepods (Mackas & Bohrer 1976, López et al. 2007), salps (Perissinotto & Pakhomov 1998) and krill (Perissinotto & Pakhomov 1996). However, to our knowledge, it has only been applied twice to fish larvae (Otake et al. 1990, Conway et al. 1996). Pigment destruction by non-fluorescent compounds during their passage through the gut is a source of uncertainties and debates for the fluorescence method (Pasternak 1994, Tirelli & Mayzaud 1998, Durbin & Campbell 2007). In our study, high phaeopigment concentrations in the larval guts (65–88 %), copepods (5–100 %) and seawater (up to 48 %) suggested either a long retention time of pigments within the gut or that the pigments originated from detrital materials. Amongst the latter, faecal pellets (Otake et al. 1990), agglutinated phytoplankton (e.g. green remains; Morote et al. 2010) or feeding filters of appendicularian houses, which sieve and concentrate particles ranging in size from 0.2 to 30 µm (e.g. Berline et al. 2011) and thus capture organisms from bacteria to microplankton (Gorsky & Fenaux 1998, Lombard et al. 2010), were identified as potential prey for fish larvae. Another possible explanation for this high phaeopigment content is that the heterotrophic prey (e.g. copepods containing degraded pigments)

contributed highly to larval fish diet (16–46%) especially for the 10–13 mm larvae which ingested copepod prey, i.e. *Paracalanus parvus*, *Oncaea* spp., *Pseudocalanus elongatus*, *Euterpina acutifrons* and *Temora longicornis*. Small copepods (*Oncaea* spp. and *E. acutifrons*), invertebrate eggs, diatoms (*Psammodicthyon panduriforme* and *Coscinodiscus* spp.) and dinoflagellates (*Dinophysis acuminata* and *Prorocentrum micans*) were in fact recognized as important prey items for small larvae (8–12 mm), whereas larger larvae (13–19 mm) appeared to feed almost exclusively on copepods (*T. longicornis, P. elongatus* and *P. parvus*) and heterotrophic dinoflagellates (*Gonyaulax* spp., Denis et al. 2016). Ingestion of soft-bodied ciliates may also have occurred as demonstrated in field and experimental studies (Fukami et al. 1999, Hunt von Herbing & Gallager 2000, Bils et al. 2017) but could not be distinguished from phytoplankton ingestion with the gut content method.

Gut evacuation rates can vary greatly in relation to a number of factors thus impacting feeding estimates (Bromley 1994). Hence, initial gut content (Perissinotto & Pakhomov 1996), prey concentration (Pasternak 1994), quality (Jobling 1980) and size (e.g. Swenson & Smith 1973, Macdonald et al. 1982, Karjalainen et al. 1991), fish size (e.g. Flowerdew & Grove 1979), feeding rhythm (Bromley 1994, Wuenschel & Werner 2004) and seawater temperature (Kiørboe et al. 1982, Irigoien et al. 2008) have all been recognized as parameters influencing gut evacuation rate estimations. In our study, gut fluorescence contents were examined from animals caught at night during a 10 d sampling period in winter. No spatial pattern in seawater temperature and pigment concentrations was observed over the sampling area; we therefore consider that gut fluorescence contents reflected the size classes and spatial differences in pigment ingestion rates of herring larvae rather than the variability of gut evacuation rate linked to seawater temperature and/or to prey concentration.

Active night feeding of winter Downs herring larvae was illustrated by the low proportion of empty guts (20–50%) and is consistent with previous studies on larval herring (Munk 1992, Denis et al. 2016, Wilson et al. 2018) and also on other fish larvae, such as spotted seatrout *Cynoscion nebulosus* (Wuenschel & Werner 2004), gulf menhaden *Brevoortia patronus*, spot *Leiostomus xanthurus* and Atlantic croaker *Micropogonias undulatus* (Govoni et al. 1983) and sutchi catfish *Pangasianodon hypophthalmus* (Mukai et al. 2010). In addition, in commercial hatcheries, most species are reared under low light conditions (<800 lux or 1.17 W m^{-2}) during their early life period while still exhibiting active feeding rates (Lee et al. 2017). Downs herring larvae may thus be well adapted to winter environmental conditions of their spawning area (low light, high turbidity, low production) and are able to actively feed consistently as was also demonstrated for the Scotland Shelf cod population (Puvanendran & Brown 1998). However, we could not ascertain whether feeding is continuous over time; thus, future analyses should investigate fish larval gut contents at higher sampling frequency and over a day/night cycle. Therefore, although suffering from uncertainties that clearly need to be identified according to each case study (species, sites, season, available prey, feeding rhythm), the gut fluorescence method works for Downs herring larvae and provides a global estimate of total ingested pigments (chl *a* and phaeopigments) from *in situ* samples.

Feeding of Downs herring larvae

As the same computation methods were used to estimate gut fluorescence contents (ng chl *a* eq. $ind.^{-1}$), a direct comparison could be made between herring larvae and copepod feeding behaviour. The gut fluorescence method demonstrated the quantitative contribution of autotrophic protists (e.g. ingestion of chl *a*) to Downs herring larval diet during winter. Although the contribution of auto- and heterotrophic protists to fish larval diet has been observed for herring (e.g. Lebour 1921, Bjørke 1976, Checkley 1982) and other species (e.g. de Figueiredo et al. 2005, 2007, Morote et al. 2010), our study provides for the first time a quantitative estimate of autotrophic protist ingestion by Downs herring larvae. Pigment concentrations (chl *a* and phaeopigments) measured in the gut should be seen as a reflection of both an effective direct consumption of autotrophic protists and an indirect consumption of chlorophyll pigments by ingestion of herbivorous zooplankton (metazoans or hetero/mixotrophic protozoans). Gut fluorescence content values for Downs herring larvae (0.15–7.30 ng chl *a* eq. $ind.^{-1}$) were within the range of those reported previously for the same species (0.03–1.64 ng chl *a* eq. $ind.^{-1}$, Denis et al. 2017) and for larvae of other species of comparable size (mackerel, 1.62 ± 0.63 ng chl *a* eq. $ind.^{-1}$; Conway et al. 1996) as well as within the range recorded for dominant copepods. Consequently, despite low food concentration, winter Downs herring larvae may exhibit nutritional levels comparable to those of dominant herbivorous copepods.

Our results demonstrated a monotonic increase in pigment ingestion rate, yet a slight decrease was observed for the larger larvae. Smaller herring larvae (8–11 mm) ingested pigments at high mean rates (52.2 ± 51 ng chl *a* eq. ind.$^{-1}$ d^{-1}), whereas lower mean rates (43.3 ± 48 ng chl *a* eq. ind.$^{-1}$ d^{-1}) were measured in larger larvae (12–13 mm). This increase combined with the spatial gradient in larval size distribution led to a clear distinction between the EEC and the SBNS. Basically, smaller larvae collected in the EEC had a more diversified diet (i.e. including auto- and heterotrophic protists) and higher pigment ingestion rates than larger larvae in the SBNS (Denis et al. 2016). The spatial variability of larval pigment ingestion revealed a paradox between higher pigment ingestion rates in the EEC where *in situ* pigment concentrations were low, and lower pigment ingestion rates in the SBNS where *in situ* pigment concentrations were high. This suggests that carbon intake by herring larvae did not only depend on *in situ* pigment concentrations, but rather on the diet composition and the size of ingested prey which increased with the size of the larvae (Denis et al. 2016). This was confirmed by the absence of a significant relationship between fish larvae pigment ingestion and autotrophic prey standing stocks. The lower pigment ingestion of 12–13 mm larvae could also be explained by their shift from omnivory to carnivory (Denis et al. 2016, 2017). This diet shift could have released the grazing pressure exerted by larvae on autotrophic protists and explain higher pigment concentrations in the SBNS. Regarding smaller larvae (8–11 mm), the highly diversified omnivorous diet they exhibited including pigments (Denis et al. 2016, 2017) appeared to allow them to feed enough to survive under poor winter trophic conditions.

Auto- and heterotrophic protists play an essential role in larval growth by enhancing the development of their digestive functions, which, in turn, facilitates the enzymatic digestion of other prey like copepods (Hjelmeland et al. 1988, John et al. 2001, Illing et al. 2015) and contributes to nutrient intake (Braven et al. 1995). These larvae can direct energy reserves towards somatic growth in particular, to reduce the risk of predation and mortality. In fact, slow-growing larvae remain vulnerable to predation for a longer period of time ('stage-duration' hypothesis), while fast-growing larvae have higher survival potential ('bigger is better' hypothesis; Houde 1987, 2008, Anderson 1988). Low daily carbon rations derived from exclusive feeding on chlorophyll pigments indicate that pigment ingestion would cover on average up to 18% of the daily metabolic needs of Downs herring larvae during winter 2014. Autotrophic protists are consequently an important part of the diet of larvae and would offset the energy requirements due to the low diversity of prey in winter, particularly regarding 8–11 mm larvae with high carbon needs. The increase in the larval gape size and maturation of the digestive tract allow growing fish larvae to feed on larger and more energetic prey such as copepods (Van Der Meeren 1991, Friedenberg et al. 2012, Denis et al. 2017). A diet shift towards copepod consumption was estimated in our study by coupling gut content measurements with optical methods (e.g. number of copepods in the larval gut content). The proportion of larvae exhibiting indirect pigment ingestion (through ingestion of herbivorous copepods) increased from 16% for smaller larvae (8–9 mm) up to 46% for the larger larvae (12–13 mm). This could be explained by the spatial co-occurrence of 12–13 mm larvae with high mesozooplankton abundance in the SBNS as shown from our results and previous studies (Boehlert & Yolklavich 1984, Theilacker 1987, Nunn et al. 2012).

CONCLUSIONS

The present study demonstrated the validity and reliability of the gut fluorescence method to quantify pigment ingestion by Downs herring larvae in the EEC-SBNS. The larval gut fluorescence method integrates both direct (phytoplankton ingestion) and indirect ingestion of pigments (ingestion of herbivorous zooplankton). The gut fluorescence content and its derived proxy (i.e. pigment and carbon ingestion) can be used as a feeding index and proxy of the larval nutritional status in future studies. We quantitatively estimated the contribution of autotrophic protists to the larval diet during the first feeding stages. At larger sizes, the proportion of autotrophic protist prey directly ingested decreased as nearly 50% of large larvae exhibited indirect ingestion of pigments (against 16% for smaller ones). This change in diet corresponds to a shift towards a less diversified, more carnivorous diet comprising mainly copepods.

The gut fluorescence content method applied to fish larvae is a very promising *in situ* method providing environmentally realistic grazing data. However, it remains difficult to identify the individual impact of forcing parameters on feeding rates *in situ* (prey size, types and abundance). This emphasizes the need to develop experimental approaches under controlled laboratory conditions to investigate and scrutinize predator–prey interactions between herring larvae and plankton targeted species of phyto- and zooplankton.

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