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# Stable isotopes as tracers can reveal resource allocation in juvenile golden gray mullets (*Liza aurata*, Risso, 1810)

Como S.[a,*,1], Carpentier A.[b,1], Rossi F.[c], Dupuy C.[d], Richard P.[d], Feunteun E.[e], Lefrançois C.[d]

[a] CNR–IAMC, National Research Council, Institute for Coastal Marine Environment, Località Sa Mardini, Torregrande 09072, Oristano, Italy
[b] EA 7316, Université de Rennes 1, Campus de Beaulieu, Av. du Gl Leclerc, Bat. 25, 35042 Rennes Cedex, France
[c] CNRS, UMR 9190, Place E. Bataillon, University of Montpellier, Montpellier 34000, France
[d] Littoral, Environnement et SociétéS (LIENSs), Université de La Rochelle, UMR 7266 CNRS-ULR, 2 rue Olympe de Gouges, 17000 La Rochelle Cedex, France
[e] CRESCO, MNHN, 38 rue du port blanc, 35800 Dinard, France

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ABSTRACT

Studies on the nutritional physiology of predator fish in the marine environment have contributed to our understanding of how they adapt to the environment and how they have evolved. Despite the fact that herbivorous and omnivorous fish species are numerous and play a significant role in the ecosystem, there is little information on how they process nutrients and how these are allocated to different tissues. This information could be particularly important for the juvenile stages, when small-sized fish are under high predation pressure and have a limited capability to intake and digest large quantities of food. The mullet *Liza aurata* ingests surface sediment and obtains its nutritional requirements from the organisms associated with the sediment, including microalgae and bacteria or small invertebrates. This paper examines how the carbon and nitrogen derived from benthic micro-organisms are allocated to the liver and muscle tissues of newborn (young-of-the-year, YOY) and one-year-old (OYO) individuals. After the animals were left feeding on $^{13}$C-enriched microalgae and $^{15}$N-enriched bacteria for 1 h, we traced the $^{13}$C and $^{15}$N in the liver and muscle tissues as well as in the blood and the gut. The YOY allocated 99% of the $^{13}$C and 88% of the $^{15}$N to the muscles, while the liver had a negligible amount of tracers (0.4% and 11% for $^{13}$C and $^{15}$N). Conversely, in the OYO experiment, the tracers were uniformly distributed throughout the muscle and liver (57% of $^{13}$C and 45% of $^{15}$N were found in the muscle, whereas 43% of $^{13}$C and 55% of $^{15}$N were in the liver). Negligible amounts were traced in the blood ($< 0.1\%$), while a part of the tracers was not assimilated and remained in the gut of both YOY and OYO fish. These results indicated a size-related shift in resource allocation during first year of growth of *L. aurata*, probably related to changes in the survival strategies among juveniles. Our results also indicated that stable isotope enrichment can be a helpful tool for studying resource allocation in fish.

## 1. Introduction

The nutritional physiology of a consumer is of considerable interest for understanding ecological and evolutionary processes (Evans and Claiborne, 2006). Apart from the baseline knowledge of nutrition for a few aquaculture fish species (Houlihan et al., 2000), most of the studies on feeding and nutrition on marine fish have been focused on different species of predators (e.g. Litvin et al., 2011; Sogard and Spencer, 2004; Stallings et al., 2010). Very little is known about the feeding and nutrition of herbivorous and omnivorous fish, despite their evolutionary and ecological diversity and the key roles they play in ecosystem processes (Clements et al., 2009; Whitfield et al., 2012). Furthermore, most of this work has focused on food acquisition (Como et al., 2014; Raubenheimer et al., 2005; Smith, 2008) and digestive processes (German et al., 2004; Moran et al., 2005; Moran and Clements, 2002). Yet, once food is digested, how the carbon and nitrogen derived from it are allocated to different tissues is unknown. This information could be particularly important for juveniles, because these stages are often considered critical for animal survival and, in turn, for population growth and fish stock maintenance (Biro et al., 2005; Sogard and Spencer, 2004; Stallings et al., 2010). Such difficulties are related to their small size and to their reduced ability to escape predators, to migrate and/or to withstand periods of food depletion (Domenici, 2010; Sogard, 1997).

* Corresponding author.
*E-mail address:* scomo71@libero.it (S. Como).
[1] Two first authors equally contributed to the paper.

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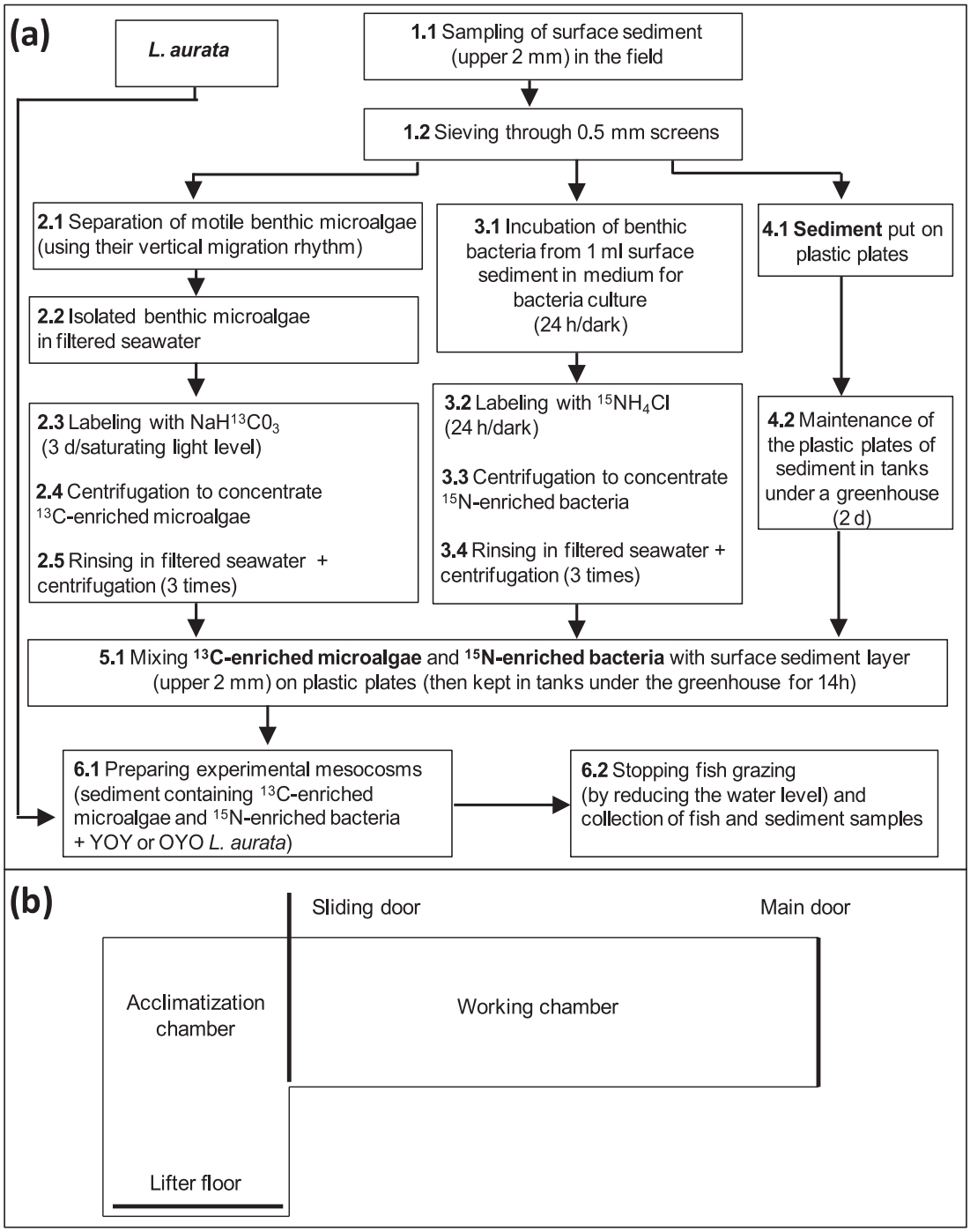


**Fig. 1.** (a) General protocol; main steps in the preparation of the experimental mesocosms and sample collection. The experimental mesocosms consist in sediment containing $^{13}C$-enriched microalgae and $^{15}N$-enriched bacteria and young-of-the-year (YOY) or one-year-old (OYO) *L. aurata*; (b) Schematic illustration of the mesocosm (viewed from the side). Not shown: the video-tracking system, which consisted of four polychrome video cameras (25 image $s^{-1}$), a multichannel video interface and a DVD recorder to follow the fish during feeding. The cameras were placed along the longest side of the working chamber in order to provide a lateral view. There was also a series of lamps connected to a timer which provided a supplementary light source in addition to the natural illumination, and a buffer tank connected to a thermostat which controlled temperature, circulation and the water level inside the mesocosm.

This study investigates the allocation of carbon and nitrogen to the somatic tissues of juvenile golden gray mullets, *Liza aurata* (Risso, 1810). This species is one of the most abundant omnivorous fish inhabiting the Mediterranean Sea and the Northwest Atlantic coasts (Carpentier et al., 2014; Hotos and Katselis, 2011; Lebreton et al., 2011). It is a euryhaline catadromous species that spawns in the open sea (Abdallah et al., 2013; Hotos et al., 2000; Parlier et al., 2006). The offspring recruit in shallow coastal areas, including estuaries and lagoons, where juveniles reside for at least 2 years of their life cycle (Hotos et al., 2000; Hotos and Katselis, 2011; Parlier et al., 2006). *L. aurata* is described as a generalist deposit feeder. After ingesting surface sediment, the animals derive their nutritional requirements, including carbon and nitrogen, from detritus and associated benthic micro and macro-organisms, particularly microalgae, bacteria and meiofauna (Carpentier et al., 2014; Lebreton et al., 2011; Vizzini and Mazzola, 2003).

In this study, we focus on how juveniles can use microbial carbon and nitrogen during the first two years after recruitment, when fish can be found in shallow coastal waters. After deliberately enriching the microbial food sources with stable isotopes (SI) ($^{13}$C-enriched microalgae and $^{15}$N-enriched bacteria), we exposed young-of-the-year (YOY) and one-year-old (OYO) juveniles to these enriched foods. We then followed the tracers in different body tissues which were selected for their functional role in the individual metabolism. We focused on the muscle, because it is implicated in somatic growth and on the liver, the most important organ for controlling the circulating levels of nutrients (Halver and Hardy, 2002; Grossell et al., 2010). We also sampled the post-hepatic blood, because it transports the nutrients released by the liver to other tissues via the circulatory system (Halver and Hardy, 2002; Grossell et al., 2010). As additional information, we measured the incorporation of $^{13}$C and $^{15}$N in the gut and in the sediment to quantify the amounts of tracers remained in the digestive content or available in the sediment.

Stable isotope (SI) tracing experiments have become a valuable tool for understanding the flow of nutrients and energy in food webs (Middelburg, 2014). So far, SI tracing in fish physiology has been used principally for understanding the best formulation of proteins, lipids or carbohydrates in manufactured feed for farmed fish (Conceição et al., 2010; Felip et al., 2015; Hamre et al., 2013). However, SI tracing could also be an excellent method for understanding how natural food sources can be allocated to the distinct functions of an individual and used by its metabolism.

## 2. Material and methods

### 2.1. Fish collection and captivity

Fifty juveniles of *Liza aurata* between 8.2 and 17.3 cm in total length (TL) and between 4.6 and 37.1 g of fresh weight (FW) were collected in Cabras lagoon, Oristano Gulf (39°55′N; 008°30′E; Western Sardinian coast, Italy) in June 2008. The fish were kept at the laboratory of the National Research Council of Oristano over the summer and at the beginning of September 2008 they were transported to the Marine Station of the University of La Rochelle (France), where the experiment was done from 17 November to 28 November 2008. In both the laboratories, fish were maintained in a 1 m$^3$ tank with re-circulating filtered seawater. The temperature and salinity of the water in the holding tank ranged between 17 °C and 24 °C (mean ± SE: 19 °C ± 0.8, n = 30) and 30 and 36 psu (mean ± SE: 33 psu ± 0.8, n = 30), respectively. After being transported to the Marine Station, the water temperature in the tank was adjusted by 0.5 °C per day, until the temperature was 20 °C. The fish were then allowed to acclimate to the temperature of 20 °C for two months (mean ± SE: 20 °C ± 0.2, n = 15). Salinity ranged between 34 psu and 36 psu (mean ± SE: 34 psu ± 0.2, n = 15). During this period, the fish were maintained under natural photoperiod (39°N), fed daily with natural periphyton and twice a week with dry pellets (BioMar®) to insure the necessary supply of proteins and vitamins. The use of natural periphyton allowed us to maintain the fish under laboratory conditions without compromising their natural foraging behavior (Richard et al., 2010). Natural periphyton (made up of microalgae, detritus and small benthic invertebrates, including meiofauna) was developed on artificial substrates in an adjacent pond (Richard et al., 2010, unpublished data).

### 2.2. Substrate preparation

The main steps in the preparation of the sediment with labeled micro-organisms are summarized in Fig. 1a. The sediment was collected from an intertidal mudflat in Aiguillon Bay (France; 46°10′N, 1°15′W) close to the Marine Station by scraping off the upper 2 mm and sieving through 0.5 mm screens to eliminate plant debris and macrofauna (steps 1.1 and 1.2). Benthic microalgae and bacteria were extracted from this sediment. To extract and enrich benthic microalgae (steps 2.1 to 2.5), 1 cm sediment layer was spread on flat trays and a series of nylon screens (63 μm mesh) was laid upon the sediment surface under natural light. Within 1–3 h, the top 2 nylon screens, on which microalgae had migrated, were gently rinsed with filtered seawater in order to detach microalgae from the screen (Riera and Richard, 1996). The microalgae were then cultured in a medium containing 0.4 g L$^{-1}$ $NaH^{13}CO_3$ (sodium bicarbonate, 4 nM, $^{13}$C, 99%, Sigma Inc.) for three days, using the procedure described by Pascal et al. (2008a). Microalgae were then concentrated by centrifugation (1500*g* × 10 min, 20 °C), washed three times with 0.2 μm filtered seawater to remove the $^{13}$C-bicarbonate and kept at 0–4 °C until being added to the sediment.

To isotopically enrich bacteria (steps 3.1 to 3.4), 1 mL of surface sediment was incubated in a 20 mL medium for bacterial liquid culture in the dark at 13 °C for 24 h (Pascal et al., 2008b). The medium was $^{15}$N-enriched using 1 g L$^{-1}$ $^{15}NH_4Cl$ (ammonium chloride, $^{15}$N, 99%, CortecNet) mixed to a solution of peptone 3 g L$^{-1}$ (BioRad), yeast extract 1 g L$^{-1}$ (BioRad), sodium glycerophosphate 0.025 g L$^{-1}$ and sequestren Fe 6 g L$^{-1}$. Bacteria were cultured for 24 h in the $^{15}$N-enriched medium, concentrated by centrifugation (3500*g* × 10 min, 20 °C), washed three times with 0.2 μm filtered seawater to remove the $^{15}$N-ammonium and preserved at −80 °C until being added to the sediment.

A total of 8 plastic plates (0.60 × 0.40 × 0.05 m) were filled with the sieved sediment and left on the bottom of a tank with the same shape and area of the working chamber of the mesocosm for 2 days, until the addition of $^{13}$C-enriched microalgae and $^{15}$N-enriched bacteria (steps 4.1 and 4.2). The tanks were positioned inside a greenhouse, so that the sediment could experience field light conditions. The sediment was also exposed to natural tidal cycles, by controlling the flow of water in and out of the tanks with a series of pumps. The water in the tanks was renewed daily to avoid excessive increases in water temperatures and salinity due to water evaporation.

The $^{13}$C-enriched microalgae and the $^{15}$N-enriched bacteria were added to the surface layer (upper 2 mm) of the sediment placed in the plastic plates (step 5.1). The surface sediment layer was removed, mixed with the enriched micro-organisms and equally re-distributed on the sediment. This method allowed for the homogenization of the added $^{15}$N and $^{13}$C on the plates as much as possible.

The plastic plates were then kept in a tank inside a greenhouse for a period of 14 h before being used in the experiments. This was considered to be a sufficient period of time to recover from disturbance for both microalgal biomass and community structure (Peterson and Stevenson, 1992).

### 2.3. Experimental set up

We conducted two separate experiments for the young-of-the-year (YOY experiment) and the one-year-old fish (OYO experiment). The gray mullet is a gregarious species and exhibits schooling behavior (Whitfield et al., 2012). We used 6 fish of a mean TL ( ± SE) of 9.23 ± 0.16 cm and a mean FW ( ± SE) of 6.18 ± 0.19 g for the YOY experiment and 6 fish of a mean TL ( ± SE) of 16.40 ± 0.20 cm and a mean FW ( ± SE) of 33.27 ± 1.01 g for the OYO experiment. The sizes are in accordance to data on population structure and age done in natural populations of *L. aurata* from a range of environments where *L. aurata* is naturally distributed (Abdallah et al., 2012; Hotos and Katselis, 2011; Lebreton et al., 2011). The fish group being tested was placed in the acclimatization chamber of the mesocosm (4 × 1 × 1 m; Fig. 1b) and left undisturbed for 16 h before the experiment was run, following the protocol described by Como et al. (2014). This method allowed fish to recover from any stress caused during handling. The fish were not fed during this period.

Two plastic plates were used for the YOY experiment and six were used for the OYO experiment. This corresponded to a foraging area in the working chamber of the mesocosm of 0.48 m$^2$ and 1.44 m$^2$ for the YOY and OYO experiment, respectively. These different foraging areas

were set-up in order to obtain a constant biomass of fish per unit area (≈100 g $m^{-2}$, in our study). In both experiments, $^{13}C$ and $^{15}N$ were added in excess to avoid competition among individuals during the experiment. We assumed that the daily feeding ration of the fish corresponds to 8% of their biomass (De Silva, 1980; Laffaille et al., 2002) and we added an amount of $^{13}C$ corresponding to 16 and 42 times more than the feeding ratio in the OYO and YOY experiments, respectively. Similarly, we added an amount of $^{15}N$ corresponding to 4 and 3 times more than the daily feeding ratio in the YOY and OYO experiments, respectively. More precisely, in the YOY experiment, 5% of the total organic carbon in the sediment was $^{13}C$ and 13% of the total nitrogen was $^{15}N$, corresponding to 2017 mg $^{13}C$ $m^{-2}$ and 323 mg $^{15}N$ $m^{-2}$, respectively. In the OYO experiment, 3% of the total organic carbon in the sediment was $^{13}C$ and 11% of the total nitrogen was $^{15}N$, corresponding to 342 mg $^{13}C$ $m^{-2}$ and 192 mg $^{15}N$ $m^{-2}$, respectively. The fish group being tested was placed in the acclimatization chamber of the mesocosm (4 × 1 × 1 m; Fig. 1b) and left undisturbed for 16 h before the experiment was run, following the protocol described by Como et al. (2014). This method allowed fish to recover from any stress caused during handling. The fish were not fed during this period.

An hour and a half before the beginning of the experiment, the plastic plates of sediment were placed on the floor of the working chamber of the mesocosm (step 6.1 in Fig. 1a). The water level was raised by about 50 cm and the fish were encouraged to move from the acclimatization chamber to the working chamber. This was done by opening the sliding door and lifting up the floor of the acclimatization chamber. In general, the fish entered rapidly (in < 3-min). The experiment began when the fish started feeding on the sediment and lasted for 1 h. This was in accordance with previous short-term tracing studies concerning resource assimilation in fish (Berge et al., 1994; Eliason et al., 2010; Rojas-Garcia and Rønnestad, 2003). At the end of the experiment, the fish were encouraged to move back to the acclimatization chamber and the water level was progressively reduced in the working chamber (step 6.2 in Fig. 1a). In this study, the fish exposed to $^{13}C$-enriched microalgae and $^{15}N$-enriched bacteria are indicated as Experimental fish (hereafter called E-fish).

The background values for isotopic signatures of different tissues were measured in similar mesocosms, where 6 fish were treated as in the experimental mesocosms, but they were not exposed to $^{13}C$-enriched microalgae and $^{15}N$-enriched bacteria (hereafter called B-fish). The same procedures regarding maintenance and transfer of fish as well as sediment preparation were followed as for the experimental mesocoms, with the exception that $^{13}C$-enriched microalgae and $^{15}N$-enriched bacteria were not added to the sediment. One background mesocosm was run for each age (i.e. YOY and OYO). For each age (i.e. YOY and OYO), the values of TL and FW of E-fish and B-fish were analyzed using one-way ANOVA with E-fish vs. B-fish as a fixed factor. The analysis did not reveal any differences in TL and FW between E-fish and B-fish (One-way ANOVA: F values not shown; $p > 0.05$).

The mesocosm was set up in a room with natural light sources in order to allow photosynthesis to be carried out by microalgae during the experiment. The mesocosm experiments were performed in the same period of the day (in the morning) in order to avoid bias due to daily variations in the fish feeding activity (Almeida, 2003). All possible precautions were taken to limit fish stress during the experiments. Each fish handling was done with extreme care. None of the fish showed abnormal behavior or died during the experiment.

### 2.4. Data collection

At the end of the experiment, the fish were immediately sacrificed with MS222 (0.48 g $L^{-1}$) and dissected for sample collection. Each fish was analyzed separately. Blood samples were immediately taken from the caudal vein using heparinized syringes, whereas the other samples for liver and muscle tissue, as well as for digestive content (DC) were removed under the stereomicroscope. In addition, three sediment cores (2 cm i.d.) were collected per plastic plate to determine the remaining $^{13}C$ and $^{15}N$ concentrations and the total biomass of microalgae and bacteria using fluorimetre and API counts, respectively. In the YOY experiment, the two plastic plates were sampled, whereas in the OYO experiment we randomly chose two out of the six plastic plates used. At the end of the experiment, the amount of microalgae (expressed as chlorophyll-*a* weight) (mean ± SE; n = 6) in the sediment was 108 ± 6 µg Chl-*a* g $sed^{-1}$ and the amount of bacteria was $3.7 \times 10^7$ $mL^{-1}$ in the YOY experiment. In the OYO experiment, the amount of microalgae was 111 ± 23 µg Chl-*a* g $sed^{-1}$ and the amount of bacteria was $2.7 \times 10^7$ $mL^{-1}$.

All samples for stable isotope analyses were freeze-dried and ground using a ball mill. Prior to the isotopic composition analysis, the samples of the $^{13}C$-enriched microalgae preparation and those of sediment and digestive content of fish were acidified using a 1 N HCl solution to eliminate carbonates. The samples of liver, blood and muscle were treated with cyclohexane to remove naturally $^{13}C$-depleted lipids, following the protocol described by Chouvelon et al. (2011).

### 2.5. Data analysis

Isotopic measurements were taken with an EA-IRMS (Isoprime, Micromass, UK). Isotopic values were expressed in the δ notation as deviations from international standards (Vienna Pee Dee belemnite limestone, VPDB, for $\delta^{13}C$ and nitrogen in air, air $N_2$, for $\delta^{15}N$), following the formula: $\delta^{13}C$ or $\delta^{15}N = [(R_{sample} / R_{standanrd}) - 1] \times 10^3$, where R is the isotope ratio in $^{13}C/^{12}C$ or $^{15}N/^{14}N$.

R was derived from the measured $\delta^{13}C$ as $R = [(\delta^{13}C / 1000) + 1] \times R_{VPDB}$ and from the measured $\delta^{15}N$ as $R = [(\delta^{15}N / 1000) + 1] \times R_{airN2}$, where $R_{VPDB}$ and $R_{airN2}$ are the isotope ratio of international standards.

The fraction (F) of the heavy isotope of C or N compared to the total carbon and nitrogen measured is computed from R with $F = R / R + 1$ for $^{13}C$ and $F = R / R + 2$ for $^{15}N$ (Mariotti, 1982).

For each tissue (muscle, liver, and blood), the incorporation of label (I) was calculated as excess (above background) isotope uptake, such as:

$$I_i = B \times O_i \times (F_{Ei} - F_{Bi})$$

with *i* the label (C or N), B the tissue biomass (expressed in g dry weight, DW), O the % organic carbon or nitrogen in the dry tissue biomass, $F_E$ the faction of heavy label in E-fish and $F_B$ the faction of heavy label in B-fish. I is expressed in µg $^{13}C$ or $^{15}N$ and as percentage of total incorporation in the animal.

The dry biomass of muscle, liver and blood was not available for E-Fish, whereas their body fresh weight (FW) was taken. The DW biomasses for muscle, liver and blood of E-Fish were estimated from their body FW and correlation coefficients which were obtained from twenty-six new individuals of *L. aurata*. These individuals of a TL between 3.1 and 16.5 cm and a FW between 6 and 24.8 g represented the size of fish used in our study. In addition, they were maintained under laboratory condition following the protocol applied for E-Fish.

Therefore, the DW biomasses for muscle, liver and blood were estimated using the following equations:

(1) $y = 0.438x - 0.859$, and $z = 0.166y - 0.032$, where y = muscle FW (g); x = body FW (g) and z = muscle DW (g).
(2) $y = 0.021x - 0.023$ and $z = 0.120y - 0.002$, where y = liver FW (g), x = body FW (g) and z = liver DW (g).
(3) $y = 0.00062e^{0.633x}$, where x = body FW (g), y = blood DW (g).

Finally, incorporation of label (I) in the digestive content (DC) and in the bulk of the sediment was calculated as the product of the excess isotope and the amounts of organic carbon and nitrogen in the remaining DC and sediment at the end of the experiment. The $^{13}C$:$^{15}N$ ratio was then calculated in the DC and in the sediment. For both YOY

and OYO, the assimilation capacity of $^{15}N$ was estimated by comparing the amount of $^{15}N$ remaining in the DC to the total amount of $^{15}N$ found in the body. The assimilation capacity was not calculated for $^{13}C$, due to the removal of lipids from the muscle and liver samples.

Data for all variables are presented as a mean ± standard error. We used a paired *t*-test (Zar, 2010) to compare (i) the proportion of tracers ($^{13}C$ and $^{15}N$) between tissues and (ii) the $^{13}C$:$^{15}N$ ratio between DC and sediment, for YOY and OYO experiments, separately. Paired *t*-test was performed using the function 't.test' in the 'stats' package for R v 3.4.2 (R Development Core Team, 2015).We analyzed the $^{15}N$ assimilation capacity of YOY and OYO using a repeated measure linear-mixed model (GLM, Crawley, 2009). We used a GLM model with one fixed factor (age) and replicate fish as the random, repeated factor. We also compared two models using AIC (Akaike's An Information Criterion), one without and the other with fish as repeated measures, assuming the fish behavior could be correlated in the same mesocosm. Unless indicated otherwise in the Results section, we did not find any differences between the two models, and there were no different results using one or the other model, indicating that fish within the same mesocosm seemed not to affect their reciprocal behavior. GLM was also used to compare (iv) the background stable isotope signatures of the tissues and the DC between YOY and OYO. GLM analysis was performed using the functions 'lme' and 'lm' in the 'nlme' package for R v 3.4.2 (R Development Core Team, 2015). We estimated the variability among individuals of each age (YOY and OYO) using the standard error (Zar, 2010).

## 3. Results

In both the experiments, young-of-the-year (YOY) and one-year-old (OYO) fish had very small amounts of $^{13}C$ and $^{15}N$ in their blood (< 1.0%; Fig. 2a–d). The proportions of $^{13}C$ and $^{15}N$ assimilated in the blood were found to be lower than the proportion of tracers incorporated in the muscle and liver, in both YOY and OYO (Paired *t*-test: blood vs. muscle + liver: t values not shown; $p < 0.05$ for all tests).

In the YOY experiment, both the tracers ($^{13}C$ and $^{15}N$) were allocated differently to the liver and the muscle (Paired *t*-test between liver and muscle: t = 339.34 and 19.99, for $^{13}C$ and $^{15}N$, respectively, p = 0.00 for both the tests; Fig. 2a, c). Most of the $^{13}C$ and $^{15}N$ were allocated to the muscle (99% and 88% for $^{13}C$ and $^{15}N$, respectively), while the remaining labels were found in the liver (0.4% and 11% for $^{13}C$ and $^{15}N$, respectively). Conversely, in the OYO experiment, the $^{13}C$ and $^{15}N$ were uniformly distributed between liver and muscle (liver vs. muscle: t = 0.54, p = 0.61, and 0.29, p = 0.78; for $^{13}C$ and $^{15}N$, respectively; Fig. 2b, d). Overall, 57% of the $^{13}C$ and 45% of the $^{15}N$ were found in the liver, while 43% of the $^{13}C$ and 55% of the $^{15}N$ were found in the muscle. There was also more variability among OYO than YOY individuals, with two out of six animals showing little assimilation by the muscle (Tables 1, 2).

In the YOY experiment, there was a higher $^{13}C$:$^{15}N$ ratio in the digestive content (DC) of fish than in the sediment (mean ± SE; n = 6; 16.6 ± 0.9 and 10.0 ± 1.0 in the DC and the sediment, respectively; paired *t*-test: t = 4.37, p = 0.01), while the ratio was slightly lower in

**Table 1**
Incorporation of microalgal-derived $^{13}C$ ($\mu g^{13}C$) and bacterial-derived $^{15}N$ ($\mu g^{15}N$) and relative proportions (%) in muscle, liver and blood of individual fish of young-of-the-year (YOY) *L. aurata*.

| | YOY | | | | | |
|---|---|---|---|---|---|---|
| | | $\mu g^{13}C$ | | | % | |
| Individual | Muscle | Liver | Blood | Muscle | Liver | Blood |
| 1 | 6.2 | 0.0 | 0.0 | 99.3 | 0.4 | 0.3 |
| 2 | 4.4 | 0.0 | 0.0 | 99.3 | 0.0 | 0.6 |
| 3 | 4.6 | 0.0 | 0.0 | 99.1 | 0.1 | 0.9 |
| 4 | 4.8 | 0.0 | 0.0 | 99.2 | 0.2 | 0.5 |
| 5 | 4.9 | 0.0 | 0.0 | 98.7 | 0.6 | 0.7 |
| 6 | 4.7 | 0.0 | 0.0 | 98.4 | 0.9 | 0.7 |
| Mean | | | | 99.0 | 0.4 | 0.6 |
| ± SE | | | | 0.2 | 0.1 | 0.1 |
| | | $\mu g^{15}N$ | | | % | |
| 1 | 0.2 | 0.0 | 0.0 | 82.4 | 17.1 | 0.4 |
| 2 | 0.5 | 0.1 | 0.0 | 87.6 | 12.1 | 0.3 |
| 3 | 0.6 | 0.0 | 0.0 | 96.6 | 2.9 | 0.5 |
| 4 | 0.2 | 0.0 | 0.0 | 86.8 | 12.8 | 0.4 |
| 5 | 0.5 | 0.1 | 0.0 | 86.6 | 13.1 | 0.3 |
| 6 | 0.8 | 0.1 | 0.0 | 89.5 | 10.2 | 0.3 |
| Mean | | | | 88.3 | 11.4 | 0.4 |
| ± SE | | | | 1.9 | 1.9 | 0.0 |

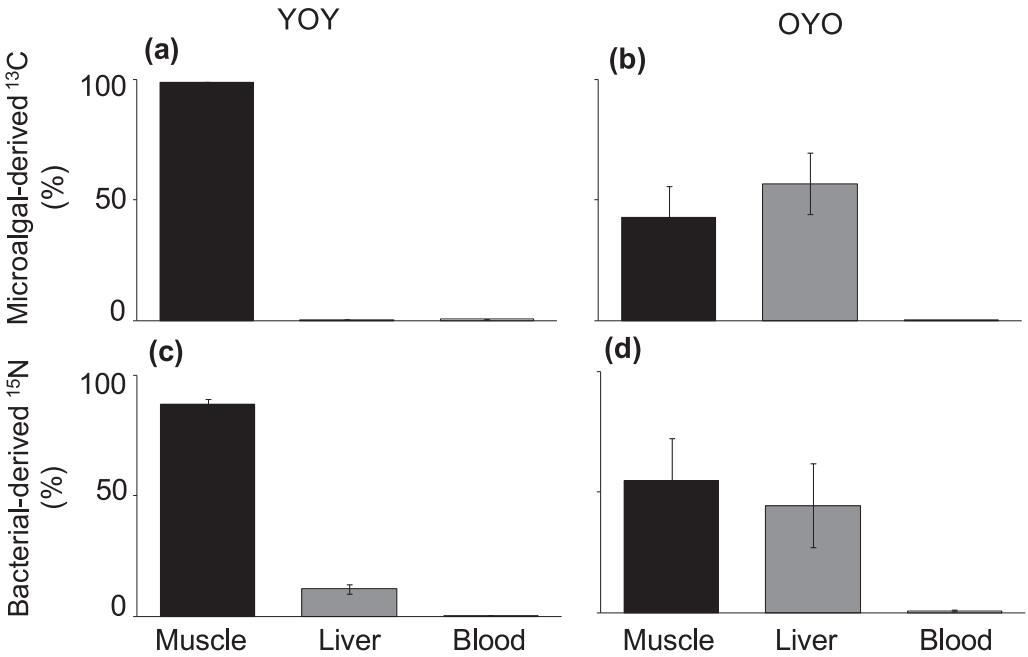


**Fig. 2.** Proportions of microalgal-derived $^{13}C$ and bacterial-derived $^{15}N$ (mean ± SE; n = 6) incorporated by muscle, liver and blood of young-of-the-year (YOY) and one-year-old (OYO) *L. aurata*.

**Table 2**
Incorporation of microalgal-derived $^{13}C$ ($\mu g^{13}C$) and bacterial-derived $^{15}N$ ($\mu g^{15}N$) and relative proportions (%) in muscle, liver and blood of individual fish of one-year-old (OYO) *L. aurata*.

| | YOY | | | | | |
|---|---|---|---|---|---|---|
| | $\mu g^{13}C$ | | | % | | |
| Individual | Muscle | Liver | Blood | Muscle | Liver | Blood |
| | $\mu g^{13}C$ | | | | % | |
| 1 | 8.2 | 2.6 | 0.0 | 76.1 | 23.9 | 0.1 |
| 2 | 1.7 | 1.5 | 0.0 | 52.7 | 47.3 | 0.0 |
| 3 | 17.7 | 5.5 | 0.0 | 76.3 | 23.7 | 0.0 |
| 4 | 0.0 | 2.6 | 0.0 | 0.0 | 99.6 | 0.4 |
| 5 | 0.7 | 4.0 | 0.0 | 14.2 | 85.8 | 0.1 |
| 6 | 2.8 | 4.4 | 0.0 | 38.7 | 60.9 | 0.4 |
| Mean | | | | 43.0 | 56.9 | 0.1 |
| ± SE | | | | 12.9 | 12.9 | 0.1 |
| | $\mu g^{15}N$ | | | | % | |
| 1 | 1.0 | 0.2 | 0.0 | 83.5 | 15.8 | 0.7 |
| 2 | 0.0 | 0.2 | 0.0 | 0.0 | 100.0 | 0.0 |
| 3 | 1.0 | 0.5 | 0.0 | 66.3 | 33.1 | 0.6 |
| 4 | 2.2 | 0.2 | 0.0 | 90.4 | 9.0 | 0.6 |
| 5 | 3.0 | 0.4 | 0.0 | 87.7 | 12.0 | 0.3 |
| 6 | 0.0 | 0.5 | 0.0 | 0.0 | 97.3 | 2.7 |
| Mean | | | | 54.7 | 44.5 | 0.8 |
| ± SE | | | | 17.6 | 17.5 | 0.4 |

the DC than in the sediment in the OYO experiment (0.9 ± 0.1 and 1.3 ± 0.0; t = 3.12, p = 0.01).

In both the experiments, there was a large amount of $^{15}N$ remaining in the DC of fish. Overall, 74% and 66% of the $^{15}N$ ingested was found in the DC of YOY and OYO individuals, respectively. These proportions did not differ between YOY and OYO (GLM: Estimated difference between ages = 8.29, SE ± 10.92, *t*-test = 0.76, p = 0.47).

With regard to the background stable isotope signatures, OYO fish showed $^{13}C$-enriched values of the muscle (−17.2 ± 0.1), blood (−18.8 ± 0.2) and DC (−18.8 ± 0.4) as compared to YOY fish (−18.7 ± 0.1, −20.4 ± 0.1 and −22.1 ± 0.2 for the muscle, blood and DC, respectively; GLM; Estimated differences between ages = −1.52, SE ± 0.14, −1.61, SE ± 0.18, −3.29, SE ± 0.42, *t*-test = −10.72, −8.87, −7.78 for the muscle, blood and DC, respectively; p = 0.00 for all tests; Fig. 3a, b). The analysis did not detect significant effects of age (p > 0.05) for the $\delta^{13}C$ values of liver, nor for the $\delta^{15}N$ values of muscle, liver, blood and the DC (GLM: results not shown; Table 3; Fig. 3a, b). While the DC of YOY (−22.1 ± 0.2) were more $^{13}C$-depleted than sedimentary organic matter (SOM; −20.5 ± 0.2), the DC of OYO (−18.8 ± 0.4) were more $^{13}C$-enriched than SOM (−20.9 ± 0.1) (Table 3; Fig. 3a, b). Furthermore, background data showed variability for $^{13}C$ (digestive content) and $^{15}N$ (muscle) among OYO individuals, as found for the enriched fish.

**Table 3**
$\delta^{13}C$ and $\delta^{15}N$ values in muscle, liver, blood and digestive content (DC) of young-of the-year (YOY) and one-year-old (OYO) *L. aurata* used as background values (mean ± SE; n = 6). $\delta^{13}C$ and $\delta^{15}N$ values of sedimentary organic matter (SOM) in each background mesocom were also reported.

| | Background values | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | YOY | | | | OYO | | | |
| | $\delta^{13}C$ | | $\delta^{15}N$ | | $\delta^{13}C$ | | $\delta^{15}N$ | |
| | Mean | ± SE | Mean | ± SE | Mean | ± SE | Mean | ± SE |
| Muscle | −18.7 | 0.1 | 12.0 | 0.1 | −17.2 | 0.1 | 11.0 | 0.8 |
| Liver | −19.9 | 0.1 | 11.7 | 0.2 | −19.8 | 0.1 | 11.8 | 0.3 |
| Blood | −20.4 | 0.1 | 11.8 | 0.1 | −18.8 | 0.2 | 10.6 | 0.6 |
| DC | −22.1 | 0.2 | 10.7 | 0.1 | −18.8 | 0.4 | 10.5 | 0.5 |
| SOM | −20.5 | 0.2 | 7.1 | 0.1 | −20.9 | 0.1 | 7.0 | 0.1 |

## 4. Discussion

There was a remarkable difference in the allocation of resources between young-of-the-year (YOY) and one-year-old (OYO) fish, despite the fact that they are both considered juvenile fish and share the same habitat, a shallow coastal area. These results could underline important, yet unknown, metabolic changes during the juvenile phases. However, part of the difference might be due to different feeding behaviors. We reasonably assumed both size classes and ages to have an omnivorous, benthic diet. It is well known that once offspring (smaller than 5 cm length) have recruited in shallow areas, juveniles change feeding habits and shift from a carnivorous diet made of pelagic animals to an omnivorous diet of benthic resources (Laffaille et al., 2000; Lebreton et al., 2011; Whitfield et al., 2012). We sampled individuals > 8 cm already recruited in shallow waters, thus relying on benthic food resources and occupying the same trophic level, as corroborated by similar $\delta^{15}N$ values found in the tissues of background fish. We focused on microbial sources, by mixing $^{13}C$-enriched benthic microalgae and $^{15}N$-enriched bacteria to sieved sediment and adding the mixture to experimental mesocosms 14 h before allowing the fish to feed. Despite having the same benthic diet, YOY and OYO individuals might show different feeding behaviors. Como et al. (2014) observed that the OYO animals feed occasionally and ingest large amounts of sediment. Instead, the YOY animals feed repeatedly and pick small quantities of surface sediment, which is visible when observing the frequent small feeding pits. This behavior could discriminate between the ingestion of food sources and probably affect the assimilation of carbon and nitrogen. Indeed, there were differences between the $^{13}C$:$^{15}N$ ratio in the bulk sediment and the digestive content (DC) of the YOY (10.1 ± 1.0 vs. 17 ± 0.9; mean ± SE), while clear-cut differences were not found between the bulk sediment and the DC of the OYO individuals (1.3 ± 0.0 vs.

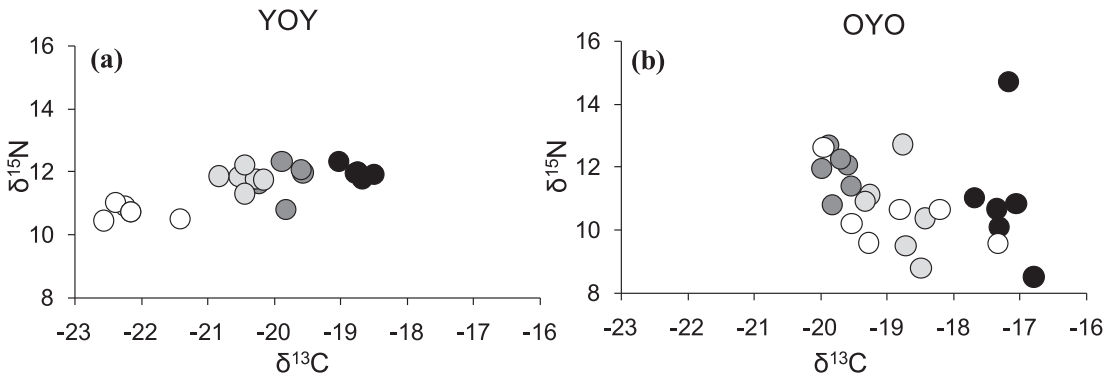


**Fig. 3.** $\delta^{13}C$ and $\delta^{15}N$ values in muscle, liver, blood and digestive content (DC) of (a) young-of the-year (YOY) and (b) one-year-old (OYO) *L. aurata* used as background values. Colors: Black = muscle; Gray = liver; Light gray = blood; White = DC.

0.9 ± 0.1). When we left the sediment mixture acclimatizing in the mesocosm for 14 h, the small sediment-dwelling invertebrates which remained in the sediment after sieving, mainly meiofauna, could have assimilated the tracers. This is because it takes 1 to 2 days to the meiofauna to start incorporating the tracers, $^{13}C$ or $^{15}N$, through bacterial and microalgal feeding (Evrard et al., 2010; Middelburg et al., 2000; Rossi et al., 2009). The differences in the isotopic composition of DC of fish and the bulk sediment might thus partly reflect differences in the abilities of picking up this labeled sediment-dwelling organisms.

Despite the fact that some of the observed differences in resource allocation could be explained by feeding behavior, about 90% of the $^{13}C$ and $^{15}N$ were measured in the muscle of the YOY while the tracers were equally partitioned between the muscle and liver in OYO fish. This large gap cannot be explained solely by slight variations in feeding behavior, but would involve metabolic changes. In fish, the liver serves as the primary regulating organ for circulating levels of C and N in the form of lipids, carbohydrates as well as free amino-acids and small peptides derived from the proteins (Evans and Claiborne, 2006; Grossell et al., 2010; Halver and Hardy, 2002). The C and N are absorbed while they are in the digestive tract and through the hepatic portal vein, they enter in the liver where they are further processed and sent to other body tissues or stored as reserve (Halver and Hardy, 2002). Free amino acids and peptides are immediately mobilized to the circulatory system or bound to lipids to form lipoproteins (Berge et al., 1994; Eliason et al., 2010; Rojas-Garcia and Rønnestad, 2003). The lipoproteins are important molecules for the regulation and transport of dietary lipids to adipose tissues where they are accumulated as energy storage (Eliason et al., 2010; Halver and Hardy, 2002; Sheridan, 1988). In our study, lipids were removed from muscle and liver samples before isotopic analysis and carbohydrates represented a fairly small fraction of the body mass (stored glycogen is < 1% of fish body; Halver and Hardy, 2002). Therefore, the $^{13}C$ and $^{15}N$ measurements are an estimate of the accumulation of the total C and N as proteins and amino-acids.

Fish were sacrificed one hour after ingesting the tracers. Rojas-Garcia and Rønnestad (2003) showed that radio-labeled dietary free amino-acids and small peptides reached a peak in the liver between 30 min and 1 h after feeding in juveniles of *Hippoglossus hippoglossus*. This suggests that in our experiments animals were given enough time to process food, extract most of the free amino acids and small peptides and have them pass through the liver into the circulatory system to be used for growth and reproduction. These molecules should then appear in the plasma soon after feeding (Berge et al., 1994; Espe et al., 1993; Rojas-Garcia and Rønnestad, 2003). Unfortunately, we analyzed blood cells and plasma together, thereby introducing a bias in capturing tracers in the plasma. One hour was probably too short a time to allow the hemopoietic organs, such as thymus, kidney and spleen to encounter the tracers and incorporate them in the blood cells. As a consequence, most of the blood cells in our samples were unlabeled, thereby hiding the $^{13}C$- and $^{15}N$-enrichment of the plasma.

Therefore, assuming that free amino-acids and small peptides have passed through the liver, the lack of accumulation of both tracers in the liver of the YOY while they accumulate in the liver of OYO suggests that young individuals start building reserves during their second year of residence in coastal habitats, before migrating and reproducing in the ocean. In addition, the accumulation of tracers in the muscle, a tissue implicated in growth, strongly suggest that younger individuals invest more in growing during their first year than in their second year. This is consistent with the fact that the *L. aurata* juveniles grow remarkably fast during their first year of life, attaining over 60% of their maximum length as observed in natural conditions (Abdallah et al., 2012; Shekk et al., 1990).

The fast growth of smaller-sized juveniles and the accumulation of reserves in larger individuals could be a strategy for increasing survival up to the age of their first sexual maturation, which is around 2–3 years (Abdallah et al., 2012; Hotos et al., 2000; Hotos and Katselis, 2011). Predation is one of the most important causes of mortality of fish in shallow coastal areas and the predation risk decreases as the fish size increases (Biro et al., 2005; Kelley, 1987; Sogard, 1997). Juvenile gray mullets have numerous predators, including adult European seabass and shorebirds (Cosolo et al., 2011; Liordos and Goutner, 2009; Riley et al., 2011). For instance, in the Cabras lagoon, Como et al. (2017) found that small juveniles *L. aurata* corresponding to YOY size contribute frequently to the diet of cormorants (Como et al., 2017). The risks of predation of the *Liza* spp. YOY in shallow coastal lagoons and the upper intertidal zones of estuaries are counterbalanced by food availability, necessary for growing and recruiting into larger size-classes (Carpentier et al., 2014; Lebreton et al., 2011). The accumulation of reserves may allow the larger sizes to migrate to the subtidal zones and survive in these zones where food availability is lower, but where they are less visible to predators, especially shorebirds. Previous studies on the population dynamics of *L. aurata* and the sympatric *L. ramada* have described a general ontogenetic change in the migratory patterns of juveniles in micro- and macro-tidal systems (Carpentier et al., 2014; Katselis et al., 2010, 2007, 2003; Laffaille et al., 2000; Lebreton et al., 2011). In the micro-tidal system of the Mediterranean Sea, for instance, periodical migrations from lagoons to the adjacent subtidal areas have been observed for juveniles larger than 10 cm in length, corresponding to the size of OYO individuals (Katselis et al., 2010, 2007, 2003). Similarly, OYO individuals are found to stay in subtidal zones of macro-tidal systems of the North Atlantic coasts and do not undertake migration during ebb tide in the upper intertidal zones as instead observed for the smaller juveniles (Carpentier et al., 2014; Laffaille et al., 2000; Lebreton et al., 2011). Future studies should simultaneously investigate the allocation of resources and migration from a range of environments where the *L. aurata* is naturally distributed.

## 5. Conclusion

In summary, stable isotope tracing successfully revealed interesting patterns in the nutrition physiology of *L. aurata* juveniles. Our study provides the first evidence that juvenile classes of *L. aurata* can shift the way they allocate the food sources, passing from a metabolism focused on rapid growth to balancing between growth and reserve accumulation. This could be a strategy for fast growth in order to be able to migrate and survive in less productive areas, where predation risk decreases. This size-dependent shift in resource allocation indicates that the first year after recruitment might represent a bottleneck for the survival of local populations. Threats to coastal areas such as warming and hypoxia could undermine the residence of these juveniles in coastal areas and reduce fish stock.

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## Conflict of interest

None of the authors has any conflicts of interest.

## Ethical approval

All experimental procedures in this work comply with the current laws in France.

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