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ORIGINAL PAPER

# New tool to elucidate the diet of the ormer *Haliotis tuberculata* (L.): Digital shell color analysis

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**Abstract** Food sources of the European abalone *Haliotis tuberculata* throughout its life cycle are still to be clarified in nature. A novel non-destructive method of digital shell color analysis to reveal the diets of European abalone (ormer) was developed in this study. The method was calibrated using ormers reared under experimental conditions in North Western Brittany in 2012 and fed a controlled monospecific diet to define the shell hues associated with various macroalgae (i.e., Rhodophyta, Chlorophyta, and Phaeophyta). General food preferences were established by comparing the shell hue of wild adult ormers and experimental adult ormers. Shell hue corresponds to the color tint in the HSL color space measured on digital pictures of the shell. Experimentally, shell hue values differed according to treatment, with the most yellow-green hue (72°) for ormers fed *Saccharina* sp. and the coral hue (25°) for ormers fed *Palmaria palmata*. High variation in shell color of wild ormers was observed according to the sampling site and/or ontogeny. The diet of wild ormers may be related to the abundance of different drifting algae in their respective habitats. Thus, this non-destructive and easy-to-use technique appears to be a promising tool for determining the diet of *Haliotis* species and, perhaps, other herbivorous mollusks.

**Keywords** Numerical color · Shell hue · HSL color space · Abalone · Food sources · Experiment

## Introduction

The diets of herbivorous marine animals are not easily determined, because observations of feeding in nature are very difficult. Gut content analyses are subject to severe biases due to differential rates of digestion and loss of identifiable characteristics of algae in guts (Day and Cook 1995) and reveal only the last meal. Stable isotope analysis requires the knowledge of isotopic fractionation between sources and the consumer which vary between species, with the quantity of food or the growth rate (Vander Zanden and Rasmussen

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2001; Caut et al. 2009; Emmery et al. 2011). Furthermore, the stable carbon and nitrogen isotope signature in macroalgae may vary over time and space (Raven et al. 2002) and does not allow clear discrimination of the different species of Phaeophyta (Dethier et al. 2013). Finally, food preference experiments have no basis for duplicating the range of available algae and the levels of starvation that may be experienced in the field. Thus, a record of what has been eaten over time would be very useful. Here, we apply a method to estimate the diet of *Haliotis tuberculata* L. which can be tested on other herbivorous mollusks.

*Haliotis* spp. are benthic grazers living in rocky shore crevices and under rocks where they capture drifting algae. Their abundance increases with the complexity of substrate which provides protection against predators (Shepherd 1973). The only European species is *Haliotis tuberculata* L., known as the ormer. Its distribution extends into the Eastern Atlantic from the southern English Channel down to the northwest coast of Africa (Mgaya 1995).

The abalone diet changes during ontogeny. Post-larvae are able to feed on benthic diatoms, usually living on coralline algae (Kawamura and Takami 1995; Won et al. 2010). Juvenile abalones eat microalgae and attached or drifting soft macroalgae (Nash 1991; Wood and Buxton 1996; Takami and Kawamura 2003; Won et al. 2010). As abalones grow, their feeding switches to drifting macroalgae (Nash 1991; Mgaya 1995; Takami and Kawamura 2003; Guest et al. 2008; Won et al. 2010). Juveniles and adults abalones feed mainly at night (Nakamura and Archdale 2001; Baldwin et al. 2007). The change in abalone diet is related to the modification of digestive enzyme activity and radula morphology mostly occurring during the post-larval stage (Takami and Kawamura 2003; Onitsuka et al. 2004).

The food preferences of abalones have long been studied, mainly in laboratory or hatchery conditions, due to their high commercial importance. These studies tested various microalgae and macroalgae and formulated diets aimed at obtaining low post-metamorphosis mortality rates and optimized growth rates in juveniles (Kawamura et al. 1998; Stott et al. 2002). Studies of abalone diet in their natural environment are scarce, mainly because their execution is complex. The food preferences of adult abalones for Rhodophyta, Chlorophyta, or Phaeophyta are still to be clarified. Abalone species are reported to feed on a wide range of algae including Phaeophyta and Rhodophyta (Shepherd and Steinberg 1992). McShane et al. (1994) observed that the toughness of algae is the main criterion for the choice of food by *Haliotis rubra*, but grazing also depends on the availability of algae in the environment, because *Haliotis* spp. are opportunistic grazers (Shepherd and Steinberg 1992; Day and Cook 1995).

Mollusk shells are composed of calcium carbonate crystals layers (aragonite and/or calcite). The shell is a durable structure that records variations in environmental conditions through the incorporation of atoms or molecules from the surrounding environment during growth (Wilbur and Saleuddin 1983). Pigments are incorporated into mollusk shells, resulting in various shell colorations within a species and between species. Shell pigments, only synthesized in the plant kingdom, are composed of polyacetylenic molecules, and carotenoids complexed with some forms of protein (Fox 1966; Barnard and De Waal 2006; Hedegaard et al. 2006). Melanin, porphyrin, and psittacofulvin are also found in the molluscan shell matrix (Comfort 1951; de Oliveira et al. 2013). Shell pigmentation results mainly from a combination of genetic factors (Winkler et al. 2001; Jackson et al. 2006; Liu et al. 2009; Roussel et al. 2013; Ge et al. 2015; Yue et al. 2015) and diet (Leighton 1961; Lindberg and Pearse 1990; Manríquez et al. 2009). A shift in food sources can lead to a species-specific modification of shell pigmentation (Olsen 1968a; Underwood and Creese 1976; Gallardo et al. 2003; Liu et al. 2009; Stemmer and Nehrke 2014; Hoang et al. 2016).

The influence of diet on abalone shell color has already been demonstrated by several authors (Leighton and Booloottian 1963; Olsen 1968b; Gallardo et al. 2003; Liu et al. 2009; Hoang et al. 2016), but no attempt has been made to use shell pigments to decipher the food sources of wild abalones. Color analysis of digital pictures is a non-destructive method already used by Tlusty (2005) to assess how diets containing carotenoid add color to the shell of white American lobsters. Hancz et al. (2003) applied this technique to evaluate the coloration of goldfish and koi carp with diet changes. Hoang et al. (2016) studied abalone shell hue in pictures to highlight shell color variation according to macroalgae supply.

The goal of the present study was to develop a new method based on digital color analysis to understand the link between shell color and algal pigments in experimental conditions. We supplied a monospecific diet to ormers to find specific shell color values according to algal sources. These values were then used to study the shell color of ormers originating from the natural environment to determine their diet. The results provide a first experimental basis to develop a novel tool to understand the diet of *H. tuberculata* populations in their natural environment.

## Materials and methods

### Experimental approach to controlling ormer food sources

In situ experiments were carried out at Aber Wrac'h ria (48°36′46N; 4°33′30W; North Western Brittany, France,

Fig. 1). The experimental individuals were born in June–July 2010 in the hatchery (France Haliotis, Fig. 1). This cohort came from the mating of 8 females and 4 males. To avoid inbreeding, either the male or female came from the natural environment for each mating, the other parent being hatchery-reared ormers. They were reared as one group for 1 year in land-based tanks containing plates (60×60 cm; pictures in Roussel et al. 2013) covered with mostly *Ulvella lens* and some benthic diatoms. Ormers aged 8 months were then fed *Ulva* sp. and *Palmaria palmata* every week, in addition to *U. lens* which remained the highly dominant food source. During summer 2011, 52,500 individuals were transferred to the sea into six growing structures composed of four square compartments (1×1×0.5 m; only 21 compartments were used) in the experimental area (Fig. 1), where they were kept until the end of January 2012. Each compartment contained eight lines of 42 oyster catchment cups as shelters for the ormers, with 2,500 juveniles per compartment. The ormers were fed *ad libitum* once a month with a mix of *P. palmata, Saccharina latissima*, and *Laminaria digitata*.

On February 9, 2012, the experiment started with 21 000 ormer juveniles (mean ± standard deviation; size 24 ± 0.15 mm; weight 2.2 ± 0.041 g) distributed equally into six farm caging structures called "Abblox". Each Abblox was composed of four 1 $m^3$ compartments (square mesh size 5 mm) that contained 18 lines of 30 oyster catchment cups and 1000 juveniles per compartment. The Abblox were immersed in the experimental area 50 cm above the seafloor (height of stand) at a depth of 8 m. After this date, ormers were fed every month at spring tides with a single macroalgal species in each compartment within the six Abblox, so that a minimum of 5% of the initial macroalga weight remained before each new feeding. The algae leftovers were removed before each feeding. Seven macroalgae were chosen on the basis of their availability in the experimental area, their palatability to ormers (Mgaya 1995), and their nutritional value (Mercer et al. 1993; Mai et al. 1996): two Rhodophyta, *Palmaria palmata* and *Gracilaria* sp.; two Chlorophyta, *Ulva lactuca* and *Ulva* (*Enteromorpha*) sp.; and three Phaeophyta, *Laminaria digitata, Laminaria hyperborea* (only the stipe), and *Saccharina* sp. Macroalgae were collected in the intertidal zone 24 h before each feeding. Each diet treatment was composed of three replicates: three compartments with the same macroalgal diet were randomly distributed in the six Abblox with four compartments (ormers in the three other compartments were used for companion studies).

At the end of the experiment (December 18, 2012), five ormers were sampled haphazardly from the three compartments for each treatment (total 35 ormers). The soft tissues were removed and the shells brushed and rinsed with water and kept away from light to preserve the pigments until the shell color analysis.

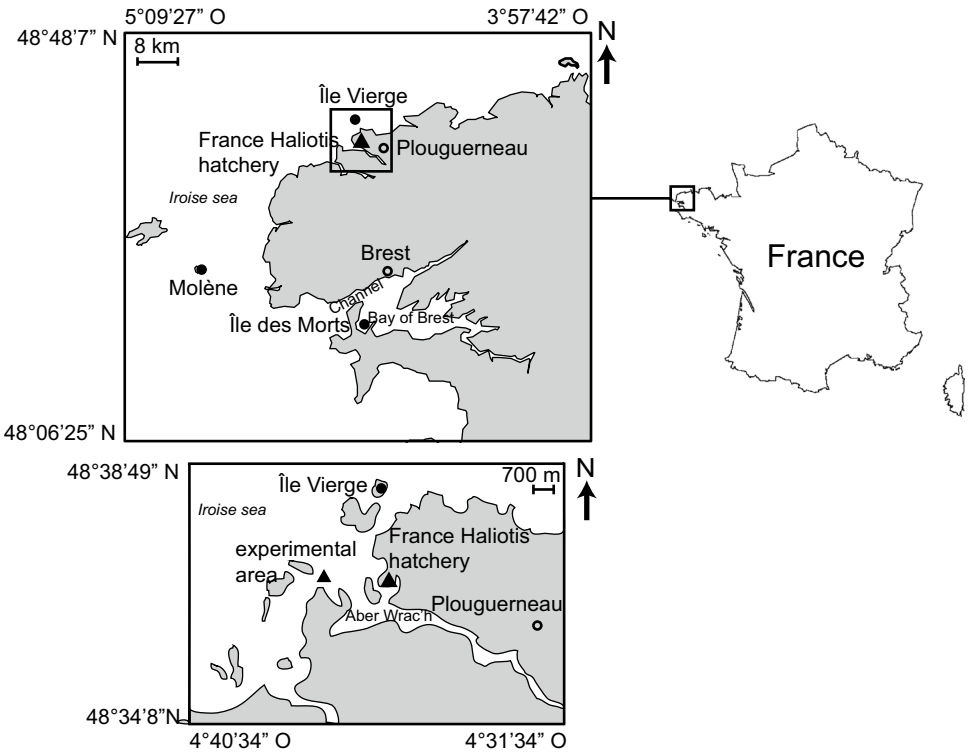


**Fig. 1** Location of the France Haliotis hatchery where *Haliotis tuberculata* were born and reared, the experimental area, and the three sites where wild ormers were sampled: Île des Morts (in the Bay of Brest), Île Vierge, and Molène. *Full circles* wild ormers; *full triangles* experimental ormers

The seawater temperature was recorded every 20 s by a probe (HOBO® U20) placed within a compartment of an Abblox from March 10, 2012, to December 10, 2012.

### The field-based approach

To assess the ormer diet in their natural environment based on their shell color, seven individuals were sampled by scuba divers: three at the Île des Morts in the Bay of Brest on March 4, 2014 (~5.7 cm in size), three at Molène Island in April 2014 (~6.5 cm in size), and one at the Île Vierge, close to the experimental area, on January 31, 2014 (~8.4 cm in size) (Fig. 1). After removing the soft tissues, the shells were brushed and rinsed with water to eliminate epibionts, which were more abundant on these shells than the experimental ormer shells. Hydrochloric acid was not used to avoid shell pigment damage, as pigments are localized in the prismatic layer in abalones (Budd et al. 2014). Shell color was measured to one side of any large epibionts. These shells are referred to as "wild" ormers.

### Shell color analysis

To assess shell color traits according to the algal diet, the entire shell was photographed using a 36 million pixel Nikon® D800 camera with a 50 mm f/1.4 Nikor lens and a f/11 aperture. To prevent dark shadows and light reflection on the shells, the ormers were photographed using two Elinchrom® Style RX600 electronic flashes equipped with neutral light boxes. This setup provided diffuse and homogeneous lighting. Colors were calibrated with the color chart QPcard® 201 (http://www.sirui-photo.us/QPcard/rgb-values.jpg) and the Adobe Photoshop® CS5 software (version 12.0).

The digital color of pixels can be described by different methods, such as the RGB and HSL color spaces. The color of digital images (photographs and scans) acquired in the RGB color space is composed of a combination of three layers (red, green, and blue), each comprising 256 levels of luminance (lightness) in 8 bits. In this color space, the slightest variations in saturation and luminance induce varying values in the three color layers, making interpretation difficult. On ormer shells, the saturation and luminance of color can vary greatly according to the lighting conditions, the shell ridges, and biological factors (e.g., shell growth; Comfort 1951). The HSL color space can provide a solution by describing the color components separately. This color space discriminates the hue (H), saturation (S), and lightness (L). The hue, or color tint, describes the color spectrum on a chromatic wheel (see Online Resource 1) from 0° (red) to 360° (red) with 120° for green and 240° for blue. The saturation represents how vivid the color is, from non-color (0%) to pure color (100%, see Online Resource 1). The lightness ranges from 0% (black) to 100% (brightest color, see Online Resource 1).

Different methods can be used to transform RGB values into hue values, giving roughly the same results in our study (Travis 1991; Cheng et al. 2001; Karcher and Richardson 2003; Gonzalez and Woods 2008). The relatively simple conversion method described by Karcher and Richardson (2003) was chosen for this study. RGB color space was converted to HSL color space as follows. The red, green, and blue values of pixels along the maximum shell growth axis (near the respiratory pores and corresponding to the spiral length) (Roussel et al. 2011) were sampled on each photograph. The values were then averaged over a 40×40 pixel square because of the high density of pixels resulting in strong variability in the RGB values between close pixels. The RGB values were transformed into a percentage by dividing each level by 255. The saturation was then calculated following equation (Karcher and Richardson 2003):

$$\text{Saturation} = [\max(R,G,B) - \min(R,G,B)] \,/\, \max(R,G,B). \quad (1)$$

If the saturation is equal to zero, the hue value cannot be obtained from the RGB value. Therefore, the percentages of RGB were converted into hue values using the following equation (Karcher and Richardson 2003):

$$\begin{aligned}
&\text{if } \max(R,G,B) = \min(R,G,B)\text{: hue} = 0\\
&\text{if } \max(R,G,B) = R\text{: hue} = 60 \times \left[(V-B) \,/\, (\max(R,G,B) - \min(R,G,B))\right]\\
&\text{if } \max(R,G,B) = V\text{: hue} = 60 \times \left(2 + \left[(B-R) \,/\, (\max(R,G,B) - \min(R,G,B))\right]\right)\\
&\text{if } \max(R,G,B) = B\text{: hue} = 60 \times \left(4 + \left[(R-V) \,/\, (\max(R,G,B) - \min(R,G,B))\right]\right)\\
&\text{if hue} < 0\text{: hue} = \text{hue} + 360.
\end{aligned} \quad (2)$$

### Dating of brown bands stopping the green color of experimental ormer shells

The experimental shells from ormers fed Phaeophyta and Chlorophyta were mainly green, interspersed with brown bands, though the food was consistent over time (see Online Resource 2). To understand the source of the brown bands, their appearance was assessed on three ormers fed *L. digitata* (individuals 1, 4, and 5; see Online Resource

2). As the growth patterns on the surface of abalone shells are difficult to read and interpret (Roussel et al. 2011), the oxygen isotopic composition of these shells in relation to the water temperature was used in order to date the time of appearance of brown and green bands (Jolivet et al. 2015).

The outer carbonate layer of the three ormer shells was sampled at each brown band, at each green band, and before the appearance of the green color, using a Dremel drill with a 0.3-mm grinding tip. Each small groove was cut following the growth line until a sufficient amount of material was obtained for a $\delta^{18}O$ analysis (70–100 µg). A total of 62 aliquots of *H. tuberculata* carbonate were collected and stored in clean glass vials until analysis. The $\delta^{18}O_{shell}$ of the carbonate samples was analyzed using a Finnigan MAT 253 isotope ratio mass spectrometer coupled with a Gas Bench II automated sampling device at Mainz University, Germany. The results were reported as per mil (‰) deviations relative to the Vienna Pee Dee Belemnite (V-PDB) carbonate standard. Repeated measures of the NBS-18 and IVA Carrara standards yielded a precision of 0.03 and 0.07‰, respectively. The $\delta^{18}O_{shell}$ was converted into temperature, allowing sample dating, following the protocol detailed in Jolivet et al. (2015). The general trends of temperature calculated from the $\delta^{18}O_{shell}$ samples coincided with the trends in temperature measured in seawater (Fig. 2). Thus, the approximation of sample dating was consistent.

### Data analysis

All graphic and statistical analyses were performed using the R software (R Core Team 2015). The significance level was 95%.

*Experimental ormers* As ormer length was not measured or marked (too stressful and too many individuals) at the beginning of the experiment, identifying the beginning of the area of interest on the shell is difficult. For the ormers fed *L. digitata, Saccharina* sp., *Ulva lactuca*, and *Ulva* sp., we considered that the appearance of the first green band corresponded to the beginning of the experiment, and the hue of the shell formed during the experiment was defined for both green and brown bands (see photographs in Online Resource 2). First, the different color bands were delimited visually on the photographs. Next, they were adjusted with hue values: when abrupt modifications of hue were observed inside a same color band, this band was divided into several distinct bands (see an example in Online Resource 3). For ormers fed *L. hyperborea, Gracilaria* sp., and *P. palmata*, shell color was visually homogenous. Thus, the experimental part was defined by the part of the shell where the hue value was consistent with the hue seen at the shell edge (see Online Resource 3). For interpolation at the population level, 95% confidence intervals were calculated for hue with the Student's *t* test and compared between treatments and color bands. Different analyses of variance (Welch's robust ANOVA tests, due to heterogeneity of variance) were performed to compare: (1) the hue values of different individuals within the same treatment for green or brown color bands for inter-individual variability, and (2) hue values of green or brown bands between dietary treatments for their effect on shell hue. For the latter, if a diet effect was significant, a pairwise test was conducted with the Holm method and non-pooled standard deviation.

*Wild ormers* The hue values of the seven wild ormers were plotted against the distance in spiral length from the apex (the oldest shell) to the edge (the most recent shell). This allows one to estimate hue variations with relative age. Hue values were compared in wild and experimental ormers to evaluate their diet. Moving medians (calculated based on 101 points) were used to smooth the hue values.

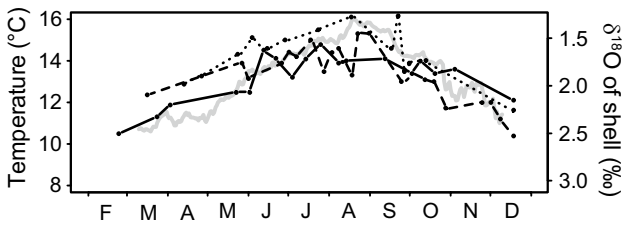


**Fig. 2** $\delta^{18}O$ of ormer shells converted into the temperature recorded in carbonates (*black lines* and *black points* for measured samples) for the three individuals fed *Laminaria digitata* (*solid line* individual 1, *dashed line* individual 4, *dotted line* individual 5). The seawater temperature in the experimental area is represented by *solid grey lines*

## Results

### Hue values of experimental ormer shells

The hue values varied within the same treatment (see Table 1, $P<0.01$ for each Welch's robust ANOVA between individuals in each treatment and each color of band). Green bands were identified on the shells of ormers fed Chlorophyta and Phaeophyta, with hue values varying from 48.5° to 72.9°, higher than those of brown bands (25.1°–41.2°; Fig. 3; Table 2). The hue values of these green bands were significantly different between each diet treatment (Table 1, $P<0.01$ for all pairwise comparisons). The hue values closest to the green color corresponded to ormers that fed on the brown algae (especially *Saccharina* sp.), in contrast to the hue values (close to the yellow color) of ormers fed *Ulva* sp. (Fig. 3). The hue values of the brown bands were significantly different between diet treatments (Table 1, $P<0.01$ for pairwise comparisons), except for *L. hyperborea* and *Gracilaria* sp. ($P=0.28$, pairwise test; Fig. 3; Table 2). Brown bands with hue values closest to 0° (red color) were observed with Rhodophyta (*P. palmata*) and those farthest from 0° were for *Saccharina* sp. (Fig. 3).

**Table 1** Results of Welch's robust ANOVA tests on hue values

| | Value of the statistic | | Degrees of freedom (*df*) | | *P* value | |
|---|---|---|---|---|---|---|
| *Tests on hue values of the different individuals within the same treatment for green or brown color bands* | | | | | | |
| | Green bands | Brown bands | Green bands | Brown bands | Green bands | Brown bands |
| *Laminaria digitata* | 838.65 | 53.25 | num. *df* = 4<br>denom. *df* = 3671.27 | num. *df* = 4<br>denom. *df* = 939.60 | <0.01 | <0.01 |
| *Saccharina* sp. | 431.99 | 39.28 | num. *df* = 4<br>denom. *df* = 3287.77 | num. *df* = 4<br>denom. *df* = 1202.84 | <0.01 | <0.01 |
| *Ulva lactuca* | 812.46 | 458.24 | num. *df* = 4<br>denom. *df* = 1942.14 | num. *df* = 4<br>denom. *df* = 1955.53 | <0.01 | <0.01 |
| *Ulva* sp. | 376.28 | 214.77 | num. *df* = 4<br>denom. *df* = 2612.66 | num. *df* = 4<br>denom. *df* = 1973.74 | <0.01 | <0.01 |
| *Laminaria hyperborea* | | 716.24 | | num. *df* = 4<br>denom. *df* = 1142.16 | | <0.01 |
| *Gracilaria* sp. | | 2504.70 | | num. *df* = 4<br>denom. *df* = 4220.26 | | <0.01 |
| *Palmaria palmata* | | 7241.34 | | num. *df* = 4<br>denom. *df* = 3965.22 | | <0.01 |
| *Tests on hue values of green or brown bands according to treatments* | | | | | | |
| | Green bands | Brown bands | Green bands | Brown bands | Green bands | Brown bands |
| | 2915.87 | 3047.54 | num. *df* = 4<br>denom. *df* = 12785.44 | num. *df* = 6<br>denom. *df* = 11463.76 | <0.01 | <0.01 |

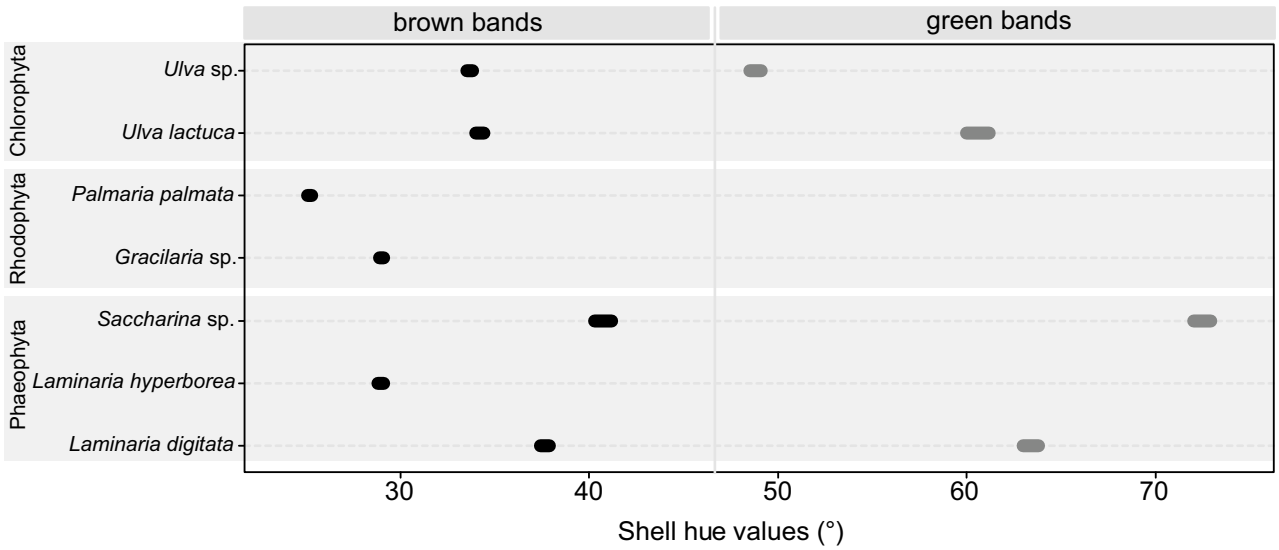


**Fig. 3** 95% confidence intervals for the shell hue of experimental *Haliotis tuberculata* for each treatment (in ordinate). *Grey confidence intervals* indicate hue values for green bands. *Black confidence intervals* indicate hue values for brown bands

### Dating of brown bands on ormers fed *Laminaria digitata*

On the three individuals assayed, visible brown bands mainly appeared during winter and spring (from February to April and from November to December; Fig. 4). For the rest of the year, the appearance of brown bands frequently matched feeding days (within a few days), with some individual variability (Fig. 4). For feeding days when brown bands were not visible on shells (mainly in summer, from July to September; Fig. 4), hue values decreased few days before or around the day of feeding.

### Hue values of wild ormer shells

The hue values of the seven wild ormer shells varied between individuals, sampling sites, and with age. For the samples from the Bay of Brest (Fig. 5a), the juvenile

**Table 2** Hue of green and brown bands on the experimental shells according to treatment

| | Treatment | Green bands | | Brown bands | |
|---|---|---|---|---|---|
| | | Mean | 95% confidence interval | Mean | 95% confidence interval |
| Phaeophyta | *Laminaria digitata* | 63.4 | 63.0–63.8 | 37.6 | 37.4–37.9 |
| | *Laminaria hyperborea* (stipe) | | | 28.9 | 28.8–29.1 |
| | *Saccharina* sp. | 72.4 | 72.0–72.9 | 40.8 | 40.3–41.2 |
| Chlorophyta | *Ulva lactuca* | 60.0 | 60.0–61.2 | 34.2 | 34.0–34.4 |
| | *Ulva* sp. | 48.8 | 48.5–49.1 | 33.6 | 33.5–33.8 |
| Rhodophyta | *Gracilaria* sp. | | | 29.0 | 28.9–29.1 |
| | *Palmaria palmata* | | | 25.2 | 25.1–25.3 |

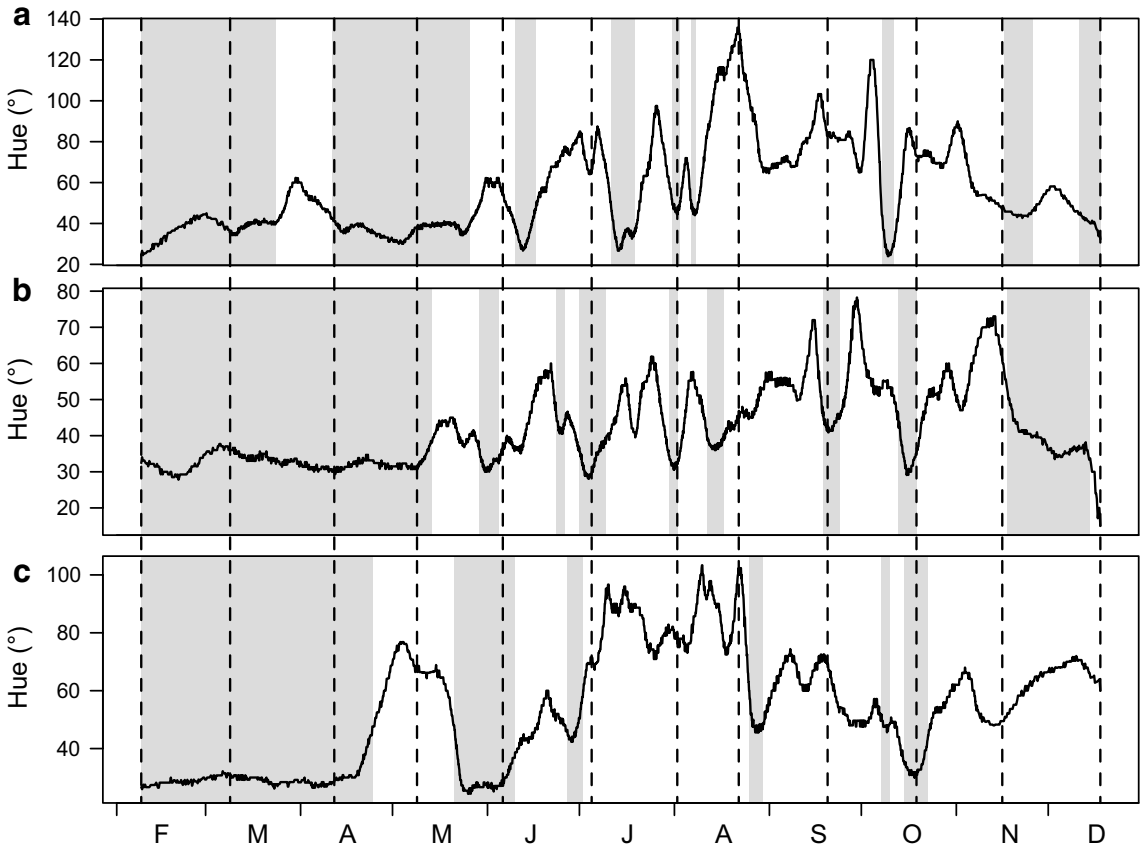


**Fig. 4** Shell hue values (*solid black lines*) according to the experimental time (from February 9, 2012, to December 18, 2012) for the three ormers fed *Laminaria digitata* (**a** individual 1, **b** individual 4, **c** individual 5). *Grey rectangles* indicate visible brown bands on the shells. *Vertical dashed lines* indicate feeding days

period (near the apex) was characterized by high individual variability in hue values (from 34° to 60° within a few millimeters and then from 60° to 20° within a few millimeters along the shell spiral, for one individual). The hue values stabilized and homogenized around 20° toward the shell edge for the three ormers (Fig. 5a). The ormer sampled at Île Vierge followed the same trend, with the hue stabilizing around 30° (Fig. 5c). For the ormers from Molène, the hue values were in the same range in the juvenile part of the shell but differed between individuals with age (Fig. 5b).

The relationship between the hue values of wild ormers and the confidence intervals found under experimental conditions varied between sampling sites. In the Bay of Brest, the hue values of the three ormers were similar to those fed Phaeophyta and Chlorophyta (Fig. 5a).

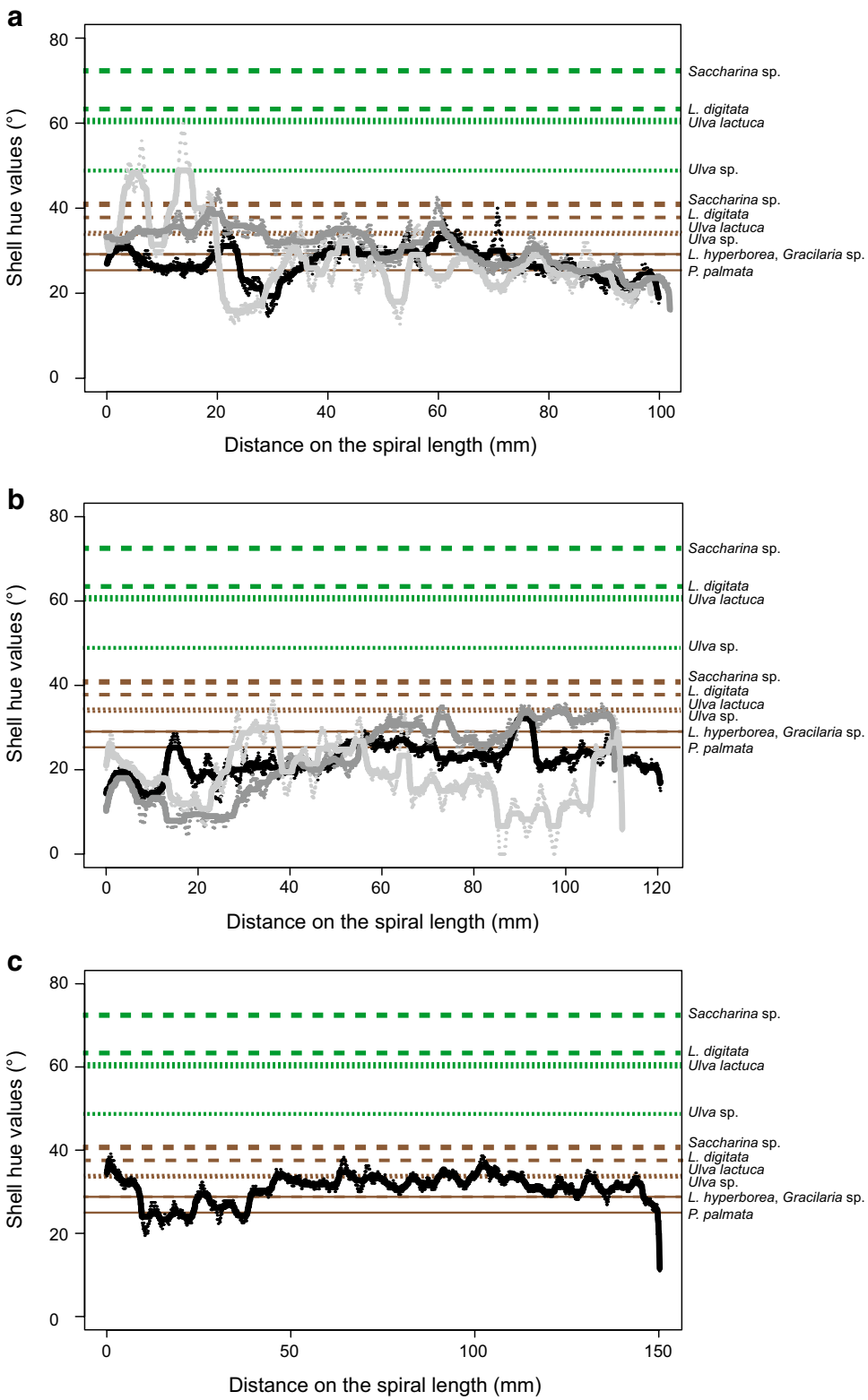


**Fig. 5** Shell hue values (points) and moving medians (*lines*; medians at 101 points) for different wild *Haliotis tuberculata* individuals (indicated by *grey scale*) sampled in the Bay of Brest (**a**), at Molène (**b**), and at Île Vierge (**c**). *Horizontal lines* indicate 95% confidence intervals of hues defined for experimental ormer shells (Table 2), for green bands (*green lines*) and brown bands (*brown lines*). *Line* thickness represents the lower and higher values of confidence intervals. *Long horizontal dashed lines* Phaeophyta; *short horizontal dashed lines* Chlorophyta; *horizontal solid lines* Rhodophyta

Close to the edge, values stabilized within the confidence intervals corresponding to *P. palmata* in experimental populations (Fig. 5a). At Île Vierge, the hue values were mostly within the confidence intervals for *H. tuberculata* fed *Ulva* spp., and sometimes within those for ormers fed Rhodophyta (e.g., during the juvenile period, Fig. 5c). At Molène, the hue values were very low compared to those calculated for experimental ormers and highly variable

during the growth, often closer to the hue of ormer shells fed Rhodophyta, especially *P. palmata* (Fig. 5b).

The hue values for wild ormers never reached the confidence intervals of green bands, except for one individual sampled in the Bay of Brest (Fig. 5).

## Discussion

Mollusk shells are biomineralized tissue whose composition records variations in environmental conditions, such as temperature (Grossman and Ku 1986; Chauvaud et al. 2005; Jolivet et al. 2015) and food sources (Wilbur and Saleuddin 1983; Chauvaud et al. 2011; Marchais et al. 2015). Pigments incorporated into mollusk shell carbonates, in the prismatic layer of juvenile *Haliotis asinina* (Budd et al. 2014), cannot be synthesized by the animals themselves, especially the carotenoids that are produced only by the vegetable kingdom (Fox 1966). The pigmentation of mollusk shells varies according to pigments obtained from the diet (Leighton 1961; Olsen 1968a; Manríquez et al. 2009) and genetics (Liu et al. 2009; Roussel et al. 2013; Ge et al. 2015; Yue et al. 2015). Jackson et al. (2006) highlighted direct relationships between gene expression and shell coloration for *H. asinina*. Our study clearly demonstrates, both visually and by a numerical approach, that the shell color of *H. tuberculata* is at least partly controlled directly by diet.

Two other techniques were considered in our study, before the analysis of hue, to characterize pigments contained in the ormer shell prismatic layer: Raman microspectroscopy and high-performance liquid chromatography with pigment extraction method for soft tissues. Due to the shell structure, the pigment concentration in the shells, and the difficulty of extracting them, we found that the characterization of shell pigment compositions following food sources using these two techniques is complicated and unreliable.

### Specific shell hue according to assimilated macroalgae

The post-treatment change in shell hue, which was often visible to the naked eye in our study, may be the result of an accumulation of macroalgae pigments in vesicles within the cells of the tubule localized within a region of the mantle (Budd et al. 2014). Green bands appeared on the shell when ormers were fed the kelps *L. digitata* and *Saccharina* sp., and the green algae *Ulva* spp., whereas shells kept a red/brown appearance after being fed the kelp *L. hyperborea* and the red algae *Gracilaria* sp. and *P. palmata* (see pictures in Online Resource 2 and Table 2). Similar shell color changes with diet have been observed on other *Haliotis* species (Leighton 1961; Olsen 1968a, b), with a banding pattern in response to various diets (Gallardo et al. 2003; Liu et al. 2009). One experimental study highlighted this phenomenon in *H. tuberculata* (Koike 1978). In these studies, the authors characterized the different shell colors with a qualitative value (e.g., pale green, olive green, bluish-green, or turquoise), which is subjective. Color perception may differ between individuals whose color vision is classified as normal due to the existence of two different long-wavelength cone mechanisms (Neitz and Jacobs 1986). Here, we provide an objective indicator of shell color for each treatment to decipher the diet of ormers in their natural environment.

In this study, shell hue is shown to be a tool for characterizing ormer shell color according to their diet, as demonstrated previously with *Haliotis laevigata* shells (Hoang et al. 2016). Hue values could not be distinguished for ormers fed *L. hyperborea* and *Gracilaria* sp. (Fig. 3; Table 2). Unlike other algae, *L. hyperborea* was only supplied as stipes. Stipe pigments in this species may differ from that of the frond, which may explain the absence of a green band, compared to ormers fed the other Phaeophyta algae.

Green shell pigmentation was previously observed for *H. rufescens* (Leighton 1961), *H. sorenseni, H. discus hannai* (Olsen 1968a), and *H. laevigata* (Hoang et al. 2016) fed Phaeophyta and Chlorophyta. Hoang et al. (2016) demonstrated that *Ulva* sp. contains more chlorophyll *a* and less zeaxanthin (carotenoid) than *Gracilaria cliftonii*, which could explain the green shell coloration for *H. laevigata* fed *Ulva* sp. and the brown shell coloration for abalones fed *G. cliftonii*. Shell color depends on macroalgae pigments (Hoang et al. 2016), but Olsen (1968a) showed that the shell color of abalones fed the same species of macroalgae differed between abalone species. Thus, the shell hues we found for *H. tuberculata* cannot be generalized to other abalones. These species require separate validation. Marine animals accumulate carotenoids from algae and can modify them through metabolic pathways, which can complicate the interpretation of shell color, but this has not been shown for abalones, (Maoka 2011). More investigations are required to match shell pigments and macroalgae pigments.

The brown bands that interspersed the green shell color of *H. tuberculata* fed *L. digitata, Saccharina* sp., and *Ulva* spp. do not appear to have been observed for a monospecific diet in other abalone species. Dating these bands based on the percentage of shell growth for the treatment with *L. digitata* indicated that the brown bands appeared during the winter and spring periods (from November to April) and around the feeding days. These brown bands could reflect the integration of damaged algal pigments. Macroalgae picked up in winter and at the beginning of spring were mainly senescent (pers obs), compared to summer (from July to September) when the large brown bands were absent. Dating of the hue values was only an

approximation, and the precision can be a few days, which could explain individual variations in the appearance of the brown bands. Just before or on the day of the fresh macroalgae supply, hue values decreased. Macroalgae in compartments could deteriorate during the month between each feeding day. Phaeophyta and Chlorophyta have specific chlorophyll pigments: chlorophyll-*b* for Chlorophyta and chlorophyll-*c* for Phaeophyta (Bianchi et al. 1997; Caron et al. 2001). These pigments are responsible for the green pigmentation of plants. Products of chlorophyll degradation, without the magnesium core, lose the green pigmentation and become yellowish in color (Matile et al. 1999). Carotenoids, pigments with an orange-yellow color, are contained in Phaeophyta and Chlorophyta and deteriorate less rapidly than chlorophyll, heightening the brown pigmentation of abalone shells (Moss 1968; Jeffrey et al. 1997). Rhodophyta have other specific pigments, such as phycoerythrins and phycocyanins, that are not present in other algal phylums (Bianchi et al. 1997); they lead to the red pigmentation of this algal phyla and probably to the modification of abalone shell pigmentation. A study of the advancement of pigment status during macroalgal degradation and their transfer into shell carbonates would be required to confirm this hypothesis of damaged algal pigments for brown bands.

Strong variability in shell hue within the same treatment was observed in our study. Partial control by the genetics of shell color (Jackson et al. 2006; Liu et al. 2009; Roussel et al. 2013) could explain this variability. More extensive studies are required to evaluate the real inter-individual variability and, thus, the reliability of the digital shell color analysis.

In the present study, shell hue values were given for a monospecific diet that does not occur in the wild, where ormers have an algal mix at their disposal. Another experiment needs to be carried out with an algal mix containing controlled proportions. With the dating of different points on the ormer shell and the confirmation from $\delta^{18}O_{shell}$, the shell hue could be compared to the percentage of macroalgae species in the mix. This experiment allows to determine if some species in the ormer diet can mask or modify the hues that are produced by other algae assimilated. Furthermore, only seven algae were tested, but other algal species can be eaten by ormers and their consequences on shell hue are still to be explored. Experiments on a wider range of the algal species are required to specify which species of Rhodophyta, Chlorophyta, or Phaeophyta have been consumed.

### Diet of wild *Haliotis tuberculata*

The previous studies have shown that ormer growth does not stop during the winter period (from December to February), which allows a continuous recording of environmental variations in shell carbonates (Clavier and Richard 1986; Roussel et al. 2011; Jolivet et al. 2015). Thus, reconstruction of the wild ormer diet can be performed throughout the year.

The food preference of the young ormers sampled in the Bay of Brest and at Île Vierge was not easy to establish due to the high variability of the shell hue, which can be explained by the many sources of diet (macroalgae species plus small filamentous or encrusting algae) and the shift in food sources between juveniles and adults (Nash 1991; Takami and Kawamura 2003; Won et al. 2010). Ormer behavior may also explain this hue variability and the convergence of hue for ormers sampled in the Bay of Brest and at Île Vierge with aging. The movements of *H. tuberculata* become less important with aging, as an annual mean range of 6.7 m was found for *H. tuberculata* in adulthood (Clavier and Richard 1982). Young ormers may exhibit foraging behavior, assimilating a wide range of macroalgae until they settle in places where food sources, though less diverse, can fulfill their requirements (Shepherd 1973). The convergence of hue with aging seems to indicate a main assimilation of red algae by wild ormers, but a complete algal inventory of this sampling site is required to conclude on ormer food preferences.

At Molène, shell hue varied between individuals and during ormer growth. The variability in shell color could be the result of high algal biodiversity at Molène (REBENT: http://www.rebent.org/documents/index.php) compared to the other sampling sites. The abalone diet seems to depend primarily on the local abundance of macroalgae (Shepherd and Steinberg 1992; Day and Cook 1995). Another hypothesis may be that the environment at Molène is more unsettled than the one in the Bay of Brest and at Île Vierge, suggesting greater mobility and variable macroalgae availability.

The shell hue of ormers sampled at Molène reached values near the hue for intense red (0°). Visually, these ormer shells were pinker than shells from other sampling sites (see Online Resource 2). Leighton (1961) observed pink pigmentation on shells of *H. rufescens* experimentally fed a mix of red and brown algae. Assimilation of both Phaeophyta and Rhodophyta cannot be excluded for ormers sampled at Molène, because they were taken in a *Laminaria* bed sheltering high macroalgae biodiversity. Abalone shell pigmentation may also mimic the color of the substrate to limit predation (Liu et al. 2009). The substrate at Molène may be composed mainly of encrusted Corallinaceae (pink/red algae), which is the substratum for abalone larval recruitment (Shepherd and Turner 1985; Mgaya 1995; Wood and Buxton 1996; Takami and Kawamura 2003).

The shell hue of wild ormers did not reach the confidence intervals of shell hues defined for green bands

(except for one individual sampled in the Bay of Brest, Fig. 5), which suggests the assimilation of damaged pigments contained in drift macroalgae. Guest et al. (2008) demonstrated stable isotopes in *H. rubra* fed mostly detritic algae containing damaged pigments. Adult abalones preferentially eat drift macroalgae during decomposition and can graze on fixed fresh algae when the current is too weak to bring drift algae (Shepherd 1973; Tutschulte and Connell 1988; Mgaya 1995). *H. tuberculata* also moves in the direction of the swell and main currents, allowing it to be in the location with the highest abundance of drift algae (Clavier and Richard 1982).

The validation of hue values versus diet for wild ormer shells is difficult due to the lack of knowledge on the distribution of biomass for every macroalgal species at each sampling site. Our hue values on wild ormer shells are preliminary results and further research is required to understand the determinants of wild ormer shell color. Macroalgae biomass and species abundance, both fresh and drift algae, in the natural environment should be assessed over a year before ormers are sampled. Then, some ormers could be sampled to measure hue values and relate them to dating based on $\delta^{18}O_{shell}$ and the seawater temperature for the comparison between shell hue and algal biomass. Finally, the method of digital color analysis appears applicable for wild ormers, but complementary experiments are required to assess the role of genetics in the shell hues produced by the three algal groups.

## Conclusion

The analysis of shell pigmentation using the shell hue (HSL color space) on photographs was the first step to developing a new method to assess the food sources of wild abalones, which could be useful for the management of fished populations and to obtain good information on their food preferences for aquaculture. This is a promising method, because distinct shell hues were obtained according to the macroalgae assimilated by ormers. These hue values provide an objective assessment of shell pigmentation compared to shell color analysis by the naked eye. The strengths of the digital shell color analysis include the fact that it is non-destructive for the shell, in contrast to stable isotope sampling, as well as its low cost and easy setup (only photographic and computer equipment). The drawbacks of this analysis are the effects of genetics on shell pigmentation (Jackson et al. 2006; Liu et al. 2009; Roussel et al. 2013) and that shell erosion with aging might disturb the interpretation of shell hue values in terms of diet. Care should also be taken to avoid degradation of the shell pigments by light. Complementary experiments are required to obtain a better understanding of pigment transfer from food to carbonates. In addition, more individuals need to be studied after that a wider range of algae was tested in an experiment to better appreciate the inter-individual variability in hue values and to provide a better representation of wild populations in hue values. Furthermore, detailed studies in natural environment are required to investigate the impacts of substrate, season, and sampling site (algal relative abundance and hydrodynamism). Our results are a first step to develop the digital shell color analysis for diet analysis of abalones or other herbivorous mollusks.

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**Compliance with ethical standards**

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**Conflict of interest** Authors declare that they have no conflict of interest.

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