

New method for presenting nutritionally defined food sources to marine organisms

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Abstract

To study nutritional ecology in aquatic and marine ecosystems, it is important to be able to perform feeding trials enabling feeding and nutrient selection behavior of organisms to be observed in situ. Current studies in marine nutritional ecology are limited to laboratory and shallow water studies using food sources of undefined nutrient concentration. Here we describe a technique for delivering standardized nutrient sources in situ to both pelagic and benthic organisms as a feeding choice array, in a way that allows the amounts eaten of different foods and nutrients to be quantified and correlated with precise descriptions of the feeding behavior of individual organisms. A pilot study proved that this feeding choice array can be successfully deployed in marine environments. It will also be possible to use this technique to explore patterns of nutrient limitation and to investigate the nutritional regulatory response of marine and aquatic organisms.

Introduction

Patterns of food selection based on the nutritional quality of the food provide important information for ecologists regarding the nutritional requirement and evolution of organisms (Jones and Flynn 2005; Raubenheimer and Simpson 2004). Studies into nutritional ecology have recently recognized the importance of, and moved toward, an integrative approach to the analysis of food selection focusing on the interactive effects of different nutrients and other food components (Raubenheimer and Simpson 2004). Knowledge of terrestrial nutritional ecology has evolved rapidly over recent years, but the marine literature remains limited to a handful of notable studies (Choat and Clements 1998; Raubenheimer et al. 2005). To date, marine studies have focused on how the nutritional, chemical, and physical properties of food sources affect choice and the consequences of this process for body condition and reproductive capacity (e.g., Duffy and Paul

1992; Chanas and Pawlik 1995, 1996; Poore and Steinberg 1999; Cruz-Rivera and Hay 2001). Few studies, however, have attempted to explore the interactive effects of key nutrients in the diet of marine animals.

Studies to date in marine nutritional ecology have offered choices of either whole animal, plant, and algal tissue (Horn et al. 1982; Neighbors and Horn 1991; Zemke-White and Clements 1999; Cruz-Rivera and Hay 2000a, 2001; Waddell and Pawlik 2000b; Choat et al. 2002; Buck et al. 2003; Pillans et al. 2004; Raubenheimer et al. 2005; Cox and Murray 2006) or arrays comprising crude extracts of animal and plant material, dried, ground, and set in block form (Steinberg 1988; Duffy and Paul 1992; Hay et al. 1994; Chanas and Pawlik 1995, 1996; Waddell and Pawlik 2000a; Cruz-Rivera and Hay 2000b; Hill et al. 2005). These approaches have provided important insights into choices made by foraging animals. The emphasis has been on the role that secondary metabolites have in deterring feeding, and in general the nutritional value of the food has been overlooked in mediating choice in ecological studies (but see Chanas and Pawlik 1995, Choat and Clements 1998, Raubenheimer et al. 2005 for notable exceptions). When standardized foods are offered to quantify preference, only rarely is the nutritional value of the food known and not a priori (Chanas and Pawlik 1996; Cruz-Rivera and Hay 2000a).

A major difficulty in the study of nutritional ecology in marine systems is providing a technique for delivering standardized nutrient sources in situ to both pelagic and benthic organisms, in a manner that allows the amounts eaten of different foods and nutrients to be quantified and correlated

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Acknowledgments

This research could not have been completed without the generosity of Woodside, who donated the remotely operated vehicle (ROV) time and supported us logistically, and the skill of the Total Marine Technology ROV crew. We are grateful for the hospitality afforded to us by the Woodside Company Man, Offshore Installation Manager, and crew of the *Guardian* during our mission. We thank Dr. Fiona Clissold for advice on chemical analysis. This is Scientific and Environmental ROV Partnership using Existing Industrial Technology (SERPENT) publication #25, contribution #2 from South East Asia (SEA) SERPENT project, Australia.

with precise descriptions of the feeding behavior of the individual organisms. Cafeteria trials, where an array of different food sources is offered to animals, are a suitable tool for these types of studies. Ideally, to test food and nutrient selection responses experimentally, such a method should meet 5 key criteria: *a*) the precise nutritional value of the food sources, whether standardized food sources or animal, plant, or algal material, is known a priori, *b*) the food items are robust enough to withstand being deployed in the habitats in question, *c*) they are accepted as food by the target study organisms, *d*) the feeding behavior of individual organisms can be observed, and *e*) if testing hypotheses that require the precise measurement of nutrient intake, feeding behavior should be able to be correlated with gravimetric measures of consumption.

The aim of the present study was to devise such a technique using standardized natural and artificial nutrient sources and to demonstrate its use at depths beyond which SCUBA can be used readily for conducting in situ experiments. We describe the techniques and provide pilot data where we used a remotely operated vehicle (ROV) from a drilling rig in the Otway Basin, Southern Ocean, Australia, in May 2006 to illustrate the method and demonstrate the breadth of information that can be gained from the technique, in particular how the feeding responses of individual organisms can be observed and correlated with measures of consumption.

Materials and procedures

Standardized food blocks—The differences in foraging behavior of marine vertebrates and invertebrates were quantified using standardized nutrient sources. Agar-based food blocks were made comprising *a*) natural protein-rich food (ground squid), *b*) natural carbohydrate-rich food (ground seagrass or kelp), and *c*) natural lipid (fish oil). In addition to these, agar food blocks containing chemically defined sources of protein (casein) and carbohydrate (dextrin) were tested, and pure agar food blocks were included to act as controls. These 6 types of food block were offered to the study organisms in situ as feeding choice arrays.

Dried squid (Hang Hing Marine Products Co., Hong Kong), fish oil (Melrose Laboratories Pty, Ltd.), casein (Sigma-Aldrich), and dextrin (Sigma-Aldrich) were purchased off the shelf. Samples of the seagrass *Zostera capricorni* were collected from North Maroubra (33.9°S, 151°E), New South Wales, and samples of the kelp *Ecklonia radiata* were collected from Bare Island (34°S, 151°E), Botany Bay, New South Wales. Samples were collected from early April to early June 2006.

Seagrass and macroalgae samples were washed and cleaned of adherent substrate in clean seawater and dried to constant mass in a Labmaster forced fan oven at 75°C. Seagrass, kelp, and squid were then ground to a fine powder in a Retsch mixer mill (MM301) and stored in polyethylene jars. The amount of protein and carbohydrate in the natural samples of seagrass, kelp, and squid were determined using the Bradford protein assay (Bradford 1976) and the phenol-sulfuric assay (DuBois et

al. 1956), respectively. The microtiter plate used in both the protein and carbohydrate assay was read by a Spectromax 384 Plus spectrophotometer (Molecular Devices), and the computer program Softmax Pro (Molecular Devices) was used to read the standard curve, protein, and carbohydrate values.

Protein was extracted from 20 mg finely ground material that was placed in a 2-mL Eppendorf tube with 500 μ L of either 0.1 M NaOH (seagrass) or 1 M NaOH (macroalga and squid) (Rausch 1981). The samples were sonicated in a Sonicclean 100T (Transtek Systems) for 30 min in distilled water, heated for 15 min at 90°C in a Julabo SW22 water bath (John Morris Scientific Pty Ltd.), and centrifuged at 11,000 rpm (2×10^4g) for 10 min in a 5415D centrifuge (Fronine Laboratory Supplies). Once centrifuged, the supernatant containing the protein was collected and stored. To extract the remaining protein, the seagrass pellet was washed with 167 μ L of 0.1 M NaOH, and 250 μ L of 1 M NaOH was used to wash the macroalga and squid pellets. The solutions were centrifuged at 11,000 rpm (2×10^4g) for 10 min and the supernatants pooled. To neutralize the supernatants, 13 μ L of 5.8 M HCl was added to the seagrass supernatant and 130 μ L of 5.8 M HCl was added to the supernatant of the macroalga and squid. Trichloroacetic acid (100%) stock was used to precipitate the protein from the supernatant; 90 μ L of 100% TCA was used for the seagrass and 98 μ L TCA was used for the macroalga and the squid, giving a final concentration of 10%. The solution was refrigerated for 30 min to allow the protein to precipitate and then centrifuged for 10 min at 11,000 rpm (2×10^4g) and the TCA/supernatant solution removed. The remaining pellet was washed with 100 μ L -20°C acetone. This was done quickly so as not to dissolve the protein pellet. The acetone was added to remove any remaining TCA, which would interfere with the Bradford protein assay. The pellet was then dried in a fume hood. Once dried, the pellet was dissolved in 1 mL 0.1 M NaOH. Heating in hot water, vigorous vortexing, and short bursts of sonication were also required to dissolve the pellet. The seagrass solution was diluted to 1 part solution, 19 parts distilled water (1 in 20), the macroalga was diluted to 1 part solution, 79 parts distilled water (1 in 80), and the squid solution was diluted to 1 part solution, 159 parts distilled water (1 in 160). These dilutions were conducted so that the protein assay could be carried out accurately. The Bradford protein assay was used to quantify the total amount of protein in the samples (Bradford 1976). Six individual samples each of seagrass, macroalga, and squid were assayed.

Total available carbohydrate was extracted from 20 mg of finely ground sample that was placed in a Kimax tube with a Teflon-coated lid. Then 1 mL 0.2N H_2SO_4 was added, and the Kimax tube was placed in a boiling water bath (Julabo SW22, John Morris Scientific Pty Ltd.) for 1 h. The solution was refrigerated for 1 h and centrifuged (Centrifuge 5810R, Fronine Laboratory Supplies) for 10 min at 11,000 rpm (2×10^4g) to remove the supernatant, which contains the carbohydrates. The phenol-sulfuric acid assay was employed to quantify the

total amount of available carbohydrate in the samples using the methods defined by Dubois et al. (1956). Six individual samples each of seagrass, macroalga, and squid were assayed.

Each food block was made using 50 mL seawater, which was heated in a glass beaker on a hotplate to 80°C (Selby magnetic stirrer hotplate N759). Nutrient sources were then added and stirred vigorously for 1 min, and 6 g agar was added. Additionally, 1 g sodium alginate was added to the lipid solution to act as an emulsifier, to evenly disperse the lipid throughout the food block (Cary et al. 1992). Nutrient sources were added at the appropriate amounts to result in final concentrations of 0.25 g protein g⁻¹ wet weight food block for high concentration squid and casein, 0.10 g protein g⁻¹ food block for low concentration squid, 0.25 g lipid g⁻¹ food block for fish oil, and 0.125 g protein and 0.125 g carbohydrate g⁻¹ food block for the dextrin/casein blocks. Final concentrations of carbohydrate and protein for seagrass were 0.06 g carbohydrate g⁻¹ food block and 0.03 g protein g⁻¹ food block, and for kelp final concentrations were 0.06 g carbohydrate g⁻¹ food block and 0.09 g protein g⁻¹ food block.

The nutrient-agar solution was then mixed for 30 s and poured into a mold fashioned from a 175-mL polystyrene cup (0.4 m × 0.36 m × 0.4 m) that contained a hollow plastic golf ball with holes in its surface (Fig. 1A). This acted as an internal frame for the food blocks and provided holes for securing with cable ties. A cable tie was then passed through the holes of the golf ball and secured to a plastic grid. The feeding blocks were placed in a refrigerator for 24 h to set before deployment.

Calibration of food blocks—Because it was planned to measure amounts consumed of the feeding blocks when in situ on the seabed and in laboratory studies by weighing them before and after deployment, it was necessary to determine the mass lost or gained by the food blocks when in the seawater. Two control experiments were conducted. In the first trials, one of each type of food block, except for low concentration squid, was placed in 3 10-L buckets containing 9 L seawater to determine the mass gained or lost in still water. The food blocks were left in the seawater for 24 h, the maximum amount of time food blocks spent on the seafloor, and weighed (0.01 g, Mettler Toledo PB3002-S) at the beginning and end of the trial.

The second experiment was designed to test the mass gained or lost by the blocks in flowing seawater, simulating deep-sea conditions. The actual experiments were to take place in the Bathyal zone, which begins at the continental shelf break. Flow speeds in the Bathyal zone tend to be <10 cm s⁻¹ and vary little from day to day at one location (Eckman and Thistle 1991). Trials were conducted in a recirculating flume (Vogel 1983) with a seawater velocity of 10 cm s⁻¹.

Three food blocks of each type, except low concentration squid, were placed randomly in the center of the flume. The food blocks were left in the flowing seawater for 24 h and change in weight determined as previously described. Still and flowing trials were run twice on consecutive 24-h periods with different food blocks in each trial.

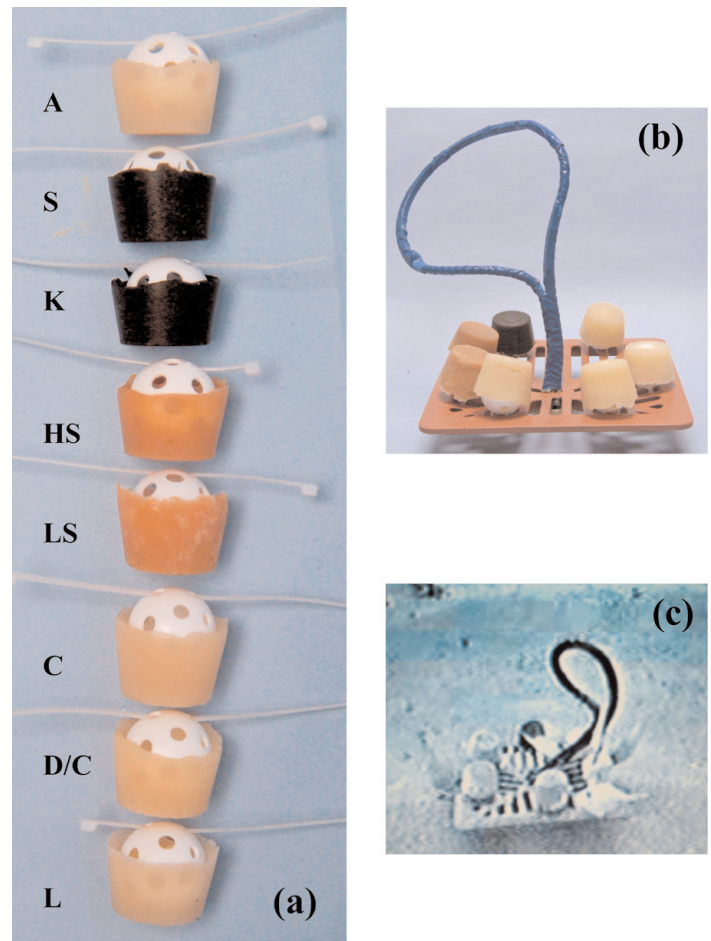


Fig. 1. (A) Agar-based standardized food blocks, set in plastic golf balls with cable ties for attachment to the feeding choice arrays. Individual food blocks are identified by letter (HS, high concentration squid; LS, low concentration squid; C, casein; S, seagrass; K, kelp; D/C, dextrin/casein; L, lipid; A, agar). (B) Feeding choice array with food blocks attached using cable ties. The grid is weighted and fitted with a rope handle for deployment on the seabed (clockwise from the back: kelp, casein, agar, dextrin/casein, lipid, high concentration squid, low concentration squid; the seagrass-based food block is not shown in this photograph). (C) The feeding choice array successfully deployed on the seabed at 92 m water depth in Bass Strait, South Australia. The array was deployed on a rocky reef environment dominated by sponge and coral communities, and the velvet leatherjacket (*Parika scaber*) was the most common fish at the array.

Regression analysis was used to determine whether there is a linear relationship between the mass gained or lost by each food block as a function of nutrient type and water velocity (Quinn and Keough 2002).

Feeding choice array deployment and monitoring—A pilot study was conducted in the petroleum and gas development field Thylacine (39°14.495'S, 142°54.234'E), Bass Strait, Australia, in May 2006. The site was characterized by a rocky sponge and soft coral reef environment with a water depth of 92 m. Work was conducted from the *Maersk-Guardian* jack-up drilling rig, and access to the ocean floor was achieved using

the ROV *CI Surveyor Plus* equipped with one 5-function arm. Video footage was obtained using a Hitachi HV-C20 camera recorded to a Panasonic DMR AS2 hard drive. Footage was then transferred to DVD for analysis.

A system was designed to ensure the food blocks could be successfully deployed on the seafloor by a ROV. One of each type of food block was attached in a random position, using a cable tie, to a 245 mm × 245 mm plastic drain cover at 50-mm intervals. The plastic grid was weighted with a 1-kg diving weight, and a rope and float were attached to allow for deployment and retrieval by the ROV (Fig. 1B).

Two feeding choice arrays were made with the high concentration squid food block, seagrass food block, kelp food block, casein food block, dextrin/casein food block, lipid food block, and agar food block attached. Food blocks were made as previously described. The 5-function arm of the ROV was used to secure the feeding choice array for transport to the sea floor. The blow-out preventer (BOP), situated at the top of the drilling well, was used as a point of reference for deploying arrays. Arrays ($n = 2$) were deployed on different compass bearings and at different distances from the BOP during the day (bearing 20°, distance from BOP 22 m) and at night (bearing 350°, distance from BOP 35 m).

Arrays were deployed on the undisturbed seabed and the ROV was positioned 3 m away. The camera was adjusted so that the feeding choice array was in the center of the frame of view and all the food blocks were visible (Fig. 1C). Recording of the feeding choice array commenced when the ROV and camera were in place and the hydraulics on the ROV were turned off. Arrays were then filmed for 1.5 h. After this period, the feeding choice arrays were retrieved and brought to the surface.

Video analysis was used to identify the individual species that were feeding, observe individual animal's feeding responses to the different nutrient sources, and observe behavioral interactions between foragers at the array deployed during daytime (and hence unaffected by the lights from the ROV). We were unable to analyze the data from the nighttime deployment, as fish attracted to the array were preyed on throughout by a juvenile New Zealand fur seal (*Arctocephalus forsteri*).

Eight species of fish were attracted to the food arrays, but the velvet leatherjacket (*Parika scaber*) were numerically dominant, and their schooling behavior on the feeding array kept all other species away from the array. *Parika scaber* is common temperate reef fish in Australia and New Zealand and, as an omnivore, samples food sources more frequently in habitats that are devoid of algae (Choat and Ayling 1987; Russell 1983). Individual fish ($n = 12$), of this species were watched from the moment the field of view was entered to the moment it was left, and individual behavior was recorded. The investigation and feeding time of each block was recorded. Food investigation was defined as a fish swimming close to (<30 mm) a particular food block for a period of longer than 3 s

without feeding. Feeding was defined as an individual fish taking repeated bites from the same food block. The selection of different nutrients was determined by recording the number of sampling events defined in this case as bites from the food blocks that were not spat out by the fish, by a particular species of fish, on a particular food block during the 30-min period after the arrival of the first fish at the array. After the arrival of the first fish, each block was watched individually for 30 min, and the number of samples taken from that block was recorded. The number of fish in the feeding group of which the individual fish was a member and whether aggression was shown during feeding was also recorded. This allowed us to start to explore whether feeding and food selection were affected by group size. A group was defined as 3 or more fish investigating or feeding at the array at any one time. Fin displays, notably the extension of the dorsal trigger, were used to determine aggression during feeding (Myrberg and Thresher 1974).

Assessment

Calibration of food blocks—All food blocks gained mass through the absorption of water after 24-h submergence in still or moving water. There was a strong positive linear relationship between initial and final mass:

$$\text{final mass (g)} = -0.343 + 1.051 \text{ initial mass (g)} \quad (1)$$

($F_{1,82} = 20152$, $P < 0.001$, $R^2 = 0.996$). This relationship held regardless of the type of nutrient source (Fig. 2A) or water velocity (Fig. 2B). Such changes must be accounted for to determine the actual weight loss due to consumption of the food block by aquatic organisms. Hence, the change in mass recorded for a food block retrieved from the sea floor will include a negative component due to consumption, but also a mass gain due to water absorption by the block. Correction for the latter involves the following formula:

$$C = W_i - W_c \quad (2)$$

where C = consumption, W_i = initial mass of food block in g, and W_c = final mass corrected for water absorption.

Because

$$W_f = -0.343 + 1.051 W_c \quad (3)$$

where W_f = final mass before being corrected for water absorption, and solving for W_c :

$$W_c = (0.343 + W_f)/1.051 \quad (4)$$

then consumption would equal

$$C = W_i - (0.343 + W_f)/1.051 \quad (5)$$

where C = consumption in g, W_i = initial mass of food block in g, and W_f = final weight of block in g.

Using the above procedure, it is possible to calculate the consumption of the food blocks by organisms over a 24-h period. Consumption can be deemed to occur when there is

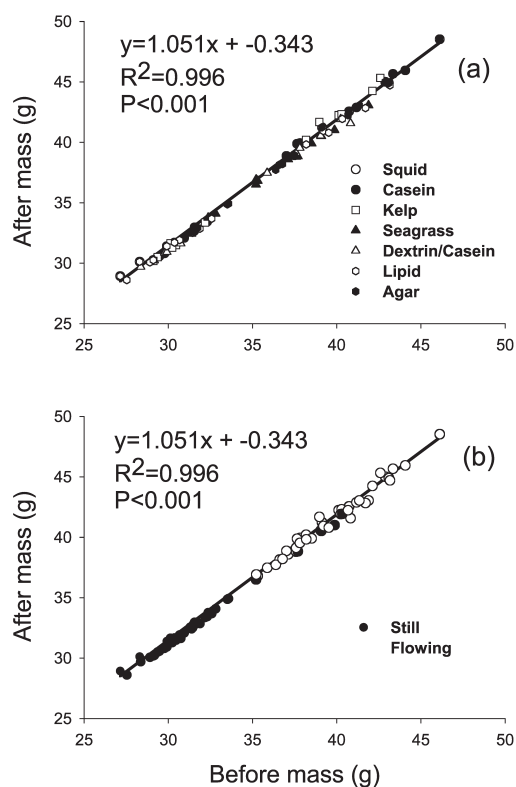


Fig. 2. Relationship between feeding blocks (A) in still and flowing seawater (B) before mass (g) and after mass (g).

physical evidence of feeding scars on the food blocks. Because no difference was observed due to nutrient sources or flow regimes, future calibrations could be done in still water; however, calibration would need to take account of the period of deployment of food blocks. The experimental potential of this is reviewed in “Discussion.”

Feeding choice array deployment and monitoring—The pilot study provided evidence that the food array technique will provide a unique and highly effective way of studying nutritional ecology in aquatic environments. Because only 2 arrays were deployed, it is not possible to conduct statistical analyses on the results, but the potential of the technique is clear. The standardized food blocks meet the 4 essential criteria (see “Introduction”), but most notably they were robust enough to survive deployment and retrieval by the ROV through the treacherous swash zone (none of the blocks was damaged on deployment or retrieval) and were palatable to a variety of fish and invertebrates.

A single fish species, the velvet leatherjacket (*Parika scaber*), dominated the feeding choice array. The only major feeding event observed, other than by *P. scaber*, was by the larger 6-spined leatherjacket (*Meuschenia freycineti*). Other organisms observed approaching the array included hermit and spider crabs, indicating benthic invertebrates are attracted to the arrays. Feeding behavior of the fish was observed, and the

length of time fish investigated each food block and the number of sampling events from food blocks in the array were recorded (Fig. 3a). The squid-based food block was sampled by fish the most, nearly 3 times more than any other food block, in a 30-min period (Fig. 3b). Individual sampling events on the squid-based food block also lasted longer than any other food block (Fig. 3c). The technique made it possible to gain information regarding the group dynamics of the leatherjackets while feeding. The mean feeding time increased with group size, and feeding times were shorter when aggressive behavior was observed (Fig. 3d and e). An overall picture can

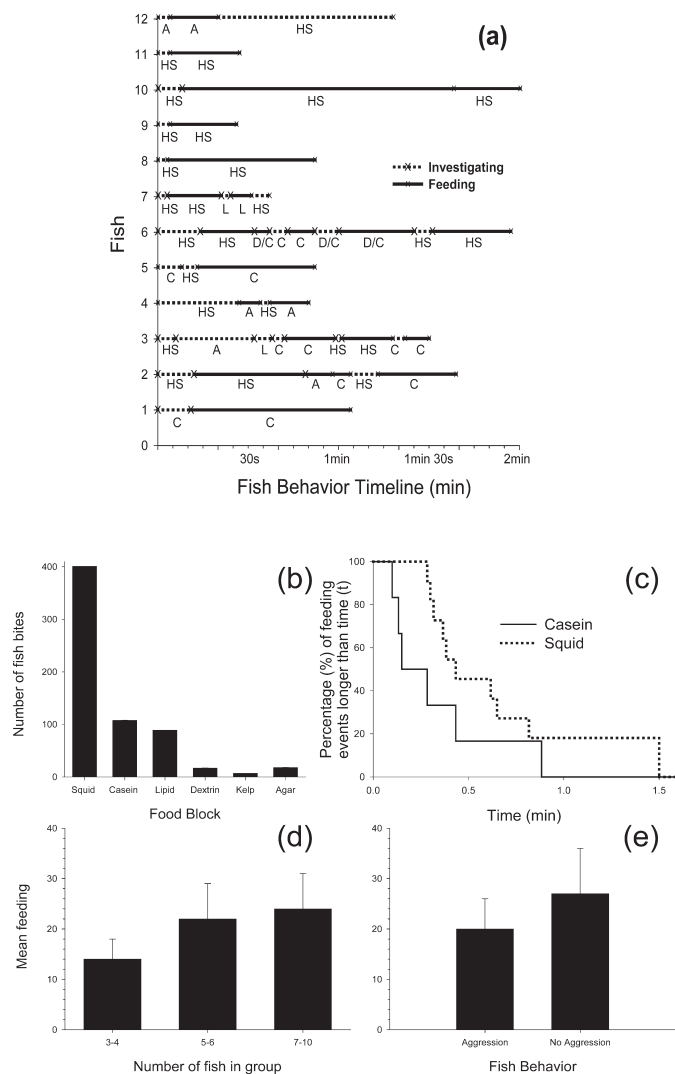


Fig. 3. Types of data collected from video analysis of the behaviors of *Parika scaber* at the feeding choice array. (a) Timeline showing investigating and feeding times on individual food blocks. Individual food blocks are identified by letter (HS, high concentration squid; LS, low concentration squid; C, casein; S, seagrass; K, kelp; D/C, dextrin/casein; L, lipid; A, agar). (b) Nutrient selection and (c) analysis of the percentage of nutrient feeding events longer than a given time over a 2-min period. (d) Mean (\pm SE) feeding times of *P. scaber* in groups with different number of individuals and (e) while displaying aggression or no aggression during feeding.

be drawn of the feeding behavior of individual species during the 1.5-h period by studying the percentage of time that was taken up by investigating and feeding and the time there was no activity at the array.

Discussion

We have reported a method that allows the deployment of an array of nutritionally defined food sources on the sea floor. Nutrient sources are set in agar blocks that are robust enough to survive immersion and palatable to both fish and benthic invertebrates. The blocks are offered as a feeding choice array, designed to be manipulated on the seafloor by ROVs, allowing data to be collected at depths and over continuous periods of time that would not be feasible for SCUBA diving. The food blocks could also be deployed autonomously by landers or benthic cameras that have traditionally used animal, plant, and algal material as food sources. Care should, of course, be taken to minimize any influence on the behavior of the target organisms by the use of appropriate artificial lighting (Raymond and Widder 2007; Widder, 2005), and to consider the potential impact of artificial structures on organism aggregation (Keenan et al. 2007; Jamieson et al. 2006).

The results from the pilot trial indicate that a broad range of information can be gained from using our technique. Observations of how animals handle the standardized food blocks need to be considered when analyzing the video data. For example, we found the leather jackets to be messy eaters, with food sometimes falling from their mouths during feeding. Additionally, more than one individual fed from a food block during an observation period. Hence, we enumerated bites taken by individual fish as "sampling events," rather than quantifying intake gravimetrically as the change in block weight during deployment. Such a measure provides important information about food selection behavior, if not actual intake. Quantifying amounts of nutrients ingested for the leatherjackets or other similarly messy feeders would require calibrating mass changes in food blocks to direct observations of the behavior of individual fish as they fed on the blocks. Spillage could also be collected using an underlying mesh collection device. The method is, however, ideal for less messy feeders, such as many mobile benthic invertebrates that are adapted to scrape feeding, for which simple mass difference will provide an accurate measure of consumption.

The feeding and food sampling behavior of solitary individuals can be observed and compared to that of individuals of the same species in a group, where social factors and competitive interactions come into play. The technique can also provide information on the response of organisms to a food source over time: how long it takes for organisms to find the array, investigate the food sources, begin feeding, and leave. The feeding array system could also easily be modified to incorporate such features as chemical and physical defenses (Steinberg 1988; Hay et al. 1994; Chanas and Pawlik 1995; Waddell and Pawlik 2000a; Hill et al. 2005).

The technique is particularly promising for studying the nutritional ecology of deep-sea scavengers, which face a number of nutritional challenges due to the unpredictable nature of feeding events. However, previous studies have sought to investigate the response of scavengers in the deep sea to food falls, in the form of animal carcasses, rich in protein and lipids, or plant and algal material comprised of protein and carbohydrates (Gooday and Turley 1990; Klages et al. 2002; Jones et al. 1998; Priede et al. 1994). There are currently two approaches to studying these food falls. The first approach involves the deployment of sources of animal, plant, and algal material in situ, which are then observed and the response of scavengers to them monitored (Lawson et al. 1993; Debenham et al. 2004). The second involves the study of scavenger guilds formed around natural food falls that have been discovered by chance (Baco and Smith 2003; Smith and Baco 2003; Goffredi et al. 2004). These techniques provide a wealth of information, such as the response of scavengers to food falls, the species of scavengers that feed, and the length of time a food fall lasts before it is devoured. However, it is not possible to manipulate the nutritional composition of the food being eaten, or to quantify the amounts eaten, and the use of standardized food blocks will allow investigators to address important questions regarding an organisms' response to food deprivation and nutrient limitation.

The use of standardized food arrays could enable direct comparisons to be made between the nutritional factors shaping the ecologies of different marine environments. Here we used it to understand feeding behavior at a site of anthropogenic disturbance. But if applied to more natural systems, it would be particularly enlightening to compare organisms from depths beyond the photic zone, where food falls are stochastic events, with similar (or the same) species living in shallow seas within the highly productive photic zone, where food is constantly available. It will also be possible to use the technique to explore patterns of nutrient limitation in the deep sea and to investigate the nutritional regulatory responses of organisms in situ—a technique that has recently been used to powerful effect in a terrestrial system (Simpson et al. 2006).

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Submitted 23 May 2007

Revised 16 March 2008

Accepted 8 May 2008