MARINE ECOLOGY


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# Nutritional input from dinoflagellate symbionts in reef-building corals is minimal during planula larval life stage

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**Dispersion of larval offspring is of fundamental ecological importance to sessile marine organisms. Photosymbiotic planulae emitted by many reef-forming corals may travel over large distances before settling to form a new colony. It is not clear whether the metabolic requirements of these planula larvae are met exclusively with lipid and protein reservoirs inherited from the mother colony or when metabolic inputs from their endosymbiotic dinoflagellates become important. Pulse-chase experiments using [$^{13}$C]bicarbonate and [$^{15}$N]nitrate, combined with subcellular structural and isotopic imaging of freshly emitted symbiotic larvae from the coral *Pocillopora damicornis*, show that metabolic input from the dinoflagellates is minimal in the planulae compared with adult colonies. The larvae are essentially lecithotrophic upon emission, indicating that a marked shift in metabolic interaction between the symbiotic partners takes place later during ontogeny. Understanding the cellular processes that trigger and control this metabolic shift, and how climate change might influence it, is a key challenge in coral biology.**

## INTRODUCTION

In (sub)tropical shallow-water reefs, most scleractinian corals live in mutualistic endosymbiosis with dinoflagellates of the genus *Symbiodinium* ("zooxanthellae") located within their gastrodermal cells. The dinoflagellates photosynthetically fix carbon (C), assimilate nitrogen (N), and translocate essential compounds (for example, lipids, glucose, and amino acids) to their animal host, thereby supporting its metabolic requirements for growth, skeletal formation, and reproduction [reviewed in (*1*)]. A large fraction (20%) of symbiotic corals reproduce by brooding, and emitting planktonic larvae (planulae), which disperse and settle to form new colonies in a process fundamental to the expansion of reefs and the maintenance of their diversity. The energy budget of planulae is critical to their longevity and dispersal potential (*2*, *3*).

It is well established that planulae, upon emission, are very rich in maternally derived endogenous lipids (up to 70% by weight) and protein reserves. Planulae from many corals also host autotrophic dinoflagellate symbionts, transferred either vertically from the mother colony or horizontally from the ambient seawater. The amount of energy stores largely determines the larval survivorship and competency period (*2*, *4*–*7*). However, in constrast to adult corals, the autotrophic contribution of dinoflagellates to the metabolism of the planula and the replenishment of its energy stores is poorly constrained, with constrasting evidence arguing both for and against the significant translocation of photosynthates (*4*, *8*–*12*).

We performed a stable isotope pulse-chase experiment on newly released (less than 12 hours since emission) symbiotic planulae of the reef-building coral *Pocillopora damicornis* (Linnaeus, 1758). These planulae contain endosymbiotic dinoflagellates, in principle, permitting an immediate autotrophic nutrient contribution to the metabolism of their host.

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## RESULTS AND DISCUSSION

The genotype composition of the coral-dinoflagellate symbiotic association is known to influence its biological functions (*13*). Therefore, we first determined the taxonomy of each symbiotic partner (that is, the "*Symbiodinium*" photosymbionts and the "*Pocillopora damicornis*" host) at the clade and type levels with multiple independent markers.

Classification of the coral host was assessed using three molecular markers, in addition to skeletal micromorphology and lunar timing of the release of planula larvae. Genetically, planula larvae and adult colony nubbins are classified as clade 1 type β lineage of *P. damicornis*, according to the sequence of their entire internal transcribed spacer (ITS), specifically their ITS2 region in nuclear DNA, and according to their open reading frame region in mitochondrial DNA (mtORF) (*14*, *15*). Morphologically, the adult skeletal corallites corresponded to the *P. damicornis* clade 1 type β morphotype (*16*). Colonies released their planula larvae a few days before full moon, corresponding to reproductive type B in Hawaii (*17*). Together, these results show that our biological material belongs to the clade 1 type β lineage of the *P. damicornis* species complex. This lineage was recently redescribed as *Pocillopora acuta* (Lamarck, 1816), a species previously synonymized with *P. damicornis* (Linnaeus, 1758) (*16*).

Taxonomical classification of the *Symbiodinium* endosymbionts was assessed at the clade and type levels on the basis of the marker sequences in the 18S and internal transcribed region, and specifically in the ITS2 region in ribosomal DNA (*18*, *19*). These nuclear markers indicated that, in both planulae and adult colonies, the dinoflagellate symbionts belonged to clade C, ITS2 type C1, without diversity detected via restriction enzyme profiling of cloned sequences.

Planulae were labeled for 6 hours in light with [$^{13}$C]bicarbonate and [$^{15}$N]nitrate (which cannot be assimilated by coral host cells), followed by a 66-hour chase period in natural seawater with normal C and N isotopic compositions under a 12-hour light/12-hour dark cycling. Photosynthetic incorporation of $^{13}$C and assimilation of $^{15}$N by the dinoflagellate endosymbionts and the subsequent translocation of labeled compounds toward planula host tissue were quantified with subcellular resolution by combining transmission electron microscopy (TEM) and nanoscale secondary ion mass spectrometry (NanoSIMS) imaging (Figs. 1 and 2) (*20*, *21*). Here, we describe the dynamic inorganic C

and N assimilation and translocation process in the planulae throughout the pulse-chase experiment, and discuss this autotrophic contribution in comparison with that in adult colonies of the same lineage of *P. damicornis*, pulse-chase-labeled independently under similar experimental conditions (*22*).

Within the planula dinoflagellate endosymbionts (Fig. 1, A and B), both $^{13}C$ and $^{15}N$ were rapidly incorporated into different cell compartments and were already detectable after 30 min into the pulse period, during which the isotopic enrichments increased quasi-linearly (Fig. 1, C to E, insets). Over the subsequent 66-hour chase period, the $^{13}C$ enrichment in starch granules and lipid droplets experienced an ~80% drop (Fig. 1D). This rate of turnover in dinoflagellate C reserves (mainly starch granules and lipid droplets) was much lower than that observed in adult *P. damicornis* colonies where, under similar

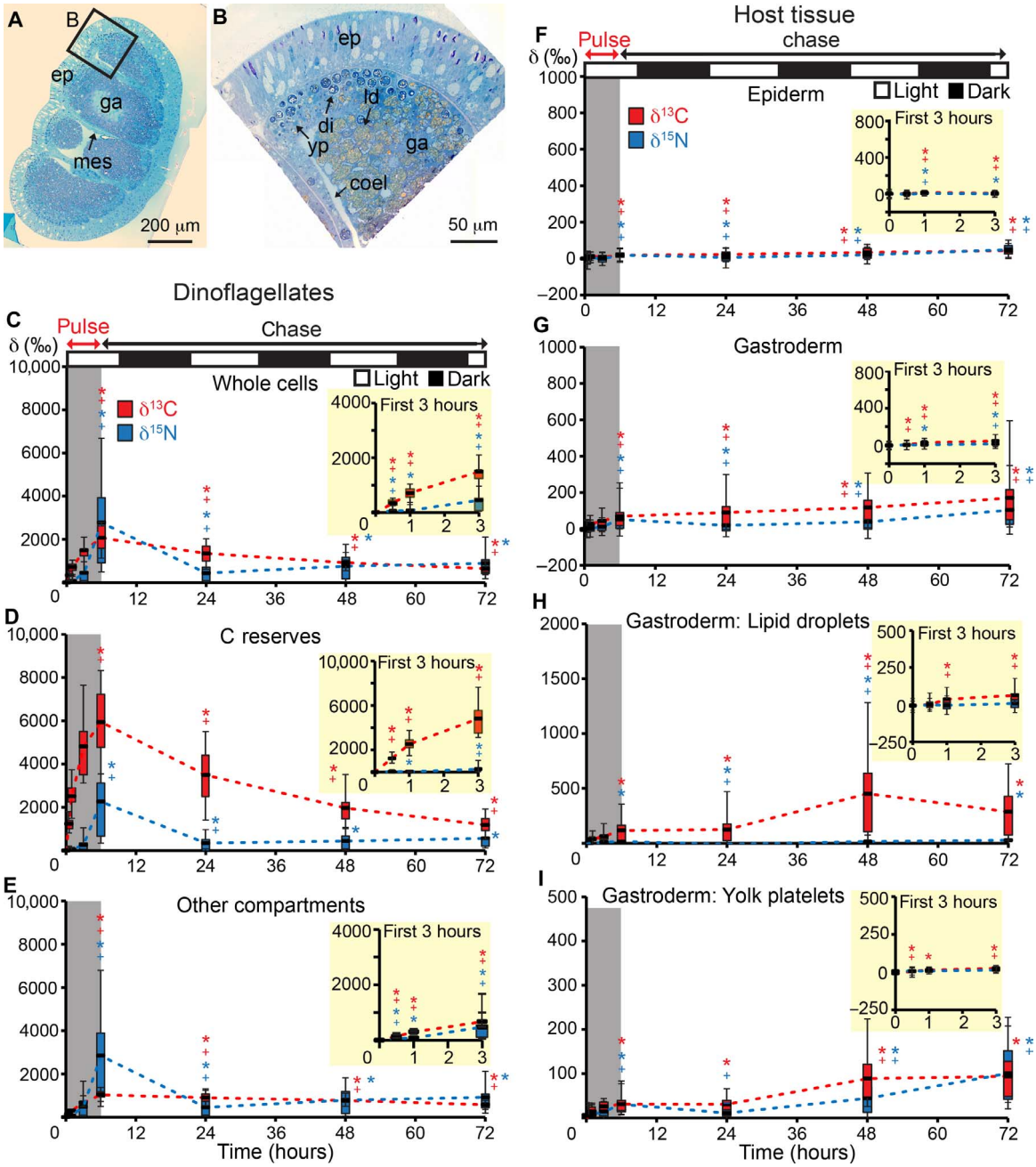


**Fig. 1. Photosynthetic C fixation, nitrate assimilation, and translocation in newly released symbiotic *P. damicornis* planulae.** (**A** and **B**) Histological sections of a planula, collected less than 12 hours after emission. coel, coelenteron; di, endosymbiotic dinoflagellate; ep, epiderm; ga, gastroderm; ld, lipid droplet; mes, mesentery; yp, yolk platelet. (**C** to **I**) Average $^{13}C$ and $^{15}N$ enrichments measured in the dinoflagellate cells (C to E) and in the planula host tissue during the pulse-chase experiment (F to I). Data are shown as box-whisker plots, with black horizontal bars indicating average values. Significant differences (Wilcoxon rank-sum test) are indicated between labeled and unlabeled control corals (*) and between samples from two consecutive time points ($^{+}$).

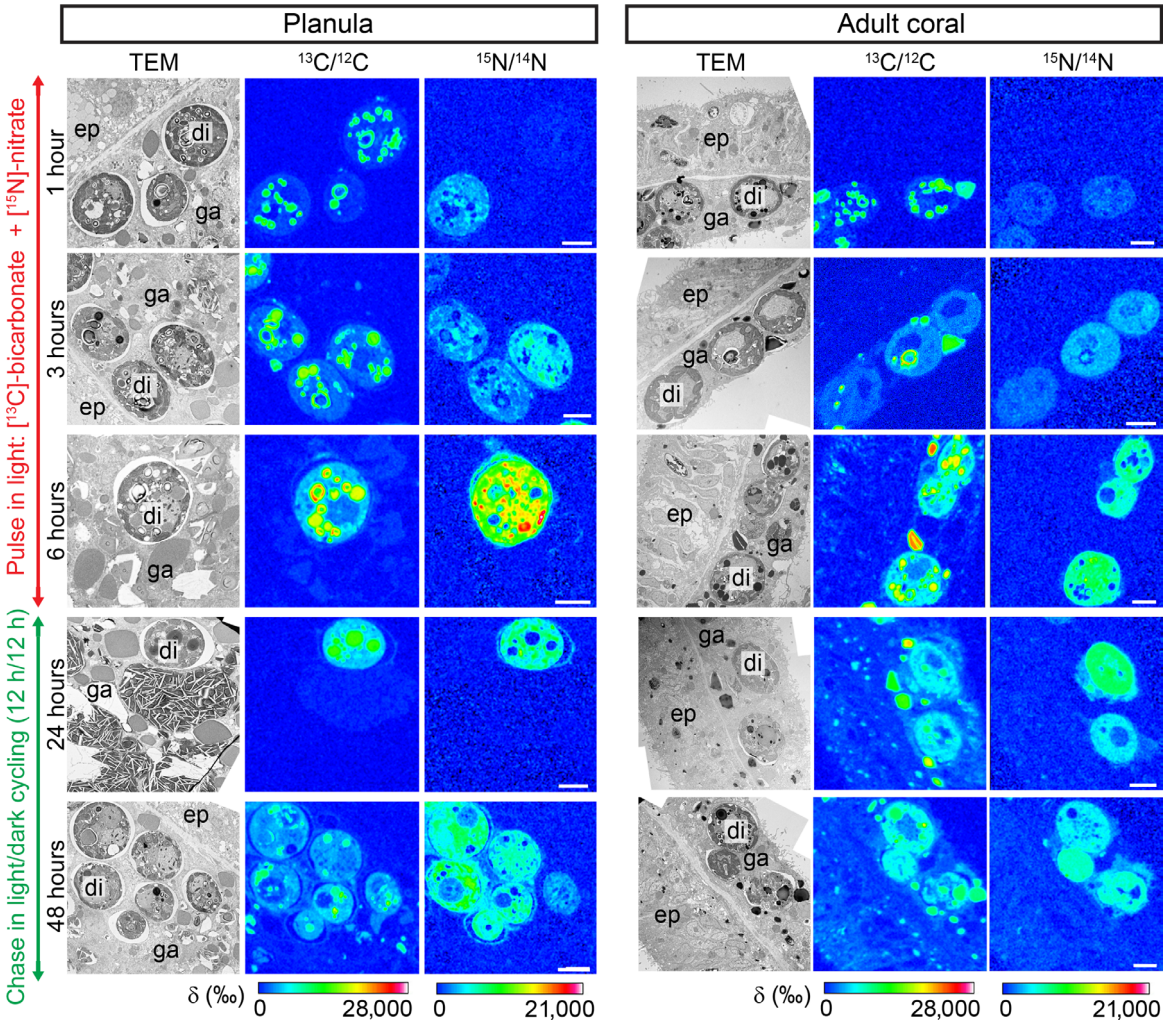


**Fig. 2. Visualization of C and N assimilation into dinoflagellates and translocation into host tissue in adult *P. damicornis* corals and their newly emitted planulae.** Each row includes a representative TEM micrograph of the dinoflagellate-containing host tissues and its corresponding quantitative NanoSIMS $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotopic maps during the pulse (6 hours) and chase (until 48 hours) under light/dark cycling. Data for adult corals are derived from a recent study (*22*). Scale bars, 5 μm. di, dinoflagellate; ep, epiderm; ga, gastroderm.

experimental conditions, a comparable (that is, ~80%) $^{13}C$ depletion was seen within just 18 hours into the chase (*23*). This difference might arise from (i) the lower translocation rate of photosynthates toward the host tissue in coral planulae compared to adults, (ii) the lower basal metabolism (respiration rate) of dinoflagellates in coral larvae, and/or (iii) the ability of gastrodermal cells in planulae to supply their endosymbionts with a substrate for respiration (for example, neutral lipids), rendering them less dependent on their own C reserves.

In contrast, the $^{15}N$ enrichment of dinoflagellates (and their subcellular compartments) decreased by ~70% within the first 18 hours into the chase, then remained essentially stable until the end of the experiment (Fig. 1, C to E). Such fast $^{15}N$ depletion was not previously observed in adult corals (*22*–*24*), and it was not accompanied by a corresponding increase in the planula host tissue, which would indicate translocation.

Within the planula host tissue, the gastroderm benefited more than the epiderm from the translocation of metabolic compounds from the dinoflagellates (Fig. 1, F and G): both $^{13}C$ and $^{15}N$ enrichments slowly increased during the chase, reaching average values of ~170 and ~100‰, respectively (Fig. 1G). In gastrodermal cells, $^{13}C$ was detected primarily in large osmiophilic lipid droplets (*25*), with a peak $^{13}C$ enrichment at 48 hours (Fig. 1H). Numerous gastrodermal crystalline granules (fig. S1), referred to as "yolk platelets" and often assumed to serve as protein storage (*25*), were found accumulating both translocated $^{13}C$ and $^{15}N$ (Fig. 1I).

Data on adult colonies of *P. damicornis* exposed to similar pulse-chase experimental conditions (except for 100 μmol photons $m^{-2}s^{-1}$ light intensity) and obtained with identical sample preparation and analytical methods (*22*) allowed for a direct comparison between the levels of translocation of compounds from dinoflagellates in planula and adult corals, respectively. Figure 2 illustrates the turnover and local translocation of photosynthetically derived $^{13}C$ and assimilated $^{15}N$ from dinoflagellate symbionts to adjacent host tissue (gastrodermis and epidermis) in both coral planulae and adult colonies. Figure 3A

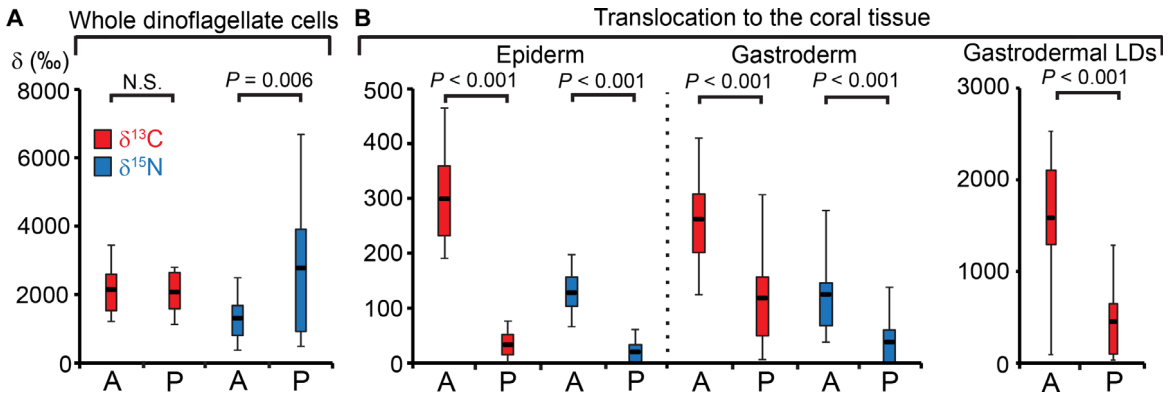


**Fig. 3. Comparison of the trophic contribution of C1 *Symbiodinium* cells to *P. damicornis* type β host in coral planulae versus adult colonies.** Data are shown as box-whisker plots, with black horizontal bars indicating average values, and are compared by means of Wilcoxon rank-sum test. $^{13}C$ and $^{15}N$ enrichments in dinoflagellates are shown at 6 hours, that is, at the end of the labeling pulse. $^{13}C$ and $^{15}N$ enrichments observed in the host tissue of planulae (P) or adult corals (A) are shown at 48 hours, during the chase. Data for adult corals are derived from a recent study (*22*). (**A**) Isotopic enrichment levels in dinoflagellates. (**B**) Isotopic enrichment levels in host coral epidermal and gastrodermal cells, as well as in gastrodermal lipid droplets (LD). NS, not significant.

shows that the level of photosynthetic C fixation is similar, and the nitrate assimilation slightly higher, in dinoflagellates from adult colonies at the end of the labeling pulse (that is, at 6 hours). This demonstrates that the symbionts in the planulae and in adult corals have essentially the same capability to incorporate $^{13}C$ and $^{15}N$ from bicarbonate and nitrate, respectively.

In Fig. 3B, the $^{13}C$ and $^{15}N$ enrichment levels in coral host tissue are compared between planulae and adult colonies at 48 hours into the pulse-chase experiment, reflecting the local translocation of compounds from the dinoflagellates. At this time point, the observed isotopic enrichments in the planula tissue generally have reached their maximum (Fig. 1, F to I), hence avoiding underestimation of translocation. From Figs. 2 and 3B, it is obvious that systematically less local translocation of both $^{13}C$- and $^{15}N$-enriched compounds took place from dinoflagellates to the adjacent planulae gastroderm and epiderm. Quantitatively, these differences amount to factors of ~9 and ~2 for $^{13}C$ and factors of ~6 and ~3 for $^{15}N$ in the epidermis and gastroderm, respectively. Lipid droplets in the gastroderm have been demonstrated to be the primary sink for translocated C-bearing photosynthates in adult corals (*22*). Figure 3B also shows that the $^{13}C$-labeling levels in these lipid droplets were higher in adult corals by a factor of ~3.5. In conclusion, the local rate (at the scale of individual NanoSIMS images) of translocation of metabolic compounds from dinoflagellates to the coral host tissue was systematically much lower in planula compared with adult coral colonies.

In further support of this finding, we note that, compared to adult *P. damicornis* corals, symbiosomal "extra-algal" lipid droplets (occurring outside the dinoflagellate but inside the symbiosome) were only rarely observed in the newly released coral planulae. These ultrastructures are thought to be involved in the extrusion of lipids from dinoflagellates toward the host tissue (*26*–*29*). The very low abundance of symbiosomal lipid droplets in planula larvae compared to adult corals is consistent with the hypothesis of a substantially lower rate of translocation of photosynthetic C from symbionts to host in this initial planktonic life stage of a reef-building coral.

In a parallel 6-hour dual-isotopic pulse of [$^{15}N$]ammonium and [$^{13}C$]bicarbonate in light, we investigated the ability of newly released planulae to assimilate ammonium (fig. S2). This experiment also independently verified the observed time scale of photosynthetic $^{13}C$ accumulation in dinoflagellates presented above. With regard to ammonium, observations on adult symbiotic reef corals (*23*, *24*, *30*) have clearly demonstrated the preference of dinoflagellates for ammonium over nitrate assimilation and the ability of the host tissue for direct ammonium assimilation. Our data for planulae corroborate these findings. Dinoflagellate and planula host cells of both epidermis and gastrodermis rapidly (within 15 min) and simultaneously incorporated the $^{15}N$ tracer, albeit with a much higher efficiency for dinoflagellates compared to coral cells (factor of ~10; fig. S2). Additionally, throughout the pulse, hotspots of $^{15}N$ enrichment were observed in the epiderm tissue of [$^{15}N$]ammonium-labeled planulae, most of them colocalized with coral cell structures such as Golgi bodies and nucleoli (fig. S1). In the gastrodermal coral cells, these hotspots were abundant and frequently corresponded to crescent moon–shaped ultrastructures with an empty center (material likely lost during sample preparation) (fig. S1). Areas of high $^{15}N$ enrichment, due to ammonium assimilation within the planula host cells, could not be unambiguously correlated to prokaryotic morphotypes, although the potential involvement of bacteria in planula holobiont metabolism is likely (*31*).

Overall, our results indicate that, in comparison with adult *P. damicornis* type β colonies, C1 *Symbiodinium* provides substantially less nutrition to the planula larvae. At the scale of the regions imaged with NanoSIMS, we have visualized the local translocation of isotopically labeled compounds into host cells in the immediate vicinity of the dinoflagellate cells. As shown in Figs. 2 and 3, this local translocation was significantly lower in planula than in adults.

However, to evaluate the total transfer of photosynthates and nitrogenous compounds to the coral, the density of symbionts in the host tissue must be considered. Indeed, a lower overall translocation from dinoflagellate symbionts to the host might be due to a lower bulk density of dinoflagellates and/or a lower rate of translocation from individual symbiont cells to their adjacent tissue layers. Therefore, the possible dinoflagellate density difference between a planula larvae and an adult coral was quantified and evaluated.

The inner gastrodermal tissue was very thick in these newly released planulae, with lipids taking up the bulk of its volume (Fig. 1,

A and B). The average *Symbiodinium* density of this planula gastrodermis was quantified to about one eighth of the density in the thin gastrodermis of an adult coral in the coenosarc region, that is, the tissue from which the corresponding NanoSIMS data in Figs. 2 and 3 were obtained. However, in the planula gastrodermis, the dinoflagellate population was strongly concentrated in a relatively narrow (<50 µm) layer located just below the mesoglea at the interface with the overlying epiderm (Fig. 1B). Here, the dinoflagellate density was in fact comparable to that of the thin gastroderm of the coenosarc area of adult colonies.

In terms of bulk transfer of photosynthates, given that the average gastrodermal density of dinoflagellates was clearly lower in planulae than in adults, the metabolic input from the photosymbionts would be lower in planulae if the translocation took place at the same rate. However, in addition, our NanoSIMS imaging of isotopic enrichment around the dinoflagellate cells also revealed a significantly lower local translocation to the host planula tissue (Figs. 2 and 3). Together, these results indicated that the lower level of trophic input from dinoflagellates to the host in the planulae, compared with adult corals, was due to the combination of a density effect and a much lower translocation rate from the photosymbionts to the host, imaged here for the first time with NanoSIMS.

Further work is required to establish the generality of this conclusion by investigating other coral and *Symbiodinium* species. However, from this specific study, it appears that freshly released coral planulae rely principally on their endogenous, maternally derived energy reserves (lipids and proteins). At this early stage, planulae's main benefit from hosting dinoflagellates seems to be primarily the transmission of *Symbiodinium sp.* populations. Nutritional metabolic fluxes between symbiotic partners seem to fully develop only at later life stages. A recent bulk-level isotopic study on 22- to 27-day-old *P. damicornis* planulae indicated that dinoflagellate endosymbionts translocate up to ~70% of photosynthetically fixed C (labeled using $^{14}C$) to the coral host tissue, which is similar to the proportion observed in adult corals (*9*). Precisely establishing the developmental stage at which transition to adult metabolic interactions takes place in symbiotic corals, and how environmental changes might affect this process, is of keen interest to marine biologists and ecologists. More generally, the occurrence of ontogenetic variations in trophic exchanges between symbiotic partners presents a unique opportunity to investigate in detail the underlying fundamental regulatory mechanisms.

## MATERIALS AND METHODS

### Planulae collection and experimental design

Experiments were carried out at the Océanopolis Aquarium (Brest, France) in May 2013. Two cohorts of planula larvae were obtained on successive days from one large (~15 cm in diameter) mother colony of the coral *P. damicornis* (Linnaeus, 1758) grown in open-system aquaria with natural seawater pumped from the Brest estuary. This coral is propagated in long-term aquarium cultures via fragmentation and natural reproduction, from initial colonies originating from Indonesia under CITES permit FR01081 00211/12-i. Buoyant planulae released overnight for 1 or 2 days, respectively, before the full moon (at the expected peak of larval release predicted from monthly records) were collected in the morning and used within 1 hour for the pulse-chase isotopic labeling experiments. The use of larvae released from a single colony minimized parental influence on the physiology of the planulae (*32*). The sexual or asexual origin of the released larvae was not established. Experimental settings were as follows: temperature, 24° ± 1°C; salinity, 35 ± 1‰; pH, 8.1 ± 0.1; 0.4-µm filtered artificial seawater [composition published by Harrison *et al.* (*33*)] or 0.4-µm filtered natural seawater with low nutrient concentrations [$NH_4^+$, <1 µM; $NO_2^-$, <1 µM; $NO_3^-$, <5 µM; $PO_4^{3-}$, <1 µM; data for seawater in Brest estuary obtained from "Service d'Observation en Milieu Littoral" (http://somlit-db.epoc.u-bordeaux1.fr/bdd.php) recorded in early June 2013]. Light irradiance (photosynthetically active radiation) was 380 µmol $m^{-2}$ $s^{-1}$ (LI-COR LI-1400 datalogger connected to a LI-193SA spherical sensor) for a 12-hour light/12-hour dark photoperiod provided by a 400-W HQI Titano-Gewiss metal halide lamp (GW84464M).

In the first pulse-chase experiment, one cohort of newly released planula larvae (less than 12 hours old) was incubated for 6 hours in light in a glass beaker filled with 500 ml of artificial seawater, initially free of bicarbonate ions and dissolved inorganic N, and supplemented with 2 mM [$^{13}C$]bicarbonate ($NaH^{13}CO_3$, 99 atomic %; Sigma-Aldrich) and 30 µM [$^{15}N$]nitrate ($K^{15}NO_3$, 98 atomic %; Sigma-Aldrich). This dual-isotopic labeling pulse started ~3 hours after the onset of the light period. After 6 hours, the labeled larvae were rinsed for 5 min in unlabeled natural seawater (500 ml) and transferred into a glass beaker filled with 500 ml of natural seawater with normal C and N isotopic abundance for a 66-hour chase period in a 12-hour light/12-hour dark cycling. Daily renewal of natural seawater (50% of the volume) and agitation (air bubbling with Pasteur pipettes) were carried out during the pulse-chase experiment to equilibrate the incubation seawater with ambient air and to avoid larval settlement. Two to three replicate planulae were sampled for TEM ultrastructural and NanoSIMS isotopic imaging, at 0 (control), 0.5, 1, 3, 6 (in the pulse), 24, 48, and 72 hours (in the chase).

In the second short-term pulse experiment, the other cohort of newly released planula larvae (less than 12 hours old) was incubated in light for 6 hours in a glass beaker filled with 500 ml of artificial seawater supplemented with 2 mM [$^{13}C$]bicarbonate ($NaH^{13}CO_3$, 99 atomic %; Sigma-Aldrich) and 20 µM [$^{15}N$]ammonium ($^{15}NH_4Cl$, 98 atomic %; Sigma-Aldrich). Planulae were sampled at 0 (control), 0.25, 0.5, 1, 3, and 6 hours into the pulse. In both experiments, planulae sampled at time 0 (controls) were not incubated in the isotopically enriched seawater but, were directly introduced from unlabeled natural seawater in the fixative solution.

Throughout the above pulse-chase experiments, coral planulae were prevented from settling by gentle air bubbling into the beaker; they remained viable and swimming, with no visible indications of stress, that is, without extensive mucus secretion or paling. When placed under static conditions, about 40% (8 of 20) of the remaining pulse-labeled planulae successfully metamorphosed, demonstrating that the labeling procedure did not compromise their competency.

### Markers for taxonomical identification of coral-dinoflagellate symbiotic partners

Taxonomical classification of the coral host and its dinoflagellate endosymbionts was assessed for planulae and adults on the basis of multiple independent markers. DNA markers were polymerase chain reaction–amplified from genomic DNA extracted with the TRIzol reagent protocol for simultaneous RNA and DNA purification, following the manufacturer's instructions. For the *Pocillopora* host, three molecular markers were used with their published primer sets and cycling

conditions: (i) the partial ribosomal RNA gene and the entire ITS region (ITS-1, 5.8*S*, and ITS-2) amplified with ITS1 and ITS4 in nuclear DNA; (ii) the ITS2 region amplified with ITSC2-5 and R28*S*-1 in nuclear DNA (*14*, *15*); and (iii) a putative control region amplified with FATP6.1 and RORF in mitochondrial DNA (*14*, *15*). The amplicons from planulae (Océanopolis) and adult colonies (ATPD) were cloned or directly sequenced in both directions for each set of primers. Sequences were aligned using ClustalW in BioEdit, and a search, using a basic local alignment search tool (BLAST), was performed in the National Center for Biotechnology Information (NCBI) database for taxonomical classification. All sequences were deposited in GenBank under accession numbers KU197078 to KU197082.

The micromorphology of the skeleton of adult colony nubbins was observed, bleached of tissue, dried, and gold-coated with a JEOL JSM-840 scanning electron microscope at the electron microscopy platform of the Museum National d'Histoire Naturelle. Rhythmicity of planulation was continuously monitored in relation to the lunar cycle over the previous 3 years.

For the *Symbiodinium* endosymbionts, two molecular markers were used with their published primer sets and cycling conditions, including the 18*S* ribosomal DNA amplified with the ssz primer set (*34*) and the internal transcribed region ITS2, amplified with itsD and its2rev primers (*19*). Sequences were aligned using ClustalW in BioEdit, and a BLAST search was performed in the NCBI database for taxonomical classification. Sequences were deposited in GenBank under accession numbers KU197083 to KU197085.

### Tissue observations

Sampled planulae (controls included) were chemically fixed for 24 hours at room temperature in Sörensen-sucrose phosphate buffer [0.1 M phosphate (pH 7.5), 0.6 M sucrose, 1 mM $MgCl_2$] containing both 2.5% glutaraldehyde and 0.5% formaldehyde (Electron Microscopy Sciences). They were then rinsed in buffer without aldehydes and post-fixed for 1 hour at room temperature with 1% $OsO_4$ in 0.1 M Sörensen phosphate buffer, dehydrated in graded series of ethanol (50, 70, 90, and 100%), and embedded in Spurr's resin. Chemical fixation induced contraction along the oral/aboral axis of the planula, resulting in shape transition (from elongated to spherical and disc-shaped) (*25*). Tissue sections were cut with a DiATOME 45° diamond knife (Ultracut microtome). Semithin sections (~0.5 µm) were stained with methylene blue–azure II (Electron Microscopy Sciences) and observed with a Zeiss Axio Imager Z2 light microscope equipped with a Zeiss AxioCam MRc 5 digital camera.

Dinoflagellate density was quantified from histological images acquired from replicate planulae ($n$ = 4) via individual cell counting and standardization to the gastrodermal surface area, using ImageJ software (National Institutes of Health). Similar counts were taken from histological images of the coenosarc area of replicate adult colony branches ($n$ = 14), previously analyzed with NanoSIMS (*22*). Ultrathin sections (~70 to 90 nm) were mounted on Formvar/C–coated alphanumeric grids, counterstained with 4% uranyl acetate and Reynold's lead citrate solution (Electron Microscopy Sciences), and observed at 80 kV with a Philips CM 100 TEM at the Electron Microscopy Facility of the University of Lausanne (Switzerland).

### Quantitative NanoSIMS isotopic imaging

The exact same areas in the planula tissue first imaged by TEM were subsequently analyzed with the NanoSIMS 50L ion microprobe (Cameca) in the Laboratory for Biological Geochemistry (EPFL, Lausanne, Switzerland), allowing direct correlation of ultrastructural TEM and isotopic NanoSIMS images. Because of the chemical fixation of planula tissue with aldehydes and osmium tetroxide, as well as ethanol dehydration procedure, low-molecular-weight soluble compounds and diffusible ions were extracted during sample preparation. Consequently, only isotopes incorporated into stabilized macromolecules were imaged by NanoSIMS.

TEM grids were mounted on 10-mm aluminum stubs and coated with ~10-nm gold. The grids were bombarded with a 16-keV primary ion beam of $Cs^+$ (1 to 3 pA) focused on a spot size of about 100 to 150 nm on the sample surface. Secondary molecular ions $^{12}C_2^-$, $^{13}C^{12}C^-$, $^{12}C^{14}N^-$, and $^{12}C^{15}N^-$ were simultaneously collected in electron multipliers at a mass resolution sufficient to avoid potentially problematic isobaric interferences on $^{13}C^{12}C^-$ and $^{12}C^{15}N^-$. Charge compensation was not necessary. Isotopic images (256 × 256 pixels) of areas ranging between 15 × 15 and 50 × 50 µm$^2$ were obtained by rastering the primary beam across the sample surface with a dwell time of 5 ms. For each sampled planula, ~7 to 10 NanoSIMS isotopic maps were obtained from an ultrathin section to image and quantify both isotopic enrichment and spatial distribution within dinoflagellates and planula tissue. Distribution maps of the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios were obtained by taking the ratio between the drift-corrected $^{13}C^{12}C^-$ and $^{12}C_2^-$ images and the $^{12}C^{15}N^-$ and $^{12}C^{14}N^-$ images, respectively. $^{13}C$ and $^{15}N$ enrichments were expressed in the δ notation

$$\delta^n X(‰) = \left(\frac{X_{mes}}{X_{nat}} - 1\right) \times 1000 \quad (1)$$

where $\delta^n X$ is either $\delta^{13}C$ or $\delta^{15}N$, $X_{mes}$ is the measured $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio, and $X_{nat}$ is the average natural $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio measured regularly throughout the day in nonlabeled, identically prepared coral samples throughout the period of NanoSIMS analyses.

### Definition of regions of interest and statistical analyses

Enrichments in $^{13}C$ and $^{15}N$ were measured in the planula tissue by defining regions of interest (ROI) in the NanoSIMS isotopic images, using the L'IMAGE software (developed by L. Nittler, Carnegie Institution of Washington, Washington, DC). For the dinoflagellates, ROI were defined by drawing the outline of individual cells. For the epidermal and gastrodermal host tissues (Fig. 1, A and B, and Fig. 2), ROI were defined as circles of 2 to 3 µm covering each epithelia and avoiding the mesoglea, intercellular spaces, and coelenteron. Isotopic enrichments of intracellular compartments, such as the coral gastrodermal lipid droplets and yolk platelets, as well as the dinoflagellate subcellular C reserves (Fig. 2), were obtained from ROI drawn in 15 × 15–µm high-resolution NanoSIMS images, following the outline of each structure. This process is illustrated in detail in fig. S2 of the study by Kopp *et al.* (*22*).

Data for each class of ROI from duplicate or triplicate coral planulae per time point were combined after no significant difference in average isotopic enrichments was observed between replicates. Individual ROI data and statistical significance are reported in data file S1. The total number of pooled ROI analyzed per time point ranged from $n$ = 25 to 40 for dinoflagellate cells, from $n$ = 500 to 1000 for epiderm and gastroderm, and from $n$ = 40 to 100 for intracellular gastrodermal lipid droplets and yolk platelets.

Statistical analyses were performed using the R software. Shapiro-Wilk and Bartlett tests were first used to assess data normality and homoscedasticity. Subsequently, for each class of ROI defined above,

isotopic enrichments between time points were compared two by two as follows: In case of non-Gaussian distributions, the nonparametric Kruskal-Wallis test was applied combined with a pairwise Wilcoxon rank-sum test, instead of the one-way analysis of variance combined with a pairwise *t* test. Holm's correction was systematically used when doing pairwise multiple comparisons tests. Summary *P* value tables of these pairwise comparisons are provided in data file S1. Results were considered significant at the 5% level.

## SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/3/e1500681/DC1

Fig. S1. High-resolution imaging of $^{15}N$ distribution in coral cells of planulae exposed to [$^{15}N$] ammonium.

Fig. S2. Ammonium assimilation by newly released coral planulae.

Data file S1. Summary tables of NanoSIMS isotopic measurements and statistical analyses.

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