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RESEARCH

Oxylipin signalling in a coral-dinoflagellate symbiosis

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Abstract The symbiotic relationship of cnidarians with dinoflagellates of the family Symbiodiniaceae is based on host-symbiont recognition processes and continuous molecular exchange between partners. However, the molecular signals involved are unresolved. Oxylipin signalling plays a pivotal role in mediating various cellular processes, including inflammation and molecular signalling. Its function in the cnidarian-dinoflagellate symbiosis, including its potential role in inter-partner molecular communication, remains unclear. Here, prostaglandin EP2 receptors 2 (EP2) and 4 (EP4) were localised and quantified using immunohistochemistry in the tissues of the coral *Acropora* sp. aff. *tenuis*. Both coral larvae and polyps of juvenile colonies were examined when in symbiosis with one of their two native dinoflagellate symbionts, *Cladocopium goreaui* and *Durusdinium trenchii*, during early (3 days) and later (30 days) stages of symbiosis establishment (relative to aposymbiotic corals). EP2 and EP4 were present in both the gastrodermis and epidermis of larvae and polyps, regardless of their symbiotic state. Abundance of EP2 and EP4 was affected by symbiotic state, symbiont identity, coral life-stage, and the age of the symbiosis. Specifically, *D. trenchii*, but not *C. goreaui*, decreased host EP2 levels in larvae and polyps, and EP4 levels in coral polyps. Conversely, *C. goreaui*, but not *D. trenchii*, decreased EP4 levels in coral larvae. This research enhances our understanding of oxylipin pathway regulation in the coral-dinoflagellate symbiosis across various life-stages, and in response to different symbiont

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species, laying the groundwork for deeper exploration into the molecular signalling mechanisms that underlie this symbiosis and the influence of coral metamorphosis on these mechanisms.

Keywords Lipid signalling · Coral larvae · Coral polyps · Immunohistochemistry · Oxylipin receptors · Symbiodiniaceae · *Acropora tenuis*

Introduction

Coral reefs are one of the Earth's most valuable ecosystems due to their productivity and biodiversity (Moberg and Folke 1999). Coral reef function and survival are underpinned by the symbiosis between the host cnidarian (e.g. corals) and dinoflagellates of the family Symbiodiniaceae that live in the gastrodermal cells of the host. Symbiodiniaceae cells provide photosynthetic products in support of host metabolism, growth and reproduction, and facilitate the recycling and conservation of essential nutrients such as nitrogen (Muscatine and Porter 1977; Falkowski et al. 1984; Rädecker et al. 2015; Rädecker and Meibom 2023). While the functional properties of such cnidarian-algal symbioses have been studied for decades, the molecular mechanisms that mediate an effective and persistent coral-Symbiodiniaceae symbiosis remain poorly understood (Davy et al. 2012). For instance, stability of the cnidarian-dinoflagellate symbiosis is founded on inter-partner molecular exchange; however, we know relatively little about the molecules and processes that mediate this crosstalk (Rosset et al. 2021).

In the cnidarian-dinoflagellate symbiosis, the best-studied molecular signalling mechanism to date involves cell–cell glycan-lectin binding; however reactive oxygen species, sterol compounds and lipids have all been proposed to play a role (Matthews et al. 2017; Rosset et al. 2021; Botana et al. 2022). Oxylipins, a class of oxygenated fatty acids, have gained particular recent attention (Rosset et al. 2021; Botana et al. 2022). Oxylipins are signalling lipids produced by the oxidation of polyunsaturated fatty acids (Du et al. 2020). These membrane-diffusible, short-lived signalling molecules are produced by many organisms including cnidarians and dinoflagellates (Onodera et al. 2004; Lõhelaid and Samel 2018; Botana et al. 2022), and are involved in cellular processes, especially inflammation, by interacting with specific G protein-coupled receptors and peroxisome proliferator-activated receptors (Hattori et al. 2008; Scher and Pillinger 2009; Nieman et al. 2016; Vleeshouwers et al. 2021). Consistent with these roles, oxylipins are upregulated in soft corals under heat stress and/or injury (Lõhelaid and Samel 2018); conversely, oxylipin synthesis genes are downregulated in the sea anemone *Exaiptasia diaphana* ('Aiptasia'), a model system for the cnidarian-dinoflagellate symbiosis, when in the symbiotic *versus* aposymbiotic (i.e. symbiont-free) state (Matthews et al. 2017). Indeed, evidence suggests that oxylipins contribute to inter-partner communication across a diverse range of symbioses, such as fungus-plant, bacterium-plant, and protist-mammal symbioses (Burow et al. 1997; Calvo et al. 1999; Pohl and Kock 2014; Yao et al. 2023).

In a previous study, we localised and quantified two prostaglandin receptor proteins, EP2 and EP4, in the tissues of Aiptasia (Gamba et al. 2024). While both receptors mediate prostaglandin E2 (PGE2) signalling, they can activate overlapping or distinct downstream pathways (Fujino et al. 2002, 2003), making it important to study them individually. PGE2s are well-known oxylipins that are important for a variety of processes such as inflammation, immune cell regulation, cellular homeostasis, tissue repair, and molecular signalling (Lundequist et al. 2010; Kawahara et al. 2015; Cheng et al. 2021; Vleeshouwers et al. 2021), and are important for inter-partner crosstalk between, e.g. fungi and plants and protists and mammals (Kubata et al. 1998; Tan et al. 2019). In our previous study, EP2 and EP4 receptor abundances decreased in the gastrodermis of anemones hosting their homologous (i.e. native) dinoflagellate symbiont (*Breviolum minutum*) *vs*. the aposymbiotic state (Gamba et al. 2024). This led to the hypothesis that, as in other symbioses (Brodhagen et al. 2008; Patkar et al. 2015; Rosset et al. 2021), the symbiont dampens host oxylipin pathways and hence the host immune response, so facilitating the symbiont's intracellular persistence. Moreover, EP2 and EP4 were localised to the symbiosome membrane, the host-derived membrane that surrounds the symbiont and hence acts as the interface between the two partners, providing more direct evidence for a role in inter-partner communication (Wakefield and Kempf 2001; Davy et al. 2012; Dani et al. 2017).

In this current study, we aimed to establish whether the oxylipin receptor patterns seen in the model Aiptasia system also occur in reef-building corals. Specifically, we localised and quantified receptors EP2 and EP4 in the tissues of the common coral *Acropora* sp. aff. *tenuis* via immunohistochemistry (see Bridge et al. 2023 for an update on *A. tenuis* taxonomy), in response to harbouring different symbiont species and coral life-stage (i.e. larva *vs*. juvenile polyp). Both symbiont species are homologous with this coral at the study site in Okinawa, Japan, although *D. trenchii* appears to be the main symbiont in juvenile corals whereas *C. goreaui* is more prevalent in adults (Ulstrup and Van Oppen 2003; Little et al. 2004; Abrego et al. 2009; Yamashita et al. 2014; Lewis et al. 2024). While the reason for the common early life-history dominance of *D. trenchii* is unknown, initial acquisition of symbionts is thought to be relatively non-selective, potentially favouring *D. trenchii*, which is often considered as an opportunist (Little et al. 2004; Stat and Gates 2011; Yuyama et al. 2018; Bellantuono et al. 2019;

Ng et al. 2019). Furthermore, previous studies have shown that cnidarian host gene expression shifts significantly in the first days and weeks post-symbiosis initiation, suggesting key physiological changes (Yuyama et al. 2018; Voss et al. 2019; Gorman et al. 2022). Based on this, we analysed receptor abundance at two time points: (1) initial symbiont uptake (3 days post-inoculation), when symbionts typically begin proliferating inside host cells, and (2) at a later stage (30 days post-inoculation), as maintaining larvae of this coral beyond then is challenging. Given these dynamics, we hypothesised that receptor abundance would reflect the opportunistic nature and early colonisation success of *D. trenchii*.

Methods

Acropora sp. aff. *tenuis* collection and spawning

Colonies of *Acropora* sp. aff. *tenuis* ($n = 7$) were collected by SCUBA diving on May 26th 2023, from a depth of ~ 5 m on the reef flat of Sesoko Island, Okinawa, Japan (N 26° 39′50.76″; E 127° 52′10.14″). Collection was conducted under permit number 31–26 issued by the Okinawa Prefectural Government. All coral colonies were placed in outdoor tanks at the Sesoko Marine Research Station, Tropical Biosphere Research Centre, University of the Ryukyus, and cone-shaped traps with mesh were positioned above each colony to capture gamete (egg-sperm) bundles. Spawning occurred between 19:30 h and 20:00 h on May 26th 2023. Gamete bundles were transferred to the laboratory, where they were placed in buckets containing 0.22 µm filtered seawater (FSW). The 0.22 µm FSW was gently mixed to facilitate dissociation and fertilisation, ensuring a thorough mixing of gametes from the seven different colonies to mitigate the effects of genetic variation. The 0.22 µm FSW was replenished twice over two hours post-fertilisation, and thereafter was replaced twice daily to maintain optimal conditions. Larvae became motile three days after spawning/fertilisation, and were subsequently placed in 5 L tanks containing 0.22 µm FSW, which was replaced once a day. Once transferred into the tanks, the larvae were maintained at a temperature of 26.8 °C (± 0.8 °C standard deviation) and an irradiance of 100 µmol photons m^{-2} s^{-1} on a light:dark cycle of 12 h:12 h (Osram Dulux 36/W890 fluorescent bulbs).

Symbiodiniaceae

Two Symbiodiniaceae species were used: *Durusdinium trenchii* (culture ID Ap2, originally isolated from an unknown sea anemone, Okinawa, Japan) and *Cladocopium goreaui* (culture ID CCMP2466, originally isolated from the sea anemone *Discosoma sanctithomae*, Caribbean Sea). Cultures of both species were maintained in f/2 medium in 0.22 µm filtered artificial seawater (ASW) (Coral Pro Salt, Red Sea, New Zealand), at a constant temperature of 25 °C and an irradiance of 100 µmol photons m^{-2} s^{-1} on a 12 h:12 h light:dark cycle (Osram Dulux 36/W890 fluorescent bulbs). All cultures were last sub-cultured 12 weeks before the experiment. To confirm the identity of these cultures, DNA extraction was performed using the cetyltrimethylammonium bromide-phenol–chloroform method. The ITS2 region of ribosomal DNA was amplified by PCR using the forward primer ITSintfor2 (5′-GAATTGCAGAACTCC GTG-3′) and the reverse primer ITS2Rev2 (5′-CCTCCG CTTACTTATATGCTT-3′). Each PCR mixture had a total volume of 25 µL and contained 1 ng template DNA, 1 mM total deoxynucleoside triphosphate, 7.5 pmol of each primer, and 1.5 U of Taq DNA polymerase. A Qiagen thermocycler was used for PCR with the following cycling regimen: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 56 °C for 15 s, and extension at 72 °C for 10 s. A final extension step was performed at 72 °C for 3 min. The amplicons were subjected to direct Sanger sequencing (Macrogen Inc., Seoul, South Korea). The obtained sequences were aligned using Geneious Prime v. 2019.2.3 (Biomatters Ltd., Auckland, New Zealand), and a custom BLAST search was conducted against Symbiodiniaceae ITS2 sequences in Geosymbio to determine their identity (Tables S1 and S2, supplementary material).

Symbiosis establishment

Acropora sp. aff. *tenuis* produces gametes and larvae that are symbiont-free. One week after spawning, larvae ($n = 200$) were inoculated with either *C. goreaui* or *D. trenchii*. The 200 larvae were divided by pipetting them across 8 × 40 mL plastic petri dishes containing 40 mL of 0.22 µm FSW with 4×10^5 cells/mL of the respective symbiont culture; symbiont density was measured with a cell counter (LUNA-FL; Logos Biosystems; USA). After 12 h, the inoculation process was repeated as described above, and following a further 12 h the inoculated larvae were transferred to 0.22 µm FSW and maintained as previously described until sampling (see below). As a control, the same process was performed with another 200 larvae but excluding the addition of symbionts.

Ten days after spawning, a separate subset of larvae ($n = \sim 300$) was induced to settle in 20 mL plastic petri dishes, with approximately 12 individuals *per* dish. Settlement was stimulated with the synthetic peptide Hym-248 (EPLPIGLW-amide) at a final concentration of 5×10^{-6} M in 0.22 µm FSW (Iwao et al. 2002). After a 12-h incubation, in which coral larvae settled, 0.22 µm FSW in the petri dishes was replaced, and thereafter 0.22 µm FSW

replacement occurred once a day. The polyps were maintained at the same temperature and irradiance as those used for larval culturing. Three days after settling, ca. 100 coral polyps (eight dishes) were inoculated with either *C. goreaui* or *D. trenchii*, by replacing the FSW in each dish with 20 mL of fresh 0.22 µm FSW containing 4×10^5 cells/mL of the respective symbiont culture, measured as above. An additional 100 coral polyps were held in the aposymbiotic state, acting as a control. The coral polyps (both inoculated and control groups) were maintained as previously described until sampling (see above).

Sampling

Five larvae were collected from each petri dish at 3 d and 30 d post-inoculation using a plastic pipette, with aposymbiotic larvae harvested simultaneously ($n = 8$ *per* treatment, 5 technical replicates). To begin the sampling process, animals were anaesthetised by immersing them in 0.22 µm FSW containing 0.5 M $MgCl_2$ for 10 min. They were then fixed in a solution of freshly prepared 4% formalin in 0.22 µm FSW and kept at 4 °C overnight to facilitate fixation. Once fixed, the larvae were rinsed in phosphate-buffered saline (PBS) and immersed in a cryoprotectant solution consisting of 10%, 20%, and 30% sucrose dissolved in PBS at 4 °C. They were then embedded in an optimal cutting temperature (OCT) (Tissue-Tek OCT, Sakura) medium and rapidly frozen in liquid nitrogen. Finally, the frozen samples were stored at − 80 °C until further analysis.

At 3 d and 30 d post-inoculation, the FSW in the dishes containing the coral polyps was replaced with 0.22 µm FSW containing 0.5 M $MgCl_2$, and the polyps anaesthetised for 10 min. This solution was then replaced with freshly prepared 4% formalin in 0.22 µm FSW, and the polyps fixed overnight at 4 °C. Fixed polyps were placed in a 10% EDTA/0.22 µm FSW solution at 4 °C for decalcification. The EDTA solution was replaced once a day until the skeleton of the polyps completely disappeared, with polyps detaching from the dish. Five fixed polyps were collected from each dish (n = 8 *per* treatment, five technical replicates), treated with cryoprotectant, and embedded and stored as described above. All samples were transported to New Zealand for immunohistochemical analysis.

Custom antibody validation

Antibodies designed in our previous study were used here (Table S3, supplementary Material) (Gamba et al. 2024). Briefly, EP2 and EP4 amino acid sequences were identified using publicly available Aiptasia genome and transcriptome databases (Lehnert et al. 2014; Baumgarten et al. 2015). The complete amino acid sequences and corresponding NCBI accession numbers for Aiptasia EP2 and EP4 receptors are provided in Fig. S1 (supplementary Materials). InterProScan software was used to find antigenic regions for the EP2 and EP4 receptors. When selecting the epitopes, criteria used were: conservation level, antigenicity, and the avoidance of transmembrane regions (Paysan-Lafosse et al. 2023). Epitope sequences were aligned against *Oryctolagus cuniculus* (European rabbit) and the Aiptasia genome (Lehnert et al. 2014; Baumgarten et al. 2015; Parkinson et al. 2016) to confirm antigenicity and avoid cross-reactivity. Antibodies were then developed using synthetic peptides.

Given that the antibodies were created using Aiptasia databases, it was necessary to ensure that they would also work with our corals. To do this, coral proteins were extracted from a single frozen coral fragment and used to verify the antibodies via immunoblotting. An airbrush fitted to a reservoir containing ice-cold protein extraction buffer (100 mM Tris, 100 mM NaCl, 10 mM EDTA, pH 7.4, 10 µg/ml protease inhibitor cocktail) (cOmplete™, Mini, EDTA-free protease inhibitor cocktail, Roche) (Dani et al. 2017) was used to separate coral tissue from the skeleton. The tissue slurry was then centrifuged (500 g; 3 min) to separate the host and symbiont fractions. The host fraction (supernatant) was transferred to a new tube and mixed with 5% β-mercaptoethanol (v/v). Samples were then heated on a heat block (70 °C; 7 min), centrifuged (20,238 g; 10 min), and the supernatant stored at − 80 °C prior to analysis and protein quantification using a Qubit® 2.0 fluorometer and protein assay kit (ThermoFisher Scientific). Protein extracts and pre-stained molecular weight standard (Thermo Scientific™ PageRuler™ Plus Prestained Protein Ladder) were resolved by TRIS–glycine SDS-PAGE. Following SDS-PAGE, a semi-dry blotting system was used to transfer the proteins onto a 0.45 µm PVDF membrane. The membrane was blocked at room temperature for 1 h using TRIS-buffered saline (TBS) containing 5% fat-free milk and then incubated with the different primary custom antibodies at a concentration of 1:500 at 4 °C on a shaking table overnight. The membranes were then incubated with a secondary antibody (1:2000, Goat Anti-Rabbit IgG H&L (Alexa Fluor® 555) (ab150078)) for 2 h at room temperature. A Typhoon FLA 9500 laser scanner (GE Healthcare Life Sciences.) was used to visualise the bands.

Because EP2 and EP4 are not yet characterised in *Acropora tenuis*, InterProScan and Geneious 2.0 (Biomatters Ltd., Auckland, New Zealand) software was used to ensure that the primary antibodies attached to the correct proteins. Reefgenomics was used to align the amino acid sequences of Aiptasia EP2 and EP4 to the genome of *A. tenuis*, to find uncharacterised proteins that have similar sequences and that matched the results from the immunoblotting analysis (Voolstra et al. 2015; Liew et al. 2016). InterProScan was then used to confirm that these uncharacterised proteins possess the same functional domains as EP2 and EP4, while

Geneious 2.0 was used to align the amino acid sequence of these uncharacterised proteins to the epitope sequences used to build the custom antibodies (Fig. S2, supplementary Material).

Immunolocalisation and semi-quantitative analysis

Using a CM3050 cryostat (Leica Biosystems), embedded coral larvae and polyps were sectioned (10 µm), mounted on positively-charged slides (Superfrost Plus Adhesion Microscope Slides, Epredia), and stored at − 20 °C. For immunolabelling, PBS buffer containing 0.1% Triton X-100 was used to permeabilise these cryosections. PBS-0.1% TWEEN (PBST) was used to wash the samples three times before immersion with the blocking buffer (PBST; 10% normal goat serum; 1% BSA) for 2 h at room temperature. Cryosections were then incubated with the primary antibody (1:100) in a blocking buffer overnight at 4 °C. The following day, the samples were incubated for 2 h at room temperature with the secondary antibody (1:500 in blocking buffer, Goat Anti-Rabbit IgG H&L (Alexa Fluor® 555) (ab150078)). DAPI mounting medium (2 µg ml^{-1}) was then applied to stain DNA, before sealing with a coverslip. Pre-absorption and secondary antibody controls were included for both coral larvae and polyps (Figs. S4 and S5, supplementary material). The immunolabelled samples were then visualised with confocal laser microscopy (Fluoview PV300, Olympus, Japan). DAPI was visualised with a DNA (430 nm) filter, oxylipin receptors with AlexaFluor555 (570 nm), and chlorophyll autofluorescence with AlexaFluor647 (650 nm). The same settings were used to acquire the z-stacks.

We applied the workflow developed in our previous study (Gamba et al. 2024) to quantify AlexaFluor555 intensity in host tissues, measure host tissue volume, and mask autofluorescence from the symbionts. Briefly, Fiji (ImageJ2) (Schindelin et al. 2012) was used to perform image analysis, while background noise, 3D Gaussian blur, and background subtraction methods were used to smooth images and manually remove background noise (Kelley and Paschal 2019; Tivey et al. 2020). A mask using thresholding intensities for the AlexaFluor647 channel was created to select symbionts and eliminate them from the AlexaFluor555 channel, to cancel out any autofluorescence signal created by the symbionts' putative lipid bodies (Nielsen and Petrou 2023). In a previous study, we demonstrated via western blotting that these custom-made antibodies do not react with symbiont proteins, confirming that the signals observed inside the algal cells are due to autofluorescence (Gamba et al. 2024). AlexaFluor555 images were manually segmented to acquire images of host epidermis tissue. A 3D object counter plugin was then used to measure signal intensities in both the unsegmented AlexaFluor555 images, representing the total host tissue, and the AlexaFluor555 segmented images, corresponding specifically to the epidermis tissue (Bolte and Cordelières 2006). Gastrodermis signal intensities were obtained by subtracting the epidermal value (segmented images) from the total host tissue value (unsegmented images). AlexaFluor555 intensities were then normalised to host tissue volume, which was calculated using the brightfield channel. Symbiont density was calculated *per* image (n = 16) by manually measuring the symbiont number using the AlexaFluor647 channel (Table S4, supplementary material), and normalising it to host tissue volume using the brightfield channel.

Statistical analysis

All data were checked for normality, homogeneity and equal variance, and transformed where necessary. To calculate the difference in the intensities of these receptors in the gastrodermis and epidermis of larvae and polyps at days 3 and 30 post-inoculation, a two-way mixed ANOVA was used. Finally, a Tukey HSD post hoc test was used to find where the differences lay between all the pairwise comparisons. A permutational two-way ANOVA test followed by Bonferroni-adjusted pairwise comparisons were used to identify differences in the symbiont density in larvae and polyps at days 3 and 30 post-inoculation.

Results

Symbiont density

Symbiont density was significantly different between symbiont species at different post-inoculation time-points and coral life-stages, and there was significant interaction between these two factors (permutational two-way ANOVA; $p < 0.0001$ for all comparisons) (Fig. 1). At day 3, the symbiont density was higher in coral polyps *versus* coral larvae when hosting *D. trenchii* ($p < 0.0001$) but not when hosting *C. goreaui*. At day 30, symbiont density was significantly higher in polyps than larvae when hosting either *D. trenchii* or *C. goreaui* ($p < 0.0001$ for all comparisons). Finally, *C. goreaui* and *D. trenchii* symbiont densities were significantly higher in coral polyps at day 30 compared to day 3 ($p < 0.0001$).

Receptor localisation and abundance

The antibodies exhibited high specificity in immunoblotting, demonstrating distinct bands for each target protein (Fig. S2, supplementary Material). EP4 and EP2 had not been previously characterised in corals, so there was no direct reference for our observations. Nevertheless, a 60 kDa band was observed for EP4 and a 38 kDa band for EP2. Upon custom BLAST analysis of the amino acid sequences of EP4 and

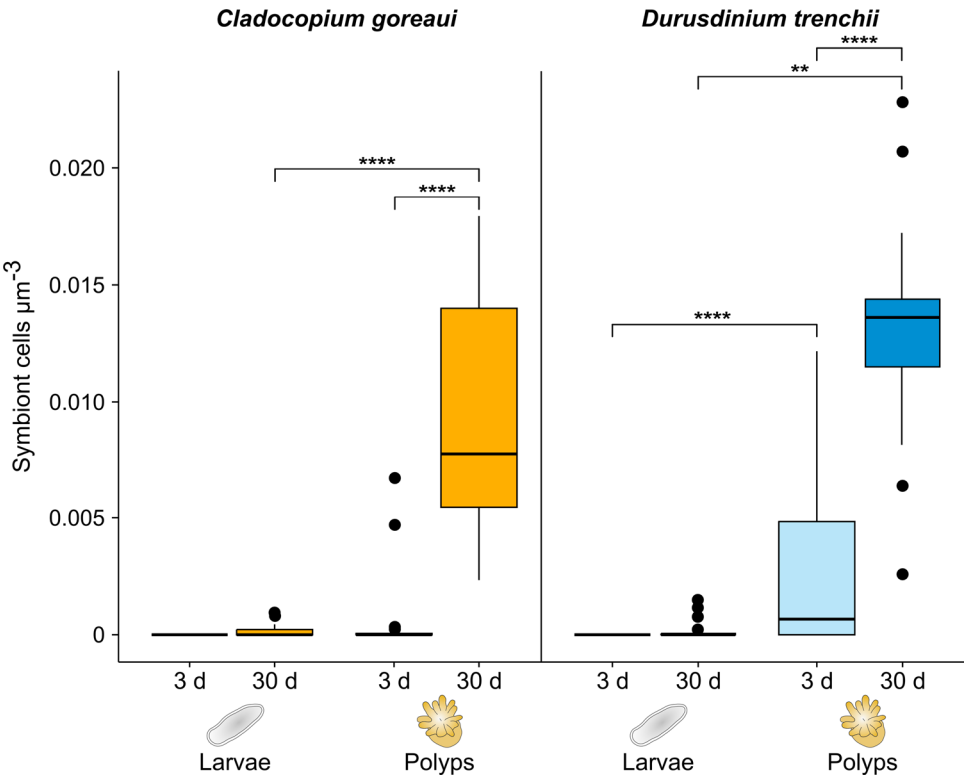


Fig. 1 Symbiont density (*per* unit area-of-interest) of different symbiont species (*Cladocopium goreaui* and *Durusdinium trenchii*) in larvae and juvenile polyps of the coral *Acropora* sp. aff. *tenuis*, 3 and 30 days post-inoculation. $N = 16$, whiskers show upper and lower quartiles, outlier values marked by points. Asterisks indicate significant differences: $*P < 0.05$; $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$

EP2 from Aiptasia against the genome of *A. tenuis*, a protein of 60 kDa (aten_0.1.m1.19428.m1) and a protein of 39 kDa (aten_0.1.m1.582) were identified, respectively (Voolstra et al. 2015; Liew et al. 2016) (full amino acid sequences of aten_0.1.m1.19428.m1 and aten_0.1.m1.582 can be found in Fig. S3, supplementary material). Further analysis using InterProScan confirmed that the functional domain of these two proteins is characteristic of Rhodopsin G-coupled receptors, which includes the EP2 and EP4 receptors. While the antibodies were designed based on Aiptasia sequences, and some uncertainty remains regarding their specificity for coral EP2 and EP4, the clear and distinct bands observed in the western blots, combined with strong bioinformatic evidence, suggest that these antibodies successfully recognise EP2 and EP4 in corals.

EP2 and EP4 were localised to both the epidermis and gastrodermis of coral larvae and polyps, independent of symbiotic state (i.e. aposymbiosis or symbiosis with different symbiont types) or time in symbiosis (Fig. 2). These two factors influenced the abundance of both receptors, however. In coral larvae, the overall abundance of EP2 was significantly affected by symbiotic state ($F_{2,42} = 10.93$; $p = 1.5 \times 10^{-4}$) and time in symbiosis ($F_{1,42} = 23.35$; $p = 1.83 \times 10^{-7}$) but not the interaction between the two (Fig. 3). More specifically, at day 3, EP2 abundance was higher in the gastrodermis *vs.* epidermis for both *C. goreaui*- and *D. trenchii*-colonised larvae, but not aposymbiotic larvae ($p < 0.0001$ for all comparisons), whereas EP2 abundance decreased from day 3 to day 30 in the gastrodermis of *D. trenchii*-colonised larvae only ($p = 5.6 \times 10^{-5}$).

Similarly, in coral polyps, the overall abundance of EP2 was again significantly affected by symbiotic state ($F_{2,42} = 6.82$; $p = 0.002$) and time in symbiosis ($F_{1,42} = 8.03$; $p = 0.007$) but there was no significant interaction between the two (Fig. 3). At day 3, the overall abundance of EP2 was higher in *C. goreaui*-colonised than aposymbiotic polyps ($p = 0.01$). In addition, EP2 abundance increased from day 3 to day 30 in aposymbiotic polyps in both the epidermis and gastrodermis ($p = 8.3 \times 10^{-5}$ and $p = 0.0014$, respectively).

In coral larvae, the overall abundance of EP4 was not significantly affected by symbiotic state or time in symbiosis, though there was a significant interaction between the two factors (two-way ANOVA; $F_{2,42} = 18.4$; $p = 1.81 \times 10^{-6}$) (Fig. 4). The overall abundance of EP4 was greater at day 3 in larvae hosting *C. goreaui* than in either *D. trenchii*-colonised or aposymbiotic larvae ($p = 3.13 \times 10^{-4}$ and $p = 3.24 \times 10^{-4}$, respectively). From day 3 to day 30, however, the overall abundance of EP4 decreased in *C. goreaui*-colonised larvae whereas it increased in *D. trenchii*-colonised larvae ($p = 2.11 \times 10^{-4}$ and $p = 0.04$, respectively). As a result, at day 30, the overall abundance of EP4 was higher in *D. trenchii*-colonised than *C. goreaui*-colonised larvae ($p = 0.029$). These trends were also seen in the individual tissues of larvae. *C. goreaui*-colonised larvae had a higher EP4 abundance in the gastrodermis than the epidermis at day 3 ($p = 0.017$), although the abundance declined

in both tissue layers from day 3 to day 30 ($p = 8.34 \times 10^{-4}$ and $p = 9.9 \times 10^{-6}$, respectively). Conversely, EP4 abundance increased in the gastrodermis of *D. trenchii*-colonised larvae only, across this same timeframe ($p = 0.038$) (Fig. 4).

In coral polyps, the overall abundance of EP4 was significantly affected by symbiotic state ($F_{2,42} = 15.122$; $p = 1.13 \times 10^{-5}$) and time in symbiosis ($F_{2,42} = 22.262$; $p = 2.64 \times 10^{-5}$), and there was a significant interaction between the two factors ($F_{2,42} = 20.33$; $p = 6.67 \times 10^{-7}$). The overall abundance of EP4 at day 3 was higher in *D. trenchii*-colonised polyps than in either *C. goreaui*-colonised or aposymbiotic polyps ($p < 1 \times 10^{-7}$ and $p = 0.011$, respectively); it was also significantly higher in aposymbiotic polyps than in *C. goreaui*-colonised polyps ($p = 4.76 \times 10^{-4}$). In contrast to the trend seen in coral larvae, however, the overall abundance of EP4 declined significantly in *D. trenchii*-colonised polyps, as well as in aposymbiotic polyps by day 30 ($p = 1.9 \times 10^{-6}$ and $p = 1.97 \times 10^{-3}$, respectively).

Similarly to coral larvae, EP4 abundance was greater in the gastrodermis than epidermis of *C. goreaui*-colonised polyps at both days 3 and 30 ($p = 2 \times 10^{-4}$ and $p = 8 \times 10^{-7}$, respectively), as well as in *D. trenchii*-colonised polyps at day 3 ($p = 9 \times 10^{-6}$). Unlike in larvae, however, EP4 abundance increased in the gastrodermis of *C. goreaui*-colonised polyps between days 3 and 30 ($p = 2.4 \times 10^{-3}$) but decreased between these time-points in both the epidermis and gastrodermis of *D. trenchii*-colonised polyps ($p = 2.7 \times 10^{-4}$ and $p < 1 \times 10^{-7}$, respectively) and the gastrodermis of aposymbiotic polyps ($p = 0.027$).

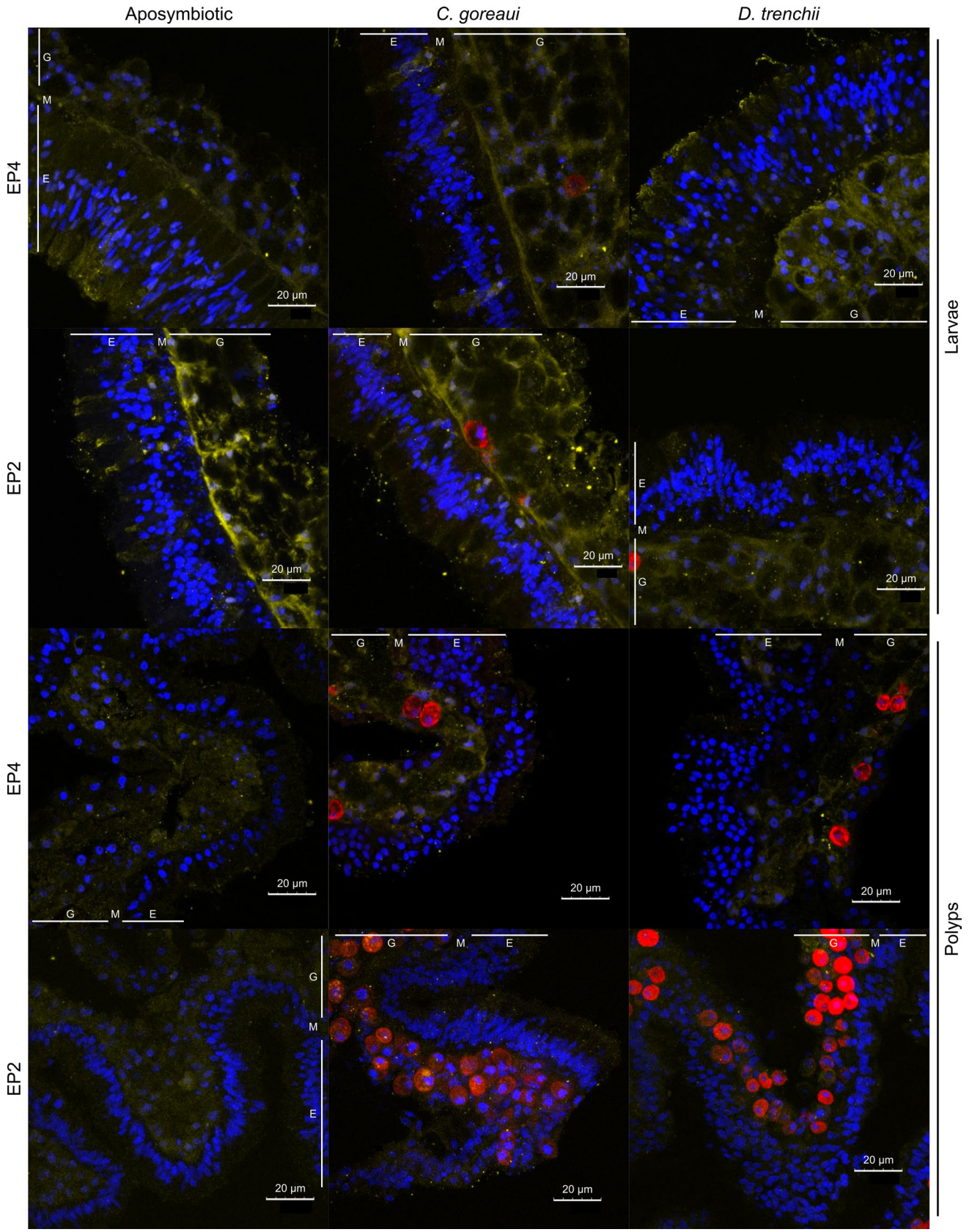
Discussion

The oxylipin receptors, prostaglandin EP2 2 (EP2) and 4 (EP4), were localised to both the epidermis and gastrodermis of symbiotic and aposymbiotic larval and juvenile corals, although their responses were dependent on symbiont type and/or coral life-stage, consistent with our previous work in the model cnidarian Aiptasia (Gamba et al. 2024). The different responses of EP2 and EP4 were not surprising, as EP2 can couple only with the Gs protein alpha subunit (Gαs) whereas EP4 can couple with either the Gαs or Gi protein alpha subunit (Gαi) (Fujino et al. 2003). Gαs is known to increase cAMP production, whereas Gαi reduces cAMP production (Vleeshouwers et al. 2021), so the two receptors have different roles in regulating cAMP (Raker et al. 2016; Vleeshouwers et al. 2021). Increased cAMP levels trigger the activation of protein kinase A, which in turn regulates key immune-related transcription factors such as CREB and NF-κB (Zanassi et al. 2001; Takahashi et al. 2002). Moreover, beyond cAMP signalling, EP2 and EP4 can independently activate the β-catenin pathway, while EP4 is also capable of stimulating the phosphatidylinositol 3-kinase pathway (Banu et al. 2009; Rundhaug et al. 2011). This suggests that EP4 and EP2 may have distinct roles in downstream immune regulation, highlighting the complexity of oxylipin GPCR-mediated signalling (Fujino et al. 2005; Vleeshouwers et al. 2021).

Consistent with previous reports, *C. goreaui* and *D. trenchii* affected host EP2 and EP4 abundance in very different manners (Yuyama et al. 2012, 2018; Mohamed et al. 2020). Differences observed for *C. goreaui- versus D. trenchii*-colonised corals could not be explained by symbiont cell density, which was similar for both symbiont species, perhaps unsurprisingly given that both are found in *Acropora* sp. aff. *tenuis* in nature and that Symbiodiniaceae uptake early in the life-cycle of many corals, including *A. tenuis*, is nonspecific (Coffroth et al. 2001; Little et al. 2004). However, it is important to emphasise that similar symbiont densities do not necessarily correlate with identical host immune responses. This was shown, for instance, by a metabolomic analysis of Aiptasia, where distinct immune responses were observed in response to different symbiont types, despite similar symbiont densities (Matthews et al. 2017). Another notable factor was the life-history stage, with different patterns observed for larvae *versus* juveniles. Metamorphosis leads to dramatic immune system changes in various animals, such as amphibians (Humphries et al. 2022), fish (Guerrero-Peña et al. 2021) and insects (Johnston et al. 2019), and previous studies have demonstrated that algal colonisation leads to different molecular responses between coral larvae and polyps (Yuyama et al. 2018; Hartmann et al. 2019; Mohamed et al. 2020). The developmental differences seen here were therefore not unexpected; while it is well-established that symbiont acquisition benefits coral polyps, there is ongoing debate about whether symbiosis is beneficial or detrimental for coral larvae (Hartmann et al. 2019; Hazraty-Kari et al. 2022). Finally EP2 and EP4 displayed distinct patterns in polyps and larvae depending on the symbiont colonisation stage (early *vs*. later symbiosis establishment). This is consistent with previously-reported physiological changes seen in both Aiptasia and *Acropora tenuis*, where symbiosis onset and establishment influence the host transcriptome, proteome and metabolome with respect to, e.g. metabolism, molecular signalling, stress, and immune-regulation, as well as the rate of host cell proliferation (Oakley et al. 2016; Matthews et al. 2017; Yuyama et al. 2018; Voss et al. 2019; Weis 2019; Gorman et al. 2022). The combined effects of symbiont identity, symbiosis colonisation stage and coral life-history stage on the abundance and localisation of EP2 and EP4 will be considered further here.

EP2

In the early stages of symbiosis establishment (day 3), EP2 receptor abundance was greater in the gastrodermis than


Aposymbiotic
C. goreaui
D. trenchii
EP4
EP2
EP4
EP2
Larvae
Polyps
G
M
E
20 µm

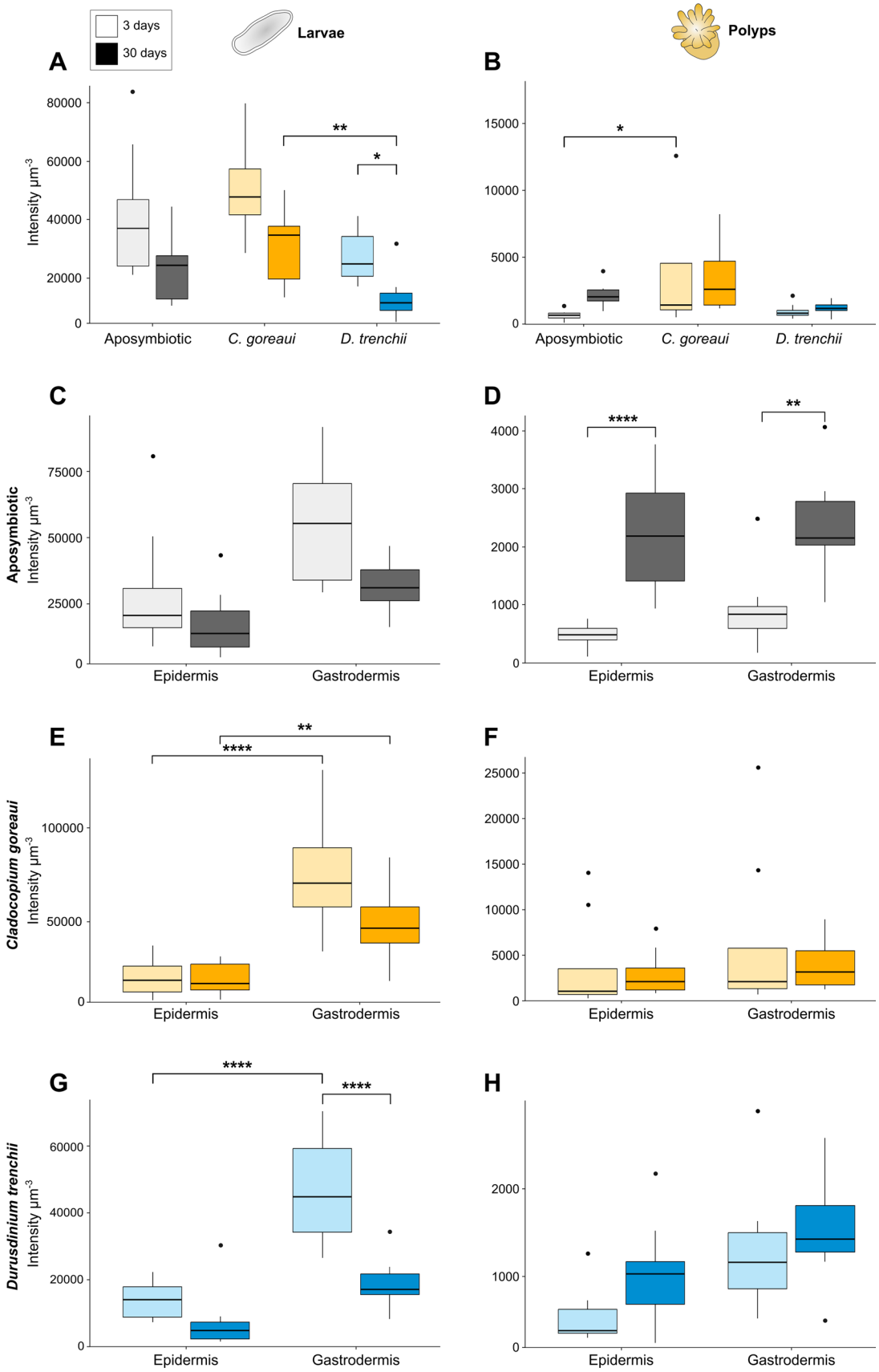
◂**Fig. 2** Immunolocalisation of the oxylipin receptors EP2 and EP4 in aposymbiotic and symbiotic coral (*Acropora* sp. aff. *tenuis*) larvae and polyps hosting either *Durusdinium trenchii* or *Cladocopium goreaui* 30 days post-inoculation. Red: chlorophyll autofluorescence of the symbionts; blue: nuclear staining with DAPI; yellow: AlexaFluor555-conjugated secondary antibody, though yellow within the symbionts represents lipid body autofluorescence. Host tissue layers were labelled as follows: E, epidermis; M, mesogloea; G, gastrodermis. Note: The images in the figure were only used for localisation and not quantification. These images were taken with different microscope settings. To quantify antibody fluorescence, minimum laser power was used to avoid background noise. Therefore, the difference in receptor levels between treatments cannot be detected with the naked eye

the epidermis of symbiotic larvae, regardless of symbiont type; this difference was not evident in aposymbiotic larvae (Fig. 3). Oxylipins are known to mediate a variety of immune response pathways via EP2 receptors, by increasing cAMP production (Jiang and Dingledine 2013; Kawahara et al. 2015; Wang et al. 2022). The elevated abundance of receptors during symbiosis onset could therefore reflect an increase in cellular oxidative stress and an immune response by the host, events that are often associated with symbiont acquisition (Reyes-Bermudez et al. 2009; Mohamed et al. 2016; Hartmann et al. 2019). Furthermore, symbiont colonisation of coral larvae is associated with an increase in apoptosis (Dunn and Weis 2009; Voolstra et al. 2009; Mohamed et al. 2016); a process regulated by EP2 (Hoshino et al. 2003; Takadera et al. 2004; Hu et al. 2013). Given the localisation of the symbionts to the gastrodermis and the potential role of EP2 in the coral's immune response, it is unsurprising that this receptor was most abundant in this tissue layer; indeed, our previous work has localised EP2, in part, to the symbiosome membrane in Aiptasia, further highlighting its possible role in host-symbiont crosstalk (Gamba et al. 2024).

EP2 abundance decreased between day 3 and day 30 of larval colonisation, although only in the gastrodermis (i.e. not the epidermis) of *D. trenchii*-colonised larvae; this decrease was not observed for *C. goreaui*-colonised or aposymbiotic larvae. It has been proposed that some Symbiodiniaceae can suppress the host's immune system to facilitate their intracellular persistence (Mansfield et al. 2017; Matthews et al. 2017; Weis 2019). Such suggestions mirror our previous study, where we observed lower EP2 levels in Aiptasia that hosted either *Breviolum minutum* or *D. trenchii* when compared to aposymbiotic anemones (Gamba et al. 2024). Together, these observations are consistent with the idea that EP2-mediated oxylipin signalling may play an immune activation role in cnidarians and may need to be suppressed for the establishment and maintenance of symbiosis. NF-kB pathways, which are mediated by EP2, are important pro-apoptosis and immunity pathways that are known to be downregulated in the host tissues of both coral larvae and adult Aiptasia when they are inoculated with homologous symbionts compared to the aposymbiotic state (Mansfield et al. 2017; Weis 2019; Helgoe et al. 2024). Similarly, symbiont genes that may regulate the host's immune system are downregulated when symbionts are in symbiosis with coral larvae (Mohamed et al. 2020). The fact that a decrease in EP2 abundance was not observed in *C. goreaui*-colonised larvae here may suggest that this symbiont is less capable than *D. trenchii* of influencing oxylipin-EP2 pathways in *Acropora* sp. aff. *tenuis*. In Aiptasia, *D. trenchii* – while not being its native symbiont and even causing a heightened host immune response – is known to possess the machinery to manipulate the host's immune system (Matthews et al. 2017), with symbiont proteins and genes associated with immunosuppression being upregulated in symbiosis (Bellantuono et al. 2019; Mashini et al. 2024). Similarly, the immune system of the coral *Orbicella faveolata* is suppressed as the population density of *D. trenchii* increases (Fuess et al. 2020).

In part agreement with the larval data, at day 3, EP2 abundance was higher in *C. goreaui*-colonised than aposymbiotic polyps. This difference was not observed between *D. trenchii*-colonised and aposymbiotic polyps, suggesting that *D. trenchii* may suppress the host's immune system via dampening of oxylipin-EP2 signalling to facilitate intracellular survival. Indeed, the consistent dampening of EP2 from day 3 onwards in polyps but not larvae suggests that this regulation is particularly effective in polyps, and highlights that polyps and larvae may employ different cellular mechanisms when establishing a symbiosis (Poland and Coffroth 2017; Hartmann et al. 2019). The behaviour of *D. trenchii* observed in our study is consistent with previous observations that analysed the transcriptome of *A. tenuis* polyps when in the aposymbiotic state *versus* symbiotic with either *C. goreaui* or *D. trenchii* for 10–20 days (Yuyama et al. 2018). These authors showed that pathways involved in immune system processes and lysosomal digestion are downregulated in *D. trenchii*-colonised polyps, but not in those containing *C. goreaui* when compared to aposymbiotic polyps, further suggesting that *D. trenchii* is particularly adept at manipulating the host's physiology (Yuyama et al. 2018). This is in line with field observations as, in nature, *A. tenuis* juveniles tend to form a symbiosis with *D. trenchii* rather than *C. goreaui* (Little et al. 2004; Yorifuji et al. 2017), and *D. trenchii* promotes a higher growth rate of *A. tenuis* juveniles than does *C. goreaui* (Yuyama and Higuchi 2014).

In aposymbiotic coral polyps, EP2 abundance increased in the epidermis and gastrodermis from day 3 to day 30; this change was not observed in symbiotic polyps (Fig. 3). Consistent with this observation, aposymbiotic cnidarians are


3 days
30 days
Larvae
Polyps
A
B
C
D
E
F
G
H
Intensity µm-3
Aposymbiotic
C. goreaui
D. trenchii
Epidermis
Gastrodermis
Cladocopium goreaui
Durusdinium trenchii
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*
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◂**Fig. 3** Oxylipin receptor EP2 fluorescence intensity (*per* unit area-of-interest) in *Acropora* sp. aff. *tenuis* tissues in different symbiotic states. A, B: Total intensity of EP2 in larvae **A** or polyps **B** either 3 days (light colours) or 30 days (dark colours) post-inoculation. C–H: EP2 fluorescence intensity by tissue layer in aposymbiotic larvae or polyps **C** and **D**, larvae or polyps hosting *Cladocopium goreaui* **E** and **F**, and larvae or polyps hosting *Durusdinium trenchii* **G** and **H**. $N = 8$, whiskers show upper and lower quartiles, outlier values marked by points. Asterisks indicate significant differences: $*P < 0.05$; $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$

known to be susceptible to cellular stress, increased immune activity, and bacterial infection when compared to symbiotic cnidarians (Oakley et al. 2016; Martínez et al. 2017; Roesel and Vollmer 2019; Valadez-Ingersoll et al. 2024). Conversely, several 'omics' studies have demonstrated a downregulation of stress- and immune-related genes, proteins and metabolites when host cnidarians are in symbiosis with their native symbionts (Lehnert et al. 2014; Matthews et al. 2017; Sproles et al. 2019; Yoshioka et al. 2021, 2023). It is unclear why this same pattern did not occur in aposymbiotic larvae, though one possible explanation is their high lipid content, which may render them less susceptible to starvation and cellular stress (Harii et al. 2007; Hartmann et al. 2017, 2019; Rivest et al. 2017).

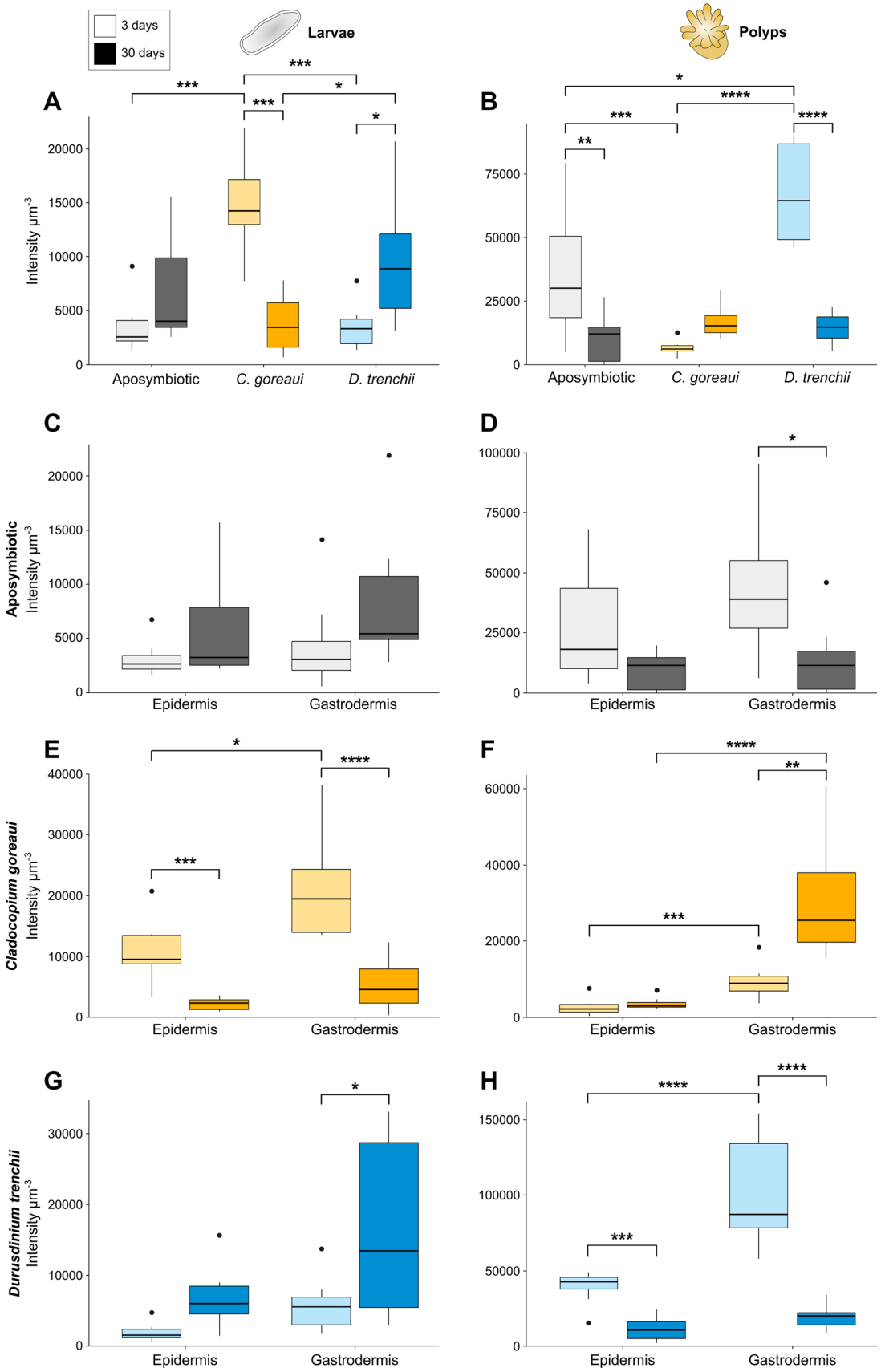
EP4

The abundance of EP4 did not change in aposymbiotic larvae over time, as with EP2. However, EP4 abundance did increase in *C. goreaui*-colonised larvae compared to *D. trenchii*-colonised and aposymbiotic larvae at day 3 (Fig. 4). This may indicate an oxylipin-EP4-mediated immune response to the initial uptake of *C. goreaui* but not *D. trenchii*, which is further supported by initially more elevated EP4 levels in the gastrodermis *versus* epidermis in response to *C. goreaui*. However, the subsequent decline in EP4 abundance in *C. goreaui*-colonised larvae across both tissue layers suggests that this symbiont may ultimately be able to regulate the host's immune response. Consistent with this interpretation, a previous transcriptomic study of *C. goreaui*-colonised *A. tenuis* larvae identified numerous immune- and stress-related genes that were downregulated in both the symbiont and host during symbiosis, which may suggest that this symbiont can suppress host pathways, at least in the larval stage (Mohamed et al. 2020). In contrast, however, EP4 abundance increased in the gastrodermis of *D. trenchii*-colonised larvae between days 3 and 30, which could indicate that *D. trenchii* is less effective at regulating oxylipin-EP4 signalling in larvae over time. Our results align with previous observations where inoculation of *A. tenuis* larvae with *D. trenchii* did not induce major changes in gene expression, while substantial changes in gene expression were seen when *A. tenuis* polyps were inoculated with this species (Yoshioka et al. 2023), perhaps indicating enhanced host-symbiont communication and integration at the polyp stage.

EP4 abundance was higher in the gastrodermis than the epidermis of symbiotic polyps, but not in aposymbiotic polyps at day 3, indicating that both symbiont species trigger an immune response at symbiosis onset. However, EP4 abundance at this timepoint differed with symbiont identity and symbiotic state, being greater in aposymbiotic and *D. trenchii*-colonised polyps than *C. goreaui*-colonised polyps (Fig. 4). These differences were no longer apparent at 30 days due to a relative decline in EP4 abundance in the epidermis and gastrodermis of *D. trenchii*-colonised polyps and the gastrodermis of aposymbiotic polyps, and an increase in the gastrodermis of polyps containing *C. goreaui*. The marked decline in *D. trenchii*-colonised polyps is perhaps associated with immune suppression by this symbiont, as also suggested in our previous study with *D. trenchii*-colonised Aiptasia (Gamba et al. 2024). Notably, these results differ from those seen in *Acropora* sp. aff. *tenuis* larvae, which may reflect changes related to shifts in host ontogeny and inter-partner specificity (see above). Nevertheless, these patterns are consistent with the common dominance of *D. trenchii* in juvenile *A. tenuis* polyps (Ulstrup and Van Oppen 2003; Little et al. 2004; Abrego et al. 2009; Yamashita et al. 2014; Lewis et al. 2024). This uncertainty highlights the need for much more work to confirm the roles of both EP2 and EP4 in this symbiosis.

Conclusions

We show how EP2 and EP4 abundances in *Acropora* sp. aff. *tenuis* are influenced by symbiotic state, symbiont species, and coral life-stage. *D. trenchii* may be more effective at mediating the host's immune response than *C. goreaui*, particularly in the polyp rather than the larval stage, perhaps explaining why this symbiont species tends to dominate juvenile *A. tenuis* polyps (Yamashita et al. 2014; Yorifuji et al. 2017). However, how EP2 and EP4 receptor abundances continue to change as the coral colony grows and matures requires further study, especially in light of further potential shifts in symbiont community composition. Indeed, *C. goreaui,* rather than *D. trenchii*, is commonly dominant in *A. tenuis* adults (Ulstrup and Van Oppen 2003; Little et al. 2004; Abrego et al. 2009). Furthermore, how these receptors and their associated signalling pathways


3 days
30 days
Larvae
Polyps
A
B
C
D
E
F
G
H
Intensity µm-3
Aposymbiotic
C. goreaui
D. trenchii
Epidermis
Gastrodermis
Cladocopium goreaui
Durusdinium trenchii

◂**Fig. 4** Oxylipin receptor EP4 fluorescence intensity (*per* unit area-of-interest) in *Acropora* sp. aff. *tenuis* tissues in different symbiotic states. A, B: Total intensity of EP4 in larvae **A** or polyps **B** either 3 days (light colours) or 30 days (dark colours) post-inoculation. C–H: EP4 fluorescence intensity by tissue layer in aposymbiotic larvae or polyps **C** and **D**, larvae or polyps hosting *Cladocopium goreaui* **E** and **F**, and larvae or polyps hosting *Durusdinium trenchii* **G** and **H**. $N = 8$, whiskers show upper and lower quartiles, outlier values marked by points. Asterisks indicate significant differences: $*P < 0.05$; $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$

respond to environmental change, including thermal stress, and the consequent effects on the symbiont population, warrant investigation.

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Author's contributions Andrea Gamba: Conceptualization (co-lead), Data Curation (lead), Formal Analysis (lead), Investigation (lead), Methodology (lead), Software (lead), Validation (lead), Visualization (lead), Writing—Original Draft Preparation (lead), Writing—Review & Editing (equal). Saki Harii, Frederic Sinniger: Conceptualization (supporting), Methodology (supporting), Writing—Review & Editing (equal). Clinton Oakley: Conceptualization (supporting), Methodology (supporting), Supervision (supporting), Writing—Review & Editing (equal). Arthur Grossman, Virginia Weis, David Suggett: Conceptualization (supporting), Writing—Review & Editing (equal), Funding Acquisition (lead), Methodology (supporting). Simon Davy: Conceptualization (co-lead), Writing—Review & Editing (equal), Funding Acquisition (lead), Supervision (lead), Methodology (supporting), Project Administration (lead), Resources (lead).

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Data availability All data associated with this article are available within this paper and in its supplementary information.

Declarations

Conflict of interest The authors declare no competing interests.

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