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Chemical aspects of mass spawning in corals. II. (–)-*Epi*-thunbergol, the sperm attractant in the eggs of the soft coral *Lobophytum crassum* (Cnidaria: Octocorallia)

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Abstract The eggs of *Lobophytum crassum* Von Marenzeller, 1886, collected at Magnetic Island (19°10′S; 146°52′E) in October or November between 1983 and 1993, contained significant amounts (6% dry weight) of (−)-*epi*-thunbergol, in addition to other terpenoid metabolites also present in the parent colony. (−)-*Epi*-thunbergol was not present in the tissues of the releasing colony. Using fluorocarbon droplets impregnated with the chemotactic molecules and a video-microscopic technique for the direct observation of sperm under the influence of attractant molecules, we showed that (−)-*epi*-thunbergol, whether isolated from *L. crassum* or from a gorgonian octocoral (*Briareum* sp.), significantly attracts sperm from *L. crassum* colonies. Attraction could be detected using direct observation at concentrations as low as 3.25 μg ml^{-1}. This is the first evidence for sperm chemotaxis in the Alcyonacea. Eggs from *L compactum*, a common alcyonacean coral at Orpheus Island (18°36′S; 146°29′E) contained (−)-thunbergol as the egg-specific compound. *L. compactum* was not found at Magnetic Island. (−)-Thunbergol from *L. compactum* and (+)-thunbergol from a Douglas fir tree both showed levels of attraction similar to (−)-*epi*-thunbergol against *L. crassum* sperm. Although *L. crassum* sperm were apparently neither stereo- nor enantio-specific in their selectivity for chemotactic molecules, (−)-*epi*-thunbergol was identified as the natural sperm attractant in the eggs of *L. crassum*.

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Introduction

Soft corals (Cnidaria, Octocorallia, Alcyonacea) are important contributors to the attached benthic communities on shallow Indo-Pacific reefs (Dinesen 1983). This is in sharp contrast to Caribbean and tropical Atlantic reefs, where gorgonians are the predominant non-scleractinian corals (Bayer 1961; Dahlgren 1989). Reproduction in alcyonaceans occurs by either sexual or asexual modes. Asexual reproduction, which involves budding of ramets from the parent colony followed by fission, may be the most important reproductive strategy in terms of increasing local numbers of individuals (Lasker 1990). Sexual reproduction with eventual larval dispersion (Babcock et al. 1986) has an important role in the recruitment of new genets to other reefs and ensures genetic vitality (Harrison et al. 1984).

Three modes of sexual reproduction have been identified in soft corals: broadcast spawning, internal brooding and surface brooding (Aliño and Coll 1989). Internal brooding was first observed in *Xenia macrospiculata* and *Heteroxenia fuscensces.* (Ehrenberg) (Gohar 1940). Broadcast spawning was described by

Hartnoll (1977), when he observed that the temperate soft coral *Alcyonium digitatum* released gametes (eggs and sperm) into the water column. The Okinawan species *Lobophytum crassum* was also identified as a broadcast spawner (Yamazato et al. 1981). *L. crassum* colonies are gonochoric, but differ from *A. digitatum* in that a 2 yr period is required to complete oogenesis. The polyps of female colonies normally contain 1 and 2 yr- old oocytes, while male colonies generally have an annual cycle of spermatogenesis. Both sexes release buoyant gametes into the water column, where fertilisation takes place. *Sarcophyton glaucum* from the Red Sea also adopts this mode of sexual reproduction (Benayahu and Loya 1986), as do several *Lobophytum* species on the Great Barrier Reef (Bowden et al. 1985; Aliño and Coll 1989). Broadcast spawning is now considered the most common sexual reproductive strategy among alcyonaceans.

The work of Gohar (1940) on *Xenia macrospiculata* was confirmed and expanded upon by Benayahu and Loya (1984 a,b, 1985), who also reported the third type of spawning in octocorals, external surface brooding, in *Parerythropodium fulvum fulvum* (Benayahu and Loya 1983). The Mediterranean *Clavularia crassa* (Weinberg 1978), the temperate *Capnella gaboensis* (Farrant 1985), and several tropical soft corals of the family Xeniidae also reproduce by this mode (Dinesen 1985; Aliño and Coll 1989). The octocoral *Heliopora coerulea*, the sole member of the order Coenothecalia, has also been shown to adopt external surface brooding as a reproductive strategy (Babcock 1990).

By far the majority of the Alcyoniidae broadcast-spawn from colonies of the separate sexes, and most do so within a week of the full moon in late spring in the central region of the Great Barrier Reef (Aliño and Coll 1989). Chemically mediated attraction between the gametes (Miller 1985 a) has recently been reported for the scleractinian *Montipora digitata* (Coll et al. 1994). The present study was undertaken to investigate the possibility of such chemically mediated interactions in the soft coral *Lobophyum crassum*.

Our research initially focussed on identifying chemical differences between the eggs and somatic tissues of the colonies releasing eggs, for our rationale was that the eggs should contain specific compounds if they are to attract sperm. We have reported examples of egg-specific compounds from *Lobophytum compactum* (Bowden et al. 1985), *L. microlobulatum* (Coll et al. 1986), and from several species of *Sinularia* (Sammarco and Coll 1988). We then attempted to determine whether the egg-specific compounds attracted sperm from the same species. Sperm are only available on two nights each year, making the chemotaxis study rather difficult, but over the last several years we have identified colonies of the soft coral *L. crassum* whose sex and chemical composition were known. We report here the chemical nature and structural identity of the sperm attractant in this species.

Materials and methods

Soft coral selection

The alcyonacean soft coral *Lobophytum crassum* Von Marenzeller, 1886 is a gonochoric (dioeceous) species whose gametes are released into the open ocean during mass, multi-species spawning events on the Great Barrier Reef, Australia. More than 20 colonies of *L. crassum* were surveyed and tagged in situ on the shallow (1 to 3 m depth) reef flat in Geoffrey Bay, Magnetic Island (19°10'S; 146°52'E), 6 mo prior to spawning. Small portions of the colonies were collected, frozen and freeze-dried prior to extraction and chemical analysis. Taxonomy was determined classically based on morphological and skeletal characteristics. The colonies chosen for study were both morphologically and chemically identical.

The colonies were subsequently examined during the weeks prior to the predicted time of spawning. Colony sex was determined in the field by tearing open one or more of the lobes. Female lobes contained mature pink/purple eggs, while male lobes contained globular whitish spermaries. These determinations were subsequently confirmed in the laboratory using low-power microscopy.

Gamete collection in situ

Conical muslin nets (radius 0.5 m, height 0.8 to 1 m) were placed over selected female colonies of known chemical composition in the late afternoon of spawning. The nets resembled plankton nets, with polystyrene floats to increase buoyancy and a transparent lightweight plastic jar as a cod-end. The jar, which was buoyed up by a little air, was attached to the net via its lid, the top of which had been excised leaving only the threaded rim. The rim was screwed over the net and onto the jar, permitting easy attachment and removal after collection of the eggs. The net was thus attached to the jar in such a way that there were no obstacles to the buoyant eggs rising up the length of the net and gathering in the jar. The base of the net was stitched to a wire loop which surrounded the coral colony. It was held down with lead weights. Eggs were removed from the collection system by unscrewing the cod-end from the net and attaching a plastic lid. On return from the collection site, the eggs were transferred to small sample vials using a Pasteur pipette and frozen until they could be freeze-dried. Sperm could not be collected in situ because of planktonic contamination.

Egg and sperm collection ex situ

To facilitate egg and sperm collection in later years, tagged female and male colonies of *Lobophytum crassum* with identical chemistry were cut into two portions, and half of each colony was left in place. The other half with attached substratum was relocated to a galvanised steel grid to which it was affixed with cable ties or plastic coated wire. These smaller clonal duplicate colonies were taken ashore, where they spawned in separate plastic buckets at the appropriate time. Eggs and sperm were collected separately, and concentrated suspensions of sperm were reliably obtained in excellent condition with no possibility of cross-contamination. Sperm water was stored in glass vials and kept at 4 °C after it had been found that plastic jars and ambient evening temperatures of 25 °C reduced sperm viability. After spawning was complete, female and male colonies were preserved in 70% aqueous alcohol for taxonomic and reference purposes.

Extraction, chromatography and structural determination

Lobophytum crassum colonies. Freeze-dried coral tissue was exhaustively extracted with dichloromethane, and the solvent was

removed in vacuo. The crude extract was subjected to vacuum liquid chromatography (Coll and Bowden 1986), and key metabolites were purified by high-performance liquid chromotography (HPLC) on silica-gel columns, as outlined elsewhere (Bowden et al. 1985). Structures of compounds were determined by nuclear magnetic resonance (NMR) spectroscopy and comparison with authentic samples.

Lobophytum crassum eggs. Frozen eggs were freeze-dried, extracted with dichloromethane (DCM) three times, and the solvent removed in vacuo to afford a crude, oily extract. The crude extract was subjected to vacuum liquid chromatography with gradient elution in five steps: petrol (light petroleum) 100%, petrol/ethyl acetate 90:10, petrol/ethyl acetate 50%, ethyl acetate 100% and methanol 100%. Five fractions were collected and concentrated by removal of solvent in vacuo. Each of the fractions was subjected to the Müller sperm chemotaxis bioassay, and the active fraction was further purified by HPLC on silica gel. Pure compounds thus obtained were retested using the bioassays described below.

Müller bioassay technique

The quantitative assay for testing sperm chemotaxis was devised by Müller (1976, 1977) for studies on brown algal gametes. Compounds to be tested for their effect on sperm were dissolved in FC-72 (3 M Company), an inert fluorocarbon which is insoluble in, and more dense than water. For each assay, a stainless steel ring (12 mm i.d, 1 mm thick) was placed on the bottom of a 49 mm plastic petri dish. The ring was filled with filtered seawater. Three droplets ($\simeq 0.1$ µl) containing the test compound and one reference droplet of pure FC-72 were placed in the centre of the ring using a 1 µl syringe. A fresh sample of sperm was added and dispersed evenly with a Pasteur pipette. After 3 min, the distribution of sperm on the surface of the four droplets was photographed using dark-field optics and Zeiss microflash equipment on a standard research microscope, and the number of sperm present within a standard area in the centre of each droplet was counted. Control tests consisted of four droplets of pure FC-72 with sperm present.

Each compound was tested several times using this assay. Eventually, an image analysis system (Image 1.4 software system supplied by A. Hasblad) consisting of a high-resolution microscope-mounted video camera (Sony DXC-151P), frame-grabber (Quick Capture), and Macintosh II Ci computer system was used for image-capture and sperm-counting. The chemotactic effect was measured by comparing the quotient of the number of cells per field in the test droplet divided by the number of cells per field in the reference droplet. Control quotients were obtained from the series of experiments using four pure FC-72 droplets. Control and experimental results were compared using the Kruskal–Wallis analysis of variance, following procedures described in Zar (1984).

Video-microscopic observation of sperm chemotaxis

Aqueous solutions of the chemotactic substance were introduced into diluted sperm solutions using a microsyringe within the field of vision of a video microscope under low-power objective (Miller 1985 a). The behaviour of the sperm was recorded on a video casette before and after addition of the attractant, and the behaviour of sperm was compared. Chemotaxis was indicated if the sperm became activated and swam towards the outlet of the syringe. Using this assay, it was possible to distinguish the relative efficacy of different compounds as sperm attractants and to determine, by using a one-half dilution series, the approximate minimum levels of compound that gave an observable attractive response. Tests were carried out on (−)-*epi*-thunbergol from *Briareum* sp. and from *Lobophytum crassum*, (−)-thunbergol from *L. compactum*, and (+)-thunbergol from Douglas fir trees, against sperm from *L. crassum*.

Results

Two distinct chemotypes of *Lobophytum crassum* grow on the reef flat at Geoffrey Bay. Representative analytical data on the chemical composition of colonies of each chemotype used in the study appear in Table 1. The levels of sarcophytoxide and the levels of sarcophytonin-A were similar in each of the two chemotypes, and one of the two also contained a eudesmanoid diterpene. The eggs from either group of female colonies contain (−)-*epi*-thunbergol as the additional egg-specific compound (Fig. 1a).

Loss of sperm viability or motility was the major limitation on the bioassays. Sperm stored at room temperature in glass jars lost viability in ~3 h, and the use of plastic vials reduced sperm viability to ~1 h. However, sperm stored at 4 °C in glass vials for up to 12 h, were viable when returned to ~25 °C.

Based on the Müller technique (Maier and Müller 1986), only Fraction 2 (petrol/ethyl acetate, 90:10) of the five fractions obtained from chromatography of the egg extract, was active as a chemotactic agent (Fig. 2; $p < 0.001$, ANOVA). Repeated HPLC separation revealed that the active component in Fraction 2 from the eggs of *Lobophytum crassum* was (−)-*epi*-thunbergol. Pure (−)-*epi*-thunbergol attracted sperm in the Müller droplet bioassay (Fig. 3). Unfortunately,

Table 1 *Lobophytum crassum.* Composition of crude extract of tissue from female (*F*) and male (*MA*) and eggs (% dry coral wt)

Compounds	Female colonies	Eggs	Male colonies
L. crassum (Type 1)	Colony F1, F2	Colony F1, F2	MA1, MA2
sarcophytoxide	3.7%	1.7%	3.0%
sarcophytonin-A[96]	0.6%	0.4%	0.5%
(−)-*epi*-thunbergol	0%	0.6%	0%
L. crassum (Type 2)	Colony F3	Colony F3	Colony MA6
sarcophytoxide	2.2%	1.6%	1.8%
sarcophytonin-A	0.4%	0.3%	0.4%
eudesmanoid-diterpene	1.8%	1.3%	1.8%
(−)-*epi*-thunbergol	0%	0.6%	0%

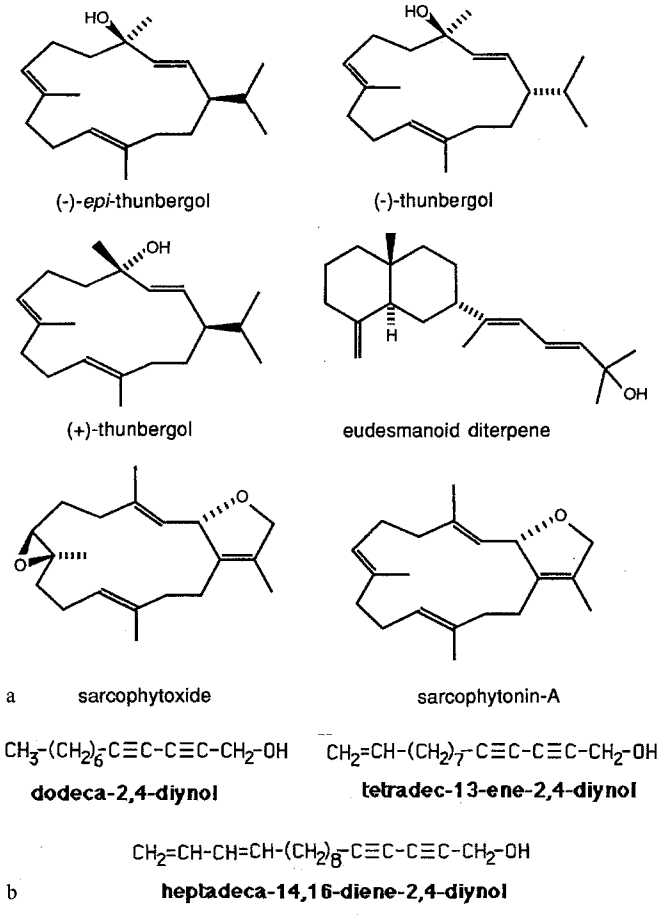

Fig. 1 **a** *Lobophytum crassum*; **b** *Montipora digitata*. Structural formulae for coral-derived secondary metabolites and sperm attractants

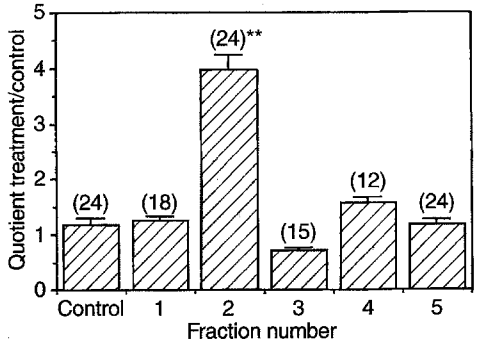

Fig. 2 *Lobophytum crassum*. Mean chemotactic effect obtained for crude extract fractions (SM86/25; Type 1) eggs at 50 mg ml^{-1}, using sperm from Colony MA1; error bars indicate standard error for each value; (*n*) represents number of assays [** Significantly different from controls ($p < 0.001$, ANOVA)

subsequent experimentation revealed that the solubility of (−)-*epi*-thunbergol in FC-72 did not exceed 300 μg ml^{-1} (Leone 1993), accounting for the poor concentration-dependence of the chemotactic effect.

Because of the extremely low levels at which chemotactic agents generally operate, it was possible

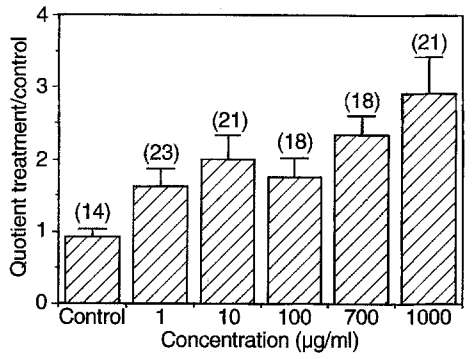

Fig. 3 *Lobophytum crassum*. Relative chemotactic effect obtained for (−)-*epi*-thunbergol at different concentrations in fluorocarbon, using sperm from Colony MA1; error bars indicate standard error for each value; (*n*) represents number of assays; all concentrations significantly different from controls ($p < 0.05$, ANOVA)

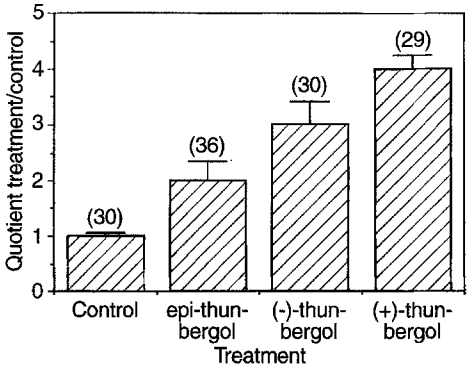

Fig. 4 *Lobophytum crassum*. Relative chemotactic effect obtained for several compounds at 1 mg ml^{-1} in fluorocarbon, using sperm from Colony MA2. Levels of attraction were not significantly different between treatments, but all were significantly attractive relative to controls ($p < 0.001$, ANOVA). (*n*) represents number of assays

that the sample of (−)-*epi*-thunbergol tested may have contained another more active component which was present below the level of detection by ^{1}H-NMR spectroscopy. A sample of (−)-*epi*-thunbergol obtained from the gorgonian octocoral *Briareum* sp. was tested in the Müller assay, and found to have comparable activity to the sample derived from *Lobophytum crassum*. A further complication came from the fact that samples of (−)-*epi*-thunbergol always contains traces of (−)-thunbergol after chromatography. Presumably, there is a slow but unavoidable interconversion of (−)-*epi*-thunbergol to (−)-thunbergol to the extent of 5 to 10%, i.e. detectable by ^{1}H-NMR spectroscopy. The reverse epimerisation does not seem to occur so readily, i.e. purified samples of (−)-thunbergol did not contain amounts of (−)-*epi*-thunbergol at levels detectable by ^{1}H NMR spectroscopy. (−)-Thunbergol from *L. compactum* (Bowden et al. 1985) and (+)-thunbergol from the Douglas fir (Kimland and Norin 1968) were tested, and the two thunbergol samples had comparable attractive abilities towards the sperm of *Lobophytum crassum* (Fig. 4).

The Miller bioassay (Miller 1985a) confirmed the results obtained in the fluorocarbon droplet assay, and showed detectable attractive effects on *Lobophytum crassum* sperm starting from a solution of 100 $\mu g\,ml^{-1}$ of (−)-*epi*-thunbergol, by serial dilution down to 3.25 $\mu g\,ml^{-1}$. The sperm swam to the point of chemical release over significant distances, certainly exceeding 20 sperm lengths.

Discussion

Sperm-attractant substances have been identified in brown algae (Maier and Müller 1986), and a number of other systems have been studied including hydrozoans (Miller 1979 a,b), molluscs (Miller et al. 1995), ascidians (Miller 1982), starfish (Miller 1985b; Punnett et al. 1992), sea urchins (Ward et al. 1985) and urochordates (Miller and King 1983; Yoshida et al. 1993). The identification of the chemotactic principal involved in sperm–egg interactions for the soft coral *Lobophytum crassum* is the first such example in the Octocorallia, and follows a recent report of the chemotactic substances from the scleractinian *Montipora digitata* (Coll et al. 1994).

In the case of *Montipora digitata*, an hermaphroditic species which releases egg–sperm bundles into the ocean, fertilisation is generally delayed for up to 30 min after spawning (Babcock and Hayward 1986). Sperm chemotaxis may be important to increase the probability of sperm/egg contact (fertilization). Specificity in this response could be important in reducing sperm wastage (Coll et al. 1994). *Lobophytum crassum*, a gonochoric species, releases eggs or sperm into the water from separately sexed colonies which are usually distant from one another. In the complex mixture of synchronously spawned gametes that occurs during mass coral spawning (Babcock et al. 1986; Aliño and Coll 1989), sperm attraction may be very important if effective fertilisation is to occur. Species-specificity of sperm chemoattraction would also be an advantage (Harrison et al. 1984). (−)-*Epi*-thunbergol has been identified as the egg-specific compound in the eggs of *L. crassum*. This made it a likely candidate as the sperm attractant, and two separate bioassay systems using *L. crassum* sperm showed that it indeed attracted sperm. In the Miller apparatus, sperm aggregation was observed down to 3.25 $\mu g\,ml^{-1}$, and weak chemoattraction was measured in the Müller fluorocarbon droplet test to 1 $\mu g\,ml^{-1}$. The lack of differentiation between the 1 and 100 $\mu g\,ml^{-1}$ tests is attributable to problems of solubility of the diterpenes in the fluorocarbon solvent.

Two confounding factors had to be clarified before this result could be reported without ambiguity. The first was that an even more active attractant might be present as a trace contaminant in the highly purified sample of (−)-*epi*-thunbergol derived from *Lobophytum crassum* eggs. This possibility was eliminated by obtaining a pure sample of (−)-*epi*-thunbergol from the gorgonian octocoral *Briareum* sp., which contained this compound as a major component in the organic extract of its somatic tissues (Bowden et al. 1989). A purified sample attracted *L. crassum* sperm.

The second problem arises from the fact that (−)-*epi*-thunbergol is not particularly stable to silica gel chromatography, and slowly interconverts to (−)-thunbergol during purification. Purified samples generally contain 5 to 10% of (−)-thunbergol, while (−)-thunbergol samples can usually be obtained free from (−)-*epi*-thunbergol. We tested (−)-thunbergol, which was the egg-specific compound for *Lobophytum compactum* from Orpheus Island, against sperm of *L. crassum* from Magnetic Island. (−)-Thunbergol was as attractive to these sperm as (−)-*epi*-thunbergol at the same concentration. Furthermore its enantiomer, (+)-thunbergol isolated from the Douglas fir tree (Kimland and Norin 1968), was also as active towards *L. crassum* sperm at similar levels to (−)-*epi* and (−)-thunbergol. All three compounds are isomers with the same functional groups, and each is comparably attractive to *L. crassum* sperm. These results showed that *L. crassum* sperm are neither stereo- nor enantio-specific in their sensitivity towards the attractants.

These experiments confirm that (−)-*epi*-thunbergol is the natural sperm attractant for *Lobophytum crassum* sperm, since it alone occurs in the eggs of *L. crassum*. Its epimer (−)-thunbergol and related enantioner (+)-thunbergol are also attractive to the same sperm. *L. crassum* [(−)-*epi*-thunbergol] and *L. compactum* [(−)-thunbergol] have not been observed to co-occur at Magnetic or Orpheus Island. (+)-Thunbergol is a terrestrial natural product and has not been reported from marine sources. This apparent lack of specificity is not extraordinary, and similar results have been noted in studies of algal chemotaxis (Moore 1977; Müller 1977). Chemotactic agents must attract conspecific sperm; if they also attract other sperm, it confers some competitive advantage, resulting in sperm wastage in other species. Although *L. crassum* sperm do not discriminate between the three compounds in the laboratory, in the field it may not be necessary because of geographical isolation of the species producing them. Furthermore, the very low incidence of interspecific cross-fertilisation in the Scleractinia has been attributed to discrimination at the time of gamete fusion, rather than to the specificity of sperm chemo-attraction (Willis et al. 1993).

It is of considerable interest that while the sperm attractant in the scleractinian coral *Montipora digitata* was a series of unsaturated fatty alcohols (Fig. 1), the sperm-attracting agent for *Lobophytum crassum* was a cembranoid diterpene. In the course of preliminary studies over several years (unreported), we confirmed that the sperm attractants for *M. digitata* had no effect

on *L. crassum* sperm, and vice versa. In each case, however, the sperm attractant was the egg-specific compound. We have identified egg-specific compounds from >10 different species over the last 10 yr. Most have been reported in the literature (Sammarco and Coll 1988). It is not unreasonable to assume that they are all chemo-attractive agents for their respective sperm. Because of the difficulty of carrying out bioassays, and of identifying soft corals to species level and sex with absolute certainty in the field (or even in the laboratory), it is unlikely that this saga will ever be fully told. This paper provides the definitive answer for *Lobophytum crassum*, but for the present, the nature of the sperm attractants in other soft coral species remains in the realm of informed speculation.

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