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MECHANISMS OF REPRODUCTIVE ISOLATION AMONG SYMPATRIC BROADCAST-SPAWNING CORALS OF THE *MONTASTRAEA ANNULARIS* SPECIES COMPLEX

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Abstract.—Many coral species spawn simultaneously and have compatible gametes, leading to controversy over the nature of species boundaries and the frequency with which hybridization occurs. Three western Atlantic corals, *Montastraea annularis*, *M. faveolata*, and *M. franksi*, typify this controversy; they all spawn sympatrically on the same evenings after the fall full moons. Here we show, in both Panama and the Bahamas for multiple years, how a variety of mechanisms may act in concert to reproductively isolate all three species. Field studies indicate that *M. franksi* spawns two hours earlier than the other two species, and the eggs released during this earlier period disperse an average of 500 m by the time the other species spawn. Field measures of fertilization indicate that peak fertilization occurs when spawning synchrony is high and that corals that spawn at the tails of the spawning distributions have greatly reduced fertilization success. Laboratory studies indicate that there is a gametic incompatibility between *M. faveolata* and the other two species. There are regional differences in the gametic compatibility of *M. franksi* and *M. annularis*. In Panama, the two species are completely compatible, whereas in the Bahamas, *M. franksi* sperm can fertilize *M. annularis* eggs but the reciprocal cross often fails. Gamete age influences patterns of fertilization, such that very young eggs seem resistant to fertilization and old sperm lose viability after two hours. In sum, the combination of temporal differences in spawning, sperm aging, gamete dispersal and dilution, and gametic incompatibility act in various combinations among the three species, making it unlikely that hybrid fertilization would occur.

Key words.—Coral reef, fertilization, hybridization, mass-spawning, speciation.

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The process of speciation depends on reproductive isolation providing a barrier to genetic exchange between populations (Mayr 1963). The origin and maintenance of species in the sea has received special attention because the barriers needed to establish reproductive isolation are more obscure than they are on land. Physical features capable of limiting gene flow are less obvious in the sea, and when fertilization is external there are many opportunities for heterospecific fertilization (reviewed in Palumbi 1994).

Gametic incompatibility and temporal and spatial differences in gamete release are the primary mechanisms contributing to prezygotic reproductive isolation between closely related marine species with external fertilization (Palumbi 1994). Taxa that spawn asynchronously (Strathmann 1987; Lessios 1991; Knowlton 1993) or in allopatry (Lessios 1984; Byrne and Anderson 1994) are not likely to cross-fertilize even when their gametes are compatible. However, many recognized species have been observed to spawn in close proximity with little temporal separation (Harrison et al. 1984; Babcock et al. 1986; McEuen 1988; Pearse et al. 1988; Babcock et al. 1992; van Vehgel 1994; Clifton 1997; Szmant et al. 1997; Hagman et al 1998a; Sanchez et al. 1999; Levitan 2002a), and evidence for incompatibility among such species is not ubiquitous (Willis et al. 1997; Pernet 1999; Levitan 2002b).

The nature of reproductive boundaries is especially confusing for corals (Veron 1995). In the Indowest Pacific, for example, more than 100 species spawn simultaneously during annual mass-spawning events (Harrison et al. 1984; Babcock et al. 1986). Most work on species boundaries has focused on the species-rich genus *Acropora*, where hybrids may be commonly produced in laboratory crosses, and clear genetic differences between described species have been difficult to establish (Willis et al.1997; Hatta et al. 1999; van Oppen et al. 2001, 2002; Marquez et al. 2002a, 2002b). High intraspecific genetic heterogeneity (Odorico and Miller 1997) and chromosomal numbers (Kenyon 1997) are also suggestive of hybridization and reticulate evolution. Other groups have been much less studied, but the genus *Platygyra* provides an important comparable example within the family Faviidae (Miller and Babcock 1997; Miller and Benzie 1997).

Mass spawning also occurs in the tropical North Atlantic (van Veghel 1993; Hagman et al. 1998b), and the smaller number of species makes it possible to investigate the reproductive biology and genetics of all potentially interbreeding taxa. This is a real advantage, because when many species remain unstudied, it can be argued that gene flow between apparently isolated taxa can still occur via genetic exchange with species for which no data exist. In the case of the three Caribbean acroporids, recent genetic studies suggest that *Acropora prolifera* is an F_1 hybrid of *A. palmata* and *A. cervicornis* (van Oppen et al. 2000; Vollmer and Palumbi 2002), with little evidence for extensive introgression between *A. cervicornis* and *A. palmata* (Vollmer and Palumbi 2002). Most reproductive studies in the western Atlantic have focused on the *Montastraea annularis* species complex (van Veghel 1994; Knowlton et al. 1997; Szmant et al. 1997; Hagman et al. 1998a,b; Sanchez et al. 1999), whose members

are among the most important reef builders of the region. However, despite the existence of several studies of spawning times and gametic compatibility, there is no consensus on the effectiveness of prezygotic isolating mechanisms in this group (e.g., van Veghel 1994; Knowlton et al. 1997; Szmant et al. 1997; Hagman et al. 1998a; Sanchez et al. 1999). Genetic analyses have also led to contradictory conclusions with respect to the degree of reproductive isolation (van Veghel and Bak 1993; Medina et al. 1999; Lopez et al. 1999).

However, all of these previous studies had fairly low sample sizes due to the limited number of evenings per year when these corals spawn, many did not quantify fertilization success in the laboratory or specify spawning times relative to actual sunset times, and none considered the effects of gamete aging, dispersal, and dilution or patterns of fertilization in the sea. What has been lacking is a comprehensive study, quantifying all aspects of the potential isolation mechanisms over several regions and multiple years using identical methods. Here we provide data for two widely separated regions (with more limited data from a third region) and show how the combination of temporal variation in spawning times, allopatric distribution of gametes, and gametic incompatibility can reproductively isolate each species from all others. Genetic and morphological data from the two primary regions that support this interpretation are presented in a companion paper (Fukami et al. 2004).

Materials and Methods

Reproductive Biology of the Montastraea annularis Species Complex

Members of the *M. annularis* species complex are hermaphroditic and reproduce annually in the late summer to early fall (van Veghel 1994; Knowlton et al. 1997; Szmant et al. 1997). Each polyp within the colony produces a single gamete bundle containing both sperm and eggs. There are significant differences in the number and size of eggs produced among species. *Montastraea franksi* produces an average of 55 eggs per bundle at a diameter of 340 μm, *M. annularis* produces an average of 105 eggs per bundle at a diameter of 313 μm, and *M. faveolata* produces an average of 120 eggs per bundle at a diameter of 320 μm (Szmant et al. 1997). These bundles become apparent as they start to pass through the pharynx of the polyp (setting phase). Setting precedes the release of the bundles into the water column (spawning or birthing phase) by 10–20 min. After release, the bundles slowly float to the surface. As the bundles reach the surface they burst into independent sperm and eggs (van Veghel 1994). The buoyant eggs float at or near the water's surface, and the sperm, while swimming, disperse neutrally (D. Levitan, pers. obs.).

Study Sites

Multiple-year reproductive studies were conducted in the Republic of Panama (San Blas Islands, 1996–1997; Bocas del Toro, 1998–2002) and in the Bahamas (Lee Stocking Island, 2000–2001). At each of these sites, we made observations of the timing of spawning (laboratory and field), gamete dispersal (field), and fertilization success with respect to gamete species, age, and dilution (laboratory and field). A single visit was also made to Curaçao for limited observations of field spawning times, because at this site all three species have been reported to spawn in complete synchrony (van Veghel 1994). These locations are widely separated and represent the northern, southern, and western edges of the Caribbean and tropical North Atlantic (Fig. 1). On the studied reefs in the San Blas (Aguadargana, 5–15 m) and Curaçao (Slangenbaai, 13–17 m), all three members of the *M. annularis* complex occur commonly side by side. In the Bahamas, *M. franksi* is primarily a deep-water species and is rare at our study site (Norman Cay, 4–6 m), and in Bocas del Toro this species dominates most reefs, including the majority of our study sites (Davey reef, 4–8 m; Javier reef, 6–12 m, but slightly less so at Hospital Point, 1–6 m; Solarte reef, 2–7 m). Consequently, in the Bahamas we have only laboratory observations of spawning times for *M. franksi*, whereas in Bocas del Toro this species dominates our field data.

Field Observations of Coral Spawning, Gamete Dispersal, and Fertilization

In the Bahamas and Panama, corals were identified; primarily by colony morphology (Weil and Knowlton 1994; but see Discussion; Fukami et al. 2004) and examined to determine if they contained mature gametes (pink eggs present in the mesenteries) one week prior to the predicted spawning time. Field observations of spawning were made at sites with a high proportion of ripe colonies. At each site, a transect line was established and marked with green chemical lights at intervals determined by water clarity. The transects ranged in length from 39 to 121 m, and divers surveyed corals to approximately 10 m on either side of this line. In the Bahamas in 2000, due to the distribution of ripe colonies, a circular area was established with lights on the perimeters of a 60 × 80-m oval. The total survey areas ranged from 780 m^2 to 4800 m^2.

Starting on the fourth or fifth day following the full moon, teams of four to eight divers observed corals starting approximately one hour after sunset. Monitoring typically continued until approximately six hours past sunset. Each pair of divers patrolled approximately a 15-m section of the transect line. The goal was to record every spawning coral within each section (rather than a more scattered sampling across the full transect). On nights with enough divers (three or four pairs) the entire transect line could be successfully monitored. When a diver encountered a coral with gamete bundles conspicuously protruding from the polyps (setting), he/she activated a red chemical light attached to a small lead sinker and recorded the light number and time. Typically, after 20 min all the corals preparing to spawn during that bout were identified. When a coral colony released its gamete bundles, the diver recorded the time and separated the chemical light (with the identifying number) from the lead sinker (similarly numbered). The diver, carrying the chemical light, chose a single gamete bundle near the center of the rising cloud of bundles and followed it as it slowly rose to the surface. Because simultaneously spawning corals were generally several meters apart, the gamete bundles of one colony usually rose to the surface as a discrete cloud. At the surface, the diver

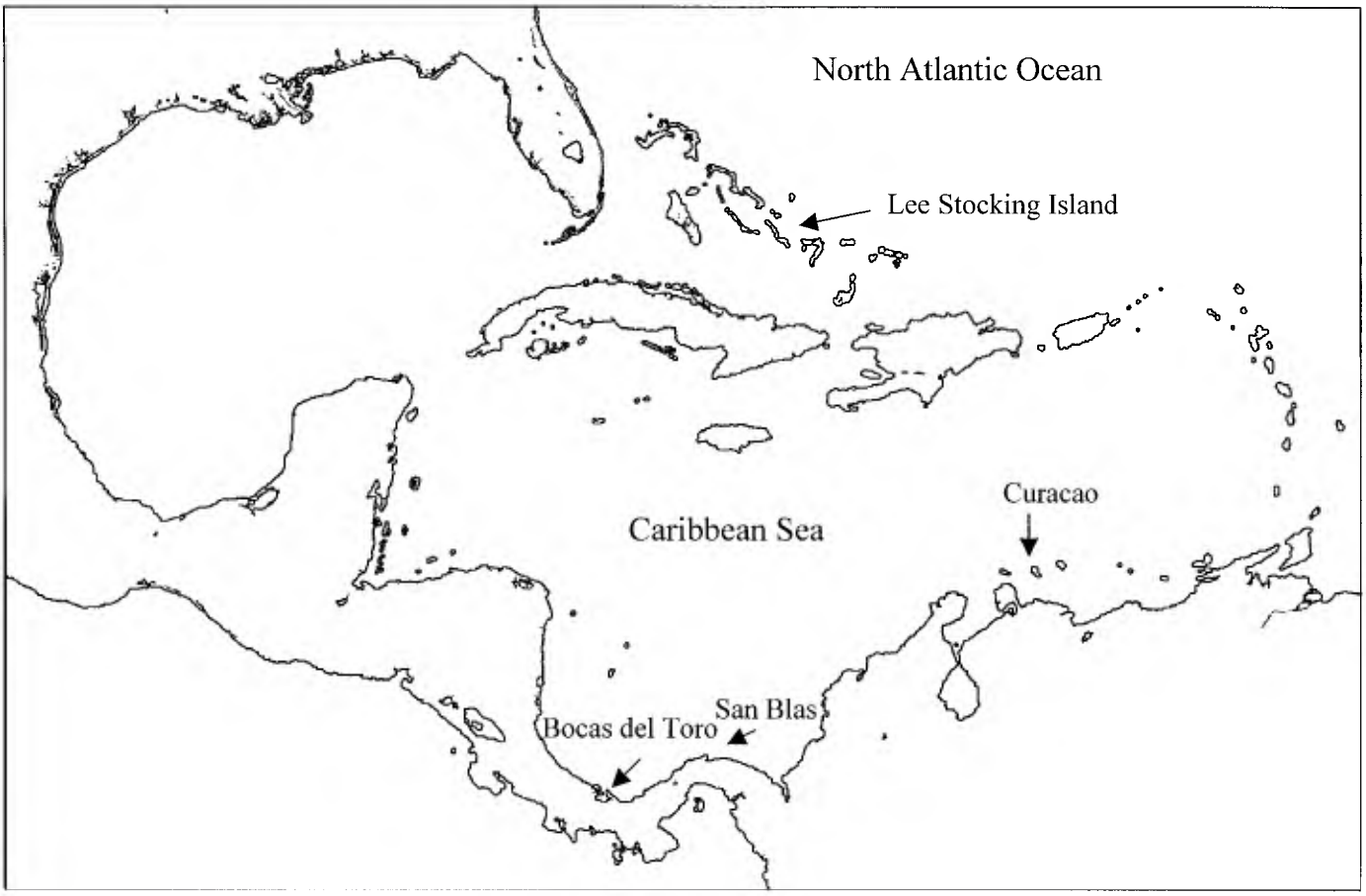

FIG. 1. Map of study sites.

recorded the time and released the buoyant chemical light. The diver then returned to the bottom to search for the next spawning colony.

Other team members in a chase boat recorded the time the chemical light reached the surface and 10–90 min later (with a goal of 30 min) approached the chemical light. When the boat was within 10 m of the chemical light a surface plankton tow was conducted. The plankton net had an 80-μm mesh, and the eggs collected from each tow were rinsed in sperm-free seawater and inspected three hours later for evidence of fertilization and early development (typically four to 16 cells). The chemical light was collected, and its number, time, and GPS position recorded. The ability of chemical lights to mark a batch of coral eggs is discussed in Appendix 1.

A second task conducted on the chase boat was to collect a 50-ml surface water sample every 15 min and place this sample in a plastic cup containing 50 ml of seawater and approximately 200 unfertilized *M. franksi* eggs. This second measure of fertilization is an estimate of fertilization potential in the water over time. The time and GPS position of each water sample was recorded, and three hours later the eggs were investigated for evidence of fertilization. The eggs for this experiment were collected from coral colonies held in the laboratory under an early sunset treatment (see *Collection, Handling, and Identification of corals*). The eggs, from a single colony spawned approximately 1.5 h before the natural spawn, were rinsed free of sperm and brought to the reef so that sampling could start about 30 min before the first colony was noted to spawn in the field. Samples were taken from approximately one hour after sunset for four to five hours. Because the chase boat was following the chemical lights, the water samples would start over the reef and then follow the gamete slick as it drifted away from the reef.

After the last night of spawning, the species identification and position of each spawning colony was noted by locating the numbered lead sinkers. Each colony was mapped on a chart, and the distance between the colony and the position of its corresponding chemical light when it was collected was calculated.

In Curaçao, the relatively deeper depths at which the three species coexist and the presence of only two divers made it impossible to monitor spawning for more than 100 min per night. On two successive nights (22, 23 September 1997), observations were made from 2000 h to 2120 h (the predicted time of exclusive *M. franksi* spawning based on other studies).

Laboratory Fertilization Assays

Collection, handling, and identification of corals

Ripe *M. annularis*, *M. franksi*, and *M. faveolata* corals were provisionally identified in the field, and a piece with approximately 100–400 cm^2 of living surface area was broken

off, cleaned of associated organisms with a wire brush, and maintained in the laboratory with fresh flowing seawater. On days 4–8 following the full moon, approximately two hours before sunset, corals were placed individually in white buckets at a location with minimal exposure to artificial light. A subset of corals were covered in black plastic to establish a two hour early sunset treatment. Previous studies established that these corals would spawn approximately two hours before conspecifics experiencing normal sunset (Knowlton et al. 1997), thereby allowing gametes from early spawning species to be tested with normally late spawning species using equal age gametes.

Corals were checked every 20 min to determine setting and spawning times. When corals spawned, approximately 100 gamete bundles were collected with a 10-ml pipette. Gamete bundles in 20 ml of seawater were placed in a plastic cup and swirled to break the bundles into individual eggs and sperm. This suspension was then filtered through 80-μm Nitex (Sefar Canada Inc., Scarborough, Ontario) mesh to separate the eggs from the sperm. The sperm were kept in a cup with a total volume of approximately 20 ml; the eggs were rinsed several times with fresh filtered seawater collected several hours prior to spawning, and then poured into another cup with 20 ml of seawater. Egg concentration from these stock suspensions was determined from three replicate counts of 0.1-ml subsamples. One milliliter of the sperm suspension was fixed in concentrated formalin, and the sperm concentration was determined from eight replicate counts using a hemocytometer. The stock gamete suspensions were used in a variety of experiments described below.

Gametes and skeletal vouchers were collected for corals used in fertilization experiments and analyzed genetically and morphologically. In Panama, where these species were first described (Weil and Knowlton 1994), field identifications were supported by concordant patterns of genetic and morphological variation (details in Fukami et al. 2004). In the Bahamas, however, colonies in shallow water exhibit morphologies not seen in Panama (or Curaçao), which initially made field recognition, especially of *M. faveolata*, more difficult. When genetic and morphological data conflicted with field identification, the former were used in assigning species names to colonies used in fertilization trials.

Matrix fertilization experiments

A series of matrices were established to determine patterns of gamete compatibility in self, conspecific, and heterospecific crosses. Corals experiencing both normal and early sunsets were used in this experiment, so that species normally spawning at different times could be accessed using freshly spawned gametes. This provides an intrinsic measure of gamete compatibility, independent of gamete age. Assays were conducted using sperm concentrations empirically shown to result in high conspecific fertilization rates. A matrix of plastic cups was established, with cups containing 48 ml of filtered seawater. When the first coral was observed to spawn, the gametes were collected and separated into eggs and sperm as described above. One milliliter of the stock sperm suspension was placed into each cup in the first column in the matrix and 1 ml of the stock egg suspension was placed into each cup in the first row in the matrix. The coral identification, time of spawning, and time of addition to the matrix was recorded. As each subsequent coral released gamete bundles, the separated sperm and eggs were added to additional columns and rows, respectively.

Independent matrix experiments were conducted 11 times (seven in Panama and four in the Bahamas). Because each pairwise test within a particular matrix is not completely independent, the data were analyzed in two ways. The first used each individual cross within and across all matrices as the data, and the second used the matrix averages for each type of cross as the data. In both cases, the individual data were square-root arcsine transformed before analysis.

Gamete age and dilution experiments

Gamete age and dilution can influence fertilization success (Levitan et al. 1991). The interaction between gamete age and sperm concentration was investigated in conspecific and heterospecific crosses of the two coral species with compatible gametes but different spawning times: the early spawning *M. franksi* and the late spawning *M. annularis* (see Results). Two experiments were conducted, all using Panamanian corals exposed to natural sunset times. The purpose of this experiment was to test the ability of sperm and eggs from the two compatible species to cross-fertilize under their naturally distinct differences in spawning times. In the first experiment, *M. franksi* sperm was added first to *M. franksi* eggs and then, after approximately two hours (when *M. annularis* spawned), to *M. annularis* eggs. The second experiment added sperm of the late spawning *M. annularis* to *M. annularis* eggs and to *M. franksi* eggs that had spawned approximately two hours earlier. Each experiment was replicated four or five times with unique corals. In each replicate, the eggs were tested with serially diluted sperm (see details in Appendix 2).

RESULTS

Observations of Spawning Times in the Field and Laboratory

In the field in Panama and the Bahamas, spawning by members of the *M. annularis* complex was observed on 28 evenings during 10 lunar events between 1996 and 2002 (Table 1). Spawning occurred during days 4–8 following the full moon (Fig. 2). At particular locations one or two species might have been rare or absent, but in every location all common species were observed spawning during the lunar event. Within a lunar event, *M. franksi* tended to spawn earlier in the month, most frequently spawning on days 5–7, whereas *M. annularis* and *M. faveolata* peaked on days 6–8 (Fig. 2). If all the corals collected in the laboratory spawned by day 7, divers typically did not dive on day 8. Because of this bias, the average proportion of corals spawning on day 8 is likely to be less than that reported in Figure 2.

Spawning times are reported as hours and minutes following sunset (Astronomical Applications Department, U.S. Naval Observatory). Spawning times in the field are accurate to within a couple minutes, as divers were constantly observing corals. If a coral was marked as a setting colony but the exact spawning time was missed, it was not included in

TABLE 1. Sites and dates of spawning events. Although all three species were present at all sites, they differed substantially in their abundance, from reef to reef, and not all species were observed to spawn in the field at every event. All three species were observed to spawn in the laboratory at every site and date where corals were collected: a, *Montastraea annularis*; v, *M. faveolata*; k, *M. franksi*; ND, no data.

Date	Site	Laboratory fertilization assays	Field observations of spawning
1996			
Sept. 3–4	San Blas, Aguadargana reef	a, v, k	a, v, k
1997			
Aug. 24–25	San Blas, Aguadargana reef	a, v, k	a, v, k
Sept. 22–23	Curaçao	ND	a, v, k
1998			
Sept. 11–12	Bocas del Toro, Javier reef	a, v, k	k
1999			
Aug. 30–Sept. 2	Bocas del Toro, Javier reef	a, v, k	k
Sept. 29–30	Bocas del Toro, Davey reef	ND	a, k
2000			
Aug. 19–21	Lee Stocking Island, Norman's Cay	a, v, k	a, v
Sept. 17–18	Bocas del Toro, Davey's reef	a, v, k	k
Sept. 19–20	Bocas del Toro, Hospital Point	a, v, k	a, k
2001			
Sept. 8–9	Lee Stocking Island, Norman's Cay	a, v, k	a, v
Oct. 6–7	Bocas del Toro, Javier reef	ND	k
Oct. 8–9	Bocas del Toro, Solarte reef	ND	a, k
2002			
Sept. 25–26	Bocas del Toro, Davey's reef	ND	k
Sept. 27	Bocas del Toro, Hospital Point	ND	a, k

the data (Fig. 3a). Laboratory observations were made at 10- to 20-min intervals, so these estimates may slightly bias the spawning observations to a later time (Fig. 3b). Overall, a three-way ANOVA testing the effects of method of observation (field vs. laboratory), species and geographic region (Panama vs. the Bahamas) indicate a significant effect of method and species but no significant effect of region or any of the interactions. Pairwise tests (SNK) indicate that each species had a significantly different mean spawning time. Field estimates of spawning were on average 18 min earlier than the laboratory estimates. This difference may reflect the bias in observation time (see above), the fact that laboratory corals were above the water's surface and may have perceived sunset at a later time compared to corals at depth (see below), or the possibility that spawning in the field is accelerated by pheromones released by the earliest spawning colonies.

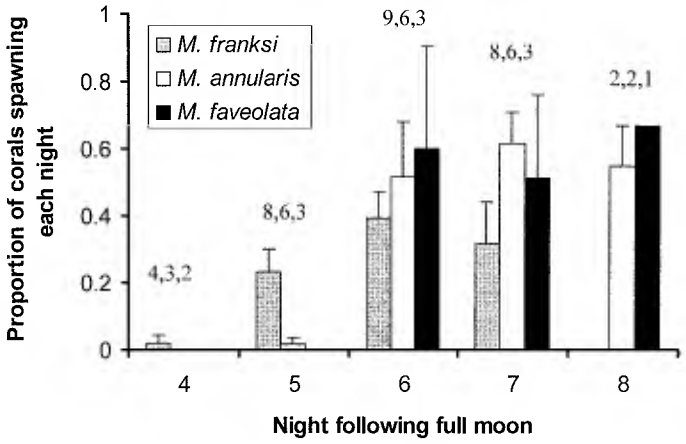

FIG. 2. Distribution of *Montastraea* spawning on days following full moon. Data are the average proportion of corals observed to spawn on one evening based on the total number that spawned during that lunar event (bars are standard error). Samples sizes (number of evenings when a particular species was present) are reported above each bar and differ because on some reefs one or two species may have been rare or absent and on some lunar events a different subset of evenings may have been surveyed.

Observations from the field across all locations indicate that *M. franksi* spawned between 1:25 and 2:36 (mean 1:55) hrs after sunset, *M. annularis* spawned between 3:04 and 4:22 (mean 3:43) hrs after sunset, and *M. faveolata* spawned between 3:43 and 4:40 (mean 3:56) hrs after sunset. *Montastraea annularis* and *M. faveolata*, the species with incompatible gametes (see below), have significantly different but overlapping spawning times at all locations. *Montastraea franksi* and *M. annularis*, the two species with compatible gametes, had a significant difference in spawning time averaging 108 min across all dates (Fig. 3b). On six occasions, spawning times for *M. franksi* and *M. annularis* individuals were recorded on the same evening. An average of 101 min (SE 5.4) separated the average spawning times of these species on those dates. The average time between the last *M. franksi* individual observed to spawn and the first *M. annularis* observed to spawn on a single location and date was 70 min (SE 8.6).

In Bocas del Toro, the depth of each spawning coral colony was noted. Regression analysis indicates that spawning time is unrelated to the depth distribution in *M. annularis* (t =

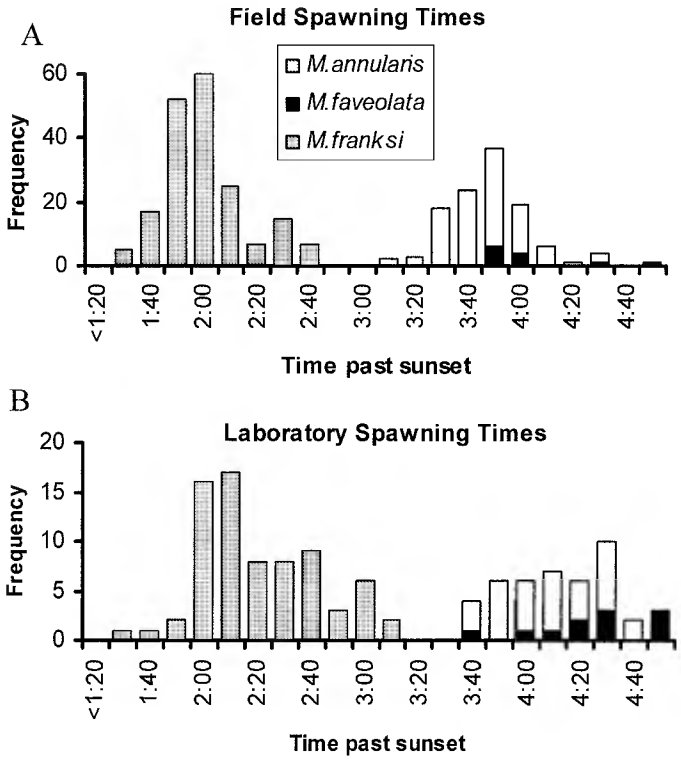

FIG. 3. Spawning times for corals in Panama and the Bahamas based on field (A) and laboratory (B) observations. Times are adjusted as time following sunset. Because there were no significant differences in spawning times across sites within species, site data were pooled.

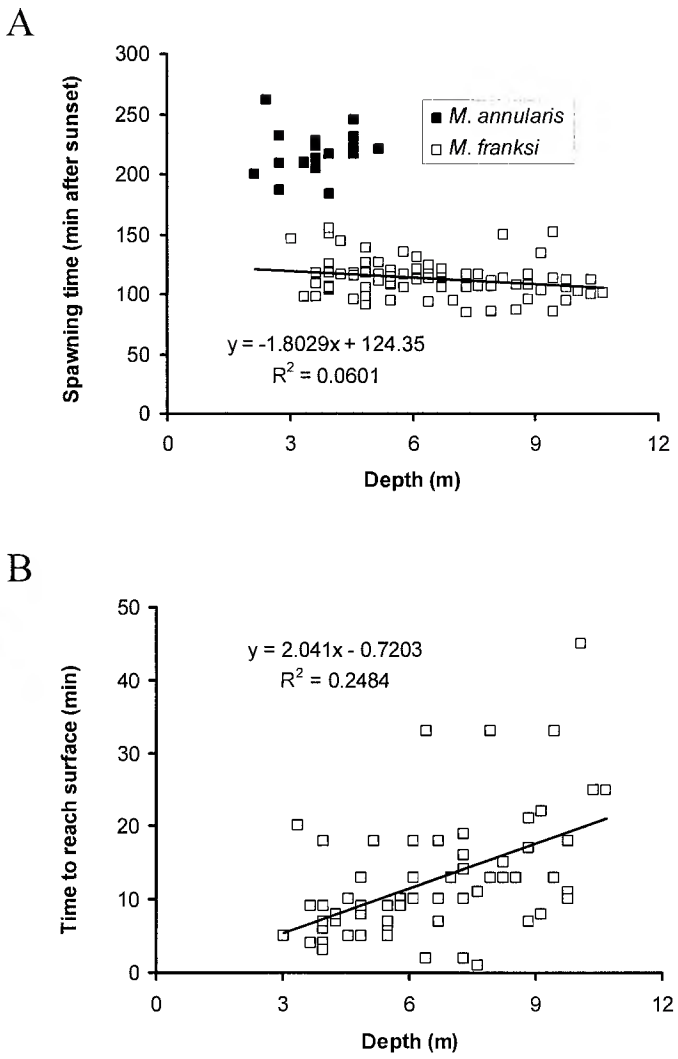

FIG. 4. Spawning and surfacing times as a function of coral depth. (A) Spawning times following sunset for *Montastraea franksi* and *M. annularis* as a function of the depth of the spawning coral colony. (B) The time interval from spawning to when the gamete bundles reached the surface as a function of water depth in *M. franksi*.

0.58, df = 18, $P > 0.5$). However, in *M. franksi*, spawning time is slightly, but significantly, earlier in deeper water ($t = 2.30$, df = 84, $P < 0.05$, Fig. 4a). This depth effect does not explain the differences in spawning times between the two species as the spawning depths largely overlap (Fig. 4a).

In *M. franksi*, the time it takes for gamete bundles to reach the surface, where they burst and fertilization can occur, increases with the depth of the spawning coral (Fig. 4b). Regression analysis indicates an average travel rate of 0.5 m/min ($t = 4.38$, df = 59, $P < 0.0001$). The increase in travel time (slope = 2.0) compensates for the differences in spawning time (slope = −1.8) such that there is no significant relationship between colony depth and the time the gametes arrive on the surface (t = 0.41, df = 59, $P = 0.68$).

In Curaçao, field observations were limited to a window of time when *M. franksi* would be expected to be the only species observed spawning based on observations elsewhere, but contrary to a previous report of simultaneous spawning by all three species at this location (van Veghel 1994). During this time, only *M. franksi* was observed to spawn, despite the fact that many colonies of the other two species occurred where spawning was monitored. Thirteen colonies were observed to spawn between 1:48 and 2:29 (average 2:02) hours after sunset. These times were not significantly different from observations of *M. franksi* in Panama ($F = 2.58$, df = 110, $P > 0.05$). In shallower water, a few colonies of *M. annularis* and *M. faveolata* were observed to spawn while returning to shore at the end of the dives at between 3:43 and 3:44 hours past sunset, times typical for these species in both Panama and the Bahamas (Fig. 3b). Although we cannot rule out additional spawning by *M. franksi* later in the evening or earlier spawning by the other two species at other depths, the most parsimonious interpretation of these data is that spawning times for the three species in Curaçao resemble those reported from elsewhere.

Dispersal of Eggs in the Field

Chemical lights traveled 9–1732 m in the 6–119 min from release to capture. On different nights the lights often drifted in different directions, but on all nights the lights drifted off the reef at the time of sampling (Fig. 5). Overall, the relationship between dispersal distance and time was best fitted by a linear function of time ($R^2 = 0.21$ for linear vs. 0.11 for log-transformed data; $y = 0.0038x - 0.0046$; $t = 6.79$, df = 195, $P < 0.0001$). The average rate (km/min) of move-

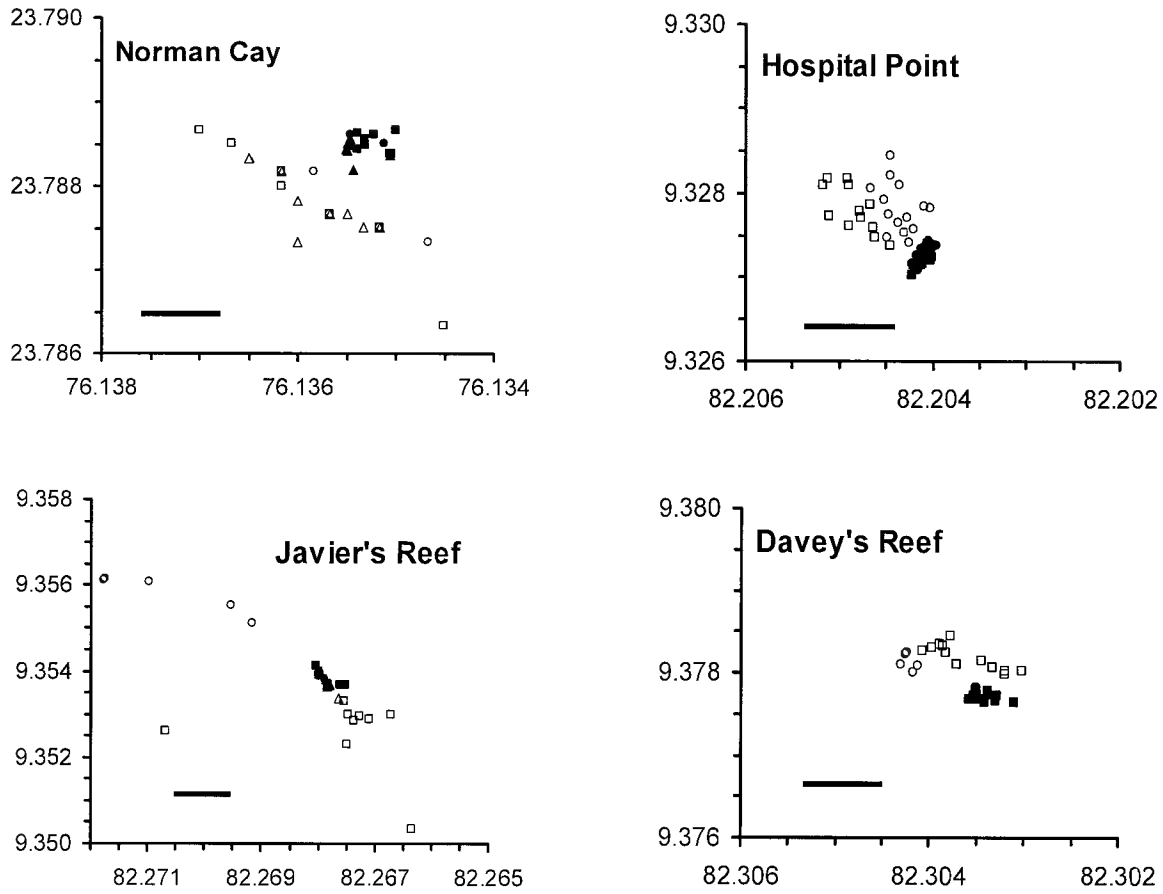

FIG. 5. Representative maps of the distribution of corals (solid symbols) and the chemical lights (open symbols) associated with each coral at the time the plankton sample and light were collected. Different shaped symbols represent different nights of spawning on the same reef. Different panels represent different reefs. Scale bar equals 1 km. Because each light was collected at a different time and there was variation in time of release to time of capture, this does not portray an exact snapshot of the relative position of the chemical lights to each other.

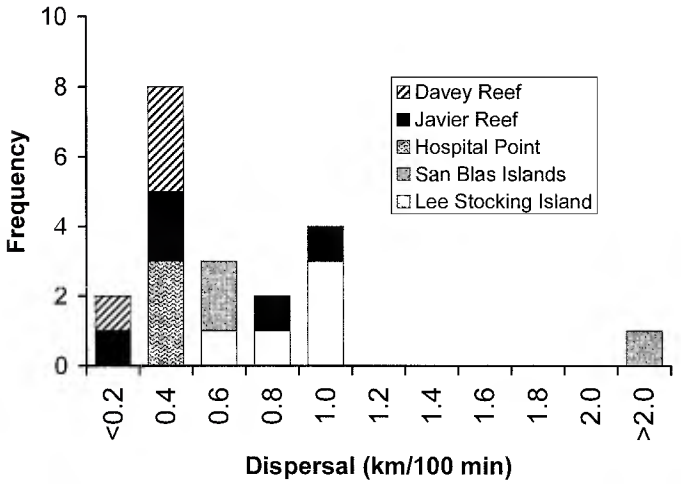

FIG. 6. The frequency of average calculated distances moved in 100 min for each night of spawning.

ment calculated for all chemical lights dispersing on any one evening was multiplied by 100 min to estimate the average dispersal distance achieved by *M. franksi* eggs before *M. annularis* spawns. Overall, eggs were estimated to travel an average of 542 m (SE 98) during a 100-min interval on the 20 nights of coral spawning when chemical light data were collected (Fig. 6). Ninety percent of the eggs were estimated to move more than 200 m, and no gametes were estimated to disperse less than 100 m in 100 min. The highest level of dispersal was observed during an evening in the San Blas Islands, when gametes were estimated to move just over 2 km in 100 min.

Fertilization Potential

Measures of fertilization of precollected eggs exposed to water samples were successfully made on eight nights of spawning (six in Bocas del Toro and two in the Bahamas). Of these, only one night was on a reef where both *M. franksi* and *M. annularis* were present and spawned. This small sample size is a result of the logistic difficulties of coordinating the collection of laboratory spawning with the field work, the dominance of single species on many reefs (*M. franksi* in Bocas del Toro and *M. annularis* in the Bahamas), and the

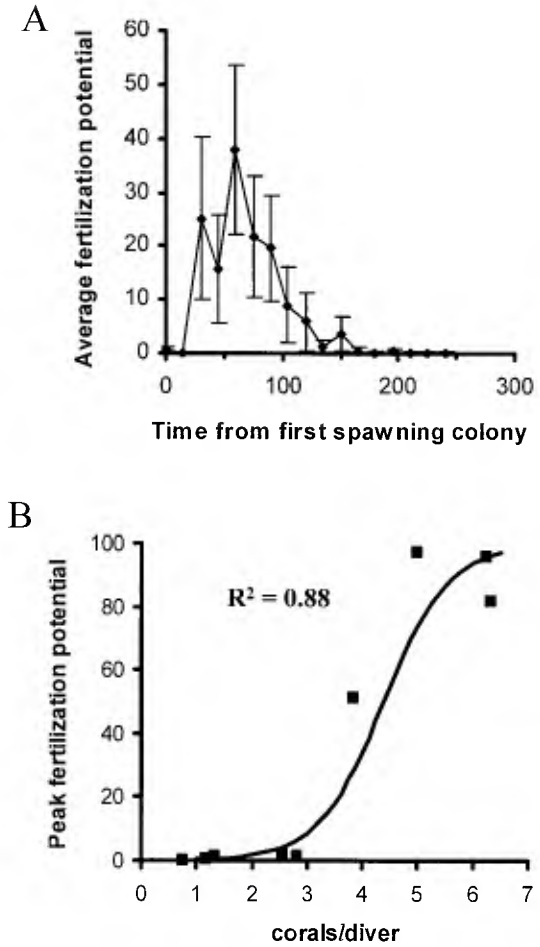

FIG. 7. The fertilization potential of sperm. (A) The percent of eggs fertilized, using precollected *Montastraea franksi* eggs and water samples collected in the field over time. Time adjusted as time from the first noted coral spawning on that particular evening. Error bars are standard errors ($N = 8$ evenings). (B) The peak fertilization potential for each evening as a function of the average number of corals noted spawning per observer. We used corals per observer rather than corals per area, because on different nights different numbers of divers were used. Fertilization data were logit transformed for regression analysis and then backtransformed for visualization.

fact that these two species often spawn on different nights (Fig. 2).

Overall, fertilization was noted in samples collected approximately 30 min after the first observation of spawning, at a time concurrent with the gametes reaching the surface of the water. Fertilization potential peaked approximately 30 min later. Fertilization potential decreased shortly afterward, and little fertilization was noted two hours following the initiation of spawning (Fig. 7a). The peak fertilization potential on any given night ranged from 0% to 97%. This variation could largely be explained by the number of corals observed to spawn on each evening (logit-transformed fertilization data, $R^2 = 0.88$, Fig. 7b). On the evening where both species were spawning, the peak fertilization potential (82%) occurred shortly after the *M. franksi* gametes reached the surface (Fig. 8). This pulse decreased to near zero as the *M. annularis* gametes reached the surface. A second smaller peak (2%) was noted after *M. annularis* gametes reached the surface (22:30 h). The peak heights for both species are consistent with the regression predictions based on the number of colonies observed to spawn for each species (6.3 and 2.8 corals/diver for *M. franksi* and *M. annularis*, respectively, Fig. 7b).

Natural Fertilization Success

Natural levels of fertilization were successfully measured on 10 evenings (five in Bocas del Toro and five in the Bahamas). Average fertilization of all corals that spawned on an evening was correlated with the number of observed spawning colonies (Fig. 9). Natural levels of fertilization were measured on two evenings that *M. franksi* and *M. annularis* were both observed to spawn at the same site (Fig. 10). On both these nights, peak fertilization for *M. franksi* was associated with the mean spawning time for that species. Corals that spawned slightly early or late compared to the mean spawning time had reduced fertilization success (significant second order polynomial, $F = 7.27$, df = 44, $P < 0.01$). *Montastraea annularis* showed the same trend, but this was not significant ($F = 0.38$, df = 16, $P > 0.05$). While some error in the ability of a chemical light to track the eggs of a specific coral colony is likely, the increased variance caused by this error would only weaken correlations of fertilization with spawning times (Appendix 1).

Matrix Fertilization Experiments in the Laboratory

This experiment used early sunset manipulations to induce a subset of corals that spawn at different times to spawn at similar times to determine gametic compatibility independent of gamete age. As a result of this manipulation, the ages of both the sperm and eggs were not significantly different between conspecific and heterospecific crosses (sperm age in Bahamas $F = 0.22$, df = 555, $P > 0.05$; sperm age in Panama $F = 2.48$, df = 133, $P > 0.05$; egg age in Bahamas $F = 0.26$, df = 555, $P > 0.05$; egg age in Panama $F = 2.48$, df = 133, $P > 0.05$). The fertilization assays used optimal sperm concentrations for high fertilization (mean = 489 sperm/μl, SE = 57; cf. *Gamete Age and Dilution Experiments in the Laboratory* results below). We examined 12 different cross types including self (three), conspecific (three), and heterospecific (six) crosses. Two-way analyses of variance of the individual fertilization values revealed significant main effects (cross-type and region) and a cross-type by region interaction (all $P < 0.0001$). A similar analysis of the average matrix values revealed significant main effects ($P < 0.0001$ and $P < 0.001$ for crosstype and region, respectively) but no significant interaction. Overall, fertilization tended to be higher in Panama. Adjusted least square means indicate an overall reduction of 6.6% of eggs fertilized in the Bahamas compared to Panama.

Independent ANOVAs within each region indicate significant differences among the types of crosses. All conspecific crosses along with most heterospecific crosses of *M. annularis* and *M. franksi* are compatible, while heterospecific crosses involving *M. faveolata* and all self crosses are largely incompatible (Fig. 11a,b).

There are two notable exceptions to these patterns. First,

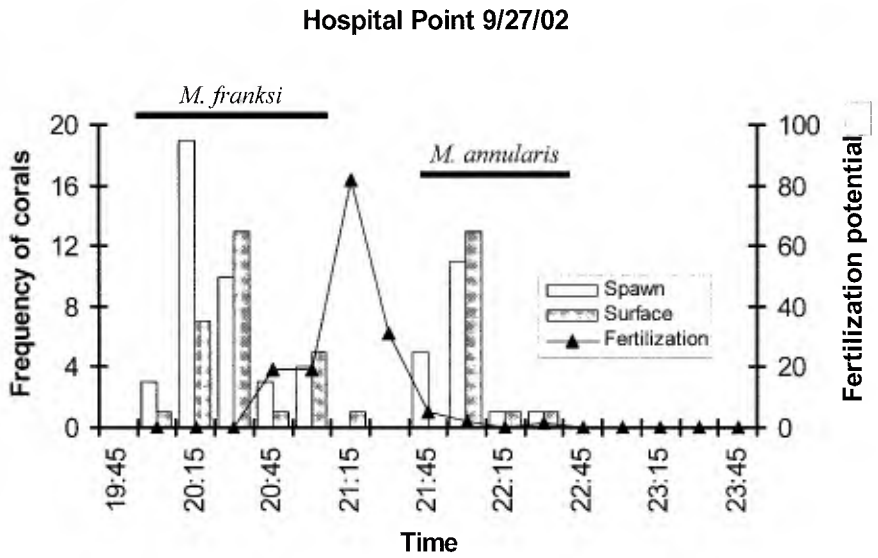

FIG. 8. Relationship between spawning time of individual coral colonies, the time the gametes reach the surface, and fertilization potential for a night when both *Montastraea franksi* and *M. annularis* spawned.

some heterospecific crosses involving *M. faveolata* are successful. The occasional fertilization noted in these crosses is correlated with the age of the eggs; older eggs were more easily fertilized ($R^2 = 0.04$, $P = 0.004$). While egg age only explains a small fraction of the overall variance in fertilization success, it does explain almost all the aberrantly high fertilization (Table 2). Only two of 77 crosses involving *M. faveolata* and *M. annularis* (the two species that spawn at similar times) exceeded 15% of eggs fertilized when the eggs were younger than 75 min. On average, less than 1% of the eggs were fertilized in this heterospecific cross.

Second, in the Bahamas there is a significant asymmetry in *M. franksi* and *M. annularis* crosses; crosses involving *M. franksi* sperm and *M. annularis* eggs largely fail, but reciprocal crosses are successful. In Panama, the same types of crosses are symmetrical and successful. This regional difference in *M. franksi* symmetry is the likely explanation for the significant interaction term of the ANOVA. The asymmetry in fertilization between *M. franksi* and *M. annularis* in the Bahamas does not appear to be an artifact of differences in egg age (Fig. 12). The average difference between egg ages of the two crosses is much greater in Panama (80 vs. 20 min), yet in Panama there is no evidence for an asymmetry in compatibility. Although some intracross variation in compatibility is apparent in the Bahamas, crosses involving *M. franski* sperm and *M. annularis* eggs generally fail across all egg ages, while the reciprocal cross results in patterns of fertilization similar to conspecific crosses.

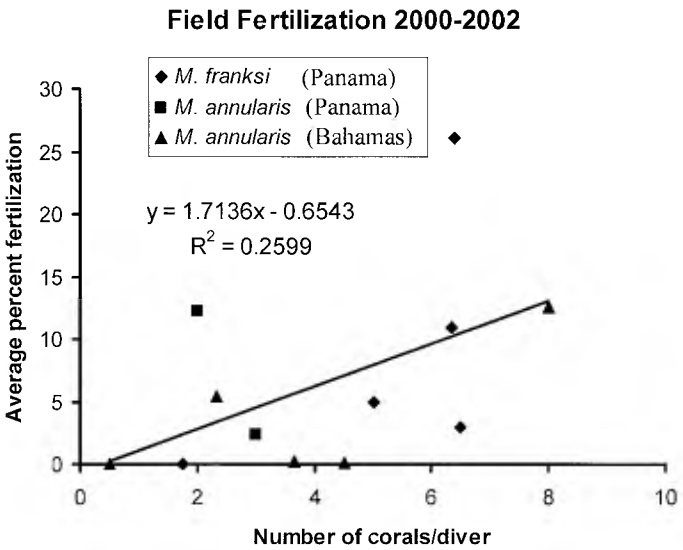

FIG. 9. The average percent of eggs fertilized as a function of the average number of corals noted spawning per observer. Fertilization data are from plankton samples collected from each released chemical light. Each datapoint is the average fertilization success for each evening. Different symbols represent different species and regions.

Gamete Age and Dilution Experiments in the Laboratory

The previous experiment used early sunset manipulations to induce corals to spawn at similar times to determine gamete compatibility independent of gamete age. However, the two compatible species spawn nearly two hours apart from one another. In the sperm age experiment, *M. franksi* sperm was used fresh (range 14–35 min after spawning) with conspecific eggs and then aged (range 115–158 min) until *M. annularis* spawned, when they were tested with the eggs of that species. Two orders of magnitude more *M. franksi* sperm were needed to fertilize 50% of *M. annularis* eggs compared to *M. franksi* eggs, using gametes released during natural spawning times (Fig. 13a). The nonoverlapping 95% confidence intervals, calculated by fitting the data to the fertilization kinetics model, indicate that the fertilization profiles of these two crosses are significantly different. Thus, in the field, *M. franksi* sperm is less likely to fertilize *M. annularis* eggs compared to conspecific eggs, assuming the water mass containing these gametes overlap and remain at similar sperm concentrations. However, because the sperm from the earlier *M. franksi*

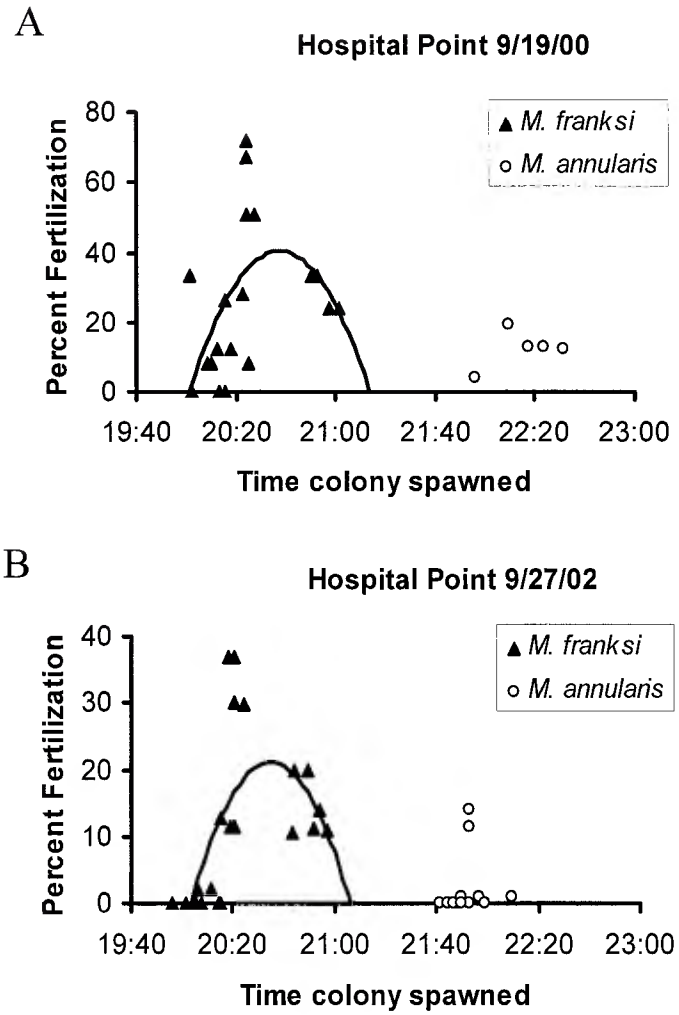

FIG. 10. The percent of eggs fertilized as a function of the time each colony spawned for *Montastraea franksi* and *M. annularis* on the two evenings, (A) September 19, 2000; and (B) September 27, 2002, when both species spawned on the same reef (Hospital Point). The polynomial regression was significant for *M. franksi* but not *M. annularis*.

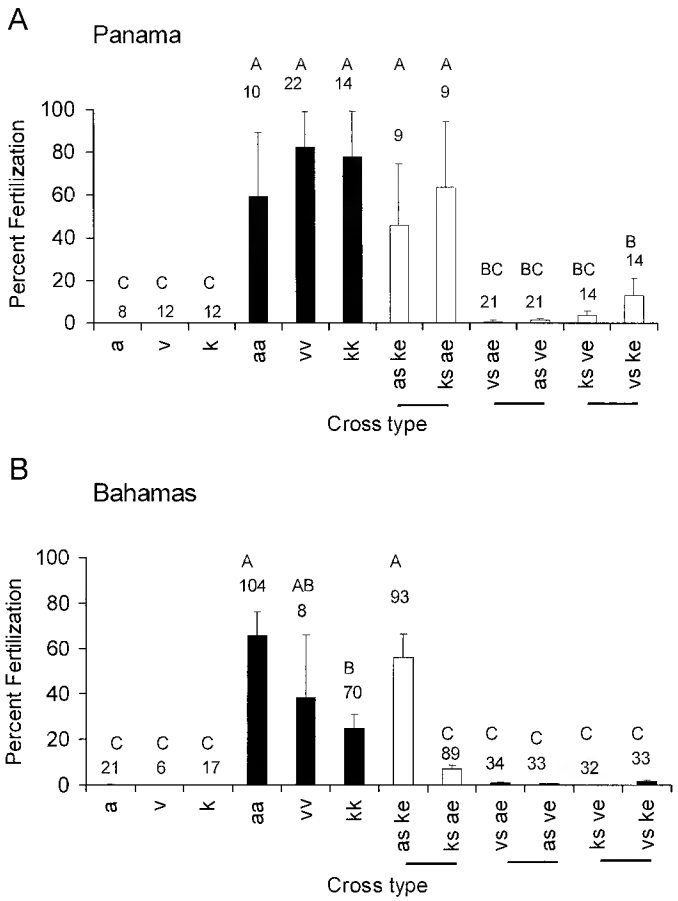

FIG. 11. Fertilization success of individual crosses under optimal sperm concentrations in the fertilization matrix experiments: (A) Panama; and (B) Bahamas. Single letters (a, *Montastraea annularis*; v, *M. faveolata*; and k, *M. franksi*, respectively) represent self crosses, double letters represent conspecific crosses and letters followed by an "s" or "e" represent sperm or eggs for each heterospecific cross. Conspecific crosses are highlighted as solid bars and heterospecific crosses are highlighted as open bars. Lines under legend group species pairs. Cross types with different letters are significantly different using SNK pairwise comparisons. Sample sizes and standard errors are plotted.

spawning event will likely move off the reef and dilute over the time interval before *M. annularis* spawns (Figs. 5–8), the likelihood of this cross becomes even lower.

In the egg age experiments, the early spawning *M. franksi* eggs were aged until *M. annularis* spawned, when *M. annularis* sperm was crossed with conspecific (egg age 24–63 min) and *M. franksi* (egg age 120–171 min) eggs. These fertilization assays indicate no significant difference in the fertilization profiles using *M. annularis* sperm with both *M. annularis* and *M. franksi* eggs spawned at natural times (Fig. 13b). The 95% confidence intervals overlap over the entire range of sperm concentrations in this comparison. Thus, in the field, old *M. franksi* eggs encountering sperm from the later spawning *M. annularis* are just as likely to be fertilized as are fresh *M. franksi* eggs encountering fresh *M. franksi* sperm, assuming *M. franksi* eggs are over a reef where *M. annularis* spawns. These two experiments used corals from Panama. At this site there was no evidence of an asymmetry

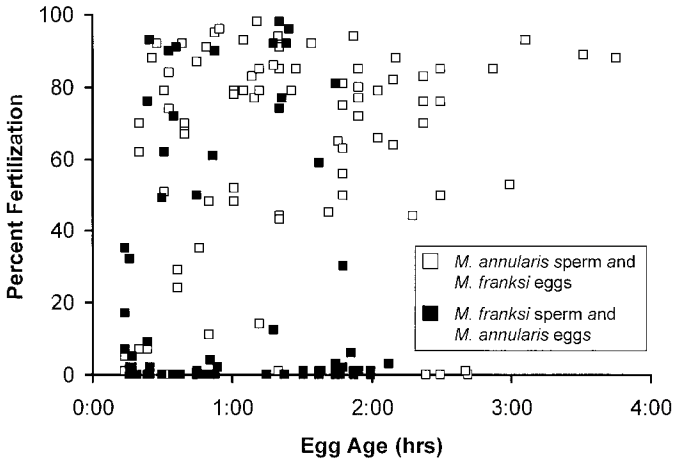

FIG. 12. Fertilization success of corals, from the Bahamas, as a function of egg age in crosses between *Montastraea annularis* and *M. franksi*.

in gamete compatibility (Fig. 11a) that might confound these results.

Discussion

Mechanisms of Reproductive Isolation

Montastraea annularis and *M. faveolata* have overlapping spawning times in the late evening but have incompatible gametes. The likelihood of this cross averaged 1% at optimal sperm conditions in the laboratory and was not significantly different from the likelihood of selfing in no-choice experiments. Typically, studies that examine heterospecific fertilization when eggs are given a choice of both heterospecific and conspecific sperm demonstrate that heterospecific fertilization becomes even less likely (Howard 1999). Therefore, it seems unlikely that under field conditions, in the presence of conspecific sperm, hybrids would be formed between these two species.

Montastraea franksi spawns nearly two hours before the other two species. In the laboratory using fresh gametes, it is completely compatible with *M. annularis* in Panama. In the Bahamas, *M. franksi* eggs are compatible with *M. annularis* sperm but not the reciprocal.

Fertilization of *M. annularis* eggs by *M. franksi* sperm is nevertheless unlikely, even in Panama where there is no evidence of gametic incompatibility. During the nearly two-hour gap in spawning times, *M. franksi* sperm disperse off the reef (Figs. 5, 6), become diluted (Figs. 7, 8, 10), and lose viability (Fig. 13). This severely limits the chances of *M. franksi* sperm outcompeting the freshly released *M. annularis* sperm for *M. annularis* eggs. Estimates of fertilization potential indicate that sperm have peak fertilization performance for about an hour before dilution and aging effects reduce the likelihood of fertilization (Fig. 7). On reefs with both *M. franksi* and *M. annularis* spawning on the same evening, the *M. franksi* fertilization potential was reduced to near zero by the time the *M. annularis* eggs reached the surface (Fig. 8). These field estimates of the likelihood of *M. franksi* sperm fertilizing *M. annularis* are probably inflated because these measures were conducted in the middle of the *M. franksi* gamete slick, and this slick disperses off the reef by the time *M. annularis* spawns. So, although *M. franksi* and *M. annularis* adults may occur in close proximity, several hundred meters (Figs. 5, 6) can separate their gametes.

Montastraea franksi eggs, on the other hand, do not lose viability in two hours and could easily be fertilized by *M. annularis* sperm in both Panama and the Bahamas. This assumes that, following the *M. franksi* spawning event, *M. franksi* eggs remain unfertilized and that those unfertilized eggs remain in close proximity to *M. annularis* colonies. The natural estimates of fertilization from plankton samples indicate that indeed many eggs remain unfertilized following a spawning event (Fig. 9). However, the gamete dispersal data indicate that *M. franksi* eggs are likely to travel several hundred meters during the interval between *M. franksi* and *M. annularis* spawning (Figs. 5, 6). What this study cannot rule out is the possibility that the unfertilized eggs of *M. franksi* could drift over another reef occupied by *M. annularis* when that species spawns, resulting in hybrid fertilization. Of all the possible combinations of hybrid fertilization among these three species, this is the most likely possibility; it would

Table 2. The percent of heterospecific crosses involving *Montastraea faveolata* that exceeded 15% of eggs fertilized in young (<75 min) and old (>75 min) eggs. Numbers of crosses in each group are given in parentheses.

	Cross	
Egg age	*M. faveolata* × *M. franksi*	*M. faveolata* × *M. annularis*
Young	6.4% (47)	2.6% (77)
Old	19.6% (46)	9.4% (32)

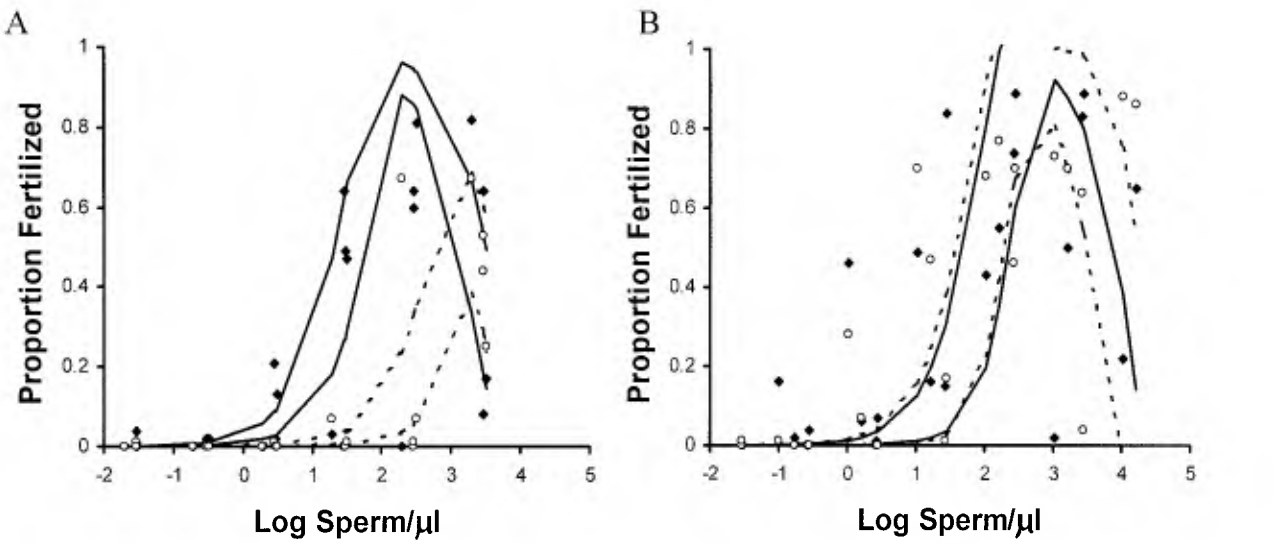

Fig. 13. Fertilization success as a function of log sperm concentration for corals spawning at their natural times. Tests comparing (A) fresh *Montastraea franksi* sperm and fresh *M. franksi* eggs (solid symbol and line) with old *M. franksi* sperm and fresh *M. annularis* eggs (open symbol and dotted line); (B) fresh *M. annularis* sperm and fresh *M. annularis* eggs (solid symbol and line) with fresh *M. annularis* sperm and old *M. franksi* eggs (open symbol and dotted line). Lines indicate the 95% confidence intervals on the mean.

TABLE 3. Published *Montastraea* spawning times throughout the Caribbean. Data are published as a starting and stopping time in the field (van Veghel 1994; Knowlton et al. 1997; Szmant et al. 1997; Sanchez et al. 1999) or from laboratory observations (Knowlton et al. 1997). The data from Colombia include setting and spawning times. Data from the Gulf of Mexico are approximate because exact dates are not provided. All times of spawning are adjusted as time (in hours) past sunset, using the astronomical calculations from the U.S. Navy for each date and location.

Site	Date	*M. franksi*	*M. faveolata*	*M. annularis*
Gulf of Mexico	1991–1998	1:16–2:36	3:11–4:56	4:06–4:31
Hagman et al. 1998b				
Florida	8–9 Sept. 1993		2:57–3:57	2:57–3:57
Szmant et al. 1997	27 Aug. 1994	0:44–1:19	2:14–3:14	2:14–3:14
	28 Aug. 1994	2:45–3:30	3:45–4:30	3:45–4:30
	16–18 Aug. 1995	1:35–2:05	3:05–3:50	3:35–4:20
	14 Sept. 1995			3:53–4:08
	15 Sept. 1995		3:33–4:03	
Bahamas	30 Aug. 1991		2:57–?	2:57–?
Szmant et al. 1997	1 Sept. 1991	2:57–3:57		2:57–3:57
Colombia	24 Aug. 1997		3:24–4:09	
Sanchez et al. 1999	22 Sept. 1997		3:01–3:51	3:01–3:51
	23 Sept. 1997		3:17–4:02	3:17–4:02
	21 Oct. 1997		3:17–3:57	3:17–3:57
	11 Oct. 1998		2:58–3:32	2:52–3:57
Panama	26–30 Aug. 1994	1:48–2:33	3:03–4:03	3:03–4:03
Knowlton et al. 1997	16–18 Aug. 1995	1:27–2:47		3:47–4:47
	14–16 Sept. 1995	1:44–3:44	3:04–5:04	4:04–5:44
Honduras	16 Aug. 1995	0:06	1:36–1:51	3:21–4:01
Knowlton et al. 1997				
Curacao	29 Sept.–1 Oct. 1991	2:33–4:33	2:33–4:33	2:33–4:33
Van Veghel 1994				
Literature average		1:52–3:00	3:05–4:02	3:17–4:15
Current study		1:25–2:35	3:43–4:40	3:04–4:22

depend on the distribution of reefs and the local flow regimes and could be expected to vary regionally (see *Regional Differences in Reproductive Isolation and Introgression*).

Comparisons with Other Studies

In our studies in Panama and the Bahamas, we individually timed and marked more than 380 spawning corals during 28 spawning events and never observed *M. franksi* spawning simultaneously with the other two species. This highly consistent pattern is largely supported by other studies from Panama, Honduras, southern Florida, the Flower Garden Banks in the Gulf of Mexico, and Colombia (Table 3). Indeed, the overall averages of spawning times for the three species from these published studies, once adjusted by time of sunset, are nearly identical to our findings (Table 3).

Two apparent exceptions to this pattern merit comment. The first, from Curaçao, represents the first published study on spawning times for these species (van Veghel 1994); although it does not present data on the three morphotypes individually, it notes that all three spawned at the same time, approximately two to four hours after sunset. This contradicts our (albeit limited) observations from the same reefs, where we observed no *M. annularis* or *M. faveolata* spawning during the period when we predicted *M. franksi* should and did spawn. The second observation comes from Andros Island in the Bahamas in 1991, where Szmant et al. (1997) noted a single event with *M. franksi* and *M. annularis* spawning together three hours past sunset in 1991. However, all later spawning events noted by the authors indicate a one- to two-hour gap in spawning times between those two species, and the authors concluded that *M. franksi* is temporally isolated from *M. annularis* (Szmant et al. 1997). The study in Curaçao involved large teams of amateur divers (M. L. J. van Veghel, pers. comm.), and in the Bahamas the morphotypes can be more difficult to distinguish (N. Knowlton, pers. obs.; see below), suggesting that identification errors may have occurred. It is also possible that in both these initial observations divers simply noted all three species spawning within a two- to four-hour bracket and did not resolve any finer differences. In any case, it remains that for the vast majority of observations across the range of these three species, the pattern of temporal differences between *M. franksi* and the others is extremely robust.

Other studies also largely support our findings on patterns of heterospecific fertilization. The results are completely consistent with an earlier study from Panama that used the number of larvae produced from crosses rather than a percent of eggs fertilized (Knowlton et al. 1997). In the Flower Gardens (Hagman et al. 1998b), heterospecific fertilization was lower than conspecific fertilization using gametes collected in the field. Crosses between *M. annularis* and *M. faveolata*, the two species that spawn simultaneously, had an average fertilization success of less than 6%. The authors noted a reduction in heterospecific fertilization between *M. annularis* and *M. franksi*. This result might be caused in part by sperm age effects, but is also consistent with our findings from the Bahamas that revealed a significant asymmetry in compatibility of this cross in the northeastern region. In southern Florida (Szmant et al. 1997), fertilization assays were conducted with the response being the number of swimming larvae produced. The overall patterns are similar to the present study; crosses between *M. franksi* and *M. annularis* produced more larvae

than crosses between *M. faveolata* and either *M. franksi* or *M. annularis*. In contrast to the present study, they noted that 75% of latter crosses produced at least some larvae. However, they also reported that more than 50% of self crosses produced at least some larvae. In part, the variance they noted in heterospecific crosses involving *M. faveolata* could be caused by egg age effects, but the result of larvae being produced from self crosses, a result we rarely found, suggests the possibility of sperm contamination. Although site differences in compatibility cannot be dismissed in this case, two nearby sites—the Bahamas and northern Gulf of Mexico—consistently demonstrated near zero fertilization between *M. faveolata* and *M. annularis*.

Regional Differences in Reproductive Isolation and Introgression

The corals investigated in the laboratory assays of fertilization were also investigated for evidence of genetic and morphological divergence in both Panama and the Bahamas. These data are presented elsewhere (Fukami et al. 2004), but, in summary, there is good evidence for divergence of all three species in Panama and some degree of morphological and molecular convergence in the Bahamas. The variance in the degree of divergence might be caused by historic or recent regional differences in the degree of reproductive isolation. The present data suggest that spawning times and gametic compatibility are species specific and similar (excepting the asymmetry in one cross; see below) across regions. However, what may differ is how these traits interact with regional differences in reef topography and water currents. The wide expanse of the Bahamas, with hundreds of small islands and thousands of patch reefs, may be more likely to have gametes drift from one reef to another during spawning events, increasing the likelihood of hybrid fertilization between *M. franksi* eggs and *M. annularis* sperm. On many Caribbean islands and off the coasts of Central and South America, where reefs are on the edge of deeper water, once eggs leave a particular reef they may have little chance of encountering additional conspecific or heterospecific sperm. As additional data from other locations in the Caribbean become available, it will be interesting to note if patterns of genetic or morphological convergence or divergence can be correlated with how these conserved reproductive traits perform on different reefs.

Relationship between Depth and Time of Spawning

Species living in deeper water experience reduced light levels earlier than shallower species and this may provide an earlier cue for spawning. This phenomenon is reflected in the laboratory corals placed in the dark two hours prior to sunset, spawning earlier than unshaded corals (Knowlton et al. 1997). However, different coral species do not respond to a single light threshold, as *M. franksi* and *M. annularis* living at the same depths spawn at different times, but within a short interval of their respective conspecifics (Fig. 4a).

Within *M. franksi*, however, increased depth is correlated with earlier spawning times. The variance in spawning times explained by depth is small, but this is not too surprising given temporal and spatial differences in water clarity that might influence light levels more than the limited seven meters depth gradient sampled. Regardless of the unexplained variance, mean spawning times are predicted to change approximately 18 min for each 10 m in depth. This difference in spawning times is compensated by the time it takes those gametes to reach the surface (Fig. 4b), at least over the range of depths studied. This suggests that the depth-related intraspecific differences in spawning times act to increase rather than decrease reproductive synchrony.

Selection for enhanced fertilization would favor corals spawning at a time such that they would mix with conspecific gametes at the surface, regardless of depth. There are at least two possible scenarios that would result in the compensation between spawning and surfacing times. The first is that there is a fixed light threshold for spawning and, coincidentally, the functional relationships between light attenuation and gamete buoyancy with depth are reciprocal. The second is that this is an evolved trait involving selection on buoyancy (e.g., by the size or number of buoyant eggs in a bundle), the way the corals respond to light attenuation in their spawning behavior, or both.

The Evolution of Reproductive Isolation

The evolution of reproductive isolation between lineages sets them on largely separate evolutionary pathways and has been one of the most studied phenomena in evolutionary biology (Howard and Berlocher 1998). Prezygotic reproductive isolation can occur in free-spawning marine invertebrates via the mechanisms seen in many other organisms (e.g., spatial and temporal differences in reproductive activity). They lack courtship in the conventional sense, as well as mechanisms associated with internal fertilization (sperm precedence and cryptic female choice), but barriers between eggs and sperm can play comparable important roles in isolating taxa (Palumbi 1994). Interestingly, the three taxa in the *M. annularis* complex exhibit prezygotic reproductive isolation via most of the mechanisms that are potentially available.

It is not clear whether gametic or temporal isolating mechanisms would evolve more rapidly. Reproductive failure caused by sperm limitation (Levitan 1998) could result in stabilizing selection on both gamete recognition proteins as well as spawning synchrony. However, reproductive failure caused by too many sperm (Styan 1998) could result in sexual conflict, chase-away selection on reproductive traits (Holland and Rice 1998), and the evolution of reproductive isolation (Parker and Partridge 1998; Gavrilets 2002; Martin and Hoskin 2003). While sexual conflict has been implicated as the mechanism driving the positive selection on gamete recognition proteins in many broadcast-spawning invertebrates (Palumbi 1999; Swanson and Vacquier 2002), there is no apriori reason why sexual conflict would not also result in diversifying selection on spawning times.

Two of our results suggest that stabilizing rather than diversifying selection may be more important to this coral system. The first is that corals spawning a little too early or too late compared to their conspecifics have reduced fertilization success compared to corals that spawn at intermediate times (Fig. 10). This suggests that stabilizing selection within populations for enhanced reproductive success at peak spawning

times may outweigh sexual conflict caused by too many sperm. Second, the general consistency in patterns of reproductive isolation across the Caribbean suggests little diversifying selection or even genetic drift among populations (although there are hints of intraspecific variation in the levels of compatibility [asymmetry of *M. franksi* and *M. annularis* only in the Bahamas] and mean spawning times [earlier in the Honduras; Table 3]).

Phylogenetic inferences drawn from both genetic and morphological data suggest that *M. annularis* and *M. franksi* are more closely related to each other than either is to *M. faveolata* (Fukami et al. 2004). Among these taxa, gametic incompatibility appears to be strongly established in crosses involving the more distantly related *M. faveolata*, independent of whether the crossed species spawns at similar times (*M. annularis*) or different times (*M. franksi*). Corals as a group, however, show no clear patterns with respect to degree of divergence and mechanism of reproductive isolation. Although timing differences separate the most closely related taxa in the *M. annularis* complex, the opposite is true for some Pacific species of *Acropora*: the most divergent taxa are separated by timing differences, whereas the more closely related taxa are separated by gametic incompatibilities (Fukami et al. 2003).

We did not examine the possibility of postzygotic barriers to introgression. It is possible that hybrids might have reduced larval or adult survival, be sterile, or produce offspring that spawn at suboptimal times. Because corals have long generation times and are difficult to rear, essentially all research has focused on prezygotic barriers. In other groups, closely related species with overlapping distributions often lack absolute reproductive barriers even when genetic analyses suggest little or no gene flow (e.g., Levitan 2002b), so in this respect these corals are not unique.

Conclusions

How can three sympatric species with external fertilization maintain their integrity when all three spawn on the same evening, often in close proximity? *Montastraea annularis*, *M. faveolata*, and *M. franksi* demonstrate a combination of gametic, temporal, and spatial prezygotic isolating mechanisms that greatly reduce the likelihood of hybridization. None of the isolating mechanisms described provides perfect blocks to hybrid fertilization, but the barriers appear to be substantial, especially considering that fertilization trials were no-choice protocols, which typically overestimate the likelihood of hybridization (Howard 1999).

We cannot, of course, rule out the possibility that hybridization can occur at biologically significant levels. At some places and times, the relative abundance and spatial distribution of species, depth profiles, water clarity, spatial configuration of adjacent reefs, and weather conditions may make cross-fertilization more likely than is suggested by our data. Thus, the rate of speciation or ongoing reticulation may be heterogeneous either locally or regionally. However, in spite of these possibilities, barriers associated with gametic compatibilities and the timing of spawning appear to be surprisingly uniform across a wide geographic area.

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Appendix 1

The Ability of Chemical Lights to Track Coral Eggs

The ability of the buoyant chemical light sticks (length = 15 cm) to mimic egg dispersal was tested during five daytime trials conducted in the San Blas Islands using small floating particles that could be seen from a boat. Chemical lights were released with one cup of perlite (small buoyant pebbles used for potting plants) as egg mimics. The GPS position of the lights and the perlite was monitored for one to two hours in each trial.

The distance from the lights to the center of the perlite egg mimics increased slightly as a linear function of the distance traveled by

the perlite ($y = 0.079x - 3.463$, $R^2 = 0.32$, $P < 0.0001$). Using the regression equation from this relationship, the chemical lights were predicted to be 8 m from the center of the perlite slick at the average distance of 147 m (SE = 19 m) moved by the chemical lights during the coral spawning (see below). This difference is well within the error of the GPS and suggests that the chemical lights provide a reasonable measure of movement of small particles floating on the water's surface at these spatial and temporal scales.

The test of these chemical lights in daytime trials indicated that they remained within the 10-m radius used for the plankton tows of during the coral spawn. During the evenings of the coral spawning, coral eggs were collected in 82% of 161 plankton tows, supporting the hypothesis that these chemical lights generally stay close to the dispersing eggs. It is of course possible that eggs from two or more corals might intermingle on the surface. However, because this is most likely among corals that spawn simultaneously and in close proximity, there should be an inverse relationship between the likelihood of intermingling and the variance it would cause to our estimates of the time a place of spawning associated with a chemical light. Variance caused by large differences in the presumed and actual coral eggs sampled would tend to obscure patterns and make our tests conservative.

Appendix 2

Details of Gamete Age and Dilution Experiments

One milliliter of the stock sperm suspension was mixed into a scintillation vial containing 9 ml of filtered seawater. One milliliter of this dilute sperm was placed into the next vial and repeated, to establish six, 10-fold serially diluted sperm vials. In the first experiment (sperm age), 1 ml from each serial sperm vial was added to two vials containing 8 ml of filtered seawater. Eggs were then added to one set of vials approximately 30 min after spawning (fresh *M. franksi* sperm and eggs). Approximately two hours later, eggs from a newly spawned coral were added to the second set of vials (old *M. franksi* sperm and fresh *M. annularis* eggs).

In the second experiment (egg age), eggs from early and late spawning species were added to the two sets of vials approximately 30 min after each colony spawned, and then serially diluted sperm from a late spawning coral (*M. annularis*) was added to the experimental vials (fresh *M. annularis* sperm with fresh *M. annularis* and old *M. franksi* eggs).

Experimental vials were swirled for three rotations and then left at ambient temperature for three hours. Approximately 100 eggs from each experimental vial were then inspected for evidence of early development (two or more cells).

Results of these experiments were fitted to a fertilization kinetics model that includes the effects of polyspermy (Styan 1998). This was done to generate 95% confidence intervals to test for differences in the fertilization profiles between treatments. Styan's (1998) model predicts the proportion of monospermic zygotes (φ_{mono}) as a function of the initial sperm concentration (S_0, sperm/μl), the initial concentration of virgin eggs (E_0, eggs/μl), the time of sperm-egg exposure (t, sec), the time required for blocks to polyspermy to become effective (t_b, sec), and rate constants describing collisions (β_0, mm^3/sec) and fertilization (β, mm^3/sec) as follows:

$$\varphi_{mono} = 1 - e^{-x} - (1 - e^{-x} - xe^{-x})(1 - e^{-b}), \quad \text{(A1)}$$

with

$$x = \beta S_0/\beta_0 E_0(1 - e^{-\beta_0 E_0 t}) \quad \text{(A2)}$$

and

$$b = \beta S_0/\beta_0 E_0(1 - e^{-\beta_0 E_0 t_b}). \quad \text{(A3)}$$

The empirical data were fitted to this model using the Marquart method of nonlinear regression and 95% confidence intervals were calculated for each treatment (SAS Institute 1996). Rather than simultaneously solve the three unknowns (β, β_0, and t_b), the collision constant was estimated using known values for the cross-sectional area of the egg (0.07 mm^2 from empirical measures) and the sperm velocity (0.2 mm/sec, typical marine invertebrate sperm velocity; Levitan 1995; Vogel et al. 1982). The model fits to these data were not dependent on the estimated value of this collision constant.