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Mounting of erratic histoincompatible responses in hermatypic corals: a multi-year interval comparison

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SUMMARY

Studies on allorecognition in the phylum Cnidaria have disclosed complex arrays of effector mechanisms, specificity and competency to distinguish precisely between self and non-self attributes, and have revealed the existence of allogeneic maturity. Here we studied allo-responses between young *Stylophora pistillata* colonies by following 517 allogeneic interactions between naturally settled kin aggregates and by establishing 417 forced allogeneic and autogeneic assays made of solitarily settled spat that were cut into two similar size subclones, of which one had been challenged allogeneically. Fused assays were exposed to a second allorecognition challenge, made of three allogeneic types. Whereas about half of the kin allogeneic interactions led to tissue fusions and chimera formations, none of the 83 non-sibling pair combinations were histocompatible. In contrast to previous results we recorded rejections between siblings at the age of less than two months. More challenging, we documented cases of fusions between interacting siblings at ages older than one-year-old partners, all differing from a previous study made on the same coral population more than a decade ago. Similar erratic histoincompatible responses were recorded in other pocilloporid species. We suggest that these results reflect reduced genetic heterogeneity caused by chronic anthropogenic impacts on shallow water coral populations where planulae originating from the same mother colony or from different mother colonies that are genetically related share increasing parts of their genomes. Offspring born to related parents may also reveal an increase in genomic homozygosity, and altogether impose erratic alloimmunity.

Key words: allorecognition, corals, histocompatibility, fusion, rejection.

INTRODUCTION

Invertebrates employ non-adaptive, germline-encoded immunity that efficiently identifies allogeneic and xenogeneic attributes through the expression of a multiplicity of cellular and morphological phenomena (Rinkevich, 1999). The literature provides ample evidence for the crucial role of invertebrates' innate immunity in manifesting these highly specified arrays of effector mechanisms (Loker et al., 2004) and the importance of high polymorphism for their efficient maintenance and expression (Rinkevich, 2004; Cadavid et al., 2004). While allorecognition is one of the major characteristics of invertebrate immunity, its qualities and the events expressed morphologically by the effector arms vary fundamentally between different taxa, although all share the hallmark nature of precise discriminatory capability between 'self' and 'non-self', even between closely related conspecifics (Grosberg, 1988; Leddy and Green, 1991; Rinkevich, 1996; Rinkevich, 1999; Schwarz et al., 2007). Historecognition of 'self' *versus* 'non-self', however, may represent two separate avenues for immunity, either by detecting the presence or absence of attributes that define self or by detecting the presence or absence of non-self attributes (Neigel, 1988).

As in other invertebrates, it is above dispute that self/non-self recognition is hallmark to cnidarian immunity (Neigel, 1988; Leddy and Green, 1991; Rinkevich, 1996; Rinkevich, 1999), albeit without being able to distinguish between the two different immunological routes. Literature on cnidarian immunity documents that allorecognition and xenorecognition are naturally expressed phenomena that result in either fusion between contacting allogeneic partners in a wide array of histoincompatible outcomes or culminating in various 'rejection' phenomena (Rinkevich and Loya, 1983; Hidaka, 1985; Chadwick-Furman and Rinkevich, 1994; Rinkevich, 1996; Rinkevich, 1999; Frank et al., 1997; Hidaka et al., 1997; Amar et al., 2008). All cnidarian's immune characteristics implicate innate immunity parameters as no true adaptive components have been identified in these innate systems (Rinkevich, 1999; Loker et al., 2004; Dunn, 2009), although elements suggesting memory and specificity were documented in several cases (Rinkevich, 1996; Rinkevich, 1999). However, as in other invertebrate taxa (Magor et al., 1999), the major obstacle for finding a true evolutionary relationship is that homologous molecules operating in non-identical systems may have different constraints on structural conservation and, therefore, may display distinct patterns of activities.

Working on hard and soft corals' immunity, definitive studies (Hidaka, 1985; Frank et al., 1997; Hidaka et al., 1997; Barki et al., 2002) showed that high proportions of allogeneic interactions between young partners culminated in fusions, an outcome not documented when branches of adult colonies were paired. This is of special interest because allorecognition is thought to reduce costly tissue fusion with individuals other than self (Rinkevich, 1999). Fusion between conspecifics is not restricted to corals and is commonly found even in hydrozoans (Frank and Rinkevich, 1994; Cadavid et al., 2004). Fusion between juveniles of scleractinian corals (the formation of chimeric entities) were first detailed by Hidaka (Hidaka, 1985) in *Pocillopora damicornis* and then in other pocilloporid corals like *Stylophora pistillata* (Frank et al., 1997), *Seriatopora caliendrum* and *Seriatopora hystrix* (Nozawa and Loya, 2005). Histocompatible

outcomes in corals may further reveal variable outcomes with time. For example, several studies documented the existence of temporal reversals in xenogeneic (Chornesky, 1989) and allogeneic encounters (Chadwick-Furman and Rinkevich, 1994; Frank and Rinkevich, 1994). These reversals were not due to other environmental factors; rather, they reveal inherent differences between interacting xenogeneic or allogeneic counterparts. Other studies revealed the existence of delayed allogeneic responses, such as cytotoxicity, overgrowth, reversal and the appearance of secondary responses that differed from primary elicited responses (i.e. Frank and Rinkevich, 1994). Delayed responses, as primary responses, manifested the immune characteristics of selectivity and reproducibility, even when examined through time (Rinkevich, 2004).

One of the most interesting issues in coral historecognition is immunological maturation (Rinkevich, 1996; Rinkevich, 1999); namely, how different is allorecognition in young, newly settled corals from adult colonies (Hidaka, 1985; Frank et al., 1997; Hidaka et al., 1997; Barki et al., 2002). However, the biological mechanisms that perpetuate these interactions have yet to be completely identified and characterised. Therefore, in the present study we further aimed to elucidate allorecognition elements characteristic to young, immunologically pre-mature genotypes of *S. pistillata*, a common Red Sea branching form. This was performed by following allo-responses developed in naturally settled kin aggregates and in forced allogeneic and autogeneic assays. Results revealed mixed fusion and rejection reactions between siblings while interactions between non-siblings have always resulted in rejections, suggesting that genetic relatedness affects allogeneic outcomes. These results differed from the outcomes of a previous study made more than a decade ago on the same coral species and on a population residing at the same geographical site (Frank et al., 1997).

MATERIALS AND METHODS

Planulae collection and spat rearing

Collections and rearing of planula larvae were performed as per Amar et al. (Amar et al., 2007). Briefly, *S. pistillata* Esper 1797 larvae were collected *in situ* during 2005 and 2006 reproductive seasons from 10 gravid colonies growing on the fringing reef, adjacent to the Interuniversity Institute for Marine Sciences (Eilat, Red Sea). Each *S. pistillata* colony represents a single genet, as colonial fragments of this species in Eilat do not develop to mature adult colonies (Shaish et al., 2006). Planulae were dispatched to the laboratory at the National Institute of Oceanography (Haifa, Israel), and groups of 50–70 kin planulae were placed in polyester-film-coated 60 mm Petri dishes containing unfiltered seawater. Under these conditions, 67% of the planulae settled in aggregations (<1 mm from each other) of at least two individuals per aggregate, while the rest (33%) settled solitarily (Amar et al., 2007; Amar et al., 2008). Upon settlement, each individual or aggregated entity was cut with its surrounding polyester surface, glued onto a 5.0 cm × 7.5 cm glass slide and transferred to a flow-through aquaria system [Mediterranean seawater, temperature controlled, 23–25°C (Amar et al., 2007)].

Allorecognition interactions in naturally settling aggregates

Allogeneic interactions were monitored for up to one year on aggregated entities, which consisted of chimeras and rejecting partners (Amar et al., 2008). Altogether, we monitored 364 randomly selected genotypes from the 2005 cohort (107 aggregates of 2 partners, 50 aggregates of 3 partners) and 153 genotypes from the 2006 reproductive season (66 aggregates of 2 partners, 7 aggregates of 3 partners) originating from 10 and 5 maternal colonies, respectively. Then, we calculated the distributions of fusions and rejections between interacting partners in each entity of siblings. Because allogeneic interactions remained stable four months after initiation (Amar et al., 2008), we referred to the position of each one-year-old allogeneic entity as representing a well established and mature alloimmune status.

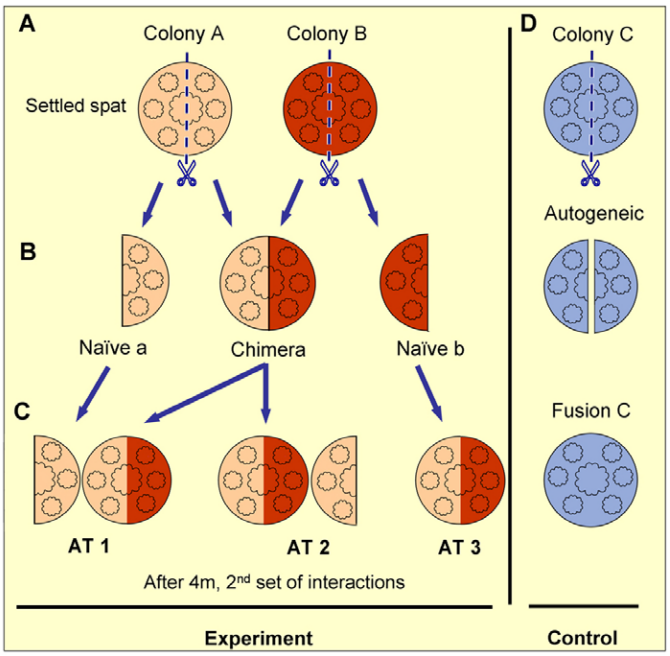

Fig. 1. Experimental set-up for establishing forced allorecognition interactions. (A) Two naïve, young, settled spat 'A' and 'B'. (B) Forced allorecognition interactions. In 2005 experiments, this act was performed on very young spat, up to five weeks post-settlement, and in 2006 experiments, on 6–8-month-old colonies. The assayed entities were divided into two subclones each. One-half from each genotype was used for the allogeneic assays and the second half was used as naïve counterpart. (C) After four months, chimeras were challenged by the different Assay Types (AT 1, AT 2) or those corresponding naïve subclones of the same allogeneic partners were used for a second set assay of the same combination (AT 3). (D) Autogeneic assays.

Forced allorecognition interactions

Solitarily settled spat were cut into two similar size subclones using fine scissors (Fig. 1A). Subclones, one from each genotype, were used for allorecognition assays by gluing them in forced pairs on a glass slide covered with a polyester surface, at a distance of less than 1 mm from each other (Fig. 1B). The remaining allogeneic naïve subclones were glued to glass slides. As controls, we employed autogeneic assays of pairs of the same genotypes (Fig. 1D). All glass slides were kept in conditions identical to those described in the spat-rearing section. We established 417 forced pairs (342 in 2005 and 75 in 2006, total of 834 genotypes) that included 297 allogeneic pairs (siblings and non-siblings) and 120 isogeneic control pairs (Table 1). In the 2005 assays, the young spat used for the initial step (Fig. 1A) were less than five weeks old (sizes of 6–8 polyps) whereas the 2006 assays were performed on 6–8-month-old genotypes (sizes of 20–30 polyps). For assays of both cohorts, the animals were maintained under constant conditions (Amar et al., 2007) for at least four months, and then only fused allogeneic pairs (chimeras) were used for the next step of the study that involved a second allogeneic challenge (Fig. 1C). In that step, naïve subclones were carefully

Table 1. Forced allorecognition interactions

	Siblings			Non-siblings			Control		
	No. of pairs	No. of pairs survived* (%)	No. of fusions (%†)	No. of pairs	No. of pairs survived* (%)	No. of fusions (%†)	No. of pairs	No. of pairs survived* (%)	No. of fusions (%†)
2005	87	56 (64)	23 (41)	155	63 (41)	0 (0)	100	42 (42)	42 (100)
2006	34	32 (94)	15 (47)	21	20 (95)	0 (0)	20	20 (100)	20 (100)

*Both partners in each pair.
†Percentages are out of survived pairs.

detached from their substrates and glued near the corresponding fused subclones in either combination of the following Assay Types (AT): (i) AT 1: juxtaposing the side of the chimera originally representing the same genotype; (ii) AT 2: juxtaposing the side of the chimera originally representing the other genotype; and (iii) AT 3: second set of the same forced-paired genotypes combination (Fig. 1C). Subclones that reached minimal size of 26 polyps (N=10) were divided into two subclones and these subclones were used to challenge the chimera with more than one of the combinations described above. After this step, colonies were observed and monitored for an additional six months.

Colonies were observed weekly under a Nikon SMZ800 stereomicroscope (Chiyoda-ku, Tokyo, Japan). Photographs (once every two weeks during the first two months, thereafter once per month) were taken with a Color View 2 Soft Imagin System camera (Münster, Germany) equipped with a millimetre grid as a scale bar.

Statistical analyses

The non-parametric Mann–Whitney U-test was used to compare fusion frequencies between the 2005 and 2006 collections, using SPSS software version 10 (SPSS Inc., Chicago, IL, USA) for Windows. Manual Chi-squared tests were performed to compare the survival rates and fusion frequencies of genotypes participating in all forced allogeneic assays. The results are presented as means ± s.d.

RESULTS

Allorecognition interactions in naturally settling aggregates

In total 517 conspecific interactions were followed (364 and 153 in the 2005 and 2006 collections, respectively). Fusion frequencies for kin aggregates originating from the same maternal colonies ranged from 37.5% to 76.8% in the 2005 collections and 46.3% to 70.2% in the 2006 collections (Fig. 2). No significant difference was documented for fusion rates between the 2005 and 2006 collections (51.1±11.5% and 59.9±10.3%, respectively, Mann–Whitney U-test, Z=–1.59, P=0.129). The same implies to comparisons performed on each of the five colonies monitored in both years for the total fusion percentages of those siblings (59.9±10.9% and 48.1±7.6%, respectively, Mann–Whitney U-test, Z=–1.76, P=0.076; Fig. 2).

Forced allorecognition interactions

Survival rates, as determined at four months after experiment initiation, differed between the 2005 and the 2006 assays (Table 1, 48.7±13.3 and 96.3±3.2, respectively; χ^2=59.71, d.f.=1, P<0.001). This difference stemmed from stress imposed on young, small-sized 2005 spat, during the procedure of subcloning into two halves (performed by a less-trained worker; 80% of the mortality occurred within the first month following this procedure). Fusions started 7.2±4.0 days from the onset of assays, characterised by the formation of a continuous layer of tissue across the contact area, followed by skeletal deposition. Approximately one month later, the original borderline demarcating the genotypes in each chimera gradually diminished and zooxanthellae were evenly distributed in the fused zones. Because, upon fusion, both partners merged morphologically, it was difficult to follow structurally the fate of each individual. Furthermore, new polyps developed in the chimeric entities in all parts, including along the fusion area, blurring the morphological distinctions between fused partners.

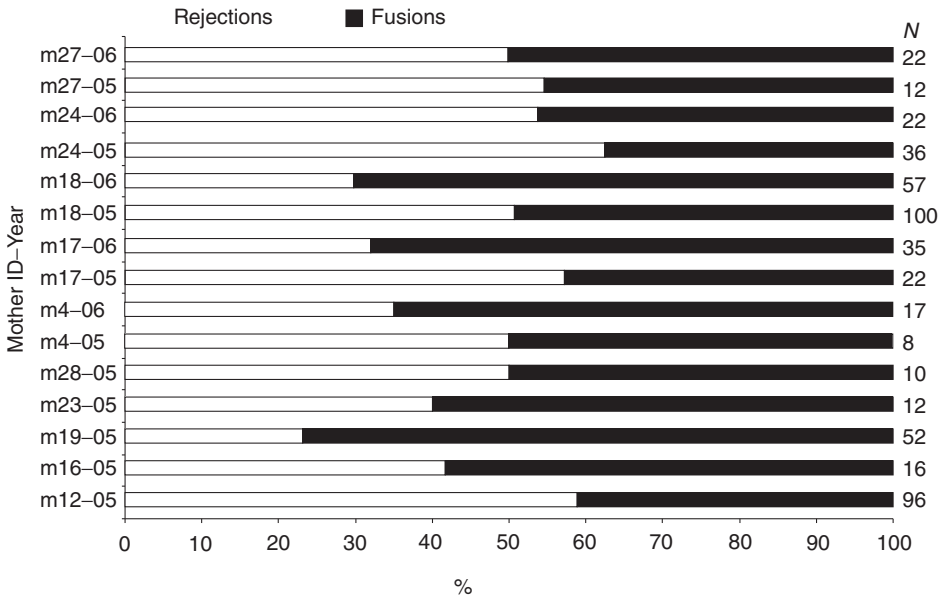

Fig. 2. Percentages of fusions and rejections between siblings in the 2005 and 2006 reproductive seasons. m = mother colony; N = number of interactions between offspring from a specific mother colony; 05, 06 = 2005 and 2006 cohorts, respectively.

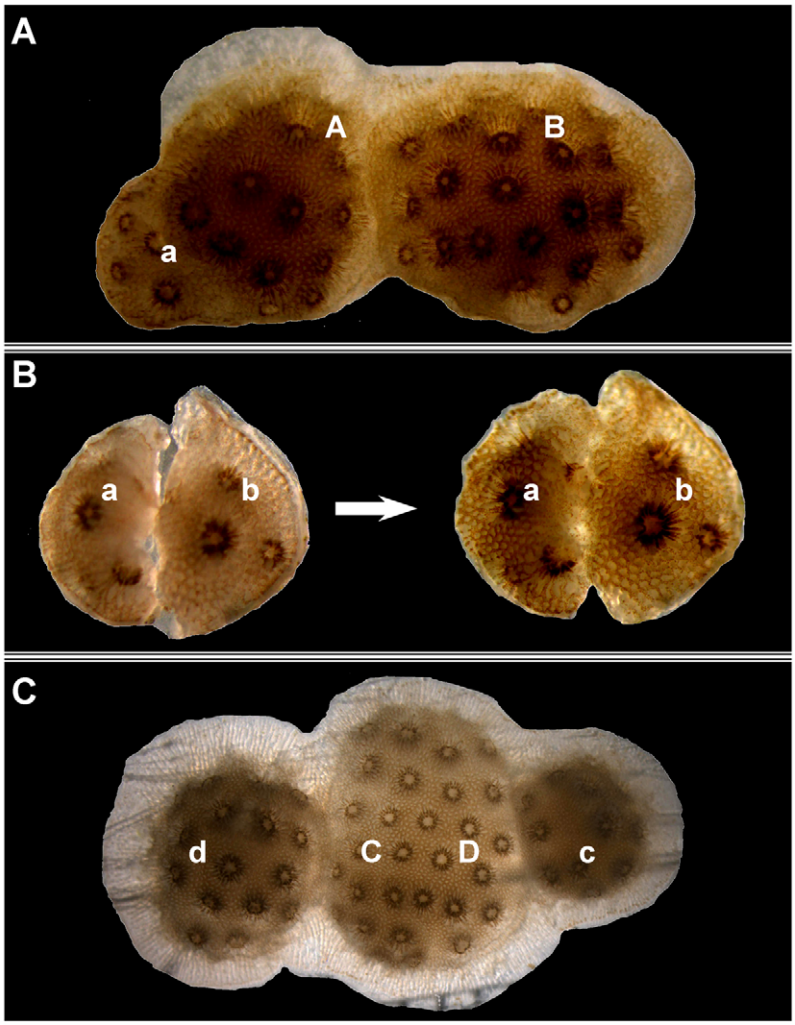

Fig. 3. Second allogeneic challenge imposed on forced-chimera. (A) Assay Type (AT) 1, challenged with allogeneic-naïve subclone of genet 'a' juxtaposed to the former subclone of the same genet 'A' that had fused four months earlier with a subclone of genet 'B' (B). AT 3, challenge with allogeneic-naïve subclone of genets 'a' and 'b', second set of the same forced-paired genotypes combination (C). A chimera formed by genotypes 'C' and 'D', double challenged by AT 2. An allogeneic-naïve subclone of genet 'd' is juxtaposed to the chimeric side where the former subclone of genet 'C' was resided and allogeneic-naïve subclone of genet 'c' is juxtaposed to the side of former genet 'D'.

Results revealed that fusion frequencies did not differ significantly between the assays of both years (χ^2=0.28, d.f.=1, P>0.05). Fusions were observed only between siblings compared with zero fusion in the non-sibling set-ups (Table 1). This outcome was further supported by observations on naturally settling aggregates of *S. pistillata* planulae collected from different mother colonies (non-sibling) in Eilat, which showed solely allogeneic rejections (B. Linden, unpublished).

In the following experimental step (four months later), we re-challenged the chimeras with naïve allogeneic partners, as described (Fig. 1C and Fig. 3). Throughout this period, six chimeras were treated as described in Fig. 1C, AT 1 and seven chimeras were subjected to AT 2 (Fig. 1C and Fig. 3A). In addition, 10 of the naïve subclones reached the minimal size of 26 polyps, which allowed us to generate dual-reciprocal assays of the same forced-paired genotypes combination; AT 1 and AT 2 combined with AT 3 (Fig. 1C) or a double re-challenge from both sides of the chimera were generated (Fig. 3C). Surprisingly, all assays performed (30 AT 1–3 and an additional three cases as described in Fig. 3C) resulted in fusions. Neither cytotoxic responses nor pseudofusions, characteristic of allogeneic interactions of adult *S. pistillata* colonies (Rinkevich and Loya, 1985; Chadwick-Furman and Rinkevich, 1994; Frank et al., 1997) or transitory fusions (Frank et al., 1997), were recorded during the additional six months of follow-up observations.

DISCUSSION

Cnidarians distinguish precisely between self and non-self attributes and react specifically to an array of allogeneic challenges (Grosberg, 1988; Leddy and Green, 1991; Rinkevich et al., 1994; Cadavid et al., 2004; Schwarz et al., 2007; Mydlarz et al., 2008; Palmer et al., 2008; Dunn, 2009). This is performed by allorecognition systems that demonstrate all of the variety of features found in the complex structure of the vertebrate immune systems (Rinkevich, 2004). *In situ* studies and laboratory experimental settings further revealed that cnidarian allorecognition systems are also highly polymorphic at the morphological level (Buss, 1981; Rinkevich, 1996), all supporting the consideration that this innate immunity may offer key insights into the complexities of higher metazoans' tissue transplantation systems (reviewed in Grosberg, 1988; Rinkevich, 1996; Dunn, 2009). Literature results attest for highly conserved components of immunological attributes in cnidarians that are more closely related to vertebrate homologues than other invertebrate model systems, indicating that these basal invertebrates are far from 'simple' in the array of immunological effector mechanisms they possess (Dunn, 2009). This conclusion for complex immunity in the cnidarians is further illuminated by documenting cell-based immune defences (expressed by granular acidophilic amoebocytes) in gorgonian corals [literature in Mydlarz et al. (Mydlarz et al., 2008)] and the presence of the phenoloxidase activating melanin pathway in two species of hard coral (*Acropora* and *Porites*), both

developing local pigmentation in response to interactions with a variety of organisms (Palmer et al., 2008).

While the invertebrate immune system is based on self/non-self recognition manifested by cellular and humoral processes, cnidarians lack any operational vasculature system. Previous studies on *S. pistillata* allorecognition reactions disclosed a complex array of effector mechanisms (Rinkevich and Loya, 1983; Rinkevich and Loya, 1985; Resing and Ayre, 1985; Chadwick-Furman and Rinkevich, 1994; Amar et al., 2008). These included fusions, transitory fusions, cytotoxic and necrotic rejections, the formation of borderlines, bleaching, overgrowths and death (Chadwick-Furman and Rinkevich, 1994; Rinkevich, 2004; Amar et al., 2008). Morphologically comparable interactions were recorded in other scleractinian coral species such as *Acropora hemprichi* (Rinkevich et al., 1994) and *Pocillopora damicornis* (Hidaka, 1985).

Studying the details of coral histocompatibility, Frank et al. suggested that the allorecognition system in young *S. pistillata* specimens matures through three time-dependent distinctive stages, all developed within four months post-metamorphosis (Frank et al., 1997). While tissue fusions are built-up between interacting spats younger than four months old, the formation of long-lasting stable chimeras were documented only in chimeras developed by the fusion of partners younger than two months old. The chimeras observed in Frank et al. (Frank et al., 1997) were created in both pairs of siblings and pairs of non-related offspring. Studying corals originating from the same coral reef site as in Frank et al. (Frank et al., 1997), the results of the present study provide a different portrait to *S. pistillata* allorecognition. Whereas about half of the kin allogeneic interactions led to tissue fusions, i.e. chimera formations, none of the 83 non-sibling pair combinations were histocompatible, and rejections between young siblings at the age of less than two months were documented, in contrast to previous results (Frank et al., 1997). Further surprising are the results of the present study documenting fusions between siblings at older ages than four months [in contrast to Frank et al. (Frank et al., 1997)], even between more than one-year-old partners.

A similar phenomenon of erratic histoincompatible responses was described for *P. damicornis* interactions. While Hidaka (Hidaka, 1985) observed fusions between all combinations of young spat (sibling, non-siblings and different colour morphs), in a subsequent study (Hidaka et al., 1997) they documented a novel rejection type between young sibling colonies, a result that has not been previously recorded (Hidaka, 1985). This rejection was termed as 'incompatible fusion', marked by a seemingly regular fusion between partners that develops into rejection and/or separation between individuals. A hypothesis was put forward (Hidaka et al., 1997) that ontogenetic changes in histoincompatibility system of *P. damicornis* were variable, occurring at earlier or later stages of development. A comparable phenomenon was described in *S. pistillata* and was termed transitory fusion (Amar et al., 2008). In contrast to the *Stylophora* characteristic outcomes of this study [as in Frank et al. (Frank et al., 1997)], alloreactions between pairs of siblings or non-sibling partners of *P. damicornis*, *Seriatopora caliendrum* and *Seriatopora hystrix* (Hidaka et al., 1997; Nozawa and Loya, 2005) were not affected by the partners' age.

Results on both *P. damicornis* (Hidaka, 1985; Hidaka et al., 1997) and *S. pistillata* (Frank et al., 1997) (this study) revealed changes in allo-responses between two consecutive sets of experiments, performed at an interval of more than a decade, by the same laboratories, on the exact coral site populations. We suggest that such scenarios can develop in cases of continuous partial inbreeding where planulae originating from the same mother colony or from different mother colonies that are genetically related share increasing parts of their genomes. Such partial inbreeding, possibly caused by the reduction in *S. pistillata*'s effective population size (K.-O.A., unpublished), is due to almost four decades of chronic anthropogenic impacts on the shallow water coral populations at Eilat (Rinkevich, 2005). This may result in reduced genetic heterogeneity (Underwood et al., 2007), directly reflecting impacts on the genetic features of local coral populations, evolving with different genotypic types, in tandem with mating systems. We hypothesise that offspring born to related parents not only share increasing parts of their genomes but will also show increased genomic homozygosity (*sensu* Charlesworth and Charlesworth, 1999), followed by changes in alloimmunity [as predicted for other colonial marine organisms (Rinkevich, 1993)]. By using defined genetic lines of the hydroid *H. symbiolongicarpus*, Cadavid et al. (Cadavid et al., 2004) have shown that allorecognition in this hydroid resides in a single chromosomal region that contains at least two loci, further illuminating the important role of heterozygosity in self/non-self expression. It is also well documented that invertebrates compensate the absence of adaptive immunity characteristics by using highly variable elements of innate immunity (i.e. FREPs, fibrinogen-related poteins; DsCAM, Down syndrome cell adhesion molecule; SR-Crs, Scavenger receptor cystein-rich domain) (Litman et al., 2005; Kvell et al., 2007).

The above outcome for the possible impact of reduced genetic heterogeneity is an alarming sign because in vertebrates such reduced heterozygosity is associated with increasing impacts on immune resistance to disease agents (Acevedo-Whitehouse et al., 2003; Whiteman et al., 2006; Reid et al., 2007). Therefore, partial inbreeding not only appears to have the potential to shape life history, behavioural, morphological and physiological traits in living species (reviewed in Keller and Waller, 2002) but may also affect expressed alloreactivity. It is unfortunate that contrary to other fields of immunity where availability of methods for studying genome-wide expression profiles has led to impressive achievements, in corals there is no accepted synthesis of what historecognition is or what it does (Rinkevich, 2004; Schwarz et al., 2007; Mydlarz et al., 2008; Palmer et al., 2008; Dunn, 2009). We (B.R., personal observation) recorded additional erratic histoincompatible responses in *S. pistillata* allorecognition. While allogeneic assays performed on adult colonies during the early 1980s (Rinkevich and Loya, 1983; Rinkevich and Loya, 1985) resulted in high proportions of rejections, two decades later, assays on adults living in the same reef area resulted in a high proportion of pseudofusion outcomes, and less numbers of vigour rejections (B.R., unpublished). Because all allogeneic responses in *S. pistillata* are highly reproducible (Rinkevich, 2004), changes of responses may not be considered as a causative outcome of immunological unrelated biological or physical parameters. The above results may also suggest that histocompatibility in *S. pistillata* is a multilocus related phenomenon (similar to allogeneic resorption phenomenon in colonial botryllid ascidians (Rinkevich, 1993)], where the number of shared alleles could reflect the expressed severity of rejection or fusion percentages between non-related partners.

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