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Ontogenetic shifts in fusion–rejection thresholds in a colonial marine hydrozoan, *Hydractinia symbiolongicarpus*

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Abstract Like many modular organisms, genetically distinct colonies of the hydrozoan *Hydractinia symbiolongicarpus* naturally fuse to produce chimeras. One of the principal cooperative benefits of fusion arises from the increased size of the resulting chimeric individual, which may enhance survivorship. However, fusion also promotes conflict through competition between cell lineages for representation in reproductive tissues. Previous studies on *H. symbiologicarpus* show that, consistent with kin selection theory, a highly polymorphic self/non-self recognition system limits fusion to close kin. However, these recognition systems are intrinsically subject to error. Conspecific acceptance threshold theory predicts that as the costs and benefits of making recognition errors change, or the frequencies of encounters between acceptable and unacceptable kin vary, the recognition system should respond. Specifically, as the benefits of acceptance decline or the frequency of encounters with unacceptable individuals increases, the acceptance threshold should become more restrictive. We tested this hypothesis by monitoring changes in the expression of fusion/rejection behaviors of *H. symbiolongicarpus* during colony establishment, a period of high mortality when the size-dependent benefits of fusion may be changing most rapidly, and the frequency of encounters with close kin declines. Across seven full-sib families, fusion frequencies between pairs of sibling colonies declined from 73% for 3-day-old colonies to 58% by day 12. This decline is consistent with optimal acceptance threshold theory. However, the period of maximum decline also corresponds to an interval during which the recognition effector mechanism becomes fully functional, suggesting that the shift to a more restrictive conspecific acceptance threshold may reflect an intrinsic constraint on recognition system maturation.

Keywords Chimera · Conspecific acceptance threshold · Kin recognition · Self/non-self recognition · Germline parasitism

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Introduction

In many social insects, colonies derived from different foundresses coalesce transitorily or permanently (Keller 1993; Crozier and Pamilo 1996). Similarly, in many colonial marine invertebrates, as well as fungi, cellular slime molds and some bacteria, genetically distinct conspecifics permanently or temporarily fuse to form a single chimeric individual (Buss 1982; Grosberg 1988; Hughes 1989; Crampton and Hurst 1994; Glass et al. 2000; Strassmann et al. 2000; Velicer et al. 2000).

A chimera represents an intimate arena in which different genotypes may cooperate or compete (Buss 1982; Haig 1997; Grosberg and Strathmann 1998). As with fusion between Argentine ant nests (e.g., Holway et al. 1998), the most obvious potential benefits of chimera formation arise from the immediate increase in size of both members of a chimera (Buss 1982). In a number of organisms, size is positively correlated with an increase in survivorship (Highsmith et al. 1980; Jackson 1985; Hughes and Connell 1987; Hughes 1989; Babcock 1991; Foster et al. 2002), competitive ability (Buss 1980), fecundity (Gross 1981; Jackson 1985; Peterson 1986; Kapela and Lasker 1999; Foster et al. 2002), and a decrease in age at first reproduction (Harvell and Grosberg 1988). In addition, a chimeric soma houses more genetic diversity than a non-chimeric one (Buss 1982): this diversity may provide a selective advantage to the chimera

in a heterogeneous environment. Finally, members of a chimera could synergistically compensate for each other's developmental deficiencies, as in cellular slime molds (Buss 1982; Dao et al. 2000) and social myxobacteria (Velicer et al. 2000).

Just as the formation of multiply queened colonies in social insects often leads to discord among matrilines (Strassmann 1989; Choe and Perlman 1997), the mixing of genetically distinct cell lineages, each of which retains the capacity to differentiate into either gametes or somatic tissue (Berrill and Liu 1948; Nieuwkoop and Sutasurya 1981; Whitham and Slobodchikoff 1981), engenders the potential for conflict between cell lineages over access to the germ line (Buss 1982, 1987; Haig 1997; Matapurkar and Watve 1997). Under these conditions, one member of a chimera could reproductively parasitize the other, by (1) biasing the differentiation of its own multipotent cells toward gametic, rather than somatic tissue (Strassmann et al. 2000; Fortunato et al. 2003), or (2) using the somatic tissues of the other member of the chimera for provisioning its gametes and embryos (Buss 1982; Dao et al. 2000). Such intraspecific reproductive parasitism occurs in the colonial ascidian, *Botryllus schlosseri* (Sabbadin and Zaniolo 1979; Pancer et al. 1995; Stoner and Weissman 1996), the cellular slime mold, *Dictyostelium discoideum* (Buss 1982; Hilson et al. 1994; Strassmann et al. 2000) and the myxobacterium, *Myxococcus xanthus* (Velicer et al. 2000), and is likely far more widespread.

Given these potentially significant costs and benefits of chimera formation, kin selection should favor the evolution of mechanisms that limit fusion to close relatives. Indeed, virtually all organisms capable of intergenotypic fusion possess highly polymorphic allorecognition systems that restrict acceptance of fusion partners to interactions between clonemates and close kin (Grosberg 1988; Buss 1990; Crampton and Hurst 1994; Crespi 2001; but see Strassmann et al. 2000). An error-free kin recognition system would allow an actor to distinguish and accept only those recipients whose relatedness exceeds the critical level specified by Hamilton's Rule (Hamilton 1964a, 1964b). However, because the genetic and environmental cues or labels used by an actor to detect its kinship to a potential recipient usually do not perfectly reflect relatedness, or because the actor's recognition template is too broad to discriminate among some classes of relatives, recognition errors occur (Crozier and Dix 1979; Getz 1981; Lacy and Sherman 1983). In particular, when the distributions of recognition cues that typify different classes of kin overlap, an actor can commit two basic types of recognition error: it can either reject individuals that belong to the appropriate (or desirable) class of kin (type I error), or accept individuals that belong to a more distantly related (undesirable) class than specified by Hamilton's Rule (type II error) (Crozier and Dix 1979; Getz 1981; Reeve 1989).

For simple binary behavioral traits, such as conspecific fusion and rejection, the acceptance threshold (sensu Reeve 1989) is the degree of phenotypic dissimilarity between the actor's recognition template and the potential recipient's labels, below which the actor accepts a conspecific as a recipient of altruistic behavior, and above which it rejects the recipient conspecific (Starks et al. 1998). In some circumstances, the optimal acceptance threshold may simply be the value of template-label dissimilarity that minimizes the incorrect assignment of potential recipients to a particular class of kin (Getz 1981). However, Reeve (1989) showed that the optimal conspecific acceptance threshold may deviate from this value, depending on the fitness costs of committing recognition errors and the frequencies of interactions with different classes of kin. For example, when the costs of accepting an inappropriate recipient are large relative to the benefits of accepting an appropriate recipient, selection generally favors a more restrictive acceptance threshold than the value specified by Hamilton's Rule. The effects of encounter rates with desirable and undesirable individuals on the optimal acceptance threshold are more complex, and depend upon the context in which recognition occurs. In the simplest cases, thresholds become more restrictive as the frequency of interactions with more distantly related, and presumably less desirable, individuals increases (Reeve 1989).

To the extent that the costs and benefits of recognition errors, and encounter frequencies with desirable and undesirable individuals, vary, so too will the optimal acceptance threshold. Indeed, some birds, amphibians, and insects facultatively adjust their acceptance thresholds according to ecological circumstances, social context, and ontogenetic state (reviewed in Gamboa et al. 1991; Blaustein et al. 1993; Keller 1993; Pfennig et al. 1993; Crozier and Pamilo 1996; Sherman et al. 1997; Starks et al. 1998). Similarly, several studies on marine invertebrates suggest that fusion–rejection frequencies vary ontogenetically (Duerden 1902; Hidaka 1985; Ilan and Loya 1990; Shenk and Buss 1991; Shapiro 1996; Frank et al. 1997).

From ecological, morphological, and developmental perspectives, intergenotypic fusion and aggression between colonies of hydroids in the genus *Hydractinia* are unusually well studied (reviewed in Ivker 1972; Buss et al. 1984; Müller et al. 1987; Buss and Grosberg 1990; Shenk and Buss 1991; Grosberg et al. 1996; Mokady and Buss 1996; Frank et al. 2001). The sexually produced, crawling planula larvae of *H. symbiolongicarpus* Buss and Yund 1989, like those of many other members of the Family Hydractiniidae, settle on gastropod shells occupied by hermit crabs, in this case *Pagurus longicarpus* (Yund et al. 1987; Buss and Yund 1989). Once attached, the larvae metamorphose into small (<1 mm), sessile feeding polyps. Through repeated and sustained asexual budding of the founder polyps and their descendants, colonies consisting of up to several thousand polyps, linked by a ramifying and anastomosing gastrovascular system, may completely envelop a host gastropod shell. This growth habit highlights one of the primary roles of colony fusion and self/non-self recognition, namely to promote the physical and genetic integrity of an individual as it grows around a three-dimensional form (a gastropod shell), or recovers from injury, and re-encounters

self tissues (Feldgarden and Yund 1992). Indeed, contact between self tissues always leads to permanent tissue fusion.

In many cases, however, multiple *Hydractinia* larvae often colonize a single, hermit crab-occupied shell, leading to encounters between non-self tissues (Yund et al. 1987; Yund and Parker 1989; Hart and Grosberg 1999). When the resulting colonies grow into contact, one of three outcomes ensues. Virtually all (>95%) interactions between half-sibs and more distant relatives (Yund et al. 1987; Grosberg et al. 1996; Mokady and Buss 1996) lead to aggressive rejection, accompanied by the production by one or both colonies of specialized, nematocyst-laden fighting structures, termed hyperplastic stolons (Schijfsma 1939; Ivker 1972; Buss et al. 1984). Aggressive rejection generally results in the competitive exclusion of all but one colony, although aggressive interactions occasionally moderate or cease and colonies co-exist on the same shell (Yund et al. 1987; Buss and Yund 1989; Yund and Parker 1989; Buss and Grosberg 1990). Alternatively, contact between full-sib colonies can lead to permanent intergenotypic fusion and the formation of a genetically chimeric individual (Teissier 1929; Crowell 1950; Hauenschild 1954, 1956; Ivker 1972). Finally, as in the colonial ascidian *Botryllus schlosseri* (Rinkevich and Weissman 1989), initially fused colonies may subsequently reject each other. In *Hydractinia*, such "transitory fusion" sometimes involves the production of hyperplastic stolons by one or both of the rejecting partners (Teissier 1929; Schijfsma 1939; Hauenschild 1954; Shenk and Buss 1991).

Fused *Hydractinia* permanently or temporarily share a gastrovascular system that transports multipotent interstitial-cells (I-cells) between members of the chimera (Nieuwkoop and Sutasurya 1981). These multipotent cells can differentiate into a range of gametic and somatic tissues and, as in the ascidian *Botryllus schlosseri* (Sabbadin and Zaniolo 1979), multipotent cell lineages may persist for long periods, perhaps even after transitory fusion has led to the physical dissolution of the chimera (Müller 1964, 1967).

Previous studies in *Hydractinia* of ontogenetic shifts in allorecognition specificity have focused on either changes prior to the completion of embryogenesis (Lange et al. 1992; Fuchs et al. 2002) or immediately preceding sexual maturity (Shenk and Buss 1991), and have not examined the ecologically decisive period directly following metamorphosis when newly founded colonies appear to be especially vulnerable to mortality (Yund et al. 1987). Moreover, no prior studies have explicitly considered the impact of variation among families. Such variation may be especially important in species such as *H. symbiologicarpus* that exhibit substantial within- and among-family variation in colony ontogeny, morphology, and spatial competitive ability (Buss and Grosberg 1990). The present study examines ontogenetic shifts in conspecific fusion frequencies in multiple families of *H. symbiolongicarpus* during the critical phase of colony establishment.

Methods

Collection, husbandry and mating procedures

In September 1999 and May 2002 we collected several hundred hermit crabs (*Pagurus longicarpus*), with shells encrusted with single *Hydractinia symbiolongicarpus* colonies, from the shallow mudflats of Barnstable Harbor, Mass., USA. We transported these colonies on their hermit crab hosts back to our laboratory in Davis, Calif., USA, where we sorted reproductively mature male from female colonies and maintained them in separate seawater aquaria at 16–17ºC (Bunting 1894; Ballard 1942). We isolated male from female colonies for at least 1 week prior to experimental matings, to ensure both that no foreign sperm survived among the females and that female colonies had spawned all eggs fertilized prior to collection (Yund 1990; Levitan and Grosberg 1993).

In 1999, we initiated matings between two pairs (families 1099D and 1099E), and in 2002 between five pairs (families 0602C, E, F, H, and L), of male and female *H. symbiolongicarpus* colonies (Table 1). Because there are no sufficiently polymorphic co-dominant genetic markers available in *H. symbiolongicarpus* to determine with absolute precision that wild-collected colonies are not chimeric, we selected parental colonies to minimize the possibility of initiating families with chimeric parents. We used only colonies that fully covered shells, were without signs of an intercolony border and that contained only male or female reproductive polyps. These precautions aside, fusion in nature is rare (Hart and Grosberg 1999). Furthermore, sibships initated from a chimeric parent(s) would simply result in a family composed of full and half-sibs, which would result in lower initial fusion frequencies in that family but is unlikely to affect an ontogenetic shift in fusion behavior. Therefore, for each mating, we chose pairs of shells fully

Table 1 Mating design and sampling protocols for ontogeny of allorecognition experiments in *Hydractinia symbiolongicarpus*. *Family* Family identifier, *Mother/Father* parental identifiers, *Distance* distance between pairs of siblings at settlement, *n* number of pairs of larvae in each treatment, *Days observed* days post-settlement when fusion/rejection behaviors were scored, *Year* year in which family was bred

Family	Mother	Father	Distance (mm)	*n*	Days observed	Year
1099D	F99D	M99D	1	112	3, 5, 7, 9, 12, 14, 16 and 18	1999
1099E	F99E	M99E	1	144	3, 5, 7, 9, 12, 14, 16 and 18	1999
0602C	F970	M988	1	191	3, 5, 7, 9 and 11	2002
			4	168	5–18	
0602E	F934	M943	1	126	3–10	2002
			4	103	4–18	
0602F	F973	M952	1	142	3–10	2002
			4	107	4–18	
0602H	F927	M967	1	138	3–6	2002
			4	127	8–18	
0602L[a]	F907	M893	1	154	3–8	2002
			4	*	8–14	

[a] Several members of family 0602L became diseased during the latter phases of the observational period. Consequently, we excluded data for larvae settled 4 mm apart

covered with a single ripe *H. symbiolongicarpus* colony, one male and the other female. We placed this isolated male–female pair in approximately 1,800 ml of aerated, 0.22-μ filtered seawater at 18–19°C. During the induction and mating period, we changed the seawater in the aquaria daily, and starved both the hermit crab hosts and the colonies themselves. We induced spawning by holding the mated pairs in complete darkness for 48 h and then exposing them to bright light for 3–4 h (Ballard 1942). Four to 6 h following light exposure, we transferred fertilized eggs into a 150-mm plastic petri dish, filled with 0.22-μ filtered seawater. We allowed the larvae to develop in these dishes at room temperature, transferring the larvae daily into clean 150-mm petri dishes containing previously unused 0.22-μ filtered seawater. Forty-eight h after fertilization, we pipetted the fully developed planula larvae into 100-mm plastic petri dishes containing a 1:1 mixture of 58 mM CsCl and 0.22-μ filtered seawater (Müller 1973). This treatment with an iso-osmotic solution of a monovalent cation in seawater apparently mimics the effects of naturally occurring bacterial inducers by closing potassium channels, depolarizing cells in the larva, and initiating a signal transduction pathway leading to metamorphosis (reviewed in Frank et al. 2001). The larvae remained in this solution for 2–4 h, when they began to metamorphose by discharging adhesive nematocysts and contracting their tails, resembling miniature, pink Hershey's chocolate kisses. At this point, for families 1099D and 1099E, we individually transferred larvae, using a modified Pasteur pipette, to 150-mm-diameter petri dishes, embossed with a 20×20-mm grid on their bases. For each of the two families, we positioned ≈160 pairs of larvae 1 mm apart, with one pair of full-sib larvae per square. In order to spread the timing of contact over an 18- to 20-day period we slightly modified our settlement procedure for the families bred in 2002 (families 0602C, E, F, H, and L), settling some colonies close together and some colonies farther apart. For the 2002 families, we transferred metamorphosing larvae to 84×57-mm glass slides, with a 4×3-grid engraved on each slide (each cell measured 21 mm×19 mm). We aimed to settle 200–250 pairs of larvae from each family at each of two distances: (1) 1 mm apart: 6 pairs of larvae within each cell (3 pairs in one column, 3 pairs in an adjacent column); (2) 4 mm apart: 2 pairs of larvae per cell (1 pair in the top of the cell and 1 pair in the bottom of the cell).

For both the 1999 and 2002 families, we allowed all settled larvae to complete metamorphosis at room temperature. Approximately 48 h after settlement, all competent larvae had completed metamorphosis into primary feeding polyps, at which point we fed them with 2-day-old brine-shrimp (*Artemia franciscana*) nauplii. Following this initial feeding, we transferred the petri dishes containing families 1099D and 1099E, with colonies facing downward, directly into a 530-l recirculating seawater system at 18–19°C. For the remaining families, we placed the slides carrying the experimental pairs of colonies in plastic racks that held the slides vertically, and stored the colonies for an additional 24 h in a shallow tray filled with aerated seawater. We then moved the racks into the same 530-l aquarium. We fed all colonies daily for 2–3 h with a dense suspension of 2-day-old *A. franciscana* nauplii.

Behavioral observations

Following settlement of the sibling larval pairs (day 0), we observed tissue interactions daily under a dissecting microscope using both reflected and transmitted light. We continued these observations for 18 days, by which time most pairs had contacted and we terminated each experiment. In 2002, for the set of larvae that we settled 4 mm apart, we measured the initial distance between primary polyps of each pair of colonies at 12×, using an ocular micrometer calibrated against a stage micrometer. Four days after settlement, we began observing colonies and scoring behavioral interactions, every other day, until colonies made contact. Behavioral outcomes were ascribed to the day first contact was established. We scored the behavioral interactions between all healthy colony pairs as follows:

1. Fusion: When gastrovascular connections formed between two colonies, accompanied by visible exchange of fluid and particles between them, we scored the contact as fusion. This behavior is usually restricted to interactions between self, full-sibs, and parents and progeny (Ivker 1972; Grosberg et al. 1996).
2. Rejection: When interacting colonies did not form gastrovascular connections we scored the contact as rejection. Rejection usually elicits the production of hyperplastic stolons by one or both colonies, and the discharge of a specialized group of nematocysts (stinging organelles), the microbasic mastigophores (Ivker 1972; Buss et al. 1984; Buss and Grosberg 1990; Grosberg et al. 1996).
3. Transitory fusion: Approximately 20–35% of full- and half-sib colony pairs that initially fuse later separate, a behavior we score as transitory fusion (Grosberg et al. 1996). Separation occurs anywhere from days to weeks following initial fusion (Shenk and Buss 1991, Grosberg et al. 1996). Because we terminated our observations 18 days post-settlement, the values we report for transitory fusion underestimate the true frequencies. For this reason, we pool fusion and transitory fusion frequencies for statistical analyses.
4. Avoidance: We occasionally observed a novel behavior that we term avoidance. In the most obvious cases, avoidance occurred as stolons from one or both colonies in a pair grew directly toward each other, then suddenly changed their direction of growth, away from the stolons of the other colony. In addition, apparently vigorous colonies produced numerous stolons in all directions except toward the other member of a pair. Avoiding colonies never made contact with each other during the period of the experiment and neither colony was apparently diseased.

Data analysis

To test the null hypothesis that frequency of fusion (fusion + transitory fusion) does not change with ontogeny, we analyzed the data using a logistic regression (PROC LOGISTIC; SAS version 8.1). We only considered fusion and rejection response types in this analysis. Contact type (fusion + transitory fusion versus rejection) was the response variable. We treated Day as a continuous explanatory variable and Family as a categorical explanatory variable. We initially analyzed the data over the entire sampling period of 18 days. Because the sample sizes decreased through time, especially over the last week of sampling, we also re-analyzed the patterns of fusion and rejection for data sets truncated at days 12 and 14. Truncated data sets included 85 and 95% of the total data points, respectively. Finally, because previous studies in the closely related *H. echinata* indicate that the allorecognition system of that species functionally matures within the first 3–4 days following metamorphosis (Lange et al. 1992), we re-analyzed the patterns of fusion and rejection over days 4–18 and 5–18. These truncated data sets included 85 and 80% of the total data points, respectively.

The occurrence of avoidance behavior in some interactions suggested that colonies may sense the outcome of an interaction prior to contact, and alter their growth to postpone or avoid contact with the other colony. We therefore tested the hypothesis that time to contact differs between those colonies that fuse versus those colonies that reject, using an analysis of covariance (ANCOVA PROC GLM; SAS version 8.1). We treated Time to contact as the response variable. We included Family and contact Type (fusion, rejection or transitory fusion) as categorical explanatory variables and Initial distance (distance between the primary polyps of each colony) as a continuous explanatory variable. Because the transitory fusion response takes days to weeks to be expressed, we postdated the time of the transitory fusion response to the date of initial contact between the colonies.

Results

In the 1,505 pairs of full-sib that we scored across seven unrelated families, 68% of contacts resulted in fusion (fusion + transitory fusion) and 32% in rejection (Table 2). Over the 18-day period post-settlement that we monitored these colonies, 7% of fusions became transitory fusions (Table 2).

With the exception of family 1099E, fusion (fusion + transitory fusion) frequencies consistently declined through time, although the magnitude of the decline appeared to vary among families (Fig. 1). Logistic regression, based on the entire data set, confirms this pattern over the first several weeks of development ($P<0.01$; Table 3, Fig. 2), irrespective of family identity ($P>0.57$; Table 3). There was also no significant interaction between family and time (Table 3). When we re-analyzed the data set truncated at days 12 and 14, we still found a significant decline in fusion frequency ($P<0.05$ and $P<0.005$, respectively, data not shown here). Thus, the significant effect of time on fusion frequency is not an artifact of a decrease in the actual number of colonies making contact towards the end of the 18-day experimental period. However, when we re-analyzed the data over days 4–18 and 5–18 post-settlement, the effect of day becomes non-significant ($P=0.4189$ and $P=0.4353$ respectively; Table 4).

Table 2 Overall fusion, rejection and transitory fusion frequencies in each family and across all families. Observed fusion frequencies on day 3 and 12 for each family and across all families. Fusion = fusion + transitory fusions

Family	*n*	Fusion	Rejection	Transitory Fusion	Fusion Day 3	Fusion Day 12
1099D	112	0.71	0.29	0.05	0.76	0.57
1099E	144	0.67	0.33	0.04	0.69	0.68
0602C	359	0.70	0.30	0.08	0.61	0.50
0602E	229	0.66	0.34	0.06	0.71	0.52
0602F	249	0.65	0.35	0.10	0.87	0.53
0602H	258	0.73	0.27	0.06	0.76	0.68
0602L[a]	154	0.64	0.36	0.08	0.73	*
All families	1505	0.68	0.32	0.07	0.73	0.58

[a] Several members of family 0602L became diseased during the latter phases of the observational period and thus day 12 outcomes were excluded from the analysis

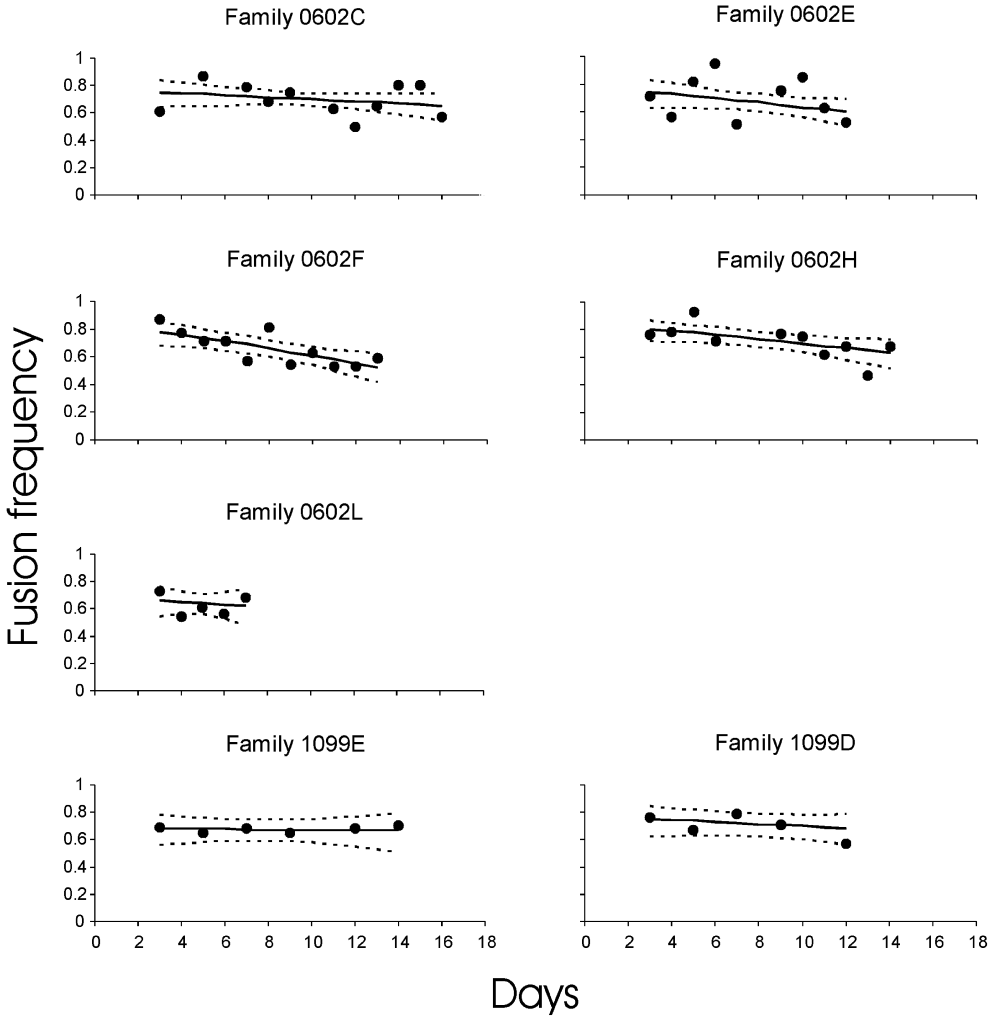

Fig. 1 Fusion (fusion + transitory fusion) frequency versus time to contact (days post-settlement) for each family. The plotted data represent the proportion of all pairwise contacts in a family on a given day that fused. We did not estimate fusion frequencies on days when the numbers of contacts was <10. *Solid lines* show the logistic regression calculated for each family from the total data for that family. *Dotted lines* correspond to the 95% confidence limits of the regression analysis

Table 3 Logistic regression analysis of the effects of ontogeny (day) and family membership (family) on fusion frequencies between pairs of full-sibling juveniles for the entire observational period. Type III analysis of effects

Effect	*df*	Wald X^2	*P* value
Day	1	7.0988	0.0077
Family	6	4.7869	0.5714
Day × family	6	5.0069	0.5429

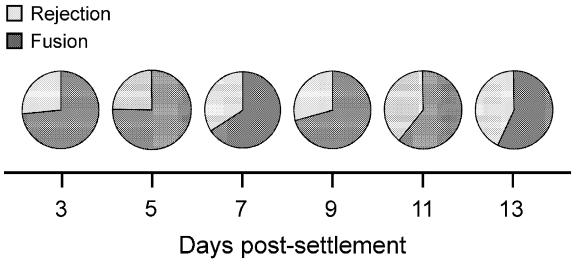

Fig. 2 Summary of rejection (*light gray*) and fusion (*dark gray*) fusion frequencies, pooled across all families, on days 3 (n=226 contacts), 5 (n=144), 7 (n=187), 9 (n=122), 11 (n=104), and 13 (n=74) post-settlement

Table 4 Logistic regression analysis of the effects of ontogeny (day) and family membership (family) on fusion frequencies between pairs of full-sibling juveniles, restricted to days 5–18 post-settlement. Type III analysis of effects

Effect	*df*	Wald X^2	*P* value
Day	1	0.6086	0.4353
Family	6	6.0192	0.4210
Day × family	6	5.2218	0.5157

Table 5 Frequency of expression of avoidance behavior. We monitored this behavior in four of the five families bred in 2002. Avoidance behavior occurs when colonies locally redirect or cease stolon growth as they grow toward each other. See text for details

Family	*n* pairs	*n* Avoidance	Frequency
0602C	168	1	0.006
0602E	103	7	0.068
0602F	107	3	0.028
0602H	127	14	0.110

Avoidance behavior occurred in all four families in which we monitored it. The frequency of avoidance varied among families, ranging from 0.6%–11% (Table 5). Nonetheless, time to contact between pairs of colonies did not differ between those colonies that fused and those that fought (Table 6). Only settlement distance (P<0.0001) and family (P<0.01) significantly affected the time that colonies took to make contact.

Discussion

The major factors influencing the evolution and expression of self/non-self recognition specificity in colonial

Table 6 Analysis of covariance of the effects of interaction outcomes (outcome = acceptance versus rejection), family, and initial distance (*idist*) between colonies on the time to contact between pairs of sibling colonies

Effect	*df*	Type III SS	Mean square	*F*	*P* value
Idist	1	391.424	391.424	112.01	<0.0001
Outcome	2	0.612	0.612	0.35	0.7105
Family	3	10.857	10.857	4.50	0.0074
Idist × outcome	2	1.943	0.971	0.28	0.7575
Idist × family	3	18.628	6.210	1.78	0.1506
Outcome × family	6	9.191	1.532	0.44	0.8532
Idist × outcome × family	6	7.701	1.284	0.37	0.8996

organisms are the costs and benefits of intergenotypic (and self) fusion versus rejection, and the frequencies with which colonies exhibit these behaviors. The primary cost of chimera formation to the individual appears to be the opportunity that fusion provides for somatic and germ cell parasitism (Sabbadin and Zaniolo 1979; Buss 1982; Hilson et al. 1994; Strassmann et al. 2000; Velicer et al. 2000). There are a variety of ways that individuals may limit these costs, the best-documented of which is passive or aggressive rejection of partners following initial tissue contact. Avoidance behavior, such as we have reported for the first time in *Hydractinia symbiolongicarpus*, represents another way that individuals can circumvent fusion, and further eliminate costs associated with direct tissue contact. Our observations of avoidance behavior in a small number of colony pairs confirms and extends previous studies on *Hydractinia* that have suggested that modifications in patterns of stolon growth and allorecognition behaviors may not require direct contact between allogenic individuals (Müller et al. 1987; but see Lange et al. 1989).

Of the potential advantages to fusion (Buss 1982), increased colony size appears to be the most general and the most likely to change ontogenetically. Some of the first accounts of ontogenetic shifts in the expression of acceptance/rejection behaviors date back to early descriptions of interactions between colonies of hydrozoan cnidarians in the genus *Hydractinia* (Teissier 1929; Schijfsma 1939; Hauenschild 1954). More recently, Shenk and Buss (1991) reported a change in the fusion behavior of 25 sibling *H. symbiolongicarpus* colonies to their parents, assayed at two points in development, long after colony establishment. In this study, we extended previous work by quantitatively demonstrating that fusion frequencies between full-sib *H. symbiolongicparpus* significantly decrease during the ecologically critical first few weeks of colony establishment. Approximately 73% of full-sib pairs fused 3 days after settlement and metamorphosis (Table 2). By day 12, fusion frequencies declined to <58%, a frequency consistent with adult fusion frequencies reported by Grosberg et al. (1996).

Several other colonial marine invertebrates apparently undergo a similar ontogenetic decline in the frequency of

fusion between allogeneic (i.e., conspecific non-self) individuals as juveniles age or grow. For example, in the coral *Pocillopora damicornis*, newly settled juveniles often accept each other and fuse, whereas adult allogeneic colonies rarely accept each other, and usually exhibit active rejection behavior (Hidaka 1985). Moreover, adolescent colonies sometimes exhibit a response intermediate between fusion and aggressive rejection (Hidaka et al. 1997). Similarly Ilan and Loya (1990) showed that larvae and juveniles of the sponge *Chalinula* sp. readily fused with allogeneic individuals, whereas grafted adults rejected each other. Allogeneic colonies of the marine bryozoan *Membranipora membranacea* also fuse to form neurally integrated chimeras when they are small; however, as colonies grow larger, this integration terminates (Shapiro 1996). Finally, Frank et al. (1997) claim that in the coral *Stylophora pistillata*, the self/non-self recognition system that regulates fusion/rejection behaviors matures through three distinct stages, with increasing specificity accompanying each transition. It remains unclear, however, whether any of these changes in specificity are statistically significant, because none of these authors performed any analyses on their data.

Conspecific acceptance and rejection behaviors in most colonial marine invertebrates, including *H. symbiolongicarpus*, appear to be controlled by highly polymorphic allorecognition systems of varying genetic complexity (Grosberg 1988). Such genetically based self/non-self recognition systems are inherently prone to error, the magnitude of which depends on the number of loci and number of alleles used to distinguish among different classes of relatives, and the matching rules that relate a potential recipient's cues or labels to an actor's template (Getz 1981; Lacy and Sherman 1983; Reeve 1989). Reeve (1989) predicted that as the costs and benefits of recognition errors change, so, too, should the optimal conspecific acceptance threshold [e.g., spadefoot toads: Pfennig et al. (1993); honey bees: Downs and Ratnieks (2000)]. A shift in acceptance thresholds can also be elicited by changing encounter rates with acceptable and unacceptable classes of kin (e.g., Starks et al. 1998).

Reeve (1989) analyzed a variety of social situations or recognition contexts in which actors interact with recipients and conspecific acceptance thresholds might be expected to shift. His "guard model: frequency-dependent context with pair-wise kin interactions" most closely approximates the nature of conspecific interactions in *Hydractinia*. This model assumes that (1) an actor will encounter two classes of kin; acceptable and unacceptable, (2) an individual will be an actor and a recipient with equal frequency, and (3) individuals do not actively seek each other but merely encounter each other. *Hydractinia* colonies growing on shells contact both close and distant kin (Hart and Grosberg 1999), and since most interactions are pairwise, *Hydractinia* colonies are simultaneously both the actor and recipient in an interaction. Previous studies further show that *H. symbiolongicarpus* larvae at best move short distances following attachment to a host gastropod shell, and that their settlement behavior is unaffected by both density and kinship of conspecific larvae (Yund et al. 1987).

Under the "guard model: frequency-dependent context with pair-wise kin interactions", the evolutionarily stable acceptance threshold should become more restrictive (i.e., a greater portion of acceptable individuals should be rejected) under the following conditions: (1) as the mean number of interactions with more highly related kin decreases and the mean number of interactions with less highly related kin increases, (2) as the relatedness of one or both classes of kin decreases, and (3) as the benefit of altruism decreases and the cost of aid increases. Several lines of evidence suggest that both the frequency of interactions with close kin and the relatedness of interactors are initially high, and then decline during the first few weeks following settlement of *H. symbiolongicarpus* larvae. First, multiple larvae often settle on a shell during a single daily recruitment event (Yund et al. 1987), and recruitment tends to be localized on the undersurface of shells, near the aperture and siphon (Yund et al. 1987; Yund and Parker 1989). Second, genetic evidence indicates that sibling larvae often settle on the same shell (Hart and Grosberg 1999). This pattern likely arises from clutches of benthic eggs quickly sinking as a group following synchronized release by a female and remaining together during larval development (48–72 h) until they attach to a passing hermit crab host's shell. However, because *H. symbiolongicarpus* live on highly mobile substrata, subsequent recruitment events likely consist of larvae unrelated to those already settled on a shell. Finally, the proportion of multiply occupied shells in a population, and therefore, the frequency of interactions with conspecifics, declines through a recruitment season, principally due to competitive exclusion (Yund and Parker 1989; Hart and Grosberg 1999).

Thus, *H. symbiolongicarpus* fulfills at least two of the three conditions for an adaptive shift to a more restrictive conspecific acceptance threshold. In addition, as recruits grow and become less vulnerable to predators and competitors, the benefits of intergenotypic fusion should correspondingly decrease. In this case, the third condition, namely that the benefits of altruistic behavior decrease, would also be fulfilled.

The ontogenetic decline in fusion frequencies exhibited by *H. symbiolongicarpus* very early in development is therefore consistent with an adaptive shift in the conspecific acceptance threshold. However, this pattern may also reflect changes in other facets of the recognition system (reviewed in Gamboa et al. 1986; Sherman et al. 1997), or constraints on the expression of specificity due to maturation of allorecognition systems (Hidaka 1985; Frank et al. 1997). For example, neonatal mice and humans are, compared to their adult conspecifics, relatively immunodeficient (Marshall-Clarke et al. 2000; Morein et al. 2002). A day-by-day analysis of the decline in fusion frequency that we report here suggests that the most important changes in the *H. symbiolongicarpus* recognition system occur during the first 3–4 days following metamorphosis (Tables 3, 4). This period corresponds to a

dramatic change in the cnidom of the closely related *H. echinata*, when microbasic mastigophores, the nematocysts found exclusively in the stolons that discharge during aggressive rejection, become detectable (Lange et al. 1989).

Shenk and Buss (1991) suggest that *H. symbiolongicarpus* also pass through a later ontogenetic shift in self/non-self recognition specificity, corresponding to the onset of sexual maturity, and manifested by increasing rates of rejection and the expression of transitory fusion. They assayed fusion behavior of 25 sibling colonies to their parents at two points in development: (1) 40 days post-metamorphosis when progeny were still reproductively immature and (2) 140 days post-metamorphosis when progeny were reproductively mature. At 40 days, all progeny fused with both parents. However, when progeny were re-assayed at 140 days, only 4 of the 25 progeny fused with both parental colonies, 10 transitorily fused with both parents and 11 fused with one parent and transitorily fused with the other parent.

Buss and Shenk (1990) interpret this decline in fusion frequency and increase in the expression of transitory fusion to be an outcome of decreasing benefits of fusion as colonies grow beyond some critical size and increasing costs of fusion corresponding with the onset of sexual maturity. Specifically, an increase in the expression of rejection or transitory fusion at or near the time of sexual maturity presumably reduces the risks of reproductive parasitism (Buss and Shenk 1990; Ilan and Loya 1990; Shenk and Buss 1991; Hidaka et al. 1997). However, the threat of reproductive parasitism could be greatest early in ontogeny for at least two reasons. First, because clonal organisms do not sequester their germ lines (Buss 1982; Buss 1987), fusion of juveniles prior to the differentiation of gametes from multipotent cell lineages is just as risky as fusion between mature colonies. For example, in the colonial ascidian *Botryllus schlosseri* (and presumably *Hydractinia*), the separated components of formerly fused individuals can retain cell lineages derived from both partners long after disconnection (Sabbadin and Zaniolo 1979; Sabbadin and Astorri 1988; Stoner and Weissman 1996). Second, the earlier that two genotypes fuse, the longer the pre-reproductive interval for a parasite's multipotent cell lineage to multiply at the expense of the other member of the chimera. Consequently, transitory fusion may provide little protection against reproductive parasitism. Indeed, our data show that there is no clear association between the expression of transitory fusion and the onset of sexual maturity (see Buss 1990). In our study, transitory fusion occurred as early as 4 days post-settlement and 1 day post-fusion, weeks before colonies would become sexually mature.

In the context of our current understanding of *Hydractinia* biology, the results of our study indicate that colonies of *H. symbiolongicarpus* undergo an adaptive shift in fusion frequency during the first few weeks following settlement that is consistent with the predictions of conspecific acceptance threshold theory. Specifically, the decreasing size-related benefits of intergenotypic fusion and increasing risks of intraspecific parasitism together increase the costs of making recognition errors as colonies grow. When coupled with a decreasing likelihood of interacting with acceptable classes of kin as colonies cover their host's shells, colonies should, and do, become increasingly restrictive in their acceptance of fusion partners. However, this shift may also reflect functional and developmental constraints on the expression of recognition specificity, rather than a shift in the acceptance threshold per se.

Although our studies did not reveal a statistically detectable effect of family identity, some families appear to exhibit a stronger ontogenetic response than others (Fig. 1). Whether more extensive studies verify this pattern or refute it, remains to be seen. However, if families do actually differ in the degree to which they express an ontogenetic shift in acceptance thresholds, it will be pivotal to determine how, or if, such differences are related to the well-documented differences among families in growth morphology and competitive ability (Buss and Grosberg 1990; Yund 1991).

Further progress toward understanding the adaptive significance of apparent shifts of conspecific acceptance thresholds in this and other systems requires both experimental manipulation of the costs and benefits of recognition errors (e.g., Downs and Ratnieks 2000), as well as a mechanistic analysis of the components of recognition systems, so that the effects of changes in labels, templates, and thresholds on the expression of fusion and rejection behaviors can be distinguished. Manipulations of recognition systems will be especially challenging in organisms such as *Hydractinia* and many other marine invertebrates in which cues and templates appear to be primarily under genetic, rather than environmental, control. The next steps therefore depend on advances in our knowledge of how the components of recognition systems operate at the level of genes, gene regulation, and gene interactions.

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