Internal brooding favours pre-metamorphic chimerism in a non-colonial cnidarian, the sea anemone Urticina felina

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The concept of intraorganismal genetic heterogeneity resulting from allogeneic fusion (i.e. chimerism) has almost exclusively been explored in modular organisms that have the capacity to reproduce asexually, such as colonial ascidians and corals. Apart from medical conditions in mammals, the natural development of chimeras across ontogenetic stages has not been investigated in any unitary organism incapable of asexual propagation. Furthermore, chimerism was mainly studied among gregarious settlers to show that clustering of genetically similar individuals upon settlement promotes the occurrence of multi-chimeras exhibiting greater fitness. The possible occurrence of chimeric embryos and larvae prior to settlement has not received any attention. Here we document for the first time the presence of natural chimeras in brooded embryos and larvae of a unitary cnidarian, the sea anemone Urticina felina. Rates of visible bi- and multi-chimerism of up to 3.13 per cent were measured in the broods of 16 females. Apart from these sectorial chimeras, monitored fusion events also yielded homogeneous chimeric entities (mega-larvae) suggesting that the actual rates of natural chimerism in U. felina are greater than predicted by visual assessment. In support of this assumption, the broods of certain individuals comprised a dominant proportion (to 90%) of inexplicably large embryos and larvae (relative to oocyte size). Findings of fusion and chimerism in a unitary organism add a novel dimension to the framework within which the mechanisms and evolutionary significance of genetic heterogeneity in animal taxa can be explored.

Keywords: chimera; fusion; genetic heterogeneity; allorecognition; marine invertebrate

1. INTRODUCTION

Our understanding of life histories largely relies on the concept of the individual, which is intrinsically associated with genetic homogeneity and uniqueness [1]. However, intraorganismal genetic heterogeneity (IGH) has now been shown to occur naturally in protists, fungi, plants and animals [2], questioning the definition of individuality, with vast implications in ecology and evolution. Until recently, the significance of IGH was essentially dismissed because of its presumed rarity, even though supporting evidence for genetic homogeneity is equally scarce [2]. While not always clearly distinguished in the literature, two main types of IGH can be defined on the basis of functional origin and genetic change. Mosaicism is the outcome of genetic variations within an organism that may be caused by somatic mutations or other processes, whereas chimerism is the result of allogeneic fusion. In this sense, chimerism is primarily restricted to algae and colonial marine organisms with pelagic propagules (sponges, hydroids, corals, bryozoans and ascidians), while mosaicism also commonly occurs in unitary animals as well as in clonal and acclonal plants [2,3]. A broad definition of a chimera states that it is an organism that simultaneously harbours cell populations from two distinct fertilization products (zygotes), whether via coalescence or through an exchange (e.g. blood twins and foetal–maternal chimeras in humans [4]).

As evidence of their natural occurrence accumulates, chimeric entities are becoming a focus of interest in trying to determine the mechanisms and evolutionary significance of genetic heterogeneity. Much like the mythological monsters, chimeras in animal taxa are often perceived as aberrant and generally found to be detrimental in terms of relative fitness costs and benefits for the chimeric state compared with genetically homogeneous conspecifics [2,5]. However, while the negative outcome of natural chimerism has been documented in juvenile colonies of soft corals [6], important rates of chimerism (2–5%) have been measured in wild adult populations of hermatypic corals [7]. Of the several potential benefits that have been suggested to explain the prevalence of chimeric entities (e.g. increased growth, reproductive output or survivorship) very few have so far been demonstrated, except greater fitness of multi-chimeras in colonial ascidians and corals [5,8]. This finding is in line with the assumption that greater genetic variability translates into more versatile physiological qualities enabling chimeras to better cope with environmental changes.

The mechanisms that might promote or prevent chimerism have been examined in the hopes of shedding some light on this puzzling phenomenon. In soft corals and colonial hydroids, a window of opportunity was

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proposed to exist for chimerism during early ontogeny, making it possible only until the complete development of allorecognition [6,9,10]. In cnidarians, chimerism is thus believed to result from a failure to discriminate self from non-self, with few positive outcomes [6,11], except in larger colonies composed of multiple fused entities [8,12]. In ascidians, and possibly bryozoans, frequency of fusion between kin was shown to be positively correlated with relatedness of colonies [13,14]. This led to the theory that spatial clustering of genetically similar individuals upon settlement will promote the occurrence of multi-chimeras, the latter exhibiting greater fitness and resistance [5].

Chimerism in animal taxa has primarily been studied in modular/colonial organisms that have the capacity to reproduce asexually (ascidians, bryozoans, corals), whereas its occurrence and significance in unitary organisms is known only from medical conditions (e.g. blood or foetal chimerism) in humans and other mammals [2]. Fusion of tunic matrices has been documented in closely aggregated adults of solitary ascidians [15,16] and surgical tissue recombination was performed to study histocompatibility in a solitary coral [17]. However, the natural development of chimeras across ontogenetic stages has not been investigated in any unitary organism incapable of asexual propagation. Furthermore, chimerism is typically studied among gregarious settlers [14,18], whereas its possible occurrence in brooded propagules prior to settlement has not received any attention. Brooded propagules represent a unique example of closely related kin that are clustered for a prolonged period of time at an early ontogenetic stage when allorecognition might be weak. So far, studies on brooding species (soft corals) have only looked at fusion among larvae following their release and, to our knowledge, never reported embryonic chimeras.

Here, we document for the first time the presence of natural chimeras in brooded embryos and larvae of a unitary cnidian, the dahlia anemone *Urticina felina*. We also provide data on fusion rates at different ontogenetic stages and discuss the possible significance of various types of chimeric entities in this species.

2. MATERIAL AND METHODS

*Urticina felina* (Actiniaria: Cnidaria) is a gonochoric species that is only known to reproduce sexually [19,20]. Like other sea anemones, it lacks true ovaries, and oocytes grow within gametogenic mesenteries between the retractor muscle and the mesenterial filaments. The embryos and larvae are brooded freely inside the coelenteron; mobile embryos and planulae can also be found inside the tentacles (figure 1a).

Evidence of chimerism in *U. felina* was initially noted after the natural release of planula larvae by laboratory-maintained adults in August 2008, when the presence of several fused settlers was observed. After three months of growth, two fused juveniles were relaxed in a solution of MgCl₂ for a few hours, preserved in 4 per cent formaldehyde and processed using standard histological procedures [21] to assess the extent of the fusion. Sections (6 μm) were stained with haematoxylin and eosin, and examined under a stereomicroscope.

Research was subsequently undertaken to assess the prevalence of this phenomenon. Adults of *U. felina* were collected at a depth of 10 m off the Avalon Peninsula (Newfoundland, Canada) between March and July 2009 and in June 2010. They were maintained in tanks (20–40 l) supplied with unfiltered running seawater, at temperatures that followed the annual ambient cycle (0–10°C), under natural photoperiod and planktic food supply. The proportion of fertile females (with a visible brood) was three out of 22 individuals in 2009 and 13 out of 46 in 2010.

During the natural larval release period (July–September), planulae were emitted by the females in several major planulation events. The larvae were gently collected at the water surface within 24 h of release and examined as outlined below. A few females also released eggs or embryos outside the major planulation period; these were collected as well. More embryos were obtained from seven females either by teasing them, which triggered emission through the mouth, or by clipping a tentacle tip to release the embryos. Sea anemones exposed to these samplings did not modify their behaviour and clipped tentacles regenerated within a few days.

Eggs, embryos and planulae were examined under a Nikon SMZ1500 stereomicroscope attached to a Nikon DXM1200F digital camera. They were photographed to be counted and measured using *Simple PCI* (v. 6.0). The number and size of naturally fused individuals in each brood was recorded. All propagules were subsequently placed in flow-through tanks where they settled and grew into juveniles.

3. RESULTS

Of the 27 169 propagules collected throughout this study, 120 were visibly chimeric. The overall ratio of chimeric propagules (combining embryos and larvae) varied considerably in the 16 females examined (0–3%). Among 1565 embryos collected from seven females, 18 were chimeric, yielding rates of embryonic chimerism of up to 3.13 per cent in a given brood (table 1). Among 25 604 planula larvae collected from 12 females, 102 were chimeric, with rates of larval chimerism in a single brood of up to 1.19 per cent (table 2). Furthermore, 23 out of the 102 chimeric planulae (22.55%) were multi-chimeras, i.e. formed by fusion of more than two entities (table 2). No fusion was ever detected among gregariously settling larvae or adjoining juveniles.

On average chimeras were larger than non-chimeric individuals from the same brood. Some entities were fused only via a narrow thread of tissue, whereas others were well fused and formed peanut-shaped larvae (figure 1b) or complex aggregates (figure 1c). When fusion occurred at the aboral extremity, the chimeric individual grew functional mouths and tentacles at both ends of the body (figure 1d). These bi-headed juveniles developed like unitary juveniles but could not settle as firmly; they tended to attach using the central part of the body (where the basal discs were originally located). One of the monitored bi-chimeras developed only one functional juvenile, with a stump partner devoid of mouth and tentacles. Larval chimeras that were weakly fused were fragile and tended to waste away during the study. Histological sections of viable chimeric embryos, planulae and juveniles showed continuous layers of ectodermal...
and endodermal tissue between the fused partners (figure 1c,f). In chimeric juveniles, the partners shared layers of endoderm, mesoglea and ectoderm, and the gastrovascular cavity (figure 1f). Teasing one partner elicited contraction of the two partners, a phenomenon not seen in adjoining unitary settlers, indicating that viable chimeras shared the same nerve web.

After observing that some visibly chimeric embryos could develop into larger yet fully homogeneous larvae, an analysis of propagule size frequencies was conducted. Histological sections of the reproductive tissues (Sun 2011, unpublished data) showed that the largest oocytes (free-moving in tentacles or along mesenteries) were typically the same size as early embryos to the blastula or early gastrula stage (0.2–0.3 mm²). However, in 11 of the 12 sea anemones examined here, up to 90 per cent of the propagules were larger than expected on the basis of mature oocyte size; with maximum sizes of 0.6–0.8 mm² for embryos and 3.5 mm² for larvae.

### Table 1. Visible chimerism detected at the embryonic stage in seven brooding *U. felina.*

<table>
<thead>
<tr>
<th>brood</th>
<th>number of bi-chimeric embryos</th>
<th>total number of embryos</th>
<th>rate of chimerism in embryos (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2009</td>
<td>2</td>
<td>120</td>
<td>1.67</td>
</tr>
<tr>
<td>1-2010</td>
<td>1</td>
<td>32</td>
<td>3.13</td>
</tr>
<tr>
<td>2-2010</td>
<td>2</td>
<td>71</td>
<td>2.82</td>
</tr>
<tr>
<td>3-2010</td>
<td>3</td>
<td>173</td>
<td>1.74</td>
</tr>
<tr>
<td>4-2010</td>
<td>0</td>
<td>490</td>
<td>0</td>
</tr>
<tr>
<td>5-2010</td>
<td>10</td>
<td>525</td>
<td>1.90</td>
</tr>
<tr>
<td>6-2010</td>
<td>0</td>
<td>154</td>
<td>0</td>
</tr>
</tbody>
</table>

### 4. DISCUSSION

It is generally assumed that species in which fragmentation or fusion are normal features of the reproductive cycle will be predisposed to chimerism [22]. Consequently, chimerism has almost exclusively been studied in modular species [2]. The present work brings evidence of natural chimerism in a non-colonial cnidarian and the first observation of chimeric embryos and larvae inside a
brooding species. These findings are examined from the evolutionary standpoint of allorecognition maturation and putative cost/benefits associated with chimerism.

Reported rates of natural fusion among recruiting colonies of botryllid ascidians vary from 8 [23] to 73 per cent [14]. In colonial cnidarians (soft corals) rates of natural fusion among settlers range from 3–5% in a field study of adult colonies [7] to 40–90% under laboratory conditions [6]. The present work on sea anemones (unitary animals) found natural fusion rates of up to 3 per cent among brooded embryos and larvae, although actual rates are presumably greater (as the chimeric state was observed to become undetectable in some propagules, apart from the much larger size). Biases from holding conditions are not likely to have occurred since rates of chimerism were measured inside the brood or upon natural release. After larval release (i.e. among gregarious settlers) no evidence of fusion outside the mother was found.

Taxon-specific and ontogenetic variations in rates of chimerism are closely tied to the mechanisms and maturation of allorecognition. In ascidians, allorecognition is controlled by a single highly polymorphic fusibility/histocompatibility locus (Fu/HC [5]), whereas in cnidarians (hydroids) it is encoded in a gene complex [24]. Control of allorecognition in corals and sea anemones is unknown but likely follows the model of hydroids. The high level of polymorphism characterizing allorecognition in marine invertebrates was proposed to have evolved in response to selection for fusion with self rather than kin [25]. We have assumed, therefore, earlier than in the other cnidarians studied so far (colonial forms). This is consistent with the belief that coloniality in marine organisms has evolved from solitary ancestors through progressive weakening of zooidal individuality in favour of increased individualization of the colony [28]. Despite this, the reproductive strategy of U. felina (internal brooding) is conducive to chimerism during early ontogeny.

A common question is whether chimerism is beneficial, and if so why does it not occur more frequently. Fusion between allogeneic entities might confer immediate advantage to the chimera by virtue of the increment in size, especially because the latter correlates with fecundity and viability in sessile colonial invertebrates [29]. Greater fitness of multi-chimeras has been confirmed in ascidians [18,30] and corals [8,12], and molecular evidence of chimerism is reported in wild coral populations [7]. However, an important distinction exists in the type of chimeras occurring in the different taxa. Ascidians are known to form cytomictical chimeras with cell lines so intermixed that original entities can no longer be distinguished, whereas corals typically exhibit sectorial chimeras, in which fused entities retain a degree of individuality [7,31]. Both types of chimerism were detected here on the basis of morphology: sectorial chimeras were observed in all stages, but some visibly chimeric embryos eventually grew into morphologically homogeneous mega-planulae. Could the former result from allogeneic fusion and the latter from isogeneic fusion? Molecular studies will be required to clarify this question. Benefits of chimerism in U. felina might then be restricted to the development cytomictical chimeras (e.g. mega-propagules) exhibiting greater fitness and dispersal abilities, whereas sectorial chimeras (e.g. biheaded propagules) would not gain any advantage. It is even possible that homogeneous entities are the primary (most common yet undetectable) chimeric product and sectorial chimeras an aberrant (easily detectable) sub-product. In support of this hypothesis, size frequencies of

### Table 2. Visible chimerism detected at the larval stage in 12 brooding U. felina.

<table>
<thead>
<tr>
<th>brood</th>
<th>number of bi-chimeric larvae</th>
<th>number of multi-chimeric larvae</th>
<th>total number of larvae</th>
<th>proportion of bi-chimeric larvae (%)</th>
<th>proportion of multi-chimeric larvae (%)</th>
<th>rate of chimerism in larvae (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2010</td>
<td>5</td>
<td>0</td>
<td>420</td>
<td>1.19</td>
<td>0</td>
<td>1.19</td>
</tr>
<tr>
<td>2-2009</td>
<td>1</td>
<td>0</td>
<td>160</td>
<td>0.63</td>
<td>0</td>
<td>0.63</td>
</tr>
<tr>
<td>3-2009</td>
<td>3</td>
<td>0</td>
<td>312</td>
<td>0.96</td>
<td>0</td>
<td>0.96</td>
</tr>
<tr>
<td>3-2010</td>
<td>0</td>
<td>0</td>
<td>3963</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6-2010</td>
<td>0</td>
<td>0</td>
<td>375</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7-2010</td>
<td>1</td>
<td>0</td>
<td>2672</td>
<td>0.04</td>
<td>0</td>
<td>0.04</td>
</tr>
<tr>
<td>8-2010</td>
<td>42</td>
<td>13</td>
<td>5638</td>
<td>0.74</td>
<td>0.23</td>
<td>0.98</td>
</tr>
<tr>
<td>9-2010</td>
<td>3</td>
<td>0</td>
<td>328</td>
<td>0.91</td>
<td>0</td>
<td>0.91</td>
</tr>
<tr>
<td>10-2010</td>
<td>0</td>
<td>0</td>
<td>405</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11-2010</td>
<td>3</td>
<td>2</td>
<td>7859</td>
<td>0.04</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>12-2010</td>
<td>13</td>
<td>6</td>
<td>2476</td>
<td>0.53</td>
<td>0.24</td>
<td>0.76</td>
</tr>
<tr>
<td>13-2010</td>
<td>8</td>
<td>2</td>
<td>996</td>
<td>0.80</td>
<td>0.20</td>
<td>1.00</td>
</tr>
</tbody>
</table>
brooded larvae in *U. felina* commonly show a substantial number of larger offspring that clearly fall outside the normal size distribution, quite probably the result of intra-brood chimerism favoured by the tight packing of embryos inside the tentacles of the mother.

The most widely stated risk associated with chimerism is the establishment of cell lineage parasitism, whereby stem cells from one partner in the chimera might outcompete the others for access to the germ lineages [32]. This is known to occur in ascidians [33] but whether it is a significant cost in sectorial colonies is unclear. Anyhow, this risk is most effectively mitigated by restricting fusion to close relatives. An allorrecognition system having one polymorphic locus (as in ascidians) would serve as a kin recognition system, but its effectiveness is expected to be a function of the number and frequency of the alleles in the population. In colonial ascidians, fusion between closely related colonies seems to be favoured by the tendency of closely related kin to aggregate upon settlement [5,18]. In hydroids (and presumably other cnidarians) where such a tendency has not been documented [19], this narrow ontogenetic window still corresponds to several weeks, leaving plenty of opportunity for allogenetic fusion. Embryonic chimeras developing into large morphologically homogeneous mega-planulae was shown here for the first time. Whether this reproductive strategy evolved as a cost-effective means of producing larger offspring and whether it is widespread remains to be determined. To our knowledge, brooding soft corals studied with respect to chimerism have not been reported to produce chimeras prior to larval release, although this has not explicitly been investigated.

While intriguing from an ecological standpoint, natural chimerism also has implications for investigations of population genetics, as clearly shown in coral populations of the broadcast spawning coral *Acropora millepora* on the Great Barrier Reef. *PLoS ONE* 4, 1–8. (doi:10.1371/journal.pone.0007751)

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While intriguing from an ecological standpoint, natural chimerism also has implications for investigations of population genetics, as clearly shown in coral populations of the Great Barrier Reef [7]. Costs/benefits and prevalence of the chimeric state definitely warrant further investigation. While this should ideally be done in wild populations, it is nearly impossible to monitor embryonic and larval fusion in the field. The review by Pineda-Krch & Lehtila¨ [2] has already indicated that further research is needed to experimentally assess the frequency of occurrence of fusion similar to previous work centreing on ascidians [30,34]. Our findings suggest that unitary species of brooding taxa may offer novel models for research in this field.

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