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ORIGINAL ARTICLE

Response of zooid size in *Cupuladria exfragminis* (Bryozoa) to simulated upwelling temperatures

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Abstract

We investigate the effect of the temperature–size rule upon zooids of the tropical American bryozoan *Cupuladria exfragminis*. Results show that mean zooid length, zooid width and zooid area vary significantly between clonal replicates of *C. exfragminis* kept under different controlled temperature conditions. Significantly larger zooids are produced during times of lowered water temperature that are comparable with the temperatures that occur during seasonal upwelling along the Pacific coast of Panama where the animal lives in abundance. Interpolation of data suggests that a drop of 1 °C causes a 5% increase in zooid size, and that almost all variation in zooid size in natural populations can be explained by temperature. Results are discussed in context of the potential use of zooid size variation in cupuladriid bryozoans to measure the strength of seasonal upwelling in ancient seas by analysing zooid size changes in fossil colonies. The technique of cloning cupuladriid colonies by fragmentation is also discussed with reference to its benefits in experimental studies where genotypes need to be controlled or replicated.

Problem

The temperature–size rule is an almost universal response of organisms to produce larger body sizes in colder ambient temperatures (Atkinson 1994; Atkinson & Sibly 1997; Angilletta & Dunham 2003). Despite being ubiquitous however, the cause of the relationship between body size and temperature has remained somewhat unclear, although recent studies have shown great promise in elucidating the underlying mechanisms (Atkinson *et al.* 2006; Walters & Hassall 2006 and references therein).

The consequence of the temperature–size rule in cheilostome bryozoans is to cause variations in the size of the individual iterated zooids of which colonies are composed, although the underlying mechanism may act upon actual cell size and even organelle density (Atkinson *et al.* 2006). Studies of bryozoan colonies, both under culture and in natural habitats, have demonstrated that larger zooids are budded in colder waters when compared with zooids budded in warmer waters (*e.g.* Menon 1972; Hunter & Hughes 1994; O'Dea & Okamura 1999; Atkinson *et al.* 2006; Lombardi *et al.* 2006).

Because of the relationship between zooid size and temperature, the size of zooids in fossil bryozoan colonies has been proposed as a method of estimating palaeotemperatures, and has been termed the 'zooid size approach' (O'Dea & Okamura 2000a). The original aim of the approach was to use zooid size changes within species over geologic time as a proxy for relative changes in palaeotemperature (Okamura & Bishop 1988; O'Dea 2000). However, because mean zooid size sometimes varies more between different genotypes of the same species than over time or between environments (O'Dea 2000), it is clear that this approach is untenable without the use of considerable amounts of replicate data. Additionally, such an approach, when applied over long time scales, requires the effect of temperature upon size to be separated from any evolutionary changes in size that may have taken

place, a procedure which is essentially impossible in fossil organisms.

Cheilostome bryozoan colonies are usually composed of hundreds to thousands of zooids that are iterated sequentially as the colony grows (McKinney & Jackson 1989), and growth often continues over a year. Thus, the amount of variation in zooid size within colonies is a correlate of the amount of variation in temperature that the colony experienced during its growth (O'Dea & Okamura 2000a; O'Dea & Jackson 2002). The relationship between zooid size variation within colonies and the mean annual range of temperature (MART) has been quantified by regression, allowing measures of zooid size variation in fossil colonies to be used to estimate MART in ancient seas retrospectively (O'Dea & Okamura 2000a). Because all the zooids within a colony are genetically identical, assessing within colony variation in this way eliminates the confounding variable of inter-colonial genotypic variations in size.

One particularly valuable application of this approach is to understand the seasonal thermal regimes that characterized the seas around the Isthmus of Panama during its formation (O'Dea & Jackson 2002). The Panama Isthmus formed during the Neogene (Coates & Obando 1996), leading to the closure of the seaway connecting the Caribbean and the eastern Pacific around 3.5 Ma, resulting in profound oceanographic, climatological and biological changes on local, regional and global scales (Collins & Coates 1999). The two coasts of the Isthmus today experience extremely different seasonal thermal regimes. The Gulf of Panama, on the Pacific coast, is subject to seasonal upwelling that results in substantial annual drops in temperature as well as driving high planktonic productivity. In stark contrast, the Caribbean coast experiences no upwelling, very little seasonal variation in temperature and low planktonic productivity (D'Croz & Robertson 1997).

As the Isthmus formed over several millions of years, it closed the connection between the Pacific and Caribbean. As a consequence, major environmental and then biological changes occurred in shallow waters of the Caribbean, including pulses of origination and a regional mass extinction in marine benthonic taxa (Jackson & Johnson 2000; Todd *et al.* 2002). The cause of the extinction has been suggested to have been the end of upwelling and subsequent collapse in planktonic productivity caused by the cessation of water transport through the once-open sea way. However, there are currently no high resolution estimates of the history of upwelling in the region during this time with which to corroborate such a theory (Todd *et al.* 2002). Independent techniques of understanding seasonal upwelling in the Caribbean Neogene are therefore imperative to understanding the geological and biological evolution of Tropical America.

Cupuladriid bryozoans are as abundant during the Neogene as they are in the seas of tropical America today. Because of their considerable profusion as fossils, O'Dea & Jackson (2002) explored the potential of the zooid size approach in cupuladriids to resolve the deficiency of environmental data during the formation of the Isthmus. The study compared responses of zooid sizes in cupuladriids from the upwelling Pacific and the non-upwelling Caribbean to the actual thermal regime that the colonies experienced. Analysis revealed cyclical changes in zooid size through ontogenetic growth in Pacific colonies but not in Caribbean colonies, thereby corresponding to the relative intensities of upwelling in both seas. Additionally, estimates of MART using the zooid size approach (O'Dea & Okamura 2000a) corresponded well with actual MART's, supporting the use of the approach in fossil cupuladriids as a proxy to estimate the intensity of upwelling in ancient seas.

Despite these results however, the study was not able to be absolutely certain that the cause of the variations in zooid size in Pacific colonies was the result of temperature fluctuations due to upwelling, rather than other corollary or interrelated factors that also fluctuate seasonally along the Pacific coast of Panama, such as productivity levels, as has been suggested by Needham *et al.* (2003) and O'Dea (2005). In addition, the study could not discount the fact that the fluctuations in size may have been part of a natural cycle of changes in zooid size during colony development, and nothing to do with changes in the environment.

This study investigates whether temperature fluctuations comparable with those that occur during times of upwelling could be responsible for the zooid size changes observed in natural populations by O'Dea & Jackson (2002) by culturing colonies of *Cupuladria exfragminis* (Herrera-Cubilla *et al.* 2006) under controlled temperature regimes.

Material and Methods

Cupuladria exfragminis (Herrera-Cubilla *et al.* 2006)

Cupuladria exfragminis (Fig. 1) is a member of the free living bryozoan family Cupuladriidae (Herrera-Cubilla *et al.* 2006). All cupuladriids are unusual among bryozoans because colonies do not live attached to rocks, plants, corals or other stable substrata, but live freely on sand, silt or mud. Cupuladriids possess long setae called vibracula that are regularly distributed among the colony and extend away from colony's surface (Cook & Chimonides 1983). Their combined movement enables colonies to remove sediment from the colony's surface thereby permitting continued feeding during times of high sedimentation. Vibracula also enables cupuladriids to move

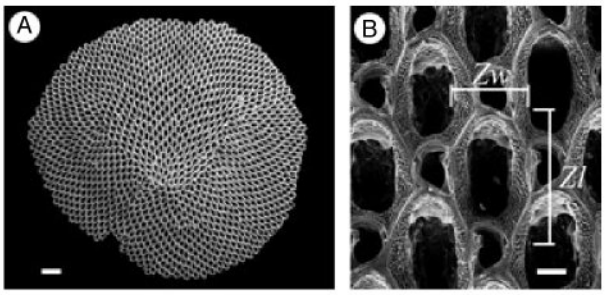

Fig. 1. *Cupuladria exfragminis* from the Gulf of Panama in the Tropical Eastern Pacific. A: Colony composed of iterated zooids and showing regenerative growth from a triangular fragment that can be clearly seen in the centre of the colony (scale bar = 1 mm). B: Measurement of length (Zl) and width (Zw) of a typical normal feeding zooid (autozooid) (scale bar = 100 μm).

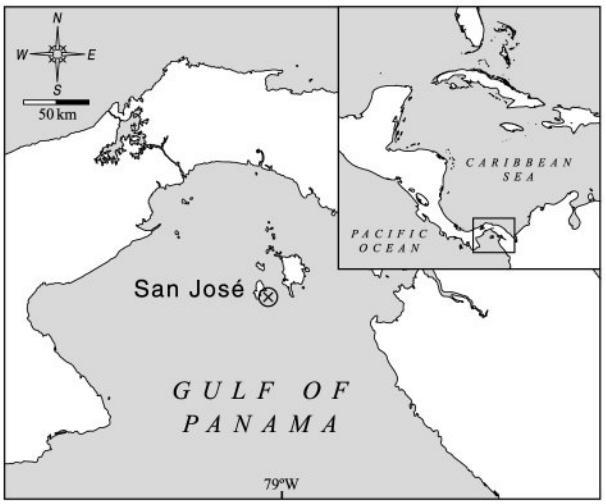

Fig. 2. Study location: The Gulf of Panama on the Pacific coast of the Isthmus of Panama showing location of sampling at San José Island.

laterally across the sediment surface, and vertically up and down through the sediment. Such a motile life habit allows cupuladriids to inhabit soft-bottomed seas where non-motile filter-feeding animals would normally become quickly and fatally smothered in sediment (Winston 1988; O'Dea *et al.* 2004).

Cupuladria exfragminis is an extremely abundant cupuladriid that inhabits sandy sediments from depths of around 10–130 m along the Pacific coast of southern Central America (O'Dea *et al.* 2004; Herrera-Cubilla *et al.* 2006). Thousands of individual colonies may be found in a single dredge sample. Out of those populations so far observed, *C. exfragminis* produces the majority of its colonies by fragmentation and regeneration (*e.g.* Fig. 1A), while only rarely are colonies produced via a sexually derived larvae (O'Dea *et al.* 2004).

We use *C. exfragminis* in this study for the very reason that it propagates mostly by fragmentation. As such, it is easy to create replicate genetically-identical clones for study by fragmenting single colonies by hand and allowing the resulting fragments to regenerate into new colonies.

Collections and study location

Large numbers of colonies of *C. exfragminis* were collected by dredging sandy areas close to Isla San José, in the islands of Las Perlas, in the Gulf of Panama (Fig. 2) (8°14.571′ N, 79°05.158′ W) at a depth of approximately 20 m. This region experiences seasonal upwelling from January to April, during which time waters can drop from their non-upwelling temperature of around 28 °C to a low of around 16 °C during years of very strong upwelling, but normally temperatures drop to between 20 and 25 °C (D'Croz & Robertson 1997; D'Croz *et al.* 2001). Collections took place in August 2004 when the region was not experiencing upwelling, and water temperatures were around 28 °C.

Experimental approach

Figure 3 illustrates the experimental approach used in this study. Following collection, one hundred individual colonies were broken by hand. For each individual colony, a pair of replicate clonal fragments was chosen for study.

Two clone groups were created, each with a replicate from each of the one hundred colonies. All replicates were placed in aquaria in individual and separate numbered boxes each with a teaspoon of sediment.

Replicates were allowed to establish regenerative growth in an open seawater system for 2 weeks before being subjected to different temperature regimes. In September 2004, the clone groups were moved into two closed seawater sys-

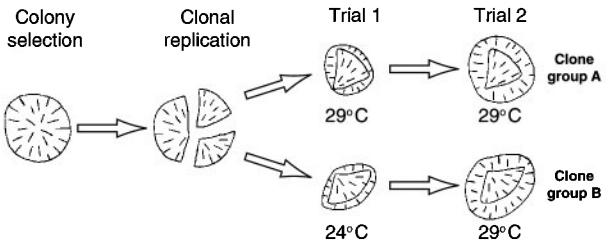

Fig. 3. Experimental procedure used in this study. Colonies were fragmented by hand to create two clonal replicates per colony. Clonal replicates were subjected to different temperature regimes and zooid size measured from the ensuing regenerative growth. Clone group A was subjected to non-upwelling temperatures in both Trials, while Clone group B was subjected to lowered upwelling temperature in Trial 1 and then non-upwelling temperature in Trial 2.

tems for Trial 1. Clone group A was maintained at 29 °C and Clone group B at 24 °C. The water temperature in the latter was lowered slowly over a 5-day period so as not to induce temperature shock to the replicates. As air temperatures were always lower than required water temperatures, water temperature was maintained using four water heaters for each aquaria, which maintained temperatures to within ±0.5 °C. Throughout the experiment, aquaria water was changed three times each week with crude sea water from the Gulf of Panama to maintain sufficient food levels. Each time, fresh sea water was brought to the appropriate temperature prior to changing.

Trial 1 ran for 2 months which provided enough time for fragments to begin regenerative growth and is a good representation of the amount of time colonies may spend in cooler upwelling waters naturally each year in the Gulf of Panama. After this time we found that a number of the replicates had failed to begin regenerative growth or had died. As a result, out of the original 100 fragment pairs, 70 were found to have both replicates living and growing healthily, and thus 70 replicate pairs were applicable for study.

Zooid morphometrics were obtained from the zooids in the newest row of regenerative growth along a single side of each replicate from both clone groups.

Five of the most recently budded autozooids (normal lophophorate zooids) from each replicate were chosen haphazardly, but zooids of strange shapes were dismissed from analysis if it was obvious that their development had been distorted by external factors (see O'Dea & Okamura 2000a). Newly budded zooids at colony margins are easily distinguished from older zooids that have simply stopped budding by their fresh, yellow to light pink color, rather than deeper brown color, and their lack of encrusting epibiota.

For each zooid, maximum zooid length (mm) and maximum zooid width (mm) were measured from which zooid area (mm) was calculated (length × width) (Fig. 1). For each replicate, mean zooid length, mean zooid width and mean zooid area were determined.

Trial 2 began immediately subsequent to Trial 1. All replicates were placed in a single aquaria maintained at 29 °C. Trial 2 ran for 9 months which gave colonies sufficient time to add additional regenerative growth so that we could be certain that further regenerative growth had occurred after Trial 1 (Fig. 3).

At the end of Trial 2 a further 43 replicate pairs became untenable for analysis as we were not able to be entirely confident that they had produced sufficient regenerative growth since Trial 1.

Data on zooid size were therefore taken from the 27 usable replicate pairs in exactly the same way as for Trial 1.

To summarize, Trial 1 measured the zooid size response between clone groups exposed to an upwelling (lowered) temperature and a non-upwelling (normal) temperature. Trial 2 measured the zooid size response in the same clone groups but with no difference in temperature between them. This allowed us to investigate if temperature controls zooid size within genotypes, quantify the effect of temperature upon zooid size, and observe if any response due to a reduction in temperature is lost following a return to normal temperatures.

Analysis of data

We calculated the overall mean and standard deviation of zooid length, width and area for all replicates in each clone group in both trials for comparison.

The difference in mean zooid length, width and area between clonal replicates was determined for each colony in each trial. We used a one-way analysis of variance (ANOVA) to see if the difference in sizes between clones varied significantly from zero, allowing us to reject the null hypotheses that temperature differences do not affect zooid size within clones.

Results

In Trial 1 the overall mean length, width and area of zooids was found to be bigger in Clone group B that was exposed to lowered temperatures than clone group A exposed to normal temperatures (Table 1). In Trial 2 zooid morphologies were not considerably different

Table 1. Mean zooid length, width and area of clonal replicates of *Cupuladria exfragminis* under different temperature treatments.

	Trial 1 (n = 140)		Trial 2 (n = 54)	
Measure	Clone group A (29 °C)	Clone group B (24 °C)	Clone group A (29 °C)	Clone group B (29 °C)
Mean zooid length (SD) (mm)	0.350 (0.046)	0.389 (0.051)	0.351 (0.024)	0.355 (0.025)
Mean zooid width (SD) (mm)	0.246 (0.031)	0.268 (0.030)	0.246 (0.020)	0.245 (0.026)
Mean zooid area (SD) (mm^2)	0.086 (0.016)	0.104 (0.016)	0.087 (0.010)	0.087 (0.010)

Trial 1: Clone group A experienced non-upwelling temperature (29 °C) and Clone group B lowered upwelling temperature (24 °C). Trial 2: both clone groups experienced the same temperature (29 °C).

between the two clone groups (Table 1). Additionally, the length, width and area of zooids in clone group B in Trial 2 was similar to that measured in clone group A in Trial 1 (Fig. 4), showing that a return to normal ambient temperature resulted in a return to the production of smaller zooids.

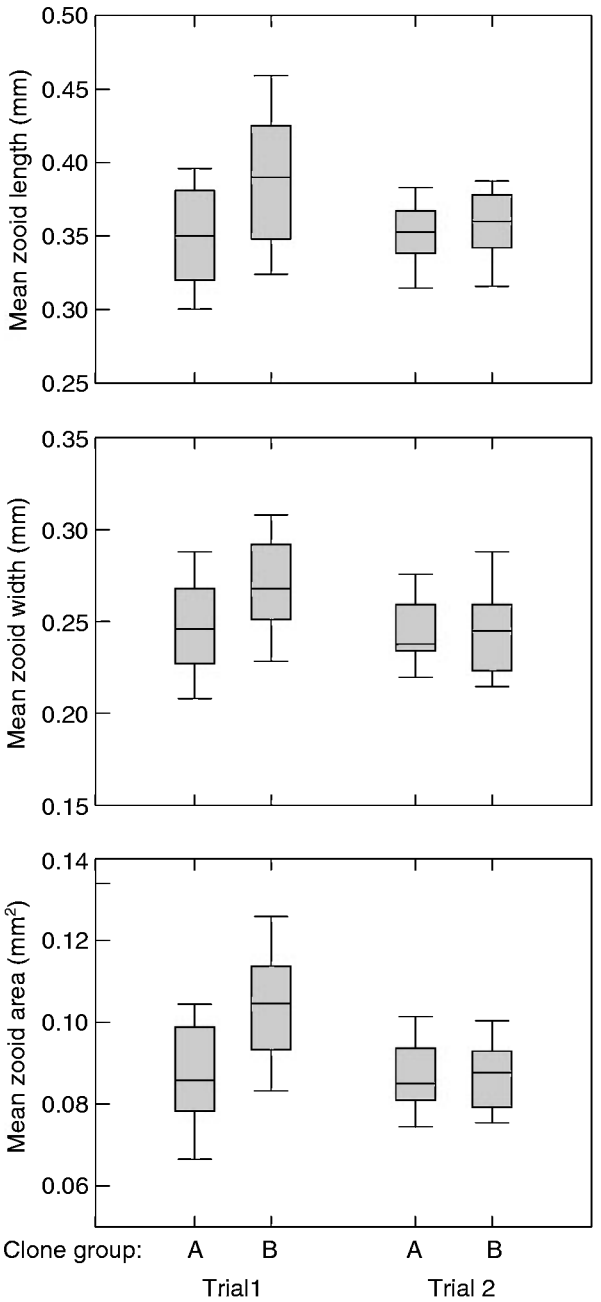

Fig. 4. Mean zooid length (A), zooid width (B) and zooid frontal area (C) of clone groups of *Cupuladria exfragminis* colonies under different temperature regimes. See text and Fig. 3 for further details.

The mean difference in length, width and area between two replicates from the same colony was found to be negative in Trial 1, showing that clones in colder water produced on average larger zooids than the same clones in the warmer water (Fig. 5). This difference was found to be highly significant different from zero for zooid length ($F = 14.64$, $P < 0.001$), zooid width ($F = 4.33$, $P < 0.05$), and zooid area ($F = 21.76$, $P < 0.001$) (Table 1). In Trial 2, individual clones showed no significant difference in zooid length ($F = 0.31$, $P = 0.577$), zooid width ($F = 0.02$, $P = 0.885$), or zooid area ($F = 0.06$, $P = 0.812$).

Replicates were found to produce zooids around 12% longer and wider and zooids around 26% larger in area under the cooler water treatment in Trial 1 (Table 2).

Discussion

Zooid size variation and temperature

Results show that the size of newly budded zooids in *Cupuladria exfragminis* is significantly larger during times of lower ambient water temperatures, such as those experienced during a moderate upwelling event in the Gulf of Panama. This data corroborates previous observations of the inverse relationship between temperature and zooid size observed in many cheilostome species (Menon 1972; Hunter & Hughes 1994; O'Dea & Okamura 1999, 2000a,b; O'Dea & Jackson 2002; O'Dea 2003, 2005; Lombardi *et al.* 2006), and provides strong evidence that zooid size in *C. exfragminis* conforms to the temperature–size rule.

After budding larger zooids during lowered, upwelling-like temperatures, colonies were found to return to produce smaller zooids once temperatures rose again. Thus, as a colony buds new zooids along the margin, zooids should respond in sequence to seasonal changes in temperature. In all cheilostomes, once a zooid is budded the calcified skeleton remains a fixed size during the entire life function of the colony, although the zooid may be used by many regenerated polypides. As a result, the colony skeleton should preserve the record of temperature changes in the zooid morphology (O'Dea & Jackson 2002). Although other factors were not controlled in this study (see later), these data suggest that the variation in zooid sizes observed in natural colonies of cupuladriids by O'Dea & Jackson (2002) was probably a signal of fluctuations in temperature because of seasonal upwelling.

The relative importance of temperature upon morphological variability

The results described here support previous suggestions that the effects of the temperature–size rule upon zooid size in cheilostomes are sufficiently strong to be

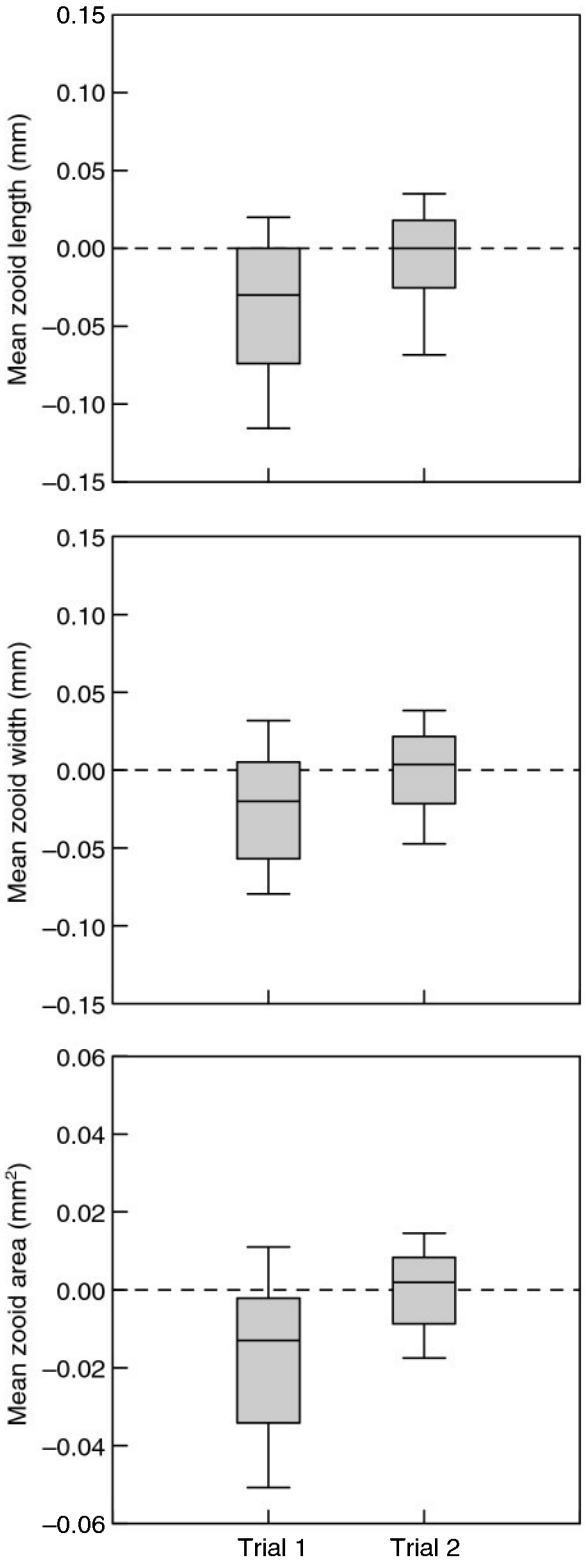

Fig. 5. Mean difference in zooid length (A), zooid width (B) and zooid frontal area (C) between clonal replicates of individual colonies of *Cupuladria exfragminis*. See text and Fig. 3 for further details.

measurable (Menon 1972; Hunter & Hughes 1994; Lombardi *et al.* 2006). One study has contradicted these findings by suggesting that temperature may not play an important role in controlling zooid size (Novosel *et al.* 2004). Unfortunately, because the study compared overall zooid size between colonies from different localities it was unable to remove the effect of genetic variability upon zooid sizes, which is known to have a significant influence upon zooid sizes among genetically different bryozoan colonies (Hunter & Hughes 1994; O'Dea & Okamura 1999). A further study showed that food significantly controlled zooid size in *Electra pilosa*, but did not investigate the response of temperature variation and was therefore unable to assess the relative importance of temperature upon size (Needham *et al.* 2003). Nonetheless, their findings were important in showing that changes in food levels could have a bearing upon zooid morphology.

Profiles of zooid size through the sequential growth of cupuladriid bryozoans from the Gulf of Panama revealed cyclical changes in zooid size (O'Dea & Jackson 2002). These cyclical patterns were presumed to have been produced by seasonal drops in temperature because of seasonal upwelling, leading the authors to propose that it was possible to estimate the level of upwelling intensity that the colonies had experienced during their development, and that the approach should be applicable to fossil cupuladriids to measure upwelling intensity in ancient seas. However, it was impossible to be absolutely certain that temperature was the main controlling factor for the zooid size changes observed. Indeed, the cycles could have been a response to another abiotic or biotic factor such as food availability or salinity that also changed seasonally.

O'Dea (2005) suggested that a combination of fluctuating food levels and temperature may produce a combined effect that accounted for larger fluctuations in zooid size in the erect bryozoan *Pentapora foliacea*. Of the three studies that have considered the relative effects of temperature and food availability upon size, each one concluded that either food levels do not significantly affect zooid size, or that they have a substantially less important effect upon size than temperature (Hunter & Hughes 1994; O'Dea & Okamura 1999; O'Dea 2005).

The present study is unable to determine the relative importance of the effect of salinity or food levels upon zooid size in *C. exfragminis* because neither phytoplankton concentrations nor salinity levels were controlled or measured during the trials. Nevertheless, because each clone group received the same mix of water, we can be fairly certain that water quality variables such as levels of food and salinity were for all intent purposes identical between clone groups at each water change, and therefore

Table 2. Mean and mean percentage difference between individual clone pairs exposed to different temperature regimes (see Fig. 3).

	Trial 1 (n = 140)		Trial 2 (n = 54)	
Measure	Mean difference between individual clones (A–B) (mm)	Mean % difference from clone A to clone B	Mean difference between individual clones (A–B) (mm)	Mean % difference from clone A to clone B
Zooid length (SD) (mm)	−0.039* (0.055)	12.78 (18.96)	−0.004 n.s. (0.033)	1.53 (10.20)
Zooid width (SD) (mm)	−0.022* (0.045)	11.00 (20.84)	0.001 n.s. (0.032)	0.20 (13.34)
Zooid area (SD) (mm^2)	−0.018* (0.022)	26.07 (38.11)	−0.001 n.s. (0.013)	1.85 (16.13)

Result of one-way ANOVA on the significance that the difference between individual clonal replicates differs from zero (ANOVA, *P < 0.001, n.s. = not significant).

ran parallel between clone groups. Thus, although we cannot be entirely conclusive that temperature accounted for all the variation observed in zooid size, the changes are more than likely the result of the factor that we did control, which was temperature.

In spite of these uncertainties, we feel that the data are sufficiently robust and that there is strong support in previous studies to suggest that the majority of the variations we measured were caused by the changes in temperature. If we make such an assumption, we are able to progress our examination into the response of zooids in *C. exfragminis* to the temperature–size rule. An increase in mean zooid length and zooid width of around 12% from a decrease in temperature from 29 to 24 °C was measured (Table 2). Zooid area is more sensitive than length or width, with zooids increasing in size around 26%, due to the compound effects of unidirectional changes in length and width as zooid area is a composite of zooid length and width (Table 2). Thus, a decrease of around 5 °C results in a roughly 25% increase in zooid area. We can therefore suggest that a 1 °C decrease in temperature results in a roughly 5% increase in zooid area.

This is the first time such an estimate has been made and it may prove useful to understand temperature-mediated responses in fossil bryozoans. However, the estimate is based upon the assumption that the relationship between zooid area and temperature is linear. Menon (1972) measured a linear relationship between temperature and zooid size in the encrusting bryozoan *Conopeum reticulum*, and O'Dea & Okamura (2000a) found a linear relationship between variation in zooid size and temperature. There is also robust evidence suggesting that the temperature–size rule in general acts in a linear way but only within threshold boundaries. Within non-extreme fluctuations in temperature, size in many organisms is altered linearly, but extreme temperatures result in non-linear size responses (Angilletta & Dunham 2003; Atkinson *et al.* 2003; Walters & Hassall 2006).

A linear relationship between temperature and zooid size is also supported by comparing zooid size variation from our cultured colonies to variation in wild colonies. In a previous study, mean zooid area in cupuladriid colonies from the Gulf of Panama was found to increase during perceived times of upwelling from around 20 to 60% between colonies (O'Dea & Jackson 2002, Figure 5). Seasonal upwelling in the Gulf of Panama results in a drop in temperature of around 4–12 °C, depending upon the strength of upwelling and depth of sampling (D'Croz & Robertson 1997). Thus, if a 12 °C drop in temperature results in a 60% increase in zooid area and a 4 °C drop in temperature results in a 20% increase in area, by interpolation we arrive at the same figure of a 1 °C drop resulting in a 5% increase in size estimated from our culture data.

Because the estimate of percentage change with temperature from this study coincides very well with the variation observed in naturally grown colonies it is not necessary to invoke other controlling factors to explain observed variation. These results therefore support the hypothesis that the temperature–size rule accounts for nearly all the eco-phenotypic variation in zooid size within cupuladriid colonies.

Although the question of how relatively important are the effects of food levels upon zooid size remains inconclusive, there is now strong evidence from a variety of sources to suggest that the effects of food are either limited or considerably overshadowed by those of temperature (Hunter & Hughes 1994; O'Dea & Okamura 1999; O'Dea 2005).

Notes on cloning cupuladriid colonies by fragmentation

Mechanically fragmenting colonies into smaller pieces and simply allowing the fragments to regenerate is an easy way to create clonal replicates which are then perfectly suited to manipulation under controlled culture conditions when one wants to control for genotype. This approach works particularly well in cupuladriids such as *C. exfragminis* because they naturally fragment and regenerate. The approach should be extremely useful when compared with other methods of ensuring clonal purity among bryozoan replicates.

Summary

By culturing cupuladriids in controlled temperature aquaria, this study showed that the temperature changes that occur during times of upwelling in the Gulf of Panama can be the cause of zooid size changes in *Cupuladria exfragminis*. Colonies were found to bud significantly larger zooids when ambient temperatures were reduced, while once temperature returned to normal levels newly budded zooids returned to their original smaller size. Although unable to remove the potential influence of food levels or salinity upon zooid size, we were able to quantify the amount of change in zooid size, and, by assuming that this change was temperature-mediated, we propose that within a normal temperature range, a change of 1 °C results in a roughly 5% change in zooid size. The study does however provide strong evidence for the assumption that temperature is the most important of the potential influences upon zooid size because a 5% increase in zooid size per 1 °C accounts for almost all variations in zooid size observed in natural populations. These results support the use of zooid size analysis in fossil cupuladriids as a proxy for measuring seasonal variations in temperature of ancient seas, but our prediction of 5% to 1 °C needs to be tested in additional taxa before being applied to palaeontological or ecological systems.

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