Predator cues alter the timing of developmental events in gastropod embryos

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Heterochrony, differences in the timing of developmental events between descendant species and their ancestors, is a pervasive evolutionary pattern. However, the origins of such timing changes are still not resolved. Here we show, using sequence analysis, that exposure to predator cues altered the timing of onset of several developmental events in embryos of two closely related gastropod species: Radix balthica and Radix auricularia. These timing alterations were limited to certain events and were species-specific. Compared with controls, over half (62%) of exposed R. auricularia embryos had a later onset of body flexing and an earlier occurrence of the eyes and the heart; in R. balthica, 67% of exposed embryos showed a later occurrence of mantle muscle flexing and an earlier attachment to, and crawling on, the egg capsule wall. The resultant developmental sequences in treated embryos converged, and were more similar to one another than were the sequences of the controls for both species. We conclude that biotic agents can elicit altered event timing in developing gastropod embryos. These changes were species-specific, but did not occur in all individuals. Such developmental plasticity in the timing of developmental events could be an important step in generating interspecific heterochrony.

Keywords: heterochrony; plasticity; sequence analysis

1. INTRODUCTION

Heterochrony, differences in the timing of developmental events between descendant species and their ancestors, has been proposed to be a pervasive evolutionary feature [1,2]. Parameters used for inferring such heterochronic patterns include age, size and shape, developmental stage and rate [3] and can include physiological processes [4]. Most recently, the relative order of events in the developmental sequence has been used to make evolutionary comparisons [2], with sophisticated statistical techniques employed to map event sequences onto phylogenies (e.g. [5]). Such analyses have confirmed the widespread occurrence of differences in developmental event timing between species, yet it is still unclear exactly how important variation in event timing within species might be for evolution (e.g. [6]), with some studies reporting a relatively low occurrence of sequence change relative to the number of possible changes [5,7].

One key aspect of the debate is the degree to which intraspecific variation in the timing of developmental events might provide the raw material for interspecific heterochrony ([3,4,8]). Plasticity in event timing within species has been documented previously, including several instances of environmentally mediated variation in the onset of specific physiological functions (reviewed in [4]). Fewer investigations focus on plasticity in event sequences (for multiple events), those that do so suggest that such variation or polyphenisms may be common, but are limited to fishes [8–10].

Here, we examine whether alterations in the timing of developmental events within invertebrates can be elicited through exposure to a biotic agent by exposing developing embryos of two closely related freshwater gastropods to cues from predatory fishes. Our questions were, first, does plasticity in event timing occur in invertebrates and, if so, does it occur through stress from biotic agents? Second, if such plasticity does exist, is it expressed consistently between species? By using two, closely related species known to exhibit interspecific differences in event timing [7], we were able to address this question within an evolutionary context.

2. MATERIAL AND METHODS

Adult Radix auricularia were collected in March 2005 from South Drain, a drainage ditch in the Somerset levels (2°52′ W, 51°10.75′ N) and adult Radix balthica in July 2005 from a pond in east Cornwall (4°27.5′ W, 27°2′ W). Snails were maintained in the laboratory under a 12 L : 12 D regime at 15°C (pH = 7.4) in plastic aquaria (30 x 20 x 21 cm) containing continuously aerated artificial pond water (APW [11]), and were fed ad libitum on iceberg lettuce and spinach; water was changed weekly. Egg masses were removed from aquaria walls within an hour of deposition. Individual eggs from five egg batches were excised from masses under low-power magnification (8-10×) and allocated randomly to wells (diameter = 15 mm, depth = 15 mm) in a cell culture tray (125 x 80 x 2 mm) (Thermo Fisher Scientific) and grown at T = 20°C either in control water or water that contained predator cues (n = 12 per treatment per egg batch). Water in each well was replaced daily.

Cue water was produced using tinch (Tinca tinca L.), a molluscivorous fish that consumes freshwater snails and elicits avoidance behaviour and morphological plasticity in Radix spp. [12–14]. Four fish were kept in continuously aerated APW (volume = 4 l) in a plastic aquarium (280 x 180 x 160 mm).

Embryos were observed under high-power magnification (80–100×) and the time of onset of 14 developmental events recorded. These events derived from an interspecific comparison of freshwater snasorial snails [7] and included the onset of a mixture of morphological stages (laying, trocophore, veliger, hippo, free swimming, hatching and migration from egg mass) and developmental events (eye formation, first heart beat, ontogeny of body flexing, mantle muscle flexing, attachment, crawling and radula action). As several events occurred close together (often 1–2 h apart), particularly between the hippo and crawling events, continual observation was required to distinguish the precise time of event onset.

We used three approaches to investigate changes in the relative timing of developmental events. First, for each individual, we scored each developmental event based on a comparison with the ‘standard’ sequences observed by Smithwaite et al. [7] for these species: events in the equivalent position scored 0 and in a different position scored 1. These binary data were analysed using a generalized linear mixed model with treatment (cues versus no cue) as a fixed factor and egg mass as a random factor; the Wald statistic, with a binomial error distribution, was used to test for differences between treatments.
scores were entered into the PCA.

Individuals exposed to predator cues using a principal components analysis (PCA); each event was allocated a score based on the degree of deviation in sequence position for the investigated event. The control treatment of *R. auricularia* had only one sequence, while the *R. auricularia* cue exposed treatment had two observed sequences (modal sequence followed by 62.3% of individuals). The events included were: A, 24 cell; B, trochophore; C, veliger; D, hippo; E, eye; F, heart; G, swimming; H, body flexing; I, mantle muscle flexing; J, attachment; K, crawling; L, radula; M, hatching (details in text).

We then used a series of basic statistics derived from ontogenetic sequence analysis (OSA) of these samples [15] to construct a ‘global’ comparison among treatments and species, calculating the average and the modal sequence position for each event, the standard error of sequence position and the sum of absolute differences between the mean and the modal sequence position for each event, the standard error of sequence position and the sum of absolute differences between the mean and the modal sequence position for each event. Although this approach does not account for the possibility that event changes may differ in terms of their ‘importance’, it does allow a basic comparison of the amount of sequence change between treatments, and provides an estimate of the relative degree of deviation in sequence position for the investigated developmental events.

Finally, we made a global comparison of the standard developmental sequences in both species (i.e. those observed under control conditions) with the altered sequences observed in some individuals exposed to predator cues using a principal components analysis (PCA); each event was allocated a score based on the order that it occurred in the developmental sequence and these scores were entered into the PCA.

3. RESULTS
Predator cues elicited changes in the sequence of developmental events in both species (figure 1), but these changes were restricted to certain events: three in *R. auricularia* and five in *R. balthica*. For

Figure 1. Sequence maps (sensu [15]) for the control and the predator cue exposed treatments of *R. balthica* and *R. auricularia*. The relative frequency of alternate sequences is indicated by segment line weights. Two distinct sequences were observed in the *R. balthica* control sample with 85.9% of individuals following the modal sequence (bold line on left). The exposed treatment of *R. balthica* included three observed sequences (modal followed by 66.7%), with one additional sequence that was predicted but not observed (broken line). The control treatment of *R. auricularia* had only one sequence, while the *R. auricularia* cue exposed treatment had two observed sequences (modal sequence followed by 62.3% of individuals). The events included were: A, 24 cell; B, trochophore; C, veliger; D, hippo; E, eye; F, heart; G, swimming; H, body flexing; I, mantle muscle flexing; J, attachment; K, crawling; L, radula; M, hatching (details in text).

Figure 2. PCA of developmental event sequences in *R. balthica* (Rb) and *R. auricularia* (Ra) showing normal development or altered development in the presence of predator cues (P).

*R. auricularia* all three event changes in cue treatments were significant and occurred together in 62 per cent of embryos: eye formation (event E) and heart beat (event F) both occurred earlier in the developmental sequence (eye—position 6 instead of 7, Wald statistic 12.1, p = 0.0005; heart—position 5 instead of 6, Wald statistic in both cases 12.1, p = 0.0005), whereas body flexing (event H) occurred later (position 7 instead of 5, Wald statistic 12.1, p = 0.0005; figure 1). Three event changes were also significant for *R. balthica* and occurred in 59 per cent of embryos: attachment (event J) and crawling (event K) occurred earlier (attachment—position 9 instead of 10; crawling—position 10 instead of 11, Wald statistic for both 9.5, p = 0.0021) and mantle muscle control (event I) later (position 11 compared with 9, Wald statistic 9.5, p = 0.0021) in the presence of cues (figure 1). A small number of event changes occurred in only one, or a few, individuals and OSA predicted one additional sequence to occur in the *R. balthica* samples exposed to cues if sample sizes were to be increased (figure 1). The predicted occurrence of this unobserved sequence was predicated on the variable sequence position for mantle muscle flexing (event I), which showed the greatest deviation of any investigated event.

Comparison of differences in sum mean and modal event positions between treatments showed that the control treatments were least similar between species, while the within-species comparisons were most similar for both mean and modal sums. Predator cue treatments between species were the next most similar for mean sum and were tied in terms of their similarity for modal sums. Hence, predator cues tended to cause a convergence in event order between species; the PCA of event sequences for controls and sequences resulting from cue exposure confirmed this convergence of the latter pair (figure 2).

4. DISCUSSION
Heterochrony is a common feature within mammalian phylogenies (e.g. [5]) and such interspecific variation in the timing of developmental events has also been shown to occur within invertebrate groups. Smirthwaite et al. [7] identified heterochronies throughout a clade of basommatophoran snails and showed that events such as the development of body flexing and the appearance of the eye occurred at different points in the developmental sequence in different species. Here, we demonstrated that two, closely related species from within this phylogeny, *R. balthica* and *R. auricularia*, both exhibit changes in the timing of developmental events in response to stress from predator cues, adding further evidence for the effects of such cues for development (see also [16]).

Within species, the particular events showing timing shifts were consistent but were not exhibited by all individuals. Such intraspecific variation in event timing in response to a biotic environmental variable could provide the raw material for selection and, potentially, species evolution through genetic assimilation (e.g. [17]). As the events shifting position differed between species, the selection pressures for such developmental plasticity, if it is adaptive, may also differ interspecifically. Predator cues are known to illicit strong behavioural responses in basommatophoran gastropods [13] and *R. balthica* uses crawling behaviour to escape predators [14]. Hence, the early occurrence of crawling behaviour in the embryos of this species in response to predators could confer an increased crawling ability in hatchlings. Clearly, this speculation needs testing, before any firm link can be made between developmental plasticity in event timing and traits expressed later during ontogeny, as would any similar conjecture for adaptive value in traits of *R. auricularia* brought about through altered event timing. Such knowledge is essential in order to investigate the key question regarding the potential importance of altered sequences within species for evolutionary differences among species, i.e. heterochrony. Here, there is a clear need for future studies that compare the fitness consequences of exhibiting different developmental event timings.

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