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Beyond being eaten or swept away: ontogenetic transitions drive developmental mortality in marine barnacle larvae

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ABSTRACT: Many marine animals produce numerous larvae, few of which survive to adulthood. While larval mortality is generally attributed to environmental causes such as predation and transport to unsuitable habitats, mortality deriving from the process of ontogeny has rarely been investigated. This study examines 2 hypotheses (Acquisition of Robustness Hypothesis and Transitional Timing Hypothesis) that relate ontogenescence (high mortality early in life that declines with age) to the biological process of development. We conducted 2 experiments with larvae of the barnacle *Amphibalanus improvisus* to test these hypotheses. In Expt 1, where the survival and duration-in-stage of hundreds of individual larvae were tracked under low stress conditions, half of the developing individuals died. Deaths were concentrated in the larval stages immediately adjacent to the 2 major transitions (nauplius-to-cyprid and cyprid-to-juvenile). 89% of deaths occurred in individuals that had delayed their transition to the next stage. In almost every stage, delays were associated with increased risk of death before reaching the next stage. In Expt 2, which examined stage-based tolerance of temperature and salinity stress, the cyprid stage was most susceptible to ecological stressors. Results of both experiments closely follow the predictions of the Transitional Timing Hypothesis, while neither support an Acquisition of Robustness across development. Stages adjacent to major transitions have reduced physiological tolerance to stressors. Simultaneously, these individuals must achieve competence for the next transition or remain in the current stage until death. The resulting suppression of adult recruitment likely plays an important and underappreciated role in the population ecology of marine animals.

KEY WORDS: Age-stage mortality · Arrested development · Biodemography · Evolutionary demography · Infant mortality · Juvenile mortality · Larval senescence · Marine invertebrates

INTRODUCTION

Because populations cannot expand indefinitely, highly fecund populations must also frequently experience high mortality. Many marine invertebrates and fish are powerful examples of this, producing millions of larvae, with few enough surviving to adulthood to preclude sustained exponential expansion. What happened to all the non-survivors has been a major question of marine population studies since the seminal works of Thorson (1946, 1950, 1966). He hypothesized that the main sources of larval mortality were extrinsic: gamete dilution, starvation, lethal temperatures, failure to find the right place for settlement, predation, and offshore transport by ocean currents. Many subsequent studies have shown that any or all of these factors can be important sources of larval mortality, with the main culprits usually assumed to

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be predation and offshore transport to unsuitable locations (see reviews in Young & Chia 1987, Rumrill 1990, Morgan 1995, Vaughn & Allen 2010). However, predation is a major source of larval mortality in only some studies, with predation rates ranging from 0 to 90% per day (e.g. Johnson & Shanks 2003, Allen & McAlister 2007). Offshore transport almost certainly sweeps multitudinous larvae out to sea (referred to as 'larval wastage'), but recent carefully designed studies have shown that larvae of at least some near-shore species are able to stay remarkably close to shore, even during a planktonic duration of weeks (White et al. 2014). If indeed predation and larval wastage kill many fewer larvae than previous believed, population arithmetic requires that many larvae are dying of other causes.

In his review on larval mortality, Rumrill (1990) added genetic abnormalities and larval diseases to Thorson's list of possible sources of larval death. Unlike Thorson's extrinsic causes, genetic abnormalities are an intrinsic source of mortality: even under ideal conditions many larvae simply do not have the tools to survive. Genetic abnormalities resulting in failed development and death have been observed in laboratory cultures of many organisms, and may cause significant mortality under natural conditions as well. In the 24 years since Rumrill's review, few studies of larvae have discussed failed metamorphosis as a possible source of mortality (Gosselin & Chia 1995, Roegner & Mann 1995, Gosselin & Qian 1996, 1997), and the subject has not received detailed examination. The significance of this hypothesized intrinsic source of larval mortality to marine animal populations remains poorly understood.

In his examination of the evolution of early life mortality, Levitis (2011) noted that at the beginning of life most eukaryotes experience high mortality that declines as development progresses, a pattern he labelled 'ontogenescence'. High pre-reproductive mortality should be strongly selected against, as dying before reproducing reduces one's lifetime reproductive success to zero (Hamilton 1966). Nevertheless, examples of eukaryotes that don't experience high mortality during development are hard to find (see Levitis & Martinez 2013 for budding *Hydra* as a counter-example). While much ontogenescent mortality occurs during embryonic development, the concepts, definitions and examples offered by Levitis (2011) explicitly extend to mortality up until adulthood, and therefore provide a framework for understanding larval mortality.

Levitis (2011) proposed a connection between high early-life mortality and the many 'transitions' during the same life stages. The idea behind this Transitional Timing Hypothesis is that organisms must survive through several major developmental transition events such as the first cell divisions, gastrulation, hatching, or metamorphosis in order to reach the adult stage. In many organisms, these key transitions are concentrated at the very beginning of life, but the theory predicts that major transitions at any stage can be dangerous. During such transition events, body parts, cellular mechanisms, and genetic pathways would need to be used or activated for the first time (e.g. Li et al. 2010), and failures in these untested pathways or mechanisms are hypothesized to be a common cause of death. Under this hypothesis, natural selection cannot eliminate ontogenescence because transitions are both necessary for the proper development of the young organism and inexorably associated with a variety of dangers.

While Levitis (2011) focuses his proposal of the Transitional Timing Hypothesis on intrinsic causes for the danger of transitions, he states that a wide variety of causalities could account for the concentration of mortality around transitions. Consistent with this, we define the danger of transitions to which the Transitional Timing Hypothesis refers to include dangers causally and temporally associated with those transitions, but also influenced by extrinsic factors. For example, many ontogenetic transitions drive individual ecological shifts (e.g. from the plankton to the benthos), exposing the transitioning individual to challenges it has not previously encountered and may not be able to survive (Gosselin & Qian 1997). The adoption of this definition is motivated by the fact that the level (Williams & Day 2003) and timing (Carnes & Olshansky 1997) of mortality is greatly influenced by the interactions of intrinsic and extrinsic factors. Taking human neonatal mortality as an example, premature newborns are intrinsically more susceptible to infections, temperature stress, and other extrinsic dangers, while the environment of a neonatal intensive care unit can greatly decrease what are generally thought of as intrinsic causes of mortality, such as cardiovascular failures and congenital morphological abnormalities. Rarely, if ever, can intrinsic and extrinsic risk factors be cleanly and completely separated. In the context of marine larvae, this suggests interactions between the extrinsic challenges highlighted by Thorson (1950) and the variable intrinsic susceptibilities discussed by Rumrill (1990).

The Transitional Timing Hypothesis is one of several non-mutually exclusive hypotheses (reviewed by Levitis 2011) to explain the evolution of ontogenescence. Another is the intuitive Acquisition of

Robustness Hypothesis, wherein young individuals acquire more robustness to potential dangers as they grow older and larger. As described by Levitis (2011), the acquisition of robustness can come in many forms. For example, young, small fish are likely to have a greater number of potential predators than older, larger fish will have. By growing, they acquire robustness to the threat of predation. Simultaneously, the development of their immune systems can make them more robust to the common pathogens they encounter, and their neurological capacity for learning may increasingly shield them from a variety of dangers. These and many other potential modalities are hypothesized to cause each developing individual to have declining frailty with increasing age. Natural selection is therefore constrained from eliminating high and declining early mortality when organisms must, over the course of their development, accumulate the ability to resist dangers and stressors.

Barnacle larvae provide an excellent opportunity to test the relative importance of acquisition of robustness and difficult transitions in driving deaths during development. First, they are generally quite robust to being cultured in the laboratory and are therefore excellent for laboratory mortality experiments. Second, estuarine barnacle species are frequently presented with temperature and salinity challenges, allowing for ecologically relevant tests of the robustness of their developmental stages. And third, a barnacle's development from planktonic larva to benthic juvenile involves a series of molts (small transitions involving rapid growth) followed by 2 major morphological reorganizations within a relatively short period of time. These major transitions near the end of development imply that transition-related larval mortality should be highest near the end of development. However, if ontogenescence is driven primarily by young individuals having not yet acquired robustness, mortality should be highest at the beginning of development. If both mechanisms are important, we would expect peaks of mortality both early in development and at its end. The barnacles' late transitions therefore allow for predictions that clearly differentiate the effects of the hypothesized mechanisms of developmental mortality.

Here we present 2 experiments on barnacle larvae intended to test the Transitional Timing Hypothesis and Acquisition of Robustness Hypothesis. The goals of these experiments were to determine the timing of deaths across larval stages and after settlement under relatively low-stress laboratory conditions and to examine the ability of each larval stage to tolerate environmental stress.

MATERIALS AND METHODS

Study organisms

Adult barnacles (*Amphibalanus improvisus* Darwin) attached to small rocks and reeds were collected from the Warnow Estuary in Rostock, Germany, in the fall and winter of 2011–2012 and reared in 2 aquaria (25 × 38 × 5 cm) at the Max Planck Institute for Demographic Research. Conditions within the Warnow Estuary are highly seasonal, with temperatures ranging from 0°C in the winter to >20°C in the summer and salinities ranging between 0 and 20 (Winkel 2003). During the spring and summer months when the barnacles are likely to be reproductive, temperatures between 15 and 20°C and salinities between 10 and 20 are typical in the section of the estuary where our collections were made. The life cycle of *A. improvisus* follows the typical barnacle pattern. After hatching, the larvae pass through a series of 6 naupliar stages (here abbreviated N1–N6), usually over the course of 1 to 2 wk (Anderson 1994). These nauplius larvae actively feed in the plankton, increasing in size and complexity at each molt, but with the basic morphology of the larva remaining the same. Following the final naupliar stage, however, the nauplius molts into a cyprid larva, representing a major morphological reorganization. Unlike the generalized nauplius, a larval form which barnacles share with many other crustacean groups, the cyprid larva is unique to barnacles. Cyprids swim differently than nauplii, have 2 sensory antennae upon which they can 'walk' along a surface, and do not feed. The cyprid's function is to locate a suitable substrate on which to attach and settle. When an appropriate location for settlement is found, the cyprid cements itself to the substrate and initiates metamorphosis, transforming into a sessile, benthic juvenile barnacle morphologically similar to an adult.

Due to their importance as fouling organisms, the laboratory protocols for maintaining the larval cultures of several barnacle species have been extensively studied (see Strathmann 1987 for a review). In the case of *A. improvisus*, several studies testing various food types and concentrations, as well as culture salinities and temperatures, give us a good idea of what are likely low-stress culturing conditions for the larvae of this species (e.g. Lewis 1975, Nasrolahi et al. 2006, 2007, 2012). However, no laboratory conditions are truly ideal and no environment can be created that is completely free of external stressors. Culture methods found to maximize survivorship were replicated here with the specific goal of minimizing

unintentional stress and accidental mortality during water changes, moving, and other manipulations. Signs of stress (e.g. decreased or altered activity) or accidental mortality were looked for after all manipulations. No handling stress was observed.

Artificial seawater (ASW) was mixed (salinity 15) in the laboratory by combining Tropic Marin Meersalz (Tropic Marin) with Milli-Q filtered water (the Milli-Q process included reverse osmosis, UV light exposure, and a 0.22 µm membrane). We performed 50% water changes in the barnacle aquaria every other day by carefully drawing down and then quickly replacing the ASW. The laboratory was temperature-controlled at 20°C. Following each water change, barnacles were fed *Artemia* nauplii to saturation. Barnacles were checked at least twice daily, and any individuals that died in our cultures were immediately removed from the aquaria. While we did experience some mortality associated with collection (approximately 10% in the first 2 d), fewer than 10 adults died in the aquaria over the next 6 mo. The total number of adults in the aquaria were not counted, but was estimated to be approximately 100 individuals.

After 1 to 2 mo under these conditions, the barnacles began regular production of larvae. We checked for newly hatched larvae released from the laboratory barnacles twice each day using a light to attract and concentrate them. These larvae were then gently removed with a pipette for use in experiments.

Expt 1: Stage-based survival

Stage- and duration-within-stage-based mortality of larvae was examined in 2 larval cohorts, collected in January and February 2012. For both cohorts, one barnacle nauplius was placed in each well of six 48-well plates, along with 1 ml of salinity 15 ASW (mixed as described above). This volume was chosen to maximize the number of larvae that could be followed during the experiment while still giving each larva adequate room to swim, feed, and metamorphose. However, given the physical and chemical changes that can occur in such small volumes of water, larvae were moved to well plates with new ASW every second day. This process involved locating each larva using a binocular microscope, carefully collecting the larva with a tapered glass pipette along with as little water as possible, and then releasing the larva into the new well. Barnacle nauplii and cyprids are covered by a hard exoskeleton and are extremely robust to this kind of gentle manipulation. We never observed an instance when the animal was damaged during pipetting or exhibited different behaviour following the transfer to the new well. Once in the new wells, larvae were fed the diatom *Chaetoceros calcitrans* (concentration 1×10^5 cells ml^{-1}) grown in f/2 medium (20°C and salinity 15) (Nasrolahi 2007). Once larvae reached the cyprid stage, well plates were treated with adult barnacle extract to maximize settlement success. Extraction involves crushing an adult conspecific barnacle, grinding and decanting the liquid several times, centrifuging the solution, briefly boiling the supernatant, and centrifuging a second time. The extract was then 'painted' onto the walls of the wells (Rittschof et al. 1984). Following settlement (when the cyprids cement themselves to the sides or bottom of the well plates), the water within each well was changed every second day instead of moving the animals themselves. This involved a brief emersion of the juvenile barnacles. However, *A. improvisus* experiences periods of emersion naturally and is highly resistant to such periods. No negative effects of the water change process were observed. Feeding continued as before. All well plates were kept in an incubator at 18°C with a 12:12 light/dark cycle (best culturing results from Nasrolahi et al. 2012).

Each larva in each well plate was examined using a binocular microscope every other day to determine which individuals were alive. If a larva was not swimming and did not respond to gentle prodding with a pipette, it was considered dead. This prodding never involved the crushing of the larva or any of its appendages, and no negative effects were observed from the procedure. The presence of molts in well plates was also noted and used to determine the stage of each larva. For those larvae that metamorphosed into juvenile barnacles, the condition of the juveniles was monitored for up to 12 d after settlement and metamorphosis to determine if any deaths occurred during this time period. The experiment continued until all individuals had either survived the period following settlement or had died.

To investigate the impact of the duration (in days) within a stage on the probability of death in that stage or transition to the next stage, conditional forward probabilities were calculated: we calculated the probability of dying in each stage i at or after each duration-in-stage x_i, conditional on having survived at least x days in stage i. As an example, if 80% of those cyprids who survived at least 31 d as cyprids went on to die as cyprids (Cp), then the conditional forward probability for i = Cp and x_i = 15 would be 0.8. For each x_i, this conditional probability of dying-

in-stage plus the conditional probability of transitioning to the next stage sum to one.

To further understand the effect that duration in stage has on the risk of dying in that stage, we defined being 'delayed' in each stage as spending longer in that stage than did 90% of those individuals who would go on to survive at least 12 d into the juvenile stage. For example, >90% of these successful individuals spent 2 or fewer days in stage N2, so any individual spending >2 d in the N2 stage was considered delayed. We categorized each death based on whether the individual was delayed in the stage in which death occurred, and whether it had been delayed in the previous stage, and tabulated the number of deaths falling into each category. A parallel analysis defining 'delayed' using 95% instead of 90% yielded substantially similar results and therefore is not presented.

Expt 2: Robustness to environment

We conducted a second experiment measuring stage-based robustness of barnacle larvae to temperature and salinity insults. Recently released larvae collected between July 2012 and January 2013 were placed in mass cultures of 200–500 larvae in 1 l beakers (ASW, salinity 15) as soon as possible after release from their parents. The common garden arrangement of the adult barnacles did not allow us to identify the exact parent or parents of the larval cohorts. In total, approximately 60 mass cultures were kept, although larvae were not used from all of them. These mass cultures allowed for culturing a greater number of larvae while also providing each larva with a larger environment in which to swim and feed than they would get if cultured in well plates. Water in larval cultures was changed every other day until larvae were used in experiments. Water changes followed standard procedure for larval cultures: the water in the beaker was gently poured through a fine-mesh filter (80 µm) that catches the larvae. Using a squirt bottle with ASW of the same salinity, the larvae were gently washed off of the filter and into a beaker of clean ASW of the same salinity and temperature as the first. Cultures received the same food, light, temperature, and water-changing regimen as described for Expt 1. Larvae continued in mass culture until the majority of individuals in a culture had reached the stage of interest (naupliar stages determined by a combination of age-since-hatching and carapace size).

Using the same pipetting method described above for transferring the larvae with tapered pipettes, we then placed 20 larvae from the culture into each of six 50 ml cell culture vials containing water of one of 6 salinity treatments (5, 10, 15, 20, 25, or 30). Great care was taken to ensure than no more than 30 µl of water was transferred with each larva (600 µl total into each vial), which even in the most extreme treatments (salinities 5 and 30) would not have changed the salinity of the 50 ml vial more than salinity 0.1. The vials were then completely filled with water so that no air bubbles remained inside. Vials were sealed such that no evaporation was possible and salinity held constant. Given the relatively small size of the larvae and limited time of exposure, oxygen levels were highly unlikely to decline significantly through the course of the experiments. This was confirmed through preliminary experiments using oxygen sensor spots (P. H. Dunn unpubl. data).

We tracked the number of larvae swimming (visibly motile) in each vial with a photo apparatus inside an incubator, initially set to 20°C. A camera (Marlin F-145 C2 Firewire), pointing downward from below a linear automated slide, repeatedly moved over and photographed each vial, with larvae showing as light spots on a dark background. We placed the 6 vials in random order below the slide and set up LED lights on either side of the line of vials (56 cm ALU LED rod waterproof 35× Power SMD LEDs white — 24 V, max. output 805 lumens). The lights were programmed to alternate turning on and off, so that the direction of the light passing through the vials changed by 180° every 30 s. Since barnacle nauplii are photopositive and cyprids are photonegative, this alternation of light resulted in the larvae swimming across the centre of the vials every 30 s. To determine which larvae were swimming, we programmed the camera-slide system to take a series of photographs for 90 s over each of the vials before proceeding to the next vial and repeating the process. Each series of images was then converted into short videos using FFmpeg (www.ffmpeg.org). An example of the resulting videos is available as Video S1 at www.int-res.com/articles/suppl/m559p103_supp/.

Following the 9 min of image-taking (90 s × 6 vials), the incubator was set to either increase or decrease the temperature by 5°C, depending on whether we were conducting a heat-tolerance or cold-tolerance trial. We then waited 40 min, a period that preliminary testing indicated was more than sufficient to allow the water in the vials to reach the new trial temperature, before the next series of photographs were taken. The process was then repeated, stopping

at 5°C intervals, until the temperature in the vials reached either 40 or 0°C. Following this last temperature treatment, vials were allowed to come back to 20°C, which usually took around 40 min. The contents of each of the vials were then emptied and examined under a binocular microscope to determine how many larvae survived the trial. If a larva did not swim or respond to being gently prodded by a pipette, it was considered dead. Replication of all trials was sequential, with each trial performed with new larvae of the appropriate age and stage (n = 3 for each temperature-salinity-larval stage combination, for a total of 42 trials).

Following the experiment, each short video was examined at slow speed by 2 independent observers who determined the number of larvae swimming in each vial at each time point. In the few cases where the 2 observers disagreed on the number of larvae swimming, a third observer acted as the tie-breaker. The number of larvae swimming at each salinity-temperature combination was then divided by the total number of larvae known to be in the vial to determine the proportion swimming. An average proportion of larvae swimming for the 3 trials was then calculated for each of the stage-salinity-temperature combinations and subsequently used for the analysis. Cold and heat trials were analyzed separately.

Controls were used to test for the effect of a potential salinity 'shock' treatment, i.e. placing larvae directly into vials containing water of a different salinity than the larvae were cultured in without allowing for an acclimation time. We placed 20 larvae of each stage in vials containing the most extreme salinity treatments (5 & 30) and left them in the incubator for the same amount of time as the experiment would normally run, but at a constant temperature of 20°C. We then emptied the vials and examined the contents under the binocular microscope to determine how many of the larvae had survived. All data analyses were performed in R (R Core Team 2013).

RESULTS

Expt 1: Stage-based survival

For Cohort 1, 136 of the 281 larvae (48.4%) died during the course of the experiment. A similar result was found for Cohort 2 (146 of 277 larvae died, 52.7%). The non-parametric cohort mortality patterns (Kaplan & Meier 1958) were not significantly different (log rank test, p = 0.92) and were therefore pooled together for subsequent analyses. We detected no pattern of spatial or temporal clustering of deaths on well plates. It is therefore unlikely that observed mortality can be attributed to an infection that was spread from one well to another.

The stage-based survivorship (Fig. 1) and mortality (Fig. 2) of the barnacle larvae revealed that danger was concentrated in the N6, cyprid and juvenile stages, which are, respectively, directly before, between, and after the 2 major morphological transitions in the barnacle life cycle. 29% of cyprids died, making this the stage with the highest mortality.

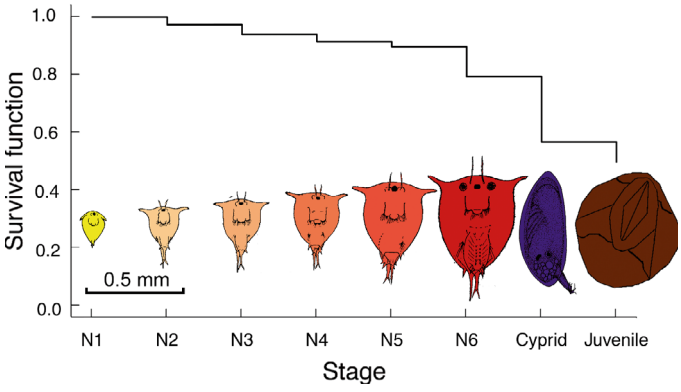

Fig. 1. Kaplan-Meier plot of survival to the end of each developmental stage in Expt 1, with to-scale line drawings of the stages. 282 of 558 individuals died during the study (50.5%), with heaviest losses occurring during the cyprid stage. Survivorship (1.0 = 100% survival) of Nauplius 1 (N1) individuals by definition = 1. Line drawings of larval stages (modified by permission from Jones & Crisp 1954) omit appendages. Colors filling these drawings are used in subsequent figures to refer to the same stages

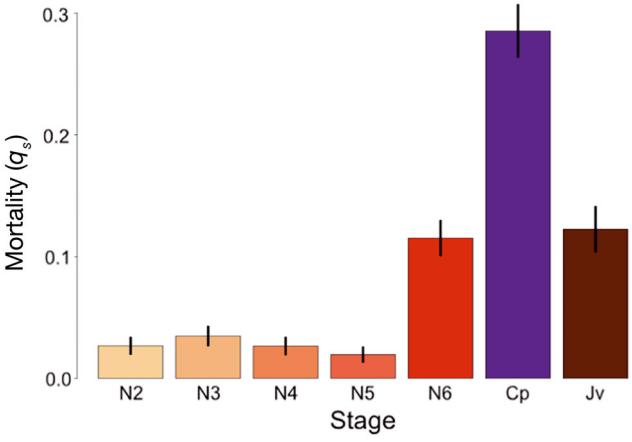

Fig. 2. Mortality in each stage clearly showing the excess risk associated with Nauplius 6 (N6), cyprid (Cp) and juvenile (Jv) stages. Mortality (q_s) is measured as the probability of dying in a stage (s) conditional upon reaching that stage. q_{Cp} = 0.285 is more than twice as high as any other stage and 10–15 times the mortality observed in the earlier naupliar stages. Error bars represent SE

There was also a clear effect of duration-within-stage on the pattern of mortality. Individuals that delayed their transition to the next stage accounted for most of the observed deaths. 215 of 243 (89%) observed larval deaths occurred in individuals that were delayed in their current stage (109 instances), in their previous stage (8 instances), or in both their current and previous stages (98 instances) (Table 1). In every larval stage the individuals that died in that stage (Fig. 3) spent longer alive in that stage than did

Table 1. Deaths during Expt 1 in Stages N2 (Nauplius 2) through Cp (cyprid), categorized by delays in the current and/or previous stage. NA: not available (duration in N1 was not measured, and therefore delay in N1 was not defined); sum excludes deaths prior to N2 and after the cyprid stage

Stage	Total number dying	Delayed in current stage	Delayed in previous stage	Delayed in current and previous stage	Delayed only in current stage	Delayed only in previous stage	Not delayed in either stage
N2	15	12	NA	NA	12	NA	3
N3	19	19	6	6	13	0	0
N4	14	13	7	7	6	0	1
N5	10	1	0	0	1	0	9
N6	58	45	0	0	45	0	13
Cp	127	117	93	85	32	8	2
Sum	243	207	106	98	109	8	28

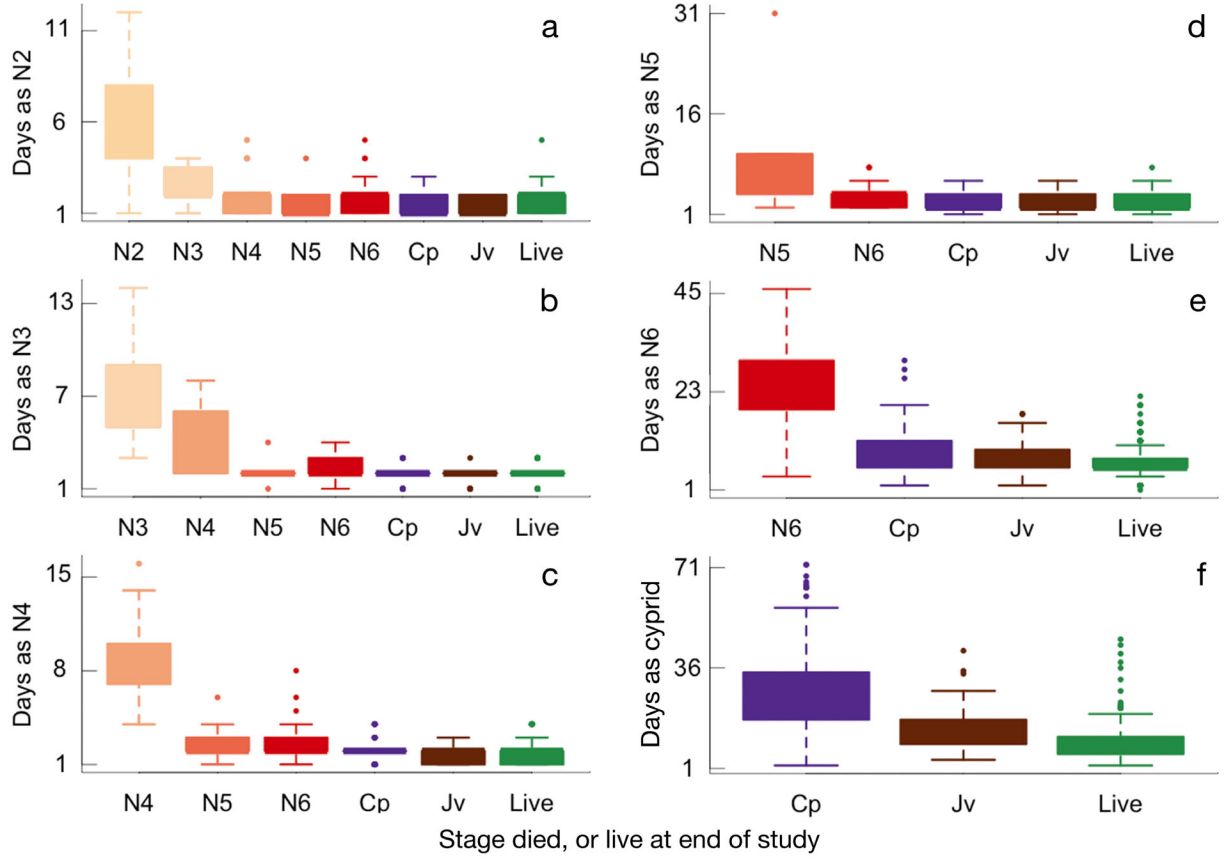

Fig. 3. Multi-part box plot showing durations in each stage (a) Nauplius 2 (N2) through (f) cyprid by individuals who died in each stage (N2 through juvenile) or were live juveniles at the end of the study. Each plot displays durations of all individuals in a single stage, grouping individuals based on their eventual fate (N2 = died as Nauplius 2 etc., Cp = died as cyprid, Jv = died as juvenile, Live = living at end of the study). Boxes: interquartile ranges; small circles: outliers, defined as falling more than 1.5 interquartile ranges above or below boxes. Boxes of the same color represent the same sub-populations defined by stage of death and changing only due to mortality. To make details more visible, axis ranges differ between plots

those who successfully transitioned to the next stage. Larvae that did make the transition after a longer stay in a particular stage often also experienced a delay in the next stage and subsequently died. Delayed transitions were particularly common in the N6 and cyprid stages as the larvae prepared to go through the 2 major morphological transition events. Most individuals dying as cyprids (85 of 127) were late in both of these stages. The longer an individual had already spent in the N6 or cyprid stage, the lower its chance of eventually making it to the following stage (Fig. 4).

Expt 2: Robustness to environment

Two repeated-measure 2-way ANOVAs (Table 2) revealed significant effects of stage, salinity, and temperature on the swimming of barnacle larvae during both heat and cold trials. Fig. 5 illustrates these data pooled across salinities, while Supplement 1 at www.int-res.com/articles/suppl/m559p103_supp/ presents each salinity level separately. In the cold trials, swimming was most impaired in cyprids in the lowest temperatures (0 and 5°C) and at the most extreme salinities (0 and 30). A high percentage of larvae recovered from the cold trials, with the cyprid stage experiencing the lowest survival (72%). In the heat trials, most larvae were unable to swim at 40°C, and cyprids were less tolerant of increasing temperature than were other stages, showing decreased swimming beginning at 25°C. The heat trials resulted in lower survival than the cold trials (78% compared to 92%), with the cyprid stage again experiencing the lowest survival (65%).

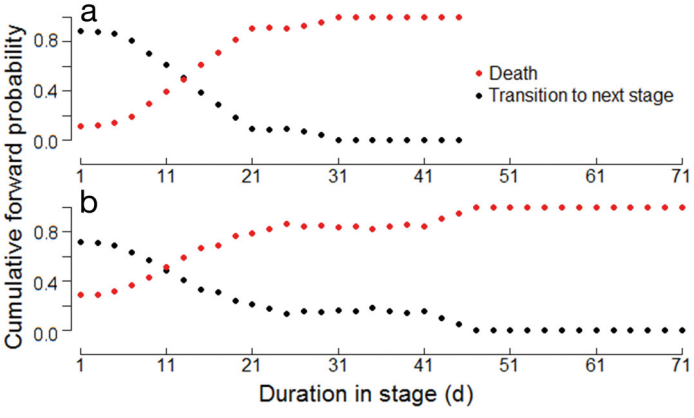

Fig. 4. Conditional forward probabilities of death or transition from stage (a) Nauplius 6 (N6), and (b) cyprid. All individuals must ultimately transition to the next stage or die in the current stage, and so the probabilities of these fates always sum to one. The probabilities shown are conditional upon having reached at least each duration-in-stage, and therefore are calculated for an increasingly self-selected subset of larvae. For example, the probability that a cyprid that has already been a cyprid for 31 d will eventually become a juvenile is 0.16. The non-feeding cyprid stage retained competence to transition longer (observed maximum = 45 d) than did planktotrophic N6 larvae (observed maximum = 29 d). Data were gathered in 2 day increments but are presented on a single-day scale for readability

Table 2. Stage-based salinity and temperature tolerance 2-way repeated-measure ANOVA results. Each 2-way ANOVA compared the swimming ability of the 7 stages of barnacle larvae (N1–Cp) exposed to 6 salinity treatments across 5 temperature treatments. Proportions were arcsin square-root transformed and checked for normality (Shapiro-Wilks test, all p-values >0.087) for the analysis. Within-subject effects (A & C) show the differences between treatment temperatures. Between-subjects effects (B & D) show the differences in swimming ability with larval stage and salinity as factors. p^{HF} is the Huynh-Feldt adjusted p-value, used because the data violated the assumption of sphericity (Zar 2010). T: temperature; Stg: stage; S: salinity; N1–N6: Nauplius 1 to Nauplius 6 stages; Cp: cyprid stage

Source of Variation	df	SS	MS	*F*	p^{HF}
A. Cold trials. Within-subjects effects					
T	5	16.281	3.256	84.376	<0.001
T × Stg	30	4.166	0.139	3.598	<0.001
T × S	25	1.399	0.056	1.450	0.078
T × Stg × S	150	5.161	0.034	0.892	0.789
Residual	340	13.121	0.039		
B. Cold trials. Between-subjects effects					
Stg[a]	6	30.153	5.025	38.055	<0.001
S[b]	5	3.558	0.712	5.389	<0.001
Stg × S	30	5.060	0.169	1.277	0.201
Residual	68	8.980	0.132		
C. Heat trials. Within-subjects effects					
T	5	111.865	22.373	706.994	<0.001
T ×Stg	30	13.885	0.463	14.625	<0.001
T × S	25	8.934	0.357	11.293	<0.001
T × Stg × S	150	5.042	0.034	1.062	0.319
Residual	420	13.291	0.032		
D. Heat trials. Between-Subjects effects					
Stg[c]	6	36.375	6.063	62.273	<0.001
S[d]	5	5.126	1.025	10.531	<0.001
Stg × S	30	3.545	0.118	1.214	0.243
Residual	84	8.178	0.097		

[a]Post-hoc Bonferroni tests (α = 0.05) on swimming by stages: Cp < N4 < all other stages & (N2, N3, N4, Cp) < N6;
[b]Post-hoc Bonferroni tests (α = 0.05) on swimming by salinities: (5, 30) < (15, 20);
[c]Post-hoc Bonferroni tests (α = 0.05) on swimming by stages: Cp < all other stages & (N5, N6) < N2;
[d]Post-hoc Bonferroni tests (α = 0.05) on swimming by salinities: 5 < all other salinities

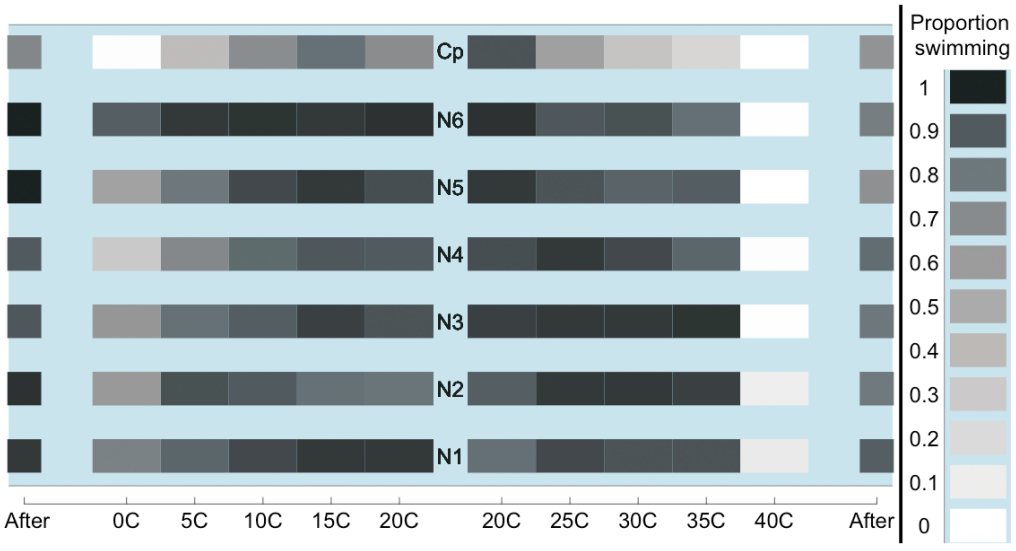

Fig. 5. Results of environmental stress trials, pooled across salinities and replicates. Tests with individuals of each stage (Nauplius 1, N1, at bottom of figure to cyprid (Cp) at top) proceeded from 20°C (in the center) to increasingly cold (on the left) or increasingly warm temperatures (on the right), before larvae were allowed to recover at 20°C (left- and rightmost columns). The darkness of each block indicates the proportion of swimming, or in after-tests, alive larvae (n = 3 for each temperature/salinity/larval stage combination)

All control trials resulted in 100% survival except when cyprids were placed in salinity 5. Here, 2 out of 20 cyprids died, still a much higher survival rate (90%) than cyprids at salinity 5 under experimental conditions (55% during cold trials and 18% during heat trials). Deaths during our experimental trials cannot be attributed to salinity shock.

DISCUSSION

Our data strongly support the hypothesis that developmental failures in barnacle larvae are temporally associated with the timing of developmental transitions, but offer no evidence of acquisition of robustness during development. These results make clear that the transitions barnacles pass through early in life, particularly their 2 major morphological reorganizations, are associated with both developmental failures and loss of stress tolerance.

In Expt 1, we followed a cohort from the N2 stage to 12 d after settlement and metamorphosis. Under laboratory conditions that excluded all sources of environmental mortality identified by Thorson (1946, 1950, 1966), 50% of all larvae died. It should be acknowledged that no laboratory conditions can completely reflect the conditions these larvae would experience in the field, particularly when the fate of individual larvae is in question. The cost of obtaining individual-level data was the unnatural, although not necessarily more stressful than natural, constrictive environment of the wells. We also note that ASW is not a perfect nutritional or chemical substitute for seawater, although it is routinely used to culture hardy marine larvae like barnacles when seawater cannot be obtained (Strathmann 1987). However, we found no evidence that would suggest the mortality observed in our experiments was the result of our chosen experimental conditions since similarly high mortality has been documented in laboratory studies of other barnacle species (Costlow & Bookhout 1957, Anil et al. 1995, Qiu & Qian 1997), and at least 1 study has also reported high stage-specific mortality rates of barnacle larvae in the field (Tapia & Pineda 2007), although it is impossible to determine how these larvae died.

Our 2 focal hypotheses, while not mutually exclusive, generated strongly diverging predictions regarding the timing of deaths in Expt 1. Even in a relatively low stress environment, larvae can be assumed to face some level of environmental challenges; if these pose significant risks and developing individuals acquire robustness to them, mortality should be highest in the earliest stages. However, if developmental failures associated with transitions are the main source of mortality, deaths should be concentrated around the later stages when major morphological transitions occur.

The late-skewed distribution of death events across both stage and duration-within-stage (Figs. 1

& 2) clearly supports only the second of these hypotheses. Deaths were concentrated in the N6, cyprid, and juvenile stages, which directly precede, bridge, and follow the 2 major morphological reorganizations of the barnacle life cycle. The interpretation that these deaths are linked to the timing of transitions is supported by the field work of Gosselin & Qian (1996) who found that cyprid mortality during the first day after settlement (38%) was almost as high as the following 44 d combined (40.1%). They found that this mortality did not correlate with grazer density, desiccation stress, wave exposure, or size-specific changes, suggesting a role for developmental failures.

To reiterate, we cannot determine causes of deaths in Expt 1, only their number and timing. While the number of deaths was likely influenced by the artificial nature of the environment as well as genetic and developmental traits of individuals, the timing of deaths within the relatively constant environment was clearly associated with transitional developmental stages, supporting the Transitional Timing Hypothesis. One could speculate that some unidentified characteristic of the N6, cyprid, and juvenile barnacles, rather than their transitional status, leads to the excess mortality we observe in those stages. However, we document a strong association between extended duration in a stage and dying in that stage (89% of deaths followed such delays), with delayed major transitions being particularly deadly (Figs. 3 & 4, Table 1). Furthermore, increased mortality risk as a result of delaying metamorphosis (sometimes referred to as larval senescence, Pechenik 1990, Wendt 1996) is well-documented in many species with marine larvae, including barnacles (Pechenik 1990, Nasrolahi 2007, Maréchal et al. 2012).

The mechanism through which delayed transitions are associated with mortality is an open question. In the case of non-feeding lecithotrophic larval stages (e.g. cyprids), this increased risk from delay has been linked to decreasing energetic reserves (Pechenik et al. 1998, Anil et al. 2001, Thiyagarajan et al. 2002). However, this argument does not apply for planktotrophic larval stages (e.g. barnacle nauplii) that continue feeding throughout the stage. A second hypothesis that could account for larval senescence in both feeding and non-feeding larvae has also been proposed wherein an 'internal clock' determines how long a larva can stay in a stage before it must either move on to the next stage or lose competence to initiate the transition and die (Zimmerman & Pechenik 1991, Zaslow & Benayahu 1996, Gebauer et al. 1998, 2003).

The reason usually given for delayed metamorphosis under both these hypotheses is that the larva has not yet found a suitable substrate or cue to initiate settlement (Pechenik 1990). Barnacle cyprids, like many other marine larvae, have been shown to respond to and settle more successfully when exposed to a conspecific settlement cue (Knight-Jones 1953). However, even in the presence of this cue, many of the cyprids in our study failed to settle. That we observed delayed transitions of nauplii (which feed and do not settle) and cyprids (which must settle but do not feed) strongly suggests that neither nutritional limitation nor insufficient settlement cues are primarily responsible for the failure to progress through development. We observed many cyprids swimming for well over a month after most others had completed settlement. Although cyprids may have had enough energy to swim for this long without having the energy to metamorphose (Lucas et al. 1979, Shilling et al. 1996, Thiyagarajan et al. 2003b), this at least suggests that energy depletion was not the only barrier to metamorphosis. We hypothesize that genetic and epigenetic failures involving transition-specific gene functions compromise the ability of many individuals to initiate metamorphosis or other major transitions. That said, if transitional life stages require more specific environments or more optimal nutritional states to succeed than do other life stages, and these more narrow tolerances lead to deaths being concentrated at these transitional stages, this also supports the Transitional Timing Hypothesis.

Expt 2, testing the resilience of larvae to temperature and salinity stress, again failed to find robustness increasing across developmental stages. We found all naupliar stages of *A. improvisus*, including N1, to be tolerant to the temperatures and salinities tested, with the exception of the lowest salinity (5) and the highest and lowest temperatures (0 & 40°C). Exposure to other salinity-temperature combinations resulted in nauplii that were mostly able to actively swim (Fig. 5). We also observed a high level of recovery for nauplii that were allowed to return to original culturing conditions following the end of the experimental exposure (Barnes 1953). While Expt 2 did not examine the environmental tolerances of juveniles and adults (non-swimming stages after the last major transition), it builds on a rich literature describing the environmental tolerances of different life stages of barnacles. The ability of each naupliar stage to cope with environmental stress is variable (e.g. Barnes 1953, Bhatnagar & Crisp 1965, Cawthorne 1978, Scheltema & Williams 1982, Harms 1986, Anil et al. 1995, Thiyagarajan et al. 2003a, Nasrolahi et al. 2006,

2012). The cyprid stage has decreased ability to tolerate temperature stress, with a marked increase in robustness following successful metamorphosis to the juvenile stage (Crisp & Ritz 1967). The adult stages of many species are remarkably tolerant to extreme temperature and salinity stress e.g. (Foster 1969, 1970, Fyhn 1976).

That the developmental stage with the narrowest environmental tolerances is the cyprid is surprising, as it is during this stage that individuals need to enter and colonize stressful environments (e.g. the rocky intertidal zone) where temperature and salinity can vary extremely and rapidly. Natural selection should tend to increase tolerances where and when they are most needed, but here they are weakest at the critical juncture. We hypothesize that this is a consequence of the physiological demands of the transitions an individual has just gone through and must prepare to go through imminently (Gosselin & Qian 1997). The cyprid's limited physiological strength, fully occupied with the metabolic demands of metamorphosis, may not be sufficient to simultaneously cope with salinity or temperature stresses. Similar failures of environmental tolerances during or just before major morphological transitions have been documented in a wide variety of organisms (e.g. fish: Ishibashi et al. 2007; corals: Negri & Hoogenboom 2011; mayflies: Wesner et al. 2014; crabs: Rey et al. 2015; and tubeworms: Lane et al. 2013) and are particularly well-studied in amphibians (Cupp 1980, Carey & Bryant 1995, Sherman & Levitis 2003, Warne et al. 2011). Increased mortality around such transitions may therefore be thought of as resulting from a temporary shortfall of physiological capacity.

Even while suffering through these physiological limitations and moving into new challenging environments, late-stage nauplii, cyprids, and new juveniles are using many genes and gene combinations for the first time in the animal's life. A barnacle's transcriptome and proteome change markedly at each major morphological transition (Thiyagarajan & Qian 2008, Li et al. 2010, Chen et al. 2011), concentrating opportunities for genetic or epigenetic defects to express themselves for the first time. Similar transcriptomic changes should be expected in any organism reorganizing its body plan (Fierro et al. 2007, Heyland et al. 2011, Ventura et al. 2013). The support we find for the Transitional Timing Hypothesis is likely due to the synergistic interactions of simultaneous physiological stress, ontological shifts in ecology, and rapid change at the molecular and cellular level.

While barnacle body-plan reorganizations are convenient test cases for the various risks associated with transitions, their underlying logic applies very broadly. The life histories of almost all biological organisms include stages of rapid change near the beginning of life. These transitions entail both stark transcriptional change (Conaco et al. 2012) and mortality risk (Levitis 2011). Tolerances to various stressors are lower in key developmental stages in a wide range of animals, including bivalves (Gobler et al. 2014), ascidians (Pineda et al. 2012), gastropods (Montory et al. 2014), sea urchins (Delorme & Sewell 2014), jellyfish (Conley & Uye 2015), insects (Woods 2013), and humans (Xu et al. 2014).

Furthermore, while the dangers of transitions are most easily detected during major developmental events, smaller and more gradual developmental changes are likely to entail mechanistically similar but more limited risks. Animals that lack major body reorganization events, such as most terrestrial vertebrates, may also suffer significant early losses due to the numerous changes occurring during early life. While other explanations, including the acquisition of robustness, likely explain ontogenescence in many cases, our results lend support to the hypothesis that the timing of transitions strongly influences the concentration of mortality in the very young.

While both an early concentration of transitions and an increased risk of mortality around these transitions are intuitively obvious and widely documented, we can't yet generalize about which transitions will be particularly dangerous, and in which ways. A better understanding of how transitional dangers cause ontogenescence could best be gained by quantifying various types of change (transcriptional, morphological, etc.) across stages in a developing cohort, and simultaneously documenting the mortality experienced by the same cohort. This would allow for quantification of dangers associated with each type of change. The detailed study of the mechanisms of ontogenescence is in its infancy.

In the context of marine population ecology, hazardous transitions compete with other causes of larval death. Most wild larvae likely die long before the risks associated with metamorphosis become relevant. However, by our admittedly rough estimate, half of those hatchlings who would otherwise reach adulthood die due to developmental failures. An additional fraction succumbs to environmental challenges that would not be deadly outside of the particularly vulnerable transitional stages. Population dynamics and density may be strongly affected by the resulting reduction in adult recruitment. Recently documented lower-than-expected larval mortality (White et al. 2014) may be counterbalanced by devel-

opmental mortality that is higher than commonly considered. Indeed, this study has examined only mortality in the larval stages; quantifying additional developmental mortality that occurs before and during hatching is the goal of ongoing additional experiments. We hypothesize that mortality is also high and concentrated around these transitions. The risks inherent to developmental transitions play an underappreciated role in the ecology of marine animals.

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