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Endostyle Cell Recruitment as a Frame of Reference for Development and Growth in the Urochordate *Oikopleura dioica*

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Abstract. In models of growth and life history, and in molecular and cell biology, there is a need for more accurate frames of reference to characterize developmental progression. In *Caenorhabditis elegans*, complete fate maps of cell lineage provide such a standard of reference. To be more widely applicable, reference frames should be easier to measure while still providing strong predictive capacity. Towards this aim, we have analyzed growth of the endostyle in the appendicularian *Oikopleura dioica* at the cellular level, and measured its response to temperature and food availability. Specifically, we test the hypothesis that age of a specific developmental stage in *O. dioica* can be predicted from the number of endostyle cells and temperature. We show that the endostyle grows by recruiting cells from the posterior tip into the lateral arms of the organ in an anterior-posterior orientation and that the rate of increase in lateral arm endostyle cells is temperature-dependent but unresponsive to nutritional intake. Endostyle cells therefore serve as an accurate and easily measured marker to describe developmental progression. Conceptually, such a method of characterizing developmental progression should help bridge life-history events and molecular mechanisms throughout organismal aging, facilitating cross-disciplinary understanding by providing a common experimental framework.

Introduction

Several approaches are used to study development and growth. At the subcellular scale, molecular studies reveal detailed mechanisms of how development is controlled (Hipfner *et al.,* 2002; Gordon *et al.,* 2005; Costa and Shaw, 2006). In life history, natural selection provides a central framework in the search for evolutionary explanations of organism traits such as growth pattern, age at maturity, number of offspring, and generation time (Stearns, 1992). Direct measurements of growth, and physiological processes such as respiration, assimilation, and feeding have led to general growth models (von Bertalanffy, 1957; West *et al.,* 2001) and elaborate species-specific simulation models (Touratier *et al.,* 2003; Nisbet *et al.,* 2004). These different approaches to the study of development and growth, from molecule to whole organism, have proceeded essentially independently. One difference between these approaches is that in contrast to many studies at the physiological and organismal level, investigations at the molecular level are generally not quantitative. Much of the problem with combining these studies is that quite different levels of organization are involved and there is no straightforward way to combine these approaches without a common reference system. Here we test the hypothesis that age of a specific developmental stage in the urochordate appendicularian *Oikopleura dioica* Fol, 1872, can be predicted from the number of endostyle cells and temperature, thus serving as such a reference system.

Recently we proposed a life-history model in which organism growth was driven by developmental progression connected to a nondimensional biological clock (Aksnes *et al.,* 2006a, b). Discrete cell cycle steps, intimately linked to genome readout and the molecular machinery of the organism, were selected to represent this clock. Data on the proliferation of cell lineages in the nematode *Caenorhab-*

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ditis elegans (Sulston and Horvitz, 1977; Sulston *et al.,* 1983) were used to determine parameters for the model. When the model was applied using species-specific parameters for *O. dioica*, the results were highly predictive for physiological rates that are commonly required as input to models (Aksnes *et al.,* 2006b). Although this model has thus far been applied only on organisms characterized by a relatively high degree of determinant development, the concept should stimulate research in bridging some of the gap between organismal growth, life-history traits, and the environment on one hand, with basic cellular mechanisms on the other (Kafatos and Eisner, 2004). Central to this approach is the framing of development in a reference system of a nondimensional sequence of events rather than to time *per se*.

Appendicularians are circumglobal filter-feeding organisms, second in abundance to copepods in marine zooplankton (Gorsky and Fenaux, 1998). Their unique filter-feeding strategy, which involves repetitive production of elaborately structured houses, allows them to capture a wide range of particle sizes, including pico- and nanoplankton (0.2–20 μm) (Flood and Deibel, 1998; Fernández *et al.,* 2004). This offers them a selective advantage in environments of low food concentration (Harbison, 1992; Acuña, 2001), and in ecosystems dominated by small particles (King *et al.,* 1980). A particularly well-studied species of appendicularian is *O. dioica.* The somatic growth of this species is programmed as a function of temperature, but it is not responsive to varying food concentrations as long as minimum food requirements are met (Troedsson *et al.,* 2002). Food resources above this level are directed to the reproductive organ, yielding clear differences in fecundity as a function of nutrition. This life-history strategy has been termed "clutch manipulation" (Aksnes and Giske, 1990), consisting of a fixed generation time in which fitness depends on a final state (Houston *et al.,* 1993)—in this case, clutch size. In contrast, "time manipulators" must reach a fixed final state, and fitness depends on how fast this is attained (Aksnes and Giske, 1990; Houston *et al.,* 1993). From the life-history strategy of *O. dioica* (Troedsson *et al.,* 2002), the idea emerged that growth could be more effectively represented by assuming that control of patterning and growth was specified according to a dimensionless developmental clock rather than by time or state variables such as mass.

Development can be defined as the change from an undifferentiated state to an increasingly more organized state. A developmental clock can be derived from detailed accounting of cell division fate maps such as that available for *C. elegans* (Sulston and Horvitz, 1977; Sulston *et al.,* 1983; but see also Schnabel, 1997, for assessment of variability in lineage determination); but for such an approach to be widely applicable, the clock should ideally be simpler and easier to measure while still providing strong predictive capacity. For *O. dioica*, increase in the number of endostyle cells as a measure of developmental progression might fulfill such requirements. The endostyle is a pharyngeal organ that is thought to be homologous with the follicular thyroid of vertebrates (Salvatore, 1969). It is present in urochordates, cephalochordates, and larval lampreys, and its function is secretion of a mucous net to retain particles during feeding (Olsson, 1965; Deibel and Powell, 1987; Hiruta *et al.,* 2005). Although it is reduced in the family Kowalevskiidae (Brena *et al.,* 2003), the endostyle in both Fritillariidae and Oikopleuriidae is an easily identifiable and well-demarcated organ positioned ventral to the mouth; it secretes a filter that traps particles for transmission to the esophagus (see Fig. 1). After metamorphosis in *O. dioica*, cell numbers in many tissues are fixed, and growth occurs principally through endoreduplication (Ganot and Thompson, 2002). The endostyle falls into this class of tissues, but the way in which endostyle cells with elevated ploidy are recruited into the lateral arms of the endostyle (see Fig. 2), and the relatively limited numbers of these lateral cells, greatly facilitates cell counting. The direct link of the endostyle to the feeding mechanism and preliminary observations during the course of other studies suggested a correlation between increased lateral arm cells and overall growth of the animal. Here we demonstrate that increase in the number of lateral arm endostyle cells provides a good reference system for developmental progression in *O. dioica*.

Materials and Methods

Culture of algae and animals

Fresh inoculates were made from static cultures of *Isochrysis* sp. (haptophyte, CCAP 927/14) and *Chaetoceros calcitrans* (bacillariophyte, CCAP 1010/11). The carbon content was 10.6 $\pm$ 0.3 pg C cell^{-1} in *Isochrysis sp.* and 4.6 $\pm$ 0.3 pg C cell^{-1} in *C. calcitrans* (Troedsson, 2005). Nutrients and silica (for *C. calcitrans*) were added, and the cultures were grown in 2-liter plastic bags under constant light (36W/20 cool white fluorescent light tube), at room temperature (20 °C) with air bubbles for agitation. The same strains were used throughout the experiments, and only algae in the exponential growth phase were used to avoid fluctuations in nutritional value.

Specimens of *O. dioica* were collected and cultured in a standard food regime as previously described (Troedsson *et al.,* 2002, 2005). To investigate the growth rate of the endostyle at varying food concentrations, animals were cultured under standard and limited food regimes at 15 °C. Animals were sampled at 24-h intervals post-fertilization. In the standard food regime, animals were fed a mixture of *Isochrysis* sp. and *Chaetoceros calcitrans*, each at a concentration of 2000 cells ml^{-1} (30.5 μg C l^{-1}) prior to 96 h and 4000 cells ml^{-1} (61 μg C l^{-1}) afterward. In the limited

food regime, the animals were fed 1/6 of the standard food regime. In a separate set of experiments to investigate the effect of temperature on the growth rate of the endostyle, animals were cultured under standard feeding regimes at 15 °C and at 20 °C, and sampled at 24-h intervals post-fertilization.

Characterization of the endostyle

BrdU pulse-chase feeding experiments were carried out to follow DNA replication in the endostyle. BrdU (Sigma) was dissolved at 100 μmol l^{-1} in 1-liter sterile-filtered (0.2 μm) seawater. Groups of 15–20 animals per age (day 2–day 4) were cultured in the presence of BrdU for 6 h and then fixed. Alternatively, animals at 48-h post-fertilization were cultured in 1 liter of 100 μmol l^{-1} BrdU for 15 h, and returned to standard culture condition (without BrdU) for an additional 36 h. The animals were then fixed in 4% paraformaldehyde (PFA) / 0.1 mol l^{-1} MOPS pH 7.4 / 0.5 mol l^{-1} NaCl / 5 mmol l^{-1} EGTA / 0.2% Triton X for 2 h at room temperature (RT, 20 °C). Samples were rinsed (5 min each) 3 times in PBS-T (phosphate buffered saline pH 7.4 / 0.1% Tween20) and DNA digested in PBS-T / 1% BSA; 1 mmol l^{-1} $MgSO_4$ / 10 units DNase I (Worthington) for 8 h at 4 °C. Digestion was stopped by adding EDTA (5 mmol l^{-1} final concentration). Animals were then incubated at 4 °C for 3–5 days with a 1:100 dilution of Rat anti-BrdU primary antibody (Accurate Chemical, BU1/75) in PBS-T / 1% BSA (Ganot and Thompson, 2002). Samples were rinsed 3 times in PBS-T, post-fixed in 3% PFA / PBS pH 7.4 for 30 min, rinsed 3 times in PBS-T, blocked in PBS-T / 1% BSA, and incubated for 48 h at 4 °C with a 1:1000 dilution of rhodamine redX conjugated anti-rat IgG secondary antibody (Jackson Immunoresearch). Samples were then washed in PBS-T, incubated 10 min in PBS-T / Topro-3 (Molecular Probes, Invitrogen) (1:1000 dilution) at RT to counterstain DNA, washed in PBS, and mounted in Vectashield medium. Confocal images were obtained with a Leica TCS-SP confocal microscope and Leica ver. 2.5 software.

DNA ploidy measurements were performed according to Ganot and Thompson (2002). After fixation, endostyle and accompanying oral glands from adult animals were manually microdissected, chromatin was stained with propidium iodide, and tissue was mounted on microscope slides. DNA content was quantified with a Leica laser scanning cytometer equipped with Win-Cyte 3-3 software, and haploid genome content was estimated by comparing DNA index values of individual nuclei with the DNA index of mature sperm (C = 1).

Counting endostyle cells

Animals were cultured at 15 °C *versus* 20 °C under a standard food regime or, alternatively, under standard *versus* limited food regimes at 15 °C (Troedsson *et al.,* 2002). At 24-h intervals, samples of 10 animals were collected from each of the experimental conditions and fixed in 4% PFA / 0.1 mol l^{-1} MOPS pH 7.4 / 0.5 mol l^{-1} NaCl / 5 mmol l^{-1} EGTA / 0.2% Triton X for 2 h at RT. Samples were washed in PBS-T for 3 min and DNA counterstained for 5 min as above. After washing for 3 min in PBS-T, samples were mounted in Vectashield, and endostyle nuclei were enumerated with the Leica TCS-SP confocal microscope as above. Developmental stage (i) was defined as the number of large (post-rapid replication) polyploid nuclei in one of the lateral rows of the endostyle (Figs. 1 and 2). The number of cells in each of the lateral arms was identical, and counts refer to one side only. The number of endostyle cells, as a representation of developmental stage, was plotted against the age of the animals, and exponential regressions were fit to the data.

Growth response

In parallel with endostyle cell counts, 10 additional animals from each sampling point were removed from each incubation beaker for analysis of trunk length. The animals were randomly collected and fixed in 4% PFA / 0.1 mol l^{-1} MOPS pH 7.4 / 0.5 mol l^{-1} NaCl / 5 mmol l^{-1} EGTA / 0.2% Triton X. The trunk length of the animals was further measured microscopically (Nikon SMZ 1500 with a Nikon camera digital sight DS-5M) using Eclipse Net software (ver. 1.16.5).

Parameter derivation of the developmental model

The developmental time model of Aksnes *et al.* (2006a) assumed cell cycling, rather than body mass, as a reference system for developmental progression. The age (t) of a developmental stage (i) at a given temperature (T) was defined by the equation

$$t = be^{xi-aT} \quad (1)$$

where (b) is related to the length of the first developmental stage at 0 °C, (a) is the temperature sensitivity, and (x) is a coefficient describing how age increases with development. Here, we used discrete steps of endostyle cell increase (i) as our frame of reference for developmental progression. The relationship between the temperature sensitivity parameter (a) and the Q_{10} coefficient is $a = (\ln Q_{10})/10$. By measuring the number of endostyle cells at 15 °C and 20 °C each 24 h throughout the life cycle, we could estimate parameters similar to those in the developmental time model. To obtain estimates of the parameters for the endostyle, we rewrite Eq. 1:

$$\ln(t) = \ln(b) + xi - aT \quad (2)$$

Rearranging Eq. 2 and making a linear regression utilizing $\ln(t)$ and i to estimate A yields

$$\ln(t) = A + xi \quad (3)$$

where, $A = \ln(b) - aT$, represents the elevation, while x represents the slope.

Statistical analysis

To test the effect of temperature and food regime in the regression between time and developmental stage, an ANCOVA (Statistica 6.1) was performed using temperature or food regime as covariates. When there was a significant effect of the covariate, linear regression (Statistix 8.0) was used to investigate whether the difference between the groups was due to the effect of slope or elevation in the regression. Parameters for the endostyle were estimated using a nonlinear, least square estimation in Statistica 6.1. To test whether the growth curves at 20 °C and 15 °C were significantly different when they were plotted as a function of time or developmental phase, a residual analysis was preformed (Suthers, 1998) by fitting polynomial regressions to the whole data sets to ensure the best possible fit of all data. Residuals from each individual data point were then calculated and a Student's *t*-test was performed comparing the two temperatures after confirmation of homogeneity of variance (Barlett's test). To ensure an unbiased representation of the two temperature regimes by the polynomial regression, the last sampling point at 15 °C (168 h) was excluded from the analysis because this point did not exist at 20 °C.

Results

Characterization of the endostyle

Endostyle nuclei increase in ploidy through endoreduplicative cell cycles (Fig. 1), as do nuclei in many tissues of *O. dioica* (Ganot and Thompson, 2002). The growth of the endostyle was principally along the anterior-posterior axis (Fig. 2) BrdU labeling experiments to visualize *de novo* DNA synthesis at various stages post-fertilization demonstrated that cells were recruited to enter endocycles and move from the posterior tip of the endostyle into the lateral arms (Fig. 2A, B). The lateral endostyle cells then slowed their rates of DNA replication, attaining final ploidies of 194 ± 26 C (Fig. 2C). This produces a symmetrical horseshoe-shaped secretory organ characterized by actively growing and replicating nuclei at the posterior tip and nuclei of very similar diameter along the anterior-posterior arms.

Increase in number of endostyle cells with respect to time, temperature, and nutrition

The simple and easily quantifiable pattern of endostyle growth led us to investigate its suitability as a reference for developmental progression. We first studied the correlation of age (t, time after fertilization) of the animal with developmental stage (i) expressed as the number of lateral endostyle cells in response to different temperatures. The model of Eq. 1 was fitted to data at 15 °C, $t = 21.8e^{0.128i}$ ($P < 0.001$) (Fig. 3). Here, variation in developmental stage (i) accounted for 94% of the variance in age (t). Similarly, at 20 °C we obtained $t = 17.9e^{0.124i}$ ($P < 0.001$), which accounted for 93% of the variance in age (t) (Fig. 3). An ANCOVA, using temperature as covariate, yielded a significant difference between 15 °C and 20 °C ($n = 243$; $F = 165.81$; $P<0.01$). Further statistical investigation by a linear regression yielded significant differences of the A parameter (elevation) in Eq. 3, while the parameter x (slope) was not statistically different between the temperature regimes (Fig. 3, Table 1). A Bartlett's test revealed that the two regressions did not have significantly different variance (Table 1).

In describing how age increases with developmental progression, a temperature-dependent relationship can be observed. This is exemplified by plotting body size increase for *O. dioica* at 15 °C and 20 °C against age as the independent variable (Fig. 4A), and comparing this to the same data plotted against developmental stage (i) as the

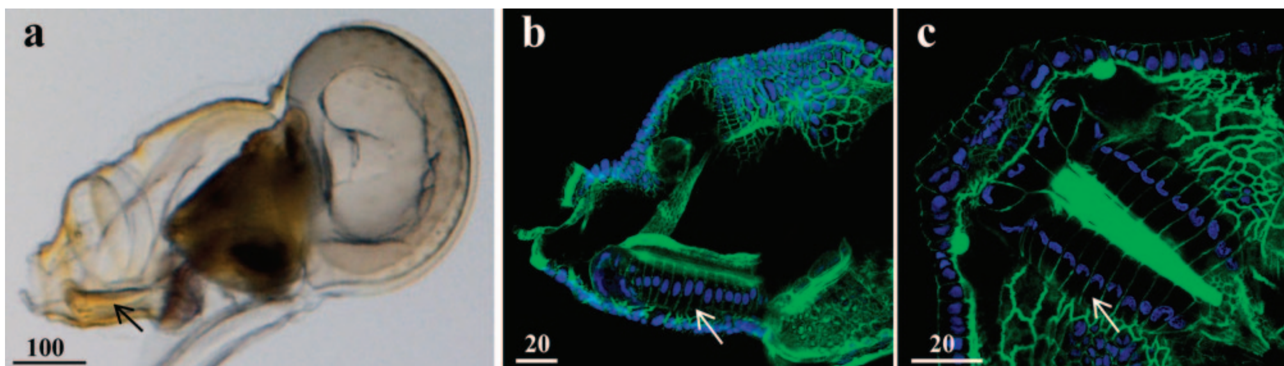

Figure 1. Location and morphology of the *Oikopleura dioica* endostyle. (a) Light micrograph of a day 4 animal, with mouth to the left and endostyle indicated by a black arrow. Confocal images show lateral (b) and dorsal (c) views, with endostyle indicated by white arrows. F-actin delimits cell boundaries (green) with nuclei stained for DNA (blue). Scale bars in μm.

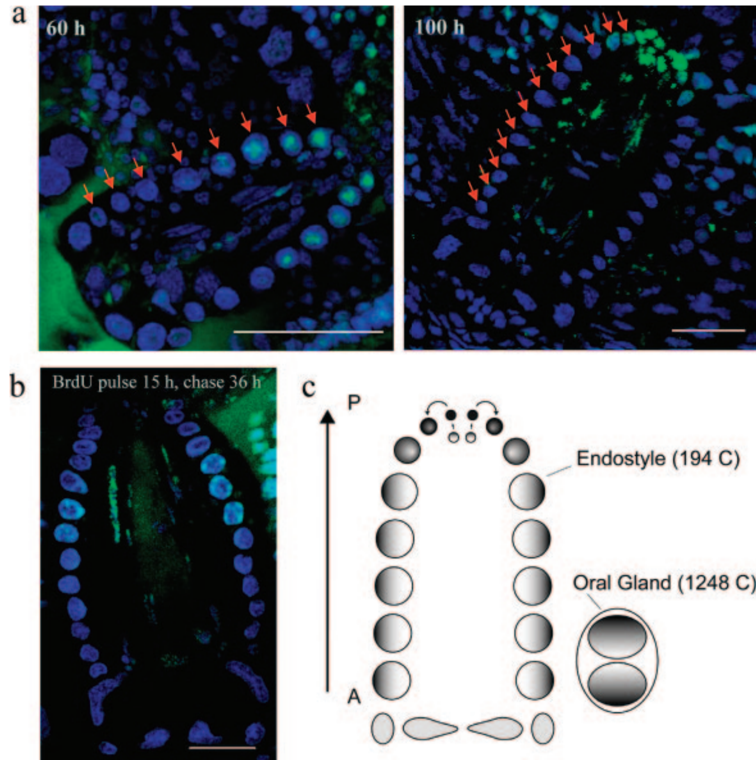

Figure 2. Growth of the *Oikopleura dioica* endostyle. Pulses of BrdU were administered at various developmental stages, and DNA replication was assessed in stacks of confocal images encompassing the endostyle (DNA, blue; BrdU, green). (a) The number of large polyploid nuclei in the lateral arms of the endostyle increased with time (60 *vs.* 100 h). Small nuclei in the posterior tip of the horseshoe-shaped endostyle were highly replicative and were symmetrically recruited into the growing lateral arms, where rates of DNA replication decreased. This resulted in chains of lateral nuclei with very similar diameters. The cells included in lateral arm counts are indicated by red arrows. (b) BrdU pulse-chase experiments demonstrate that tip nuclei exposed to BrdU overnight (D2-3) have migrated to a mid position along the lateral arms by D5. Unlabeled posterior nuclei were from cells in a pre-replicative state at D2-3, while those in more anterior positions had already been recruited to the lateral arms during the D2-3 pulse and had dramatically slowed their rates of BrdU incorporation. (c) Schematic representation of the endostyle (only nuclei of individual cells are drawn). Growth is along the anterior-posterior axis of the tissue. Throughout development, cells at the posterior tip were progressively recruited and entered a phase of rapid endocycles. As their ploidy increased, pairs of cells at the tip moved laterally to form symmetric sides of the endostyle. Once the cells were established in this lateral position, the rate of DNA replication decreased (indicated approximately by differential degree of shading). This growth mode was in striking contrast to the adjacent oral gland, in which DNA continued to replicate throughout most of the life cycle. The average ploidies of lateral endostyle and oral gland nuclei in adult animals are expressed in C (multiples of haploid genome content). Scale bars = 20 μm.

independent variable (Fig. 4B). Whereas the two growth curves are significantly different in the former plot (polynomial regression, [$y = -0.0002x^3 + 0.0937x^2 - 2.8332x + 174.35$], $r^2 = 0.765$; residual *t*-test between 20 °C and 15 °C, $P<0.0001$), they are similar in the latter (polynomial regression, [$y = -0.0328x^5 + 1.4371x^4 - 22.973x^3 + 169.96x^2 - 562.82x + 826.35$], $r^2 = 0.805$; residual *t*-test between 20 °C and 15 °C, $P = 0.988$), fulfilling the desired criteria for lateral endostyle cell numbers to serve as a temperature-insensitive reference system for developmental progression.

In conformity with one of the criteria for the developmental clock, we observed that differential food availability had no influence on the time required to attain a given developmental stage (i) (Fig. 5). The model of Eq. 1 was fitted to data under the standard food regime, yielding $t = 20.9e^{0.127i}$ ($P < 0.001$). Here, developmental stage (i) accounted for 93% of the variation in age (t). Similarly, data from the limited food regime were fitted to Eq. 1, yielding $t = 20.8e^{0.130i}$ ($P < 0.001$), which accounted for 91% of the variation in age (t). An ANCOVA, using food regime as covariate, yielded no significant difference between the standard and the limited food regime ($n = 182$; $F = 1.454$; $P = 0.23$). Since there was no significant difference between the food regimes in the ANCOVA analysis, no further statistical analysis was conducted.

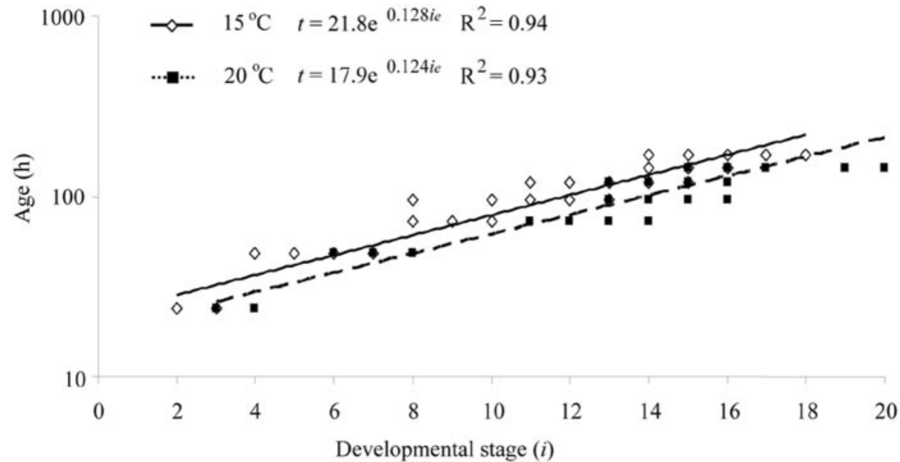

Figure 3. Age (h post-fertilization) as a function of developmental stage (*i*), defined as the number of lateral endostyle cells in animals cultured under standard food conditions at 15 °C (open diamonds) and 20 °C (filled squares). By fitting the indicated exponential functions to the data at 15 °C (solid line) and 20 °C (broken line), variation in developmental stage accounted for 94% and 93%, respectively, of the variance in age. There was a significant difference in the elevation between the two temperature regimes (Table 1).

The parameters for the developmental time model (Aksnes *et al.,* 2006a) (Eq. 1) were estimated for the whole data set (Table 2), using endostyle cells as a frame of reference. This yielded the following model to describe how age changes with developmental progression and temperature:

$$t = 53.1e^{0.107i-0.043T} \tag{4}$$

This model adequately explained the observations from all experiments in this study ($P < 0.001$). Variation in all significant ($P < 0.001$) parameters—*b*, *x*, and *a*—accounted for 92.5% of the variance in age (Table 2). This indicated that the whole body developmental time model (Eq. 1) can be applied to a simpler organ-based reference system.

Discussion

Integrating biochemical and cellular mechanisms into an understanding of growth and life-history strategies remains an important objective in biology (Kafatos and Eisner, 2004). Toward this objective, it is important to define convenient, accurate reference systems that describe developmental progression. Most models of growth and life history use body mass as the state descriptor of the organism (Stearns, 1992; Fiksen, 1997; West *et al.,* 2001; Touratier *et al.,* 2003; Nisbet *et al.,* 2004). In these models, life-history events are commonly assumed to be linked to organism size. However, it seems biologically more plausible to link life-history events to developmental stage. In the modeling framework of Aksnes *et al.* (2006a, b), it was hypothesized that age could be derived from developmental progression and temperature (Eq. 1). In the present study, we tested this hypothesis for *Oikopleura dioica*, by equating the nondimensional developmental clock (*i*) with cell recruitment into the lateral arms of the endostyle and the ambient temperature.

The growth of the endostyle is characteristic for two reasons. First, a pair of cells at the organ's tip is recruited for a limited number of rapid endocycles (between six and seven rounds of genome replications without intervening mitosis). The cell pair will then migrate into the lateral arms, where the rate of replication decreases. This is in striking contrast to the oikoplastic epithelium of the animal, where all cells endocycle with increasing rates throughout the life cycle (Ganot and Thompson, 2002). Second, the endostyle increases in size in parallel with increased size of the trunk of the animal and increasing size of the repetitively secreted filter-feeding houses. Thus, the increased capacity to filter more food particles from the water column is coupled to the increased capacity of the endostyle mesh to capture these particles and introduce them to the digestive tract. The growth of the endostyle is therefore linked to overall growth and the animal's ability to capture prey particles. Both somatic growth and house production have

Table 1

Regression analysis of logarithmic age (t) versus *endostyle stage* (i) *at 15 °C and 20 °C*

Parameter	*F*	df	*P*
Equality of variances	1.01	96/143	0.4732
Comparison of slopes	1.04	1/239	0.3084
Comparison of elevations	165.81	1/240	<0.001

F-distribution value; df, degrees of freedom; *P*, probability.

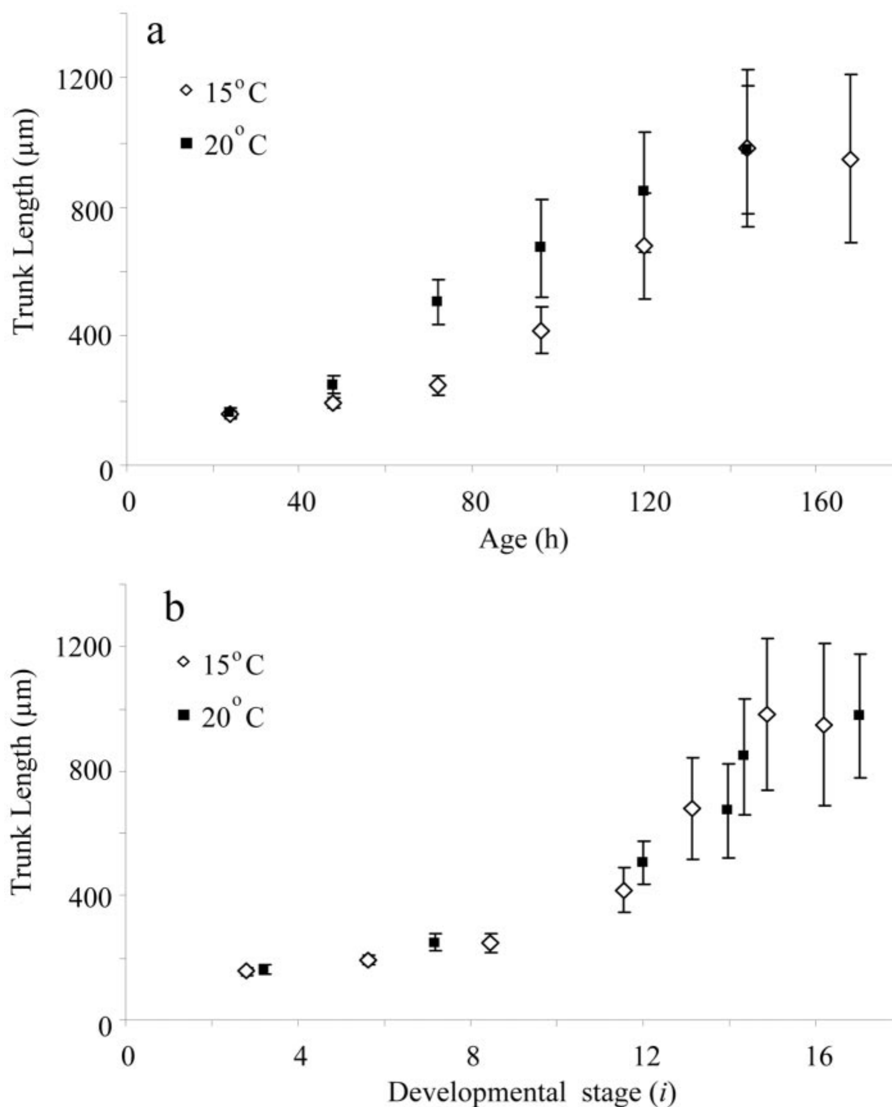

Figure 4. Growth (trunk length) of *Oikopleura dioica* expressed as a function of: (a) age (h post fertilization) or (b) developmental stage (i), at 15 °C (open diamonds) and 20 °C (filled squares). Means with standard deviations are plotted.

previously been suggested to be programmed as a function of temperature (Sato *et al.,* 2001; Troedsson *et al.,* 2002), a criterion well suited for a developmental clock. Although these systems provide a non-invasive reference, house production as a developmental clock has obvious practical limitations, while body length, as shown below, introduces larger variation in estimations of age.

The developmental progression derived from endostyle cells was highly dependent on ambient temperature (Fig. 3). In addition, when trunk length of *O. dioica* was plotted as a function of developmental stage (i) (Fig. 4B) as opposed to time (Fig. 4A), the growth curves at 15 °C and 20 °C became much more closely aligned. This demonstrated that neither age nor temperature affects the developmental sequence, but translation of the developmental sequence into age must take into account the temperature effect. Thus, in our framework, age expresses the amount of time required to reach a precisely defined developmental stage at a given temperature.

Endostyle cell recruitment as a frame of reference for development and growth in O. dioica

A common reference system to describe developmental progression of an organism has been through day degrees (Belehrádek, 1935). In this model, time and temperature from one developmental stage to another is parameterized. However, there is no implicit representation of the devel-

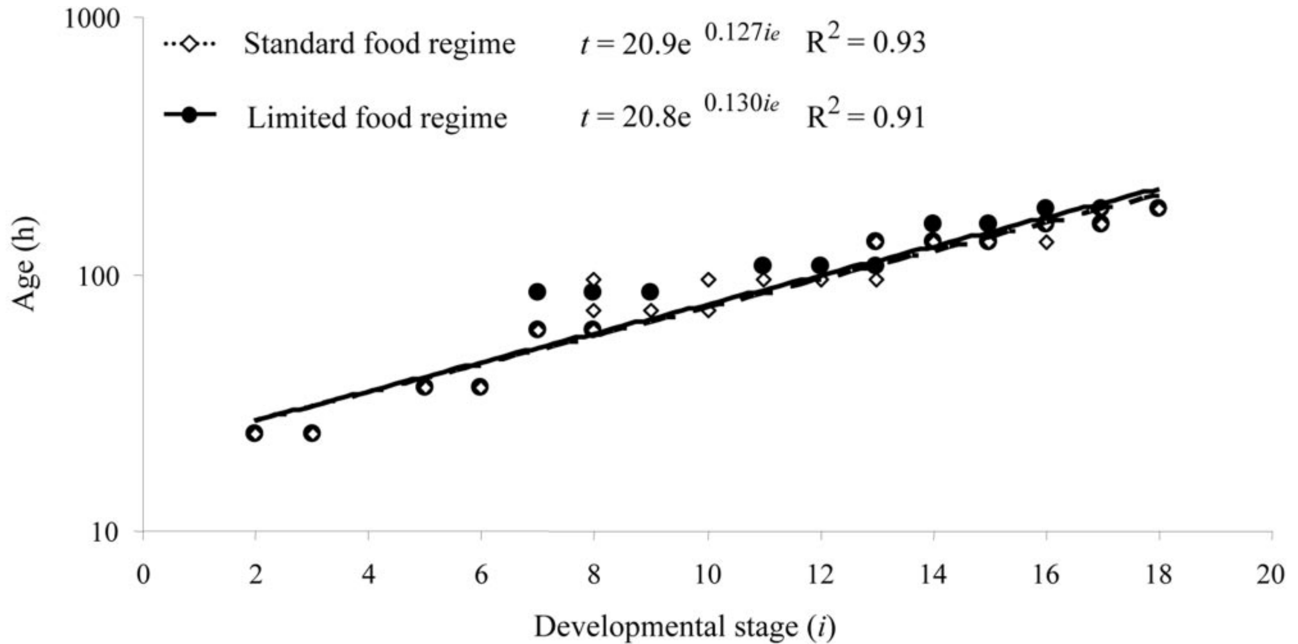

Figure 5. Age (h post-fertilization) as a function of developmental stage (*i*), in animals cultured under standard (open diamonds) and limited (filled circles) food regimes at 15 °C. By fitting the indicated exponential functions to the data for animals cultured under standard (broken line) and limited (solid line) food regimes, variation in developmental stage accounted for 93% and 91% of the variance in age. An ANCOVA analysis yielded no significant difference between the two food regimes.

opmental stage. Recently, a model was developed integrating mass, temperature, and time (Gillooly *et al.,* 2002). In this model, age is determined through knowledge of ambient temperature and body mass. In parallel with measurements of endostyle cells, we measured the body length of the animals. This allowed us to apply the model of Gillooly *et al.* (2002) to our data, using body mass to represent developmental stage. However, the models of Aksnes *et al.,* (2006a, b) and Gillooly *et al.* (2002) used a different parameterization of the temperature coefficient, so each temperature was tested separately. Included in our analysis, we used the experiment at 15 °C with both food regimes (Fig. 5) because there was no significant difference between the two. Because we do not take the temperature effect into consideration, we rewrite Eq. 5 in Gillooly *et al.* (2002) as

Table 2

Parameters for the developmental time model (Eq 1) using the endostyle as a frame of reference

Parameter	Value[1]	95% CI[2]	P[3]
b	53.1 ± 2.9	47.4–58.7	<0.001
x	0.107 ± 0.002	0.104–0.110	<0.001
a	0.043 ± 0.003	0.037–0.049	<0.001

[1] Estimated value with standard error; [2] confidence interval; [3] probability.

$t = k{*}m^{1/4}$, where t is time, m is body mass, and k is a parameter that is constant at a given temperature (k represents the right side of Eq. 5 in their model). Converting total body length to mass using the regression of King *et al.* (1980), $l = 520W^{0.3806}$, variation in mass accounted for 77.7% ± 7.3% of the variance in age. The use of endostyle cells as the state descriptor, on the other hand, accounted for 91.1% ± 4.3% of the variance in age (Note that we are using Eq. 1 without the temperature expression $b_{0*}e^{-aT}$). Previous studies have demonstrated that the total body length (trunk + gonad), and therefore mass, was dependent on both food concentration and temperature in *O. dioica* (Troedsson *et al.,* 2002). In contrast, the increase in endostyle cell number did not depend on nutritional intake (Fig. 5), and therefore seems to be a more accurate predictor, together with temperature, for age in *O. dioica*. Furthermore, many organisms do vary significantly in mass due to external factors such as varying nutritional intake. Mass would then be a relatively poor descriptor of developmental stage. Much of the variance in models using mass as a state descriptor of an organism could therefore be due to variation in nutritional intake—as, for example, would be evident in any survey comparing human mass and age. Thus, there is a clear need for more accurate state descriptors in these types of models.

Although the endostyle seems to be an attractive morphological criterion for describing developmental progres-

sion in *O. dioica*, this particular organ has a limited phylogenetic distribution and thus cannot serve as a universal reference frame. Furthermore, although the counting of lateral arm endostyle cells serves as an accurate descriptor of developmental stage, it is an invasive method. Live imaging of the endostyle using low concentrations of DNA stains such as Hoechst could be envisaged. This method, unlike the one used in our study, would avoid the need to kill the animal to determine endostyle counts, but it still is not fully noninvasive, because the animals are exposed to the DNA dye and to wavelengths of light required to excite these fluorochromes, as well as to microscopic observation. Nonetheless, it is an example of an easily measurable developmental clock, providing a reliable and accurate frame of reference for a range of studies.

Endostyle cell recruitment as a frame of reference for cell biology and gene expression studies in O. dioica

The endostyle clock appears to be a valid frame of reference for development in life-history modeling of *O. dioica*. It should also prove useful in providing an invariant reference chronology for molecular developmental studies. Age (*i.e.,* time post-fertilization) is frequently used as a reference scale (Boehm and Slack, 2005; Søviknes *et al.,* 2005; Ganot *et al.,* 2007), but variability in experimental conditions, particularly with respect to temperature, introduces inaccuracies that may complicate and even invalidate comparisons between studies. Indeed, relative time has been used by many developmental biologists. This is a useful reference scale for developmental stages where discrete morphological signposts (*e.g.,* hatching and metamorphosis) are recognized, so it has primarily been used for embryonic stages. Often, however, such reference scales are lacking for later stages of development. Describing developmental progression as discrete steps of endostyle cell recruitment provides an accurate morphological criterion upon which to frame molecular events throughout the life cycle of *O. dioica*. For example, a developmental cue (a specific gene or gene cascade) might be interpreted as being somewhat flexible because it is activated in a relaxed window of time post-fertilization. However, this might not be true throughout development. In actuality, the same cascade might be tightly bound to a specific developmental stage. Because of individual variation in time to reach a given developmental signpost—variation that might be due to external factors—the investigator erroneously concludes that activation of the gene cascade is somewhat flexible. This error will complicate models aiming to assemble molecular pathways. However, if a reference system for developmental progression, such as the number of endostyle cells in *O. dioica,* is used, the cascade would be anchored to a specific developmental stage. Independent studies of the same model organism could also be more easily, accurately, and directly compared, leading to improved understanding of restrictions and requirements for defined developmental pathways. A more accurate coupling of developmental stage and life-history characteristics will provide better understanding of molecular mechanisms underlying key life-history events.

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