Time course for tail regression during metamorphosis of the ascidian larvae

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1. Abstract

In most ascidians, the tadpole-like swimming larvae dramatically change their body-plans during metamorphosis and develop into sessile adults. The mechanisms of ascidian metamorphosis have been researched and debated for many years. Until now information on the detailed time course of the initiation and completion of each metamorphic event has not been described. One dramatic and important event in ascidian metamorphosis is tail regression, in which ascidian larvae lose their tails to adjust themselves to sessile life. In the present study, we measured the time associated with tail regression in the ascidian *Ciona intestinalis*. Larvae are thought to acquire competency for each metamorphic event in certain developmental periods. We show that the timing with which the competence for tail regression is acquired is determined by the time since hatching, and this timing is not affected by the timing of post-hatching events such as adhesion. Because larvae need to adhere to substrates with their papillae to induce tail regression, we measured the duration for which larvae need to remain adhered in order to initiate tail regression and the time needed for the tail to regress. Larvae acquire the ability to adhere to substrates before they acquire tail regression competence. We found that when larvae adhered before they acquired tail regression competence, they were able to remember the experience of adhesion until they acquired the ability to undergo tail regression. The time course of the events associated with tail regression provides a valuable reference, upon which the cellular and molecular mechanisms of ascidian metamorphosis can be elucidated.

2. Introduction

Metamorphosis is a representative event for indirect developmental species to change their body-shape and life-style. Ascidians are a major group of the subphylum Urochordata (Tunicate), the sister group of the Vertebrata, in the phylum Chordata (Delsuc et al., 2006; Lemaire, 2011). The ascidians are famous in undergoing metamorphosis to be adult (Cloney, 1978). The tadpole-like swimming larvae of ascidians have similar body structures to those of vertebrate larvae such as the dorsally located nerve cord and notochord (Lemaire et al., 2002). However, ascidians dramatically change their body plans to develop into sessile adults during metamorphosis, and larval tadpole-like structures apparently degenerate and disappear (Cloney, 1978). Ascidians are the only chordates to adopt a sessile lifestyle. This suggests that ascidians acquired the sessile lifestyle after divergence from the common ancestor of chordate (Wada, 1998). The mechanisms of evolution in the ascidian lineage can partly be explained by the idea that ascidians acquired unique genes such as the gene encoding cellulose synthase in order to live in the sessile style (Dehal et al., 2002; Nakashima et al., 2004; Sasakura et al., 2005). However, most of genes in the ascidian genome are shared with other chordates (Dehal et al., 2002), and these shared genes may have also been recruited for use in ascidian metamorphosis (Nakayama et al., 2001, 2002; Chambon et al., 2002, 2007). I am interested in understanding how ascidians acquired their unique lifestyle. For this purpose, it is important to elucidate the molecular mechanisms of metamorphosis in extant ascidians.

The metamorphic events of ascidians can be roughly divided into 10 (Cloney, 1982), and the total time required for completing metamorphosis is about two days (Cloney, 1978), which is considerably less time than that required by *Xenopus laevis* (43

days; Nieuwkoop and Faber, 1994) and *Drosophila melanogaster* (7 days; Bainbridge and Bownes, 1981). Due to the quick metamorphosis and transparency of the animals, ascidians provide excellent experimental systems in which to uncover the mechanisms of animal metamorphosis. (Cloney, 1966, 1969; Hirano and Nishida, 1997; Satoh et al., 2003; Karaiskou et al., 2015).

The overview of development of ascidians is as follows. The fertilized eggs of ascidians quickly develop within the chorion, and developed larvae hatch from the membrane. The hatched larvae swim for a certain period (Cloney, 1982; Degnan, 2001). The swimming larvae adhere to substrates such as rocks with their adhesive papillae located at the anterior-most part of the trunk (Cloney, 1982; Degnan, 2001). This adhesion is regarded as the first event in ascidian metamorphosis. Following the adhesion, other metamorphic events are initiated, such as tail regression, body axis rotation and the development of adult organs (Nakayama-Ishimura et al., 2009). After a certain amount of time has elapsed after adhesion, tail regression occurs, and the tail cells move into the trunk region (Cloney, 1961; Degnan, 2001). After tail regression, the body axis rotates through an arc of about 90°, and adult organs such as the gill slits, endostyle, and digestive tube are formed as the ascidians develop into juveniles that closely resemble adults (Cloney, 1982; Chiba et al., 2004).

In the metamorphosis of some mollusks and amphibians, it has been reported that the animal acquires metamorphic competence: the ability to start metamorphic events, according to specific developmental timings (Tata, 1968; Nadeau et al., 1989; Pechenik and Gee, 1993; Shi et al., 1996; Avila, 1998; Bishop and Brandhorst, 2003; Bishop et al., 2006). It has also been suggested that ascidians must acquire competence for metamorphosis (Ishikawa and Numakunai, 1972; Davidson and Swalla, 2001). In

the ascidian *Boltenia villosa*, the transcription and activation of particular genes, for example *Cnib*, regulate the acquisition of early metamorphic competence (Davidson and Swalla, 2001). *Cnib* is expressed in the papillary region, which is thought to be important for the acquisition of competence in several ascidians (Eri et al., 1999; Davidson and Swalla, 2001; Nakayama et al., 2001). It is also reported that in the ascidian *Halocynthia roretzi,* the time-dependent activation of acid phosphatase in microblasts, including many large granules similar to lysosomes, around the notochordal shelf contributes to the acquisition of tail-regression competence (Ishikawa and Numakunai, 1972). In most ascidian species, many factors are thought to be involved in the acquisition of metamorphic competence (Degnan et al., 1997; Eri et al., 1999; Bishop et al., 2001; Davidson and Swalla, 2001; Chambon et al., 2002, 2007; Kimura et al., 2003; Zega et al., 2005), although the knowledge is fragmental, and further investigations are necessary to comprehending mechanisms how ascidians acquire the competence for metamorphosis.

Prior to that, however, it is necessary to describe the basic information associated with the metamorphosis of ascidians as research on early ascidian development such as the fate determination required accurate cell lineages (Nishida, 1987; Kumano et al., 2014). Particularly, the total accurate time course of the events including when the metamorphic events start, how long they take, and when they finish must be described because this information has not been well reported in ascidians.

For this purpose, I first investigated the timings associated with *C. intestinalis* tail regression; the representative event of ascidian metamorphosis which converts the swimming larval body into its sessile adult form. The sub events in tail regression are as follows. First, swimming larvae are thought to acquire competence for adhesion at a

certain developmental stage after hatching (Ishikawa and Numakunai, 1972). After this event, larvae adhere to the substrate with their papillae which are protrusions at the anterior tip of the trunk. The adherent larvae, however, usually do not start tail regression immediately, suggesting the need to acquire another competence-factor for tail regression and/or a requirement for continuous adhesion to induce tail regression (Ishikawa and Numakunai, 1972). After larvae meet these conditions, their tails start to regress toward their trunks. To investigate this, I recorded larval behavior by means of videography and measured the time required for the initiation and completion of each event constituting tail regression. From these data, I estimated the time required to acquire adhesion and tail regression competence. In addition, I showed that *C. intestinalis* larvae have the ability to remember the experience of adhesion for several hours until the competence for tail regression is acquired.

3. Material and methods

3.1. Animals

Wild-type *C. intestinalis* was collected from or cultivated at Misaki (Tokyo), Maizuru (Kyoto), Mukaishima (Hiroshima) and Usa (Kochi). Animals were maintained under constant light to induce oocyte maturation. Eggs and sperm were isolated surgically from the gonoducts, and they were mixed in seawater in petri dishes to obtain fertilized eggs for the following experiments.

3.2. Temperature and density experiments

Fertilized eggs were reared at 16°C, 18°C or 20°C in 3 ml seawater. A total of 1,000 hatched larvae were transferred to new dishes, and the starting times of adhesion and tail regression were measured by observing the samples in 15-min intervals. In the temperature and density experiments, I estimated the times of the initiation of adhesion and tail regression as the respective times when I first observed a larva adhering to the substrate or starting the process of tail regression. To observe the effect of the larval density on the progression of metamorphosis, 1, 10, 50, 100, 500, 1,000 and more than 1,000 larvae were reared at 18°C in 3-cm diameter petri dishes containing 3 ml seawater.

3.3. Assessment of adhesion

50 hatched larvae were anesthetized with 0.02% MS-222 (Sigma-Aldrich Co., LLC.) in seawater to prevent swimming. 50 larvae were then transferred to a 3-cm diameter petri dish filled with seawater containing 0.02% MS-222. After larvae reached

the bottom of the petri dish, the dish was swirled gently. The movement of larvae during swirling was observed by a microscope to see whether larvae adhered or not to the bottom plane of the petri dish. Adhered larvae could not move from the point of adhesion.

3.4. Assessment of tail regression

I used 3-cm diameter petri dishes containing 700 larvae in 3 ml of seawater for recording. During recording, the plastic covers of the petri dishes were removed. To prevent seawater evaporation during the recording, the seawater surface was covered with silicon oil (Shin-Etsu Chemical Co., Ltd.). The petri dishes were set on a black plastic sheet, and they were lighted from the lateral side. Recording was carried out for 24 hours at 18°C using an OLYMPUS SZX12 microscope connected with a SONY HDR-CX590V video camera via a MAHDS-CX270/590VM and a C mount adapter (MeCan Imaging Inc.; Figs. 2A and B and Movie 1). Recorded larvae were observed one by one, and the time at which adhesion began and the times at which tail regression began and was completed were measured. The start of tail regression was defined as the time when a larva stopped swinging its tail and showed bending of the tail. The angle of bending was measured using MicroMeasure software v.1.07.1 (Scalar Co., Ltd.). The end of the tail regression was defined as the time when all of the tail region had regressed into the trunk (Fig. 2C).

3.5. Examination of the relationships between the durations of adhesion and tail regression

Swimming larvae that acquired tail-regression competence were cultured in 6-cm diameter petri dishes whose surfaces were covered with 2% agar to prevent adhesion. The larvae were transferred into non-coated same size dishes to start adhesion, and soon after that the petri dishes were washed three times with seawater to remove non-attached larvae. This process was done to leave larvae attached to the bottoms of the petri dishes immediately after transfer. These larvae were allowed to adhere for a limited time, and then they were detached by pipetting. Detached larvae were transferred into agar-coated dishes to prevent further adhesion, and the occurrence of tail regression was observed five minutes after adhesion. As a negative control, swimming larvae which did not experience adhesion were examined.

3.6. Verification of memory of experienced adhesion

In order to examine whether larvae can memorize the experience of adhesion, swimming larvae at the pre-competent stage with regards to tail regression were allowed to adhere for a limited time as described above. Approximately half the tail of each adhering larvae was removed with a razor to prevent further adhesion as previously described (Nakayama-Ishimura et al., 2009), followed by detachment. After the time to acquire tail-regression competence had elapsed, the occurrence of tail regression was observed. As a negative control, swimming larvae which did not experience adhesion were also examined by cutting their tails.

3.7. Statistical analysis

Student's t-test, the one-way ANOVA test and Tukey's test were used to show the significance of the differences. The time required to acquire tail-regression competence was analyzed by a segmented program involving the Akaike Information Criterion (AIC) and Evidence Ratio (ER) using R-software v.3.2.0 (R statistical software, Ihaka and Gentleman, 1996). AIC is a program that determines whether the linear or curved line fits best against the data plots (Akaike, 1973). The model with the lowest AIC value is considered to be the best descriptor of the data (Motulsky and Chrisopoulos, 2004; Knell, 2009). The ER was calculated to test the likelihood of a model fitting better than another model (Burnham and Anderson, 2002). A model is supported when its ER score against another model exceeds 2.7 (Burnham and Anderson, 2002). In this study, I tested the AIC and ER for six types of regression lines judged on the basis of their shapes; these lines corresponded to continuous linear or non-linear models with no break point (BP), one BP or two BPs (Table 4). For the valid model, the significance of its slopes was also tested using Student's t-test. The test was also carried out for the following molecular analysis

4. Results

4.1. Determination of culturing conditions of larvae

Before the experiments involving in-depth measurement of the timing of tail regression were begun, it was necessary to determine how culturing conditions, namely the temperature and density of the larvae, affect the timing of metamorphic events. These experiments were performed by direct observation under a microscope, not by video recording, which was adopted in the subsequent sections. To examine the influence of temperature, I estimated the timing of the initiation of adhesion and tail regression in larvae cultured at 16°C, 18°C and 20°C beginning after fertilization. For this estimation, I used the time at which the first animal in the population was observed to initiate adhesion or tail regression. I observed the larvae hatched at different times under different temperatures, suggesting that the difference in the temperature affected the progression of embryogenesis (Fig.1A; t-test, $p<0.001$). In contrast, I found that the time taken from hatching to adhesion and that from hatching to the start of tail regression were constant regardless of the difference in the temperature (Fig.1A and Table 1), suggesting that tail regression is not significantly affected by a 2-degree difference in temperature. In the following experiments I cultured the animals at 18°C.

Next, I examined the effect of the density of the larvae on the tail regression. When I cultured animals at different densities, the timing of hatching and that of tail regression were unaffected (Fig. 1B). I found that the timing of adhesion could be affected by the density: there was a tendency that larvae cultured in denser conditions showed quicker adhesion. Indeed, the starting time of adhesion gradually grew earlier by 44 min on average when the number of larvae in the petri dish was increased from 1

to 500 (Fig. 1B and Table 2; ANOVA, $p<0.001$). A significant reason for this result is that increasing chance to have a larva close to the plane of petri dish in a dense culture than a sparse culture. In the following experiments, I decided to add 700 larvae to each dish to minimize the effect derived from the density, given that any significant differences of the timing were not detected in the experience with 500, 1,000 and >1,000 animals (Fig. 1B).

4.2. Estimation of the timing with which the competence for adhesion was acquired

Under the culturing conditions mentioned in the previous section, I performed video recording of the larvae to investigate the time course of the events associated with tail regression (Figs. 2A and B). First I addressed the timing for acquiring adhesion competence. Soon after hatching, the larvae did not attach to the petri dishes, suggesting that the larvae do not have the ability to adhere at that time. Therefore, larvae must acquire the competence after hatching. Even after acquiring the competence for adhesion, larvae cannot attach to a substrate if they do not reach the substrate, meaning that the actual timing for larvae to begin adhesion should be different among individuals. Therefore, I could not estimate the time required to acquire the competence for adhesion on the basis of the average time of adhesion in multiple larvae. Thus, I decided to estimate the time required to acquire the competence for adhesion on the basis of the earliest time I observed any larva attached to the petri dish. This time was 4 hours and 44 min after hatching on average (n=5 populations), ranging from 4 hours post-hatching (hph) to 5 hours and 30 min post-hatching.

One critical issue in the above experiment was the influence of swimming to the adhering position. To eliminate this, I anesthetized larvae with an agent MS-222,

and these larvae were placed on the bottom plane of petri dish. The anesthetized larvae quickly reach the bottom of the petri dish, and if they touched the dish with adhesive papillae, they could adhere. By carrying out this adhesion experiment periodically after hatching, I could see the time when larvae tended to have the competence for adhesion. As shown in Fig. 3 and Table 3, larvae started to adhere at 3 hours and 15 min after hatching, and the frequency of adhered larvae dramatically increased at 3 hours and 25 min after hatching. As a negative control, larvae whose papillae were removed could not adhere (Fig. 3, blue bars), suggesting that the adhesion in this experiment was accomplished by adhesive papillae. To confirm that MS-222 does not affect the timing of the competence, I carried out the same experiment with anterior tip of the trunk isolated from larvae. The partial larvae showed the same tendency of adhesion with MS-222-treated larvae even if MS-222 was not added (Fig. 3, magenta bars). From these results, I concluded that *C. intestinalis* larvae start to acquire the competence for adhesion around 3 hours and 15 min after hatching, and the ability is significantly enhanced around 3 hours and 30 min after hatching.

4.3. Acquisition of the competence for tail regression

Next, I assessed the time tail regression competence was acquired. I observed the larvae one-by-one as they started adhesion, initiated tail regression, and completed tail regression (Fig. 4A, 5 populations). Compared to adhesion and the completion of tail regression, the initiation of tail regression is difficult to observe, and I had to find a characteristic phenomenon that would mark the initiation of tail regression. I found that the larval tail bent near the trunk prior to regression (Fig. 4B). I used this bending as the marker of the initiation of tail regression. Tail started regression at 15 min after bending

on average (Fig. 4C), and I added this bending time to the time required for tail regression.

As mentioned above, the time for larvae to start adhesion was quite variable among individuals, and this variation was thought to be a result of the larvae needing to reach the location (in the case of my observations, the bottom of the petri dish) where they were to adhere. According to the time when tail regression began, I divided the larvae into two groups (Fig. 4A). In group A (marked by the blue vertical line in Fig. 4A), the time at which tail regression started was almost constant among individuals (the junction between the magenta and green bars in Fig. 4A) compared to the very variable timing of adhesion, suggesting that the larvae acquired the ability to undergo tail regression just before they started tail regression. I saw from the graph that the time from adhesion to the start of tail regression decreased sequentially in group A, suggesting that the timing for larvae to acquire tail-regression competence is not affected by the length of adhesion. In group B (marked by the orange vertical line in Fig. 4A), the larvae started tail regression after a relatively short and constant period of adhesion. I assumed that the larvae categorized in group B adhered after they acquired tail-regression competence.

To examine whether the above grouping is supported by mathematical analyses and at the same time to strictly estimate the time required to acquire the tail-regression ability, I plotted the time taken from adhesion to the start of tail regression with respect to the time between hatching and adhesion, and drew regression lines (Fig. 4D, the same 5 populations). The best-fit line, which was supported by the statistical analyses (Table 4; see Materials and Methods section), was composed of two linear lines with different slopes rather than any of the other linear or curved lines, confirming

that larvae can be divided into two groups (A and B). From the shape and the statistical analyses, I found that the two linear lines have one break point (BP); thus they make up a segmented model representing a particular change (Table 4; Iguchi, 2013). In the case of *C. intestinalis* larvae, the particular change corresponds to the acquisition of tail-regression competence. The time from hatching to tail regression of the BP at which the two linear lines intersect was 11 hours and 55 min post-hatching on average (n=5 populations), suggesting that larvae begin to acquire tail-regression competence at 11 hours and 55 min post-hatching. Secondly, the slope of the right-side graph (group B) in all of the populations was 0.001 on average and was not different from 0 (t-test, p<0.05). This suggests that the larvae categorized in group B started tail regression after a period of adhesion with a constant length (Fig. 4D). The constant length of this period may be required for inducing the tail regression. The average length was 32 min in this experiment.

4.4. Length of adhesion necessary to induce tail regression

In the above section, I estimated that larvae need to adhere for 32 min before tail regression begins. I then tested whether this estimation could be supported by another analysis. Larvae that had acquired tail-regression competence were allowed to adhere for certain amounts of time, and then they were detached from the substrate. Afterward, their tail regression was observed. I found that larvae that were allowed to adhere for 28 min or longer underwent tail regression, while larvae that were allowed to adhere for less than 28 min did not (Fig. 5). I conclude that larvae need to adhere for 28 min to induce tail regression.

4.5. Tail regression is induced by the experience of adhesion

In my video observations, I found that the tails of larvae regressed soon after 32 min of adhesion was completed. There are two hypotheses to explain this observation. The first is that larvae require a seamless transition between adhesion and tail regression, and interruption of the two events may reset the experience of adhesion. The second is that larvae do not require adhesion and tail regression to be uninterrupted and that larvae can retain the experience of adhesion until they acquire the competence for tail regression. In the latter case, larvae apparently start tail regression soon after adhesion because larvae seldom became detached from their substrates in my observations. I examined these possibilities using the following experiment: Larvae at the age before the acquisition of tail regression competence (from 4 hours and 45 min to 12 hph) were allowed to adhere for 30 min and were then detached from the substrate by pipetting. After detachment, a portion of their tails was cut to inhibit them from adhering again. Tail-cut larvae could not swim efficiently, and therefore they usually fail to adhere to the substrate (Nakayama-Ishimura et al., 2009). I then observed their tail regression after the time to acquire tail-regression competence had elapsed.

Larvae that experienced 30 min of adhesion after 6 hph exhibited tail regression around 12 hph, when the time required to acquire tail-regression competence had elapsed (Figs. 6A, B, and D, Table 5). This suggests that the larvae somehow memorized the experience of adhesion until tail-regression competence was acquired. The later metamorphic events through which they develop into juveniles were clearly normal in the larvae (Fig. 6F and G, Table 6), suggesting that the 30-min adhesion during the pre-competent stage for tail regression was sufficient to induce all metamorphic events. In contrast, the tails of larvae before 6 hph did not regress after the

same experiment (Fig. 6C), suggesting that they did not have the ability to remember the experience.

Next, I addressed whether larvae need 30 min of uninterrupted adhesion to induce tail regression, or whether tail regression can be induced through interrupted adhesion for 30 min in total. To examine this, 6-hph-old larvae were allowed to adhere for 15 min, detached, and allowed to adhere for 15 min more, after which further adhesion was inhibited by cutting their tails. The tails of these larvae did not regress even though the time acquired the tail-regression competence had passed (Fig. 6E and Table 5). Therefore, larvae need to experience an uninterrupted period of adhesion for tail regression to be induced. The importance of uninterrupted adhesion was also suggested by the result that the post- competent larvae continued to swim for 12 hph without adhesion, after which were allowed to adhere for 27 min, detached and subsequently allowed to adhere for 15 min more did not exhibit tail regression (Fig. 6H and I, Table 5).

4.6. Time required for completing tail regression

In my observations, the time taken for completing tail regression varied among individuals (Fig. 7A, 58 min on average). From the video recording, I noticed that the angles of the bent tails were different among individuals. I suspected that the differences among these angles indicated the variation in the time required for tail regression, and so I examined the relationship between the angle of tail bending and the time required for tail regression. The angles between the midlines of the trunk and tail were measured (Fig. 7B) to score the angle of tail bending. I revealed that the time required for tail regression tended to be longer as the angle became greater (Fig. 7C).

The changing of plots was analyzed using R-software, and the best regression model was a segmented linear model with one BP (Table 7; see Materials and Methods). The plots were divided into two groups at the BP of 85^{o} (green and magenta in Fig. 7C). The times of tail regression in larvae whose angle was more than 86^{o} (magenta) and less than 85^{o} (green) were 75 min and 56 min on average, respectively.

I had the impression that aged larvae swimming for a longer period tend to start regression their tails more quickly than larvae which adhered earlier. To examine this possibility, the papillae of aged larvae, which lived as swimming larvae for 48 hph, were injured with razors to induce tail regression as reported previously (Fig. 8A-C; Kamiya et al., 2014). The time required for tail regression in aged larvae was 28 min, which is about half the time observed in 24-hph-old larvae (Fig. 8D; t-test, $p<0.001$). In the observation, I found that aged larvae also bent their tails. However, the time from tail bending to the start of tail movement into the trunk in aged larvae was 5 min on average, which was ~10 min shorter than the time observed in 24-hph-old larvae (Fig. 8E). I also revealed that the tail bending angle in aged larvae was less than 85^{o} (Fig. 8F, magenta plots). The time required for tail regression of 48-hph-old larvae was found to be significantly shorter than that of 24-hph-old larvae whose tail bending angle was less than 85^{o} (Fig. 8F; ANOVA, $p<0.001$).

5. Discussion

This study addressed the time course associated with tail regression during the metamorphosis of *C. intestinalis*. I thoroughly determined the time requirements for each sub-event of tail regression and constructed a time chart of the events (Fig. 9). I also revealed that *C. intestinalis* larvae can memorize the experience of adhesion when the adhesion lasts long enough to induce tail regression. The information provided by this study will be used as a framework for investigating the molecular and cellular mechanisms underlying the metamorphosis of ascidians.

5.1. Acquisition of the competence for adhesion

This study showed that larvae of *C. intestinalis* acquire the competence for adhesion after hatching. The timing of the acquisition of this competence may involve avoiding adhesion to the chorion. If larvae acquired the competence before hatching, they would adhere to the chorion, and this adhesion might prevent hatching. In addition, the adhesion might cause untimely metamorphosis before hatching, as is seen in some *Molgula* species that exhibit direct development (Tagawa et al., 1997). I concluded that larvae of *C. intestinalis* start to acquire the competence for adhesion by 3 hours and 15 min after hatching. My observation also suggests that the ability is enhanced during the next 10 min. This may indicate that a mucous compound required for adhesion starts to be secreted around 3 hours and 15 min after hatching, but at this time point the amount is not sufficient for many larvae to adhere. The amount of the compound is accumulated during the next 10 min, so as to allow for most larvae to adhere. In my videos, larvae tended to start adhesion ~1 hour later than the time acquired the adhesion competence examined accurately. As I mentioned in the result section, larvae that have acquired the

competence need to swim to the location for adhesion. For this reason the measured timings include the time spent for swimming after the acquisition of adhesion competence.

It is important to elucidate the molecular mechanisms through which the larvae develop the ability to adhere. The papillae need to mature and a mucous compound must be synthesized/secreted for adhesion. The papillae of *C. intestinalis* begin their extension after hatching, and this extension is completed at around 4 hph (Katz, 1983; Chiba et al., 2004). This timing is similar to my estimated timing of the acquisition of the competence for adhesion. On the molecular level, cellulose is a strong candidate for the mucous substrate used for adhesion. The gene encoding cellulose synthase is expressed in the epidermis of the entire larval body including the papillae (Nakashima et al., 2004). Cellulose synthesized in the epidermal cells is secreted into the outer layer and becomes a main component of tunic (Nakashima et al., 2004). Mutant larvae without cellulose synthase rarely adhere (Sasakura et al., 2005). Therefore, the extension of the papillae and the synthesis of cellulose are major components required to provide adhesion competence. However there must be additional components. For example, cellulose is produced throughout the body, while adhesion occurs exclusively at the papillae. Therefore there must be an additional factor that is necessary for adhesion. Identifying this is necessary to gain a complete understanding of the mechanism of adhesion and of the acquisition of the competence for adhesion.

5.2. Acquisition of the competence for tail regression

I estimated the time required to acquire the competence for tail regression. The timing is dependent on the time after hatching but is not influenced by adhesion. Therefore, the acquisition is regulated by innate developmental mechanisms similar to the genetic cascades that are initiated by fertilization. *C. intestinalis* larvae are in a premature state for metamorphosis at the onset of hatching, and they may complete their development at the time that they are ready for metamorphosis.

I propose two mechanisms that probably contribute to the acquisition of competence for tail regression. Firstly, larval neurons are considered to be important to induce tail regression (Kimura et al., 2003; Zega et al., 2005). It is possible that the neuronal network is still premature soon after hatching, and that larvae cannot induce tail regression until the neuronal maturation is completed. Secondly, it has been reported that the downstream genes of mitogen-activated protein kinase (MAPK) signaling including *Ci-CSP*, *Ci-Mx*, and *Ci-Sushi* induce tail regression (Chambon et al., 2002, 2007; Tarallo and Sordino, 2004). The time required for transcription and translation of the downstream genes of MAPK signaling may also be responsible for determining the time needed to acquire the competence for tail regression. To reveal all of the mechanisms of tail regression, identifying the larval neurons that induce the event and the relationship between the neurons and the signaling events in the tail cells should be considered.

Larvae require continuous adhesion for 28 min to start tail regression. This adhesion time may be the direct trigger for tail regression. Obviously the requirement for adhesion functions to ensure that larvae start tail regression after settlement; in other words, the requirement prevents swimming larvae from undergoing tail regression

before adhesion. The requirement of a relatively long adhesion time for inducing tail regression may enable larvae to start tail regression only when they surely adhere to substrates, protecting the larvae from transient and weak stimuli given to papillae. When larvae are detached, the time accumulated during the previous adhesion is forgotten, and they again require ~28 min of adhesion. This system is also useful in that it helps larvae make the correct judgment regarding adhesion. The continuous stimulation of the papillae may also contribute to changing the cellular conditions of the nervous system and tail cells so as to be ready for tail regression.

5.3. Time of tail regression influenced by tail bending and age

I found that the tail bends near the border with the trunk at the onset of tail regression. A similar change was described in another ascidian, *Herdmania curvata* (Degnan and Johnson, 1999), suggesting that this is a general phenomenon among ascidians during tail regression. In *H. curvata*, the bending is caused by dissolving cell adhesion and reconstructing cytoskeletons in muscle cells (Degnan and Johnson, 1999), which strongly suggests that the qualitative change in the tail cells is important for the event.

In *C. intestinalis*, it has been suggested that the epidermal layer moves and pushes the other tissues toward the trunk during tail regression (Cloney, 1966). Therefore, the bending of the tails in *Ciona* species might also be accomplished by the movement of the epidermal cells that is characteristic of tail regression. The speed of cell movement may be influenced by the angle of bending, with the time of tail regression becoming longer depending on the increase of the angle. I also found that aged larvae show quicker tail regression than younger larvae, a phenomenon that was

also reported in another ascidian, *H. roretzi* (Ishikawa and Numakunai, 1972). I suggest that the accumulation of various factors that function in the rearrangement of the cytoskeleton and in cell movement accelerates the tail regression after the reception of adherent stimulation. The younger larvae may be in the midst of this accumulation, while the accumulation may be almost complete in the aged larvae.

5.4. Larvae can remember the experience of adhesion

Surprisingly, *C. intestinalis* larvae can retain the experience of adhesion for at least a few hours until they acquire the ability to undergo tail regression. As adhesion is necessary to start tail regression, it is suspected that the stimulus of settlement evokes some changes in the nervous system, such as strengthening synaptic junctions and long-term potentiation by various molecular cascades, which contribute to continue the excitatory transmission toward the tail cells. Alternatively, substantial changes in the tail cells might be triggered by the stimulus of adhesion, and these changes may remain until the acquisition of the ability to undergo tail regression. The larvae that experienced adhesion for 30 min before 6 hph could not start tail regression. A previous study has suggested that the maturation of larval neurons takes 6 hph (Katz, 1983; Nicol and Meinertzhagen, 1991). Based on the similarity of the timing between these two events, I currently favor the idea that larval neurons are the main cells responsible for establishment of the memory of adhesion.

As a possible molecular mechanism underlying the memory, the cAMP response element binding protein (Ci-CREB) is important for memory formation in the larval neurons of some animals (Yamada et al., 2003; Imai et al., 2004; Kandel, 2004; Chen et al., 2010; Miyashita et al., 2012). Nitric oxide synthase expressed in the central

nervous system (CNS) is another candidate, as it is involved in synaptic potentiation to form memory in mice (Comes et al., 2007; Lange et al., 2012). In future studies, I aim to investigate the origin and evolution of the systems of memory formation that probably functioned in the ancestor of chordates and explore how this system was incorporated into the metamorphic systems of *C. intestinalis*.

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8. Tables

Table 1. Average time spent for hatching and metamorphic events at different temperatures.*

	Fertilization to hatching	Hatching to adhesion	Adhesion to tail regression
16 °C	22 h 1 min	4 h 32 min	7 h 15 min
18 °C	17 h 26 min	4 h 40 min	7 h 40 min
20 °C	15 h 30 min	4 h 30 min	7 h 45 min

* The time required for the earliest larva to hatch, adhere, and start tail regression. The experiment was performed 50 times.

Table 2. Average time spent for hatching and metamorphic events at different densities of larvae.*

*n***	Hatching	Starting adhesion	Starting tail regression
1	17 h 29 min	25 h 13 min	29 h 20 min
10	17 h 29 min	24 h 28 min	29 h 7 min
50	17 h 29 min	23 h 42 min	29 h
100	17 h 29 min	23 h	29 h 6 min
500	17 h 29 min	22 h 17 min	29 h
1000	17 h 29 min	22 h 12 min	29 h 5 min
>1000	17 h 29 min	22 h 11 min	29 h 7 min

* The time required for the earliest larva to hatch, adhere, and start tail regression. The experiment was repeated 50 times.

** *n*, number of larvae in a petri dish.

Table 3. Rate of adhesion in anesthetized and papillae-cut larvae[*].

Experimental conditions	% of adhesion in each time of post hatching (n[**]), N[***]=50						
	3 hph	3.08 hph	3.16 hph	3.25 hph	3.33 hph	3.42 hph	3.5 hph
Intact larvae with MS222	0.0 (0)	0.0 (0)	0.7 (1)	12.3 (6)	18.7 (9)	60.0 (30)	71.0 (36)
Papillae-cut larvae with MS222	2.0 (1)	2.7 (1)	2.7 (1)	2.0 (1)	1.3 (1)	4.3 (2)	3.7 (2)
Papillae region[****] with MS222	0.0 (0)	0.0 (0)	0.0 (0)	11.0 (6)	14.3 (7)	50.7 (25)	60.3 (30)
Papillae region without MS222	0.0 (0)	0.0 (0)	0.0 (0)	11.3 (6)	13.0 (7)	52.3 (26)	60.7 30)

[*] The larvae were allowed to adhere in each time of post hatching under conditions that would prevent tail movement. The number of adhered larvae was counted in each time.

[**] *n*, number of adhered larvae.

[***] *N*, number of examined larvae

[****] Larvae were cut their papillae using razor and the only papillae region was analyzed.

Table 4. Statistical analyses to select the best regression line model for Fig. 4D.[*]

AIC[**]						
Model	Linear line			Non-linear (curved) line		
Population number (*n*[***])	BP[****]0	BP1	BP2	BP0	BP1	BP2
1 (*n*=141)	477.1	29.6	33.6	160.2	111.8	48.6
2 (*n*=155)	490.1	143.9	170.8	246.3	177.3	163.4
3 (*n*=207)	691.9	208.2	202.9	346.5	253.4	225.1
4 (*n*=185)	647.9	87.0	90.7	647.9	191.9	120.3
5 (*n*=146)	379.5	201.4	205.4	295.2	211.6	220.2
ER[*****]						
Model	Linear line			Non-linear (curved) line		
Population number (*n*)	BP0	BP1	BP2	BP0	BP1	BP2
1 (*n*=141)	1.5.E+97	1	7.2	2.3.E+28	7.1.E+17	1.4.E+4
2 (*n*=155)	1.5.E+97	1	7.0.E+5	1.7.E+22	1.8.E+7	1.7.E+4
3 (*n*=207)	5.9.E+106	1	4.0	6.0.E+31	3.6.E+11	2.6.E+5
4 (*n*=185)	6.3.E+121	1	6.5	4.8.E+44	6.1.E+22	1.7.E+7
5 (*n*=146)	4.6.E+38	1	7.1	2.3.E+20	160	1.2.E+4

[*] The best regression lines from the plots shown in Fig. 4D and Supplementary Figure S2 were determined using calculated values of AIC and ER using R software (see Materials and Methods).

[**] AIC, Akaike information criterion.

[***] *n*, number of larvae in the populations.

[****] BP, break point.

[*****] ER, evidence ratio.

Table 5. Rate of tail regression in larvae allowed to adhere for a limited time.[*]

Experimental conditions	% of tail regression (n[**])	N[***]
No adhesion until 12 hph	5.4 (5)	92
Adhesion for 30 min from 5.75 hph	9.2 (7)	76
Adhesion for 30 min from 6 hph	87.0 (72)	83
Adhesion for 15 min twice from 6 hph[****]	12.9 (11)	85
Adhesion for 28 min from 12 hph	84.1 (69)	82
Adhesion for 27 and 15 min from 12 hph[*****]	10.3 (8)	78

[*] The larvae were allowed to adhere for each given time period from the pre- or post-competent stage and then were detached and held under conditions that would prevent further adhesion. The number of tail-regressed larvae was counted after 12 hph.

[**] *n*, number of tail-regressed larvae.

[***] *N*, number of examined larvae.

[****] The larvae adhered for 15 min, were detached, and were allowed to adhere for 15 min more.

[*****] The larvae adhered for 27 min, were detached, and were allowed to adhere for 15 min more.

Table 6. Rate of development into juveniles by adhesion at the pre-competent stage.[*]

Experimental conditions	% of metamorphosed animals (n[**])	N[***]
Swimming until 72 hph	6.6 (8)	122
Adhesion for 66 hours from 6 hph[****]	90.7 (97)	107
Adhesion for 30 min from 6 hph[*****]	82.6 (95)	115

[*] The number of juveniles was counted at 72 hph.

[**] *n*, number of juveniles.

[***] *N*, number of examined larvae.

[****] A positive control in which larvae were allowed to continue adhesion from 6 hph until the juvenile stage.

[*****] Larvae which were allowed to adhere for 30 min from 6 hph, were detached, and were prevented from undergoing further adhesion.

Table 7. Statistical analysis to select the best regression line model for Fig. 7C.[*]

AIC[**]						
Model	Linear line			Non-linear (curved) line		
Population number (*n*[***])	BP[****]0	BP1	BP2	BP0	BP1	BP2
1 (*n*=141)	350.3	128.4	185.1	250.8	183.5	142.1
2 (*n*=155)	332.6	173.6	190.6	238.2	221.1	227.9
3 (*n*=207)	332.2	159.7	186.6	252.1	236.8	180.3
4 (*n*=185)	359.4	163.2	172.5	283.8	251.3	231.6
5 (*n*=146)	368.5	115.8	132.4	268.5	224.2	197.6
ER[*****]						
Model	Linear line			Non-linear (curved) line		
Population number (*n*)	BP0	BP1	BP2	BP0	BP1	BP2
1 (*n*=141)	1.5E+48	1	2.1E+12	3.8E+26	9.2E+11	943.9
2 (*n*=155)	3.4E+34	1	4914.8	1.1E+14	2.1E+10	6.2E+11
3 (*n*=207)	2.9E+37	1	6.9.E+05	1.2E+20	5.5E+16	3.0.E+04
4 (*n*=185)	4.0E+42	1	104.6	1.5E+26	1.4E+19	7.1E+14
5 (*n*=146)	7.5E+54	1	4023.9	1.4E+33	3.5E+23	5.8E+17

[*] The best regression lines from the plots for the time of tail regression against the angle of tail bending shown in Fig. 7C were determined with calculated values of AIC and ER using R software (see Materials and Methods).

[**] AIC, Akaike information criterion.

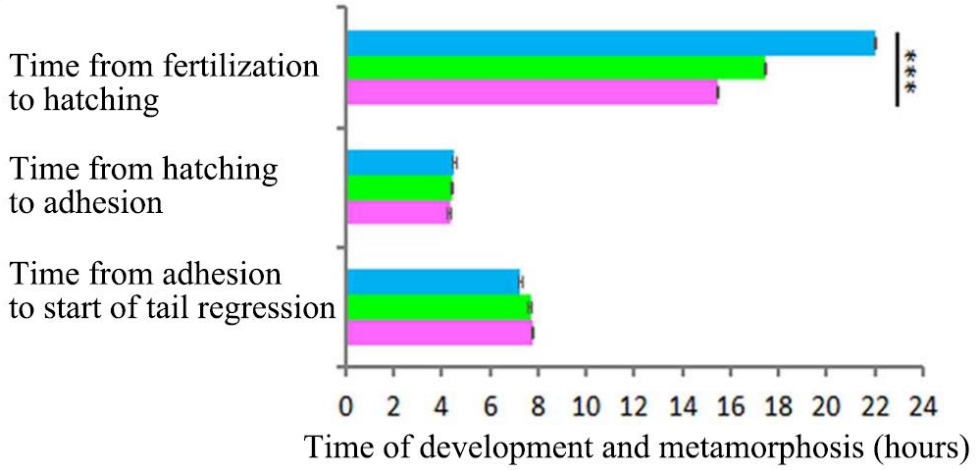
[***] *n*, number of larvae in the populations.

[****] BP, break point.

[*****] ER, evidence ratio.

9. Figures

A


Time from fertilization to hatching
Time from hatching to adhesion
Time from adhesion to start of tail regression
***
0 2 4 6 8 10 12 14 16 18 20 22 24
Time of development and metamorphosis (hours)

B

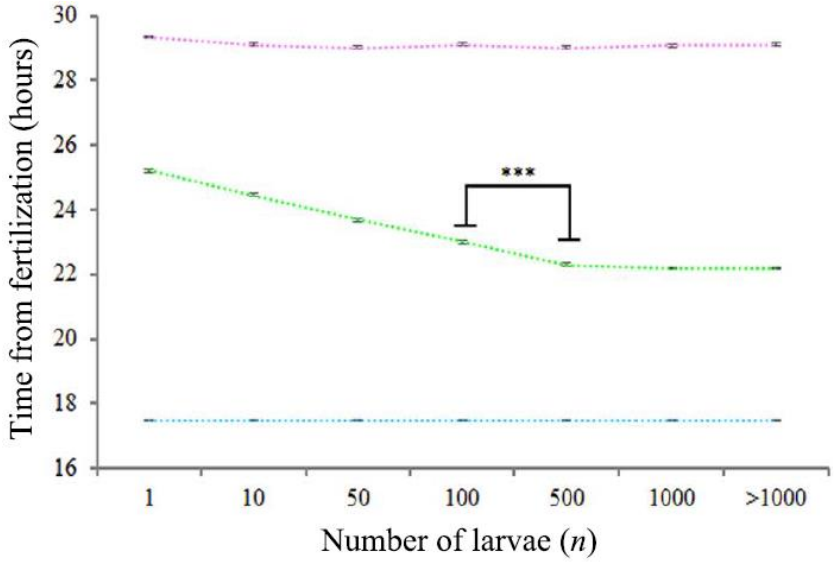

30
28
26
24
22
20
18
16
Time from fertilization (hours)
***
1
10
50
100
500
1000
>1000
Number of larvae (n)

Fig. 1. Times of hatching, adhesion and tail regression under different temperatures or larval densities.

(A) Effect of temperature. Colored bars represent the results at 16 ºC (blue), 18 ºC (green) and 20 ºC (magenta), respectively. Horizontal axis shows the time taken for development and metamorphosis (hour). The time from fertilization to hatching is affected by temperature (ANOVA, $p<0.001$***), but the time from hatching to adhesion and tail regression is constant. (B) Effect of density. Vertical and horizontal axes show the time after fertilization (hour) and the number of larvae, respectively. Colored lines represents times of hatching (blue), the start of adhesion (green) and the start of tail regression (magenta), respectively. Differences in density affect the starting time of adhesion, but the timing of hatching and that of the start of tail regression are unaffected. The timing of adhesion was significantly earlier when 500 larvae were added to a petri dish than when 100 larvae were added to a petri dish (t-test, $p<0.001$***). For each condition, the experiment was repeated 50 times.

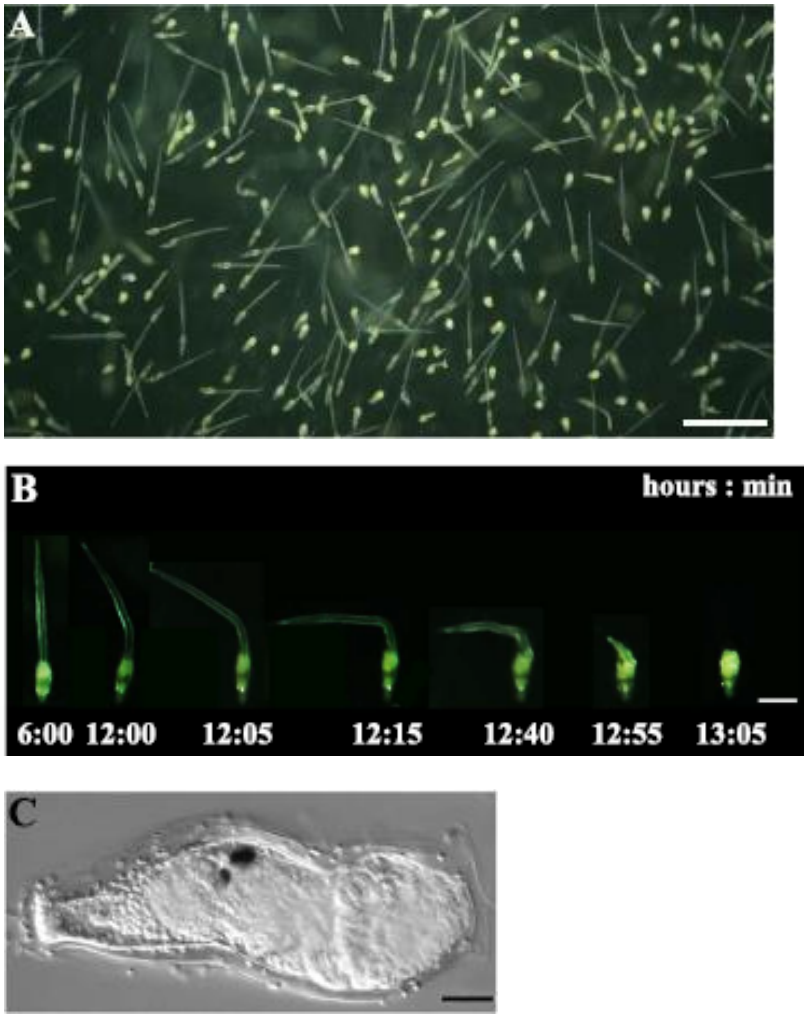


Fig. 2. Video imaging and definition of initiation and completion of tail regression. (A) A recorded video image. Scale bar, 800 μm. (B) Time lapse imaging of a larva from starting adhesion to completing tail regression. A larva adhered at 6 hours post-hatching (hph), stopped its tail motion and started tail regression at 12 hph. After the tail regression started, the larva bent its tail for 15 min, and the tail cells moved into the trunk region until 13 hours 5 min after hacthing. Scale bar, 200 μm. (C) A juvenile that just finished tail regression. Scale bar, 40 μm.

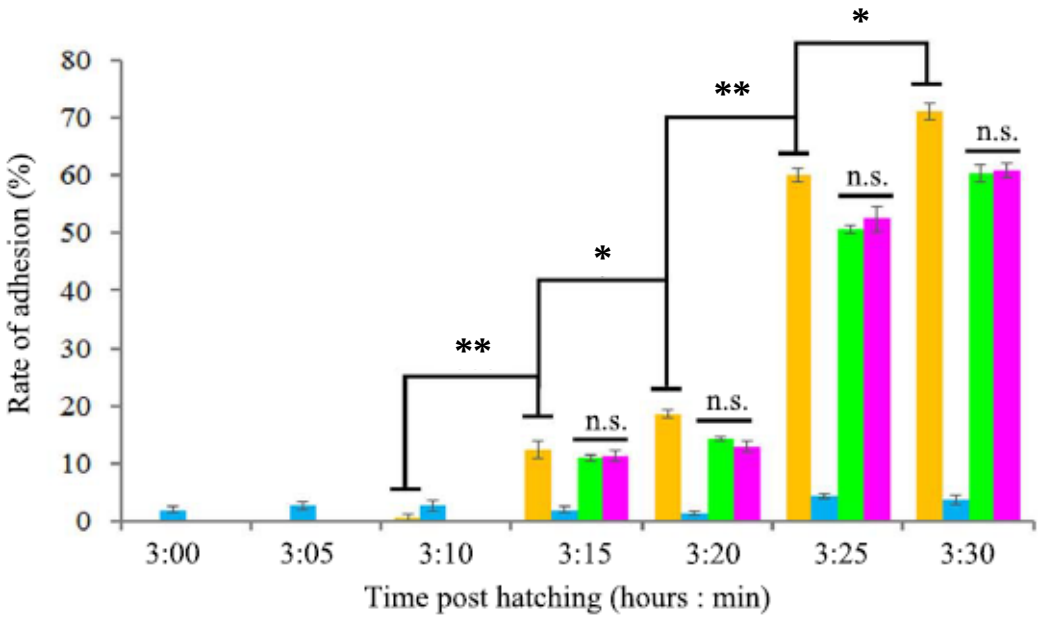


Fig. 3. Larvae start to acquire competence for adhesion around 3 hours and 15 min after hatching.

The graphs illustrate the percent of larvae or larval anterior fragments adhered to the bottom plane of petri dishes at each time point described in the horizontal axes. Colors indicate the results of intact larvae anethetized by MS-222 (orange), papilla-removed larvae anethetized by MS-222 (blue), anterior tip of larvae treated by MS-222 (green), and anterior tip of larvae without MS-222 (magenta). For each condition, the experiment was repeated three times. At 3 hours and 10 min and at 3 hours and 15 min, the adhered specimens increased significantly compared to the results of previous time points and the rate significantly increased. (Turkey's test, $p<0.01$**; $p<0.05$*; n.s, not significance).

Figure 4

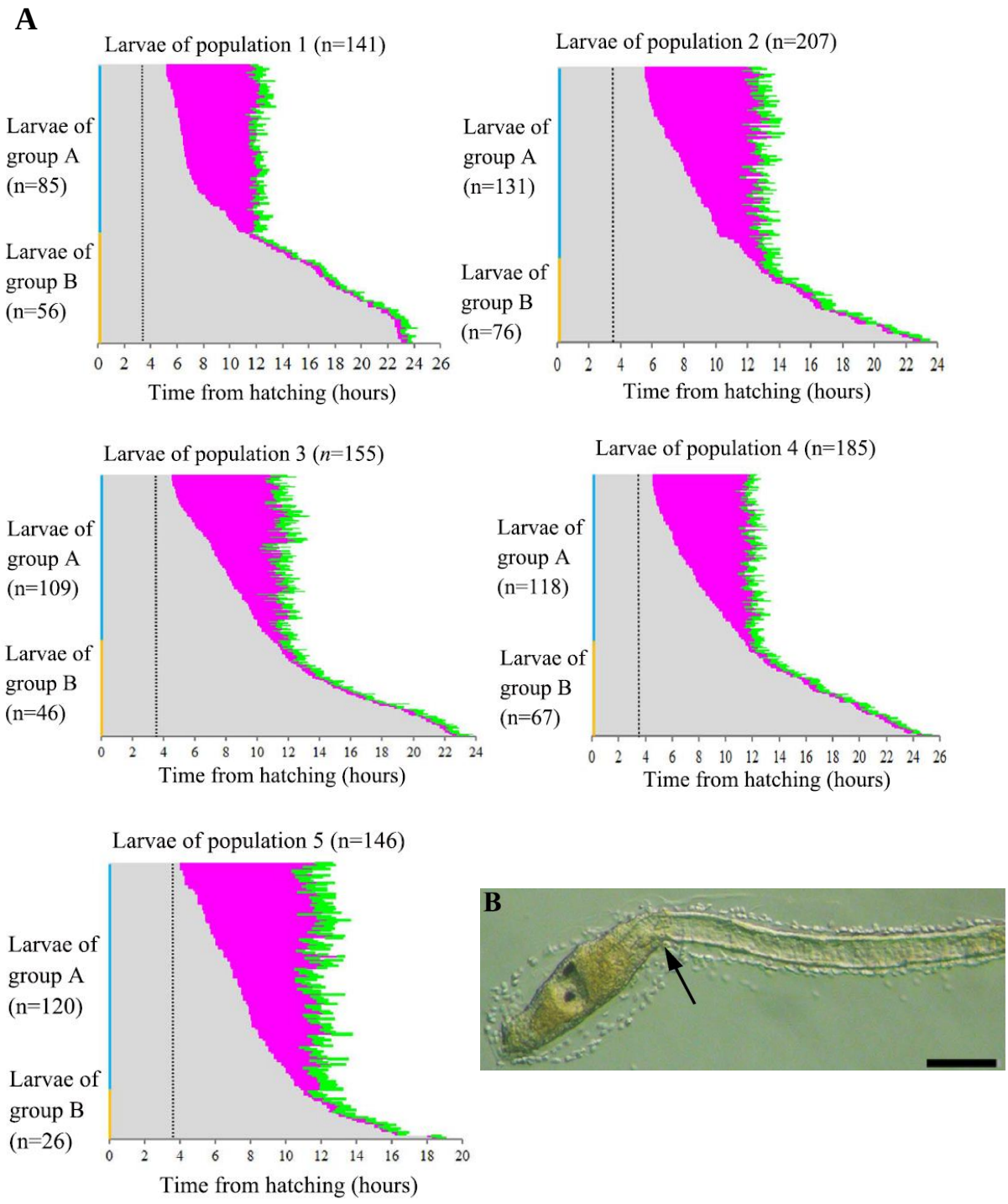
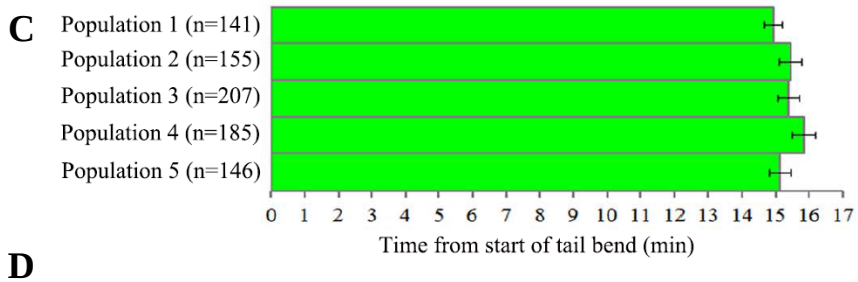

A
Larvae of population 1 (n=141)
Larvae of group A (n=85)
Larvae of group B (n=56)
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Time from hatching (hours)
Larvae of population 2 (n=207)
Larvae of group A (n=131)
Larvae of group B (n=76)
0 2 4 6 8 10 12 14 16 18 20 22 24
Time from hatching (hours)
Larvae of population 3 (n=155)
Larvae of group A (n=109)
Larvae of group B (n=46)
0 2 4 6 8 10 12 14 16 18 20 22 24
Time from hatching (hours)
Larvae of population 4 (n=185)
Larvae of group A (n=118)
Larvae of group B (n=67)
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Time from hatching (hours)
Larvae of population 5 (n=146)
Larvae of group A (n=120)
Larvae of group B (n=26)
0 2 4 6 8 10 12 14 16 18 20
Time from hatching (hours)
B

Figure 4


C
Population 1 (n=141)
Population 2 (n=155)
Population 3 (n=207)
Population 4 (n=185)
Population 5 (n=146)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Time from start of tail bend (min)

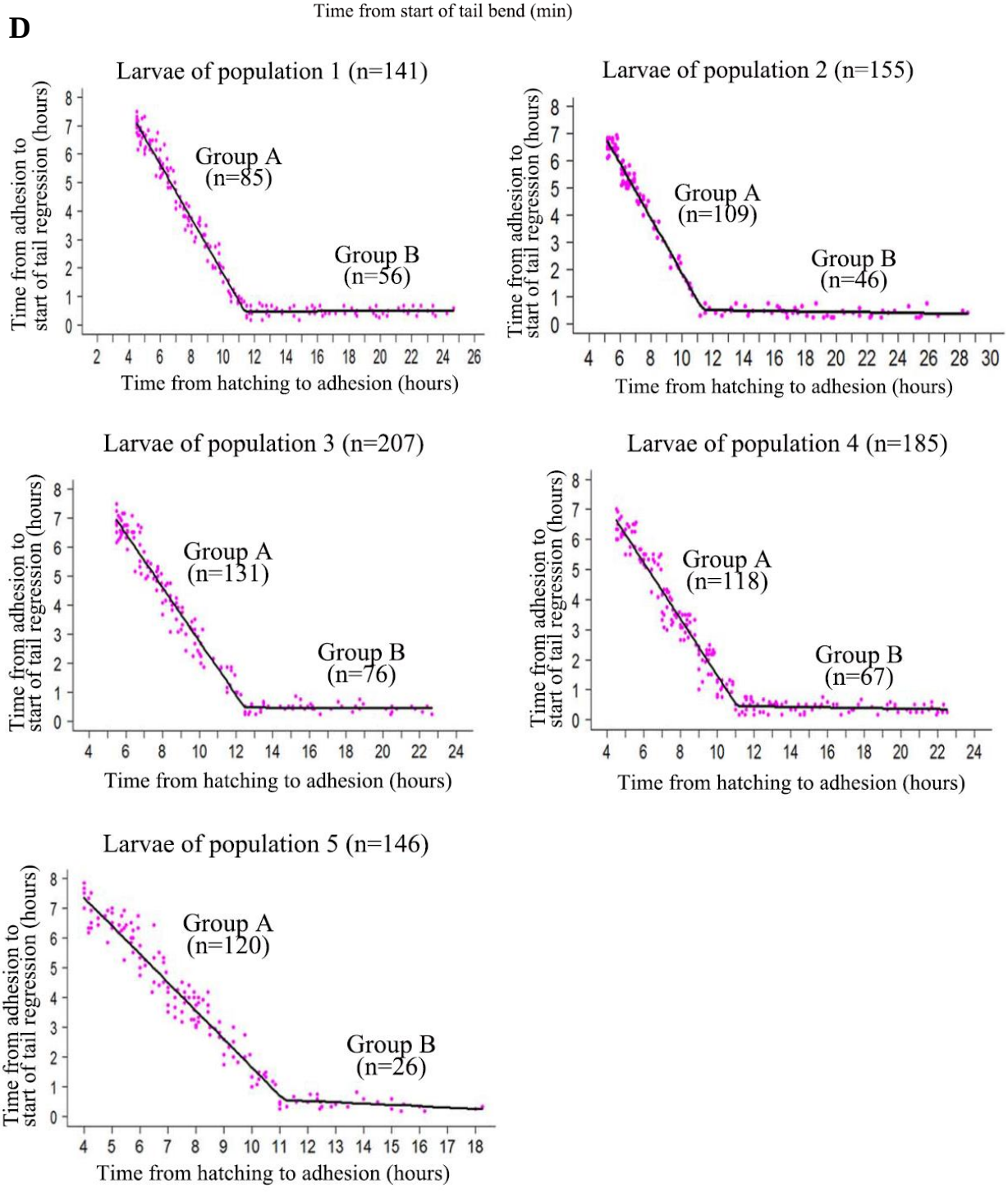

D
Larvae of population 1 (n=141)
Time from adhesion to start of tail regression (hours)
Group A (n=85)
Group B (n=56)
Time from hatching to adhesion (hours)
Larvae of population 2 (n=155)
Group A (n=109)
Group B (n=46)
Larvae of population 3 (n=207)
Group A (n=131)
Group B (n=76)
Larvae of population 4 (n=185)
Group A (n=118)
Group B (n=67)
Larvae of population 5 (n=146)
Group A (n=120)
Group B (n=26)

Fig. 4. Estimation of the time taken to acquire the competence for adhesion and that for tail regression.

(A) Recorded times of adhesion and of starting and completing tail regression in a population. The 5 groups contained 141, 155, 207, 185, and 146 larvae respectively. Horizontal axis shows the time from hatching (hours). Colored bars represent the time from hatching to adhesion (grey), from adhesion to the start of tail regression (magenta) and from starting to completing tail regression (green), respectively. Each bar graph represents the duration of metamorphosis in individual larvae and is arranged according to the time at which adhesion began. I divided the populations into two groups, groups A (*n*=85, 109, 131, 118, and 120 respectively; the larvae marked by a vertical blue line) and B (*n*=56, 46, 76, 67, and 26 respectively; the larvae marked by a vertical orange line). In group A, the start time of adhesion (indicated by the left-hand end of the magenta bar) is variable among larvae, but the start time of tail regression (indicated by the left-hand end of the green bars) is constant in almost all of the larvae. The time taken to acquire competence for adhesion is represented by a dotted line. (B) A larva starting tail regression. The position of tail bending is shown by black arrowhead. Scale bar, 100 μm. (C) The time spent from tail bending to the start of tail cell movement toward the trunk. Horizontal axis shows the time from the start of tail bending (min). (D) Estimation of the time required to acquire the competence for tail regression in the 5 populations (*n*=141, 155, 207, 185, and 146). The graph was made using R-software. Vertical and horizontal axes show the time from adhesion to the start of tail regression (hours) and the time from hatching to adhesion (hours), respectively. The regression line is a segmented line with one BP. The line is divided into two parts with different slopes. Dots (groups A and B) constituting the segmented line correspond to groups A and B shown in Fig. 4A.

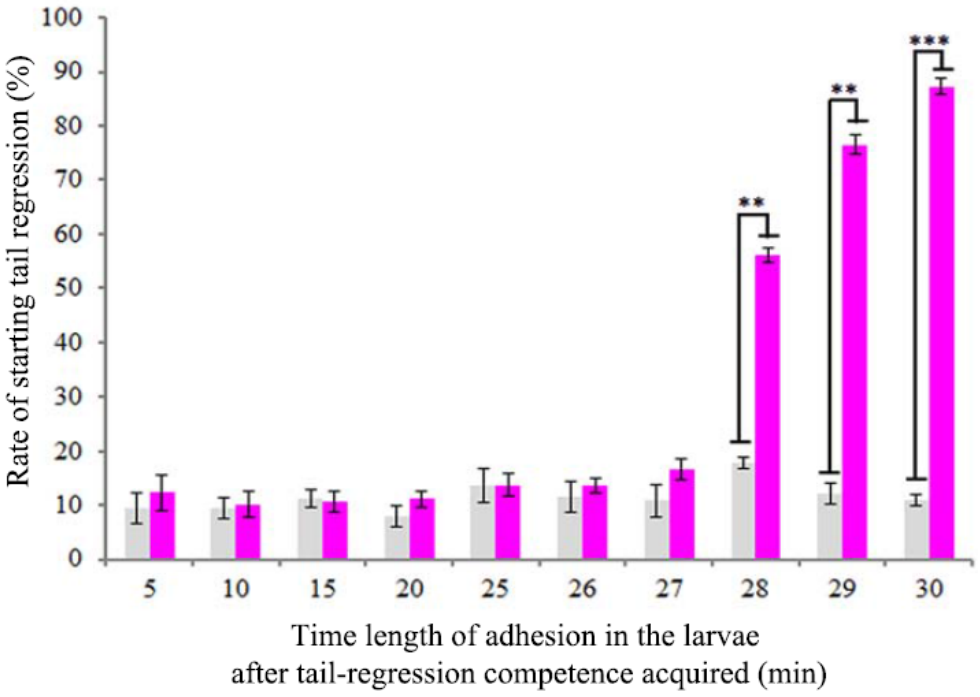


Fig. 5. Duration of adhesion sufficient to induce tail regression.

Larvae at the post-competent stage for tail regression were used. The vertical and horizontal axes represent the rate of tail-regressed larvae and the duration of adhesion (min), respectively. Gray bars represent the results of non-adhered larvae as negative controls, and magenta bars represent the results of larvae that experienced adhesion. The rates of tail-regressed larvae of the populations that had adhered for more than 28 min are significantly different from the rate of the controls. (t-test; 28 min, $p<0.01$**; 29 min, $p<0.01$**; 30 min, $p<0.001$***).

Figure 6

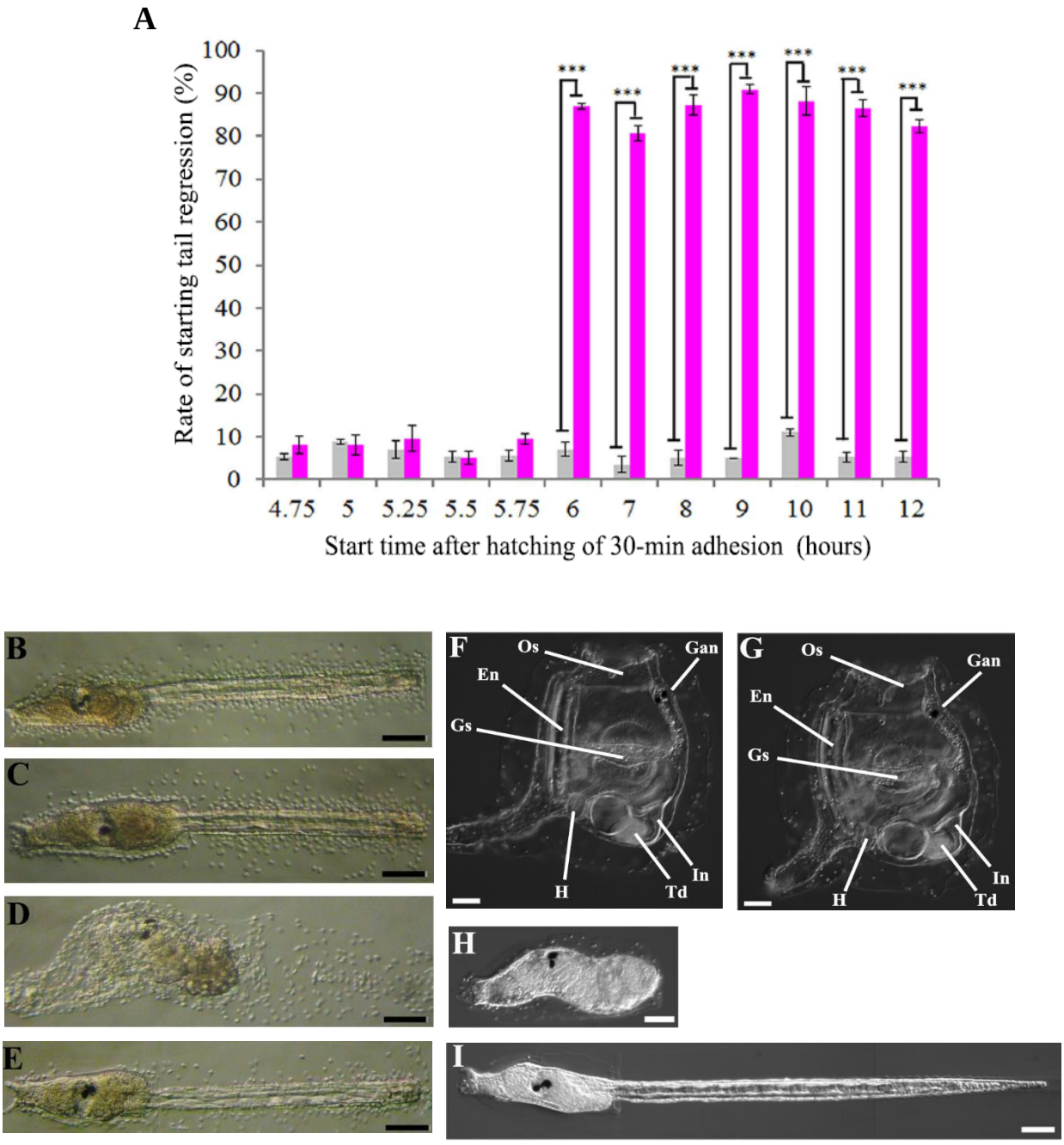

A
Rate of starting tail regression (%)
100
90
80
70
60
50
40
30
20
10
0
***
4.75
5
5.25
5.5
5.75
6
7
8
9
10
11
12
Start time after hatching of 30-min adhesion (hours)
B
C
D
E
F
Os
Gan
En
Gs
H
Td
In
G
Os
Gan
En
Gs
H
Td
In
H
I

Fig. 6. Larvae memorize the experience of adhesion.

(A) Vertical and horizontal axes show the rate of tail-regressed larvae and the starting time of larvae that had experienced 30 min of adhesion after hatching (hour), respectively. Grey bars represent the results of non-attached larvae as negative controls, and magenta bars represent the results of larvae that experienced 30 min of adhesion. The rates of tail-regressed larvae of populations that had adhered for 30 min after 6 hph are significantly different from the rate of the controls (6-12 hph; t-test, $p<0.001$***). (B-E) The morphology of swimming and adhered larvae at the post-competent stage of tail regression. Half of their tails were cut off to prevent further adhesion. Scale bar, 50 μm. (B) A swimming larva at 12 hph (negative control). (C) A larva that had adhered for 30 min before 6 hph. The tail had not regressed. (D) A larva that had adhered for 30 min from 6 hph. The tail had regressed into the trunk. (E) A larva that adhered for 15 min, detached, and adhered for 15 min again, for a total of 30 min of adhesion after 6 hph. The tail did not regress. (F and G) Occurrence of later metamorphic events after tail regression induced by 30-min adhesion at the pre-competent stage. En, endostyle; Gan, cerebral ganglion; Gs, gill slits; H, heart; In, intestine; Os, oral siphon; Td, tail debris. Scale bar, 50 μm. (F) A juvenile at 72 hph, which continuously adhered for 66 hours from 6 hph, as a positive control. (G) A juvenile at 72 hph developed from a larva that experienced 30 min of adhesion at the pre-competent stage. This animal adhered for 30 min from 6 hph and then detached. (H and I) Failure of induction of tail regression due to interrupted adhesion. Scale bar, 50 μm. (H) A larva adhered for 28 min from 12 hph. The tail regressed into the trunk. (I) A larva adhered for 27 min from 12 hph, detached, and again adhered for 15 min. Its tail did not regress.

Figure 7

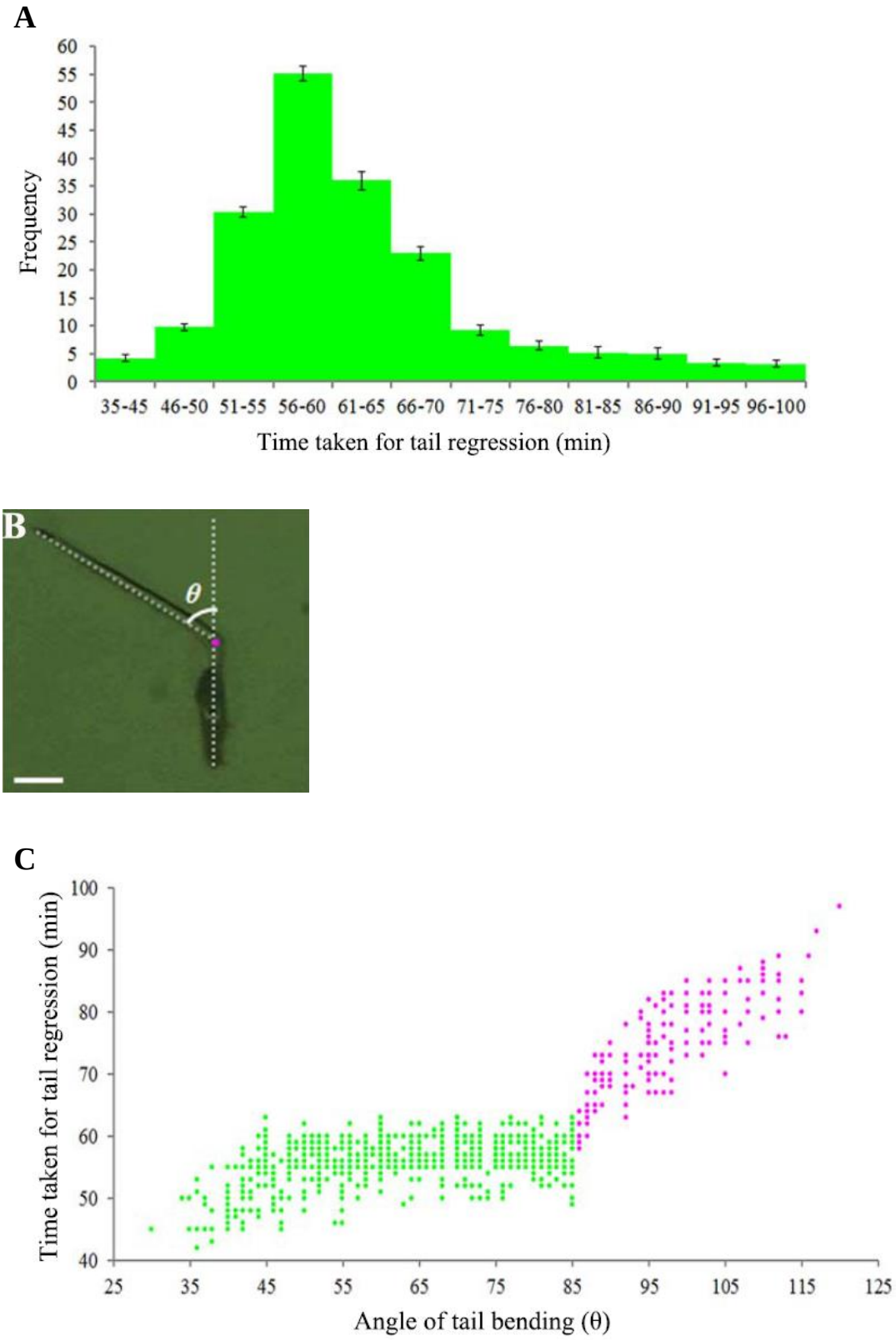
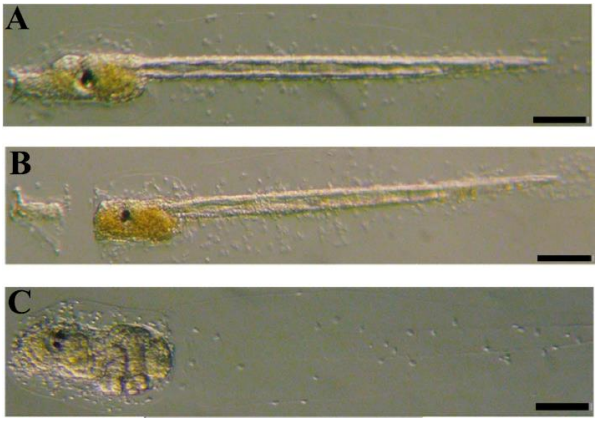
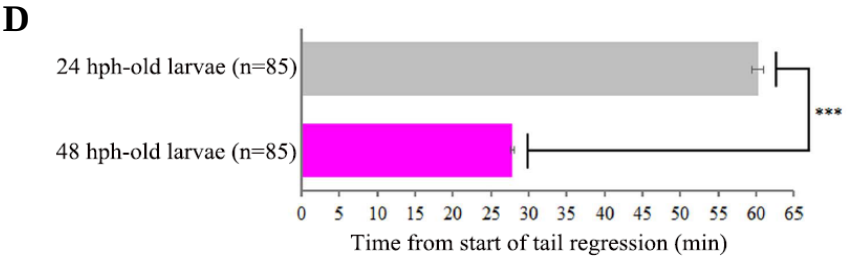

A
Frequency
60
55
50
45
40
35
30
25
20
15
10
5
0
35-45 46-50 51-55 56-60 61-65 66-70 71-75 76-80 81-85 86-90 91-95 96-100
Time taken for tail regression (min)
B
θ
C
Time taken for tail regression (min)
100
90
80
70
60
50
40
25 35 45 55 65 75 85 95 105 115 125
Angle of tail bending (θ)

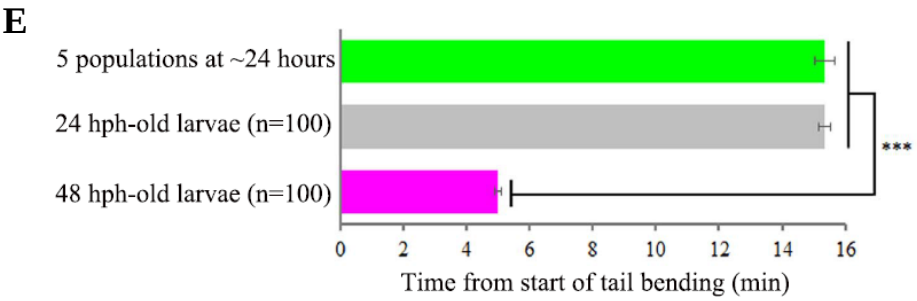
Fig. 7. The relationships between the angles of tail bending and the time spent to complete tail regression.

(A) The histogram of the time spent by larvae in tail regression. The five populations of larvae (n=141, 155, 207, 185, and 146) were analyzed. The vertical and horizontal axes respectively show the frequency and the range of time required for tail regression (min). The error bars mean the average of frequency in each of the range of five populations. (B) Measurement of the angle of tail bending. The angle of tail bending from the anterior-posterior axis as the fulcrum of the bending point was measured after tail bending stopped. The magenta circle represents the fulcrum of the bending point. Scale bar, 100 μm. (C) The relationship between the angle of tail bending and the time taken for tail regression. The vertical and horizontal axes show the time taken for tail regression (min) and the angle of tail bending, respectively. The plots were divided into two groups. Green plots represent the group in the angle range of $25^{o}<\theta<86^{o}$ and magenta plots represent the group in the angle range of $85^{o}<\theta<125^{o}$.

Figure 8


A
B
C


D
24 hph-old larvae (n=85)
48 hph-old larvae (n=85)
***
0 5 10 15 20 25 30 35 40 45 50 55 60 65
Time from start of tail regression (min)


E
5 populations at ~24 hours
24 hph-old larvae (n=100)
48 hph-old larvae (n=100)
***
0 2 4 6 8 10 12 14 16
Time from start of tail bending (min)

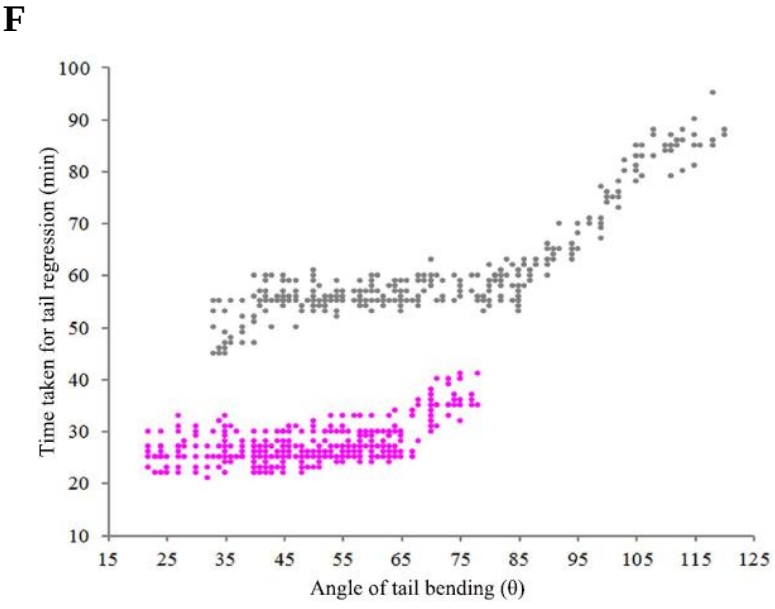

F
Time taken for tail regression (min)
100 90 80 70 60 50 40 30 20 10
15 25 35 45 55 65 75 85 95 105 115 125
Angle of tail bending (θ)

Fig. 8. The age of the larvae accelerates the time taken for tail regression.

(A-C) Tail regression induced by injury to papillae. Scale bar, 100μm. (A) An aged larva at 48 hph. (B) A papilla-cut aged larva. (C) Occurrence of tail regression after cutting of papilla. (D-F) The 24- hph-old and 48-hph-old larvae (aged larvae) were prepared, and both of their papillae were cut to induce tail regression. (D) Comparison of the time taken for tail regression by 24-hph-old and 48-hph-old larvae. The horizontal axis shows the time from the start of tail regression (min). The time taken by the aged larvae was about half that of the 24-hph-old larvae (t-test, $p<0.001$***). (E) Comparison of the time taken for tail bending among five populations at ~24 hours, the 24-hph-old larvae and the 48-hph-old larvae. The horizontal axis shows the time from the start of tail bending (min). The time from tail bending to the start of tail movement into the trunk in the aged larvae was about three times shorter than in the 24-hph-old larvae (t-test, $p<0.001$***). (F) The difference in the angle of tail bending and the time of tail regression between 24-hph-old and 48-hph-old larvae. The vertical and horizontal axes show the time taken for tail regression (min) and the angle of tail bending, respectively. Grey and magenta plots represent the results for 24-hph-old and 48-hph-old larvae, respectively.

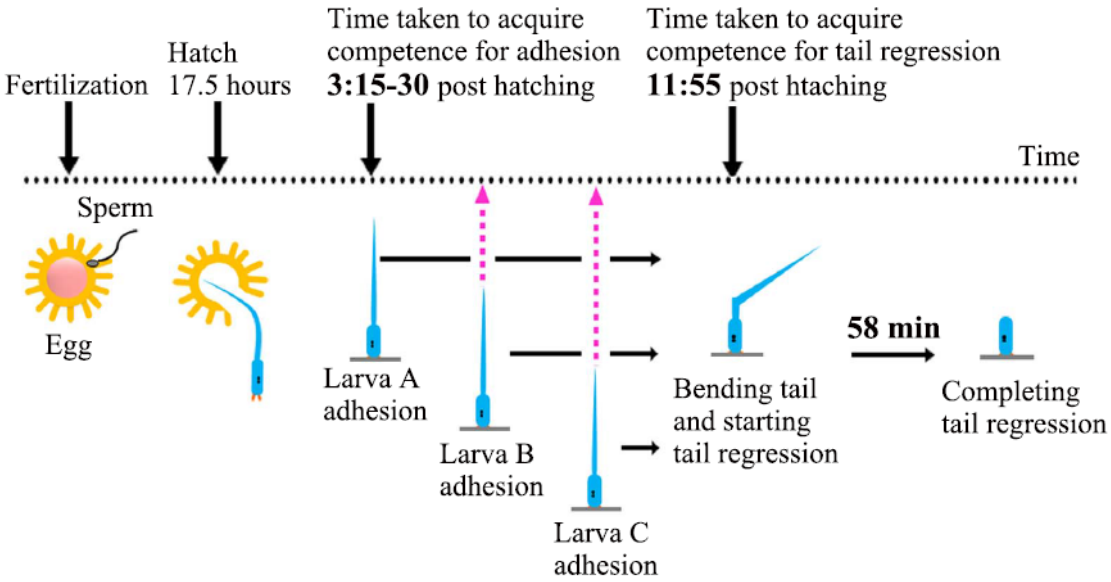


Fig. 9. A schematic diagram of the time course of metamorphosis at 18 ºC in *C. intestinalis*.

Larvae can undergo adhesion at any time after the acquisition of competence for adhesion, and this is illustrated by the three larvae A-C.