Heterochrony in the Germ Ring Closure and Tail Bud Formation in Embryonic Development of Rainbow Trout (*Oncorhynchus mykiss*)

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ABSTRACT   Due to their large yolk size, salmonid embryos take a longer time for epiboly movements and germ ring closure compared with most other teleost species. Here we analyzed the germ ring closure, tail bud formation and development of the notochord and somites in rainbow trout using live embryo imaging and in situ hybridization with the *rt-ntl* probe. *Rt-ntl* is expressed in the germ ring (blastula, gastrula and somitogenesis stage), notochord, tail bud and somites (somitogenesis stage). When epiboly covers half the yolk, a tail bud-like structure is formed and somitogenesis starts. By the time epiboly is completed, the yolk covered and the germ ring closed, the embryo has already reached the 20 somite stage. Therefore, the timing of germ ring closure and tail bud formation is reversed in trout embryos compared with zebrafish and other small model fish embryos (heterochrony). Based on this result, we re-examined the definition of tail bud formation. *J. Exp. Zool. (Mol. Dev. Evol.) 314B:187–195, 2010.* © 2009 Wiley-Liss, Inc.

The embryonic development of rainbow trout has been studied for many years and the cell fate map of trout embryos using chalk granule implantation has been established (Pasteels, '36; Ballard, '73b). In these maps, it has been shown that lateral, ventro-lateral and ventral sides of the germ ring at the early gastrula stage give rise to trunk somite, tail and lateral plate and yolk sac mesoderm, respectively. These lateral and ventro-lateral germ ring cells migrate to the dorsal side during gastrulation to form the dorsal axis at the late gastrula to early somitogenesis stage (Ballard, '73a). Besides cell fate mapping analyses, several embryonic manipulation experiments have been carried out. For example, the rainbow trout was the first teleost species in which the organizer activity that induces the head to tail axis was discovered using cell transplantation techniques (Luther, '35).

Compared with other model fish species such as zebrafish, medaka, loach and fathead minnow, salmonid embryos have an exceptionally large yolk size (~5 mm in diameter for rainbow trout and ~1 mm for these other species) (Ballard, '73c; Manner and Dewese, '74; Iamatsu, '94; Kimmel et al., '95; Fujimoto et al., 2004). Despite the size of the yolk, as with other teleost species,

trout embryos also undergo typical epiboly movement (Ballard, '73a; Iwamatsu, '94; Kimmel et al., '95). Epiboly consists of embryonic cells elongating from the animal pole towards the vegetal pole, and eventually covering up the yolk. However, possibly because of the yolk size (and also partly due to the low temperatures in which trout eggs naturally develop (Ballard, '73a), it takes a longer time for the epiboly to reach the vegetal pole in trout (e.g. 9 hr for zebrafish compared with 9–10 days for rainbow trout). This time difference causes a striking difference in the sequence of developmental events occurring in early embryonic development (heterochrony). In zebrafish, epiboly reaches the vegetal pole, then the tail bud-like structure begins to form, and

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subsequently somitogenesis starts and tail elongation follows (Kimmel et al., '95). However in the rainbow trout embryo, while epiboly is still only half covering the yolk, somitogenesis starts. By the time epiboly covers the whole yolk and the margin of epiboly (the germ ring) is closed, the embryo has already formed 15–20 somites (Ballard, '73c). This seems to suggest that somitogenesis does not need to wait for the end of germ ring closure.

Somite formation is activated from signals emanating from the area around the tail bud in teleost and other known vertebrate species such as chick, mouse, snake, frog and fish (Palmeirim et al., '97; Holley et al., 2000; Pourquie, 2003; Gajewski et al., 2006; Gomez et al., 2008). Therefore the fact that somitogenesis starts before germ ring closure in the rainbow trout may suggest that the tail bud is formed before germ ring closure as well. There are extensive studies on tail formation and anatomy of the tail bud in fish using zebrafish as a model. Cell fate mapping and time lapse analyses in zebrafish, tracking gastrula to bud stages, revealed that the major part (except the notochord and floor plate) of the future tail structure is derived from the ventral side of the gastrula embryos (Kimmel et al., '90; Kanki and Ho, '97; Kudoh et al., 2004). When the germ ring is closed, ventrally and dorsally-derived cells merge with each other to form the tail bud (Kanki and Ho, '97). However, in the trout embryo, if the tail bud is formed before germ ring closure, the tail bud could have formed without the contribution of the ventrally-derived germ ring cells. To examine this possibility, we analyzed gene expression patterns in the germ ring and tail bud using the trout *ntl* (*no tail*) gene as a probe.

Among the genes expressed in the germ ring and tail bud, *beachyury/ntl* gene is the most popular marker gene; expression has been analyzed in many vertebrate species (Wilkinson et al., '90; Smith et al., '91; Schulte-Merker et al., '94; Kispert et al., '95; Sauka-Spengler et al., 2003; Coolen et al., 2008). In zebrafish, *ntl* is expressed at the gastrula stage in the germ ring that becomes narrower as gastrulation proceeds (Schulte-Merker et al., '94). At the end of gastrulation, the germ ring of *ntl* expression is closed and the closed ring forms a large dot of *ntl*-expressing cells in the vegetal pole. These cells become the central part of the tail bud that mainly gives rise to the tail notochord and somites.

In this study, we analyzed the morphological changes during epiboly, germ ring, tail bud and somite development in rainbow trout embryos using live imaging and *rainbow trout-ntl* (*rt-ntl*) in situ hybridization. Our data emphasize the timing-difference (heterochrony) of epiboly movement, tail bud formation and associated *ntl* gene expression between trout and other fish species, and also raise a question about the definition of tail bud formation.

MATERIALS AND METHODS

Dissection of Live Rainbow Trout Embryos

Rainbow trout eggs and milt were stripped at Hatchlands trout farm (Rattery, Devon, United Kingdom) and transferred to aquaria at the University of Plymouth on ice. Eggs were then fertilized with milt and placed into the experimental set-up that consisted of plastic containers supplied with running water (7.4 ± 0.02 pH; 98.6 ± 0.3% dissolved oxygen; 8.6–9.0°C). Eggs were held in a 12 hr light:dark cycle where "light" was shaded lighting. To take photographs of live embryos, embryos were manually removed from the chorion membrane in PBS (phosphate buffered saline) using tweezers and dissection microscopy. The pealing of the chorion was initiated from the vegetal pole in day 2 to day 8 pf embryos. After day 9 pf, pealing was done from the ventral side to minimize damage to the embryos. Staging of the embryos was based on previous literature (Ballard, '73c).

The staging of the trout embryo has previously been carried out using fixed embryos with detailed diagrams (Ballard, '73c) and, to our knowledge, live images for early stage embryos in salmonids have not been recorded. This is possibly because of technical difficulties associated with removing the embryo from a thick chorion membrane and the large size of the fragile yolk. In salmonid embryos, the space between the yolk and chorion membrane is very narrow or absent and, therefore, it can be very difficult to remove the chorion membrane without damaging the yolk. If the yolk is damaged in normal fresh water, the yolk material denatures and produces white precipitates that affect further dissection and photography. To avoid such yolk precipitation, the chorion membrane was removed in a high salt buffer, PBS (phosphate buffered saline). In this condition, if the yolk is damaged, it remains in a transparent, jelly-like state so it was possible for us to dissect embryos and to take clear photo images.

In Situ Hybridization

A full length trout *ntl* cDNA was obtained from the trout genomics AGENAE program (Govoroun et al., 2006) in France (Genbank accession No. GQ241688). Using the cDNA, a digoxigenin-labelled antisense RNA probe was synthesized. Trout embryos were pealed from the chorion membrane along with removal of more than 70% of yolk materials and fixed in 4% paraformaldehyde/PBS at 4°C overnight. The embryos were briefly washed with PBS, dehydrated in 50% methanol/PBS for 30 min at room temperature and then kept in 100% methanol at −20°C for at least 12 hr. In situ hybridization of the embryos was performed as previously described (Strahle et al., '93) with modifications. Embryos in methanol were washed with 50% methanol/PBS and PBSTw (PBS + 0.1% tween20) for 30 min each. The embryos were then treated with proteinase K (Sigma, Gillingham, Dorset, UK) at 28°C for 15 min with different concentrations depending on the stages (100 μg/mL until day 9 pf and 200 μg/mL after day 10 pf). Embryos were next washed with PBTw (10 min × 2), and prehybridized with Hyb mix (50% formamide, 5× SSC, 5 mM EDTA, 0.1% Tween20, 50 μg/mL heparin (Sigma), 1 mg/mL torula RNA (Sigma)) at 65°C for 2 hr. The embryos were subsequently mixed with RNA probe in the Hyb mix (the probe was diluted in the Hyb-mix, heated at

80–90°C for 10 min and then chilled on ice for a further 10 min) and incubated overnight at 65°C. The embryos were then washed with 50% formamide in 2× SSCTw (2× SSC+0.1% Tween20) once, in 2× SSCTw twice and in 0.2× SSCTw (0.2× SSC+0.1% Tw20) twice for 30 min each. The embryos were washed with PBSTw at room temperature and preabsorbed in the blocking solution (2% blocking reagent (Roche, Burgess Hill, West Sussex, UK) with 5% Goat serum in PBSTw) for 1 hr. Embryos were added to antidigoxygenin antibody (Roche) at a 1:5000 dilution in the blocking solution and incubated at room temperature for 2 hr. After five washes in PBSTw and one wash in alkaline buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM MgCl₂, 0.1% Tween20) for 30 min each, bound antibody was detected by the alkaline phosphatase-staining reaction in the BM-purple (Roche).

RESULTS

Live Observation of Cleavage to Gastrula Stage Embryos

Live embryos were photographed from the lateral view. Early cleavage to the gastrula stage is highlighted in Figure 1. At stage 5 (32 cell stage, day 1 for our culture condition), each cleaving cell is still visible (Fig. 1A). At stage 6 (Fig. 1B), the shape of blastoderm is between spherical and cylindrical. At stage 7 (Fig. 1C), the blastoderm becomes flatter and wider and at the late stage 7 (Fig. 1D), the blastocoel is formed and the internal region

of the blastoderm becomes lighter in color. At stage 8 (Fig. 1E), the embryonic shield is formed at the prospective dorsal side. Subsequently, the hypoblastic part of the shield elongates toward the animal pole and at this stage, the surface of the yolk facing the hypoblast changes in shape from flat to round (Fig. 1D, E).

At stage 9 (Fig. 1F), the dorsal axis becomes longer and reaches close to the animal pole. By stage 10a (Fig. 1G), epiboly expands but still does not reach the equator (30–40% epiboly). The prospective head domain becomes obvious at the animal pole and a tail bud-like structure is also formed at the dorsal margin of the epiboly. This suggests that the tail bud is formed before germ ring closure.

Live Observation of Somitogenesis and Organogenesis Stage Embryos

In between stage 10a and stage 10b (Fig. 1G and 2A), somitogenesis starts. At this point, epiboly covers half of the yolk (50–80% epiboly, Fig. 2A, K) and the eyes, midbrain, hindbrain and midbrain–hindbrain boundary become obvious. Between stages 11 and 14, the number of somites increases (by roughly 9 somites/day) (Fig. 2A–D). During this period, epiboly finally covers the yolk and the germ ring is closed (stage 12, Fig. 2C). At stage 16, the tail elongation starts and a distinct tail structure becomes obvious (Fig. 2E). At stage 17, the trunk and tail start to show slow movement (Fig. 2E). It is noteworthy that
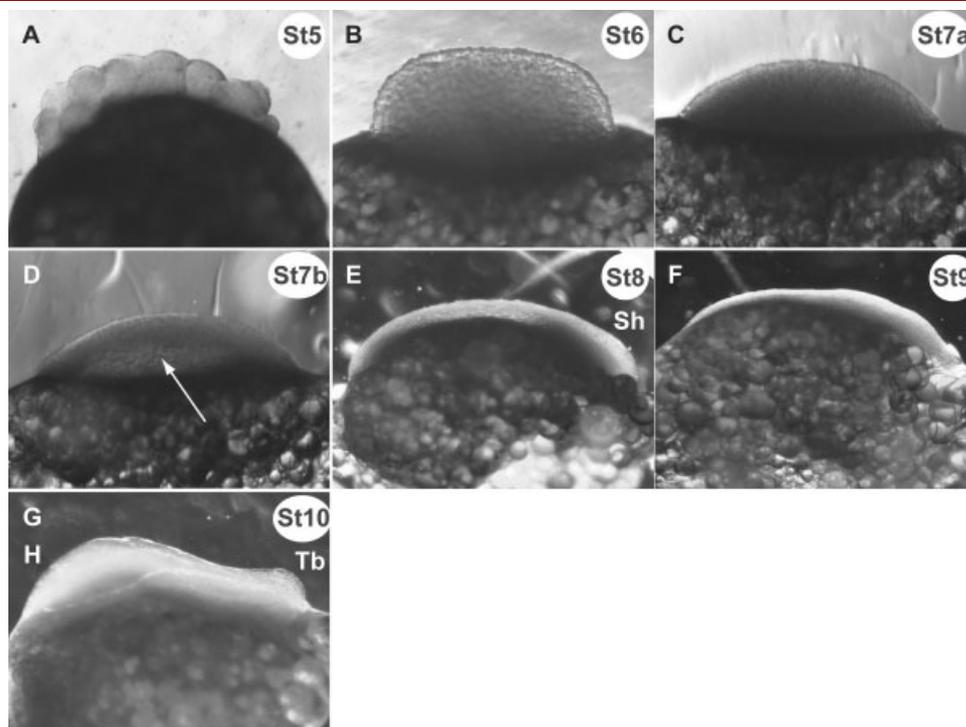

Figure 1. Live image of dissected rainbow trout embryos: Stage 5 to 10a. Lateral view. (**A**) 1dpf/St.5, (**B**) 2dpf/St.6, (**C**) 3dpf/St.7a, (**D**) 4dpf/St.7b, arrow indicates formation of a cavity in the blastula embryo, (**E**) 5fpf/St.8, (**F**) 6dpf/St.9, (**G**) 7dpf/St.10a. Sh, shield; H, head; Tb, tail bud.
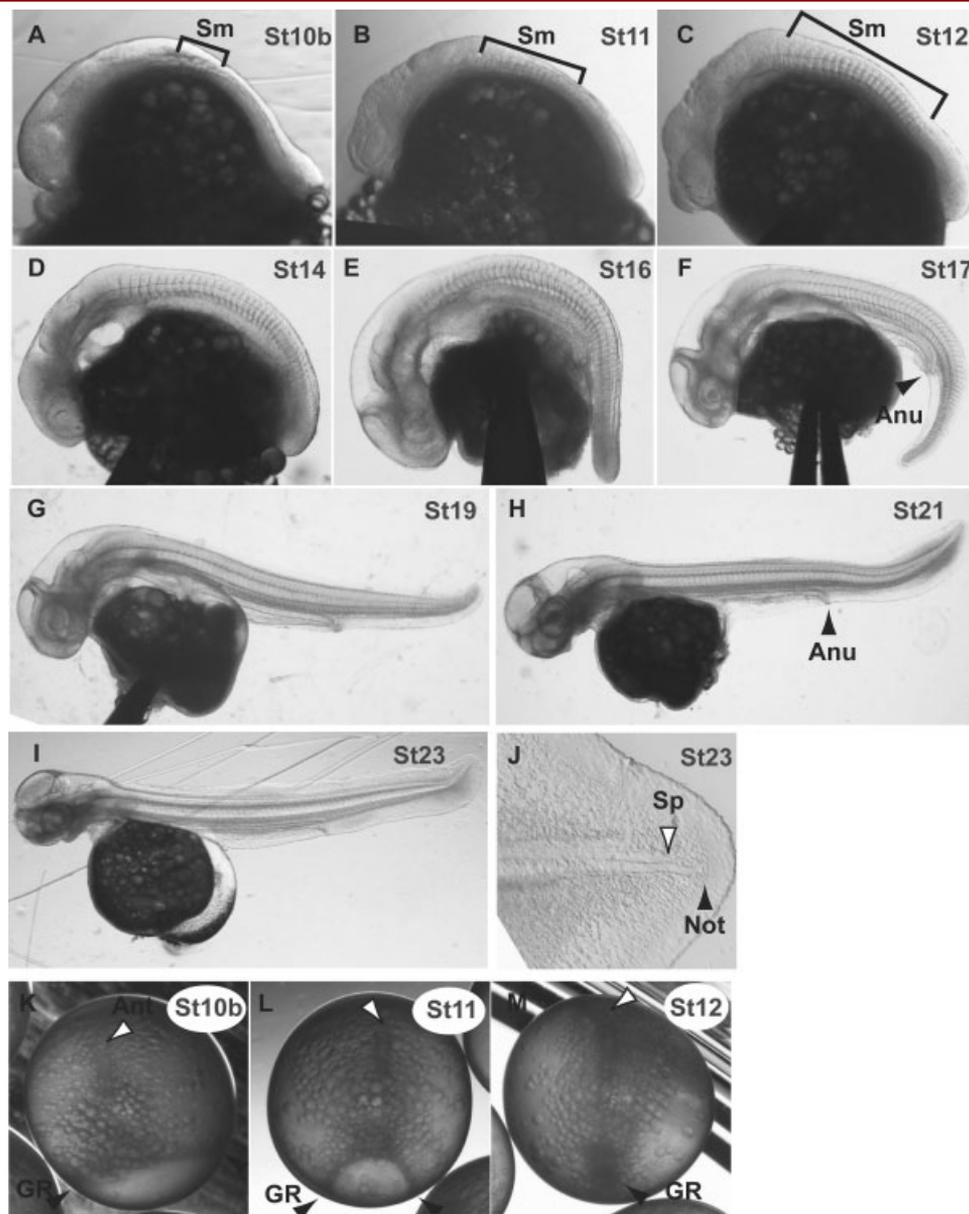
Figure 2. Live image of rainbow trout embryos: Stage 10b to 23. Lateral view of dissected embryos (**A–J**) and dorsal view of embryos with chorion membrane (**K–M**). (A,K) 8dpf/St.10b, (B,L) 9dpf/St.11, (C,M) 10dpf/St.12 (D) 12dpf/St.14, (E) 14dpf/St.16 (F) 16dpf/St.17, (G) 18dpf/St.19, (H) 22dpf/St.21. (I,J) 26dpf/St.23, Sm, somite; Anu, anus; Sp, spinal cord; Not, notochord; GR, germ ring.

zebrafish embryos show first muscle movement around the 18 somite stage whereas rainbow trout embryos do not start moving until stage 17 when more than 50 somites have formed.

At stage 19, the tail becomes straight (Fig. 2G) and at stage 21, the proliferation of tail fin cells is increased and a thick cell mass is formed at both the dorsal and ventral side of the tail (Fig. 2H). At stage 23 (Fig. 2I), the tail fin becomes wider and the end of the notochord and spinal cord are clearly visible with high

magnification. Interestingly the ends extend to different lengths (the notochord extends further toward the end of the tail) and they are not connected to each other (Fig. 2J).

Rainbow Trout ntl (rt-ntl) is Expressed in the Germ Ring, Notochord and Tail Bud During Early Development

To analyze the morphology of the germ ring, tail bud and notochord, we examined the gene expression pattern of *rt-ntl* in a

series of rainbow trout embryos. *Rt-ntl* is initially expressed in a small area (possibly at the dorsal organizer) at the blastoderm margin (germ ring) at stage 7a (Fig. 3B). The expression spreads along the germ ring at stage 7b (Fig. 3C). At stage 8, *rt-ntl* expression domain in the germ ring becomes thicker and the embryonic shield becomes obvious as a domain with particularly wide expression of *rt-ntl* (Fig. 3D). Subsequently *rt-ntl* expressing shield cells elongate and start to form the notochord (stage 9, Fig. 3E). At stage 10, the germ ring marked by *rt-ntl* becomes larger as epiboly expands along the yolk whereas the germ ring itself
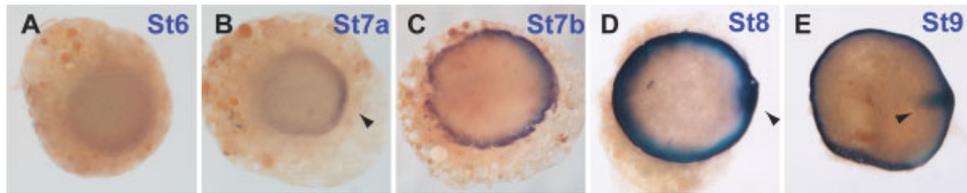

Figure 3. *Rt-ntl* expression in the blastula to gastrula embryos: Stage 6 to 9. Animal pole view. (**A**) 2dpf/St.6, (**B**) 3dpf/St.7a, (**C**) 4dpf/St.7b, (**D**) 5dpf/St.8. (**E**) 6dpf/St.9, Sh, shield; Not, notochord.
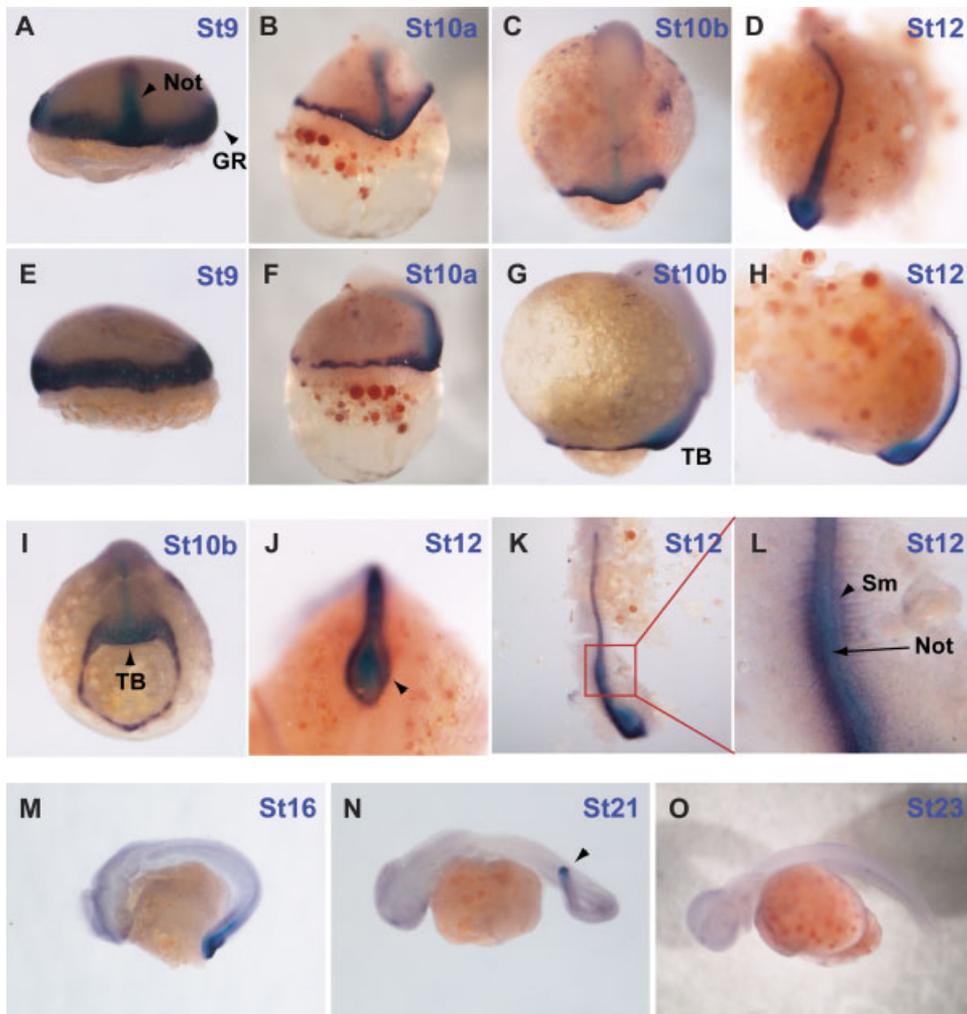

Figure 4. *Rt-ntl* expression in the gastrula to somitogenesis embryos: Stage 9 to 23. (**A–D**) dorsal view, (**E–H, M–O**) lateral view, (I, J) posterior view (K, L) flat mount view (A, E) 6dpf/St.9, (B, F) 7dpf/St.10a, (C, G, I) 8dpf/St.10b, (D, H, J, K, L) 10dpf/St.12, (M) 14dpf/St.16, (M) 22dpf/St.21, (N) 26dpf/St.23, GR, germ ring; Not, notochord; Sm, somite; Ant, anterior end; TB, tail bud.

becomes significantly thinner (Fig. 4B, F). A thick tail bud-like structure is already obvious (Fig. 4G). At stage 12 (21 somites), the germ ring is finally closed and the *rt-ntl* expression domain in the tail bud becomes leaf shaped (Fig. 4D, H, J). To analyze the expression domain in more detail, we flat mounted the stage 12 embryos stained with *rt-ntl*. In an enlarged view, we found that *rt-ntl* is expressed in posterior somites in addition to the tail bud and notochord (Fig. 4L). This is unique to trout embryos as expression of *ntl/bra* in somites has not been reported in other species. In the stage 10 embryos, somites and the margin of the tail bud are stained purple while the notochord and central part of the tail bud (end of the notochord), are stained blue. This is possibly because NBT+BCIP substrates react with alkaline phosphatase to stain with a purple color at the surface of the embryos whereas, BCIP alone predominantly reacts in the deeper layer that generates a blue color. After stage 12, *rt-ntl* expression gradually decreases from the anterior notochord and becomes restricted more to the posterior notochord and tail bud

(Fig. 4M, N). At stage 23, *rt-ntl* expression is no longer observed (Fig. 4O).

DISCUSSION

Massive Expansion of Germ Ring in the Rainbow Trout Embryos at the Epiboly Stage

Figure 5A illustrates morphological changes of the *rt-ntl* expression domain. *Rt-ntl* is expressed as a ring structure at the blastoderm margin (germ ring) around stage 7, and subsequently expression becomes wider in the embryonic shield (organizer) at stage 8. The germ ring was widest when epiboly reached the middle of the yolk (50% epiboly). Between these stages, the *rt-ntl* expression domain undergoes significant expansion (4–5 times in diameter) in the germ ring without breaking the continuous ring structure. This expansion is significant compared with other model fish species (zebrafish show 1.1 times expansion and
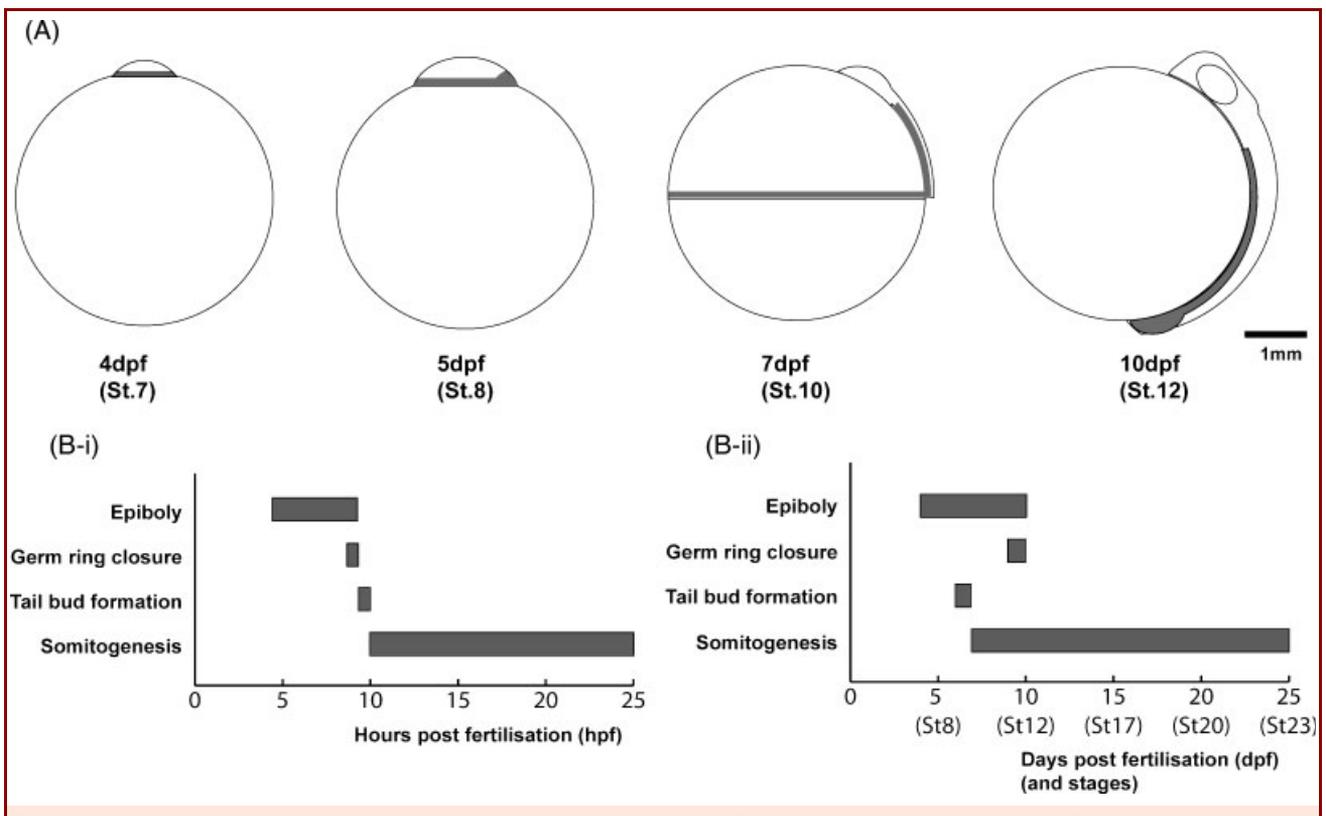

Figure 5. Heterochrony of germ ring closure and tail bud formation in zebrafish and rainbow trout embryos. **(A)** Expansion of germ ring in the rainbow trout embryo. Diagram of rainbow trout embryos with *rt-ntl* expression domain (gray). Developmental stage and date after fertilization is indicated below the embryo. When *rt-ntl* initially was expressed as a ring at the late blastula stage (stage 7), the diameter of the ring was approximately 1 mm. The ring diameter was greatest at stage 10 (4–5 mm), and eventually decreased and closed around the tail bud at stage 12. **(B–i)** In the zebrafish, when the epiboly covers the yolk, the germ ring is closed and the tail bud is formed (bud stage). Subsequently, somitogenesis starts (Kimmel et al.,'95). **(B–ii)** In the rainbow trout, it takes longer for epiboly to cover the yolk and the germ ring to close. While the germ ring is still open, the tail bud is already formed and somitogenesis starts.

medaka 1.5 times (Schulte-Merker et al., '94; Araki et al., 2001; Kudoh and Dawid, 2001). As seen in the diagram, the germ ring marked by *rt-ntl* had to move about 4 mm in distance during the epiboly stage, which is 8 times longer in distance compared with zebrafish. This difference may explain the prolonged period of the epiboly stage and the overlap of epiboly and somitogenesis in the rainbow trout.

Germ Ring Expansion as an Evolutionary Strategy to Increase Yolk Size

The expansion of the germ ring in teleosts may be a unique strategy for embryos to facilitate forming a large yolk size. The other strategies that may facilitate forming a large yolk size include those seen in dogfish. In dogfish, when epiboly expands to cover the yolk, the germ ring mesendoderm that is marked by *ntl/brachyury* family genes are split left and right (Sauka-Spengler et al., 2003). By breaking the ring structure, the embryos could be freed from the size restriction of the germ ring, and could allow the formation of a large yolk size. Rainbow trout may be an extreme case for a teleost-type of yolk expansion that expands the germ ring without breaking the ring. Such a strategy is possibly an evolutionary development from an ancestral teleost with smaller yolk and germ ring. However, the strategy of expanding yolk in teleosts would still have a limitation for the germ ring size, therefore, dogfish type strategies would possibly be more efficient.

Heterochrony in the Germ Ring Closure, Tail Bud Formation and Initiation of Somitogenesis Between Rainbow Trout and Other Model Fish Species

As seen in Figure 5B-i, in zebrafish, epiboly elongation, germ ring closure, tail bud formation and somitogenesis are sequential events that occur during the gastrula and somitogenesis stages. However, in the rainbow trout, these events are obviously not sequential. While the germ ring is still open and epiboly is continuing the tail bud has already formed and somitogenesis is starting (Fig. 5B-ii). By the time the germ ring is closed, the trout embryo has already generated more than 20 somites. These differences raise a question about the definition of the tail bud. Cell fate mapping in zebrafish has revealed that, from the gastrula to bud stage, cells that are fated for future tail structures are localised at the ventral side of the spherical gastrula embryo (Kimmel et al., '90; Kanki and Ho, '97; Kudoh et al., 2004). However, in the rainbow trout, because of the longer time for germ ring closure, ventrally located cells have not merged to the tail bud-like structure at stage 10 (Fig. 4F, G). This may mean that, in trout embryos, the tail bud is formed only from dorsally derived cells. Alternatively, some ventral germ ring cells at the blastula and early gastrula stages may have already moved to the dorsal side by stage 10 and, therefore, the majority of prospective tail cells may be relocated at the dorsal side by that stage. The latter case seems more likely for two reasons: First, cell fate mapping data indicate that prospective tail cells are originally located at the ventral and lateral side of the germ ring at the early gastrula stage (Pasteels, '36), and these cells undergo extensive cell movement toward the dorsal side before germ ring closure (Ballard, '73a). Second, our *rt-ntl* in situ data indicates that *rt-ntl* is widely expressed in the ventral germ ring at early gastrula (stage 8 and 9) whereas the ventral germ ring marked by *rt-ntl* at stage 10 is significantly thinner (Fig. 4E and I). This suggests that most of the cells have already moved away from the ventral and lateral side of the germ ring at a later epiboly stage and contributed to form the tail bud at that stage. However, a small number of cells still remain in the lateral and ventral side of the germ ring until the germ ring closes. It seems that these cells join the later tail bud or near by in the ventral side of the tail when the germ ring is closed.

Tail Formation as an Extension of the Trunk

Gene expression pattern in the tail bud has been extensively studied in *Xenopus* embryos (Gont et al., '93; Beck and Slack, '98). Gont et al. (1993) proposed that the tail bud is a derivative of the dorsal blastopore lip that expresses similar sets of genes including the *bra/ntl* homolog, *Xbra*. The tail bud grafts has activity for Spemann's tail organizer as seen in the gastrula blastopore lip in the *Xenopus* embryo. From these data, they discussed that the tail bud is not an undifferentiated blastema but rather consists of distinct cell populations that arise during gastrulation (Gont et al., '93). These cell populations for the tail bud (and gastrula tail organizer) may consist of several cell types. It has been shown in *Xenopus* that the tail bud consists of multiple domains that include the posterior end of the notochord, somites and spinal cord (Gont et al., '93; Beck and Slack, '98). Interaction of these three domains facilitates construction of a functional tail bud (Tucker and Slack, '95; Beck and Slack, '98). It is likely that induction and interaction of the notochord, paraxial mesoderm (prospective somite) and neural plate (prospective spinal cord) are equally important for trunk formation at the gastrula stage. Considering the morphological similarity between the trunk and tail in the spinal cord, notochord and somites, formation of these structures in the trunk and tail is possibly a continuous process of induction and interaction of these tissues and because of the morphological similarity between the trunk and tail neural tube, somites and notochord, it is difficult to define the border between trunk and tail in vertebrate embryos. One of the best definitions of the border between trunk and tail in the vertebrate embryo is the position of the anus. However, depending on the embryonic stage, the relative position between the anus and somite muscle dynamically shifts. For instance, in rainbow trout embryos, at stage 17 the anus is located at roughly the 40th somite from the anterior end. However, at stage 21, the anus is located at roughly the 35th somite. This indicates that there is a shift of five somites from the trunk to tail between stage 17 and 21. Therefore, the definition of the trunk and tail border can be highly variable between stages.

Definition of the Tail Bud

There could be three possible ways to define the tail bud: (1) Tail bud-like activity and structure might already be formed at the gastrula stage and involves the elongation and induction of tail structures (notochord, muscle and spinal cord) from the gastrula stage. This definition might scientifically be the most sensible as the tail organizer activity, gene expression pattern and cell fates seem to all be established at the gastrula stage. However the bud-like structure is not morphologically obvious at this stage. In addition, the tail bud at the somitogenesis stage seems to have a unique function such as signalling the somitogenesis clock (Palmeirim et al., '97; Holley et al., 2000; Gajewski et al., 2006; Gomez et al., 2008). Therefore, it is still not clear if the functional tail bud observed in the somitogenesis stage is already fully specified at the gastrula stage. (2) Tail bud is formed just before the somitogenesis stage. At this stage, a prominent bud structure becomes obvious in both zebrafish (bud stage) and trout (stage 10) (although epiboly is not closed in the trout embryos). (3) Tail bud is formed when germ ring is closed by merging the ventrally and dorsally derived germ ring cells (bud stage for zebrafish and stage 12 for trout).

Considering the timing of somitogenesis and the development of other tissues, trout stage 7 seems most equivalent to the zebrafish bud stage, therefore the definition (2) seems the most convenient to compare tail bud formation in different fish species. However at stage 7, even if a tail bud is formed, *rt-ntl* gene is expressed in the germ ring that is still widely open (30–50% epiboly) and, therefore, it is possible that although a functional tail bud may be formed at stage 7 in the trout embryo, the real completion of tail bud formation occurs at stage 10 when the germ ring is completely closed and cells from the ventral side of the germ ring merge to form the tail bud.

Tail Bud Formation May Link to the Initiation of Somitogenesis

Our data suggest that the tail-bud like structure is formed before epiboly closure, slightly preceding the first somite formation (Fig. 5B-i,ii). This data in rainbow trout seems to have a correlation with zebrafish and medaka data in that the first somite is formed after the tail bud stage (Iwamatsu, '94; Kimmel et al., '95). This correlation may suggest common mechanisms in teleosts in which tail bud formation precedes the initiation of somitogenesis, and formation of a functional tail bud might be necessary for signalling the formation of somites. This idea is consistent with the finding that the signal for the wave of somitogenesis emanates from the posterior presomatic mesoderm located around or within the tail bud in different vertebrate species (Palmeirim et al., '97; Holley et al., 2000; Pourquie, 2003; Gajewski et al., 2006; Gomez et al., 2008). It is likely that *ntl*-expressing cells in the lateral germ ring at the gastrula stage contribute to the formation of presomatic mesoderm in the tail bud that initiates the signal to form somites in the anterior presomatic mesoderm. This idea would explain the link between the formation of the tail bud-like structure and initiation of somitogenesis. Therefore, tail bud formation would possibly need to precede somite formation. It would be of interest to know the molecular signalling that links tail bud formation and initiation of the somitogenesis clock.

Possible Heterochrony Between Initiation of Somite Boundary Formation and Suppression of *rt-ntl* Expression

In fish and other vertebrate species examined so far, *ntl/brachyury* is not expressed in the somites (Wilkinson et al., '90; Smith et al., '91; Schulte-Merker et al., '94; Kispert et al., '95; Sauka-Spengler et al., 2003; Coolen et al., 2008). For instance, in zebrafish, *ntl* is expressed in the lateral germ ring that mainly gives rise to somites. However, when these cells are involuted in the hypoblast and migrate along both sides of the axial mesoderm, *ntl* expression is mostly suppressed except in the posterior presomatic mesoderm and in the tail bud (Schulte-Merker et al., '94). In contrast, in the rainbow trout, *rt-ntl* expression was observed in newly formed somites and gradually disappeared when the somites matured. Therefore, it seems that, in trout, the somite boundary starts forming before presomatic mesoderm stops expressing *rt-ntl*. This event seems to be a heterochrony of the suppression of *rt-ntl* expression and initiation of the somite boundary formation between salmonids and other fish and vertebrate species examined so far (Wilkinson et al., '90; Smith et al., '91; Schulte-Merker et al., '94; Kispert et al., '95; Sauka-Spengler et al., 2003; Coolen et al., 2008). This data also suggest that, although *ntl/brachyury* expression marks undifferentiated presomitic mesoderm in most species, this expression is still compatible with the progress of somite development and its boundary formation. In other words, cell differentiation regulated by *ntl/brachyury* and signalling to form the somite boundary occur in parallel as separable events. The possible reason why *rt-ntl* expression is attenuated until the stage when the somite boundary is formed may be due to the large yolk size and/or low temperature development of the trout embryos. Because of the large yolk size, germ ring closure is delayed until the 20 somite stage in the rainbow trout. Such a delay may cause some delay in cell differentiation in the posterior presomitic mesoderm which may coincide with the delay of the suppression of *rt-ntl* expression. It is also possible that embryonic development at a low temperature may cause attenuation of *rt-ntl* expression in the rainbow trout. RNA molecules have a higher ability to maintain a three dimensional structure at lower temperatures (Baumruk et al., 2001; Blose et al., 2007), therefore, RNA at higher temperatures (28°C for zebrafish) may quickly lose three dimensional structure and could be degraded quickly compared with a low temperature environment (9°C for rainbow trout).

Our findings suggest heterochrony, both in morphological and gene expression changes, during germ ring closure, tail bud formation and somitogenesis in a vertebrate species for the first time. These findings clarified germ ring closure and tail bud formation as separable events, as well as mesoderm development and somite boundary formation.

LITERATURE CITED

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