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**Analyses of defense morph formation of predator-induced
polyphenism in *Daphnia pulex***

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GENERAL INTRODUCTION

During the past decade, breakthroughs in molecular biology that led to our exponentially expanded genomic knowledge have innovated our current understanding of molecular mechanisms of developmental processes, but the evolution is still far away to be described by the molecular language. Currently, a new research field, evolutionary developmental biology (Evo-Devo) to provide the view that evolution is caused by heritable changes in the development of organisms was built. From the idea that changes in developmental processes give rise to the novel phenotypes and thereby drive evolution, environmentally triggered phenotypic plasticity is focused as a study model to get insights into evolutionary processes (Gilbert, 2010). Phenotypic plasticity is a phenomenon in which organisms change their phenotypes in response to environmental information into more adaptive ones in most cases, and is a universal property of living things. Eberhard (2003) defined phenotypic plasticity as the ability of an organism to react to an environmental input with a change in form, state, movement, or rate of activity.

Many organisms are able to change their morphology and/or behavior in response to the threat of predation. The phenotypic alteration by modulating developmental processes in the presence of predators is called “inducible defense” or “predator-induced polyphenism”, and these modifications can increase the fitness of prey organisms (Gilbert, 2010). Various members of the cladoceran genus *Daphnia* (commonly called “water fleas”) display a variety of phenotypic changes mainly in head shape (Tollrian and Harvell, 1999). Among predator-induced polyphenisms of *Daphnia* species, the best-studied system is the induction of neckteeth (or necktooth) in *D. pulex* by a kairomone (or kairomones) released by predatory phantom midges, *Chaoborus* larvae (Tollrian and Harvell, 1999). A kairomone is a class of semiochemicals. The general term semiochemicals applied to a chemical or mixture of chemicals that act as

messengers within or between species. Kairomones are substances, released into the environment by an organism, that induce reactions in another species in a way that the recipient rather than the emitter receives benefit (Grasswitz and Jones, 2002). Unfortunately, the molecular structure and the action mechanism of the *Chaoborus* kairomone are still unknown.

Daphnia species are important in pond and lake ecosystems, and their field ecology has been well studied (Dodson et al., 2009). *Daphnia pulex* is a potentially useful model organism for studying predator-induced polyphenism at the genetic and molecular levels (Stollewerk, 2010), for several reasons. It was the first crustacean to have its genome sequenced (Colbourne et al., 2011), and a genetic linkage map has been constructed (Cristescu et al., 2006). In addition, molecular techniques such as in situ hybridization and RNAi have now been extended to daphnid species (Sagawa et al., 2005; Kato et al., 2011). Finally, individuals with the same genetic background can express a variety of phenotypes in response to environmental cues (Tollrian and Harvell, 1999). Interdisciplinary studies of predator-induced polyphenism in *D. pulex* thus have a potential to elucidate the mechanisms underlying the evolution of plasticity (Jenner and Wills, 2007; Tollrian and Leese, 2010).

The first step in kairomone-mediated adaptive morphological change of *Daphnia pulex* may be the reception of the chemical signal by specialized sensory structures of the prey. After that, a putative physiological cascade for the defense morph formation consisting of the following steps is suggested: physiological changes through endocrine mechanisms and morphogenesis triggered by pattern formation genes. At this point, however, there is fragmented information about the developmental mechanisms of predator-induced polyphenism in *D. pulex*.

In Chapter 1, to establish the basis for studying predator-induced polyphenism in *Daphnia pulex* developmentally, the time course of embryonic development was observed and described in detail. And then, I devised the rearing method to define

kairomone sensitivity for defense morph induction in *D. pulex*. Further, I succeeded in reaffirming the protective effect by video recording. In Chapter 2, to clarify the detailed timing of the kairomone sensitive period, I designed some rearing experiments and argued the embryonic influences in response to the kairomone signals during embryonic stages. Additionally, I conducted histological analyses of the cell proliferation and of morphological changes in the process of necktooth formation. Finally the hypothesis of the cytological alteration in necktooth formation of *D. pulex* is presented, together with ideas that will help to develop future studies of predator-induced polyphenisms in *Daphnia* species.

Chapter 1

***Daphnia pulex* as a model organism for the study of predator-induced polyphenism**

INTRODUCTION

“Inducible defense” or “predator-induced polyphenism” are phenotypic changes induced by cues associated with biotic agents; most can measurably diminish the effects of subsequent attacks by the agents. The study of shifting phenotypes in response to predators is not only the focus of ecologists, but analysis of predator-induced polyphenism may make a contribution to understand about mechanisms in environment-driving evolution of novel phenotypes by integrating multidisciplinary studies from developmental biology, genetics, chemistry and so on.

The water flea *Daphnia* (Crustacea, Cladocera, Anomopoda) provides a good model system for studying inducible defenses. Several *Daphnia* species have been known to be either polymorphic or undergo some sort of seasonal change in form (Hutchinson, 1967). But researchers in the late Twentieth Century have found that the different forms experience different degrees of mortality in the presence of predators (reviewed in Tollrian and Harvell, 1999). The best-studied system is the relationship between *Daphnia pulex* and their predatory phantom midges *Chaoborus* (Diptera, Chaoboridae), which has become a model system for the study of predator-induced polyphenism. Krueger and Dodson (1981) showed for the first time that exposure of egg-bearing *D. pulex* to the third or fourth larval instar of *Chaoborus americanus* results in offspring characterized by a toothed dorsal crest called “neckteeth (necktooth)”. Necktooth effectively decreases the risk of predation by obstructing predators to capture *Daphnia* juveniles that have this outgrowth, and is completely absent in adult instars (Tollrian and Dodson, 1999; Laforsch and Tollrian, 2004) .

Daphnia pulex is a large (up to 3 mm long) pond-dwelling cladoceran. In a normal growth season *Daphnia pulex* generates diploid eggs by asexual reproduction (parthenogenesis). These eggs develop directly into larvae in the female brood chamber and are released into the water. Several investigations on *D. pulex* showed the

sensitivity to *Chaoborus* kairomone during their embryonic development (Krueger and Dodson, 1981; Parejko, 1992; Imai et al., 2009). Furthermore, the morphogenesis of necktooth appears to start during the embryogenesis (Laforsch and Tollrian, 2004; Imai et al., 2009). Although there are still few studies on the embryogenesis of *Daphnia pulex*, the timing or process of developmental regulations have not been cleared.

In this chapter, first, I described the time course of embryogenesis, which should be used as the standard in future studies. I report fine necktooth inducing methods using *D. pulex*. This necktooth inducing system enables us to develop *D. pulex* as an experimental model for studying inducible defense. Additionally, I observed predatory activity of *Chaoborus* larvae against *D. pulex* in order to verify function of necktooth.

MATERIALS AND METHODS

Animals

A single clone of *D. pulex*, which had been collected from a pond on the Hokkaido University campus, Sapporo, Japan, was used throughout this study. Animals were maintained in dechlorinated tap water at 18°C under artificial light conditions of 14 h light and 10 h dark to induce and maintain reproduction. They were fed with a concentrated monoculture of the green alga *Chlamydomonas reinhardtii*, as described in Sueoka (1960). In all experiments, *D. pulex* embryos were picked from dissected maternal brood chambers and cultured (Fig. 1, Imai et al., 2009). I defined embryonic stages using the scheme of Laforsch and Tollrian (2004).

Fourth-instar *Chaoborus flavicans* larvae were collected from a pond at the National Institute for Environmental Studies, Tsukuba, Japan.

DiI tracing of the nerve arising from first antennae

The nerve cells were labeled with the lipophilic tracer DiI (Molecular Probes). Embryos were soaked in 1% saturated solution of DiI with 1 µg/ml Hoechst 33342 (Molecular Probes) for 45 minutes during embryonic stages 2, 3 and 4 (Fig. 4B). They were then washed in dechlorinated tap water five times and observed as whole mount preparations under a fluorescence microscope (BX-50, BX-FLA; Olympus).

Kairomone medium

Chaoborus larvae were cultured in dechlorinated tap water at a density of 1–5 larvae/100 ml for 1 week in a temperature- and light-controlled incubator (18°C; 14 h light and 10 h dark), and were fed daily with sufficient *D. pulex*. Water in which *Chaoborus* larvae had been incubated was passed through a 1.2 µm filter (RAWPO4700; Millipore), dispensed into a 15 ml or 50 ml conical tube (352097 and

352098; BD Falcon), and then stored at -20°C. After thawing at room temperature, the water was filtered through a Whatman GF/C filter before use; this *Chaoborus*-conditioned water is hereafter referred to as ‘kairomone medium’ or just ‘kairomone’. Dechlorinated tap water was used as the control medium. I evaluated kairomone quality before and after freezing and only used kairomone in which the necktooth induction rate was at least 80% when embryos were reared in undiluted kairomone medium.

Induction of defenses

Juveniles reared in kairomone were examined at each instar under a stereomicroscope, and I recorded whether they had formed neckteeth, and how the neckteeth formed. The chi-square test was used to assess differences in the proportions of individuals with neckteeth between kairomone-treated and control animals.

Scanning electron microscopy (SEM)

For SEM, a method modified from Laforsch and Tollrian (2000) was used. Animals were killed in a 600 W microwave for 7 sec and immediately transferred into 70% ethanol for 3 h. Fixed specimens were dehydrated in an acetone series (70%, 80%, 90%, 95%, 99%, 99%) for 10 min each, incubated in hexamethyldisilazane for 70 min, and transferred to a desiccator. Dried specimens were glued to a brass holder and gold-coated for observation with a S3000N scanning electron microscope (HITACHI).

Observations of predatory activity of *Chaoborus* larvae against *D. pulex*

D. pulex Juveniles were divided into 3 groups based on their body length. A single *Chaoborus* larva starved for 1 day was put into a plastic dish (60 mm) with 4 ml tap water aged at least 4 days and then *D. pulex* with or without neckteeth were thrown in. After 1 hour, I counted the number of surviving individuals and checked their morph

under stereomicroscope.

Next, I captured the motion movies of the predatory activity of *Chaoborus* larvae, described as below. Three individuals of *Chaoborus* larvae starved for 1 day were put into a plastic case (60 mm × 50 mm × 8 mm). Then *D. pulex* with 1.0-1.3 mm of body length were thrown into the plastic case. Their behavior was observed and recorded by DVD video (D2-MV580; HITACHI) for 15 min.

RESULTS AND DISCUSSION

Time course of embryogenesis and early post-embryogenesis

Top row of figure 1.1 shows the time course of embryogenesis and early post-embryogenesis of *D. pulex*, which proceeded along a strict time course at 18°C. Although embryos within a clutch developed at nearly the same pace, the timing of hatching from the egg chorion varied somewhat from egg to egg. Therefore, I set the starting time of experiment when a half of the embryos in a clutch had hatched. In embryonic stage 1, the egg was covered by three membranes between oviposition and hatching (0 h): the egg chorion (the outer-most membrane), and the second and third membranes underlying the egg chorion (Laforsch and Tollrian, 2004; Imai, 2007). In stage 2, lasting from 0 h to 24 h, the embryo released the egg chorion but still kept the second and third membranes. In stage 3, lasting from 24 h to 30 h, both of the second and third membranes began to peel off, and then immature organs such as first antennae became visible. In stage 4, lasting from 30 h to 42 h, the appendages became functional, and the animals could swim with their second antennae. Based on the DiI tracing of the nerve arising from first antennae (Fig. 1.1, bottom row), the neuronal cells have extended the neuritis into the deutocerebrum in stage 4 embryos, suggesting that the sensory systems started working. Around 42 h, the animals completely shed the third membrane, and then developed into first-instar juveniles. One of the characteristic features in the first-instar was complete elongation of the tail spine. By three days from hatching (after 72 h), many individuals had reached the second-instar stage.

Defense morph induction

I first established the experimental systems to induce neckteeth formation in *D. pulex* (Fig. 1.2A). When embryos were exposed directly to kairomone, the induction rate during the first to third instars was significantly higher than the indirect maternal

exposure (Imai et al., 2009). It is known that a kairomone is a chemical compound released from *Chaoborus* larvae into the water when *Chaoborus* larvae eat *Daphnia*. Kairomone medium gradually diluted with aged water become less and less effective for the neckteeth formation. There is the concentration dependency of kairomone. (Fig. 1.2B)

Figure 1.3A shows a second-instar juvenile lacking neckteeth. The neckteeth comprise several tiny spikes (Figs. 1.3B and C). The dorsal carapace ridge between the insertion points of the first and second antennal muscles transformed into these alternately arranged tiny spikes (Fig. 1.3D). The epithelium at the base of neckteeth became thicker to form a structure called the “crest”. Some control juveniles in the first instar, and very few in the second instar, developed neckteeth although they were reared in control medium (Fig. 1.3E) as described in Tollrian (1993). Juvenile reared with kairomone formed neckteeth during the first to the fourth instar stages (Figs. 1.3B-D). The second-instar stage showed the highest frequency of forming neckteeth and the average number of spikes (Fig. 1.3E). From these results, I defined kairomone sensitivity for defense morph induction based on the frequency of forming neckteeth in the second instar juveniles.

Predatory activity of *Chaoborus* larvae against *D. pulex*

Figure 1.4 shows the neckteeth morph has the greater escape efficiency in the 1.0-1.3 mm body size. Also, the body length corresponds to escape efficiency in second to fourth instar juveniles. A single clone of *D. pulex* which was used in this study showed that the induction rates of neckteeth were the strongest in the second instars, and the number of spike produced reached the maximum in same stage (Fig. 1.3E). Our result show that there were some *D. pulex* possessed neckteeth in the first instar even if they were reared in control medium (Fig. 1.3E). However, in the first instar, comparison between the normal and neckteeth morphs had revealed that no significant differences in

the predation rates (Figure 1.4; Tollrian, 1995). Therefore I assessed kairomone sensitivity for defense morph induction by the scores counted in the second-instar larvae.

In the video clips (Supplementary Files S1 and S2) , six out of twenty juveniles without neckteeth were preyed by *Chaoborus* larvae. In contrast, juveniles with neckteeth were not preyed at all, while they were sometimes grasped (but not engulfed). Neckteeth offer protective effects against *Chaoborus* because of neckteeth interfere with the predator's ability to handle and manipulate the prey, which is in agreement with Krueger and Dodson (1981), Havel and Dodson (1984), and Tollrian (1995A).

Conclusion

It was confirmed that *D. pulex* embryos that were dissected out from maternal brood chamber could develop normally. And the time course of embryogenesis and early post-embryogenesis in *D. pulex* cultured in vitro was described (Fig. 1.1). Because of the exposure of dissected embryos to *Chaoborus* kairomone showed the high efficiency of neckteeth induction (Fig. 1.3), it was revealed that *D. pulex* embryos receive kairomone directly to develop neckteeth. Although the water in which *Chaoborus* larvae were cultured could be used as “kairomone medium”, it was suggested that it needs a certain concentration of kairomone to induce neckteeth in *D. pulex* (Fig. 1.2B). The frequency of induction neckteeth and the average number of spikes in the second-instar was the highest (Fig. 1.3E). Furthermore, in particular size of *D. pulex* instars, necktooth became the difference between success and failure of predation by *Chaoborus* because of the necktooth may make *D. pulex* the hard-to-swallow shape for *Chaoborus* larvae (Fig. 1.4, Supplementary Files S1 and S2). It seems that necktooth is produced only when it has defensive effect.

FIGURES

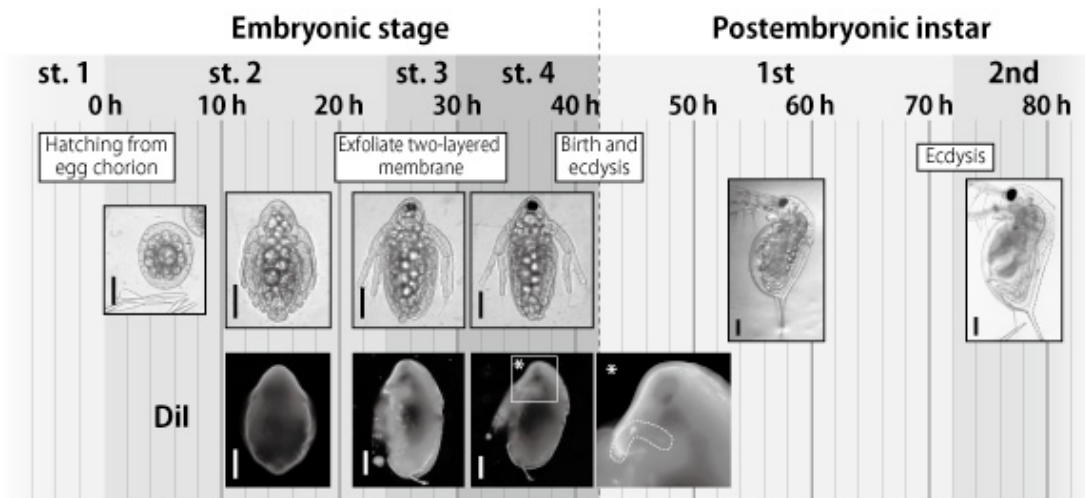


Fig. 1.1. Time course of embryogenesis and early post-embryogenesis (top row) and the nerve arising from first antennae (bottom row) in *D.pulex* at 18°C (see Results for a description). Scale bars = 100 μ m.

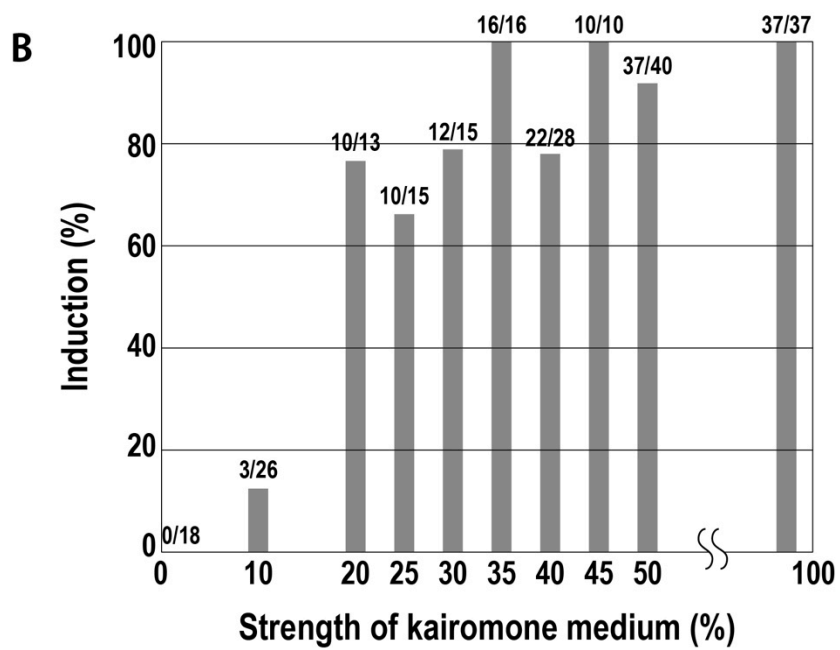
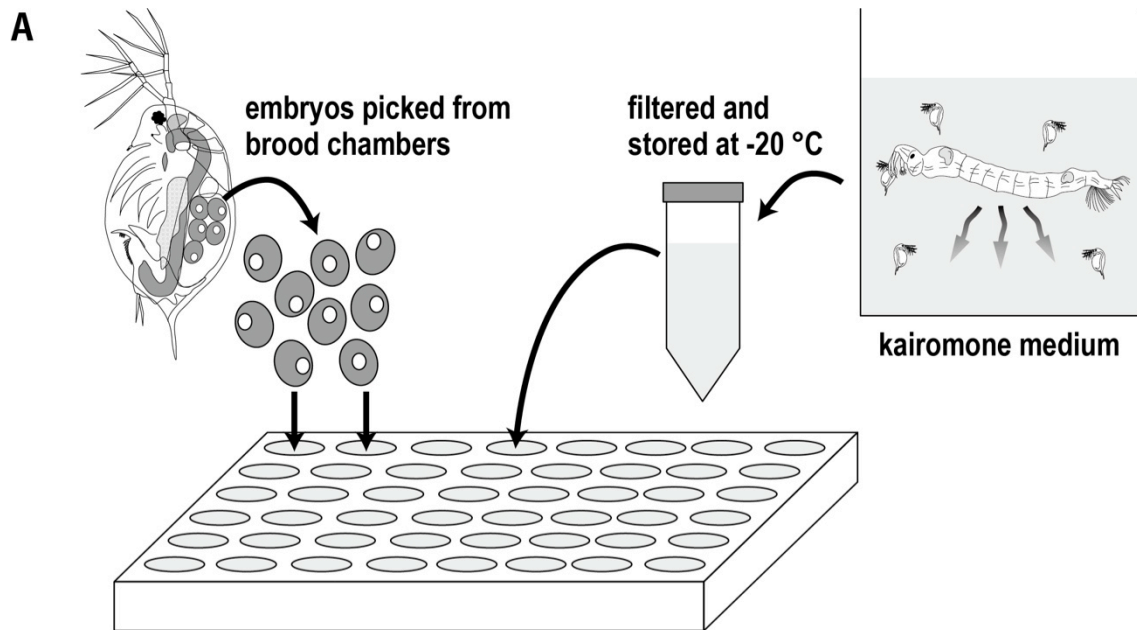


Fig. 1.2. (A) Defense morph induction system. (B) Relationship between the concentration of kairomone and the induction rate of neckteeth in *D.pulex* juveniles.

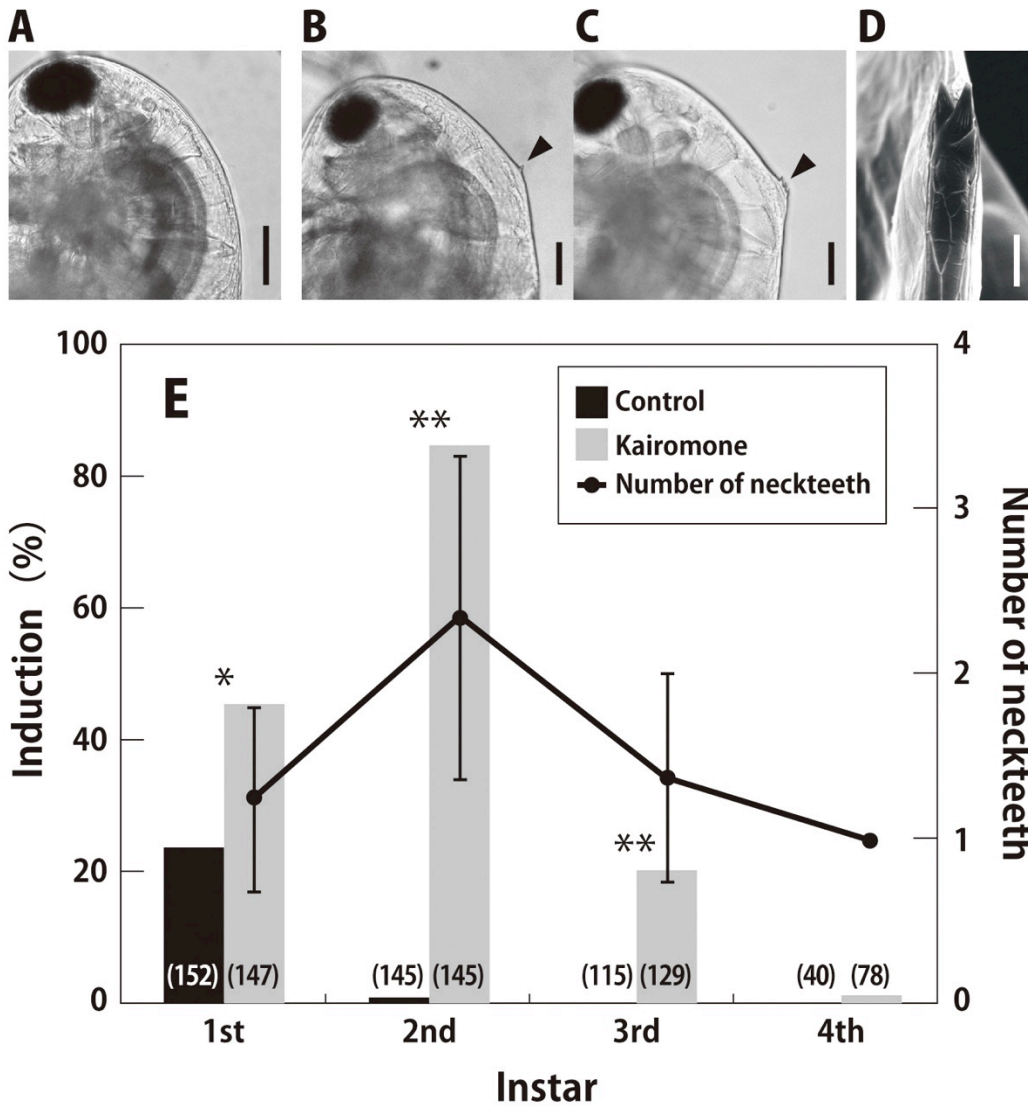


Fig. 1.3. (A) A second-instar juvenile lacking neckteeth. (B) First- and (C) second-instar juvenile with neckteeth (arrowheads). (D) SEM image showing neckteeth in dorsal view. Scale bars: 50 μm (A–C), 10 μm (D). (E) Induction frequency (bars) and number of neckteeth (line) at each instar. The proportion of individuals bearing neckteeth was compared between treated animals exposed to kairomone medium (kairomone treatment) and controls exposed to dechlorinated tap water (asterisks indicate a significant difference between treated and control animals; *, $P < 0.05$, **, $P < 0.005$, chi-square test). Data are shown as mean values \pm SD. Numbers in parentheses

indicate the sample size.

Experimental group	average size (lnetar)	Normal morph	Neckteeth morph
1	0.8 mm (1st)	⊗ ⊗ ⊗ ○	○ ⊗ ⊗ ⊗
2		⊗ ⊗ ⊗ ⊗	○ ○ ⊗ ⊗
3	0.9 mm (2st)	⊗ ⊗ ○ ○	⊗ ⊗ ⊗ ⊗
4		⊗ ⊗ ○ ○	○ ⊗ ⊗ ⊗
5		⊗ ⊗ ⊗ ⊗	○ ⊗ ⊗ ⊗
6	1.0-1.3 mm (3rd-4th)	⊗ ⊗ ○ ○ ○	○ ○ ○ ○ ○
7		⊗ ⊗ ⊗ ○ ○	○ ○ ○ ○ ○
8		⊗ ⊗ ⊗ ⊗ ○	○ ○ ○ ○ ○
9		⊗ ⊗ ⊗ ⊗ ⊗	○ ○ ○ ○ ○
10		⊗ ⊗ ⊗ ⊗ ○	○ ○ ○ ○ ○
11		⊗ ⊗ ⊗ ○ ○	○ ○ ○ ○ ○

○ escape ⊗ prey

Fig. 1.4. Predation number for normal and neckteeth morph of different body size in *D. pulex*.

Chapter 2

Identification of the kairomone-sensitive period and the histology of neckteeth formation in predator-induced polyphenism in *Daphnia pulex*

INTRODUCTION

Prey organisms have many defense strategies against their predators to increase their fitness. Many studies reported that several prey species develop inducible defenses in response to predators (Adler and Harvell, 1990; Tollrian and Harvell, 1999; Gilbert, 2010). Among them, defense strategies of *Daphnia* such as defensive morphs, life history, and behavioral changes in response to chemical cues released by predators were well studied and their adaptive significance was discussed (Tollrian and Harvel, 1999). The predator-induced polyphenism in *Daphnia pulex* represents the potential to greatly advance our understanding of plasticity because of the accumulation of genomic, genetic, ecological, and toxicological data (Eads et al., 2007; Jenner and Wills, 2007). Recently, the genome sequence of *D. pulex* has been released (Colbourne et al., 2011). In addition, *D. pulex* has been more convenient to analysis in developmental biology fields due to establishment of molecular techniques (Sagawa et al., 2005; Kato et al., 2011).

Daphnia pulex juveniles develop small protuberances in the neck region, which are termed “neckteeth (necktooth)” when *Chaoborus* kairomone are present. The necktooth effectively decrease the predation risks, because for the predators it is difficult to swallow the *Daphnia* juvenile with necktooth (Tollrian and Harvel, 1999). Kairomone is released into the water from *Chaoborus* larvae feeding on *Daphnia*, while crushed *Daphnia* do not induce defensive reaction (Walls and Ketola, 1989). In spite of many years of efforts, chemical identity has not been proven for *Chaoborus* kairomone (Tollrian and Dodson, 1999).

Morphogenesis of neckteeth appears to start during embryogenesis (Laforsch and Tollrian, 2004; Imai et al., 2009). Previous studies have suggested that in *D. pulex*, kairomone reception during embryonic development is necessary for necktooth induction (Krueger and Dodson, 1981; Parejko, 1992), and Imai et al. (2009) reported

that exposure to a kairomone after the first-instar juvenile stage is necessary to maintain the neckteeth. However, it is not known to date whether the ability to respond to kairomone(s) is limited to a certain period of embryonic development. Likewise, although Beaton and Hebert (1997) reported that polyploid cells at the region of necktooth formation are possibly involved in the formation of neckteeth, little is known about the histological basis underlying the development of the defensive morph. Recently, candidate molecular pathways have been identified that appear to be primarily involved in morphological defense formation in *D. pulex* (Miyakawa et al., 2010; Spanier et al., 2010), but additional histological and morphological information will still be necessary to fully interpret the molecular studies.

In this chapter, I applied pulse treatments of predator-conditioned medium of varying duration and intervals to *D. pulex* during the embryonic and early juvenile stages to detect the period of sensitivity to kairomones. I observed the uptake of dextran-tetramethylrhodamine during embryogenesis to reveal when embryos absorb external solutions, possibly including kairomone(s). Using BrdU (5'-bromo-2'-deoxyuridine), I also observed the timing of cell proliferation in the occipital region during necktooth formation. Finally, for clues to molecular mechanisms underlying defense morph formation, I focused on Wnt pathways that play a key role in several steps in animal development, for example, in establishing the polarity of insect and vertebrate limbs and in stem-cell proliferation (Gilbert, 2010; Goto and Hayashi, 1999). I treated animals with LiCl, which inhibits glycogen synthase kinase 3 β and affects the transcription of Wnt target genes (Klein and Melton, 1996).

MATERIALS AND METHODS

Pulse treatments to determine the kairomone-sensitive period

For kairomone pulse treatments, 48-well culture plates (353078; BD Falcon) filled with 1 ml of kairomone or control medium per well were used. Embryos or juveniles were transferred individually (one animal/well) from a well containing control medium to one containing kairomone medium. Whenever embryos or juveniles were transferred from kairomone into control medium, they were first rinsed five times in control medium in a 60 mm plastic dish (353002; BD Falcon). At first, I designed induction experiments in which embryos and juveniles were exposed to kairomone to reveal which stage is important to induce neckteeth (Fig. 2.1-C and F-V). After obtaining the results, I then conducted the remaining treatments to confirm and highlight the importance of stage 4 (Fig. 2.1D, E and PA-PQ). Second-instar animals were examined under a stereomicroscope, and I recorded whether they had formed neckteeth. The induction values cannot be compared between treatments because the kairomone concentration was not exactly the same within experiments.

Uptake of dextran-tetramethylrhodamine

Embryos were soaked in 0.1 mg/ml dextran-tetramethylrhodamine (Invitrogen) for 1 h during embryonic stage 3 (Fig. 2.2A), for 2 h during stages 3 and 4 (Fig. 2.2B), or for 30 min during embryonic stage 4 (Fig. 2.2C). They were then washed in dechlorinated tap water three times and observed as whole mount preparations under a fluorescence microscope (BX-50, BX-FLA; Olympus).

BrdU immunohistochemistry

Starting in each developmental stage, animals were incubated in kairomone or control medium containing 5 mM BrdU (5'-bromo-2'-deoxyuridine; SIGMA) for 20 h

prior to fixation (Fig. 2.3A). After treatment, they were fixed in Carnoy's solution (1:3 acetic acid to methanol) at room temperature for 15 min, dehydrated in ethanol and xylene, embedded in paraffin, and serially sectioned. The 5- μ m sections were then rehydrated, washed in 0.1% Triton X-100 in Phosphate-buffered saline (PBS), treated with 2 N HCl for 20 min, washed again in PBS, and incubated in a blocking solution of 10% fetal calf serum (FCS) for 15 min, followed by rat monoclonal anti-BrdU antibody (1:250 in blocking solution; Abcam) for 2 h. After another wash in PBS, the sections were incubated with Cy3-conjugated anti-rat IgG goat antibody (1:500; Jackson ImmunoResearch) for 1 h at room temperature. After three washes in PBS (5 min/wash), the sections were stained with 1 μ g/ml Hoechst 33342 (Molecular Probes) in PBS for 5 min to visualize nuclear DNA, and then washed three times (5 min/wash) in PBS. Finally, the sections were mounted in VECTASHIELD Mounting Medium H-1000 (Vector) and observed under a fluorescence microscope (BX-50, BX-FLA; Olympus). The number of BrdU-positive cells per necktooth-forming region was compared between kairomone-treated and control animals, using the *t*-test.

Hematoxylin and eosin (HE) staining

Animals were fixed in Bouin's fluid, dehydrated in an ethanol series (70%, 80%, 90%, 95%, 99% and 99%) and xylene twice for 30 min each, embedded in paraffin, and serially sectioned. The 5- μ m sections were deparaffinized with xylene three times for 5 min each, transferred to an ethanol series (99%, 99%, 95%, 90%, 80%, and 70%) for 5 min each, and then stained with Delafield's hematoxylin and eosin.

Phalloidin staining

Second instar juveniles with neckteeth were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH7.4) overnight at 4°C. The fixed animals were then embedded in paraffin and serially sectioned at 5 μ m. The sections were then

rehydrated and treated with 20 µg/ml TRITC-conjugated phalloidin for 2 h at room temperature. After washing three times in PBS, the sections were stained with 100 µg/ml Hoechst 33342 (Molecular Probes) in PBS for 5 min. Finally, the sections were mounted with glycerol and observed under a fluorescent microscope.

Azan staining

First instar juveniles with neckteeth were fixed in Bouin's fluid, embedded in paraffin, and sliced into 5-µm-thick sections. The sections were then rehydrated and stained with 0.1% azocarmine G for 20 min. After washing in tap water and Aniline-ethanol, the specimens were treated with acetic acid-ethanol two times for 3 min and 5 min. The sections were then incubated in mordant 5% phosphotungstic acid for 1 h, followed by a wash in tap water and staining with Aniline blue solutions for 1 h. Finally, the sections dehydrated and mounted in a mixed solution of Canada balsam and xylene.

LiCl treatment

Animals were incubated from stage 1 embryos to the juvenile stage in kairomone or control medium containing 3 mM LiCl (Nacalai Tesque), a well-known inhibitor of glycogen synthase kinase 3β in the Wnt signal transduction cascade (Klein and Melton, 1996), and observed daily under a stereomicroscope.

Real-time quantitative PCR

Total RNAs of about 80 just turned first-instar juveniles that had been exposed to the kairomone and control media were extracted using ISOGEN (Nippon Gene) and reverse-transcribed. Because I found the homologous sequences of Wnt family and β-catenin genes of *D. pulex* in JGI *Daphnia pulex* v1.0 I designed the primers, which should give a product size of 50-150 bp, for real-time PCR (Supplementary file S3).

Primers were designed using the software, Primer3Plus (Andreas Untergasser and Harm Nijveen). Relative quantification was performed using a SYBR Premix Ex Taq (Takara Bio inc.) and ABI Prism 7500 sequence detection system (Applied Biosystems). As endogenous control of constitutive expression, I used EF1 (elongation factor1). The baselines were set respectively, based on the standard samples that were cloned plasmids (diluted 10^6 copies / μ l) of each gene.

β -catenin antibody and immunostaining

Animals were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH7.4) for 2 h at room temperature. The samples were then embedded in paraffin and serially sectioned at 5 μ m. The sections were rehydrated, washed in PBS, and pretreated with 0.1% hydrochloric acid. They were then incubated in blocking solution (10% fetal calf serum in PBS) for 20 min at room temperature. The sections were then treated with anti- β -catenin mouse antibody at 0.01% dilution in blocking solution overnight at 4°C. After being washed in PBS, the sections were incubated with Alexa 488 anti-mouse IgG goat antibody (0.01%) for 70 min at room temperature. The nuclei were then counter stained with Hoechst 33342. Finally, the sections were mounted in VECTASHIELD Mounting Medium H-1000 (Vector) and observed under a fluorescence microscope.

RESULTS

Kairomone sensitive period

To determine the kairomone-sensitive period, embryos and juveniles were incubated in kairomone medium at different intervals and for different durations (Fig. 2.1). Long exposures from stage 2 to the first instar tended to increase the induction of neckteeth (Fig. 2.1, rows A-C). In contrast, animals exposed to kairomone only earlier or later than stage 4 developed no neckteeth (Fig. 2.1, rows P-V). Despite a long treatment interval, animals transferred from kairomone medium to the control medium during embryonic stage 4 showed a reduced frequency of necktooth formation (Fig. 2.1, rows D, E). Animals treated with kairomone during and around embryonic stage 4 for a short period (Fig. 2.1, rows PA-PQ) tended to form neckteeth when they had been exposed to kairomone toward the end of this stage (Fig. 2.1, rows PA, PB, PC, PF, at around 40-42 h). Moreover, even when embryos were treated for the same 4 h during embryonic stage 4, the early treatment induced no neckteeth (Fig. 2.1, rows PG-PJ) in contrast to the late treatment (Fig. 2.1, row PK).

To examine whether embryos acquired the ability to absorb external solutions, presumably including kairomone, at stage 4, I examined the uptake of fluorescent dextran during embryonic stages 3 and 4. I found no dyes in stage 3 embryos, but found them in a stage 4 embryo. At stage 4, fluorescence signals were observed in the first antennae, dorsal organ, and intestine (Fig. 2.2). The results suggest that embryos hardly absorb kairomone at all in stage 3, but readily absorbed it in stage 4.

Immunohistochemical observation of cell proliferation in necktooth formation

To examine the contribution of cell proliferation to necktooth formation, I performed immunohistochemistry following BrdU incorporation (Fig. 2.3). No active cell proliferation was observed in parts of the body other than the region of necktooth

formation. There was no significant difference in BrdU-positive cells/necktooth region between kairomone and control medium for Treatment I, confined to the embryonic stages. In contrast, epidermal cell proliferation was significantly higher in animals exposed to kairomone medium in postembryonic instars (Treatments II-V) than in the control. The number of BrdU-positive cells reached a peak in the second instar (Treatment V) following a decrease between the latter half of the first-instar stage and the beginning of the second-instar stage (Treatment IV).

Histology of necktooth

I observed cell structure changes inducing by the kairomone treatment using three staining method. Hematoxylin and eosin stain (HE, Fig. 2.4A, B) is a popular staining method. Hemalaun colors nuclei of cells blue. The eosin Y, colors other eosinophilic structures in various shades of red, pink and orange. Phalloidin (Fig. 2.4C) is a family of toxins isolated from the deadly mushroom "*Amanita phalloides*", and is commonly used in imaging applications to selectively label F-actin in cells. In azan (Fig. 2.4D) stain, nuclei are stained bright red, collagen, basement membrane and mucin are stained blue, muscle and red blood cells are stained orange to red. This stain is used as good for staining connective tissue and epithelium.

The results of these three staining, clear it show that the epidermal cells lining the cuticle beneath the necktooth were of high density and single-layered crest which consisted of loose connective tissue.

Chemical treatments

LiCl-free controls did not develop neckteeth (Fig. 2.5, upper left). LiCl treatment without kairomone resulted in a slightly swollen crest-like structure without neckteeth (Fig. 2.5, lower left). Although the number of neckteeth was not affected, the crest of the neckteeth in kairomone and 3 mM LiCl treatment was more swollen in some

individuals (Fig. 2.5, lower right) compared to that in kairomone treatment without LiCl (Fig. 2.5, upper right). Eight out of 11 individuals subjected to the combined kairomone medium and LiCl treatment developed neckteeth, and five showed a highly swollen crest comprising of loose connective tissue (Fig. 2.4B, C, D). All animals treated with LiCl died before reaching the second-instar stage.

Expression profiles of the Wnt gene family

Among the Wnt gene family, identical sequences of 9 genes were found in the JGI *Daphnia pulex* v1.0. β -catenin gene that acts an intracellular signal transducer in the Wnt signaling pathway sequence was also identified in the same way. As the results, the lengths of the Wnt pathway genes fragments were elongated in those cases. The resultant fragment lengths were shown in Supplementary file S3.

The relative expression levels of the Wnt pathway genes were quantified using real-time quantitative RT-PCR to examine whether these genes were differentially expressed after exposure to the predator kairomone in the postembryonic first-instar. However RT-PCR revealed that those 10 Wnt pathway genes were expressed in first-instar juvenile, no difference in the level of expressions between treatments (Fig. 2.6 and Fig. 2.7A).

The presence of β -catenin proteins in second-instar *Daphnia pulex* juveniles was detected by immunohistochemical staining (Fig. 2.7B). Signals of β -catenin were observed in the cells consisting crest, but it was not sure whether β -catenin proteins transferred into the nucleus.

DISCUSSION

A developmental window for necktooth induction during late embryogenesis

I found that the kairomone-sensitive period of *D. pulex* was embryonic stage 4 to first instar. This result is in agreement with previous studies which suggested the existence of a sensitive period during embryogenesis (Krueger and Dodson, 1981; Parejko, 1992; Imai et al., 2009). This critical period for necktooth induction in the *D. pulex* embryo can be viewed as a “developmental window”. Animals treated with kairomone medium during this developmental window developed neckteeth, and the longer they were treated around the window, the more neckteeth they were likely to develop.

Under natural conditions, daphnids are subject to considerable environmental fluctuations (e.g. temperature, food concentration, predation risk) during embryogenesis and subsequent juvenile instars. In addition, necktooth formation has disadvantages, resulting in the avoidance of useless necktooth formation in the absence of predators (Havel and Dodson, 1987). Therefore, it is quite reasonable for daphnids that the developmental window is just before releasing from the brood chamber, indicating the time immediately prior to encountering predators.

Kairomone action in *D. pulex*

The basic assumption of our study is that a kairomone released by the predator into the ambient water constitutes the chemical cue for necktooth induction. Considering the pulse-treatment and dextran-tetramethylrhodamine experiments, I speculate that the kairomone is actively transported into the body mainly in embryonic stage 4, and possibly accumulates in the body during this stage. If kairomone disappears from the environment, *D. pulex* seems promptly to lose the kairomone stimulus from the body. I hypothesize that the proportion of individuals that form neckteeth depends on

the total amount of the stimulus received or accumulated at the end of embryogenesis. The results partly supporting these interpretations are as follows. In the experiment using fluorescently-labeled dextran, stage 3 embryos showed practically no uptake of the external solution despite 2 h of treatment. In contrast, the dye was detected in stage 4 embryos after just 30 min of treatment. After the third embryonic molt, the influx of various chemicals in the water appears to have increased, perhaps because of the loss of physical barriers and/or alterations in permeability. Furthermore, since the *Chaoborus* kairomone (< 500 Daltons; Tollrian and von Elert, 1994) is much smaller than dextran-tetramethylrhodamin (10,000 MW), the kairomone should be able to enter the body much more freely than the dye during embryonic stage 4.

The mechanism of kairomone reception during the developmental window remains an open question. Barry (2002) showed that the development of *Chaoborus*-induced neckteeth in *D. pulex* is affected by drugs that act on hormone-secreting neurosecretory cells, and Weiss et al. (2012) showed that cholinergic stimulation mediates signal transmission of *Chaoborus* cues leading to morphological defenses. Besides, Miyakawa et al. (2010) found that genes involved in the juvenile hormone actions and the insulin signaling pathway are up-regulated in kairomone-treated first-instar juveniles. Additionally, in some insects, plastic morphological traits were reported to be triggered by juvenile hormone (Miura, 2005; Suzuki and Nijhout, 2006). Thus, the endocrine system may be involved in initiating the development of neckteeth soon after the developmental window in *D. pulex*.

Cellular changes during necktooth formation

Necktooth spikes and the crest appear to be formed by different mechanisms, even though the same predator-released kairomone induces formation of both these defensive structures. The BrdU immunohistochemistry showed that epidermal cells in the region of necktooth formation divided rapidly in postembryonic juveniles that had

been exposed to kairomone, with the number of BrdU-positive cells in this region apparently correlated with necktooth formation. I observed that the crest consisted of loose connective tissue, whereas the epidermal cells lining the cuticle beneath the neckteeth were of high density and single-layered (Fig. 2.4). Moreover, LiCl treatment indicated that LiCl affected only crest development rather than necktooth formation (Fig. 2.5).

Figure 2.8 illustrates a hypothetical model for necktooth development. After the embryo receives the kairomone stimulus, epidermal cells lining the future necktooth region proliferate intensely in the postembryonic instars. The proliferated cells then secrete the cuticle of spikes, and epidermal cells underlining the spikes enlarge as a loose connective tissue, leading to thickening of the crest. In the crest, some epidermal cells may lose cell-cell adhesion and dramatically increase their cytoplasmic volume, leading to the swollen occipital region.

Among *Daphnia* species, crest or helmet growth in the defensive morph is positioned in various regions of the head. The morphological differences across taxa could be established by the same induction and cell proliferation mechanisms as in *D. pulex*, with variation in the form and position of defensive structures determined by downstream regulatory genes specifying the position and pattern of cell proliferation.

Molecular mechanisms of the crest formation

Because LiCl inhibits GSK-3, allowing β -catenin to accumulate and affect the transcription of Wnt target genes (Klein and Melton, 1996), the effect of LiCl on crest formation suggests that crest growing process might be mediated by GSK-3 β . The results in the present study results possibly implicate a Wnt signaling pathway in the crest development.

In order to elucidate the involvement of the Wnt signaling pathway in the neckteeth formation, I checked the expression levels of Wnt 1, 4, 5, 6, 7, 8, 9, 10 and 11

and β -catenin genes in first-instar juveniles by using real-time PCR in the presence or absence of predator kairomones (Figs. 2.6 and 2.7A). I observed the gene expression of Wnt family but no significant difference of the expression level during production of the defensive phenotype. Unfortunately, it seems difficult to detect the difference in these genes' expression, because cell divisions were not so active in necktooth region in first instar. Also, in order to confirm the reproducibility of quantitative PCR results, the re-examinations were performed with another reference genes such as 18S ribosomal RNA, *actin*, *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* which had a past record as endogenous controls.

In the future, it is necessary to examine Wnt family genes expression in second-instar juveniles in which responsive cell proliferation and crest formation occur. In figure 2.7B, immunofluorescence microscopy in second-instar juveniles shows the presence of β -catenin proteins in the crest. Although it is commonly known that β -catenin proteins translocate to nucleus when the Wnt signaling pathway is activated, there was not enough evidence to support nuclear transit of β -catenin proteins in this observation (Fig. 2.7B). It is needed that future studies using in situ hybridization and RNA interference (Kato et al., 2011) to show the direct involvement of the Wnt pathway in defense morph formation.

FIGURES

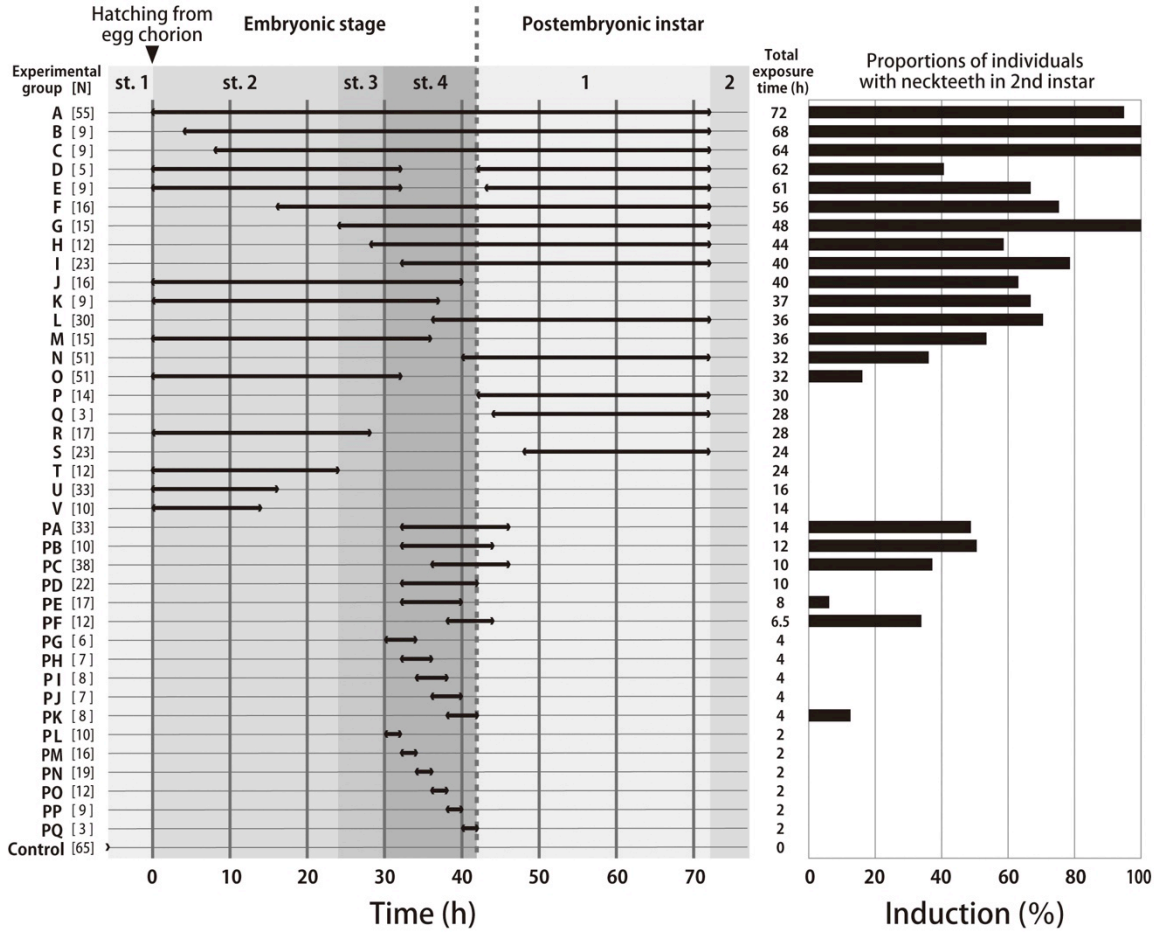


Fig. 2.1. Kairomone pulse treatments of various durations from embryonic stage 2 through the first instar. Heavy horizontal lines represent periods of kairomone treatment. Induction frequency of neckteeth scored in the second instar.

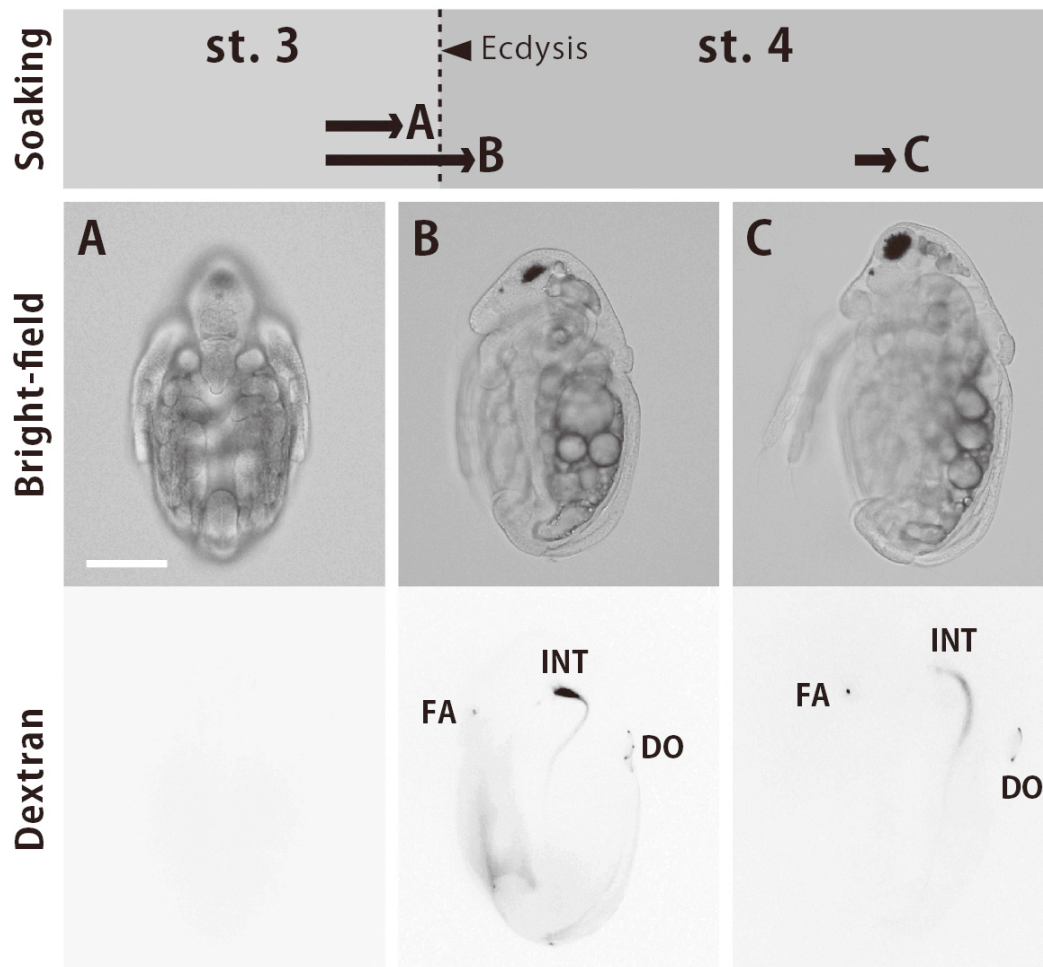


Fig. 2.2. Water influx into embryos detected by dextran-tetramethylrhodamine. Arrows indicate the treatment periods (A-C). FA, first antennae; INT, intestine; DO, dorsal organ. Scale bar = 20 μ m.

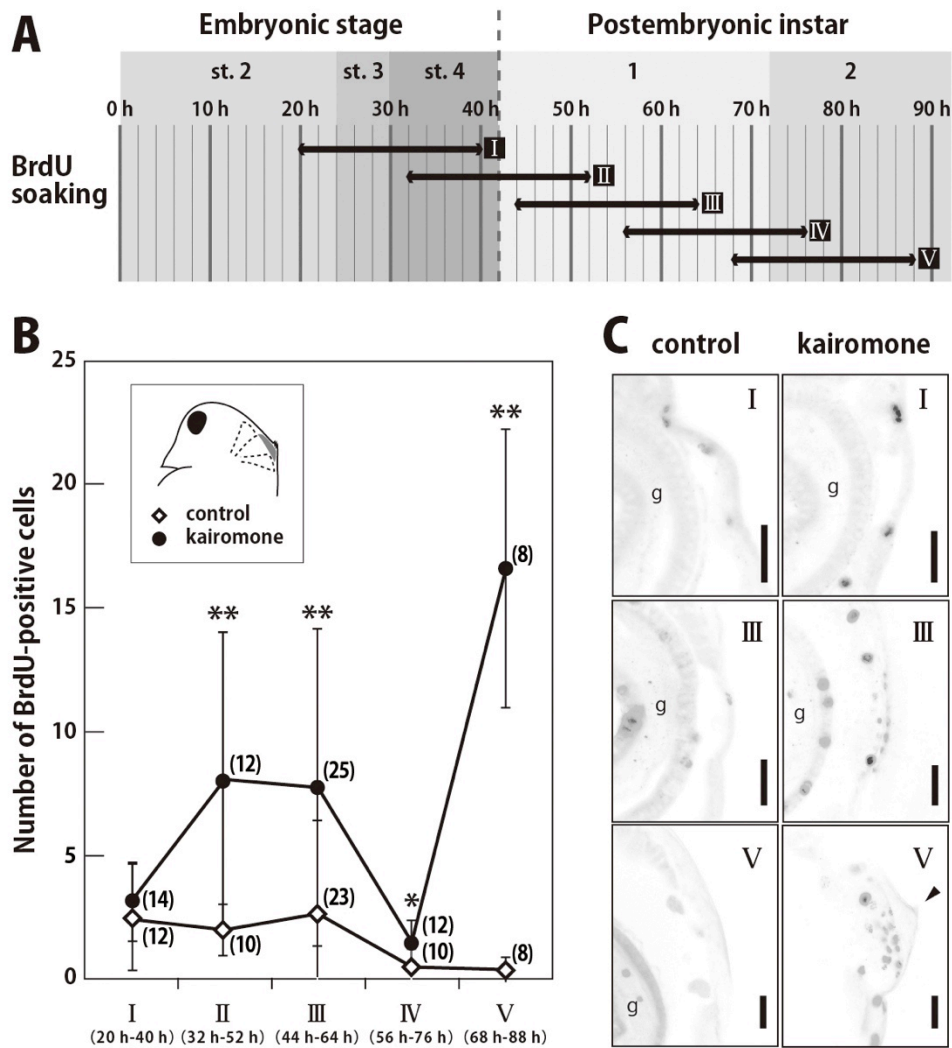


Fig. 2.3. (A) Experimental scheme. BrdU was incorporated for 20 h prior to fixation at five different points during the embryonic stages and postembryonic instars. (B) Number of BrdU-positive epidermal cells in the region between the insertions of the first and second antennal muscles (gray region in inset at upper left). Data are shown as means \pm SD (* $P < 0.02$, ** $P < 0.005$, t -test). Numbers in parentheses indicate sample sizes. (C) Images of the occipital region in sagittal sections showing cells positive for BrdU incorporation. Arrowhead indicates neckteeth; g, gut. Scale bars = 10 μ m.

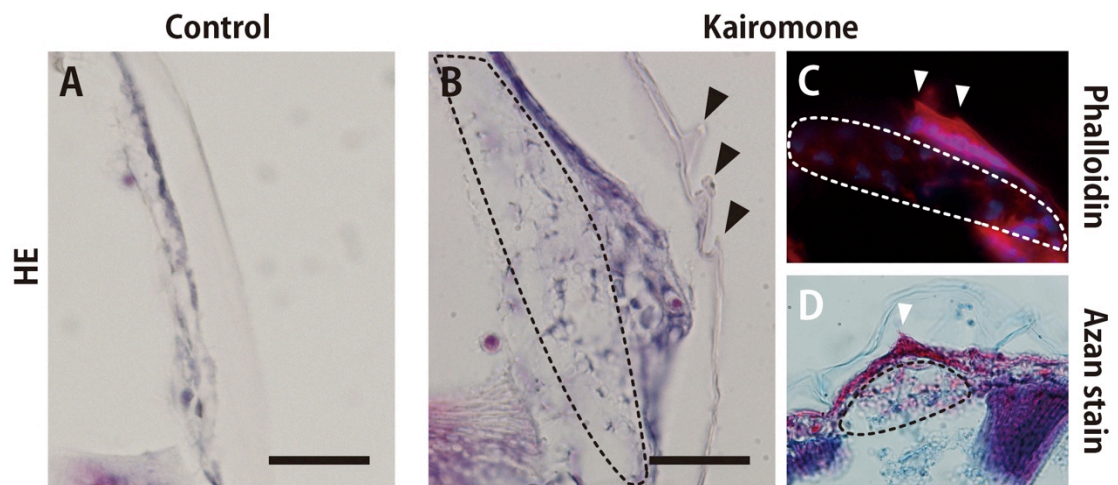


Fig. 2.4. Stained sagittal sections through the region where neckteeth form, from second-instar animals. **(A.B)** Hematoxylin and eosin staining. **(C)** F actin stained with Phalloidin-TRITC (red). Nuclei were stained with blue-fluorescent. **(D)** Azan stain. Arrowheads indicate neckteeth; dashed outline indicates the crest. Scale bars = 20 μ m.

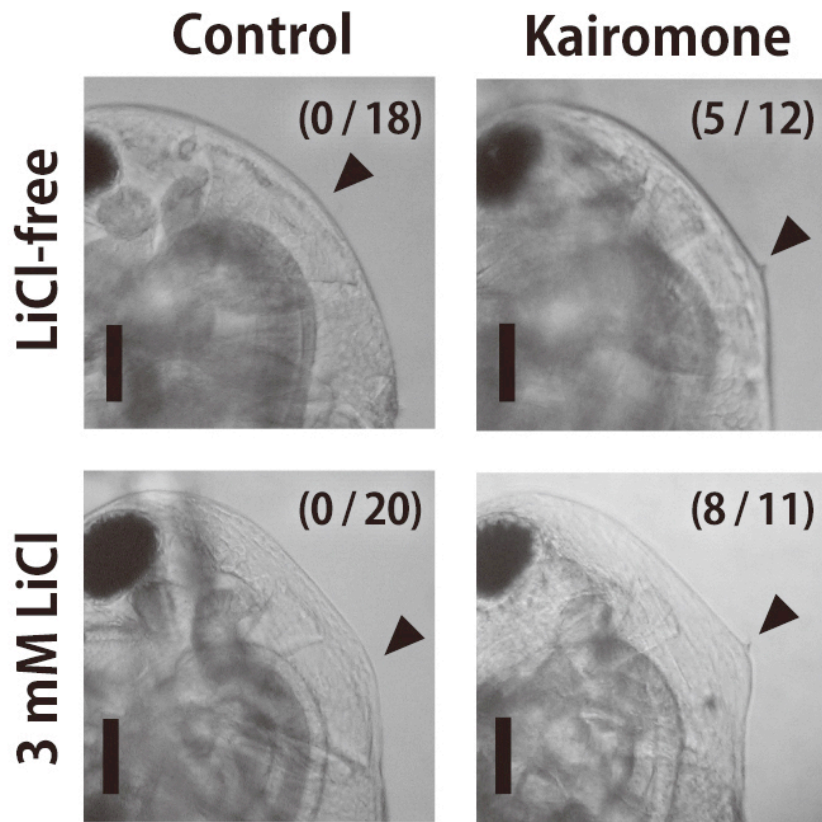


Fig. 2.5. Combined treatment with kairomone and LiCl. The fractions in parentheses indicate the proportion of individuals that formed neckteeth. Arrowheads indicate the site of necktooth formation. Scale bars = 50 μ m.

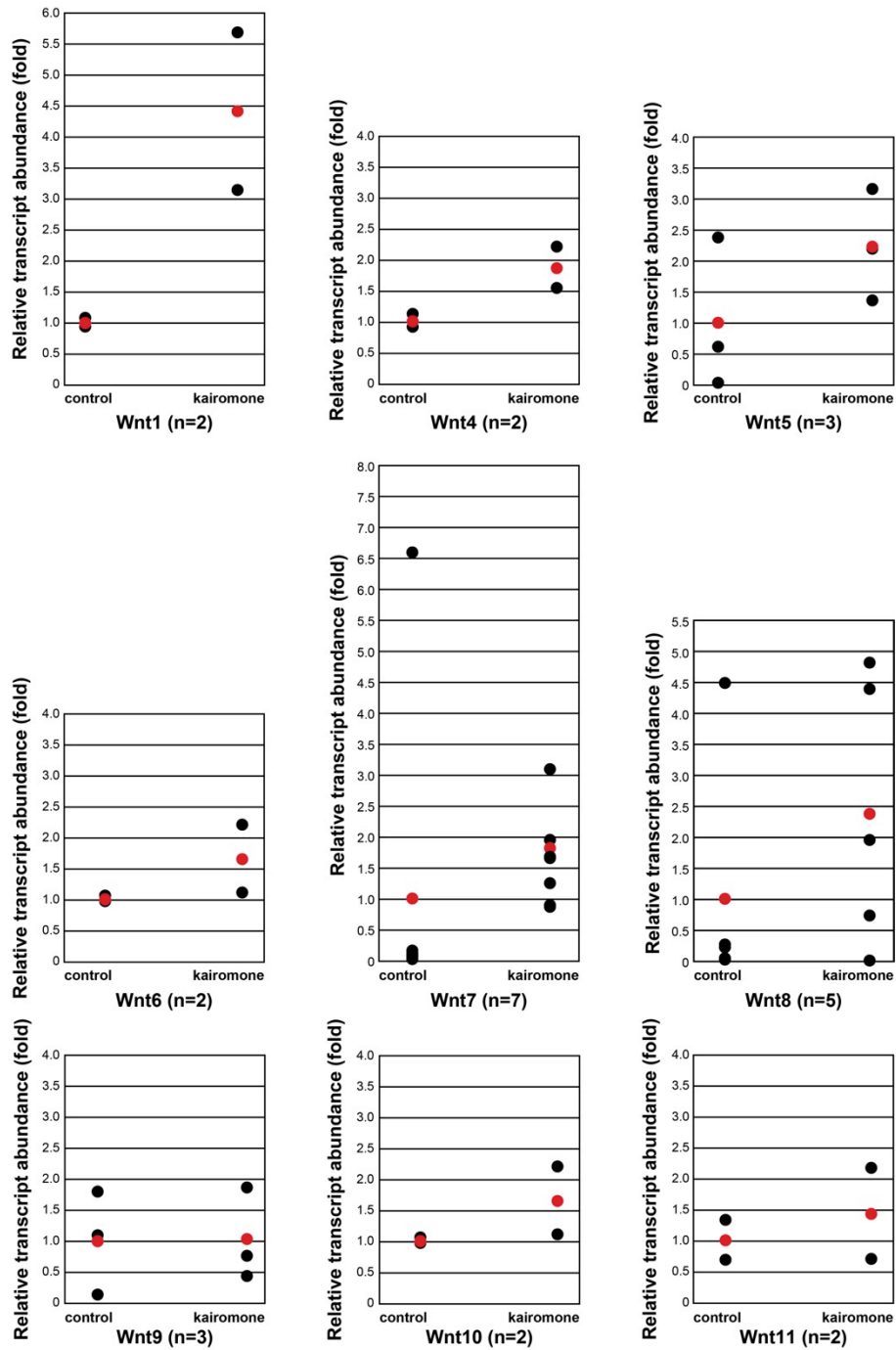


Fig. 2.6. Relative expression levels of Wnt genes in first-instar juveniles. Black circles indicate relative transcript abundance of each sample. Red circles represent the average.

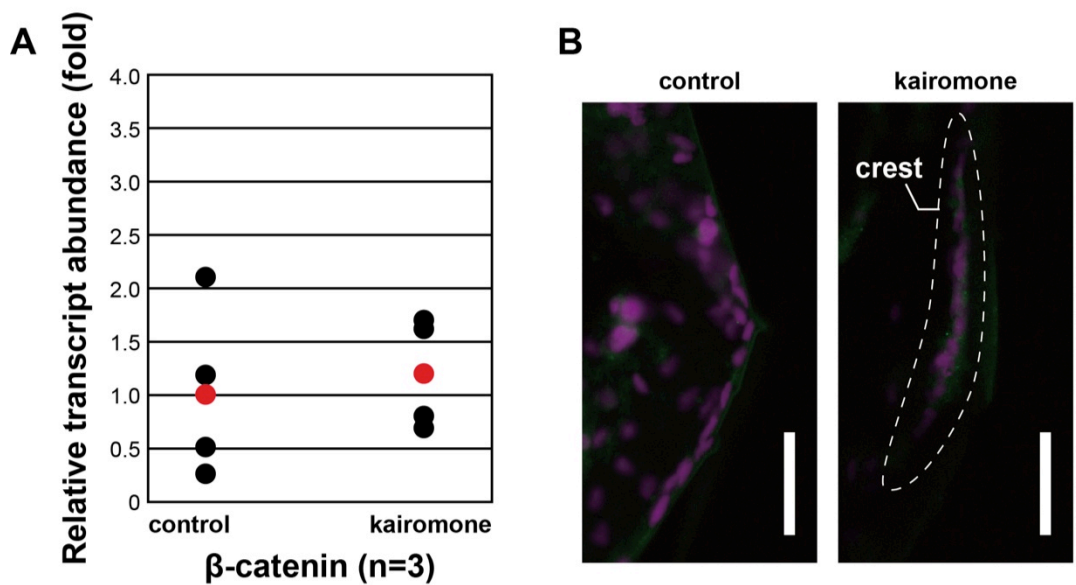


Fig. 2.7. (A) Relative expression levels of β -catenin genes in first-instar juveniles. (B) β -catenin immunohistochemistry (green) observed in second instars. Nuclei were stained with Hoechst 33342 (magenta). Dashed outline indicates the crest. Scale bars = 20 μ m.

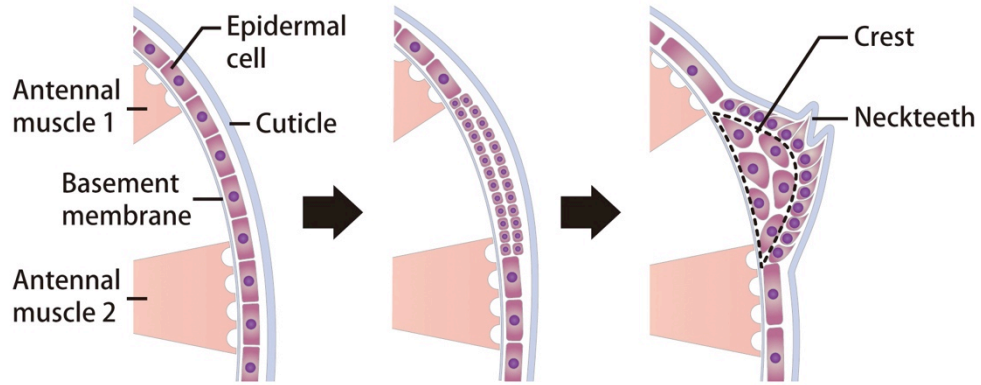


Fig. 2.8. Schematic diagram showing the process of necktooth formation (see Discussion).

GENERAL DISCUSSION

In this study, I approached the mechanisms of predator-induced polyphenism in *Daphnia pulex* through developmental biology. In Chapter 1, I described the time course of embryogenesis and established the rearing methods in which embryos were picked from dissected maternal brood chambers and cultured *ex vivo*. They should be used as the standard experiment in future studies. In addition, I confirmed the instar-dependent *Chaoborus*-induced defense morph formation and the effect of necktooth for predatory activity of *Chaoborus* larvae against *D. pulex*.

In Chapter 2, I investigated the period of kairomone sensitivity and the process of neckteeth formation in *D. pulex* through extensive treatments with pulses of kairomone(s). Then I demonstrated that the kairomone sensitive period was relatively short, extending from embryonic stage 4 to postembryonic first instar. Furthermore, I observed cell proliferation and changes in cell structure in response to the kairomone treatment. Finally I proposed the detailed model for necktooth formation in *D. pulex*.

Preliminary LiCl treatment implies that the Wnt signaling pathway involved in crest formation, and could be one possible molecular mechanism for the neckteeth formation. My study provides physiological and developmental basis to understand the mechanisms underlying adaptive polyphenism in *D. pulex*.

Developmental events of the predator-induced polyphenism in *Daphnia pulex*

In addition to previous studies, the present study provided several developmental events in the process of defense morph formation in *D. pulex*, including maternal, embryonic, and postembryonic regulations (Fig. G1).

It has been suggested that the treatment of daphnid females with *Chaoborus* kairomone induced changing their reproductive strategies to produce larger eggs in smaller numbers (Imai et al., 2009). The maternal (indirect) kairomone effects for egg

production, which generates larger juveniles, would be important for reducing the risk of predation by *Chaoborus*. My study revealed that kairomone reception during the end of embryonic stage 4 is critical for the future development of neckteeth, although the defensive traits only appear in postembryonic instars, particularly the second- and third-instars. Additionally, a kairomone signal was required for the continual neckteeth formation even in the postembryonic stages (first to third instars) (Imai et al., 2009). Taken together, the kairomone would induce the defensive phenotype of daphnids against predator by two different mechanisms for “fate determination” and “maintenance of neckteeth”.

It has been shown that the genes in the embryonic and first-instar juvenile stages exhibit different expression levels in the presence or absence of predator kairomones (Miyakawa et al. 2010). The expression of morphogenetic factors, such as Hox3, extradenticle and escargot, were up-regulated by kairomones in the postembryonic stage, suggesting that the kairomone probably controls the responsibility for defense morph formation by regulation of these genes. In addition, the expression of juvenile hormone pathway genes (JHAMT and Met), and insulin signaling pathway genes (InR and IRS-1) were up-regulated in the first-instar stage. It is well known that these hormonal pathways are involved in physiological regulation following morphogenesis in many insect species. Furthermore it has been shown that the expression level of novel genes was regulated by a differential display method when necktooth induction was determined during the embryonic stage (Miyakawa et al., 2010). However, further experiments are necessary to identify the function of these genes for the neckteeth formation.

When *D. pulex* embryos were exposed to kairomone medium, the proliferation rate of epidermal cell was significantly higher in early postembryonic instars than in the control. Therefore, the proliferation of epithelial cells may play a role in morphogenesis and cuticle secretion of necktooth. Necktooth was observed during the first to 3rd instar

juveniles, and the most apparent in the second instar. These necktooth offers effective protection against *Chaoborus* when Daphnids has approximately 1.0 mm of length. Necktooth disappeared in the further instars because the size of later instars is almost exceeds the maximum size of *Chaoborus* preys (~1.3 mm). In addition to the neckteeth formation, the predatory chemicals also affect overall body shape including tail spine (Jacobs, 1966; Krueger and Dodson, 1981; Tollrian, 1995B; Spitze and Sadler, 1996; Laforsch and Tollrian, 2004; Imai et. al, 2009).

There were some previous studies examining the cost of defensive morph formation (Tollrian, 1995B). The decreasing body size of embryos and juveniles occurred simultaneously with neckteeth formation, suggesting that the decrease in body size might be one of the costs of defensive morph.

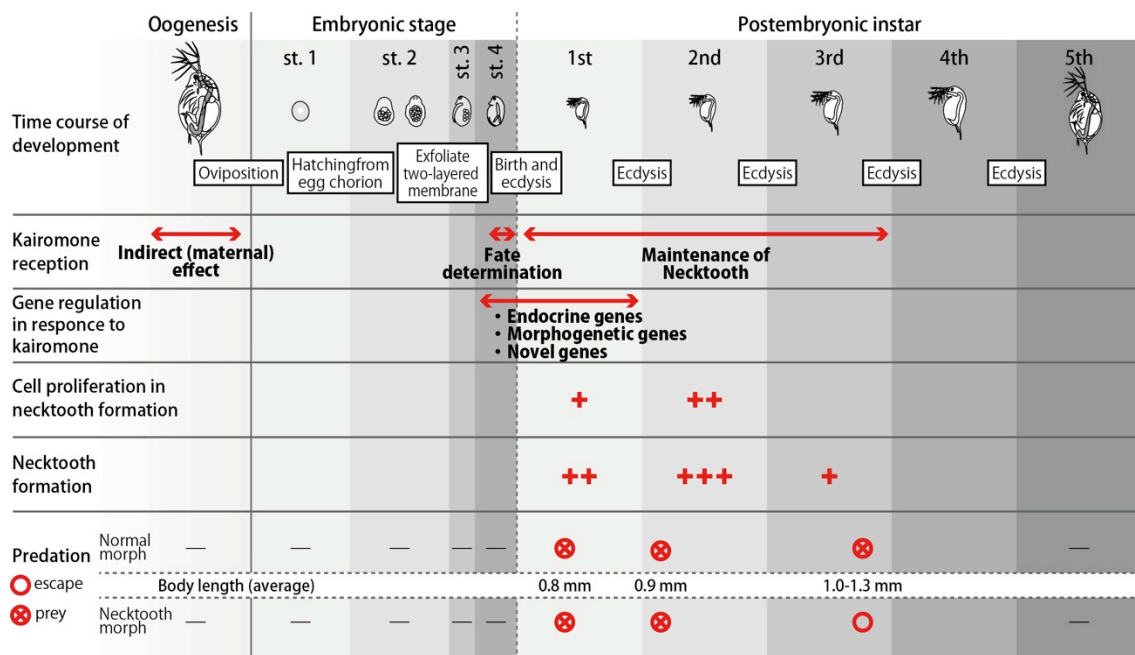


Fig. G1. Schematic diagram of the developmental events in the process of the defense morph formation in *D. pulex*

Future direction

In general, the phenotypic plasticity responding to environmental changes include the sensory mechanisms, endocrine regulations, and result developmental changes, such as cell proliferation and morphogenetic change (Nijhout 2003, Miura 2005). To understanding these sequential processes, future research should focus on to concatenate above fragments of information about the predator-induced polyphenism in *D. pulex*. For example, localization and functional analyses (i.e. in situ hybridization and RNA interference) for the Wnt signaling, which may implicate in crest development, are necessary to be performed to further clarify the mechanism of inducible defense. I hope that the histological observations in this study will be used to considerations new growing molecular studies. On the other hand, we should focus not only on endocrine and morphogenetic systems but also on upstream reception mechanisms of kairomone. However, molecular substances of kairomones released from *Chaoborus* or from other predators still remain unknown despite long history of efforts (reviewed in Tollrian and Dodson, 1999). Although identification of molecular substances of kairomones is a very difficult topic even in recent years, it would be essential for understanding neurological mechanisms of kairomone reception and, furthermore, interactions between predator and prey.

An experimentation of predator-induced polyphenism of *D. pulex* is suitable to comprehend the mechanisms of temporal or spatial changes in gene expression throughout development due to the interactions with environment. In this study, it might be suggested that variety of crest or helmet growth among *Daphnia* species could be established by the same induction cell proliferation mechanisms as in crest formation of *D. pulex* with variation. Daphnids are also known to have different thresholds of kairomone concentration among different clones (Parejko and Dodson, 1991; Spitze, 1992). Furthermore, even in same species, clones derived from ponds with high threat predation are more likely to form defensive traits than with low threat (Dennis et al.,

2011). Therefore, the comparisons between species or within species may allow us to understand the evolution of polyphenisms or novel phenotypes.

REFERENCES

- Adler FR, Harvell CD (1990) Inducible defenses, phenotypic variability and biotic environments. *Trends in Ecology & Evolution* 12: 407-410
- Barry MJ (2002) Progress toward understanding the neurophysiological basis of predator-induced morphology in *Daphnia pulex*. *Physiol Biochem Zool* 75: 179-186
- Beaton MJ, Hebert PDN (1997) The cellular basis of divergent head morphologies in *Daphnia*. *Limnol Oceanogr* 42: 346-356
- Colbourne JK, Pfrender ME, Gilbert D, et al. (2011) The ecoresponsive genome of *Daphnia pulex*. *Science* 331: 555-561
- Cristescu MEA, Colbourne JK, Radivojac J, Lynch M (2006) A microsatellite-based genetic linkage map of the waterflea, *Daphnia pulex*: On the prospect of crustacean genomics. *Genomics* 88: 415-430
- Dennis SR, Carter MJ, Hentley WT, Beckerman AP (2011) Phenotypic convergence along a gradient of predation risk. *Proceedings of the Royal Society B: Biological Sciences* 278: 1687-1696
- Dodson SL, Cáceres CE, Rogers DC (2009) Cladocera and other branchiopoda. In "Ecology and classification of North American freshwater invertebrates, third edition" Ed by JH Thorp and AP Covich, Academic Press, London, pp 774-828

Eads BD, Andrews J, Colbourne JK (2007) Ecological genomics in *Daphnia*: stress responses and environmental sex determination. *Heredity* 100: 184-190

Gilbert SF (2010) *Developmental Biology*. 9th ed, Sinauer Associates Inc, USA

Goto S, Hayashi S (1999) Proximal to distal cell communication in the *Drosophila* leg provides a basis for an intercalary mechanism of limb patterning. *Development* 126: 3407-3413

Grasswitz TR, Jones GR (2002) *Chemical Ecology*. Encyclopedia of Life Sciences. John Wiley & Sons Inc, USA

Havel JE, Dodson SI (1984) *Chaoborus* predation on typical and spined morphs of *Daphnia pulex*: behavioral observations. *Limnology and Oceanography* 29: 487-494

Havel JE, Dodson SI (1987) Reproductive costs of *Chaoborus*-induced polymorphism in *Daphnia pulex*. *Hydrobiologia* 150: 273-281

Hutchinson, G.E. (1967) *A Treatise on Limnology*. Volume 2. John Wiley & Sons Inc, USA

Imai M (2007) *Developmental regulations and underlying molecular mechanisms of the predator-induced polyphenism in the water flea *Daphnia pulex**. A thesis submitted for the degree of doctor of philosophy. Division of biosphere science, Graduate school of environmental sciences, Hokkaido University

- Imai M, Naraki Y, Tochinai S, Miura T (2009) Elaborate regulations of the predator-induced polyphenism in the water flea *Daphnia pulex*: kairomone-sensitive periods and life-history tradeoffs. *J Exp Zool Part A* 311: 788-795
- Jacobs J (1966) Predation and rate of evolution in cyclomorphic *Daphnia*. *Verh Int Ver Limnol* 16: 1645-1652
- Janssen R, Le Gouar M, Pechmann M, et al. (2010) Conservation, loss, and redeployment of Wnt ligands in protostomes: implications for understanding the evolution of segment formation. *BMC Evol Biol* 10: 374
- Jenner RA, Wills MA (2007) The choice of model organisms in evo-devo. *Nat Rev Genet* 8: 311-319
- Kato Y, Shiga Y, Kobayashi K, et al. (2011) Development of an RNA interference method in the cladoceran crustacean *Daphnia magna*. *Dev Genes Evol* 220: 337-345
- Klein PS, Melton DA (1996) A molecular mechanism for the effect of lithium on development. *Proc Natl Acad Sci USA* 93: 8455-8459
- Krueger DA, Dodson SI (1981) Embryological induction and predation ecology in *Daphnia pulex*. *Limnol Oceanogr* 26: 219-223
- Laforsch C, Tollrian R (2000) A new preparation technique of daphnids for scanning electron microscopy using hexamethyldisilazane. *Arch Hydrobiol* 149: 587-596

- Laforsch C, Tollrian R (2004) Embryological aspects of inducible morphological defenses in *Daphnia*. *J Morphol* 262: 701-707
- Miura T (2005) Developmental regulation of caste-specific characters in social-insect polyphenism. *Evol Dev* 7: 122-129
- Miyakawa H, Imai M, Sugimoto N, et al. (2010) Gene up-regulation in response to predator kairomones in the water flea, *Daphnia pulex*. *BMC Dev Biol* 10: 45
- Nijhout HF (2003) Development and evolution of adaptive polyphenisms. *Evolution & development* 5: 9-18
- Parejko K, Dodson SI (1991) The evolutionary ecology of an antipredator reaction norm: *Daphnia pulex* and *Chaoborus americanus*. *Evolution*: 1665-1674
- Parejko K (1992) Embryology of *Chaoborus*-induced spines in *Daphnia pulex*. *Hydrobiologia* 231: 77-84
- Sagawa K, Yamagata H, Shiga Y (2005) Exploring embryonic germ line development in the water flea, *Daphnia magna*, by zinc-finger-containing VASA as a marker. *Gene Expr Patterns* 5: 669-678
- Spanier KI, Leese F, Mayer C, et al. (2010) Predator-induced defenses in *Daphnia pulex*: Selection and evaluation of internal reference genes for gene expression studies with real-time PCR. *BMC Mol Biol* 11: 50

- Spitze K (1992) Predator-mediated plasticity of prey life history and morphology: *Chaoborus americanus* predation on *Daphnia pulex*. *American Naturalist*: 229-247
- Spitze K, Sadler TD (1996) Evolution of a generalist genotype: multivariate analysis of the adaptiveness of phenotypic plasticity. *American Naturalist*: S108-S123
- Stollewerk A (2010) The water flea *Daphnia* - a “new” model system for ecology and evolution? *J Biol* 9: 21
- Sueoka N (1960) Mitotic replication of deoxyribonucleic acid in *Chlamydomonas reinhardi*. *P Natl Acad Sci USA* 46: 83-91
- Suzuki Y, Nijhout HF (2006) Evolution of a polyphenism by genetic accommodation. *Science* 311: 650-652
- Tollrian R (1993) Neckteeth formation in *Daphnia pulex* as an example of continuous phenotypic plasticity: morphological effects of *Chaoborus* kairomone concentration and their quantification. *J Plankton Res* 15: 1309-1318
- Tollrian R (1995A) *Chaoborus crystallinus* predation on *Daphnia pulex*: can induced morphological changes balance effects of body size on vulnerability?. *Oecologia*, 101: 151-155
- Tollrian R (1995B) Predator-induced morphological defenses: costs, life history shifts, and maternal effects in *Daphnia pulex*. *Ecology*: 1691-1705

- Tollrian R, Harvell CD (1999) *The Ecology and Evolution of Inducible Defenses*.
Princeton University Press, Princeton
- Tollrian R, Leese F (2010) Ecological genomics: steps towards unraveling the genetic basis of inducible defenses in *Daphnia*. *BMC Biol* 8: 51
- Tollrian R, von Elert E (1994) Enrichment and purification of *Chaoborus* kairomone from water: Further steps toward its chemical characterization. *Limnol Oceanogra* 39: 788-796
- Walls MH, M Ketola (1989) Effects of predator-induced spines on individual fitness in *Daphnia pulex*. *Limnol. Oceanogra.* 34: 390-396
- Weiss LC, Kruppert S, Laforsch C, Tollrian R (2012) *Chaoborus* and *Gasterosteus* anti-predator responses in *Daphnia pulex* are mediated by independent cholinergic and gabaergic neuronal signals. *PLoS ONE* 7: e36879

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SUPPLEMENTARY FILES

Supplementary file S1. Video clip of predatory activity of *Chaoborus* larvae against *D. pulex* juvenile without neckteeth.

See the <http://youtu.be/CWZe8SUSuiw>

Supplementary file S2. Video clip of predatory activity of *Chaoborus* larvae against *D. pulex* juvenile with neckteeth.

See the <http://youtu.be/7qFwrmOjGd8>

Supplementary file S3. Homologous sequences of Wnt family and β -catenin genes of *D. pulex* and primers for real-time quantitative PCR. In each sequences, forward primer is indicated in yellow and reverse primer is indicated in blue.

Wnt1

Location: scaffold_8: 1160125-1175065

```
1 ATGAGTTCGACGCGGACGACCGTTGTTTTCGTTGTGATGTTGATGATGGTCCTCCCGTAT
61 CTCGGCACGGCCAAAGGGCGAGGAAGAGGGCAGCAAATGGTGGGTCGTATCTTACCCACCC
121 AGCCCCGATTTACAACAGCTGAATGATGTGCGCGGCGGGGGAATCGCCCCGAGCTGGTGAA
181 CCCAACAAATTTGGTGTGCAATTCATCAAGGAAAATTCAATCACGAATTCGTACTIONGGAT
241 CCCGACGTTTCATGCAACTCTGAGACGCAAGCAGCGTCGCTTGGTTCGCGACCACCCAGGC
301 GTCGTCGTGGCCGTGGCCAAAGGGCGCCAAACAGGCCATTTCCGAGTGCAAACATCAATTC
361 AAAAATAGGCGCTGGAAGTCCCCACCAAGGACTTCCATCGCGGCCGCAATCTCTTCGGA
421 AAGATCGTCGATCGAGCTTGCCGTGAAACTGCCTTCATCTACGCCCTGATGTCAGCAGCG
481 GTGACCCATTCGGTGTCTCGGGCCTGTTCCGGAAGGAGCCATCGAGTCTGCACTTGCGAC
541 TACAGCCAACGCGGACCGTCTGGAGCCGACTGGGAATGGGGCGGCTGCTCGGACAACATC
601 CAATTCGGCGTCAAGTTCTCCCGGGAGTTTGTGACGCGCCGGCGAG AAGGGCCGCGACATC
661 CGTTACATGATGAACCTCCACAACAACG AAGCCGGTCGAGTGTATAAGACCCTGGAAGAT
721 CTTGACATGATGAAGACACTCCCGCGCCAGTTCGAGAAGATGAGGGAAATAAAAAAGAAA
781 GAAACAGAGTTAGAAACGCATGAGATTGAAAGAAACCCGTCATTTTCAGCTGAGGAGTCG
841 AAAATGGGCGGCTCGTCAATCGCAACGGCTCAATTTAACGAGATAAATCTCGCACACGTT
901 TCGACGGAGATGAGACAAGAGTGCAAATGTCACGGAATGAGCGGCTCTTGACGGTGCGG
961 ACCTGCTGGATGAGACTGCCGTTCTTCCGCGTCGTCGGCGACAATTTGAAGACCGTTTC
1021 GACGGAGCCTCGCGGGTCTCATCAGCAACACGGGCCGGACGCCGGGCAACGGTCGCAAG
1081 AAGTACAACCTACCAGCTGAAGCCTTACAATCCGGAGCACAAGGCCCCCGTCCAAGAAAGAT
1141 TTGGTCTACTACGAGAACTCGCCCAACTTTTTCGAAAAGAACCTGAAATTGGGCATCGCC
1201 GGGACGCAGGGGCGCGTCTGCAACGAGACGTGATCGGCGTCGACGGCTGCGATCTGATG
1261 TGCTGCGGCAGAGGCTACAAAACGGAGGTGAAGGAGGTGGTCGAGCGCTGCGCTTGCAC
1321 TTCCACTGGTGCTGCGAAGTCAAGTGCAAAGTTTGCCGGACCAAGAAAACCATCCACACT
1381 TCAGCAGCAGCGACCGTGTCCACATGAGACCAGAGCCAGCTATCATAACCACACAATAT
1441 GCAACATCCCTCTTGATTGGAGAACTACCGATGTCGGCCGAGTTACGGATCTCTCTTTT
1501 TCCCTTTCTTTAAATGCCCTTGTGTACGCCCATATAACCAAGTACTGTACACAGTTTCAG
1561 ATTTGA
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Wnt4

Location: scaffold_78: 223807-242044

1 ATGAAGCGAATGGAGTGGTTCAACTTGGTCGTCGTCGGCTGTCTCAACTTTGGCTATT
61 CAATCCGCTTCGGCTATACAGTGGCTGGCTTTGTCACGGTCGAGCGCGGTAGCCTCGGCC
121 GCGATCGGGACGCCATCCATCTGCGACCAGCTGGTCGAGACGCTGGACACGAAGCAAGTG
181 CAGTTCTGCAAGAAGCACCCGGGTTTCATGATGAGCGTCCAGACGGGCGCCAGCCGGGCC
241 ATCGACGAATGCAAGTCCAGTTTCGACCAGACGCTGGAATTGCTCCACCTTGGACGAG
301 ACGGCCGGAGCGGCCAGCAGCGGCACTCGGGAGGGCGGCCTTCGTGCACGCCATTTGGGCC
361 GCCGGCGTGGCGCACGCTCTGACTCGGGCCTGCTCGGCCGGCGAGCTGGACGACTGTGGC
421 TCGACCGGACGGTGC GCGGAGCTTCGCCGAAGGATTCCAGTGGGCCGGCTGCTCGGAC
481 AACGTCCACTTTGGCACCAGTTTCTCGCGGACGTTTGTGACGCCCGCGAGCACCGATTC
541 TCCAAGCGCAAACCCAAAGCCAATCTCAACGGGACGGCGGCCGCCAGCAGCAAATGCAA
601 ATCTGGCGGGCACTCATGAACCTGCACAACAACGAAGCCGGACGCAAGATCATCGAGAAA
661 AGCATGAGGATTGAATGCAAATGCCACGGCGTGT CAGGTTCTTGTGAGCTGAGGACCTGC
721 TGGAAAGCCATGCCGTCTTCCGCGAGATCGGCGAGCAGTTGAAGGAGAAATTTGATAGC
781 GCCACGGAGGTGGAGCCGCGGACGTCGCATTCCGGCTCGCGACGCCACCTGGTGCCGCGC
841 AGTCCAACCTTACAAGCCGCACACGGACGCCGATTTGGTCTACCTGGATCGCTCGCCAGAT
901 TACTGCGAGTACGACTCCAAAACGGGATCGCTCGGCACCCACGGACGCCCTTGCAATAAA
961 ACGTCGAAAGCCATCGATGGATGCGACCTGCTATGCTGCAATCGAGGCTACAAGAGCCAC
1021 CGGGAAAGGAGGGCCGAGCGGTGCATGTGCAAATCCACTGGTGCTGCTCTGTCCACTGC
1081 AAAACGTGTCTGCGCGAAGTCGAAGTGCACACCTGCATGTGA

Wnt5

Location: scaffold_182: 45646-87067

1 ATGACTCGACCGCAGAAGAGGAACCAGGAATTGACGACGGACAGTGAACAGTCTCCTAGA
61 GTGCAACCACAACAACAACAACAACAGTGTGACAGAGAAGAGTAGCAGATGAAGCAGCC
121 CCAGACTGTCGGACCCCTTCTTGCTCGCCGTCGTCTTCGAGTTCTTCTTCTTATTACTA
181 TTCTCCTGGACGATCCTGCTGCTGATCGCAAGCGCTCCAGCCTCTTCCAGCACGGCCATC
241 GGCAACTGGATGAATCTCGGCGTCCAGGGCTACGAGGTGTGGCGGAATCCTCAGCTCTAC
301 ATGGTGGGAGCCCAGCCGCTGTGCACGCAAATCCCTGGGCTGTGCCTGGCCAGGCCAAA
361 TTGTGCCAACTCTACCAGGATCACATGAGCTCGGTGGGTGCGGGGCCAGGGCCGGCATC
421 GCCGAGTGCCAGTGGCAGTTCGGCTACAGGAGGTGGAATTGTTCCACCGTCGAAGATTCC
481 ACCGTCTTCGGACCCGTCTTACAAATCGGGAGCCGTGAAGCGGCGTTCGCCATTTCGATC
541 GCCGCCGCCGGTGTGTCGTTCACTCGATTT**CACGGGCTTGCCGAGAAGGT**CAACTCTCGTCT
601 TGCGGCTGCTCCCGAGCCCTCCGACCCAAGAATCTCAACCAG**GAATGGATTTGGGGCGGC**
661 **TG**CGGCGATAACATTGAATATGGATACAAATTCACGCAAGGCTTCGTGACGTCCGCGAA
721 CGTGAGAAGAACTACAAGCGAGGATCACGCGAACAAGGACGCAGCTTGATGAACCTACAC
781 AATAACGAAGCCGGACGTGCGGGCCGTGATTAAGAAAGCGCGTGTACATGCAAATGCCAT
841 GGAGTCTCCGGCTCATGCTCGTTGGTAACGTGCTGGCAGCAAAGTGGCGTTCGTTAGAGAA
901 ATCGGTGATTTTCATGAAAGACAAGTACGACGGAGCCACCGAAGTGAGAGTGAACAAACGA
961 GGCCGCCTCCAAGTGAAAAATGCCAGTACAATTTGCCGACAGCTAACGATTTGGTTTAC
1021 CTGGACGAGTCGCCGGATTACTGCCTCCGCAACAAAACCATCGGATCTTTCGGTACTTAC
1081 GGCAGGCCGTGCAACCGGACGTCTGCCGGGATGGACGGCTGCAATCTCATGTGTTGTGGA
1141 CGAGGTTACAACACGCTCAAGACGACGGTCAAAGAGCGTTGCAAGTGCAAATTCCTACTGG
1201 TGCTGTCACGTGAGTGCAAGACTTGCATCAAACGGTTCGACATTCACACCTGCAAGTAA

Wnt6

Location: scaffold_8: 1147766-1152980

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1 ATGATGATGGAACCGATGCGCCTGTGCCGTCAGCGGCGGAGGATAAAGAGCCGCCAGGTG
61 GAATCTGCCGCAAGGAGCCCAAAGTGTTCAGGAGATCGTCAAGGGGGCCCAACTGGGC
121 ACGAAGGAGTGCCAGCATCAGTTCCGCAACCGGCGATGGAATTGCACCACGGCCCCGCAAG
181 AGTCTCCGCAAAGTCATCGCTAGAGATACGCGCGAAACGGCGTTCGTCAATGCTGTCGTG
241 GCGGCGGGCGTGACCTACACCGTGACGCAAGCCTGCAGTTCCGGCCACCTCCTGCAGTGC
301 ACCTGCGATAAACTATGAAAGGCGTGTACCCGATGGTGATTGGGAATGGGGCGGCTGC
361 GCTGACAACGTCCAGCACGGATACAAAAATCACGCGAGTTTATGGACGCAAAGTACCGT
421 AAGCGGAGTGACCTCAAGACGCAAGTCATGCTGCACAACAACGAAGCTGGACGACTGGCG
481 GTCAAGAACTTTATGCGGACCGAATGCAAGTGTACGGATTGTCCGGTTCGTGCACACTG
541 CGCACCTGTTGGCGTAAATTGCCTCTGTTTCGTGACGTGGCCACCCGGCTCAAAGAGAAG
601 TTTGACGGAGCTGCCAAAGTGATTCCGGGAAATGATGGCAAGACGATCATTCCGGAAGTG
661 GCGAGCATCAAACCGCCCGGACGCGAGGATTTGATCTACTCGGAGGAATCGCCCGATTC
721 TGCAATCCGGATCGAGTCACCGGATCGCTGGGCACGGCCGGCAGGGTGTGCAACAGCACG
781 TCACCGGGAGTCGAGGGTTGCGAGCTGCTCTGCTGCGGGCGGGGCTACGAAACGCGGACG
841 ACCAAGACGCGCGTCAATTGCTACTGTGCGTTCAAATGGTGTTCGAAGTCACCTGCAAA
901 ATTTGCACCGTCAAGAAACACGTCAACACCTGCCGCTGA
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Wnt7

Location: scaffold_182: 34759-43669

1 ATGCTGGCACATGTGCATCAAACCTTCCGATTACAAGGTCACAACTGTGGTGTGTAGAG
61 CTGCGATTGTCCGGTATGGCTTTGAGCGCTGACCTGATATGCGGCAAGATACCGGGATTG
121 ACGTACAAGCAGCGGCTGCTCTGCGCCGCCAAGCCCGACGCCATGGTGGCCATCAGCAAC
181 GGAGCCAACTGGGACTAGCCGAGTGTGAGGAGCAATTCAAGTACCACCGATGGAATTGC
241 ACGGCCATCGGCAGCCGAAACGGATTCCGGTCACGTCGTCGTTGTCGGAAGCCGAGAAGCG
301 GCTTATCTGTATGCAGTGTGGAGCTCCGGCCTGACGTACGCTATCGCCCAGGCGTGCAGC
361 CAAGGAGCTATCAGCTCTTGCGGTTGCGATCCGACCAAACGAGGCGGCAAGGACCGCGGA
421 AACGGATGGAAATGGGGCGGCTGTTCCGGCCGACGTCCGATCGGGAGCCTCGCTGGCCAAA
481 CGATTCGCCGACTCGAGAGAAACCGAAGGAGACGATAGGAGCTTGATGAATCTGCACAAT
541 AATAAAGCCGGTAGAAAAATTGTCAAATCGATGTTGAAGAAGGAATGCAAATGCCACGGA
601 GTCAGCGGCAGTTGCTCACTCAAACCTGTTGGGAGAAATTGCCGGCCTTCAGGGACATT
661 GGCGATGCCCTGATGAAGCAGTACCGTGAAGCCAAGGCCGTCGTCGCCAAAGAGAGTCGC
721 AGCGGCAACGACAGCAAGCCACGCAAACTGTTGACGCTGCAGTTGCGTAGGAAGCCGCAC
781 AACAAGCCCCGTCTCTCGGAGCTGGTGTCTCAACTCTTCGCCCACTACTGCGAGGCG
841 AACCCGTCGACGGGGTCGCTGGGTGTCGTCGGCAGGAGGTGTAATCGAACCTCAACAGGT
901 GCTGATGGCTGCAACCTGCTCTGCTGTGGACGGGGGTACAACACCCACCAGTTCAACCAC
961 GTCTCTCATCAGTGCAACTGCAAATCCACTGGTGTGCGAAGTCAAGTGCCAGACTTGC
1021 ACCATCAAGAGCGAAGAGTACACCTGCAAATAA

Wnt8

Location: scaffold_15: 1358008-1360980

1 ATGAATTGTTTAGTTCGCTCGTTTTTCGCCTTGCTGGTGTATGCAAATTTTCTACTTCA
61 TCAGCTTGGTCTTGAATAATCTGTTGATGACAAGCACAAGGATCAATTTGGAATGGCG
121 GAATCGGTAGCGGCCGGCGCTGAACTTGCGATGGGCGAATGCCAATATCAATTCCGATGG
181 GAGCGCTGGGCATGTCCACGCAGCGCCTTACCAAAAAGAAGGTGTTTAGATCACTGATT
241 CGAGAATCTGCGGCTCTCCACTCTTTCTTTTCGGCTGGAATCACTTACACGTTAACGCGC
301 AACTGTTCTCGCGGCCAACTGGAAGGATGCGGATGCGCCAACATACATTCTCAACATCCA
361 AACGAATCTCCTTCAGCCACATGGCGGTGGGGAGGATGCAGCGACAACATCAAGATGGGA
421 GAACAATACTCTGTCCGAGTACTGGATTCTCTCGAGTCTGGACAGGATGCCCAGGCTTTA
481 GCTAATTTGCACAACAACCTTTGCTGGCCGTTTGGCTGTTGTCATTCAATGCGCCAGTCG
541 TGCAAATGCCACGGAGTGTCCGGCTCGTGCGCCATGCAGACTTGTTGGATTCAATTGCCT
601 CCATTCCGCACAGTTGGCCAAGCCCTCAAACGACAATACGACACAAGTGTGAGGGTTGGC
661 TATAGCAATGGCATCCTACAGATCGACGGCGGCAGCTCATCGTCCAACGGCGCCAAGACC
721 TCGTCAGGCTCGTCACAGATTTTACGCGTCTCGCCACAAACGCTCGTCTACTTGGAACTG
781 TCGCCCGACTTTTGCCGAGCAAACGTACGGCCGGAACGAGCGGCACGAAAGGCCGGACA
841 TGCTCACGAGACAAAGGTAAAGGCCTTTCCGCATCTCAACGGCGCAGCTGCATTCATCTG
901 TGCAAGAACTGCGGTCACAAAGTCAAGAAGCGCGTCGTCACTGTCATGACCTCATGCAAC
961 TGCACTTTCCAGTGGTGCTGTCAAGTCCAGTGCCAGACGTGCAACACAACGAAAAGTATT
1021 TACTCTTGCGCTTAA

Wnt9

Location: scaffold_8: 1123265-1340338

1 ATGTGCGTCCAAGGAGGAGACGGCCTTGCGGAAACACTCCTAGAAGCCATTGCGATGTCC
61 GTGTACACATGTCAGCAGCAATTCGAGTACGAACGATGGAAGTGCACACCACACGGCCAC
121 TCGCGTATGCTGACCCTGAAGAAAGCGTACAAGGAAACAGCGGCACGTACGCCTTCTCG
181 GCGGCTGGCGTCGCTCACGCTGTCGCCCCGAGCTTGTGCCGCCGGCAGACTCCGCAAGTGC
241 CACTGCGCCGAGTGGAGATGGGGCGGCTGCGGCGATAATCTCGCCTATGGCAGGACATTT
301 GCAACGAAATTTCTGGACTCGGGTTCTATTGATAAGGCCCGATCCAGTAGCGGACATTTG
361 AAGAAAGGGGAAGAAGAGAGATCCGTGGTCGAGCAGTTGGTCAAACGGACGTGCAAATGC
421 CACGGCGTCAGTGGTTCGTGTTCCGGTCAAGACGTGCTGGAATCAACTGGCAGCTTTCAGC
481 GAGACGGCCCGCCGTCATCAAGAACAGATACGACAACGCCGTTCCAGGTCAACGTCGAGATC
541 CGCGAAAACGCCAGCCACTTGAGGACGGTTTTCCGTGAGCGCGGGCGATCATCGAGTCGAC
601 CGGAAAGAGTTGATCTACCAGCAGAGATCGCCGATTTCTGCGTCGCCAATCCGCTAAGT
661 CCGGGCACGACGGATCGCCAGTGTCTGCGCGGTGAGAATTGCGACATCCTGTGCTGCGGG
721 CGCGGCTACAACGTTCCACCGACTAACCGTTTTGACGCCGTGCAAATGCAAACATCCTC
781 TGCTGTCAAGTGAATGTCAGAATTGTTTGGTGGAGAAGACCATCCACACTTGCAAATGA

Wnt10

Location: scaffold_8: 1123159-1128543

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1 ATGCGCCGTTCTGATTTGTTGCTGAGCAGTAATTCGCTGATGAATATCCGCCGGAAGCCG
61 GATGAGAAGAGACGCCAGAAAGAGGACAAATATTTCTTCAATTCGGTGATGGAGCGACAG
121 CGCCGGAACCTCGACCGACCAGCGCCATTTGCAAGAATTTCCCGGGTCTCAGCAAAAAG
181 CAGTTGGAGCTCTGCTTTCGCTACCCTGACGTCATGTCGGCCGCCATCGGAGGTCTGCAG
241 TTGGCCGTCAACGAATGCCAGTTCCAGTTCCAGAAGCACCGCTGGAAGTCTCGGCCCTG
301 GACAGGAAGAACCGCAATCCTCACAGCAGCAATTTCTGCAGAAAGGCTATCGAGAAACA
361 GCGTTCGCTTACGCCGTCTCATCTGCCGGAGTGGCGCATTCCGTGTCCAAGGCTTGCGGC
421 CAAGGTAAATTGGAATCGTGCGGATGCGATCCCAAGAGCCAGCGCGGTAACGGCGGTTTC
481 GGATCGTCGTCGACGCTGGCCGATTGGCGCTGGAGCGGCTGCTCGACAATATGGATTC
541 GGCGTCAAGTTCTCGCGCTTCTTGCTCGATTGCGCCAGCGCGGACAGGACATTCACTCG
601 CGGATTCACTTGACACAACAGCCACGTCGGCCGAACCGTAGTTGGAGCCAATTCGGAGATC
661 CGATGCAAATGCCACGGCATGTCAGGCAGTTGCGAGATGAAGACCTGCTGGAAGGCGGTC
721 CCGGAATTCCGGCGCGTTCGGCACCGTCTCAAGGAGCGCTTCAACCAGGCCGTCTTGGTG
781 GATCAATCTCAGCTGGGAAACGAGGCTGGCAGCAGCAGCAAAGGGCGGCAGTTCGCCCGG
841 CGCATCCGAGACACCGACCTGCTTCTACGAACGGTCGCCCAATTTCTGCGAGGAGCGG
901 CCGGACGTCGACTACCCGGGAATCACGGGGCGACGGTGCAACAAGACGGGCGACGAGCTG
961 GACAACCTGCCAGTCGCTGTGCTGCGGGCGCGGCTACAACGTCGTCCGCCAAAAGAGGACG
1021 GAGCGCTGCCACTGTCGCTTCCACTGGTGCTGCTCCGTCGTGTGCAACAACCTGCACAGTC
1081 GAGCAATGGGTCACCGTATGCAAATAA
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Wnt11

Location: scaffold_40: 129806-131673

1 ATGGATGGCTGTCAATTGATCGGGCTGCTCATTCTTTGCCATCTGATGGCCACGCCTGCA
61 CTCTCCATCAACTGGATAGGTTTACACAGGCACTGGGATAGTGAGTTTTGGAATTGGACT
121 ACGCTCAATTTCGTCCAGTCGCCAGATTAACAACATCAAATCGACTTGCTGGTCGGCCGTC
181 CGTCAGAAACTGTTGACCAAGGAGCAAGTTAAAGTGTGCCTCCAGCAGCCAGCTACGATG
241 CTCTCCGTTTGGGAGGCGGGCCAATCCAGCAGTCAAGCCTGTCGGCAAGCCTTCGCCGAG
301 CGCAGATGGAAGTGCAGCTCGATCGACTTCCTCCCACGGAAGGCACCCGATCTATCTCAC
361 GGAACCAGGGAACAAGCTTGGGTGTATGCGCTCACTTCGGTGCCTAACTCGTGTCTGTT
421 GCACGAGGTTGTTCTTCGGGAATTTTGAAGCAGTGCGCCTGCGGCTCGTTCCCGCGCCAC
481 CCTCCCGATGGTCAATTCAAGTGGGGAGGTTGCGGTGATGACATCAAATATGCTAAACAG
541 TTCGCCAAATCATTACGGATGCATCCCTGAAACGACACTGGAAAAACCTCACGTCATT
601 TCCGCTGTGAACATGCACAACAACCGCGTCGGTCGTAAAGTGGCCGAGCAAAGTCTACAG
661 ACGCAATGCAAATGCCACGGCGTTTCTGGTTCGTGTCAAATTTAAACCTGCTGGCGATCG
721 CTGCCACCTATTGGGGAAATAGCCAGTCGAATGAAAATGCTGTACGGATTGGCTGTGGAA
781 GTTACGCCTCGTCGACCAAACGACATGCGCCGCCGAGTGCCGTAGCTTCCGGAAGACTG
841 GCGCAAGACCTTATCTACATCACTAAATCACCTGACTTTTGTGCGCCGGATAAACAATCA
901 GGCAGTTTGGGTACGGCCGGAAGGATGTGCAACGCAACCCACGAAAGTAGCAGCGGGAGT
961 TGCCTTCCCTGTGTTGCGGTCGTGGTTACATCACCGTTATCCGTGAACAAGTCGAACGA
1021 TGCAATTGCAAATACATTTGGTGCTGCACGGTTCGCTGTAAAACGTGTGACGTCGAAGTC
1081 GAAGTGCACATGTGCAAGTAG

β -catenin

Location: scaffold_11: 373544-377305

Start/stop codons were not detected. A predicted Start codon is indicated in red and 5' UTR is indicated in gray.

```
1 GTGTCACATGGGCAATATGTTAGCAATAGCCCTGCTGATCTTCCTATGCCAGCGTTAAG
61 GAACATACATTGATGTGGCAAGGAGTTGGGCAACCTGGATATGTAGGAGATTCAGGAATC
121 CAAAGTGGGGCAACCACTCAGGCCCATCACTCTCTGGAAGAGGTGGTATGATGATATG
181 GATACTTCTGAAGGAGGAGTTATGCTTAACATGGGTGGATCAAGTGGCTATTCAGCTGGT
241 TATACTCAAGACCAAGTTGAAGACATGAATGCTCAGTTAAACCAGACTCGCTCCCAGCGT
301 ATTCGTGCTGCCATGTTCCAGAGACACTGGATGAAGGAATGGAAATTCATCTACTCAA
361 TTTGATACCACACAGCCAACCTGCTGTTCCAGCGTTTAGCCGAACCGTCGCAAATGTTGAAA
421 CATGCCGTTGTTAATTTGATCAACTATCAGGATGATGCTGATCTAGCTACAAGAGCTATT
481 CCCGAATTGATCAAGTTGTTGAACGATGAAGATCAAGTTGTCGTATCTCATGCTGCCATG
541 ATGGTTCACCAGTTGTCCAAGAAGGAAGCGTCCCGCCATGCTATCATGAATAGCCCACAG
601 GCAACCAAATGATTGCGGCTCTCGTTTCGAGCACTGTCCAATAGCAATGACCTGGAAACC
661 ACCAAAGGTGCCGTTGGTACCCTGCACAACCTATCCCATCATCGCCAAGGATTGTTGGCT
721 ATTTTAAATCTGGGGGAATTCCAGCTTTGGTGAAGCTGTTGAGCTCGCCCGTGGAAATCT
781 GTTCTTTTTATGCTATCACGACGCTGCACAACCTGCTGCTCCATCAAGAAGGCTCAAAA
841 ATGGCCGTTTCGTTTGGCGGGTGGTTTACAGAAGATGGTTGCTCTCCTTCAGCGCAACAAT
901 GTCAAATCTTGGCAATTGTTACTGATTGTCTTCAAATCTGGCGTATGGCAATCAAGAA
961 TCCAAGTTGATCATTTTAGCTTCACAGGGGCCGATGGAACCTGTTTCAATTCTTCGGTCA
1021 TATACTTACGAAAAGCTGCTGTGGACCACTTCACGAGTTTTGAAAGTGTGTGGTCTGT
1081 TCCAGCAACAAACCAGCTATTGTTGAAGCTGGAGGTATGCAAGCGTTGGCAATGCACTTG
1141 GGCCACCAAAGTCATCGCCTAGTTCAGAAGTGTATGGACCTGCCGCAATCTTCCGAT
1201 GCTGCGACCAAGCAGCAAGAAGGCTTAGAAACCCTGCTGCAAGGACTTGTTTCAGCTGTTA
1261 GGTTCTAGTGATGTTAACGTAATCACTTGCCTGCCGGAATTCTCTCTAACTTGACTTGC
1321 AACAAACCAACGCAACAAGACTGTTGTGTGTCAAGTCGGTGGTATCGAAGCGCTGGTTCCG
1381 ACCATTATTCATGCTGGCGATCGTGAAGAAATCACCGAACCTGCCATATGTGCTCTGCGT
1441 CACATGACATCACGTCACGCTGAAGCTGAAATGGCTCAAATGCCGTTTCGTCTTAGTGGT
1501 GGACTGC CCGTCATCGTCCGTTTATTACAACCCCTCTCGTTGGCCACTCATCAAGGCT
1561 CTGATCG GGTGGTTCGTAATCTGGCTTTATGCCCTGCTAACCACGCTCCGCTGCGAGAA
1621 CAAGGAGTTACTCGTTTGGTGCAGCTTTTATCCGTTGCTTTCCAAGACATCCAACGA
1681 CGTTCGTGGTTGTCAGCTCAGGCAGCGTCCAGCCACAAGGATCCTACGCCGATGGAGTA
1741 AGGATGGAAGAAATTGTCGAAGGTGCTGTAGGCCCCCTCCATATTTAGCTCGCGAGTCC
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1801 CACAATCGGGCTGTTATTCGTGGCTTGAGTGTTATCCCTATTTTCGTCCAGTTGTTGTAC
1861 AACGAGATAGAAAATATTCAACGTGTTGCTGCCGGCGTACTGTGCGAGCTGGCCTCTGAT
1921 AAGGAAGGAGCCGACATGATCGAACAGGAGGGTGCAACTGCTCCTCTGACAGAACTACTT
1981 CATTCTCGCAATGAGGGTGTTGCTACGTATGCGGCTGCTGTACTTTTCCGCATGTCTGAA
2041 GACAAGCCCCAAGATTATAAGAAGAGGCTATCTATGGAGCTGACCAATTCCTTGTTCCGC
2101 GAAGACCATCATTGTGGAATGGAGATCTGCCTTTGATGCCTCCCGATCTTCAGCAGGAT
2161 ATTCTTGACCTGACCAGCCTTATGAAGGTCCTTATGGGCAAGGCCACCCAGTGTCCAC
2221 TCCAACCAAGATGGAGGCAGGTCTTATCGTCAAGGATATGACGCCCTACCCGTAGAATCG
2281 ATGCAAGGATTGGAGCTCGGTTACACCATGGCGGCCACCCACCTTCTGCCACATCTCCG
2341 ACCATGGGCATGGGGATGAATATGGGGCATTCAATGGATCACATGGGTTCTGATTTGGAT
2401 TTCGATCCTCTGAACCACATGGGTCAGGCCACACCTCCCCAACCGCCCCACGATGCCAG
2461 GCCGCTTGGTATGATACCGACCTT