Comparative Early Developments in Winter Spawned Three Pre-larval Fishes (*Gadus macrocephalus*, *Liparis tanakae*, *Hexagrammos agrammus*)

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**ABSTRACT** This study investigated how the larvae of three winter-spawning fishes are adapted in a cold water environment by examining the morphology, digestive system, and swimming ability of larvae from three winter-spawning species (*Gadus macrocephalus*, *Liparis tanakae*, *Hexagrammos agrammus*). Data were collected at hatching and first feeding. The results were compared with reported data on several non-winter-spawning species. Mean total lengths at hatching (±SD) were 4.35±0.11 mm, 5.26±0.08 mm, and 7.48±0.35 mm for *G. macrocephalus*, *L. tanakae*, and *H. agrammus*, respectively. Three winter-spawning fishes had well-developed digestive tracts after hatching than those of non-winter-spawning fish larvae. Yolks were intact until 5 days post-hatching in all three species, indicating that they had longer mixed-feeding periods compared with fishes spawned during other seasons. *G. macrocephalus*, *L. tanakae*, and *H. agrammus* larvae had superior cruise and burst speeds (measures of swimming ability) than non-winter-spawning larvae. We conclude that the unique characteristics of these three winter-spawning species are naturally selected adaptations under lower water temperature in winter.

**Key words:** Fish larvae, first feeding, winter-spawning, swimming ability, cold water adaptation

**INTRODUCTION**

The larval phase is the period of the highest vulnerability in a fish life cycle, when the primary nutrient source shifts from internal (yolk sac) to external (foraging) (O’Connell, 1976; Theilacker, 1978; Strüssmann and Takashima, 1990). Internally, drastic developmental changes occur that make this shift possible, including the swimming ability and the development of digestive organs. At the same time, the tremendous external pressures of starvation and predation lead to mass mortality during the larval phase (Yin and Blaxter, 1987a).

These selective pressures are particularly strong during winter, when zooplankton concentrations are relatively low (Kang, 2008), live-prey size increases (Ware, 1977), and development of swimming ability and growth rate are reduced (Murphy, 1961; Leis, 2010). These unfavorable conditions during winter may have led to unique adaptations for the larvae of winter-spawning fishes. Accessing early development of larval stages of fishes are particularly suitable for observing such adaptive behaviors and physiologies because both are more apparent at this stage, given that natural selection diminishes in influence as the fishes age (Williams, 1957).

Several previous studies have examined various aspects of winter-spawning larvae including distribution, early feeding habitats, and morphology. Pacific cod (*Gadus macrocephalus*) spawns in December-January (Gwak, 2010), and its distribution and feeding ecology are well known (Takatsu et al., 2002), as well as its morphological development during the egg and larval phases (Kim et al.,
Similarly, researches on Tanaka’s snailfish (Liparis tanakae) have clarified the egg and larval development (Aoyama, 1959; Kim et al., 1986) and spawning time as in December-February (Kim et al., 2005). The spotty belly greenling (Hexagrammos agrammus) is known to spawn in November-January (Fukuhara, 1971; Lee et al., 2015) and its ontogenetic intervals have been identified according to the development of feeding and swimming characteristics (Kohno et al., 2000). However, no study has investigated the adaptations of winter-spawning fishes via various comparative methods.

In this study, we assessed the morphologies, digestive tracts, and swimming abilities of G. macrocephalus, L. tanakae, and H. agrammus. We then compared the measured traits across these winter-spawning fishes and with the data on non-winter-spawning fishes.

**MATERIALS AND METHODS**

1. **Fertilization and Rearing**

Gadus macrocephalus broodstock were net-caught on a shore in Oepo, Geoje. Eggs collected from these samples were artificially fertilized using the dry method and hatched in a 20 t water tank. The rearing period lasted from January 17-21, 2015. Water temperature was maintained at 9.56±0.37°C (approximating natural seawater) and was replaced 3-4 times in each day.

Liparis tanakae fertilized eggs were collected from a fish pot installed in the sea near Chudo. Larvae were hatched from these eggs in an 80 L water tank. The rearing period lasted from February 23, 2015 to February 27, 2015. Tank temperature was maintained at 10.4±0.67°C (approximating natural seawater) and replaced 1-2 times daily.

Fertilized eggs of Hexagrammos agrammus were collected via scuba diving from the algae covered rocks ca. ~5 m deep, near Yeongun, Tongyeong. Similar to L. tanakae, they were hatched in an 80 L water tank. The breeding period lasted from January 20-24, 2015. Water temperature was maintained at 9.78±0.32°C (approximating natural seawater) and replaced 3-4 times daily.

Experiments were performed in larvae-hatching tanks from Gyeongsangnam-do Fisheries Resources and Research Institute. Immediately after hatching, larvae were supplied 5-10 enriched rotifers/mL water; rotifer density was maintained through a twice-daily supply.

2. **Measurements of Digestive Tracts**

Thirty larval samples were fixed in 5% neutral formalin to observe morphology via a stereoscopic microscope (Olympus SZX10) and an imaging device (Q Imaging GO-21). Total length, standard length, head depth, eye diameter, myotome height, yolk length, and yolk height were measured (to the nearest 0.01 mm) using Q Capture Pro 6. Yolk volume was calculated following Blaxter and Hempel’s (1963) equation (i.e. yolk volume = π/6 x h³, where I is the yolk length and h is the yolk height). For histological analysis, 10 samples were first fixed in Davidson’s solution for 24 h, and then transported in 70% alcohol for storage. Samples were then paraffin-sectioned at 5-7 μm. Next, the sections were stained with Hansen’s hematoxylin and 0.5% eosin for histological observation using stereoscopic microscope.

3. **Larval Swimming Ability**

Larval swimming speeds were measured following a previously developed method (Masuda et al., 2002). One larva from each species was added to 1 L beakers (diameter: 10 cm), covered with a black vinyl sheet. After 3 min of acclimation, each larva (10 total) was filmed for 2 min. Cruise swimming was filmed for the first 1.5 min. The beaker was then hit three times (in 10 s intervals) with a 20 g weight attached to a string, startling the larvae and triggering burst swimming for the remaining 30 s. Filming was done with a DSC-RX100 (frame frequency: 30 frames/s; Sony Corp., Tokyo, Japan).

| Table 1. Morphological characteristics of three larvae species on the day of hatching and first feeding. Data represents mean ± (SD) (n = 30) |
|---|---|---|---|
| Species | G. macrocephalus | L. tanakae | H. agrammus |
| Days | 0 | 3 | 0 | 2 | 0 |
| TL (mm) | 4.35 ± 0.10 | 4.91 ± 0.16 | 5.26 ± 0.09 | 5.38 ± 0.10 | 7.48 ± 0.36 |
| SL (mm) | 4.19 ± 0.09 | 4.71 ± 0.14 | 5.04 ± 0.10 | 5.11 ± 0.10 | 7.09 ± 0.32 |
| BH (mm) | 1.06 ± 0.04 | 1.12 ± 0.05 | 1.14 ± 0.04 | 1.20 ± 0.06 | 1.35 ± 0.05 |
| HH (mm) | 0.57 ± 0.03 | 0.74 ± 0.04 | 1.01 ± 0.05 | 1.09 ± 0.06 | 1.10 ± 0.04 |
| ED (mm) | 0.29 ± 0.01 | 0.34 ± 0.03 | 0.46 ± 0.03 | 0.49 ± 0.04 | 0.67 ± 0.02 |
| MH (mm) | 0.24 ± 0.01 | 0.27 ± 0.02 | 0.46 ± 0.03 | 0.45 ± 0.03 | 0.59 ± 0.04 |
| YV (mm³) | 0.25 ± 0.03 | 0.13 ± 0.04 | 0.16 ± 0.04 | 0.04 ± 0.01 | 0.56 ± 0.08 |

TL, total length; SL, standard length; BH, body height; HH, head height; ED, eye diameter; MH, musculature height; YV, yolk-sac volume
Video records were converted to 30 images per second using Vegas Pro 10.0 for determining cruise and burst speeds. Next, a beam projector was first used to mark the movement paths of larvae in the 1 L beakers, and travel distances were measured with a Vernier caliper to the nearest 0.01 mm. Cruise speeds of continuously moving larvae were calculated from the mean speed during 5 s, whereas cruise speeds of periodically moving larvae were calculated from the mean speed over distance travelled. Burst speeds were measured based on the distance travelled by the larva after being startled. Mean swimming duration during 90 s was measured to assess continuous swimming ability.

Three types of swimming were recorded. Stationary

Fig. 1. Photomicrographs of three fish larvae on the day of hatching showing the whole-body and sagittal section of the body axis showing the digestive tract of larvae of *Gadus macrocephalus* (a, b), *Liparis tanakae* (c, d), and *Hexagrammos agrammus* (e, f). Scale bars = 1 mm. YO, yolk; ST, stomach; IN, intestine; RE, rectum; OE, oesophagus.
swimming was defined as a swimming duration of < 20 s, semi-sustained swimming as 20-70 s, and continuous swimming as > 70 s.

4. Statistical Analysis

Among-species differences in cruising and burst speeds were compared using a one-way ANOVA. Significant means were separated using a post-hoc Fisher’s LSD test. Analyses were performed in SPSS 23.0. Significance was set at P < 0.05.

RESULTS

1. Larval Digestive Tracts

1) The First Day of Hatching

Mean total lengths at hatching were 4.35 ± 0.10 mm (± SD), 5.26 ± 0.09 mm, and 7.48 ± 0.36 mm for *G. macrocephalus*, *L. tanakae*, and *H. agrammus*, respectively (Table 1; Fig. 1). Larval eyes in all three species were pigmented with melanophores; the dorsal, anal, and caudal fins were connected via a membrane (Fig. 1). The esophagus, stomach, intestines, and rectum were well-differentiated, indicating the establishments of early digestive tracts. Whereas, the three species exhibit some differences in the morphology and digestive system. The mouth of *G. macrocephalus* was closed at hatching, whereas the other two species had open mouths and developed jaws. In addition, *G. macrocephalus* exhibited a primordial fin-fold form, whereas *L. tanakae* had developed pectoral fins and *H. agrammus*, fan-shaped caudal fins. Mucosal-fold development was the most prominent in the convoluted mid-intestines of *H. agrammus*, followed by those of *L. tanakae* (also with convoluted intestines), while *G. macrocephalus* had linear intestines without mucosal folds (Fig. 1). Finally,
only *H. agrammus* larvae were observed to eat foods at the first day of hatching.

2) The Days at First Feeding

First feeding occurred at 3 and 2 days post-hatching for *G. macrocephalus* and *L. tanakae*, respectively, and at the first day after hatching for *H. agrammus*. Mean total lengths at first feeding were 4.91 ± 0.16 mm, 5.38 ± 0.10 mm, and 7.48 ± 0.36 mm for *G. macrocephalus*, *L. tanakae*, and *H. agrammus*, respectively (Table 1; Fig. 2). All three species had yolks at first feeding, but no morphological differences between the post-hatching and first-feeding periods. *G. macrocephalus* larvae mucosal folds were developed by this time, whereas mucosal folds in *L. tanakae* were reduced, and the yolk had decreased to yield a wider intestinal lumen.

2. Larval Swimming Ability

1) The First Day of Hatching

*Hexagrammos agrammus* showed the highest cruise speed, followed by *G. macrocephalus* and *L. tanakae*. Fisher’s LSD post-hoc test revealed that the speeds of *H. agrammus* and *L. tanakae* differed significantly (P < 0.05), but no significant difference was found between *G. macrocephalus* and the other two (P > 0.05). The burst speed of *G. macrocephalus* was significantly lower than those of the other two species (P < 0.05). *H. agrammus* and *L. tanakae* indicated similar burst speeds as 7.55 standard length (SL)/s and 7.51 SL/s, respectively (P > 0.05). As a results, *H. agrammus* had the highest cruise and burst speeds at hatching, whereas *L. tanakae* had faster burst speed and lower cruise speed than *G. macrocephalus* (Table 2 and Fig. 3a).

Mean continuous swimming durations were 90 s, 64 s, and 2 s for *H. agrammus*, *L. tanakae*, and *G. macrocephalus*, respectively. *H. agrammus* swam continuously from hatching day, but *G. macrocephalus* exhibited stationary swimming until the experiment terminated.

2) The Days at First Feeding

Cruise speeds were 2.72 SL/s, 1.98 SL/s, and 1.94 SL/s for *G. macrocephalus*, *L. tanakae*, and *H. agrammus*, respectively. These speeds rose relative to hatching-day speeds for *G. macrocephalus* and *L. tanakae*. Burst speeds were 9.15 SL/s, 7.84 SL/s, and 7.55 SL/s for *G. macrocephalus*, *L. tanakae*, and *H. agrammus*, with the fastest larvae differing significantly (P < 0.05) from the two other species (P < 0.05), which were similar in cruise speed (P > 0.05).

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**Table 2.** Swimming speeds of six species on larval stage

<table>
<thead>
<tr>
<th>Species</th>
<th>DAH</th>
<th>SL (mm)</th>
<th>Cruise speed (SL/s)</th>
<th>Burst speed (SL/s)</th>
<th>Data source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gadus macrocephalus</em></td>
<td>3</td>
<td>4.71</td>
<td>2.72</td>
<td>9.15</td>
<td>Present study</td>
</tr>
<tr>
<td><em>Liparis tanakae</em></td>
<td>2</td>
<td>5.11</td>
<td>1.98</td>
<td>7.84</td>
<td>Present study</td>
</tr>
<tr>
<td><em>Hexagrammos agrammus</em></td>
<td>0</td>
<td>7.09</td>
<td>1.94</td>
<td>7.55</td>
<td>Present study</td>
</tr>
<tr>
<td><em>Scomber japonicus</em></td>
<td>9</td>
<td>8</td>
<td>1.58</td>
<td>4.4</td>
<td>Masuda, 2009</td>
</tr>
<tr>
<td><em>Trachurus japonicus</em></td>
<td>15</td>
<td>5.2</td>
<td>2.2</td>
<td>10.3</td>
<td>Masuda, 2009</td>
</tr>
<tr>
<td><em>Engraulis japonicus</em></td>
<td>11</td>
<td>6.1</td>
<td>1.2</td>
<td>3.4</td>
<td>Masuda, 2009</td>
</tr>
</tbody>
</table>

DAH, days after hatching; SL, standard length

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**Fig. 3.** Comparisons of swimming speeds of three larvae species on the day of hatching (a) and first feeding (b). *Gadus macrocephalus* (■), *Liparis tanakae* (▲), *Hexagrammos agrammus* (●). Plots with different letters are significantly different (P < 0.05 Fisher’s LSD test).
At 3 days post-hatching, *G. macrocephalus* burst speed increased two-fold from 5.12 SL/s at hatching to 9.15 SL/s, possibly contributing to the success of its first feeding. In contrast, *L. tanakae* burst speed increased only slightly, from 7.51 SL/s to 7.84 SL/s (Table 2 and Fig. 3b).

Mean continuous swimming duration was 90 s for both *H. agrammus* and *L. tanakae*, but only 5 s for *G. macrocephalus*. Thus, *L. tanakae* significantly increased its swimming duration after the first feeding, whereas *G. macrocephalus* larvae did not, continuing with stationary swimming even after the first feeding.

**DISCUSSION**

1. Larval Appearance and Digestive Tracts

In this study, we recorded mean total lengths at hatching of 4.35 ± 0.10 mm, 5.26 ± 0.09 mm, and 7.48 ± 0.36 mm for *G. macrocephalus, L. tanakae*, and *H. agrammus*, respectively. The results were comparable to mean total lengths at hatching of non-winter-spawning fish species. *Paralichthys olivaceus* (spawn period: March-June), *Scomber japonicus* (spawn period: March-May), and *Epinephelus septemfasciatus* (spawn period: May-September) have 2.80 mm, 3.35 mm, and 1.74 mm of mean total length, respectively (Dou et al., 2002; Park et al., 2015; Park et al., 2016). Those results indicated that winter-spawning fishes tended to have larger hatched larval size than non-winter-spawning fishes. Similar to our results, large-sized hatching larvae were also observed in several winter-spawning fishes such as in *G. morhua, Platichthys flesus* (Yin and Blaxter, 1987b), *Melanogrammus aeglefinus* (Laurence, 1974), *Clupea harengus* (Blaxter and Hempel, 1963), and *Pleuronectes platessa* (Blaxter and Ehrlich, 1974). These findings are consistent with data showing that unfavorable conditions for offspring growth decrease egg production, but increase fertilized-egg and larvae size (Smith and Fretwell, 1974). Moreover, hatched eggs also increase in size when plankton size increases during winter (Ware, 1977).

In most teleosts, the digestive system is undifferentiated at hatching. Before first feeding, the esophagus, stomach, intestines, and rectum become differentiated, fully establishing an early digestive system (Iwai, 1967). The three fish species analyzed not only hatched at larger sizes, but also had more advanced digestive systems than non-winter-spawning fishes immediately post-hatching. On the other hand, *P. olivaceus* early digestive systems are established within ca. 7 days after hatching (Ochiai and Tanaka, 1986), and *S. japonicus* and *E. septemfasciatus* digestive systems both develop 3 days post-hatching (Park et al., 2015, 2016). Likewise, the early digestive systems of non-winter-spawning, *P. major* and *Acanthopagrus schlegeli*, are developed at 4 days post-hatching (Tanaka, 1973). According to Tanaka (1975), the hatching larvae from demersal eggs complete differentiation of digestive tract while the larvae from pelagic eggs do not complete the differentiation when they are hatching. As a result of this study, three fishes were hatching from demersal eggs and have complete differentiation of digestive tract. Generally, demersal eggs have longer size and incubation time than pelagic eggs (Thresher, 1984). This phenomenon could be seems to compensate for slow development rates in cold winter waters after hatching.

First feeding occurred at 0, 2, and 3 days post-hatching for *H. agrammus, L. tanakae*, and *G. macrocephalus*, respectively. However, we note that our favorable laboratory conditions, with a constant food supply of rotifers, may not fully represent the actual marine environment. Previous field studies found that *G. macrocephalus* and *L. tanakae* began to forage successfully at a smaller size than in this experiment (Takatsu, 1995; Plaza-Pasten et al., 2002). Therefore, both species seem to reach first feeding more quickly in the ocean than in captive condition. Comparative results showed that the three species tested here begin first feeding earlier than non-winter-spawning fishes: *P. olivaceus* feed at 5 days post-hatching (Dou et al., 2002), *S. japonicus* at 3 days (Park et al., 2015), and *E. septemfasciatus* at 4 days (Park et al., 2016). Earlier first feeding behavior will positively contribute to faster growth for larvae, and is thought to be advantageous for survival during winter.

Yolk was absorbed after 5, 8 and 12 days for *H. agrammus, L. tanakae*, and *G. macrocephalus*, respectively (Fukuhara, 1971; Ochiai and Tanaka, 1986; Kim et al., 1986). In contrast, for *P. olivaceus* and *S. japonicus*, yolk absorption was almost completed by 3 days post-hatching, when first feeding also occurred (Dou et al., 2002; Park et al., 2015). Similarly, yolk was nearly entirely absorbed at 4 days post-hatching in *E. septemfasciatus*, with first feeding observed a day later (Park et al., 2016). These findings indicate that most non-winter-spawning species begin eating only after their yolk absorption is almost complete, unlike three winter-spawning fishes in our study. Thus, three winter-spawning fishes exhibit a longer mixed feeding period, when larvae obtain nutrition from both the yolk reserves and external sources. The length of this period varies depending on species and water temperature (Kamler, 1992), with longer mixed feeding periods resulting in stronger resistance to starvation (Yokota et al., 2016).
Therefore, the observed feeding behavior of larvae from three winter-spawning fishes improves survival chances during prey-scarce winters.

In summary, the morphology and digestive-track development of the three winter-spawning fishes indicated a compensatory strategy for slow growth in lower water condition during winter via earlier first feeding. This strategy prolongs the mixed feeding period and subsequently increases the probability of larvae survival in the cold.

2. Larval Swimming Ability

Of the three fish species, *H. agrammus* had the longest continuous swimming duration and fastest speeds, owing to fully developed pectoral fins at hatching. High swimming speeds improve larval post-hatching foraging success and elevate their chances of fleeing from predators. As *H. agrammus* lays fewer demersal eggs, advanced swimming ability of their larvae likely compensates for such a strategy. Similar to *H. agrammus*, *L. tanakae* larvae could swim vigorously and continuously, due to fully developed pectoral fins at hatching. Their swimming duration significantly increased after the first feeding, as did their cruise speed (from 1.33 SL/s to 1.98 SL/s). These patterns suggest that *H. agrammus* and *L. tanakae* demand large amounts of energy on highly mobile swimming behaviors associated with foraging. In contrast, *G. macrocephalus* had the lowest burst speed at hatching, leading to a more rapid increase (relative to the other species) at the first feeding; as a result, larval prey-capture success rose. The mean swimming duration of *G. microcephalus* was 2 and 5 s at hatching and at 3 days post-hatching, respectively. These data indicate that *G. macrocephalus* larvae minimize continuous swimming and maximize prey-capture through bouts of burst swimming (assisted by water currents), thus reducing the amount of energy spent on eating.

Cruise speeds at first feeding of three winter-spawning fishes were faster than those of non-winter-spawning fishes such as *S. japonicus*, *T. japonicus*, and *E. japonicus* (Masuda, 2009) (Table 2). *G. macrocephalus*, *L. tanakae*, and *H. agrammus* had also higher burst speeds than the above three non-winter-spawning fishes (Table 2). These variations in the cruise and burst speeds may be attributed to differences in the properties of fertilized eggs, specifically whether the eggs are pelagic or demersal. As explained earlier, pelagic eggs hatch when organogenesis is still incomplete, while demersal eggs hatch when organogenesis is advanced (Iwai, 2005). The proposed link between the swimming abilities of three winter-spawning fish larvae and fertilized-egg properties is consistent with previous reports. During the initial post-hatching period, larvae from demersal eggs exhibit greater swimming speeds than larvae from pelagic eggs (Leis, 2010). In summary, the larvae of *G. macrocephalus*, *L. tanakae*, and *H. agrammus* have well-adapted to the cold environment by hatching after swimming appendages fully developed. This characteristic feature may compensate the slower early development during winter, allowing the larvae to forage successfully for prey.

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REFERENCES


겨울철 산란하는 대구, 꼬치, 노래미 전기 자어의 발달 비교

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요 약 : 겨울철 환경에 대한 초기 적응을 조사하기 위해 대구(Gadus macrocephalus), 꼬치(Liparis tanakae), 노래미(Hexagrammos agrammus) 자어의 부화 직후와 첫 섭식 시 외부형태, 소화기관, 유영능력의 발달을 확인하였으며, 다른 계절에 부화하는 어종과 비교하였다. 평균 전장(±SD)은 대구 4.35±0.11 mm, 꼬치 5.26±0.08 mm, 노래미 7.48±0.35 mm로 부화 시 다른 계절 부화자어에 비하여 컸으며, 세 어종 모두 초기소화기관을 갖춘 후 부화하여 다른 계절 부화자어에 비해 초기소화기관 확립시기가 빨랐다. 대구, 꼬치, 노래미의 첫 섭식 시기가 부화 후 3일, 2일, 0일이었고, 난황을 갖고 있는 기간이 5일 이상으로 다른 계절에 부화하는 자어에 비해 혼합섭이기간이 비교적 길었다. 이 3종 자어의 유영능력은 다른 계절 부화자어에 비해 순항유영속도와 돌진유영속도 모두 부화 직후부터 빨랐다. 이러한 특징들은 자어가 생존하기 어려운 겨울철 환경에 대한 자연선택 및 환경적응의 결과로 생각된다.

 찾아보기 낱말 : 자어, 첫섭식, 겨울산란, 유영능력, 저수온적응