Embryonic Development of Goldfish (Carassius auratus): A Model for the Study of Evolutionary Change in Developmental Mechanisms by Artificial Selection

Hsin-Yuan Tsai,1,2 Mariann Chang,1 Shih-Chieh Liu,1 Gembu Abe,1 and Kinya G. Ota1*

Background: Highly divergent morphology among the different goldfish strains (Carassius auratus) may make it a suitable model for investigating how artificial selection has altered developmental mechanisms. Here we describe the embryological development of the common goldfish (the single fin Wakin), which retains the ancestral morphology of this species. Results: We divided goldfish embryonic development into seven periods consisting of 34 stages, using previously reported developmental indices of zebrafish and goldfish. Although several differences were identified in terms of their yolk size, epiboly process, pigmentation patterns, and development rate, our results indicate that the embryonic features of these two teleost species are highly similar in their overall morphology from the zygote to hatching stage. Conclusions: These results provide an opportunity for further study of the evolutionary relationship between domestication and development, through applying well-established zebrafish molecular biological resources to goldfish embryos. Developmental Dynamics 242:1262–1283, 2013. © 2013 Wiley Periodicals, Inc.

Key words: domestication; normal developmental stages; model organisms

Key findings:
- This study provides the first reliable descriptions of normal embryonic stages of wild-type goldfish.
- The embryonic features of goldfish and zebrafish are almost directly comparable.
- Goldfish embryos provide a novel model for the investigation of the evolutionary relationship between domestication and development.

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INTRODUCTION

In recent years, the genomic background of divergent morphological features have been intensively studied in domesticated dogs and pigeons, the classic model organisms for the study of evolutionary biology and artificial selection (Lindblad-Toh et al., 2005; Wayne and Ostrander, 2007; Akey et al., 2010; Stringham et al., 2012; Shapiro et al., 2013). Such investigations have contributed significantly to our understanding of how genes, loci, and alleles were selected for by breeders and fanciers during artificial selection of morphological features (Wayne and Ostrander, 2007; VonHoldt et al., 2010; Akey et al., 2010; Shapiro et al., 2013), while simultaneously prompting researchers to ask how the evolution of developmental mechanisms and artificial selection are related (Morey, 1994; Trut et al., 2009; see also Gilbert, 2010). To address this question, it is important to identify additional model organisms with the following properties: established morphologically divergent strains, as seen for dogs and pigeons (Lindblad-Toh et al., 2005; Wayne and Ostrander,
The goldfish (Carassius auratus) is a well-known domesticated species (Fig. 1). According to analyses of Chinese archives, breeding of this teleost fish as an ornamental animal dates back around 1,000 years ago (Chen, 1925, 1956; Smartt, 2001).

During its domestication, several strains have been isolated and genetically fixed in the goldfish population by breeders and fanciers (Matsui and Axelrod, 1991; Smartt, 2001; Komiyama et al., 2009). Although there is no standardized classification method (Matsui and Axelrod, 1991; Smartt, 2001), one textbook categorized modern goldfish strains into 16 groups, based on the trunk shape, eye morphology, coloration, and the number and length of the fins (see Smartt, 2001; Fig. 1).

These morphological varieties of goldfish strains support the conceptual understanding of divergent morphological features, originally proposed by 19th century biologists (Darwin, 1868; Bateson, 1894). Based on its historical background and morphological features, goldfish may be a suitable model organism for the study of artificial selection, along with domesticated dogs and pigeons (Lindblad-Toh et al., 2005; Wayne and Ostrander, 2007; Akey et al., 2010; VonHoldt et al., 2010; Stringham et al., 2012; Shapiro et al., 2013). Moreover, because goldfish are commercially available and a large number of eggs can be obtained from a single female (Fig. 1C,D), goldfish embryos have been used for studies of developmental biology (Yamaha et al., 1998, 1999, 2001, 2002, 2003; Mizuno et al., 1997, 1999; Otani et al., 2002; Tanaka et al., 2004).

These reports indicate that goldfish have the above mentioned properties required for the investigation of evolutionary change of developmental mechanisms by artificial selection. In addition, goldfish are phylogenetically closely related to zebrafish (Saitoh et al., 2003, 2006; Komiyama et al., 2009). Indeed, certain molecular developmental studies of goldfish embryos used zebrafish techniques, which enabled investigation of germ cell lineage and retinal development (Passini et al., 1997; Otani et al., 2002; Chen et al., 2009). These reports suggest that the developmental processes of these organisms may be comparable, enabling molecular developmental studies in goldfish with reference to existent zebrafish data and tools, including mutants, morphants, and gene expression patterns, recorded in “The Zebrafish Model Organism Database” (Bradford et al., 2011). However, our knowledge remains insufficient to compare zebrafish and goldfish embryos, on account of a lack of reliable developmental descriptions of wild-type goldfish, the morphology of which resembles the ancestral state (Matsui, 1934; Smartt, 2001).

Previous publications have reported on the developmental processes of goldfish (Watase, 1887; Khan, 1929; Battle, 1940; Harvey and Hems, 1948; Li et al., 1959; Kajishima, 1960; Sharma and Ungar, 1980; Yamaha et al., 1999; Nagai et al., 2001; Otani et al., 2002; see also Smartt, 2001). Three of these researchers provided detailed descriptions of embryonic development and staging tables (Li et al., 1959; Kajishima, 1960; Yamaha et al., 1999) (Table 1). Two early goldfish staging tables, by Li et al. (1959) and Kajishima (1960), covered different embryonic stages from fertilization to hatching. A comparative staging table between goldfish and zebrafish was subsequently published (Yamaha et al., 1999), but its covering stage is limited to the early embryonic stages (from zygote to mid-gastrulation). Moreover, the identity of the goldfish strains used for the observation of development in these studies are not entirely clear (Li et al., 1959; Kajishima, 1960; Yamaha et al., 1999); it is possible that some of these staging tables contain mixed embryonic descriptions of wild-type and morphologically divergent strains. In fact, there is no specific description of the morphological traits of the goldfish strains used to generate the staging table of Yamaha et al. (1999). Moreover, although Li et al. (1959) and Kajishima (1960) mentioned that several different strains were used for the embryological observations, the strains used for recording the developmental stages were not specified. As such, the previously generated staging tables may be promiscuous, in that they are derived from multiple goldfish strains. Consequently, there is essentially no wild-type goldfish staging table that is comparable with

Fig. 1. Adult goldfish specimens. A: Lateral view of the common goldfish (the single fin Wakin) strain. B: Illustrations of two goldfish strains by Watase (1887). B-1: Ventral view of the bifurcated anal fin of Ryukin strain. B-2: Dorsal view of Ryukin strain. B-3, 4: Ventral and dorsal views of Ranchu strain; the dorsal fin is absent in this strain. B-5: Lateral view of the Ryukin strain. C: A pond with several goldfish in Taiwan. D: Artificially squeezed goldfish eggs on Teflon dishes. Approximately 500 to 1,000 eggs can be obtained from a single female (approximately 10 cm in length). 2007; Akey et al., 2010; VonHoldt et al., 2010; Stringham et al., 2012; Shapiro et al., 2013; and ease of handling and observation of embryos, such as for zebrafish and medaka (Kimmel et al., 1995; Takeda and Shimada, 2010).
### Table 1. Comparison of Published Staging Schemes of Goldfish

| Scheme                        | Zygote Period | Cleavage Period | Blastula Period | Gastrula Period | Segmentation Period | Pharyngula Period | Hatching Period |
|-------------------------------|---------------|-----------------|-----------------|-----------------|---------------------|-------------------|-----------------|
| Kimmel et al., 1995           | 1-cell (0h)   | 2-cell (0.75h)  | 128-cell (2.25h)| 75%-epiboly (8h)| 1-somite (10.3h)   | Prim-5 (24h)      | Long-pec (48h)  |
| Yamaha et al., 1999 (20°C)    | 1-cell (0h)   | 2-cell (1h)     | 256-cell (2.5h) | 50%-epiboly (5.25h)| 5-somite (11.6h) | Prim-15 (30h)    | Hatching (100 h)|
| Kajishima 1960 (21±1°C)       | Fertilized egg (30min) | 2-cell stage (1 h) | 512-cell (2.75h) | Germ-ring (5.6h) | 14-somite (16h) | Melanophores first appear on embryo (50h) | Pec-fin (60h) |
| Li et al., 1959 (25°C)         | 1-cell (0.73h) | 2-cell (0.9h)   | Early high blastula (2.64h) | Embryonic Shield (15h) | Optic vesicle develops (26h) | Heat beat/25 somite (25.50h) | Protruding-mouth (72h) |

#### Developmental Dynamics
the normal developmental stages of zebrafish (Kimmel et al., 1995).

Therefore, we describe here the developmental processes of the common goldfish (the single tail Wakin, which has highly similar morphology to the wild-type; Fig. 1A) (Matsui and Axelrod, 1991; Smartt, 2001) from fertilization to hatching, referencing the zebrafish staging table. Our present observations indicate that the embryos of zebrafish and goldfish are highly similar in terms of embryonic morphology, suggesting that mutant goldfish and zebrafish can be compared by their phenotypes. Such similarity also suggests that the established techniques and knowledge of zebrafish molecular developmental biology may be directly applicable to goldfish embryos, throughout development.

RESULTS

General Description

Male and female goldfish with no morphological mutations (generally referred to as common goldfish) were purchased from an aquarium shop in Taiwan (Fig. 1A) (see Experimental Procedures). Fertilized eggs were obtained by artificial fertilization, spread onto plastic dishes, and incubated at 24 °C. To confirm that embryos derived from the common goldfish parents retained their phenotype, the morphological features of more than 50 hatched embryos of each cross were examined under light microscopy.

Developmental stages are summarized in Table 2. These stages are categorized into seven periods (Zygote, Cleavage, Blastula, Gastrula, Segmentation, Pharyngula, and Hatching periods) based on the zebrafish staging table (Kimmel et al., 1995). The goldfish zygote, cleavage, blastula, gastrula, and segmentation periods were relatively easily defined by using the indices of the zebrafish developmental staging system (Kimmel et al., 1995). However, the stage corresponding to the zebrafish pharyngula period was less apparent, as staging based on the position of the lateral line primordia could not be directly applied to goldfish embryos (Kimmel et al., 1995). Thus, we redefined the stages in this period, categorizing them into three stages (see below). Moreover, the high-pec stage was not used as we were unable to define it in goldfish embryos (Table 2).

The relationship between the stages and time after fertilization at 24 °C are also presented in Table 2. We succeeded in collecting a large number of sampling points from the zygote to the segmentation period (208 points in total), to analyze the developmental rate (Fig. 2). In the goldfish embryos, hours post fertilization (hpf) showed a linear correlation with the developmental stages in the cleavage, blastula, and segmentation periods (Fig. 2). In addition, the developmental rate of these goldfish embryonic stages was largely consistent with previous reports (Li et al., 1959; Kajishima, 1960; Yamaha et al., 1999) (Fig. 2). These results suggest that our goldfish staging table is reliable for these three periods (Table 2; Fig. 2). However, we could not obtain accurate data for the gastrula period, due to difficulties in applying the zebrafish staging system (Kimmel et al., 1995) (see below for details). Moreover, in the pharyngula and hatching period, the developmental rate fluctuated dramatically between batches and individual embryos. These fluctuations seem to derive from the condition of female individuals, the timing of egg laying, and handling during the observation process at early embryonic stages. Of particular note, frequent opening and closing of the incubator for embryo observation is highly likely to have resulted in temperature changes in the incubation chamber. In fact, the embryos which were used for observing early stages showed extreme fluctuations in developmental rate at late stages. To avoid, or at least minimize, artifacts from handling, we prepared goldfish embryos for observation of late stages only, and re-sampled a few late stage embryos (31 points; plotted as black circles in Fig. 3). Based on these re-sampled late stage embryos, we estimated an approximate developmental rate, for which the estimated time of hatching is largely consistent with that reported by Li et al., (1959) (Table 2; Fig. 3).

Zygote, Cleavage, and Blastula periods

These periods were previously observed by three authors independently, and the morphological descriptions and developmental rates are generally similar (Li et al., 1959; Kajishima, 1960; Yamaha et al., 1999) (Table 2). Moreover, relatively convincing and reliable descriptions have also been reported (Yamaha and Yamazaki, 1993; Yamaha et al., 1998, 1999, 2001, 2002, 2003; Mizuno et al., 1997, 1999; Nagai et al., 2001; Otani et al., 2002; Tanaka et al., 2004). As such, we only provide brief descriptions of the developmental process, and goldfish-specific features in these embryonic periods, for comparison with earlier reports (Li et al., 1959; Kajishima, 1960; Yamaha et al., 1999) (Figs. 4–6).

One-cell stage

After goldfish eggs are exposed to water, their perivitelline space appears, and their chorion develops strong adhesiveness to the substrate (Fig. 4A). The eggs do not easily detach from the substrate, and eggs will occasionally attach to one another (Fig. 4A). On account of its thickness and adhesiveness, the surface of the goldfish chorion appears white under the microscope, and its transparency is lower than zebrafish eggs, especially at this stage (Fig. 4B).

Cleavage period (2-cell to 64-cell stage)

We defined the cleavage period as consisting of six stages: 2-, 4-, 8-, 16-, 32-, and 64-cell stages (Fig. 5). Cleavage in fertilized goldfish eggs is meroblastic, as in other teleost fishes (Swarup, 1958; Armstrong and Child, 1965; Benzie, 1968; Ballard, 1973; Kimmel et al., 1995; Martinez and Bolker, 2003; Yamamoto, 2004; Hall et al., 2004; Fujimoto et al., 2004; Fujimura and Okada, 2007; Hinaux et al., 2007). The first cleavage of the blastodisc cytoplasm takes around 40 min postfertilization at 24 °C, and forms two equal-sized blastoderms (Figs. 2, 5A). Subsequent cleavages occur every 25 to 30 min during this period (Table 2; Figs. 2, 5B–H). The vertically orientated furrow cleaves the blastodisc up to the 32-cell stage (Fig. 5B–G). The size and shape of the blastoderm cells are almost equal at the 4-cell stage (Fig. 5B). From the 8-cell
stage, the cells gradually diverge in size and shape, based on their location within the blastoderm (Fig. 5D–H). From the 32-cell stage, blastodermal cells begin to form a three-dimensional formation, with vertically elongated shapes (Fig. 5G). After dividing to form the 64-cell stage, the blastoderm has three layers when viewed from the side (Fig. 5H).

**Blastula period (128-cells; 30%-epiboly stage)**

Based on the description of zebrafish embryos in this period (Kimmel et al., 1995), the blastula period of goldfish
embryos was divided into nine stages, as follows: 128-, 256-, 512-, 1k, high, oblong, sphere, dome, and 30%-epiboly stages (Fig. 6). The blastoderm forms a hemisphere, and 128-cell blastomeres are arranged into a three-dimensional high mound of cells. The surface of the blastoderm is smoother than that at previous stages (Fig. 6A). From the 128-cell to the 1k stage, the boundary between the blastodisc and the yolk has similar features, with a clear contour (Fig. 6B–E). To distinguish between these stages, the number of blastodermal cell layers can be used as an index (Table 2) (Fig. 6A–E).

Although this period of goldfish development has been investigated by Yamaha and colleagues, the descriptions of the 1k to the dome stage were inconsistent (Yamaha et al., 1999, 2001; Otani et al., 2002). Previous reports suggest that the embryos of goldfish are different with those of zebrafish in that the yolk of goldfish is larger; as a result, the high to dome stages are not clearly distinguishable in goldfish embryos (Yamaha et al., 1999). However, we were able to distinguish these stages under darkfield microscopy, by focusing on the appearance of the blastoderm region and its boundary with the yolk (Fig. 6E–H). Moreover, histological analysis of blastula period embryos revealed that the yolk syncytial layer is similar between goldfish and zebrafish (Fig. 7) (see below). The rate of development during this period is 25 to 30 minutes (lasting until the dome stage), and embryos enter the 30%-epiboly stage after a duration exceeding 30 min (Fig. 2).

The shape of the boundary between the blastoderm and the yolk becomes smoother at the high stage (Fig. 6E), and is almost unrecognizable by the oblong stage (Fig. 6F). This smooth boundary between the blastoderm and the yolk was confirmed at the histological level (Fig. 7B). As observed in high-stage zebrafish embryos (Kimmel et al., 1995; Rohde and Heisenberg, 2007; Carvalho and Heisenberg, 2010), the yolk syncytial layer forms a thin ring with a smooth outer edge (Fig. 7).

The yolk outline is compressed at the sphere stage, as compared to the oblong stage (Fig. 6F). The overall shape of the embryo at the high and
oblong stages is slightly elongated along the animal–vegetal axis, and by the sphere stage, the outline is a “compressed pear shape” (Fig. 6G); this is contrary to zebrafish embryos at the equivalent stage, which are more spherical (Kimmel et al., 1995).

The prominent yolk, the “dome”, can be observed in goldfish embryos as well as in zebrafish embryos (Fig. 6H). The dome-like structure is more visible in later stage embryos, of around the 30%-epiboly stage (Fig. 6I). In some goldfish embryos, a dome-like structure can be observed at the oblong stage. However, the goldfish dome and oblong stages can still be distinguished relatively easily based on the shape of the internal blastoderm margin; the outline of the internal blastoderm margin of the dome stage is more highly curved than that of the oblong stage (white arrowhead and arrow in Fig. 6F,H).

**Gastrula Period**

Based on the indices of zebrafish embryos in this period, goldfish embryos can be categorized into seven stages (50%-epiboly, germ ring, shield, 60%-90%- and 95%-epiboly, and bud stages) (Fig. 8). Although percent-epiboly was used for staging of the zebrafish embryo due to its constancy (Kimmel et al., 1995), it is difficult to apply the same index to goldfish embryos; difficulties arise from the softer and larger yolk of goldfish as compared to zebrafish, and the variance in yolk size between individual goldfish embryos. In fact, although it has been reported that the rate of advance of the blastoderm margin over the yolk shows a linear increase after the 50%-epiboly stage in zebrafish (Kimmel et al., 1995), we did not observe such a linear increase of the blastoderm margin in goldfish embryos (Fig. 2).

To overcome this difficulty, we defined the “percentage of blastopore closure” as an additional index for the staging period; equivalent indices (for example, “yolk plug closure” and “area of blastoderm”) were used in earlier studies of zebrafish, other teleost species, and amphibians (Kimmel et al., 1989; le Pabic et al., 2009; Latimer and Jessen, 2010; Kim et al., 2010). This index refers to the ratio between the maximum diameter of the blastopore and that of the blastoderm-covered yolk region along the DV axis (Fig. 9).

**50%-epiboly**

The thickness of the blastoderm begins to standardize at the 40–45%-epiboly stage (Fig. 8A) and is almost fully uniform by the 50%-epiboly stage (Fig. 8B). At this stage, most embryos have a slightly oval or roundish pear shape (Fig. 8B).
Germ ring

The percent-epiboly is almost the same as that at the 50%-epiboly stage. Under darkfield and oblique light, the germ ring can be observed as a stripe at the most vegetal pole-side of the blastoderm margin along the equatorial plane (Fig. 8C).

Shield

The embryonic shield can be observed as a prominence on the inner-side of the blastoderm margin, at the dorsal side of the embryo (Fig. 8D,E). From the 50%-epiboly stage to the shield stage, involution at the blastoderm margin seems to cause changes in the shape of the uncovered yolk.

60%-epiboly

More than half of the yolk is covered by the blastoderm (Fig. 8F). The percentage of blastopore closure is around 25–30%. The dorsal side of the blastoderm, including the embryonic shield, is obviously thicker than at the previous stage. The percentage of blastopore closure begins to increase (Figs. 8F–G, 9). As the shape of the yolk plug is highly variable during the late gastrula stage (from 60% to 90%-epiboly stages) (Kimmel et al., 1995), it is difficult to apply the percent epiboly index to these stages (Fig. 8G–M). This difficulty is one of the major reasons why we were unable to fit a reliable regression line to the hour post fertilization-stage plot (Fig. 2). However, the percentage of blastopore closure seems to be consistent with the other staging indices (for example, the rudiments of brain and polster) (Fig. 8F–M).

90%-epiboly

The tail bud prominence can be observed at the dorsal aspect of the blastopore after the 85%-epiboly stage (Fig. 8I–M). The yolk uncovered by the blastoderm takes the yolk plug shape, which can be observed in amphibian embryos (Keller et al., 1985; Kim et al., 2010). The neural plate is formed as a thick dorsal epiblast at the animal pole (Fig. 8I–L). Blastopore closure is around 70–80%.

Fig. 6. A–I: Side views of goldfish embryos at the blastula stage. The black arrows indicate the boundary between the blastoderm and the yolk on the surface of 1k, high, oblong, and sphere stage embryos. White arrowheads and arrows indicate the internal margin between the blastoderm and the yolk in sphere, dome, and 30%-epiboly embryos. Scale bar = 0.5 mm.

Fig. 7. Yolk syncytial layer of an early stage goldfish embryo. A: Histological section of a high embryo. B: Magnified view of the boxed area in A. The outer edge of the yolk syncytial layer is indicated by a bracket. bl, blastoderm; y, yolk; ysl, yolk syncytial layer. Scale bars = 100 μm in A, 10 μm in B.
95%-epiboly

Early polster can be recognized in most embryos (Fig. 8M). Blastopore closure is around 85–90%. From the ventral view, the developing neural plate and exposing yolk can be observed (Fig. 8N).

Bud stage

The tail bud and polster are distinctly prominent (Fig. 8O). The blastopore is completely closed (100%-epiboly) in most embryos (Fig. 8O). One to five somites exist even before 100%-epiboly in a few embryos, as reported in the Atlantic cod (*Gadus morhua*) and Nile tilapia (*Oreochromis niloticus*) (Hall et al., 2004; Fujimura and Okada, 2007). In fact, a small amount of yolk is not covered by the blastoderm and is excluded from the embryo even during somite genesis (Fig. 10; also described in the next section).

Segmentation Period

Using somite number, we categorized the segmentation period of goldfish embryos into five stages (6-, 10-, 14-, 18-, and 22-somite stages, Fig. 9). As goldfish embryos at the late gastrula stage possess one to five somites, the gastrula stage and segmentation period overlap slightly (Fig. 2). In fact, somite development begins before the yolk is completely covered by the blastoderm, and the exposed yolk can be recognized at the 8- to 10-somite stage in a few embryos (Fig. 10A,B). With the exception of the exposed yolk, goldfish embryos share similar morphology and developmental processes with zebrafish embryos during the segmentation period (Kimmel et al., 1995). From the 6- to 25-somite stage, the rate of somite appearance is approximately two somites per hour in the goldfish (Fig. 2). This rate is largely consistent with that observed by Li et al. (1959).

6-Somite stage

Developing somites are cubical in shape. Horizontally elongated optic primordia are visible (Fig. 10A). The
rudiment of the brain begins to subdivide.

10-Somite stage
The rudiment of the brain can be recognized as a prominence in the outline of the embryo head region (Fig. 10B). Subdivision of the telencephalon, diencephalon, mesencephalon, and rhombomeric region is evident. Anterior somites demonstrate a rounded rectangular shape. Kupffer’s vesicles are readily visible in a posterior view of 10–12 somite embryos (Fig. 10E).

14-Somite stage
Somites have a V-shape (Fig. 10C). Subdivisions of the mesencephalon,
is slightly constructed and extended posteriorly, and begins to form a yolk extension (asterisks in Fig. 10C,F). Kupffer’s vesicles can be seen in the lateral and posterior view of the protruded tail bud (white arrowhead in Fig. 9C,F).

18-Somite stage

The prominent tail bud is more evident than at earlier stages (Fig. 10C,D).

22-Somite stage

The brain primordia are highly sculptured. Lens primordia and otic vesicles are visible in live embryos (Fig. 10G) and in histological sections of embryos at approximately the equivalent stage (Fig. 11A,B). The floor plate is also visible at the dorsal aspect of the notochord (Fig. 10G). The rudiment of the median fin fold can be observed at the dorsal side of embryos at the somite levels. The yolk extension takes an elongated rod-like shape at approximately this stage (Fig. 10G). The median fin fold can be recognized at the posterior levels of the yolk extension (Abe et al., 2007). The yolk syncytial layer is visible at the yolk extension in histological sections (Fig. 11C). Twitching of the trunk muscles can be observed at this stage. Examinations of horizontal histological sections also reveal that the notochord is not vacuolated at this stage, and certain somite cells attached to the notochord are elongated in an anteroposterior direction (Fig. 11D).

Pharyngula Period

From the late segmentation stage (Fig. 10G), the yolk extension and post-cloacal region begin to elongate posteriorly, and this is followed by pigmentation and budding of the pectoral fin primordia (Fig. 12). The goldfish embryos in this period were staged using the pigmentation patterns in the retina and embryo surface, and the fin budding patterns described by Kajishima (1960). Conversely, zebrafish embryos in this period were designated based on the degree of extension of the lateral line primordium (prim stages) (Kimmel et al., 1995).

The prim staging index is one of the best objective staging indices for thin and highly transparent teleost fish embryos. However, with the exception of the zebrafish staging system (Kimmel et al., 1995), no reported teleost system uses the prim index (Swarup, 1958; Armstrong and Child, 1965; Benzie, 1968; Ballard, 1973; Kimmel et al., 1995; Martinez and Bolker, 2003; Iwamatsu, 2004; Hall et al., 2004; Fujimoto et al., 2004; Fujimura and Okada, 2007; Hinaux et al., 2011). This staging system seems to be suitable for the eggs of flounder, tilapia, and cavefish (Martinez and Bolker, 2003; Fujimura and Okada, 2011). This staging system seems to be suitable for the eggs of flounder, tilapia, and cavefish (Martinez and Bolker, 2003; Fujimura and Okada, 2007; Hinaux et al., 2007), but descriptions of the lateral line primordium are not found in reports of these organisms. This may be due to the difficulty and effort required for optimizing observations of the lateral line primordia, as indicated in Kimmel et al. (1995). We were also unable to apply this staging index and these observation methods to goldfish embryos directly, because of their reduced transparency, whitish color, thicker body, and relatively large yolk; these properties impeded optimization of the conditions for differential interference contrast (DIC) microscopic observation of the lateral line primordia.

In lieu of DIC microscopy, we used a stereomicroscope with oblique light to observe lateral line primordia (see the Experimental Procedures section). We were able to trace lateral line primordia in late pharyngula stage goldfish embryos embedded on agar plates; the lateral embryonic sides of these embryos could be observed with the same depth of focus. However, we could not identify lateral line primordia in early pharyngula stage embryos, because at this stage, the anterior half of the embryonic part is curved, thick, and less transparent (Fig. 12). Thus, for stage identification during this period, we examined “otic vesicle closure” (OVC) (Fig. 13). This index corresponds with “otic vesicle length” (OVL) (Kimmel et al., 1995). OVC increases from approximately 20% at the beginning of the pharyngula period to over 60% at the end, being inversely proportional to OVL.
Based on OVC, we categorized goldfish embryos in the pharyngeal period into three different stages (25% OVC, 35% OVC, and 65% OVC; Table 2; Fig. 13). We also examined histological sections of 60% OVC embryos, to inspect the development of tissues and organs (Fig. 14).

25% OVC

Retina and skin show early pigmentation (Fig. 12A). Otic vesicles form an ellipse (black arrow in Fig. 12A’). Blood flow in the common cardinal vein (duct of Cuvier) (Reib, 1973; Zhong, 2005) and a heartbeat are present. The pectoral fin buds appear (black arrowhead in Fig. 12A’). The curved yolk extension and caudal region straighten by the late segmentation stage (Figs. 10G, 12A). More than 30 somites are visible in total. At the post-cloacal level, 14 somites can be observed (Fig. 12A,A”). Median fin folds appear at the dorsal and ventral side of the caudal area (Fig. 12A”), as in zebrafish (Abe et al., 2007). From this stage, the embryo length (EL) evidently changed. At this stage, EL = 2.8 mm. Based on these characteristics, this stage in goldfish seems to be equivalent with the Prim-5 stage in the zebrafish staging table (Fig. 12A–A”) (Kimmel et al., 1995).

35% OVC

The appearance of goldfish embryos at this stage is also similar to that of zebrafish embryos of the prim-10 to –20 stages (Fig. 12B–C”) (Kimmel et al., 1995). In several individuals, lateral line primordial can be recognized at the yolk extension level. The retina is strongly pigmented at this stage (Fig. 12B,B’). A strong heartbeat can be observed. The pectoral fin bud is more prominent by this stage (arrowhead in Fig. 12B’,C’) and melanocyte distribution is broader (compare Fig. 12B–B” with Fig. 12C–C”). The caudal part of the median fin fold dramatically changes in size during this stage; the caudal fin fold of 60% OVC embryos (Fig. 12C”) is broader than that of 35% OVC embryos (Fig. 12B”). The otic vesicle also changes from an ellipse to a round shape (black arrows in Fig. 10B’,C’).

Histological examination of later stages (equivalent to 60% OVC; Fig. 14) confirmed the presence of the telencephalon, developing nasal pit, liver, and heart ventricle and atrium (Fig. 14A–D) (Stainier and Fishman, 1992; Field et al., 2003). The yolk syncytial layer (from the mid trunk region to the end of the yolk extension) can be readily distinguished.
from the other tissues (Fig. 14E–G). In addition, the yolk syncytial layer is noticeably thicker at the posterior end of the yolk extension than elsewhere (Fig. 14G). Pronephric ducts can be observed at the lateral aspect of the intestinal tract, from the mid trunk to the cloacal level (Fig. 14E–H) (Ng et al., 2005). Median fin folds consist primarily of ectodermal epithelial cells, but also contain some mesenchymal cells (Fig. 14G–I). Myotomes are located at the lateral aspect of the neural tube, notochord, and dorsal aorta (Fig. 14D–I). Horizontal myoseptum can be identified in several transverse sections (Fig. 14F–H), and anteroposteriorly elongated myotomes can be observed in horizontal sections of the trunk region (Fig. 14J). In a single myotome, several multinucleated muscle fibers can be observed (Fig. 14J). Furthermore, the notochord is vacuolated by this stage (Fig. 14C–J). Several mesenchymal cells are distributed at the lateral aspect of the notochord at the head level (Fig. 14C). At this stage, the EL is around 3.4–3.5.

65% OVL

At this stage, the lateral line primordia extend to the trunk region at the 20th to 26th somite (or myosepta) level. There is greater contrast between pigmented and nonpigmented areas (Fig. 12D–Y’). The melanocyte on the yolk ball tends to distribute around the common cardinal vein, and pigmented regions extend from ventral to lateral (Fig. 12D’). A gap in the melanocyte pattern at the caudal level in the ventral aspect is clearly recognizable in most of the embryos (white arrowhead in Fig. 12D’’) (Parichy et al., 2009). The pectoral fin bud takes an anteroposteriorly asymmetric shape; the height to width ratio of the pectoral fin bud is greater than 0.5 (black arrowhead in Fig. 12D’). The anterior face of the pectoral fin bud slopes relatively smoothly (Fig. 12D’). At this stage, EL = 3.6.

Hatching Period

Hatching time cannot be used as an index for staging in goldfish, because it differs greatly between individuals, and is also affected by several environmental factors (for example, temperature, egg density, oxygen concentration, and others) (Kimmel et al., 1995; Yamaha et al., 1999). Goldfish tend to hatch out at around 3 days post fertilization (60 to 72 hpf) at 25 °C (Li et al., 1959; Fig. 3), but their hatching time and stages are not always synchronized among individuals, even for the same batch of embryos (Yamaha et al., 1999).

The goldfish staging system described by Li et al. (1959) follows development of the vein and median fins. On the other hand, staging of zebrafish embryos and larvae involves pigmentation patterns (distribution of melanophores, xanthophores, and iridophores), the shape of the pectoral fin bud, and the head–trunk angle (HTA) (Kimmel et al., 1995). After 48 hpf at 24 °C, goldfish embryos and larvae also show xanthophore pigmentation patterns and evident elongation of the fin bud and fins (Figs. 15, 16), which cannot be observed in the pharyngula period (Fig. 12). By applying the above characteristics and following the zebrafish nomenclature systems (Kimmel et al., 1995), we categorized goldfish embryos and larvae in this period into three stages: long pec, pec-fin, and protruding mouth (Figs. 15–17). We also conducted histological analysis of late-stage larvae in this period (Fig. 18).

Long pec

The dorsal side of the larvae, from the head to anterior half trunk regions, are pale yellow (xanthophores) (Fig. 15A). Melanophores were increased on the dorsal side of the head and the common cardinal vein. The common cardinal vein can be recognized as a black tube in the lateral view (Fig. 15A’). From this stage, the pectoral fin bud is elongated posteriorly, extending the height-to-width ratio to approximately 1.5 (Fig. 16A). The caudal part of the median fin fold is enlarged, and begins to expand in a dorsoventral direction (Fig. 15A). The HTA is approximately 40°, although there is variation between individuals. The angle in goldfish embryos at this stage is narrower than that of the zebrafish equivalent (Kimmel et al., 1995). Several embryos at this stage have already opened their mouth on the ventral aspect of the head (Fig. 15A).

Pec fin

The xanthophore colored area extends to the caudal level (Fig. 15B), and the melanophore-pigmented region of the common cardinal vein extends
ventrally (Fig. 15B). The head of this stage tilts upward slightly more than that at the long pec fin stage; the HTA is approximately 30°. The pectoral fin bud forms a blade-like shape, and has actinotrichia which will develop into the fin ray (Fig. 16B–D). At this stage, an approximate two-fold increase in the size of the apical fold of the pectoral fin was observed, as for zebrafish (Fig. 16C,D) (Yano and Tamura, 2013).

In addition, the mouth opens anterolaterally (Fig. 17B). In the eyes at this stage, six different layers (ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, retinal pigment epithelium) can be observed, as in zebrafish (Fig. 18A) (Gross et al., 2005). At the cranial level, chondrogenesis of parachordal and trabecula cartilage can be observed; at the otic vesicle level, mesenchymal cells at the lateral aspects of the notochord secrete Alcian blue-positive extracellular matrix (Fig. 18B) (Schilling and Kimmel, 1997).

**Protruding mouth**

The dorsal side of the larvae is pigmented by xanthophores (Fig. 15C). The outline of the dorsal side of the
head is almost straight; the HTA is approximately 20°. The position of the heart is evidently different between the former two stages and this stage; the heart moves to the anterior, and is located at the anterior aspect of the yolk at this stage (black arrows in Fig. 15A′, B′, C′). Under oblique transparent light, a cartilaginous structure can be observed in the ventral cranial region in the live larvae (Fig. 15C′).

Horizontal sections of the anterior part of the larvae reveal cartilaginous tissues at the anterior aspect of the otic vesicles, the lateral side of the notochord, the pectoral fins, and the hyoid and branchial arches (Fig. 18C, D) (Kimmel et al., 1995; Schilling and Kimmel, 1997). Mouth openings at this stage are more anterior than at the pec fin stage (Fig. 15B′, C′). The developing swim bladder was observed in transverse sections at the level of the posterior end of the pectoral fin (Fig. 18C) (Ng et al., 2005). The outline of the ventral median fin fold is not continuous; a gap at the cloacal level divides the pre- and post-cloacal fin folds (black arrowhead in Fig. 15C). This gap is not observed at the long-pec or pec fin stage (black arrowheads in Fig. 15A, B). The caudal median fin fold is larger than at earlier stages (Fig. 15C). Alcian blue-positive tissues are observed in all median fin folds (Fig. 18F–K). Well-developed muscle tissues and vertical myosepta are present in horizontal sections at the trunk level (Fig. 18L). Almost all embryos have hatched by this stage.

**DISCUSSION**

In contrast to previous studies that used unspecified goldfish strains (Li et al., 1959; Kajishima, 1960; Otani et al., 2002), we have described the embryonic and larval morphology of the common goldfish strain, which exhibits wild-type goldfish morphology (slender body and short fins) (Figs. 1A, 4–6, 8, 10, 12, 15). In addition, we have described the histology of embryonic and larval tissues at several stages (Figs. 7–18). Our present staging table is the first normal development table of the wild-type goldfish, covering the zygote to hatching stages (Table 2). These results suggest that the embryonic morphology of goldfish and zebrafish are, in general, comparable (Kimmel et al., 1995). However, we also identified several different embryonic features between these two teleost species.

One of the most significant differences between zebrafish and goldfish is the texture of the yolk. The yolk of goldfish embryos is softer and larger than that of zebrafish embryos.
(Figs. 4–6, 8, 10). This goldfish-specific yolk texture caused difficulties in stage identification during the late blastula stage in previous studies (Yamaha et al., 1999; Otani et al., 2002); despite these challenges, we succeeded in categorizing goldfish late blastula stages embryos into high, oblong, sphere, and dome stages (Fig. 8). Inconsistencies between our study and previous studies seem to be related to whether the descriptions were based on dechorionated embryos or not; previous studies primarily used dechorionated embryos (Yamaha et al., 1993, 1998, 1999; Mizuno et al., 1997, 1999; Otani et al., 2002), while we used nondechorionated blastula, gastrula, and early segmentation stage embryos for the embryonic descriptions (Figs. 6–10). Given that yolk shape can be easily changed by its own weight, it is conceivable that stage identification is more difficult using dechorionated goldfish embryos at the late blastula stages.

The most significant specific feature of goldfish yolk is the way its texture and size varies between individual embryos (Fig. 8G–N) (Yamaha et al., 1999). In fact, we were able to identify variations in the epiboly process between individual goldfish embryos (Fig. 8G–N), which were reflected as exposed yolk at the posterior aspect of the tail bud in early segmentation stage embryos (Fig. 10A,B). Thus, although the development of segmentation stage goldfish embryos is similar to that of zebrafish (Fig. 10), it is feasible that variations in the yolk size affect other embryonic features. For example, the relative position of the Kupffer’s vesicles may differ between large yolk and small yolk goldfish embryos, on the basis of posterior body development in zebrafish (Kanki and Ho, 1997). Moreover, while it is still unclear how variations in yolk size affect the formation of the yolk syncytial layer, our histological observations suggest that the yolk syncytial layer pattern in high-stage embryos is similar between zebrafish and goldfish (Fig. 7) (Kimmel et al., 1995; Carvalho and Heisenberg, 2010). Further investigation will be required to confirm whether variations in yolk size affect subsequent embryonic morphology, and developmental rate.

From the pharyngula to hatching periods, the following three characteristics are clearly different between goldfish and zebrafish: hatching gland, melanocyte pigmentation patterns, and mouth opening (Figs. 12, 15). We were unable to identify the distributing location of the hatching gland through observations of live goldfish using stereomicroscopy (Fig. 12). This seems to be due to the broad and indistinct distribution patterns of the hatching gland in goldfish embryos (Ouji, 1955; Ishida, 1985). Examination of melanocyte distribution patterns revealed differences in the “dorsal stripe,” “ventral stripe,” and “anterior horn” in goldfish and zebrafish (Fig. 15) (these terms are defined in Kimmel et al., 1995). Melanophores begin to cluster, and the dorsal stripe subsequently appears at the dorsal aspect of pharyngula stage embryos in zebrafish (Kimmel et al., 1995), while such a distinctive
melanophore stripe was not observed in goldfish embryos (Fig. 15). On the other hand, the ventral stripe at the caudal level became visible earlier in goldfish than in zebrafish (Fig. 12A–D, B00, C00, D00). In late pharyngula goldfish embryos, melanophores distribute at the ventral aspect of the notochord at the caudal level, forming a stripe associated with the dorsal aorta and vein (Fig. 12C, D00, 12D); this is not observed in their zebrafish equivalents (Kimmel et al., 1995). In addition, the timing of anterior horn appearance seems to be slightly later in goldfish than in zebrafish embryos (Fig. 15). In zebrafish embryos, the anterior horn can be recognized as an extension of the ventral stripe at the dorsal aspect of the yolk, and this melanocyte stripe emerges at the levels of the eyes in prim-25 stage zebrafish embryos. However, the goldfish anterior horn begins to form at the equivalent stage (late pharyngula stage; 65% OVC), and finally appears at the early hatching stage after the appearance of the xanthophore (Figs. 12D, D00, 15A, A0). Although there is variation between individuals, most of the populations exhibit the above tendencies.

In contrast to the development of pigmentation patterns, the timing of mouth opening is earlier in goldfish than in zebrafish (Fig. 17). A small open mouth was observed in pec-fin stage zebrafish embryos (Kimmel et al., 1995), while goldfish larvae at the equivalent stage exhibit a significantly larger opened mouth (Fig. 17B). These cranial embryonic morphological differences seem to reflect the adult morphology and feeding behavior. Although no significant difference was detected in the larval cranial structure in our study, these above-mentioned embryonic and larval morphologies should be carefully compared between goldfish and zebrafish (Schilling and Kimmel, 1997).

We suggest it is also worth considering the similarities (or otherwise) between the embryonic development of wild-type and mutant goldfish strains. Of the three previous reports of goldfish developmental stages (Li et al., 1959; Kajishima, 1960; Yamaha et al., 1999), only Kajishima (1960) mentioned differences in embryonic morphology between the common goldfish and mutant strains. This
Developmental Dynamics anticipated that the development of hindered its genetic analysis, it is Hammerschmidt et al., 1996; Asano et al., 1996; van Eeden et al., 1996a,b; Mullins et al., 1994, 1996; Neuhauss gently morphologies (Koh, 1931, 1932; previously isolated zebrafish mutants with prompted others to compare previ-
sion (Holland and Garcia-Fernández, 1996; Amores et al., 1998; Hoegg et al., 2004; Jaillon et al., 2004; Meyer and Van de Peer, 2005; Sémon and Wolfe, 2007; Sato and Nishida, 2010).

Comparative studies of goldfish and other teleost species may increase our understanding of genome duplication, as well as other evolutionary processes. For example, comparison of the morphology of goldfish and spontaneous mutants of medaka may determine whether or not similar mutant phenotypes arose from independent mutations of the same developmental mechanisms in different lineages. Such comparisons may be facilitated by the large number of spontaneous medaka mutant strains available (Takeda and Shimada, 2010). Furthermore, it may be possible to contrast the effects of artificial and natural selection by comparing goldfish strains to teleost species with highly divergent morphology arising from natural selection (such as stickleback, cichlid, and cavefish; Yamamoto and Jeffery, 2000; Peichel et al., 2001; Jeffery, 2001; Colosimo et al., 2004, 2005; Yamamoto et al., 2004, 2009; Kimmel et al., 2005; Albertson et al., 2005; Cresko et al., 2007; Kitano et al., 2008; Chan et al., 2010; Shapiro et al., 2004). We hope that our developmental stages will facilitate studies into the molecular developmental biology of goldfish, thereby providing insights into the evolutionary relationship between selection and developmental mechanisms.

EXPERIMENTAL PROCEDURES

Fish Samples

Adult individuals of the common goldfish strain (the single tail “Wakin”) were purchased from the aquarium fish agency in Taiwan on March 2011. This strain is Japanese in origin. To avoid confusion derived from differences in goldfish nomenclature systems used by breeders and researchers (Matsui, 1934; Smartt, 2001), goldfish individuals with a slender body and a short single fin are referred to as the common goldfish (Fig. 1) in accordance with Smartt (2001); this definition includes Funa-wo Wakin and the single fin Wakin, as defined by Matsui (1934) and Matsui and Axelrod (1991). In total, 50 male and 50 female goldfish were separated and maintained in different cages in the same aquarium tanks (4000 liters in water volume). The aquarium tanks were kept under natural temperature conditions (14–24°C).

Artificial Fertilization

On April 2011, sperm were taken from several males and preserved in Modified Kurokura’s extender 2 solution (Magyary et al., 1996) at 4°C, and eggs were squeezed out from mature females into Teflon coating dishes. Artificial fertilization was performed using dry methods. The fertilized eggs were spread onto 9-cm Petri dishes and 3-L aquarium tanks, containing tap water (22–24°C), as appropriate. Serial ID numbers were provided for male and female individuals used for artificial fertilization. One or two 9-cm Petri dishes containing 50–100 individuals were incubated until hatching, and the morphology and survival rate of the hatched larvae were recorded.

Microscopic Observation and Photography

For the observation of early stage embryos, fertilized eggs were spread onto 9-cm Petri dishes at a low density (less than 200 eggs/Petri dish). Eggs attached tightly to the surface of the Petri dish were surveyed. The position of each egg was marked to enable repeated monitoring of the same embryo. For the observation of late embryos, the egg chorion was removed by fine forceps or enzyme treatment modification zebrafish protocols, as appropriate (Westerfield, 2000). The dechorionated embryos
and hatched larvae were anesthetized using MS222 (tricaine methanesulfonate). The anesthetized samples were immediately placed on a plastic dish containing low melting temperature agarose, and positioned at the bottom of the plastic dish. After the agarose set, the dishes were inverted and samples were observed from the flat bottom. Images were acquired using an Olympus SZX16 stereomicroscope with an Olympus E-5 digital camera (Olympus, Tokyo, Japan) under dark-field and oblique light conditions. Anesthetized embryos and larvae were immersed in 4% paraformaldehyde in phosphate buffered saline, rinsed in buffer, and observed under the microscope.

Measurement of Developmental Rate

To find appropriate and suitable experimental conditions for estimation of the developmental rate of goldfish, we first incubated fertilized eggs under five different temperatures (12, 16, 20, 24, and 28°C) by using a gradient temperature incubator TG-5 (FIRSTEC, Taipei, Taiwan). Microscopic observations were conducted at room temperature (22–24°C). We adopted 24°C as the incubation temperature, on account of the stable results obtained, and relative ease of maintaining constant temperature during observation. To estimate developmental timing, fertilized eggs were incubated at 24°C, and early (cleavage and gastrula) and late (segmentation to hatching period) stage embryos were observed at 30 min and 1 hr intervals, respectively. Approximately 10–12 embryos were collected and numbered from one batch of fertilized eggs. Embryo stages were visually inspected and recorded. To increase the accuracy of the developmental time table, observation and recording were repeated in four batches, for a total of 217 points: 2012-04-17 (145 points), 2012-04-18 (19 points), 2012-04-18-02 (16 points), and 2012-04-25-01 (37 points). To observe pharyngula and hatching period embryos without exposing embryos repeatedly to room temperature, a separate batch of fertilized eggs were spread onto over 10 plastic Petri dishes. The separately spread embryos were incubated without further manipulation until harvesting. At 24 to 72 hours post fertilization (hpf), embryos and larvae were harvested and anesthetized with MS 222, and then fixed in 4% paraformaldehyde for 1 to 2 hr. Stages were determined, and used to estimate the approximate developmental timing at 24°C (Table 2).

Histology

For detailed observation of tissues and organs, embryos and larvae were harvested and anesthetized with MS 222, fixed in Bouin’s fixative, embedded in paraffin, and sectioned at 5 μm. Sliced embryonic sections were stained with hematoxylin and eosin. Larval sections were stained with Alcian blue for visualizing cartilaginous tissues at the hatching stages.

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