RESEARCH ARTICLE

In Vitro Culture of Embryos of the Guppy, *Poecilia reticulata*

Ulrike Martyn,† Detlef Weigel, and Christine Dreyer*

The rich variation in adult color patterns of male guppies (*Poecilia reticulata*) has attracted the attention of geneticists and ecologists for almost a century. Studies on their embryogenesis, however, have been limited by the fact that guppies are live bearers. We have observed normal development after explantation of guppy embryos from the ovary of pregnant females at various times after last parturition, and found that development of each batch of eggs is slightly asynchronous, most likely due to asynchronous fertilization. We have cultured explanted embryos in vitro and continuously observed their development. Although embryos explanted a few days after fertilization survived up to 4 weeks in culture, they did not complete their development. In contrast, embryos explanted at late stages of gestation could hatch and develop to fertile adults. Our embryo culture techniques overcome some of the limitations of using livebearers as study objects, and they allow continuous observation of and accessibility to live embryos at all stages. *Developmental Dynamics 235:617–622, 2006.* © 2005 Wiley-Liss, Inc.

Key words: *Poecilia reticulata*; embryo culture; asynchronous development

Accepted 11 November 2005

INTRODUCTION

The highly variable male color morphs of the guppy (*Poecilia reticulata*) have been studied for more than 80 years, and the guppy was one of the first vertebrates in which sex-linked inheritance of color loci was demonstrated (Winge, 1927). More recently, the guppy has become a model organism for studying behavioral traits such as courtship and mate choice (Evans et al., 2003; Magurran and Henderson, 2003), as well as for understanding ecogeographic adaptation (Endler, 1991, 1995; Reznick, 1997).

Guppies are live-bearers, who retain their fertilized eggs within the follicle throughout gestation (Turner, 1940; Lambert, 1970). The synchronously growing diplotene oocytes store nutrients in oil droplets and yolk, before their maturation and fertilization. This developmental program is termed lecithotrophic, and it contrasts matrotrophic programs where nutrients are provided by means of the maternal circulation throughout gestation. Matrotrophy and lecithotrophy can contribute simultaneously to embryonic nutrition in other species of poeciliid fish (Reznick et al., 2002). In guppies, the interface between the embryonic yolk portal system and the maternal follicle allows for efficient gas exchange and waste disposal, while maternal food provisioning does not seem to be required after fertilization (Turner, 1940; Thibault and Schultz, 1978).

Unfortunately, studying the early development of live bearers is more complicated than that of oviparous species, due to the inaccessibility of developing embryos for experimental manipulation. Therefore, despite a wealth of classic genetic studies on the male color polymorphism found in guppies, knowledge of both the ontogeny and the molecular mechanisms underlying this polymorphism is virtually nonexistent.

In vertebrates, trunk neural crest cells (NCCs), which become precursors of pigment cells, most likely start their migratory pathways at neurula and tail bud stages (Jacobson, 1991).

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Grant sponsor: the Max Planck Society.
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DOI 10.1002/dvdy.20664
Published online 21 December 2005 in Wiley InterScience (www.interscience.wiley.com).

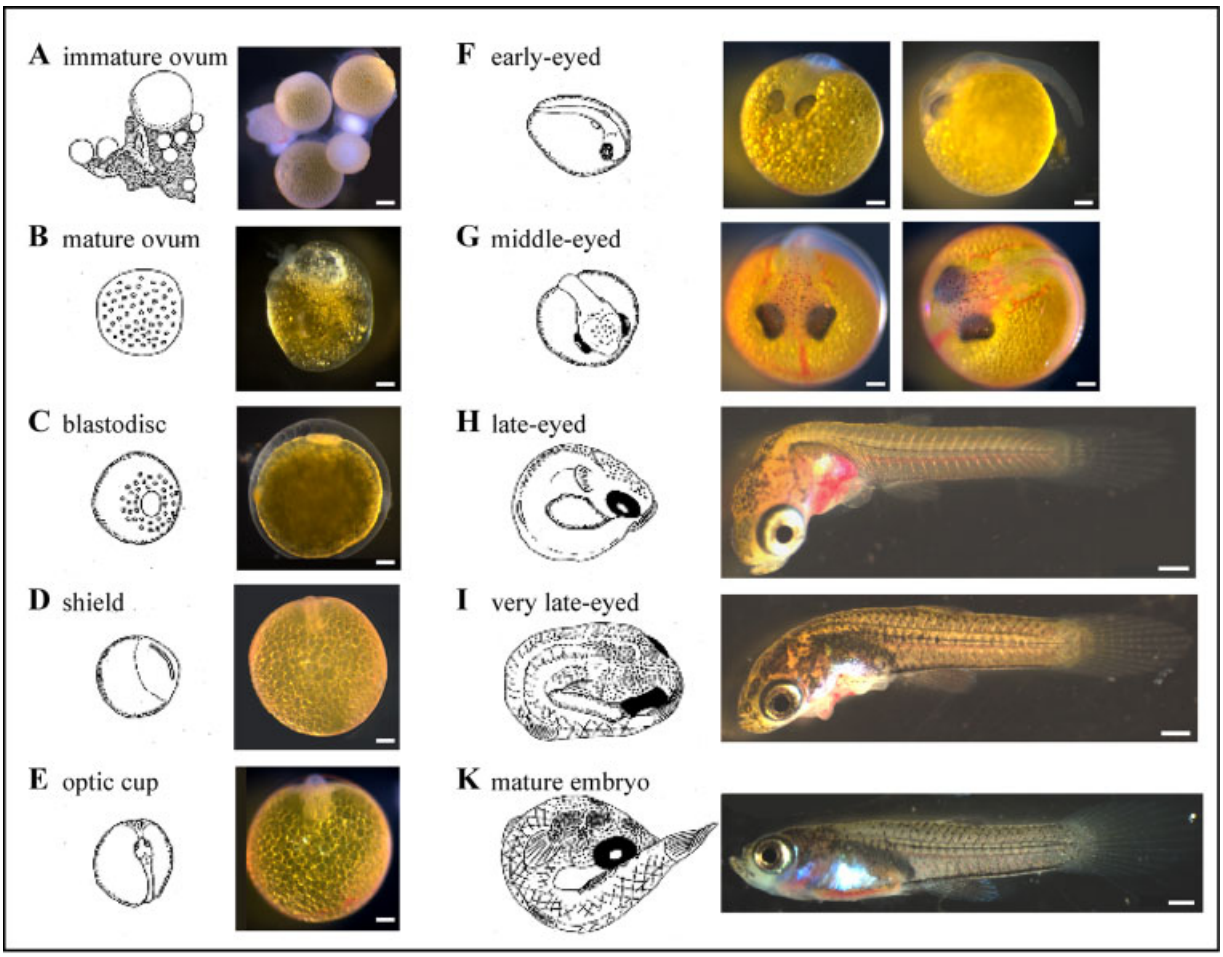
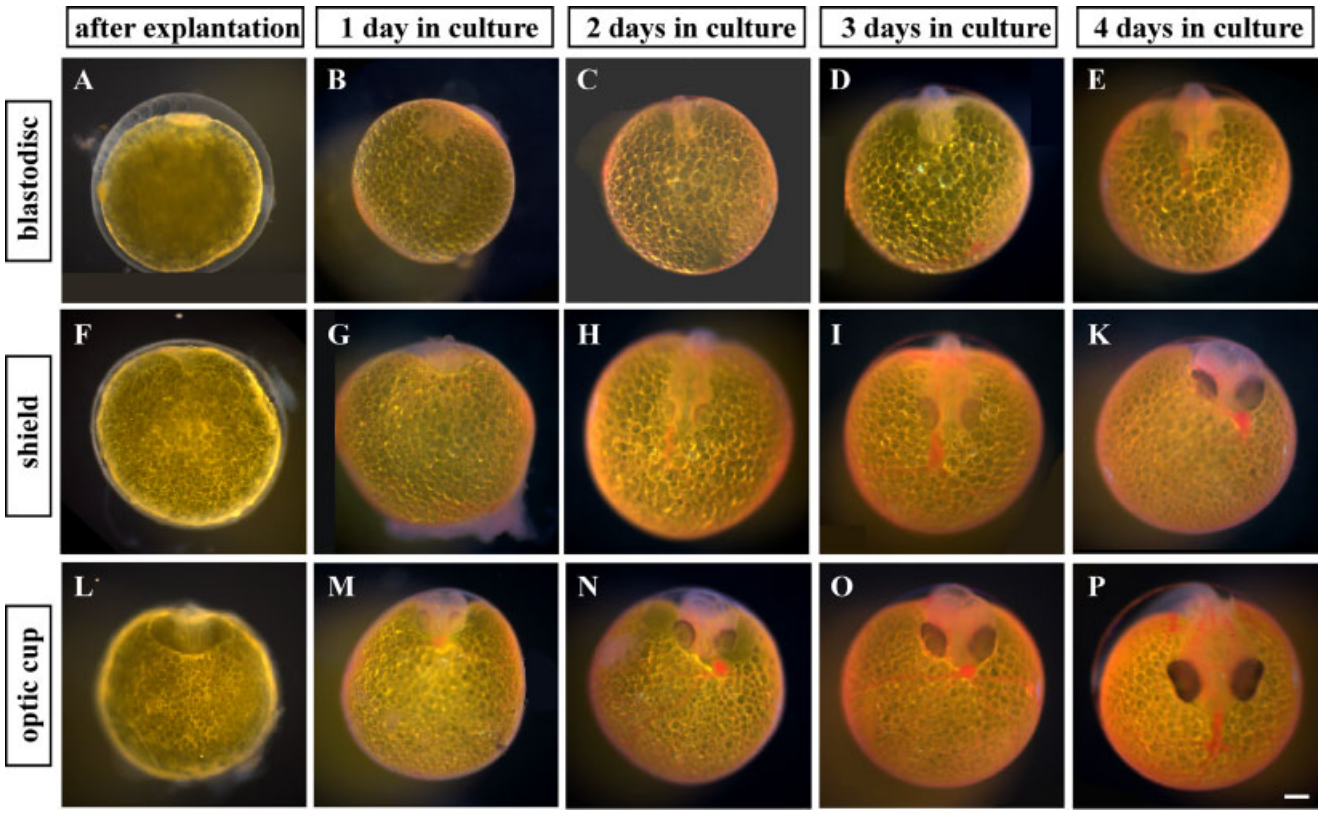

A immature ovum
B mature ovum
C blastodisc
D shield
E optic cup
F early-eyed
G middle-eyed
H late-eyed
I very late-eyed
K mature embryo

Fig. 1.


after explantation
1 day in culture
2 days in culture
3 days in culture
4 days in culture
blastodisc
shield
optic cup
A
B
C
D
E
F
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H
I
K
L
M
N
O
P

Fig. 2.

A detailed description of the formation of the embryonic pigment pattern in guppies, therefore, requires observations on embryos during early gestation. As a first step toward studying NCC differentiation and migration in embryos, we have established techniques for in vitro embryo culture, which allow the continuous observation of early development.

RESULTS AND DISCUSSION

Survey of Guppy Embryonic Development

Haynes (1995) has described the embryonic development of Poeciliids using specific developmental stages derived from *Gambusia* (Haynes, 1995). Previously, Tavolga (1949) has published a more detailed description of platyfish (*Platypoecilus*) and swordtail (*Xiphophorus hellerii*) embryogenesis.

Here, we briefly describe guppy embryonic development (Fig. 1). We withdrew embryos from pregnant guppy females and preliminarily staged them according to Haynes (1995), based on external criteria. Figure 1A shows an ovary fragment containing translucent previtellogenic, as well as opaque early vitellogenic oocytes. During stage 3 (Fig. 1B), the mature ovum contains oil droplets that are evenly distributed over the yolk surface. After fertilization, the oil droplets coalesce underneath the embryo proper, which forms a blastodisc (stage 4; Fig. 1C). During stage 5 (Fig. 1D), the primitive streak is seen on top of the shield, and this appears smaller in guppies relative to *Gambusia* (drawing at the left). At the optic cup stage (stage 6; Fig. 1E), the eyes remain unpigmented, blood vessels of the portal system are visible in the lower part of the yolk sac, and the heart becomes perceptible as a pulsating tube. The early-eyed embryo (stage 7; Fig. 1F) is pigmented light brown, first at the dorsoanterior margin and subsequently throughout the retina. Melanophores are undetectable on the head or trunk at this stage. During the early-eyed period, pigmentation of the eye, including the choroid, gradually increases, the pectoral fin buds emerge, and somitic as well as nonsomitic muscles differentiate.

Classification of the subsequent middle-eyed, late-eyed, and very late-eyed stages (stages 8–10; Fig. 1G–I) is based on further differentiation of the eyes, which parallels an increase in the skin pigmentation of the head and trunk. The middle-eyed embryo has fully pigmented eyes (Fig. 1G), whereas, in the late-eyed embryo, the choroid covers most of the retina, and rays of the dorsal, ventral, and caudal fins have differentiated. The myotome consists of approximately 30 somites (Fig. 1H).

During the middle-eyed stage, melanophores first appear above the midbrain and subsequently behind the midbrain–hindbrain boundary (stage 8; Fig. 1G). In the subsequent late-eyed stage, a line of dark pigment cells appears that demarcates the horizontal midline, and the mostly stellate black pigment cells on the head increase in number, size, and density, and become more dendritic in their appearance (stage 9; Fig. 1H). Different regions of the embryo are often covered by melanophores of different shapes (Goodrich et al., 1944; Tavolga, 1949). Furthermore, paired dorsal and ventral pigment lines emerge, followed by a second, weaker horizontal line of melanophores above and parallel to the midline. Trunk pigmentation remains inconspicuous until the very late-eyed stage, when a rhombic melanophore pattern emerges between the dorsal and upper horizontal melanophore lines, and this pattern gradually develops from the dorsoanterior to the ventroposterior region of the trunk (stage 10; Fig. 1I). Melanophores and iridophores accumulate over the lateral patches that cover the yolk. Between the very late-eyed and mature embryo stages, iridophores also differentiate on the choroid of the eyes (stage 12; Fig. 1I,K).

Simultaneous with the disappearance of the yolk, the almost rectangular flexure between the head and the trunk is gradually straightened (Fig. 1H–K). The mature embryo has resorbed completely its yolk and retracted the yolk sac. Finally, the follicle ruptures before birth (stage 11; Fig. 1K).

Asynchronous Fertilization Suggested by Asynchronous Development

Guppies are considered to show synchronous development of a single batch of embryos during each reproductive cycle, in contrast to species with superfetation, such as *Heterandria formosa*, whereby smaller broods at significantly different stages can coexist (Constantz, 1989). Nevertheless individuals of the same brood could be fertilized asynchronously, as described by Thibault and Schultz (1978), who estimated a time span of up to 6 days within the same brood. In agreement with this, we found that specimens explanted from the same mother at 8 days after last parturition (dap) fell into three groups: blastodisc, shield, and optic cup embryos (Fig. 2A,F,L).

To assess whether the specimens at earlier stages were arrested in development, we cultured representatives of each stage separately in vitro, which demonstrated that all specimens proceeded in development. Embryos cultured in vitro from the blastodisc stage (Fig. 2, upper row A–E) reached the optic cup stage after 3 days in culture. When the culture started at the shield stage (Fig. 2, middle row F–K), embryos showed the first signs of eye pigment 2 days later.

Fig. 1. Normal embryonic development of *P. reticulata*. Explanted immature oocytes (A), egg (B), or embryos (C–K) are shown on the right of each panel, together with drawings of oocytes and embryos of *Gambusia affinis* at the left (from Haynes, 1995). **A:** Immature oocytes (opaque) and early yolked oocytes. **B:** Mature egg. **C:** Blastodisc pointing to the top of the panel. **D:** Embryonic shield with primitive streak on top of yolk. **E:** Optic cup. **F:** Early-eyed embryo (top). **G:** Middle-eyed embryo. **H:** Late-eyed embryo. **I:** Very late-eyed embryos. **K:** Mature hatched embryo. In F and G, anterior dorsal and lateral aspects are shown. In H and I, the follicle wall, vitelline envelope, and yolk have been removed to facilitate a lateral view. Scale bars = 500 μm in A–G, 1 mm in H–K, respectively.

Fig. 2. Asynchronous early development. A,F,L: Embryos explanted from a single female approximately 1 week after last parturition were at the blastodisc (A), shield (primitive streak; F), or optic cup stage (L). **A–L:** Each horizontal row documents the developmental progress of an embryo explanted at the blastodisc (A–E), shield (F–K), or optic cup stage (L) during the 4 subsequent days. A,F,L: Fixed embryos; all other panels are live embryos. Scale bar = 500 μm.

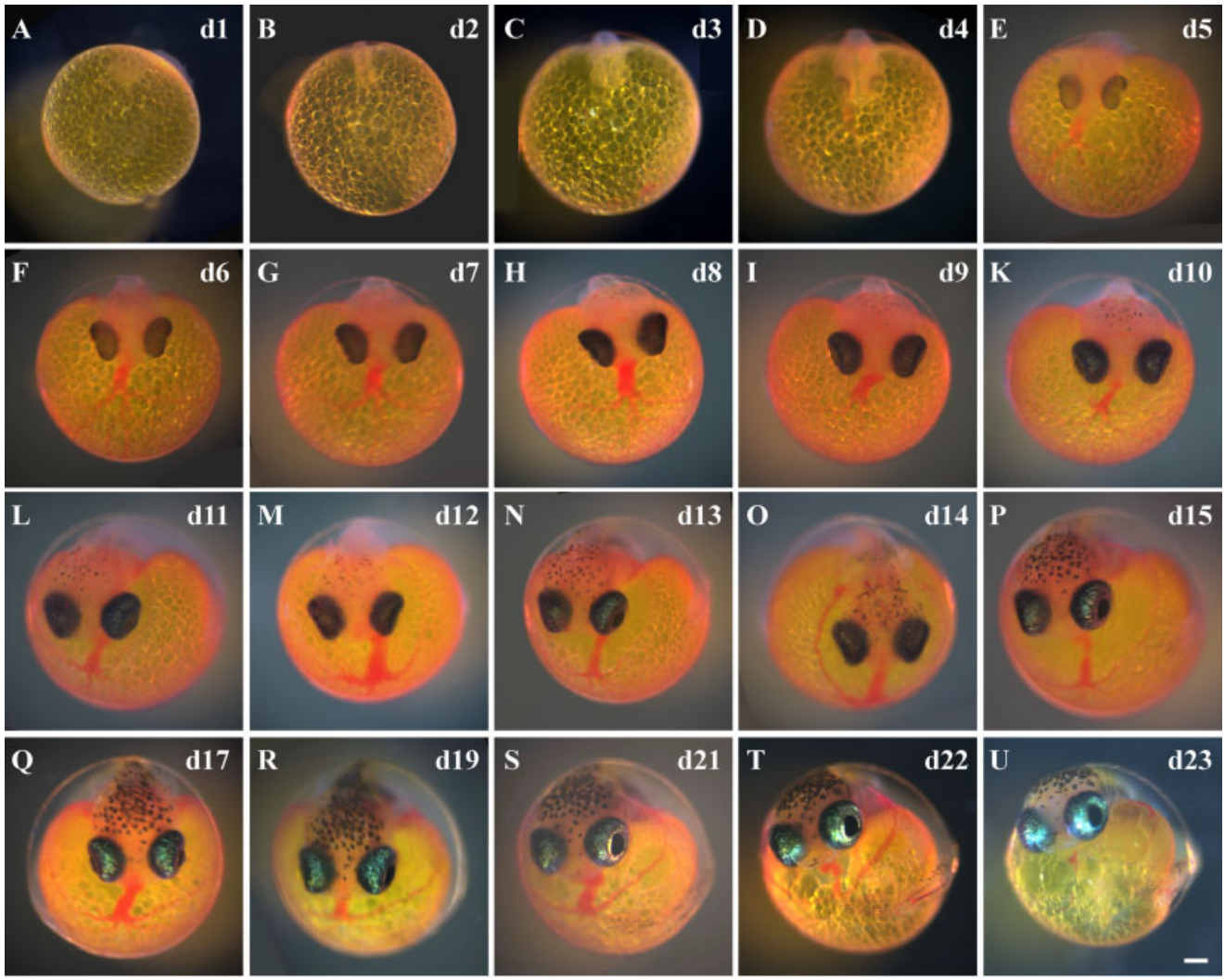

Fig. 3. Embryonic development in vitro. **A–U:** Embryos explanted at blastodisc stage (A) were cultured as described in the Experimental Procedures section and photographed on each subsequent day (B–U). See text for details. Scale bar = 500 μm.

Embryos cultured from the optic cup stage onward (Fig. 2, lower row, L–P) developed fin buds and some melanophores on top of the head 4 days later (P). These kinetics are very similar to those of early platyfish and swordtail development in vivo (Tavolga, 1949). By comparing our observations with the kinetics of early development in platyfish and swordfish, which have only slightly longer interbrood intervals (Tavolga, 1949), we suggest that fertilization of a batch of eggs occurs 3 to 8 days after last parturition in the guppy. Explanting embryos at advanced developmental stages confirmed that embryos remain asynchronous throughout their development, as seen by the differences in their body length, pigmentation, and yolk consumption (data not shown). We repeatedly have observed asynchronous growth of yolking oocytes in the guppy and therefore suggest that consecutive maturation of individual eggs could be a plausible reason for asynchronous fertilization, in analogy to observations in the platyfish (Tavolga, 1949).

Long-Term In Vitro Culture of Guppy Embryos

The lecithotrophic strategy of development (Reznick et al., 2002) entails the provisioning of embryos with resources from the maternal yolk deposit rather than from a placenta. It allows the extracorporal culture of guppy embryos. After showing that guppy embryos can continue development in culture (Fig. 2), we investigated whether we could grow explanted embryos in culture for the entire period of embryonic development, and whether they would hatch in vitro. Figure 3 shows the development of embryos explanted at blastodisc stage for 23 subsequent days. The embryonic streak stage was reached on day 2 after explanting (Fig. 3B), and the optic cup stage, with perceptible heartbeat, on day 3 (Fig. 3C). The yolk portal system became more pronounced between days 3 and 6 (Fig. 3C–F).

Eye pigmentation started at day 4 (Fig. 3D). Pigment cells on the head first appeared between days 7 and 8 (Fig. 3G,H), and they gradually increased in number and size throughout the experiment (Fig. 3G–R). After 13 days in culture, iridophores were

mainly seen on the choroid of the eyes (Fig. 3N–U), and after 14 days they were also observed over the midbrain (Fig. 3P–U). On day 21 to 23, melanophores could be seen in the tail and on the tail fin, which is wrapped around the yolk (Fig. 3S–U). However, after day 19 in culture, the yolk became very heterogeneous in structure and it appeared to contain some large, coalesced oil droplets. A considerable amount of yolk remained after 23 days of culture (Fig. 3U), when the embryo was still alive but whose development was delayed relative to embryos developing in the follicle (see below). In culture, the development of pigmentation, somites, and appendages was symmetrical, but heart development revealed obvious differences between individuals. In general, cultured embryos moved their eyes, tails, and pectoral fins within the vitelline envelope. Although normal morphology often became compromised after more than 2 weeks in culture, several embryos remained alive for a period of up to 29 days in culture, corresponding to 37 dap, although these individuals failed to completely resorb the yolk and retract the yolk sac. Although embryos developed significantly slower in vitro than in the ovary, it was impossible to exactly quantify the developmental delay in culture, due to the obvious spread in developmental stage within each batch of eggs (data not shown).

Why embryos could not be cultured for the entire period of their embryonic development remains unclear. Varying the concentration of fetal calf serum in the medium and mimicking a diurnal cycle of dim light did not significantly improve development. That some individuals survived in vitro for longer than the normal gestational period suggests that survival in vitro is not the limiting factor. Although guppy embryos are considered as completely lecithotrophic (Thibault and Schultz, 1978; Reznick et al., 2002), we cannot exclude the possibility that specific factors required for normal development are delivered by means of the maternal circulation and, therefore, that these would be lacking in vitro.

Haas-Andela (1976) succeeded in rearing *Xiphophorus* embryos in vitro, starting at neurulation, and obtained fertile fish. Similar to guppies, *Xiphophorus* embryos develop more slowly in vitro than in the follicle, and often fail to retract the yolk sac. Of interest, both survival and yolk sac retraction could be significantly improved by addition of fish-conditioned water to the culture medium and by exposure of fully developed embryos to conditioned water by means of a dialysis bag (Haas-Andela, 1976). These findings suggest that retraction of the yolk sac may be the most critical step of in vitro culture in both species and that it may require as yet unidentified low molecular weight factors from the mother, which could be contained in or substituted for by the aquarium water.

Whereas guppy embryos cultured from 8 dap onward (blastodisc to beginning early-eyed stage) showed arrested embryonic development, some embryos explanted during late gestation (17 to 21 dap) consumed and internalized the remaining yolk, and became mature and fertile fish (data not shown). When embryos were explanted during the last third of gestation, individuals whose vitelline envelope was removed survived for longer on average. Moreover, swimming embryos occasionally became fertile adults, indicating that positive effects of active movement on gas exchange and waste disposal may exist, indicating that these parameters may need future optimization.

Our success in culturing guppy embryos in vitro demonstrates that some of the drawbacks of live bearing fish as objects of early developmental studies can be overcome. Extension of in vitro culture for the entire gestation period would allow for experimental procedures not normally possible in live bearers, including lineage tracing and genetic manipulations, such as RNA interference or application of morpholino oligonucleotides.

EXPERIMENTAL PROCEDURES

Fish Culture and Observation

Guppies were maintained at 25°C in a 12-hr light and dark cycle. The following strains were used: guppies from a pet shop (Konstanz), Endler's guppies from a pet shop (Peter Erkes, Wernau), Oropuche-2, Blue, and Quare-6. Embryos were staged according to Haynes (1995) and observed on a Leica MZFLIII dissecting microscope, connected to a Zeiss Axiocam Hrc. Images were acquired using Axiovision software. Iridophores and xanthophores and their precursors were visualized using incident normal and ultraviolet light (Odenthal et al., 1996). Iridophores and their precursors were detected irrespective of the wavelength of the incident ultraviolet, whereas green autofluorescence is characteristic of xanthophore precursors.

Explantation and In Vitro Culture

Adult female guppies were anaesthetized in 0.05% MABA (m-aminobenzoic acid ethylester methane sulfate) and decapitated. Using scissors and fine forceps, the peritoneum was opened from the abdomen, and then ovaries were explanted under a dissecting microscope and transferred into 90% phosphate buffered saline (PBS). The ovarian tissue was removed to isolate individual embryos. For in vitro culture, up to 15 embryos were incubated in 8 ml of sterile embryo medium (L-15 [Leibovitz] medium [GIBCO, cat. no. 11415-049], supplemented with 15% fetal bovine serum (Biochrom AG, Berlin, Germany), 70 units/ml penicillin, and 70 g/ml streptomycin) in a dark incubator at 25°C. Alternatively, up to three staged embryos were reared in 2 ml of medium in a 24-well tissue culture plate. Remaining follicles detached from the embryos within the first 24 hr of culture and were removed. Embryos were inspected daily and medium was changed twice a week. To compare the developmental kinetics between embryos growing in the ovary and in tissue culture medium, embryos were explanted at 12, 16, and 20 dap and compared with embryos that were explanted at 8, 12, or 16 dap and then cultured for another 12, 8, or 4 days. These experiments were done using different strains that all developed significantly slower in vitro. The yolk sac was removed with forceps from some of the embryos explanted at

late stages of gestation (17–20 dap), to allow movement in culture.

ACKNOWLEDGMENTS

We thank Stephen Russell, Sureshkumar Balasubramanian, and two referees for helpful comments on the manuscript; Gertrud Scheer and Huelya Wicher for editorial help; Sureshkumar Balasubramanian, Margarete Hoffmann, Yasushi Kobayashi, and Dieter Labusch for helpful discussions and help with animal care; and David Reznick, and Axel Meyer for guppy strains. D.W. is a director of the Max Planck Institute. This paper is dedicated to the memory of our dear colleague Ulrike Martyn.

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