AACL BIOFLUX
Aquaculture, Aquarium, Conservation & Legislation
International Journal of the Bioflux Society

New contributions to knowledge of embryonic malformations in guppies

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Abstract. This paper is an amendment added to our older studies which were previously disseminated worldwide through various ways. We hereby withdraw the conclusion that teratogenic effects observed in guppy, in 2006, were due to chloramphenicol. The paper also describes some cases of Siamese twins, their ontogenetic development and highlights their similarities with the cases seen in humans.
Key words: conclusion correction, cloramphenicol, teratogenic effect, guppy Siamese twins.

Introduction. Guppy, as other poeciliids (Bourne & Watson 2009; Amiri-Moghaddam et al 2010), is one of the best model organisms used in studies of sex-determination (Lindholm & Breden 2002; Petrescu-Mag 2008, 2009; Păsărin 2010), classical (Winge 1922, 1927, 1934) and molecular genetics (Dreyer et al 2006), color patterns (Shaddock 2009), evolution of the color patterns (Hughes et al 1999), speciation and evolution (Alexander & Breden 2004), behavioral ecology (Houde 1997), spinal deformities (Gorman & Breden 2006), anatomical (Jalabert & Billard 1969), physiological (Wenkatesh et al 1992abc), embryological (Martyn et al 2006) and pollution studies (Petrescu-Mag et al 2010; Petrescu-Mag & Petrescu-Mag 2010). The current study reviews the opinion presented in our older papers (Petrescu-Mag et al 2008; submitted and accepted for publication in 2006; prepared for printing in Acta Ichtiologica Romanica 2008; not printed yet and therefore withdrawn) and substantially completes them. Also, in the current study, we bring new contributions to knowledge of embryonic malformations in guppies.

Our Previous Study. *Poecilia reticulata* is an ovoviviparous fish species. They retain their fertilized eggs within the follicle throughout gestation (Turner 1940; Lambert 1970). Even Poeciliids include many matrotrophic species (Reznick et al 2002), guppy embryogenesis has a lecithotrophic developmental program. In other words, the synchronously growing diplotene oocytes store nutrients in oil droplets and yolk before their maturation and fertilization, while maternal food provisioning does not seem to be required after fertilization. However, the interface between the embryonic yolk portal system and the maternal follicle allows for gas exchange and waste disposal (Turner 1940; Thibault & Schultz 1978; Martyn et al 2006).

Beside resistance to natural chemical factors, vertebrates developed also physiological and cellular mechanisms to counteract the effect of synthetic chemicals (Trifa et al 2009). However, even much studied, the toxic effect of such chemicals is far from being known.

Chloramphenicol is one of the most frequent used antibiotic in guppy hatcheries (Aftabbudin et al 2009) and also one of the potential teratogenic antibiotics (Fritz & Hess 1971; Guntakatta et al 1984; Flint & Orton 1984). That is why, in 2005 (after several consecutive cases of accidental teratogenic effects), we tested the effect of chloramphenicol on guppy embryos, administered at different oral doses to gravid females (69444, 41667 and 0 mg antibiotic kg food^{-1}), and also discussed and classified the observed results.

Three lots of guppyfemales (six individuals/lot) were oral feed with 69444, 41667, and 0 mg kg food^{-1} chloramphenicol (Arena, Romania) for 30 days during a whole gestation. The mixture of antibiotic with food was made in dry form (powder). Fish were kept in aquariums at 25±1ºC under a 14/10 h light/dark cycle. They were fed with a commercial diet Tetramin® bioactive formula (Tetra Gmbh, Germany), with 48% protein, three times a day *adlibitum*. Water quality parameters (O_2, pH, ammonia, nitrite and nitrate) were monitored every 10 days during the experiment. Water was well aerated and heated using a compressor and thermostatically controlled heaters (Aquael, Poland). All water tanks were cleaned daily by siphoning the excreta and uneaten food. Females were anaesthetized in 0.05% m-aminobenzoic acid ethylester methane sulfate, decapitated and ventral sectioned, 3-6 days before parturition. Embryos were examined and photographed using a binocular mycroscope (Optika) and a digital camera (Canon). The experiment was housed by USAMV Cluj (Cluj-Napoca, Romania, European Union).

After fish dissection and examination, we found five out of 199 embryos as being teratogenically affected by various types of malformations, all of them observed in the first lot. The percentages of affected individuals were 11.63% (five out of 43) in the first lot and 00.00% in both the second and control lots. Further microscopic examination revealed a strong similarity between human cases of Siamese twins and cases observed in fish (see Figs 1-5).

Classification of the five observed malformations was done after the anatomical type of deformity, as follows: bicephalic embryo malformation, ventro-ventral median (abdominal) fusion, ventrolatero-ventrolateral distal (caudal) fusion. The bicephalic embryo malformation (Figure 1) is similar to human two headed embryo malformation, or *dicephalus* (Figure 4). The ventrolatero-ventrolateral distal (caudal) fusion (Figure 3) is somehow similar to human *parapagus* (Figure 5, b). The ventro-ventral median fusion (Figure 2) is similar to human *omphalopagus* (Figure 5, a). It has a particular case where one of the two monovitelin twins is partly resorbed or atrophied and the other one is normal developed (*heteropagus* twins). This particular case was observed also in humans and other vertebrates.

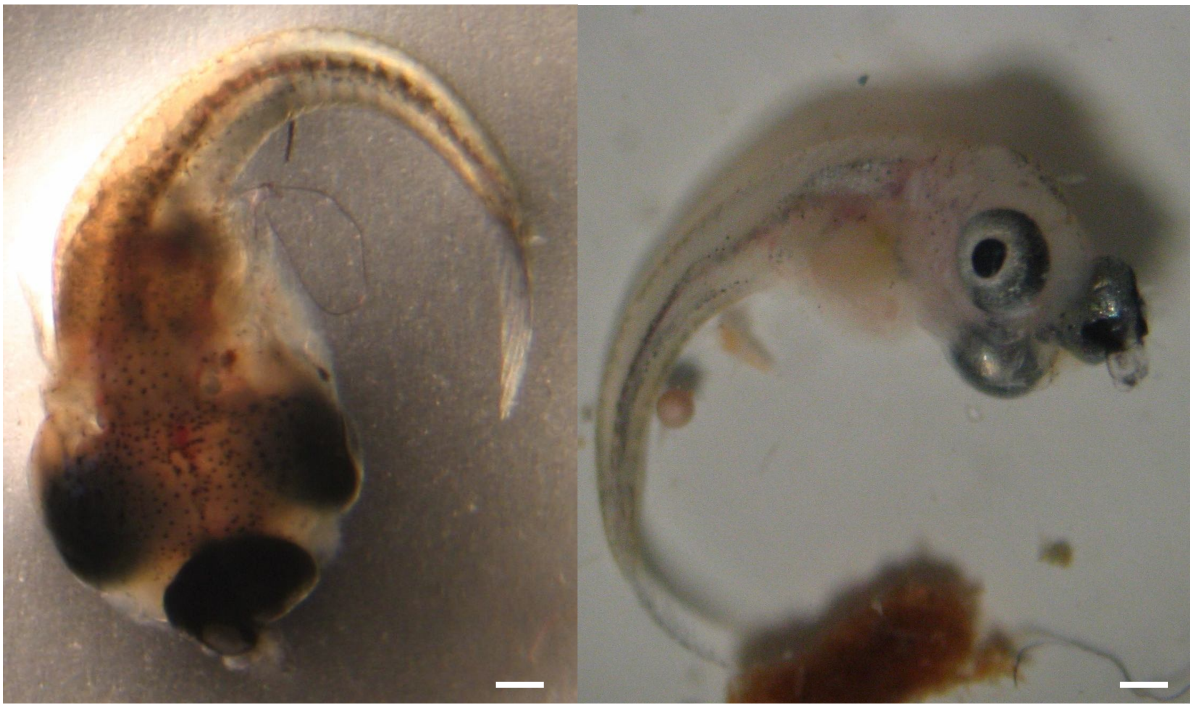


Figure 1. Bicephalic embryo malformation observed in the guppy (scale bar = 500 µm).

In humans, the incidence of conjoined twins is reported to be in the range of one in 50000 to one in 100000 (Spitz & Kiely 2003) but, as 60% from them are stillborn or die shortly after birth, the real incidence is about one in 200000 live births (Spitz & Kiely 2002). Surgical separation of Siamese twins is sometimes feasible and guppy can be a very good model for this kind of practice.

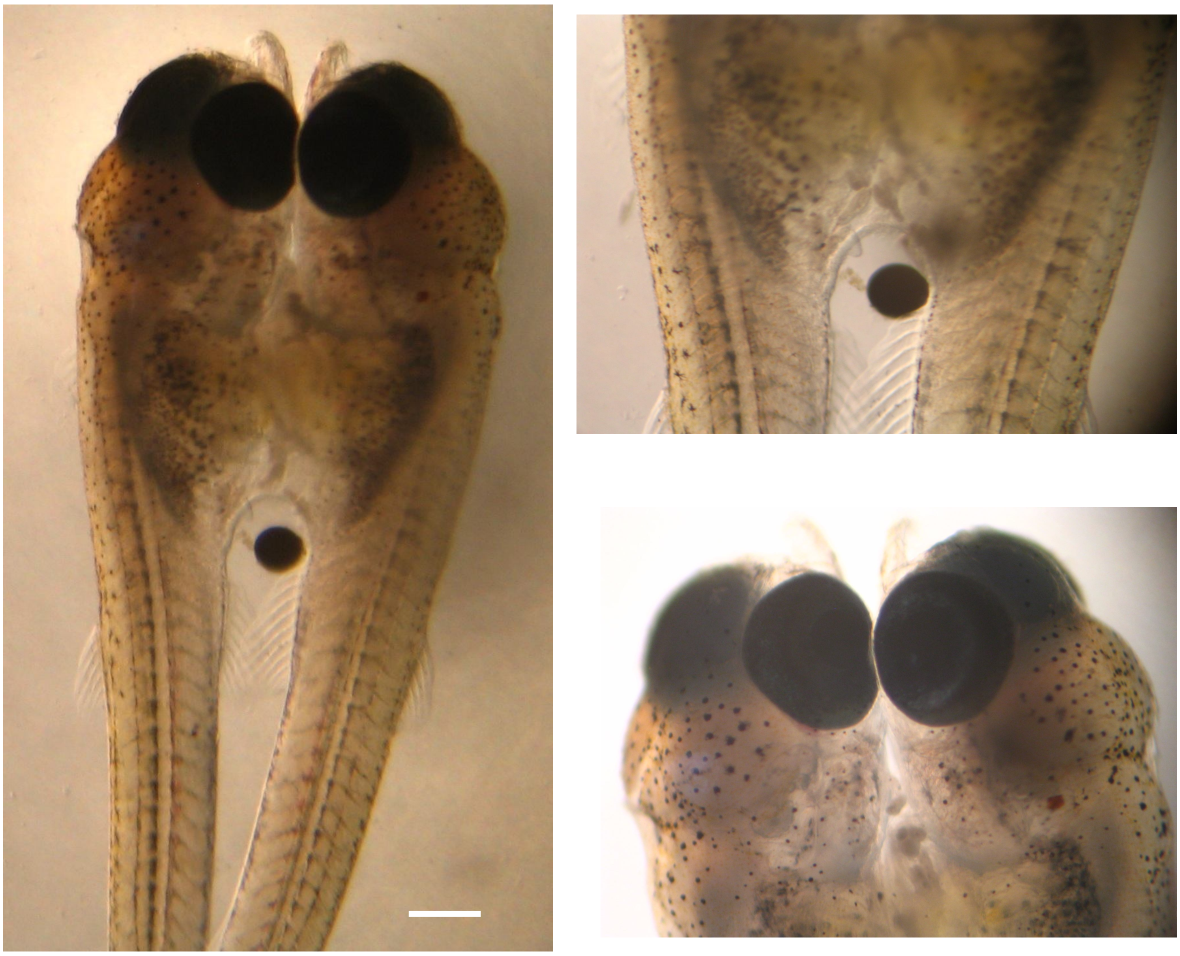


Figure 2. A case of ventro-ventral median fusion, observed in the guppy (scale bar = 500 µm).

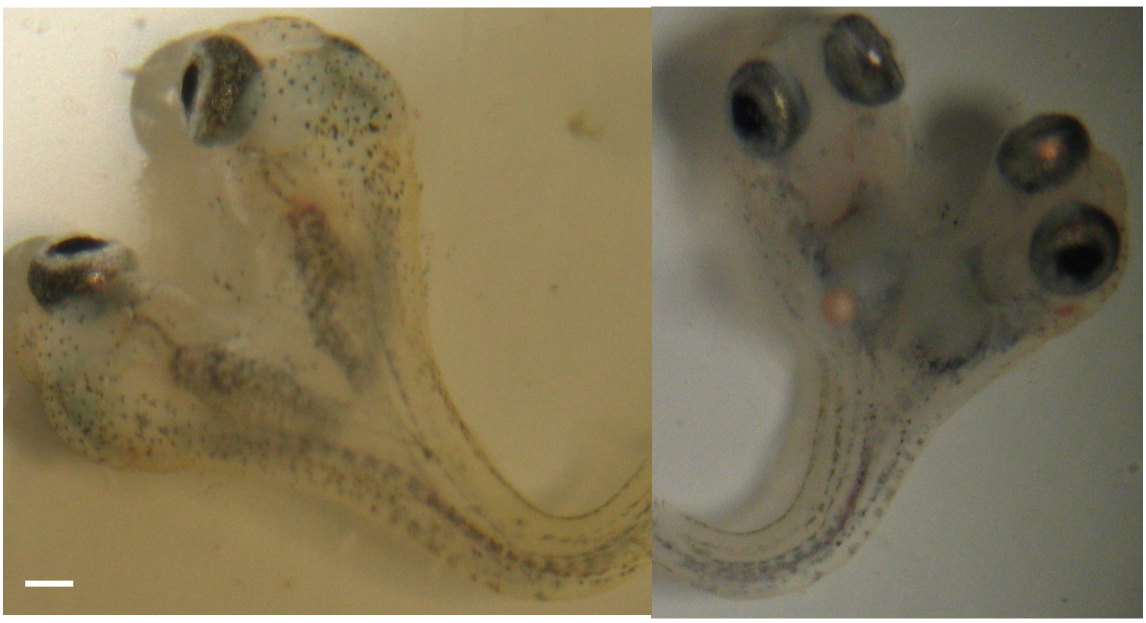


Figure 3. Ventrolatero-ventrolateral distal fusion observed in *P. reticulata* (scale bar = 500 µm).

Explanation for conjoined twins occurring is so called embrionary axial bifurcation. There are many physical, chemical, and informational factors affecting cellular signal in the early embryonary stages. These factors disturb the natural pattern of cell division, and finally cellular differentiation. According to our results, one such a chemical factor affecting normal development of embryos seemed to be chloramphenicol. At least, that was our final conclusion in the 2007 paper. Our recent opinion is much different.

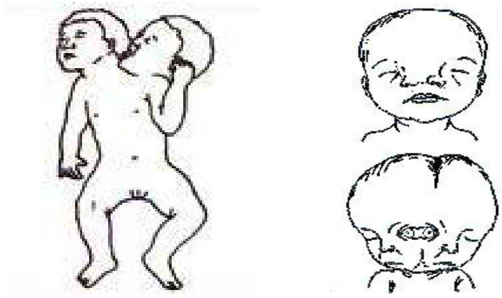


Figure 4. *Bicephalus* in humans, with different degrees of head duplication: dicephalic *parapagus* (a) and diprosopic *parapagus* (b) (www3.telus.net/tyee/multiples/4conjoined.html)

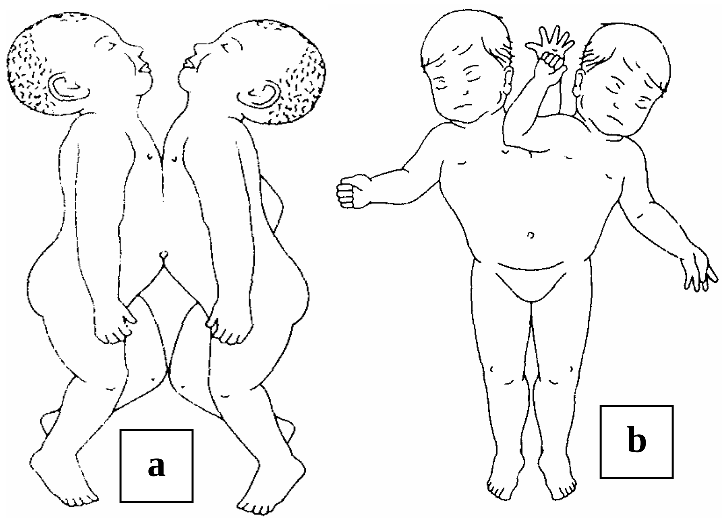


Figure 5. Common human forms of conjoined twins (Andrews et al 2006): *omphalopagus* (a) and *parapagus* (b).

Recent Observation: Wrong Conclusion. Two years later, the same team (in a rather newer formula) observed new cases of conjoined twins out of experimental treatments. We decided to repeat the initial experiment. The second and third experimental repetitions (in the same conditions, this time housed by SC Bioflux SRL, using the same guppy population from USAMV Cluj biobasis) revealed there is no correlation between cloramphenicol concentration in food and frequency of teratogenic effect in population. A number of two cases were recorded again (one for each repetition), but this time only in the control lot, while treated lots developed normally in percentages of 100%. We concluded that the high frequency of teratogenic effects is specific to the study population; in other words, the malformation has a genetic basis to appear. Thus, the results of Petrescu-Mag et al (2008) were due to hazard and the small sample size in that study can explain the results and wrong conclusion. The teratogenic effect observed in guppy (in 2006) was not due to the dose of cloramphenicol administered.

Description of One Case of Living Heteropagus. Sometimes, heteropagus starts as a classic case of omphalopagus (see Figure 6). One new born case of siamese twins (at day of life) evolved step by step (in five days) from omphalopagus to heteropagus (see Figs 7-8). We present here short morphological, anatomical and functional descriptions of this case (Tables 1-3; see also Figs 6-8).

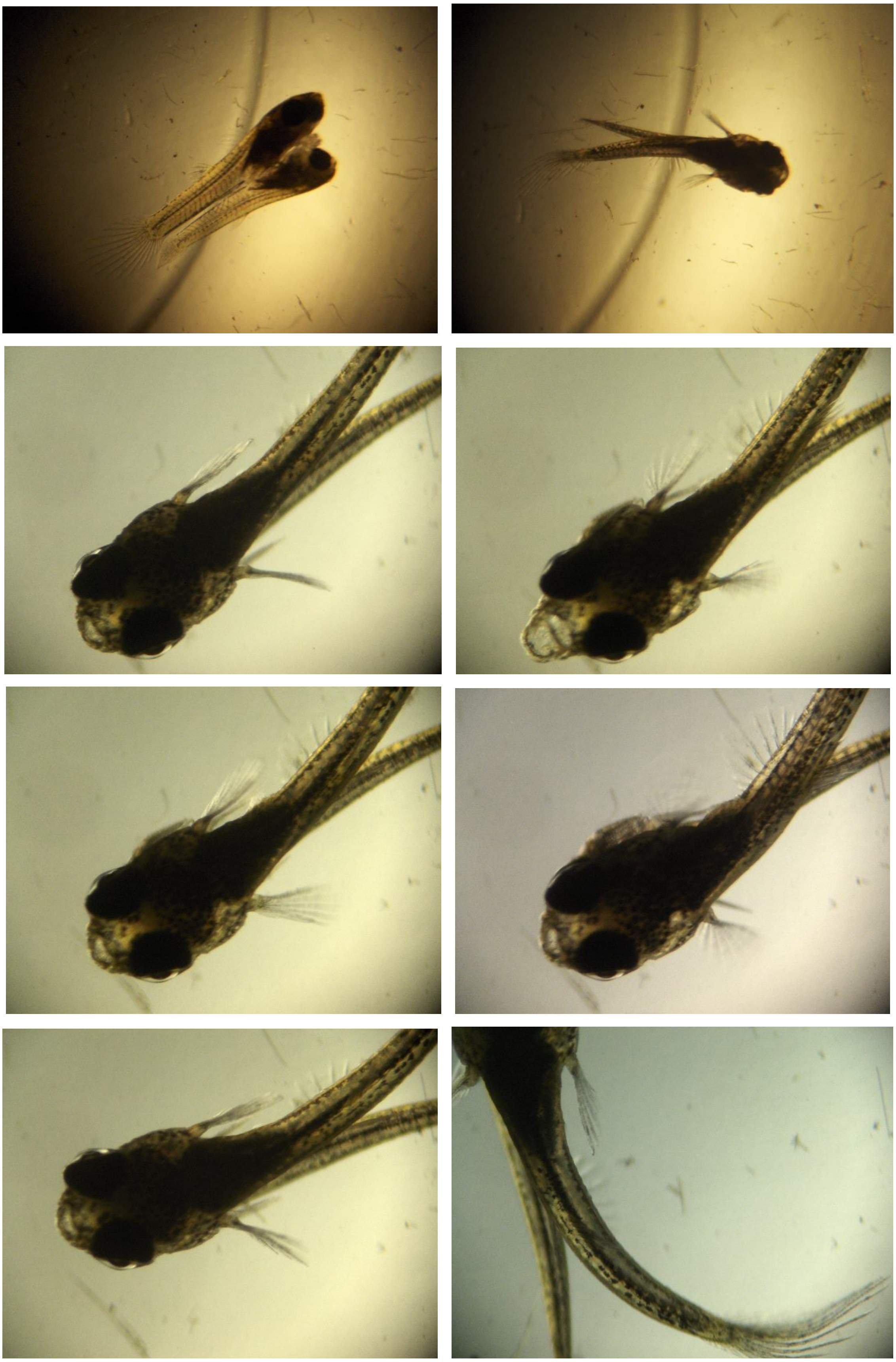


Fig. 6. Siamese guppy (born alive) at day of life.

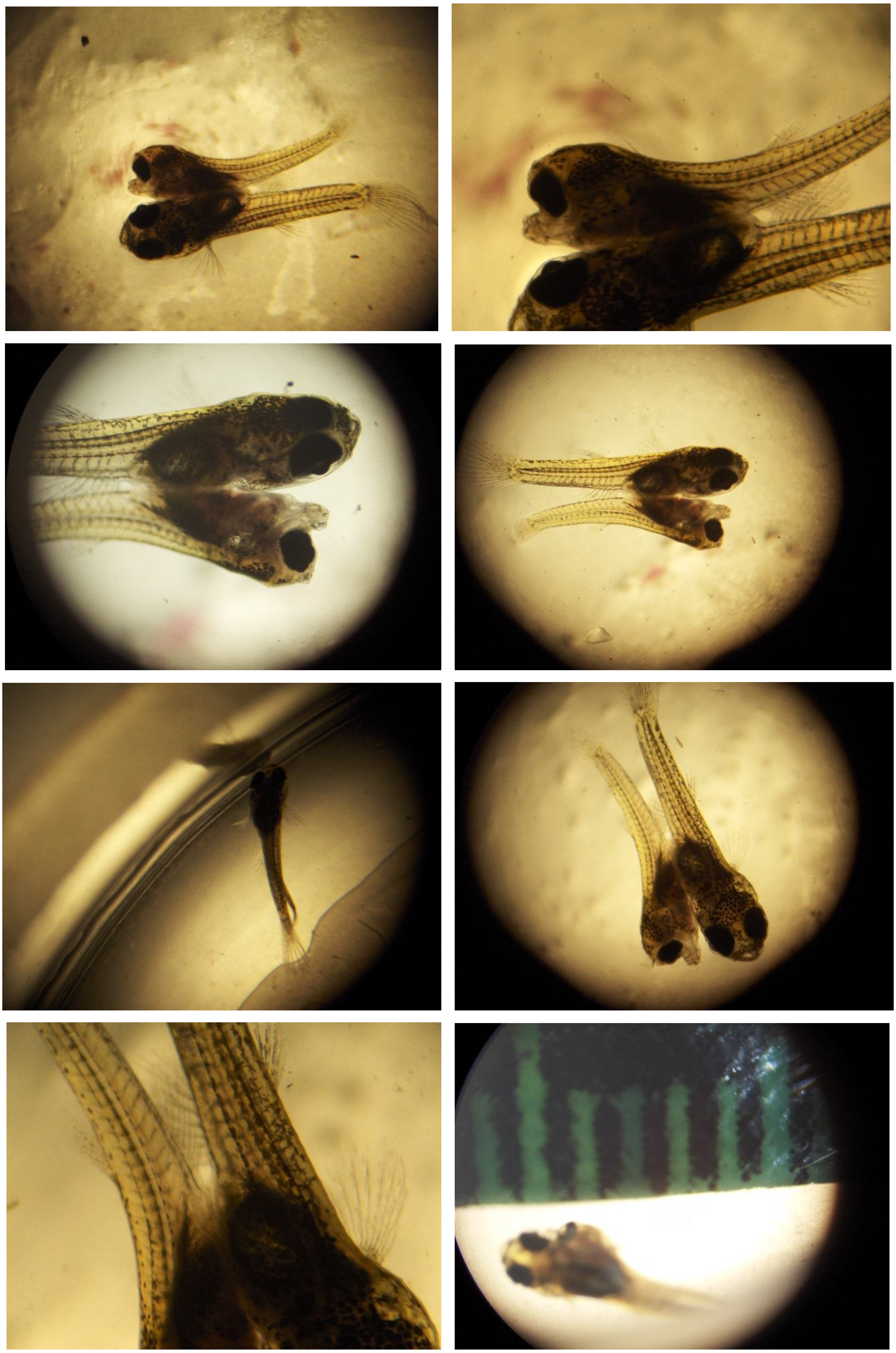


Fig. 7. The same pair of Siamese guppy, three days after birth.

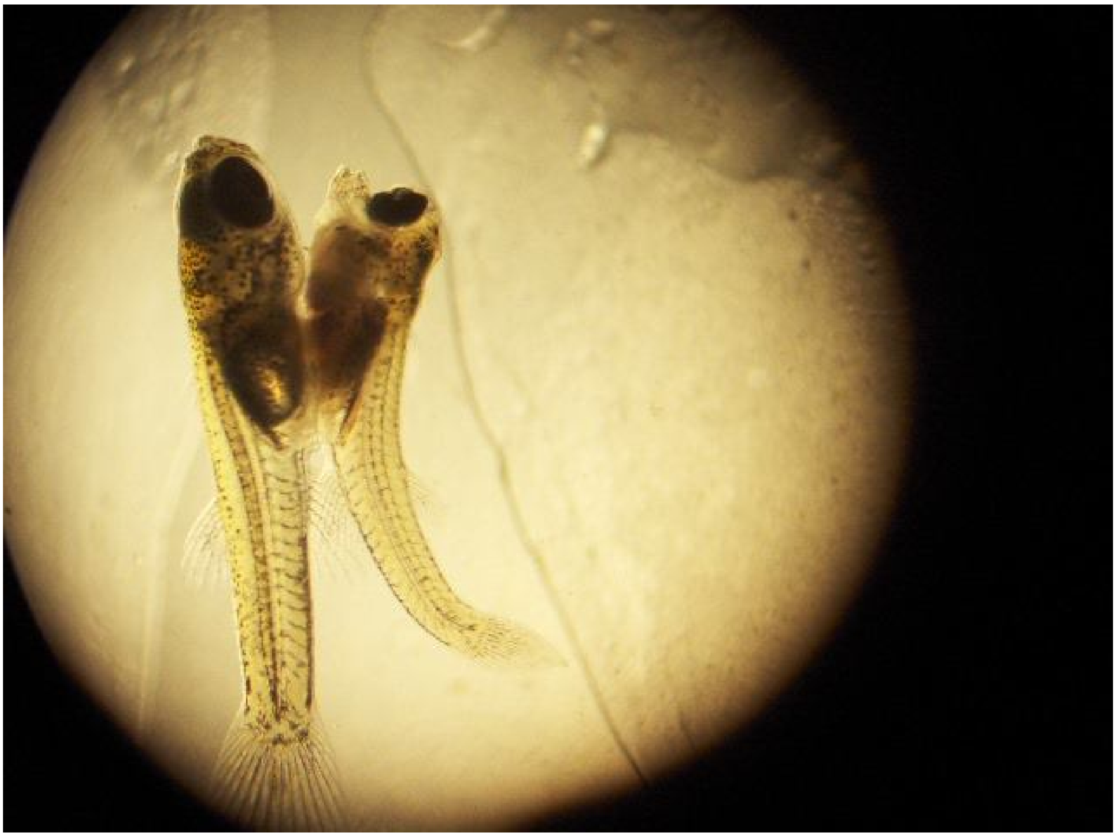


Fig. 8. The same pair of Siamese twins, five days after birth.

Table 1

Day old omphalopagus: description

Morphological, anatomical and functional observations	*Omphalopagus twins*	
	Individual 1	Individual 2
Body aspect (a very small assimetry between the two conjoined twins)	Normal and complete developed	Incomplete developed
Pigmentation (almost the same in both)	Present (melanophores)	Present (melanophores). Lacking pigmentation around the nostrils and mouth opening.
Body length	Longer. Almost equal to 2	Shorter. Almost equal to 1
Mouth	Normal	Trunk or beak like mouth
Eyes	Normal (two in number)	Cyclop
Caudal peduncle	Normal developed	Incomplete developed
Circulatory system	Complete and functional	Incomplete developed and apparently nonfunctional (yet) or weak functional
Respiration	Gill breathing, normal	Not clear
Movement	Fin fish running movements	Static

Table 2

Three days old omphalopagus: description

Morphological, anatomical and functional observations	*Omphalopagus twins*	
	Individual 1	Individual 2
Body aspect (assimetrical)	Bigger (visible surpassed the size of individual 2)	Smaller
Pigmentation (almost the same in both)	Melanophores islands around the mouth opening, around the nostrils and head. On operculae, pigmentation in the form of stars with ramifications; enhanced pigmentation dorsal-aboral (normal).	Melanophores islands on head and operculae. Lacking pigmentation around the nostrils and mouth opening. On operculae, pigmentation in the form of stars with ramifications; enhanced pigmentation dorsal-aboral (normal).
Body length	Longer	Shorter
Head	Bigger. Enhanced pigmentation dorsal-aboral	Smaller. Enhanced pigmentation dorsal-aboral
Mouth	Normal	Trunk or beak like mouth
Eyes	Normal (two in number)	Cyclop (only one eye)
Caudal peduncle and fin	Normal developed. Caudal fin: about five times bigger than observed in individual 2)	Incomplete developed (tail strike crippled)
Fins	Normal	Smaller (all of them)
Circulatory system	Functional. Normal heart beats	Functional. Fast heart beats
Respiration	Gill breathing, normal	Gill breathing, very fast
Digestive tube	Functional. Defecation trough separate anal sphincter/vent	Apparently not functional
Swim bladder	Filled with air	Not filled with air
Swimming	Is made in the normal position. Fin fish running movements. Calmer than 2.	Stand upside down; not contribute to swim, but it is very agitated. Fin fish running movements

Individual 1 tends to "cannibalize" the individual 2, this being possible due to communication of the two circulatory systems. On the other hand, the fast atrophy of individual 2 could be due to its impossibilty to eat and live a normal life. Both explanations could be true at the same time. The Siamese twins we studied died suddently on the seventh day of life due to unknown physiological causes.

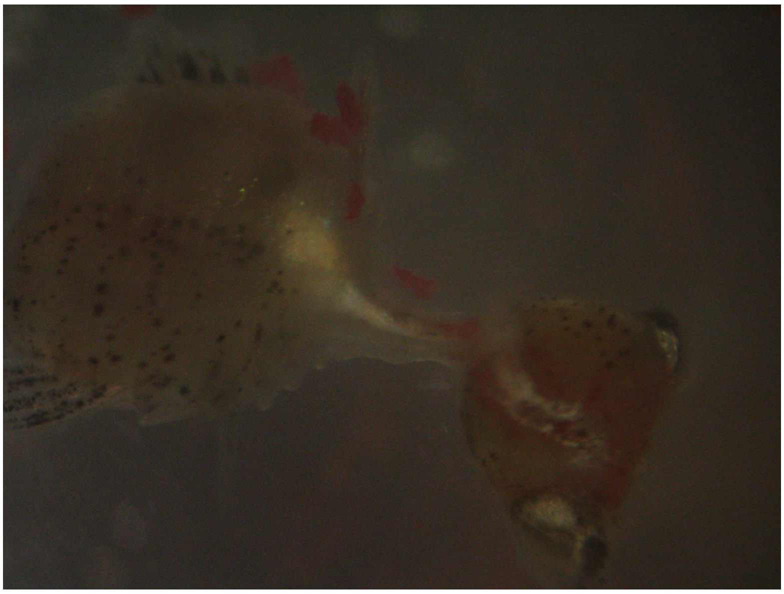
Other Types of Malformations Observed in the Same Guppy Strain. Beside Siamese twins, in the same guppy strain, we could observe some other types of interesting embryonary malformations. Some abnormal embryos were alive and still kept an aspect of vertebrate (see the case presented in Figure 9, a – dorsal view, b – ventral view), while others were tissue remains from some already dead and resorbed embryos (see Figure 9, c).

Table 3

Five days old omphalopagus, looking like a heteropagus

Morphological, anatomical and functional observations	*Omphalopagus twins*	
	Individual 1	Individual 2
Body aspect (very assimetrical)	Bigger (much bigger than 2)	Smaller and very curved back
Pigmentation (almost the same in both)	Melanophores islands around the mouth opening, around the nostrils and head. On operculae, pigmentation in the form of stars with ramifications; enhanced pigmentation dorsal-aboral (normal).	Melanophores islands on head and operculae. Lacking pigmentation around the nostrils and mouth opening. On operculae, pigmentation in the form of stars with ramifications; enhanced pigmentation dorsal-aboral (normal).
Body length	Much longer	Shorter
Head	Much bigger	Smaller
Mouth	Normal	Trunk or beak like mouth
Eyes	Normal (two in number)	Cyclop
Caudal peduncle and fin	Normal developed. Caudal fin: eight times bigger than observed in individual 2)	Incomplete developed (tail strike crippled)
Fins	Normal	Much smaller (all of them)
Circulatory system	Functional. Normal heart beats	Functional. Fast heart beats
Respiration	Gill breathing, normal	Gill breathing, very fast
Digestive tube	Functional. Defecation trough separate anal sphincter/vent. Abdominal cavity: normal. Organs: normal.	Apparently not functional. Abdominal cavity: small. Organs: atrophied.
Swim bladder	Filled with air.	Not filled with air.
Swimming/movement	Is made in the normal position. Fin fish running movements. Calmer than 2	Stand upside down; not contribute to swim, but it is very agitated. Fin fish running movements

Discussion. We are not able to tell whether cloramphenicol has any teratogenic effect in vertebrates or not. Our cases of malformations were not due to cloramphenicol. Sure is the fact, our guppy strain has a strong bias to produce Siamese twins and many other types of embryonary malformations due to genetic causes; see similar interpretations for vertebral abnormalities in Ando et al (1995) and Lodi (1978) (see also a picture with vertebral deformity from our own laboratory in Figure 10). Unfortunately for the millionfish, such hereditary properties make them study organisms for one more research subject. Surgical separation of Siamese twins is sometimes feasible and *P. reticulata* can be a very good model for this kind of practice.



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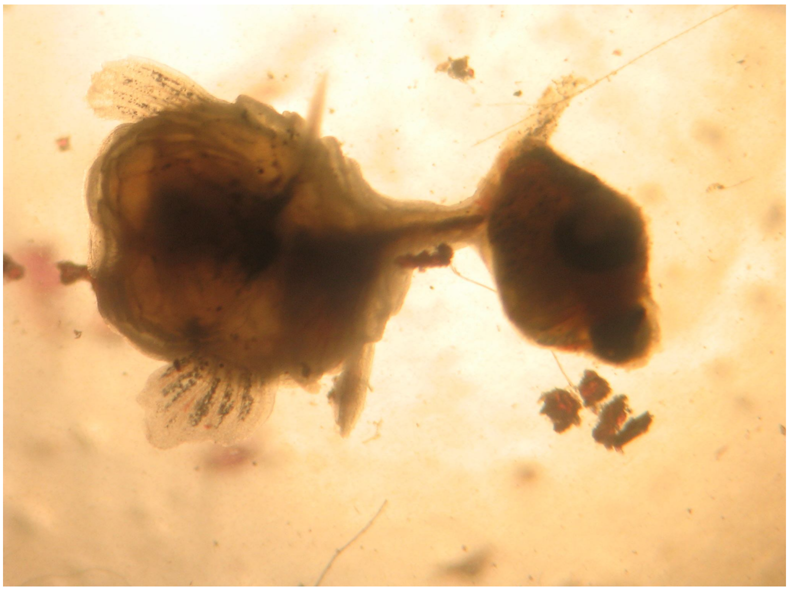

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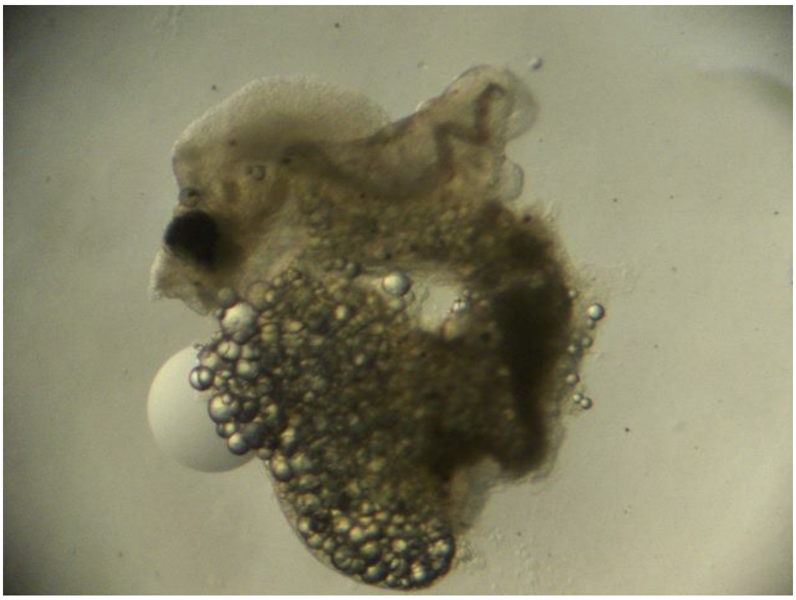

c

Fig. 9. Other types of malformations observed among the new born guppies.

Fig. 10. Vertebral deformity: another disease often studied in guppy (guppy strain: Red Blond; photo: Dragoș Ludușan).

Acknowledgements. Results of this paper were not funded by grants or other projects. Reconsidering, editing and discussion of these results are due to postdoctoral research project *Correlation of phenotypic markers with the concentration of some xenoestrogen pollutants, using as model organism the guppy fish, Poecilia reticulata Peters, 1859* (part of POSDRU Programme – Postdoctoral School in Agriculture and Veterinary Medicine Area). "This work was cofinanced from the European Social Fund through Sectoral Operational Programme Human Resources Development 2007-2013, project number POSDRU/I.89/1.5/S62371 *,,Postdoctoral School in Agriculture and Veterinary Medicine Area".*

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Received: 01 December 2010. Accepted: 30 April 2011.
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How to cite this article:
Petrescu-Mag I. V., Păsărin B., Hoha G., Hărșan R., Odagiu A. M., 2011 New contributions to knowledge of embryonic malformations in guppies. AACL Bioflux **4**(2):216-228.