THIS IS A TRANSLATED VERSION OF THE ORIGINAL ARTICLE.
RECOMMENDED CITATION AS FOLLOWS:

Calviño PA, Alonso F, Torres JS (2007). Llenado de gas de la vejiga natatoria de post-larvas de peces anuales sudamericanos (Cyprinodontiformes; Rivulidae). ***Boletín del Killi Club Argentino*****, BIBKCA No. 13: pp 18-39.** Available at: https://bit.ly/2JYUBqs

Gas filling of the swimming bladder of south American annual fish larvae (Cyprinodontiformes; Rivulidae)

Pablo A. Calviño[1], Felipe Alonso[1] and Jorge Sanjuán de Torres[2]

[1]Study Group of Killi Club Argentino (KCA), Independencia 443, Villa Ballester 1653, BuenosAires, Argentina. e-mail: pablocalvin@yahoo.com.ar. [2] SEK, Spanish Killis Society.

Abstract

The South American annual fish of the Rivulidae family bury their eggs in the substrate (generally with predominance of the slime). The eggs remain in a diapause state during the dry season until the hatching takes place with the first rains of the "wet season". When the larvae hatch, they carry out retrograde movements through the substrate until they reach the surface of it. At the time of the hatch, the gas bladder is empty and in the next 24 hs takes place the filling of it, mainly with oxygen and carbon dioxide from blood. The appearance of "belly sliders" is a phenomenon that occurs frequently in annual Killifish (Cyprinodontiformes) of the Rivulidae family of South America. This denomination comes from the fishkeeping hobby and refers to the post-larvae that after the hatching do not manage to establish a normal swimming by problems in the gas filling of the bladder gas. In the present work the influence of the substrate and the maximum standard length (SL) of different species in the "belly sliders" appearance was studied. For this an experiment was made (n-5) in which the percentage of "belly sliders" of a set of post-larvae that hatched buried in the slime (condition A) was compared with the percentage of "belly sliders" of a set of post-larvae hatched without the slime (condition B). Another experiment was made as well in which the percentage difference of belly sliders between conditions B and A, for different species, was compared against the maximum standard length (SL). It was concluded that the appearance of "belly sliders" in the condition A is minor than B and that it does exist a tendency to the increase of post-larvae with a defective filling of the bladder gas (belly sliders) with the increase of the maximum SL that the species can reach. A hypothesis of how this could happen at a physiological level and another one about the evolutionary origin of this characteristic are given. A series of other related experiments are shown and discussed. Based on these hypothesis it is considered that the exercise that the post-larvae do to get out of the substrate after the hatching and the availability of oxygen are factors that could affect the adequate filling of the bladder gas.

Resumen

Los peces anuales sudamericanos de la familia Rivulidae entierran sus huevos en el sustrato (generalmente con predominancia de limo). Los huevos permanecen en estado de diapuasa durante la época de estiaje hasta que ocurre la eclosión con las primeras lluvias de la época estival. Cuando las larvas eclosionan efectúan movimientos retrógrados a través del sustrato hasta alcanzar la superficie del mismo. Al momento de la eclosión, la vejiga natatoria se encuentra vacía y dentro de las 24 hs posteriores se produce el llenado de la misma, principalmente con oxígeno y dióxido de carbono provenientes de la sangre. La aparición de “alevines rampantes” es un fenómeno que se da con frecuencia en Killis (Cyprinodontiformes) anuales de la familia Rivulidae de Sudamérica. Esta denominación proveniente del acuarismo se refiere a las post-larvas que luego de la eclosión no logran establecer una natación normal por problemas en el llenado de gas de la vejiga natatoria. En el presente trabajo, la influencia del sustrato y la talla máxima de diferentes especies en la aparición de “alevines rampantes” fue estudiada. Para esto un experimento (n=5) fue realizado en el que el porcentaje de “alevines rampantes” de un conjunto de post-larvas que eclosionaron enterradas en limo (condición A), fue comparado con el porcentaje de “alevines rampantes” de un conjunto de post-larvas eclosionadas sin limo (condición B). A su vez otro experimento fue realizado en el cual la diferencia porcentual de rampantes entre las condiciones B y A, de distintas especies, en función de la longitud estándar (LE) máxima, fue comparada. Se llegó a la conclusión que la aparición de “alevines rampantes” en la condición A es menor que en B y que existe una tendencia al aumento de post-larvas con un llenado defectuoso de la vejiga natatoria (rampantes) con el aumento de la LE máxima que puede alcanzar la especie. Una hipótesis sobre cómo podría ocurrir este fenómeno a nivel fisiológico y otra sobre el origen evolutivo de esta característica son elevadas. Una serie de otros experimentos relacionados son presentados y discutidos. En base a estas hipótesis se considera que el ejercicio que realizan las post-larvas para salir del sustrato luego de la eclosión y la disponibilidad de oxígeno son factores que podrían afectar el adecuado llenado de gas de la vejiga natatoria.

Introduction

Fish use their swimming bladder to vary their density and facilitate their movements in the water. This organ is located in a ventral position with respect to the vertebral column and dorsally with respect to the intestine. In some fish it has a connection to the intestine (physocysts), an ancestral condition, and in the teleost this connection in general is lost during post-embryonic development (physocysts). These fishes when hatching fill their swimming bladder through a gas gland, which is attached to the swim bladder dorsally relative to the intestine (Kardong, 1998). This process occurs with the passage of glucose to lactic acid and the consequent acidification of the medium, which causes a blood release of oxygen from oxyhemoglobin and carbon dioxide from bicarbonate, which then diffuse into the swim bladder. Gases that do not immediately reach the swim bladder pass into the network through the efferent capillaries and diffuse from there to the afferent system and circulate again in the gas gland (Maceda, 2007). Most of the gas inside the swim bladder is extracted by specialized cells located on the walls of it that direct it to the circulatory system by means of capillaries. The system of filling and emptying the swim bladder through the gas gland is dynamic and controlled by the nervous system. Commonly in Teleostean fish when larvae hatch does not have their swimming bladder full of gas so they must fill it through the gas gland (Kardong,1998).

The annual South American fish of the family Rivulidae (Cyprinodontiformes) bury their eggs a few centimeters below the substrate (usually predominantly silt). The eggs remain there, and the embryo develops during the dry season until hatching occurs with the first rains of the summer season. In different species of these fish bred in captivity, some of the newly hatched larvae do not achieve adequate filling of gases from their swim bladder which then prevents them from normal swimming. In these fish this process can take from at least one hour to twenty-four hours maximum so that the larvae can normalize their swimming (obs. pers.). Fish with this problem usually fail to normalize their swim bladder throughout their lives and are known among aquarium enthusiasts as "belly sliders".

Traditionally, vague hypotheses have been established about the cause that this phenomenon could produce, such as: lack or excess in the "maturation time" of eggs, incubation with "excess humidity" in peat, the temperature of water not"sufficiently cold", etc., but no study has been conducted methodically in order to elucicide the principles of the phenomenon and the influence of these or other factors.

At the time of hatching, when mature eggs come into contact with water, the larvae come out of their immobility inside the egg: they move the eyes and tail and rotate inside the egg. The chorion "thins" and the egg swells. Half an hour later approximately, the end of the tail breaks the chorion and through brusque contractions the entire embryo is released from the chorion. Always with the tail forward, the embryo advances through the mud with undulating movements vertically. In this “backward progress" the pectoral fins are kept at the sides of the head until finally reaching the surface of the substrate. After a lapse swimming begins, which can be interrupted at varying times and even observed, in some cases, tail movements such as those that characterized the initial movements through the substrate (Vaz Ferreira et *al.*,1963). See Figure 1.

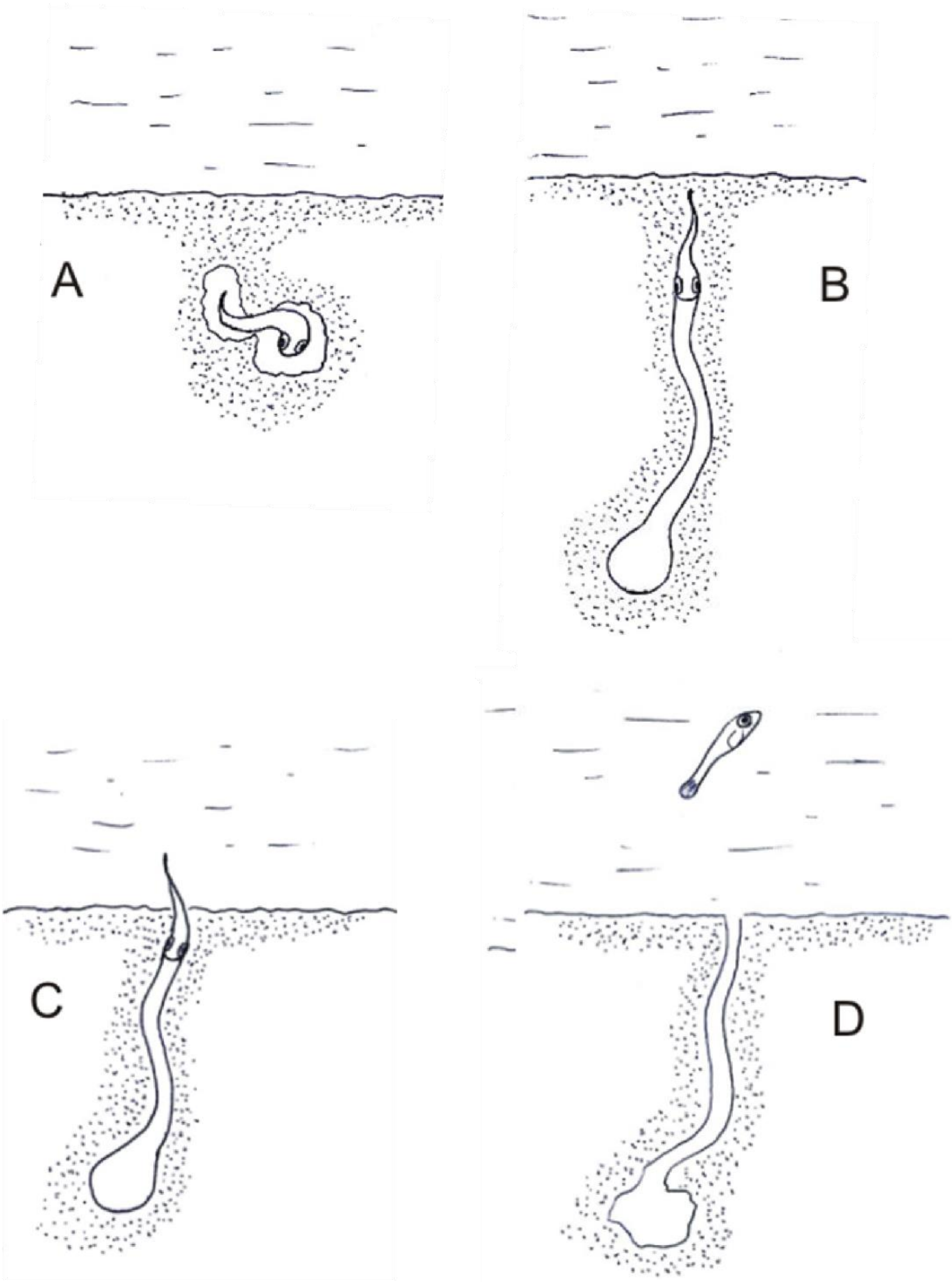

Fig. 1. A- The end of the tail breaks the corion and seeking support in the particles at the bottom, performs abrupt contractions until the entire larva leaves the corion. **B-** The larva advances through the mud with vertically undulating retrograde movements. It isa "backward progress" in which the pectoral fins are kept on the sides of the head. **C-** The larvae reach the surface of the substrate. **D-** After a lapse they start swimming. At varying periods they can perch on the bottom or perform the movements that characterized their initial displacements through the substrate. Illustration: P. Calviño.

Two hypotheses were confirmed in this work:

1) A set of eggs of annual species (Cyprinodontiformes; Rivulidae) hatched with silt (from a biotope where some of these species live) have fewer individuals with problems filling their swimming bladder with gas than those born in the same conditions but without silt.

2) Species of higher standard length (SL) have a higher number of "belly sliders fry" than those with a lower maximum SL.

Also, a hypothesis that would explain how this phenomenon could occur at the physiological level is proposed, related to a greater availability of carbon oxide to fill the bladder given by the exercise carried out by the post-larva to leave the substrate, where it is worth highlighting the importance of the oxygen available in the medium to perform an aerobic breathing and produce said carbon dioxide and reduce the metabolic path of fermentation (anaerobic) in which no carbon dioxide is released and lower energy performance of glucose is obtained.

Continuing with this line of thoughts, another hypothesis about the causes at the evolutionary level that this characteristic could have and why it could be of greater influence on the largest species is raised.

At the same time, a series of related experiments and a discussion of them are presented.

MATERIALS AND METHODS

To see the influence of the presence of silt at the time of hatching on the "appearance of belly sliders fry" experiment 1 (n=5) was performed with two series of tests in which eggs were used (incubated in peat for variable periods) in 5 different species: *Austrolebias bellottii* (Steindachner,1881), *Austrolebias nigripinnis* (Regan, 1912), *Austrolebias toba* Calviño 2005, *Austrolebias elongatus* (Steindachner, 1881) and *Trigonectes balzanii* (Perugia 1891). These eggs were hatched in separate containers. For each species, two different series of tests were carried out taking equal parts of the egg incubation peat.

In the first series of tests, peat (3 cm thick) containing the eggs was introduced and silt (3.5 x 0.5) cm was added from a biotope where some species of this group of fish live (condition A), while for the second series the same procedure was performed but no silt was added (condition B). A column of 10 cm of water was added to the containers containing the substrate and the entire contents were mixed, then the substrate decanted. It was observed that eggs, denser than most of the substrate, decant before it and were buried.

In all cases the preparation of the water used was carried out with dechlorinated running water and at room temperature, without other treatment or filtration. The water temperature in all cases was equal to or close to the ambient temperature of the peat con the eggs. The vessels used for birth observation consisted of glass jars 200 mm high and 80 mm in diameter, which were incorporated moderate "aeration" through an air pump.

The used peat was previously ground, boiled, and washed to remove impurities such as "dust" or soil that may be present in it.

The containers with the post-larvae were observed daily for one week to account for the number of belly sliders. During this period, they were fed with *Artemia* sp. nauplii newly hatched.

To analyze the results, the mean of the percentages of "belly sliders fry" in condition A was calculated and compared with the mean of the percentages in condition B.

To see the influence of the length of the species on the "appearance of belly sliders fry" eggs (incubated in peat for variable periods) of 4 different species were used for the experiment:

Austrolebias nigripinnis (Regan, 1912), *Austrolebias toba* Calviño 2005, *Trigonectes balzanii* (Perugia 1891) and *Austrolebias elongatus* (Steindachner, 1881). These eggs were hatched in separate containers. For each species, two different series of tests were carried out taking equal parts of the egg incubation peat.

The two series were conducted similarly to those carried out in the case of the experiment of the influence of slime on the "appearance of belly sliders fry".

To analyze this data, the percentage difference between conditions A and B, defined as the percentage of belly sliders fry in condition B, minus the percentage of belly sliders fry in condition A, was analyzed for each species based on its maximum standard length (SL). Where the maximum SL is considered as the maximum SL that the species and the SL reach as the distance between the snout and the posterior end of the caudal peduncle.

Results

It was observed that fry with normal swimming possessed their gas-filled swim bladder (see Fig. 2) wherefore those who failed to stabilizetheir swimming had not gas-filled their swimming bladder (Fig 3).

Fig. 2. Post-larva of *Austrolebias nigripinnis* 24hs post-hatching, with normal swimming, presents the swimming bladder full of gas. Photo: P. Calviño.

Fig. 3. Post-larva of *Austrolebias nigripinnis* 24 hs post-hatching, with abnormal swimming("belly sliders"), presents an empty gas bladder. Photo: P. Calviño.

Experiment 1 shows a tendency to increase the percentage of "belly sliders fry born without silt (condition B) compared to those born without silt (condition A). See Figure 4.

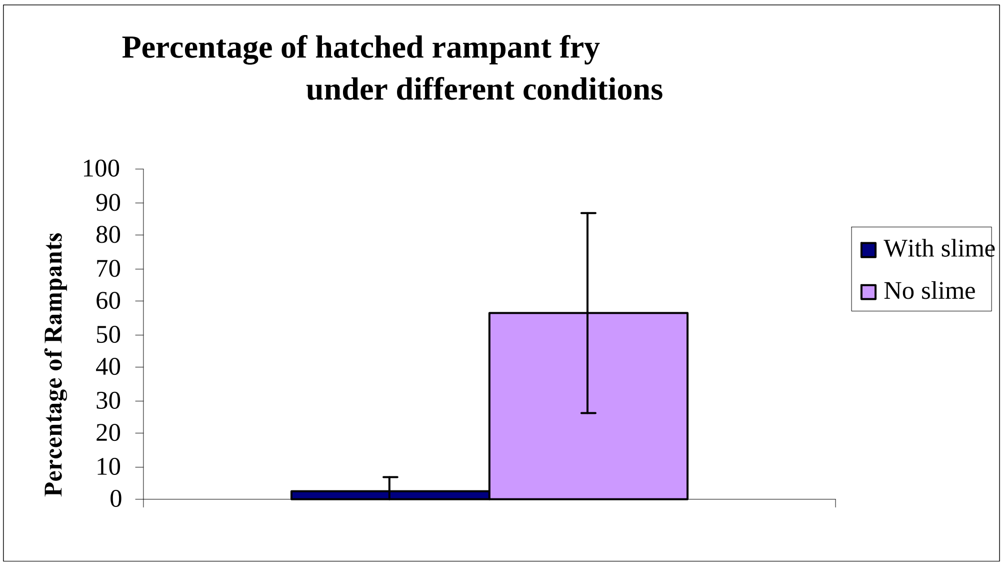

Figure 4. Percentage of belly sliders "belly sliders fry" of larvae hatched with silt and without silt. (No. 5). Error bar: ED

Experiment 2 shows a tendency to increase the percentage of "belly sliders fry" with increased LE of species.

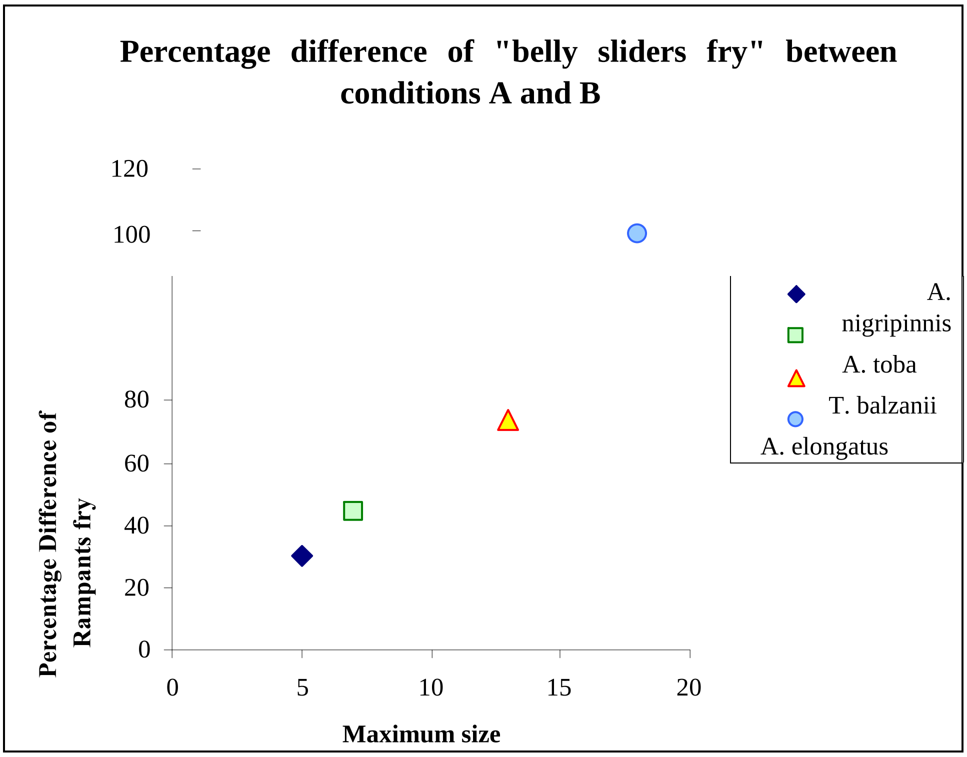

Figure 5. Percentage difference of belly sliders fry of different species between a set of larvae hatched with silt (condition A) and an hatched set without silt (Condition B) depending on the maximum size of the species.

Discussion

A short period after reaching the surface of the substrate, the post-larvae swim towards the surface of the water. This action has been misinterpreted as an attempt to get "the first mouthful of air" to fill their swim bladder, but it has been demonstrated in numerous experiments that normal initial filling of the swim bladder occurs even when post-larvae are prevented from direct access to the surface of the water. This instinctive reaction could be interpreted as a strategy to reach an area with higher oxygen levels near the surface of the water (G.C.K.A, 2003; obs. pers.).

The retrograde underground propulsion of the larvae to reach the surface of the substrate after hatching was observed (Fig.1), a phenomenon mentioned by Vaz Ferreira *et al.* (1963). Eventually the post-larvae can push the substrate with the head and not with the tail, especially when they encounter an obstacle (obs. pers.).

In the case of annual African fish of the *genus Nothobranchius* Peters, 1868 (Cyprinodontiformes, Nothobranchiidae), as in the South American genus *Papiliolebias*, this phenomenon of belly sliders appearance is not as common among amateurs. These fish, when they lay their eggs, have the particularity of not burying themselves as deep in the substrate as in the case of most South American annuals so they are usually called "sliters". In this way, it is likely that the "substrate" factor on the post-larvae is not so determinant as these eggs would be buried at very shallow depths and for reasons related to their evolution would not be as sensitive to this factor.

This evolutionary factor would also explain, within the annual South American fish (Rivulidae), why the species with the greatest size, which bury their eggs deeper than those with lower size, have a greater sensitivity to the "substrate" factor. For example, fish of the genus *Trigonectes* and large *Austrolebias*. In these species the post-larva should travel a greater path through the substrate and consequently perform a greater exercise to reach its surface area.

The following hypotheses are raised from the above:

1) The exercise performed by post-larvae after hatching to exit the substrate is necessary for proper filling of gas from the swim bladder.

2) The larger species (those with a higher maximum LE) require greater exercise for the proper filling of their swim bladder and consequently the eggs must be buried at a greater depth for this to happen.

During intense exercise, the muscles produce lactic acid by fermentation and abundant carbon dioxide (CO_2) by aerobic breathing that is released into the blood. Lactic acid will occur under anaerobiosis conditions when intense exercise demands a large amount of energy and therefore anaerobiosis-induced fermentation will begin to occur by oxygen consumption in the electron transport chain of aerobic breathing in the muscle (Glucose → Glycolysis → (fermentation → lactic acid) or (aerobic respiration → carbon dioxide + water) . Carbon dioxide released into the blood will be transported in part by hemoglobin (forming carbaminohemoglobin), and another part will be diluted in the blood. Some of the carbon dioxide dissolved in the blood will be combined with the water present in it to give carbonic acid and bicarbonate according to equation 1, which represents the buffer balance of the blood.

Equation 1. Blood buffer balance**:**

$$\mathbf{CO_2 + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow H^+ + HCO_3^-}$$

Therefore, if the concentrations of carbon dioxide dissolved in the blood are very large, an acidosis phenomenon can occur in which the pH of the blood falls (approx. 7.4) and other things are released from oxyhemoglobin according to equation 2:

Equation 2. Hemoglobin (Hgb) - Oxygen

$$\mathbf{HgbO_2 + H^+ \Leftrightarrow HHgb + O_2}$$

It follows that probably the fact that at the time after hatching the post-larvae perform this exercise through the substrate to reach its surface performing an intense exercise would produce large amounts of carbon dioxide and also some lactic acid. High concentrations of carbon dioxide in the blood could lead to acidosis that could be lethal. In such a case it is likely that these fish will avoid this by using this "excess" of carbon dioxide to fill the swim bladder.

In turn, the need for oxygen to obtain a higher energy performance of glucose by aerobic breathing and release of carbondioxide is highlighted, since fermentation to lactic acid has a much lower energy yield and no carbon dioxide is released during this process.

In this way these fish would have evolved in such a way that those individuals able to eliminate more efficiently this "excess" of carbon dioxide by the previously exposed form would have an adaptive "advantage" and then this strategy would have been preserved.

Larger species, which bury their eggs more deeply, require greater exercise to adequately fill their swim bladder. This could be explained because post-larvae would require more carbon dioxide because these species would have been "adapted" to greater release of carbon dioxide.

The fact that oxygen is an important factor in the "initial" filling of the swim bladder has been reported on numerous occasions by aquarists who obtain fewer "belly sliders fry" using for hatching water with high concentration of oxygen. This in turn is consistent with the fact, also reported by amateurs, that relatively cold water would have a positive influence on this phenomenon given that, in those conditions, oxygen has a mayor solubility in the water.

On the other hand, it cannot be clearly explained why individuals who fail to fill their swimming bladder in the first few days of life usually never manage to do so. However, it could be assumed that this would be related to the "larval hemoglobin" of these fish that could adequately carry out the transport of large amounts of carbon dioxide in the blood in such conditions and release it more easily in the vicinity of the swim bladder than the hemoglobin of the adult fish. In fact the hemoglobin of the adult fish should not necessarily be very efficient for this process since when, these fish, inhabit shallow environments (usually no more than 1m) would not need to make large variations in the volume of the swim bladder when moving through the water column and consequently would not need large amounts of gases present in the blood or an "efficient in this sense" hemoglobin to perform it. In this way a hemoglobin, in the adult fish, "less effective" in the sense of transport and release of gases in the bladder (pH effect) would not affect the life of these fish.

Following this line of research future work should focus at the physiological and anatomical level to study whether these hypotheses are corroborated or not and in turn should be studied to occur in the habitat of these species at the time of the hatching of the larvae.

Conclusions

1) The exercise performed by post-larvae after hatching to exit the substrate is necessary for proper filling of gas from the swim bladder.

2) The larger species (those with a higher maximum SL) require greater exercise for the proper filling of their swim bladder and consequently the eggs must be buried at a greater depth for this to happen.

3) Exercise could lead to increased "physiological availability" of carbon dioxide in the blood that will be usedfor swimming bladder filling.

4) Evolutionarily, it can be understood as a "strategy" to avoid a situation of acidosis, which could be lethal, the fact that these fish "require" intense exercise to fill theirbladders

5) Higher SL species would be adapted to greater exercise at birth, as they bury their eggs deeper into the substrate, and consequently to greater availability of carbon dioxide in the blood, so it would explain the fact that they "require" their eggs to be at greater depth for the proper filling of their swimming bladder.

Thanks

We appreciate the critical review of Marcelo Loureiro (FCM) and Hugo Castello (MACN). This work is dedicated to the memory of Dr. Raúl Vaz-Ferreira (1918-2006) who showed great interest in this topic by reviewing this work. Unfortunately, he died before he could give us feedback on it.

Contribution of the Authors: Fr. Calviño was responsible for experimental design. F. Alonso was responsible for data analysis. P. Calviño, F. Alonso and J.S. De Torres discussed the experiment and edited the manuscript.

SOURCES CONSULTED

Froese, R. and D. Pauly. 2007. FishBase. [online]. Electronic publication on the World Wide Web, available at: <www.fishbase.org>, version (08/2007).

G.C.K.A. 2003. Cincinnati Killifish Association. *Newsletter*, February-March 2003 **Huber, J.H.** Killi-Data 1994-2006 [online]. Electronic publicationon the World Wide Web, available at: <www.killi-data.org>, version (11/2007)

Kardong, K.V. 1998. *Vertabrates. Comparative Anatomy, Function, Evolution*. Second Edition. WCB McGraw-Hill

Maceda, A., I. Gonzalez. 2007. *The swim bladder* [online]. AlacuaIrum. Available at: <http://www.alaquairum.net/vejiga_natatoria.htm>

Lehninger, A. L. 1984. *Biochemistry. The molecular basis of cell structure and function..* Edicones Omega, S.A. Bracelona

Ophart C. E. 2003. *Respiratory acidosis* [online]. Virtual Chem Book. Elmhurt College. Available on the World Wide Web at: <http://www.elmhurst.edu/-chm/vchembook/263respiratoryacid.html>

Sorín, S. 1989. *Installation and maintenance of aquaarios.* Volume I. Editorial Albatros. Buenos Aires, Argentina

Vaz Ferreira, R., B. Sierra de Soriano and S. Scaglia de Paulete. 1963. Retrograde underground flow hatching subterranea and propulsion of *Cynolebias* postlarvae (Pisces, Cyprinodontidae) Neotropic 9 (30), 111-112

Received July 25, 2007; Accepted November 10, Noviembre 2007.

ANNEX I

The following experiments took place in November and December, coinciding with the highest temperatures
The green color in the tables indicate positive results; yellow tables neutral results; celestial tables are twonegative.

Case A	Case A-2
Austrolebias bellottii "Ezeiza, KCA 01/02" Collected 10/05/2005 Wet 22/11/05" **"with slime"** Incubation time: 7 months (excessive) Peat humidity: quite dry Gh 8od Kh 6od pH: 7 - 7.2 Water temperature: 25oC Air temperature: 25oC Total births: 12 **Normal: 11 (90%)** **Belly sliderss: 1 (10%)**	*Austrolebias bellottii* "Ezeiza, KCA 01/02" Collected 10/05/2005 Wet 22/11/05" **"limo-free"** Peat moisture: quite dry Incubated: 7 months (excessive) Gh 8od Kh 6od pH: 7 - 7.2 Water temperature: 25oC Air temperature: 25oC Total births: 6 **Normals: 4 (66.6%)** **Belly sliderss: 2 (33.3%)**

Case B	Case B-2
Austrolebias toba	*Austrolebias toba*
Collected 31/07/2005	Collected 31/07/2005
Wet 15/11/05" **"with slime"**	Wet 15/11/05" **"without silt"**
Incubated: 3.5 months	Incubated: 3.5 months
Gh 8od	Gh:no data
Kh 6od	Kh:no data
pH: 7 - 7.2	pH: 7
Water temperature: 26oC	Water temperature: 26oC
Air temperature: 26oC	Air temperature: 26oC
Total births: 65	Total births: 18
Normal: 65 (100%)	**Normal: 10 (55%)**
Belly sliderss: 0 (0%)	**Belly sliderss: 8 (44%)**

Case C	Case C-2
Trigonectes balzanii KCA 20/03	*Trigonectes balzanii* KCA 20/03
Collected: 04/ 07 /2005	Collected: 04/ 07 /2005
Wet 20/11/05" **"with slime"**	Wet 20/11/05" **withoutsilt"**
Gh 8od	Gh: syndatos

Kh 6od	Kh:no data
pH: 7 - 7.2	pH: 7
Water temperature: 26oC	Water temperature: 26oC
Air temperature: 26oC	Air temperature: 26oC
Total births: 24	Total births: 16
Normal: 23 (95.8%)	**Normal: 4 (25 %)**
Belly sliderss: 1 (1%)	**Belly sliderss: 12 (75%)**

Case D	Case D-2
Megalebias elongatus "Fanazul"	*Megalebias elongatus* "Fanazul"
Collected 04/09/2005	Collected 04/09/2005
Wet 10/12/05" **"with slime"**	Wet 26/11/05" **" limo-free and peat-free" sin**
Incubated:	Gh:no data
Gh:no data	Kh:no data
Kh:no data	pH: 7.5
pH: 7	Water temperature: 25oC
Water temperature: 20oC	Air temperature: 27oC
Air temperature: 21oC	Total eggs: 6 (after 48 hours., two hs
Total births: 5	were not born and were re-incubated
Normal: 5 (100%)	Total births: 4
Belly sliderss: 0 (0%)	**Normal: 0 (0%)**
Observations:	**Belly sliderss: 4 (100%)**
	Remarks:The first embryo was born at the time of wet, the second at the hour and a half and the fourth late 11hours.

Case E	Case E
Austrolebias nigripinnis	*Austrolebias nigripinnis*
Collected 31/07/2005	Collected 31/07/2005
Wet 15/11/05" **"with slime"**	Wet 15/11/05" **"without silt"**
Incubated: 3.5 months	Incubated: 3.5 months
Gh 8od	Gh:no data
Kh 6od	Kh:no data
pH: 7 - 7.2	pH: 7
Water temperature: 26oC	Water temperature: 26oC
Air temperature: 26oC	Air temperature: 26oC
Total births: 23	Total births: 20
Normal: 23 (100%)	**Normal: 14 (70%)**
Belly sliderss: 0 (0%)	**Belly sliderss: 6 (30 %)**

The experiences carried out here made it possible to find that the aggregate of soil or silt produces a substrate pressure that the postlarva needs for the natural exercise of struggle and displacement to the surface of the bottom.

Otherreal-time experiments

Below are the results of other experiments conducted under various conditions. In each case, the number of eggs arranged in the experience was accounted for. In the following cases the peat in which the eggs had been incubated wasexcessively wet which may have caused a lack of maturation of the embryo at the time of hatching theeggs, which would explain that some eggs were not born despite having been incubated for 4 months. Although on the other hand, it is known that all individuals from the same litter are never born at the same time. It is likely that the state of excessively wet peat delayed this process as even some fertile but still transparent eggs were found, a sign of "little maturation"

Case 3	Case 1
Austrolebias toba "Chaco" F2 Collected 24/09/2006 Wet 20/01/07" **“with 1.5cm slime"** Incubation: 119 days (4 months) In very wet peat With 3 cm of corn oil on the surface and without aeration the first 36 hours.. Gh:no data Kh:no data pH: 7 Water temperature: 26oC Air temperature: 27oC Total eggs to hatch: 50 Total births: 43 **Normal: 33 (76.7%)** **Belly sliderss: 10(23.2%)**	*Austrolebias toba* "Chaco" F2 Collected 24/09/2006 Wet 20/01/2007 **" withoutslime or peat"** Incubated: 119 days (4 months) días (In very wet peat Bottle without aeration Gh 8od Kh 6od pH: 7 Water temperature: 26oC Air temperature: 27oC Total eggs to hatch: 45 Total births: 40 **Normal: 0 (%)** **Belly sliderss: 40 (100%)**

Case 2	Case 4
Austrolebias toba "Chaco" F2 Collected 24/09/2006 Wet 20/01/07" **withoutslime or peat"** Incubation: 119 days (4 months) días (In very wet peat **Mineral water enriched with conchilla calcium and aeration is added** Gh:no data Kh:no data pH: 8 Water temperature: 26oC Air temperature: 27oC	*Austrolebias toba* "Chaco" F2 Collected 24/09/2006 Wet 20/01/2007 **" withslime, no peat"** Incubated: 119 days (4 months) In very wet peat **Mineral water enriched with conchilla calcium and aeration is added** Gh 8od Kh 6od pH: 8 Water temperature: 19oC Air temperature: 27oC

Total eggs to hatch: 45 Total births: 22 **Normal: 2 (9%)** **Belly sliderss: 20 (91 %)**	Total eggs to hatch: 51 Total births: 22 **Normal: 12 (54%)** **Belly sliderss: 10 (45%)**

Case 5	Case 6
Austrolebias toba "Chaco" F2 Collected 24/09/2006 Wet 20/01/07" **"with 1cm slime"** Incubation: 119 days (4 months) In very wet peat With moderate aeration and never direct light Gh:no data Kh:no data pH: 7 Water temperature: 26oC Air temperature: 27oC Total eggs to hatch: 54 Total births: 54 **Normal: 44 (81%)** **Belly sliderss: 10(18%)**	*Austrolebias toba* "Chaco" F2 Collected 24/09/2006 Wet 20/01/2007 **" withoutslime or peat"** Incubated: 119 days (4 months) In very wet peat Bottle with moderate aeration Gh 8od Kh 6od pH: 7 Water temperature: 26oC Air temperature: 27oC Total eggs to hatch: 45 Total births: 35 **Normal: 1 (2%)** **Belly sliderss: 34 (97%)**

Important care: If the amateur wants to implement these principles, it is very important that the thickness of peat, soil or silt used is not excessive and has enough sponginess, never compact it, since otherwise the fry are trapped quedan without being able to penetrate the substrate to reach the surface of the bottom. Experiences made with bentonite clay, silt or soil, using any of these three pure elements in the water without the use of peat, demonstrated that it producesa"caponage"effect i.e. a seal thatprevents the fry from advancing to the surface, dying unfailingly drowned or suffocated.
In fact, in nature, eggs are found in a surface area of the sustrator, where it contains not only mineral matter, but also organic matter, water and air equally.
The substrate should not exceed 5 cm thick and the peat should be well ground leaving a soft texture. If the peat is not thin, it produces grandobstacles for the fry, being able to get trapped without being able to reach the surface.
The experiences carried out showed us that the mixture of either humus, soil, or silt with peat in a ratio of 80% peat and 20% slime or humus or soil, helps to reduce thecohesion or cluping of the substrate, that is, helps to alleviate the unfavorable structural characteristics produced by clays or pure soil with water, increasing the granulation and aeration of the substrate.
On the other hand, experiences made with fine sand, the hardness and pressure of the silicon exerted on the larva, produced the detachment of the mucosa of the skin in the fry. So the use of sand must also be discarded.

The preferred elemento should be slime, as land use must be very prudent (20% maximum and 80% peat) and make sure that it does not have fertilizers of any kind as it would immediately poison fry. In addition, the earth hasa greater cantida d of anaerobic matter than silt in such a way that it can produce an accelerated development of theanaerobic microorganisms that accompany this component, but if they are not introduced in remarkable quantities, they will not be able to thrive if the conditions areclearly aerobic. This is achieved only if the specimen or bottle contains sufficient volume of water and is added to the moderate aeration bottle. Consequently consecuencia there is no doubt that the substrate of the specimen, it is necessary to avoid the production of anaerobic bacteriathat produce phenomenaof decomposition of organic matter, which result in the formation of compounds and gases harmful to the survival of fry. For safety reasons, it would be best to get slime instead of dirt.

Hypothesis not ruled out. It would not be ruled out that any mineral substance or trace element has any beneficial function or helps trigger bladder filling. For example, in plants, there is obvious evidence that these elements operate as catalysts, i.e. acel beingthe chemical reactions that take place in the body, with much lower energy consumption than would be necessary without these catalysts. Variable factors such as water temperature, aeration and ph increase or decrease the absorption of different micronutrients.
It is not ruled out that peat-limo mezcal or soil with water produces an exchange of cations that helps trigger bladder filling.

Sometimes there are eggs that despite being apparently ready to be born, they are not born. This phenomenon may be due to two factors: (1) due to a high osmotic value of water and (2) excess tannin produced by peat can over-harden the egg wrap, which they cannot then hatch.
In the case of oviparous fish, eggs have an inner medium of higher saline concentration than the water where they are deposited. If the outer concentration is very low, the liquid may pass into the eggs, causing sor swelling and subsequentrupture. The same goes for sperm, which are more sensitive than eggs to osmotic value. In this is often the success of a process of reproduction of fish considered difficult (Sorín, 1989)
When eggs are not born, it is possible to reduce the osmotic value of water, adding distilled water that is poor in minerals.

A peat that gets wet early and without substrate pressure runs the risk that the embryos have not fully formed their bone system, organs, etc. If the peat is "past" time, the fry or embryo, already weakened, may have expansed too much energy and lost its ability to fill the swim bladder.

Some experiences presented in the annex, demostraron that substrate pressure is not the onlydetermining factor, since if there is oxygen deficiency either in water as in the substrate or anaerobic contamination with nitrites also produces a high percentage of belly sliders fry beyond recreating the optimal substrate pressure. In short, the origin of the appearance of belly sliders fry is likely not to be linked exclusively to a single reason, since oxygen-poor waterand the absence of slight substrate pressure (medi or adequate) are the two mainfactors affecting the appearance of belly sliders fry in buried fish (South American annuals).

Observations

Historically, some fans mentioned succeeding and decreasing the percentage of belly sliders fry "imitating nature" by putting the peat wet on rainy days or spraying the peat with a sprayer so that the peat is slowly moistened as the embryo was supposed to need some time to "fill with oxygen". In essence, these positive experiences are not at odds with those set out here, but on the contrary, an explanation is found. Not because of the belief that they need to be wet slowly (since here all the experiences were made by filling the bottle with water abruptly) but because in this way the embryo has a thicker and more appropriate means to initiate its retrograde movements and not as dissolved as when the peat is dispersed to "wet" in a fish tank.

The need to whisk the bottle tightly by covering it with his hand allowed a natural decanting of the eggs and slime with the peat. This idea arose because que in a previous first experience, the peat substrate was excessively compacted in the bottle and addedor with alsocompacted soil, which produced the birth of the fry that were shown squeezed against the bottle (almost stamped) without being able to mobilize to reach the surface and died suffocated.

Shaking the contents with silt, peat and water, the eggs decant in a medium with a fair consistency (neither very compact, nor very loose) allowing a retrograde route of the fry to the surface of the bottom in a natural way. The minimum percentage of belly sliders fry that appeared despite using this method may have been given for two reasons: The first that the egg has not been well covered by the slime layer and deposited very superficially. The second is that this minimum percentage exists in nature even if they do not survive forlong.

It had initially been assumed that peat could produce a degree of acidity in the medium that affected the filling of gas to newly hatched post-larvae. This hypothesis loses vigour with the results obtained in experiment**D,** where nopeat was corporat. This experiment would indicate that the appearance of "belly sliders fry" was probably due to the lack of a suitable substrate and not a factor present in the peat.

- Experience **A shows** that an excessively long period of incubationis not an incident factor in the appearance of belly sliderss.

- The water temperature used for hatching eggs in cases **A** and **D** was quite high for the nature of these species. This showed us that the temperature rises tois also nota decisive factor if the eggs came from a peat with similar temperature (within a certain normal tolerance for life) although its importance is not ruled out for better benefit, it is recommended to use a mas lower temperature in the case *of some Megalebias and Austrolebias.*.

The experience of Case 3 (already performed by English killliophiles many years ago) showed that fry does not take air from the surface to inflate its bladder. Oviparous Cypronids are physocylists, so there is no connection between the swim bladder and digestive system, which prevents air from eating. In physocylists, to fill gas in the bladder, the gas gland produces lactic acid; the resulting acidity forces the blood's hemoglobina to release oxygen; from there it passes into the bladder as it passes through a complex structure known as rete mirabile..

In the experience made to verify that killis are not physomos, most fish with normal swimming were observed after 36 hours but with symptoms of lack of oxygen (boqueaban notoriously), so after this time, the oil film was removed and moderate aeration incorporated. At 48 hours,the fry were transferred from the specimen to another aquarium where they developed perfectly. In this experience sunlight was never direct.

In Case 1 all the fry died even the embryos that were not born. An embryo died when it started to break the shell and the tail loomed. This phenomenon was due to bacterial contamination due to the breakdown of a pinch of artemia that after 24 hours died in the specimen. Here the importance of water oxygenation and the fatal effect of nitrites was demonstrated, sothe cleaningof the bottom was done in all other cases every 12 hours.

Some reports of cases of "belly sliderss in other fish such as the Discs (*Symphysodon* spp.),mention that the problem was due to a lack of calcium in the water. The experience of Case 2 showed that this is not the problem for our case, since equally with calcified water were born belly sliders and never normalized. In this case the sunlight was direct in some hours of the morning.

Case 4 is very particularsince u by tyling the same water in case 2 and despite the improvement of normal births by the addition of slime, the high percentage of belly sliderss could be caused here by the stress of a sudden change of different temperaturesfrom the warm environment to cold water, then sunlight was direct to the specimen for a few hours in themorning. This could also have hurt in a certain way.

The ability of liquids to maintain dissolved gases depends, among other things, on pressure and temperature, is forthis reason that changing these factors can suddenly result in a release of dissolved gases into the blood by staying in fishtissues, causing sometimes irreversible damage. A sudden decrease in temperature or atmosphericpre-injury results in the release of dissolved gas, with the consequences indicated (Elacuarista.com). This may relate to some positive results in experiences here with temperature changes, which could help the releaseof the gas to fill thebladder.

For practical purposes we should consider that:

1. During incubation, it is advisable to handle eggs as little as possible and avoid direct sunlight
2. It is known that excess moisture in the peat and low temperatures greatly lengthen incubation times
3. It is preferable thatthe peat where the fish laid the eggs be rinsed with clean water to remove waste and toxins. Filling water to a contaminated peat at the time of hatching can quickly form anaerobic conditions that favor the appearance of belly sliderses.
4. Regardless of the wet temperature, it is advisable to use oxygen-rich water. Filling with water by blow, dripping, using oxygen pills or mild or moderate aeration, are all methods that ensure the presence of enough oxygen in the water.
5. When fry quickly looks for the surface at birth, it is not to take a breath of air, possibly this instinct is linked to the higher concentration of oxygen in the surface area
6. It is concluded that oxygen-poor water and the absence of mild substrate pressure (adequate medium) are the two main factors affecting the appearance of belly sliders fry in the burying fish (South American annuals). The latter factor especiallyimpatient in large-scale species, those that bury their eggs deeper.

Finally we consider that there is much to be delved into, but in short, this series of experiments tries to make a contribution as to the concept of
"substrate pressure on fry" or "appropriate medium"

ANNEX II

Third series of experiences, including different factors:

Case 1a	Case 2a

Austrolebias periodicus "Don Pedrito" F1 Collected 16/12/2006 Wet 23/07/07" **with2 cm of soil" No peat, no aeration and smoothie for natural decanting** Incubation: more than 7 months (excessive) Peat humidity: very wet almost saturated Gh: 10od Kh: 6od pH: 7.2 Water temperature: 10oC Air temperature: 11oC Total eggs to hatch: 31 Total births: 31 **Normal: 4 (13%)** **Belly sliderss: 27 (87 %)** **At 36 a.m.,all the dead fry were found**	*Austrolebias periodicus* "Don Pedrito" F1 Collected 16/12/2006 Wet 23/07/07" **"with 3cm more peat 1cm earth on the surface"** **With aeration and no beating, filled by dripping** Incubation: more than 7 months (excessive) Peat humidity: very wet almost saturated Gh: 10od Kh: 6od pH: 7.2 Water temperature:10oC Air temperature: 11oC Total eggs to hatch: not counting Total births: 65 **Normal: 26 (40 %)** **Belly sliderss: 39 (60 %)** **At 36 a.m.,signs of poisoning were found.** **Belly sliderss died at 48am**

Case 3a	Case 4a
Austrolebias periodicus "Don Pedrito" F1 Collected 16/12/2006 Wet 23/07/07" **"with 3 cm of pure soil"No peat, no aeration and drip filling** Incubation: more than 7 months (excessive) Peat humidity: very wet almost saturated Gh: 10od Kh: 6od pH: 7.2 Water temperature: 10oC Air temperature: 11oC Total eggs to hatch: 25 Total births: 11 **Normal: 3 (27 %)** **Belly sliderss: 8 (73 %)** **At 36 a.m.,they found signs of intoxication**	*Austrolebias periodicus* "Don Pedrito" F1 Collected 16/12/2006 Wet 23/07/07" **"with** only **1mm of dirt"** **No aeration and blow filling** Incubation: more than 7 months (excessive) Peat humidity: very wet almost saturated Gh: 10od Kh: 6od pH: 7.2 Water temperature: 10oC Air temperature: 11oC Total eggs to hatch: 2 55 Total births: 20 **Normal: 2 (10 %)** **Belly sliderss: 18 (90 %)**

Case 5a	Case 6a

Austrolebias periodicus "Don Pedrito" F1 Collected 16/12/2006 Wet 23/07/07" **"with 2 cm of peat" No soil, with aeration and moistening the peat an hour at saturation point, then filled with peat water from another aquarium.** Incubation: more than 7 months (excessive) Peat humidity: very wet almost saturated pH: 7.0 Water temperature: 10oC Air temperature: 11oC Total eggs to hatch: not counting Total births: 39 **Normal: 3 (8 %)** **Belly sliderss: 36 (92 %)** Dead: 12 to 48hs	*Austrolebias periodicus* "Don Pedrito" F1 Collected 16/12/2006 Wet 23/07/07" **"with 3 cm of peat "No dirt, with aeration and filling of blow with distilled water** Incubation: more than 7 months (excessive) Peat humidity: very wet almost saturated pH: 6.0 Water temperature: 10oC Air temperature: 11oC Total eggs to hatch: not counting Total births: 30 **Normal: 6 (20 %)** **Belly sliderss: 24 (80 %)** Muerto: 1 to 48 hs

Case 7a	Case 8a
Austrolebias periodicus "Don Pedrito" F1 Collected 16/12/2006 Wet 25/07/07" **"with 2 cm substrate" (80% peat and 20% clay soil) With aeration and filling of blow with distilled water** Incubation: more than 7 months (excessive) Peat humidity: very wet almost saturated pH: 7.0 Water temperature: 10oC Air temperature: 11oC Total eggs to hatch: not counting Total births: 52 **Normal: 7 (12%)** **Belly sliderss: 46 (88 %)**	*Austrolebias periodicus* "Don Pedrito" F1 Collected 16/12/2006 Wet 25/07/07" **"with 2 cm substrate" (80% peat and 20% clay soil) With aeration and filling of blow with dechlorinated running water** Incubation: more than 7 months (excessive) Peat humidity: very wet almost saturated pH: 7.4 Water temperature: 10oC Air temperature: 11oC Total eggs on to hatch: not counting Total births: 57 **Normal: 16 (28 %)** **Belly sliderss: 41 (72 %)** Dead: 1 to 48 hs

Case 9a	Case 10a

Austrolebias nigripinnis "Punta Lara" F1 Collected 24/09/2006 Wet 29/07/07 **with 5 cm substrate (80% peat plus 20% original biotope soil) With aeration and filling of blow with dechlorinated running water** Incubation: more than 10 months (excessive) Peat humidity: very wetalmostsaturated pH: 7.0am to 48pm pH: 6.0pm Wet water temperature: 32oC Stabilized water temperature at: 26oC Air temperature and incubated peat: 11oC Total eggs to hatch: not counting Total births:8 **Normal: 7 (87,5%)** **Belly sliderss: 1 (12.5 %)** Very vigorous and healthy	*Austrolebias nigripinnis* "Punta Lara" F1 Collected 24/09/2006 Wet 29/07/07 **with 7 cm of substrate (80% peat plus 20% original biotope soil) With aeration and filling of blow with dechlorinated running water** Incubation: more than 10 months (excessive) Peat humidity: very wet almost saturated pH: 7.0am to 48pm pH: 6.8pm Wet water temperature: 13oC Water temperature stabilized at: 13oC Air temperature and incubated peat: 14oC Total eggs to hatch: not counting Total births:30 **Normal: 19 (63%)** **Belly sliderss: 11 (36 %)** A dead fry trapped 1 cm from the compact bottom.

Case 11a	Case 12a
Austrolebias nigripinnis "Punta Lara" F1 Collected 24/09/2006 Wet 3/08/07 **without dirt or peat. With aeration and filling of blow with dechlorinated running water** Incubation: more than 10 months (excessive) Peat humidity: very wet almost saturated pH: 7.2 -7.4 Wet water temperature: 25oC Air temperature and incubated peat: 11oC Total eggs hatched:12 At 24 hs only 2 fry and belly sliderss were born, theego were suddenly passed to otherconditions: hs Ph: 6 (acidified with phosphoric acid) Water temperature: 12oC and return to bath until 26oC C02: 15 ppm? **After 24 hours it is observed:** C02: 5 ppm Total births: 9 **Normal: 0** **Belly sliderss: 7** **Dead: 2**	*Austrolebias nigripinnis* "Punta Lara" F1 Collected 24/09/2006 Wet 3/08/07 **with 1 cm of original biotope soil.** **With aeration and filling of blow with dechlorinated runningwater, the eggs were placed superficially, not buried.** Incubation: more than 10 months (excessive) Peat humidity: very wet almost saturated pH: 7.2am to 24pm pH: 7.0pm Wet water temperature: 25oC Stabilized water temperature at: 25oC Air temperature and incubated peat: 11oC Total eggs to hatch: 12 at 24 hs. 2 belly sliderss and 2 dead weredisplayed. Then returned to gradual cold water up to 12oC Total births:4 **Normal: 0 (0%)** **Dead: 4(100%)**

Case 13a	Case 14a
Austrolebias nigripinnis "Punta Lara" F1 Collected 24/09/2006 Wet 3/08/07 **without dirt or peat. With aeration and filling of blow with dechlorinated running water** Incubation: more than 10 months (excessive) Peat humidity: very wet almost saturated pH: 7.2 -7.4 Wet water temperature: 12oC Air temperature and incubated peat: 12oC After 1hs put in water bath until the temperature is stabilized at 25oC in 15 minutes. At 2 a.m. the day after they began to be born (neverso inflated cone 14a) Total eggs hatched:12 At 6 hs were hs born 4 belly sliders fry, at 24 hours 6 belly sliderss and 5 somewhat inched eggs were displayed.. Ph: 4 (acidified with phosphoric acid) Total births: 6 **Normal: 0** **Belly sliderss: 6**	*Austrolebias nigripinnis* "Punta Lara" F1 Collected 24/09/2006 Wet 3/08/07 **with peat water from another aquarium with *A. overcrowded nigripinis.* No aeration and no dirt or mob** Incubation: more than 10 months (excessive) Peat humidity: very wet almost saturated pH: 7.2 KH: 0.95 od (very soft water) GH: 2.24 od (very soft water) C02: approx. 8 ppm Wet water temperature: 13oC Stabilized water temperature at: 13oC Temperature aire and peat incubated: 13oC Total eggs hatched: 9 at 2 hs all overly inflated eggs are observed, started to be born and died immediately Total births:9 **Normal: 0** **Dead: 8** **Only one egg wasn't born.**

Case 15a	Case 16a
Austrolebias nigripinnis "Punta Lara" F1 Collected 24/09/2006 Wet 3/08/07 **without dirt or peat. With aeration and filling of blow with water Distilled** Incubation: more than 10 months (excessive) Peat humidity: very wet almost saturated pH: 7.0 KH: 0.1 od (excessively soft water) GH: 0 od (excessively soft water) C02: 0 ppm Wet water temperature: 13oC Air temperature and incubated peat: 13oC After 1hs put in water bath until the temperature is stabilized at 25oC in 15 minutes. At the following 2 hours, no inflated births or eggs were observed.	*Austrolebias nigripinnis* "Punta Lara" F1 Collected 24/09/2006 Wet 3/08/07 **with DESTILATED water. With aeration (4.2 cm of substrate composed of 80% new peat plus 20% original biotope soil) Shake.** Incubation: more than 10 months (excessive) Peat humidity: very wet almost saturated pH: 6.3 KH: 0.15 od (very soft water) GH: 0.84 od (very soft water) C02: approx. Less than 5 ppm Wet water temperature: 9oC Water temperature stabilized at: 9oC Air temperature and incubated peat: 11oC Total eggs hatched: 12 at 48 hs are observed 7 normal and very still, look somewhat intoxicated or perhaps

Here 2 cm of biotope soil was added and 24 hours later 3 normal fry are born. Total eggs to hatch: 9 **Normal: 3** **Belly sliderss: 0** **Dead: 1**	caused by the cold. Total births displayed:7 **Normal: 7** **Belly sliderss: 0** **Dead: 0**