Dissolved oxygen saturation is crucial for gas bladder inflation in turquoise killifish (*Nothobranchius furzeri*)

Jakub Žák · **Abhishek Nair Anil** · **Iva Dyková**

Abstract Providing optimal conditions for early-life gas bladder inflation of captive fish is one of the biggest challenges in fish culture. It also applies to laboratory fishes. Turquoise killifish (*Nothobranchius furzeri* Jubb, 1971) is a popular research model in biogerontology due to its short lifespan. Annual killifish in laboratory culture frequently suffer from an inability to inflate their gas bladder which may stem from suboptimal environmental conditions in captivity. Here, we investigate (1) the effect of dissolved oxygen (DO) saturation and (2) access to the water surface on gas bladder inflation and hatching success of turquoise killifish. We further histologically examine the gas bladder development from its primordial form to full inflation. In accordance with physoclistous nature of turquoise killifish, access to the water surface is not necessary for gas bladder inflation. We found that hatching success was highest in the treatment with constant or decreasing DO saturation. In contrast, the highest proportion of larvae with inflated gas bladders was found in the treatment with DO oversaturated water (130%) which was induced by the addition of an oxygen tablet. Larvae inflated their gas bladders within 2 to 48 h post-hatching. These findings represent a major step toward a solution to a persistent problem in laboratory culture of this increasingly important model organism.

Keywords Air bladder · Swim bladder · Hatching conditions · Laboratory fish · Sinker syndrome · Belly slider

Introduction

The gas bladder is a multifunctional organ that plays a crucial role in body buoyancy, hearing, sound production, and gas exchange in some fishes (Blaxter and Tytler 1978; Lumsden 2006). Its inflation is therefore a critical developmental step for proper functioning in the aquatic environment (McCune and Carlson 2004; Woolley and Qin 2010). Fishes have a limited time window during ontogeny when they can inflate their gas bladder (Marty et al. 1990; Jacquemond 2004). Inflation is achieved through a permanent (physostomi) or temporal (euphysostomi) connection of the digestive tract with the gas bladder or through gas production by specialised glands (physoclisti) (Lumsden 2006). Individuals with non-inflated gas bladders suffer from high mortality and deformities and only rarely survive in the natural environment (Goolish et al. 1999; McCune and Carlson 2004; Czesny et al. 2005). Gas bladder non-inflation decreases survival and general performance in

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laboratory fish (Jacquemond 2004; Czesny et al. 2005; Pandelides et al. 2021).

Optimal environmental conditions during embryonic development and hatching are necessary for proper gas bladder inflation (Wourms 1967; Marty et al. 1995; Goolish et al. 1999; Woolley and Qin 2010). Oily films on the water surface, overstocking, genetic mutations, and infections are among factors contributing to gas bladder non-inflation in captivity (Marty et al. 1995; McCune and Carlson 2004; Czesny et al. 2005). Thus, gas bladder non-inflation is a frequent challenge in aquaculture (Woolley and Qin 2010) and in fish research facilities (Marty et al. 1990; Goolish and Okutake 1999; Podrabsky 1999; McCune and Carlson 2004; Pandelides et al. 2021). Physostomous species and physoclistous species, which have an interconnection (ductus pneumaticus) of the gas bladder with the digestive tract early in life, typically require access to the water surface soon after hatching to inflate their gas bladder by gulping air bubbles (Woolley and Qin 2010; Pandelides et al. 2021). In true physoclistous species, the ductus pneumaticus is absent throughout their whole post-hatching life. They do not require access to the water surface but dissolved oxygen saturation seems to be crucial for gas bladder inflation (Doroshev and Cornacchia 1979; Perlberg et al. 2008). How access to the water surface and dissolved oxygen (DO) contribute to gas bladder inflation in some model fish species, including turquoise killifish *Nothobranchius furzeri* Jubb, 1971, is an unresolved question.

The turquoise killifish is a research organism with increasing importance in various fields of life sciences including aging research (Cellerino et al. 2016; Hu and Brunet 2018; Reichard and Polačik 2019; Thoré et al. 2021). The turquoise killifish gas bladder is a single-chamber organ with anteriorly positioned capillary system and a gas gland (Dyková et al. 2022). Unlike during the larval stages of zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), where a link between the digestive tract and gas bladder is initially present (ductus pneumaticus; Goolish and Okutake 1999; Marty et al 1990), the turquoise killifish lacks this connection even during the larval stage (Dyková et al. 2022). This anatomic prerequisite means that turquoise killifish do not need atmospheric air to fill their gas bladder, but this has not been verified experimentally yet.

Environmental cues control the synchronisation of embryonic development and hatching of turquoise killifish in its natural environment, ephemeral pools of African savanna (Vrtílek et al. 2018; Reichard and

Polačik 2019; Polačik et al. 2021). They hatch from embryos buried in a mud substrate after pools are flooded during the rainy season. Flooding leads to a decrease in oxygen availability for embryos in comparison to atmospheric air in the semi-dry conditions of the substrate (DiMichele and Powers 1984). It was found in various aquatic vertebrates that a decrease in oxygen availability facilitates hatching (DiMichele and Powers 1984; Rogge and Warkentin 2008). This condition has the potential to facilitate a higher hatching rate in laboratory reared annual killifish without detrimental physiological effects (Podrabsky and Wilson 2016). Nonetheless, there are still knowledge gaps in the effects of environmental conditions on the initial life stages of laboratory-reared turquoise killifish, and frequent occurrences in research facilities of larvae without inflated gas bladders remain to be explained (Wourms 1967; Podrabsky 1999; Peres da Fonseca et al. 2018).

Proper understanding of how environmental conditions are linked to gas bladder inflation and hatching of turquoise killifish has direct consequences for improving laboratory culture of this species. Here, turquoise killifish access to the water surface and (DO) saturation are investigated in relation to gas bladder inflation and hatching success. We hypothesised that access to the water surface is not essential for gas bladder inflation. Instead, higher DO saturation is predicted to positively contribute to gas bladder inflation because turquoise killifish lack a ductus pneumaticus throughout their entire post-hatching life. In contrast, lower DO saturation is predicted to positively influence the hatching success of turquoise killifish.

Material and methods

Nothobranchius furzeri (strain MZCS 222; Cellerino et al. 2016) embryos ($N = 372$) from multiple parental pairs were incubated in peat moss at 18 °C for at least 6 months in a dormant state (Diapause II; Polačik et al. 2016). To continue their development, they were removed from the peat substrate and disinfected. Disinfection consisted of three successive 5-min baths in 2‰ peracetic acid solution (0.5 mL of 5% peracetic acid in 999.5 mL of reverse osmosis (RO) water), with a rinse in RO water between baths. They were then placed in six 10-cm plastic

Petri dishes with moist filtration paper (two layers covering the dish bottom, one layer covering the embryos). Capped Petri dishes were placed in a 3-L sealed plastic airtight container and incubated for another 4 weeks at 28 °C to complete their embryonic development and reach the pre-hatching state (Polačik et al. 2016).

Hatching was initiated by placing the embryos in cool 17 °C water as recommended by standard protocol (Polačik et al. 2016). Water temperature was decreased to 17 °C by the addition of ice into 27 °C water. Water for hatching was mixed from RO water and tap water, 1:1 (resulting in conductivity 200 $\mu S \times cm^{-2}$ measured by Dist EC/TDS meter, Hanna Instruments, USA), 2 days prior to watering the embryos.

Ready-to-hatch embryos were separated into three treatment groups to investigate the role of environmental oxygen and access to atmospheric air in gas bladder filling in hatchlings. The three treatment groups were: (1) no access to the water surface ($N = 120$), set up by placing a single embryo in a 25-mL sample container (APTACA, 14120, Italy) filled with water and the lid tightened (wearing surgical gloves) under water to prevent generation of bubbles, (2) access to water surface and no oxygen tablet ($N = 126$), set up by placing embryos individually in 25 mL of water in 50-mL flat-bottom centrifuge tubes (P-Lab, R.XH99), and (3) access to water surface and oxygen tablet ($N = 126$) treatment. The embryos were placed individually in 25 mL of water in 50 mL flat-bottom centrifuge tubes with an 8-mm hole in the wall enabling access to atmospheric air. Increased DO saturation was achieved through the addition of a 6.97-mg piece (range 5.93–8.75 mg) of commercial oxygen tablet used in the ornamental fish trade ($Na_2CO_3 \times 1.5H_2O_2$; Oxytabs, JBL, Germany, manufacturer's recommended dose: 1 tablet (1.5 g) per 10 L) through the 8-mm hole. The piece of tablet was added 6 h after placing the embryo in the tube irrespective of the hatching status of the embryo.

Additionally, the effect of postponed administration of the oxygen tablet in the treatment without access to the water surface was examined. An oxygen tablet piece was added at 24 hpw (hours post watering = hours from placing embryos into water) to a subsample ($N = 52$) of hatched larvae immediately after moving them to 50-mL tubes with an 8-mm hole. The proportion of swimming larvae in this subsample was scored 4 h (28 hpw) and 24 h (48 hpw) after addition of the tablet.

Embryos were submerged in 17 °C water in the morning in an air-conditioned room with low light intensity (single 15 W light bulb in a 15 m^2 room) and air temperature 28 °C. Water in the tubes was not replaced over the course of the experiment and was not aerated because there was no risk of hypoxia (see Fig. 1A). Groups of 8–9 tubes were placed upside down into 46 plastic 2-L containers with a 3-cm water bath to maintain similar water temperature among tubes. Water temperature was measured with a glass thermometer (JBL) with precision of 1 °C at 2, 4, 6, 8, 10, and 24 hpw. Dissolved oxygen saturation in the water was measured in 60 additional tubes. A subsample of tubes without embryos ($N = 6$, water temperature = 17 °C), was used for measurement of the initial DO saturation. The remaining 54 tubes contained embryos in a pre-hatching stage, and DO saturation was cross-sectionally measured in 6 tubes per treatment at 2 hpw (water temperature during DO measurements = 25.6 °C), 6 hpw (25.0 °C), and 8 hpw (25.0 °C). All tubes for DO measurements were in a single water bath. Dissolved oxygen saturation (%) was measured for 3 min after 8-min calibration using an Oxygen Sensor Spot (SP-PSt3-NAU-D5YO; PreSens GmbH, Germany) and logged in Auto Resp v 2.1.3. software (Loligo Systems, Denmark).

Embryo hatching status and gas bladder inflation were visually checked at 2, 4, 6, 8, 10, and 24 h postwatering. Larvae with inflated and non-inflated gas bladders were distinguished based on their position in the water column inside the tube where they hatched. Larvae with an inflated gas bladder swam normally in the water column. Larvae with non-inflated gas bladders were performing surfacing behaviour (repeated attempts to reach the surface) or were laying on the bottom of the tube. The validity of this approach was confirmed by histological analysis.

A subsample of larvae ($N = 13$) was histologically examined to describe any anatomic peculiarities of the transition from non-inflated to inflated gas bladder. Haphazardly selected larvae were euthanised and fixed with 2% osmium tetroxide at 8 and 24 hpw, dehydrated in a graded series of acetone, and embedded in Spurr resin. Semi-thin parasagittally oriented sections through the entire larval body were stained with toluidine blue. Histological images were edited
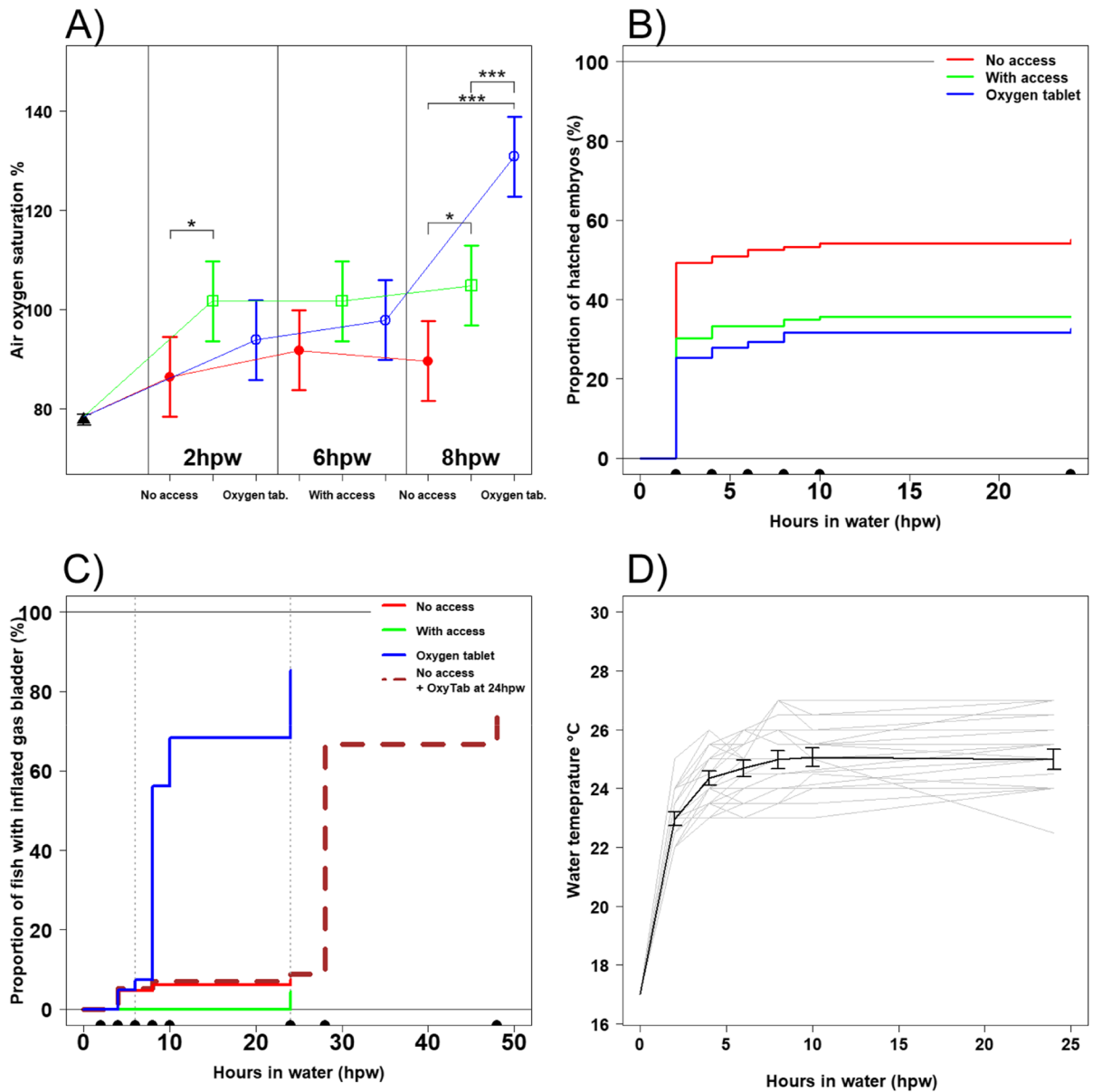
Fig. 1 Hatching progress, gas bladder inflation, and environmental parameters during the experiment on early life stages of *Nothobranchius furzeri*. **A** Treatment-specific DO saturation. The column with 8 hpw shows values 2 h after the addition of an oxygen tablet in oxy tab treatments (blue). Red represents treatments with no access to the water surface. Green represents treatment with access to the water surface and no addition of oxygen tablets. Dots show means and error bars are at 95% confidence intervals (CI). * Corresponds to significant difference $p < 0.05$; *** corresponds to significant difference $p < 0.001$. **B** Temporal pattern of the hatching success of tur-

quoise killifish embryos in relation to experimental treatment. **C** Time progress of gas bladder inflation success in relation to treatment. The vertical grey lines represent points in time when the oxygen tablets were added. **D** Thermal regime during the first 24 hpw. The black line represents average. The water temperature is lower than the room temperature (28 °C) due to the evaporation effect of air circulation in the room. Error bars represent 95% CI. Grey lines are container-specific temperature measurements. Black semi-circles on the x-axis of B) and C) represent times when the larvae were checked for hatching and gas bladder status

for consistency of brightness and contrast and converted into grey scale using Corel Draw software.

Larvae were not fed over the course of the experiment. At the end of the experiment, all unhatched embryos were discarded, and normally swimming larvae were used for breeding purposes at the Institute of Vertebrate Biology, Czech Republic.

Data analysis

The proportion of larvae with an inflated gas bladder among treatments was compared for hatched fish ($N = 152$) using binomial GLMM with *gas bladder inflation* (1, inflated; 0, non-inflated) as a response variable and *treatment* (3 levels) as predictor. *Container ID,* where the tubes were kept (46 levels), was treated as a single random factor because *experimental day* (5 levels) did not improve model fit ($\Delta AIC < 2$). Time progress in accumulative number of embryos with an inflated gas bladder (normally swimming larvae) was explored using Kaplan–Meier plots. We did not apply statistical modelling due to the irregular frequency of checks between treatments. *Treatment*-dependent hatching success was analyzed using Cox mixed model (Coxme), and *container ID* nested within *experimental day* was added as random factor.

Oxygen saturation among treatments was compared at three time points (2, 6, and 8 hpw) by a single linear model (LM). *Oxygen saturation* was a response variable and *treatment* (3 levels) in interaction with *hpw* (3 levels) were used as predictors. Pairwise comparisons of interaction terms were performed by simultaneous contrasts within *hpw* only, in package *emmeans* v 1.5.4 (Lenth 2021). Initial (0 hpw) DO concentration is only plotted for visual comparison and not statistically compared with other time points. *Water temperature* comparison among experimental treatments was compared by LMM with predictors *treatment* and *measurement time* (continuous with cubic spline). *Container ID* nested within *experimental day* was treated as random factor.

Statistical analysis was done in the R v4.0.4 environment (R Core Team 2021) using *lme4* v 1.1.26 (Bates et al. 2015), *car* v3.0.10 (Fox and Weisberg 2019), *coxme* v2.2.16 (Therneau 2020), *survival* v3.2.7 (Therneau and Grambsch 2000), *splines* v4.0.4, and *emmeans* v1.5.4 (Lenth 2021) packages.

Results

Hatching success within 24 hpw was significantly higher in the treatment without access to the water surface (54% of hatched embryos) compared to the other treatments with access to the water surface (with access 35%, and prior oxygen tablet addition 30% respectively; Coxme, $X^2_2 = 16.06$, $p < 0.001$, Fig. 1B). Hatching peaked 2 hpw and nearly ceased at 10 hpw (Fig. 1B). The water dissolved oxygen saturation at 2 hpw inversely mirrored the differences in hatching probability between treatments, where the lowest DO saturation was in water within the treatment without access to surface atmospheric air (LM, no access vs. with access, t-ratio$_{45} = 2.71$, $p = 0.025$; no access vs. prior oxygen tablet addition, t-ratio$_{45} = 1.31$, $p = 0.394$, Fig. 1A).

Gas bladder inflation was not related to time from hatching but rather to oxygen tablet addition. The highest proportion of larvae with an inflated gas bladder (89%) was found in the treatment where an oxygen tablet was added at 6 hpw. Treatments without addition of the oxygen tablet had a very low proportion of larvae with inflated gas bladders (3% with access to the water surface, 5% without access to water surface, binomial GLMM, $X^2_2 = 19.89$, p < 0.001, Fig. 1C). Similarly, a high proportion of larvae with inflated gas bladders (79%, $X^2_1 = 3.32$, $p = 0.068$) was recorded in the treatment with the addition of an oxygen tablet at 24 hpw and previous absence of access to the water surface (Fig. 1C). Accordingly, the treatment with the addition of an oxygen tablet had a significantly higher DO saturation in the water at 8 hpw than other treatments (LM, oxy.tab-no access, t-ratio$_{45} = 7.29$, $p < 0.001$; oxy.tab-with access, t-ratio$_{45} = 4.59$, $p < 0.001$, Fig. 1A). The majority of larvae inflated their gas bladder within the first 4 h after the addition of the oxygen tablet irrespective of the timing of oxygen tablet addition (6 hpw vs 24 hpw; Fig. 1C). Thermal regime was similar for all experimental treatments (LMM, $X^2_2 = 0.009$, $p = 0.995$, Fig. 1D).

Light microscopy of semi-thin sections through the gas bladders clearly distinguished (1) inflated bladder (Fig. 2a) with tightly packed epithelial cells in the anterior and the voluminous thin-walled posterior cavity dominating (2) gas bladder in the transition phase from non-inflated to inflated gas bladder,
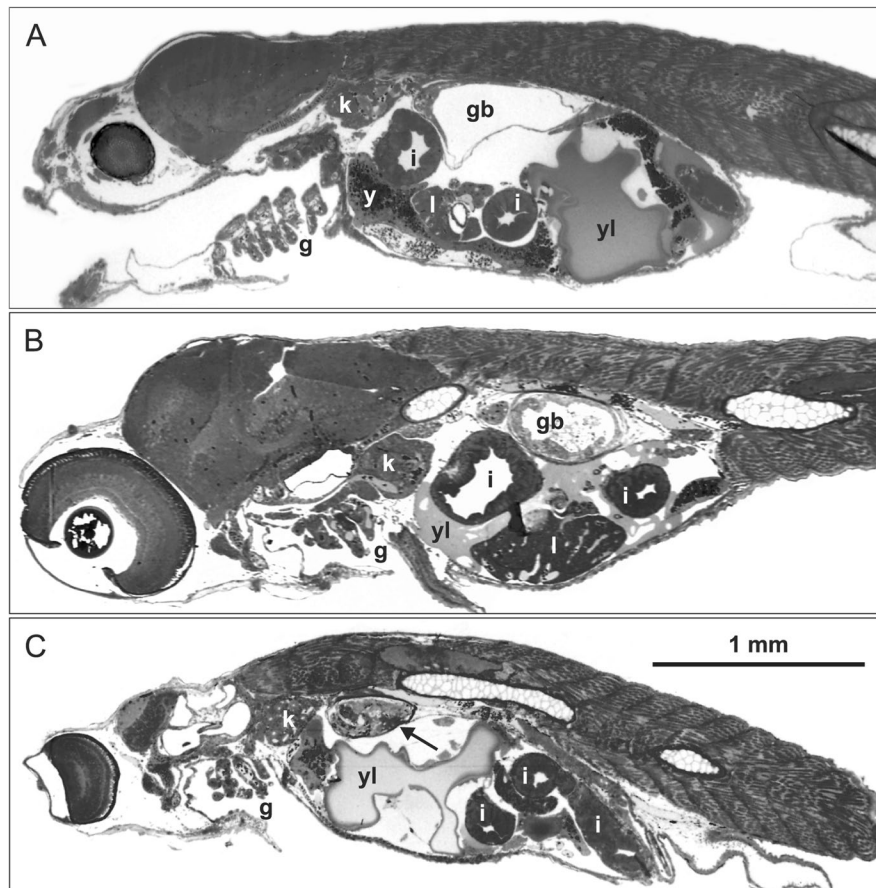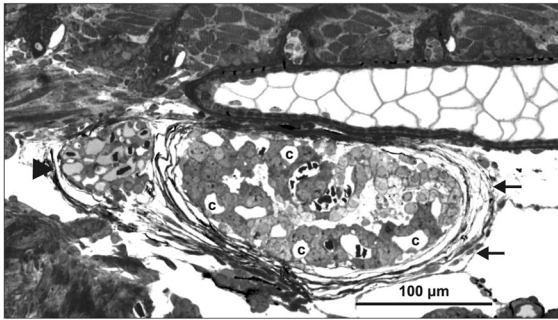
Fig. 2 Gas bladder of a turquoise killifish (*Nothobranchius furzeri*) larva examined at 8 to 24 hpw. **A** Parasagittal section through larval body with fully inflated gas bladder at 8 hpw. The anterior thick part of the gas bladder contains secreting and resorbing epithelial cells, whereas the thin-walled part of this organ after fixation remains as a large cavity (filled with gas in vivo). **B** Partially inflated gas bladder found in fish at 24 hpw. It has a relatively thick layer of large epithelial cells and a small cavity part with remnants of desquamated cells. **C** Non-inflated gas bladder (marked with arrow) seen as a cell cluster (see Fig. 3 for better detail). Fish sampled at 24 hpw. Scale bar is the same in A, B, and C. Internal organs are marked for better understanding of topology: gb, gas bladder; l, liver; i, intestine; g, gills; k, kidney; y, yolk sac with lipid droplets; yl, yolk liquid

gas bladder in this stage had a thick wall (Fig. 2b) consisting of large epithelial cells with large nuclei (resembling primordial epithelial cells found in non-inflated gas bladders as documented in Fig. 3) that surrounded a small, probably still inflating gas bladder cavity, and (3) non-inflated gas bladder (Figs. 2c and 3) containing a mass of large epithelial cells permeated by capillaries, and the thin-walled cavity part was completely absent. The gas bladder was clearly discernible in all histological slides from examined larvae as early as the first sampling point at 8 hpw.

Discussion

Environmental conditions significantly affected hatching and gas bladder inflation success in turquoise killifish larvae. Administration of an oxygen tablet led to an increase in water dissolved oxygen saturation and was crucial for turquoise killifish gas bladder inflation. We histologically identified the transition from the primordial gas bladder through its enlargement, cellular reorganisation, and inflation. Hatching success was highest in the treatment without access to the

Fig. 3 Structure details of non-inflated gas bladder of turquoise killifish (*Nothobranchius furzeri*). No cavity was observed in a series of semi-thin sections through the gas bladder shown in Fig. 2C. The semi-thin section shows several layers of connective tissue (marked with arrows) which envelopes a cell cluster permeated by blood capillaries (marked with c). The anterior is marked with an arrow and contains a capillary network structure, important for proper function of the gas bladder

water surface where DO concentration was constantly lower than in the other two treatments. Turquoise killifish larvae were able to inflate their gas bladder at any time in the first 48 h after hatching when environmental conditions were favourable. These findings significantly improve our understanding of the factors influencing hatching and early post-hatching performance of this model organism and have significant consequences for its laboratory husbandry.

High water oxygen saturation was previously hypothesised as a prerequisite for gas bladder inflation in some physoclistous species (Doroshev and Cornacchia 1979; Perlberg et al. 2008). This was confirmed in *Oreochomis mosambicus,* where the lowest successful gas bladder inflation was found in the treatment with low dissolved oxygen saturation (Doroshev and Cornacchia 1979). In our study, the administration of an oxygen tablet produced DO oversaturation up to 177% and contributed to gas bladder inflation of turquoise killifish larvae. The proportion (> 70%) of normally swimming larvae after oxygen tablet administration (irrespective of hpw) is in the upper range reported for normally swimming larvae of laboratory annual killifish (Calvino et al. 2007; Peres da Fonseca et al. 2018). Our experimental findings thus confirmed the importance of DO saturation for gas bladder inflation in turquoise killifish.

Inflation of the gas bladder in true physoclistous species is enabled by gas transport from the blood

through the capillary network and by gas secreting cells in the walls of the gas bladder (Blaxter and Tytler 1978). In addition to gill respiration, a relatively high proportion of gas transport in small larval fishes is achieved through their body surface (Foscarini 1989; Rombough 2002). Increased DO concentration in the water enables easier transfer of oxygen to the bloodstream of the larvae and its subsequent transport to the gas bladder. Oxygen is an important component in the gas bladder because here it is in higher concentration compared to the blood vessels and aquatic environment (Blaxter and Tytler 1978). This cascade may explain why increased DO saturation contributed to successful gas bladder inflation in our experiment. In the wild, oxygen oversaturation up to 154% (supporting initial gas bladder inflation) is common in shallow ephemeral pools (Meintjes et al. 1994) and is further supported by intense precipitation coinciding with killifish hatching (Reichard et al. 2017).

The time window for gas bladder inflation varies considerably among species. For example, the medaka normally inflates its gas bladder within 24 h post hatching and 5% of fish are still able to inflate their gas bladder after a 9-day access restriction to the water surface (Pandelides et al. 2021). Eurasian perch (*Perca fluviatilis*) are able to inflate their gas bladder up to a month after hatching (Jacquemond 2004). Here, we demonstrate that provided the water is sufficiently oxygen saturated, turquoise killifish can inflate their gas bladder during the first 2 days after hatching. Future studies may investigate the maximum time restriction from oxygenated water until turquoise killifish are unable to inflate their gas bladder. Outcomes of the present study demonstrate that oxygen supplementation is an essential step in the first day or two after killifish hatching.

Our finding regarding the importance of DO saturation for gas bladder inflation is in accordance with procedures recommended in hatching protocols for turquoise killifish. These protocols acknowledge the importance of proper water oxygenation for successful raising of killifish larvae (Polačik et al. 2016; Dodzian et al. 2018). An alternative to oxygen tablets is to increase DO saturation by aerator (Harel et al. 2016; Dodzian et al. 2018). Support of water oxygenation should be done carefully because too strong aeration may decrease the success of gas bladder inflation (Battaglene and Talbot 1993) and

diluting a larger piece of oxygen tablet than recommended by the manufacturer may poison the fish.

Available husbandry protocols for *Notho-branchius* killifish recommend hatching embryos in shallow water (Polačik et al. 2016; Dodzian et al. 2018; Muck et al. 2018; Philippe et al. 2018; Astre et al. 2022). Annual killifish perform surfacing behaviour after hatching and may appear like they want to gulp air from water surface (Calvino et al. 2007; Polačik et al. 2016). This behaviour likely serves as a mechanism for reaching oxygen-rich surface layers to support gas bladder inflation. A low water level means a high water-surface-to-volume-ratio that facilitates oxygen permeation into the water. The effect of different water levels for hatching and gas bladder inflation success remains to be tested.

Histological analysis was performed at a point in time when larvae were still able to inflate their gas bladder (8 and 24 hpw). All larvae had an already discernible gas bladder. In annual killifish, the gas bladder develops in the pre-hatching stage (Wourms 1972). This is in contrast to some other physoclistous species such as *Pterophyllum scalare*, where the gas bladder is not discernible until 1 day after hatching (Perlberg et al. 2008). In accordance with a previous histological study (Dyková et al. 2022), no connection of the gas bladder with the digestive tract was found in turquoise killifish. This is despite evidence from another annual killifish, *Austrofundulus myersi*, where this connection is present during the embryonic phase (Wourms 1972). The connection likely disappears in turquoise killifish prior to the onset of the gas bladder inflation process, as is the case for another true physoclistous species, *Hemichromis bimaculata* (McEven 1940). The histological structure of the turquoise killifish's inflated gas bladder at 24 h post-hatching is similar to the gas bladder in 11-day-old juveniles (see Dyková et al. 2022 for details). This finding suggests that the gas bladder develops fast under suitable environmental conditions. We did not observe any degenerative changes of the gas bladders in our histologically investigated subsample. However, previously degenerative changes were identified in annual killifish with non-inflated gas bladders (Peres da Fonseca et al. 2018). We cannot exclude that gas bladder degeneration can occur in turquoise killifish later than within the first 24 h of inability to inflate the gas bladder.

Hatching success was dependent on treatment setup which differed in DO saturation. Presented hatching success (20–50%) is in accordance with the variable hatching success observed in our facility (Polačik et al. 2016). We demonstrated that nearly twice as many embryos hatched in the treatment with no access to the water surface where DO saturation (around 79–86%) was constant. Maybe the decrease of oxygen partial pressure caused by submerging the embryo in water (from aerial conditions of semi-dry peat or filtration paper) together with constant or slightly decreasing DO saturation of water was a hatching trigger. On the other hand, DO saturation of 79–86% is within normal range (corresponding to concentration of 7.0–7.8 mg×L^{-1}); therefore, it is hard to ascribe lower DO concentration to higher hatching success (Lieke et al. 2021). The slight increase of DO saturation in treatments with access to water surface is likely related to oxygen partial pressure equalisation between atmosphere and water (known as the Henry's law). Previously, it was found in another killifish, *Fundulus heteroclitus*, that hatching is suppressed when there is an oxygen supply, which covers the metabolic costs of the embryo (DiMichele and Powers 1984). This may be the reason why lower hatching success was observed in the two treatments where a slight increase in DO saturation was observed.

To improve hatching success, some turquoise killifish protocols suggest additions of peat extract (Genade et al. 2005) and, more recently, humic acid (Dodzian et al. 2018). How the addition of humic substances influences the early life of turquoise killifish needs to be further investigated. However, it was found that at moderate concentrations they accelerate hatching in zebrafish (Lieke et al. 2021). It should be noted that whether or not lower DO concentration contributes to hatching, it cannot persist for long in the hatching water because it would likely prevent gas bladder inflation of turquoise killifish.

The conflicting roles of oxygen concentration in the early life of turquoise killifish (low DO hatching, DO oversaturation-inflation) reflect the natural conditions of ephemeral savanna pools. Embryos of annual killifish in the wild supress their hatching by developmental dormancy in the substrate until inundation (Polačik et al. 2021). A sudden flooding event may lead to a decrease in oxygen partial pressure, which stimulates hatching (DiMichele and Powers 1984).

Besides an immediate decrease in oxygen partial pressure after substrate flooding, the oxygen concentration in the substrate surrounding an embryo would undoubtedly continue to decrease due to the bacterial decomposition process of the organic matter at the pool bottom. Killifish embryos hatch within 1–3 days in the wild (Vrtílek et al. 2018); thus, they probably cannot escape the drop in substrate oxygen availability. After hatching, the larvae work their way through the water-softened muddy substrate to reach the water column (Calvino et al. 2007) with oxygen-rich rainwater (Meintjes et al. 1994), which provides ideal conditions for swim bladder inflation.

Conclusion

Knowledge of laboratory conditions supporting hatching and initial gas bladder inflation is essential for successful breeding of laboratory fish. In the presented experimental setup, the DO saturation seems to be crucial for successful gas bladder inflation of turquoise killifish. This is supported by similar findings in other true physoclistous species and common laboratory practice of hatching turquoise killifish in aerated shallow water. Constantly DO undersaturated water (or low DO saturation) supports embryo hatching, and high DO saturation is necessary for swim bladder inflation. Such contrasting DO concentrations are in accordance with conditions faced by pre-hatching embryos and larvae in the wild. Embryos are buried in DO poor substrate, and the larvae hatch into a freshly rainfed and DO supersaturated pool. The presented empirical findings may help to increase and stabilise the laboratory production of annual killifish.

Declarations

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