REVISTA ESPAÑOLA DE FISIOLOGIA, 44 (3), 323-330, 1988

Oxygen Regulation Capacity in *Discoglossus pictus* Tadpoles Between Moderate Hyperoxia and Acute Hypoxia in Water

G. Barja de Quiroga and M. Alonso-Bedate

Departamento de Fisiología Animal
Facultad de Biología
Universidad Complutense
28040 Madrid (Spain)

(Received on May 16, 1988)

G. BARJA DE QUIROJA and M. ALONSO-BEDATE. *Oxygen Regulation Capacity in* Discoglossus pictus *Tadpoles Between Moderate Hyperoxia and Acute Hypoxia in Water.* Rev. esp. Fisiol., 44 (3), 323-330, 1988.

The oxygen dependence of aquatic oxygen consumption was measured in active and anesthetized stage XVIII *Discoglossus pictus* tadpoles (Amphibia, Anura). The active tadpoles are good oxygen regulators in moderate hyperoxia and moderate hypoxia, whereas they are oxygen conformers in acute hypoxia. Critical oxygen pressure was 52 mmHg O_2. Anesthetizing the larvae changes them to perfect oxygen conformers between moderate hyperoxia and moderate hypoxia (249-63 mmHg O_2). At stage XVIII the aquatic respiratory organs are still capable of producing oxygen regulation when free access to air is denied. The marked capacity for oxygen regulation in *D. pictus* tadpoles is concordant with the strong hypoxic environments in which these animals usually live in nature.

Key words: Oxygen consumption, Hypoxia, Hyperoxia.

The bimodal respiration of amphibious vertebrate is receiving increasing attention recently (5, 9, 8) because of its need from a complex respiratory regulation, the strongly different physico-chemical characteristics of water and air as respiratory environments, and because its understanding can contribute to clarify how the transition from water to air breathing occurred during vertebrate evolution. The amphibian tadpoles constitute an ideal model since they interchange gases through three respiratory organs: skin, lungs and gills. Nevertheless the more complete works of this kind have been conducted in the extraordinarily great (around 5 g of wet weight) tadpoles of *Rana catesbeiana* (5, 6, 15) due to limitations imposed by size. The aim of this work was to ascertain if some of these conclusions were also valid for amphibian tadpoles of a moderate and more common size (around 350 mg of wet weight) as in *Discoglossus pictus.*

Amphibian tadpoles are frequently exposed in nature to very different oxygen

* To whom all correspondence should be addressed.

tensions (pO_2). Thus, diurnal variations of water pO_2 from very low values to levels as high as 450 mmHg have been measured in pools and ponds were amphibians live (7). The amphibian tadpoles can compensate these variations by modulating both lung and gill ventilation. The predominant role of lung ventilation in O_2-regulation has been recently reported at stages XVI-XIX in *R. catesbeiana* tadpoles (5). Nevertheless, it would be interesting to know if at these stages amphibian tadpoles can also compensate pO_2 water variations just by changing gill ventilation. This would be advantageous since the risk of predation strongly increases when the animals swim to the surface in hypoxic waters for a breath. Thus, we studied the O_2 consumption ($\dot{V}O_2$) of stage XVIII *D. pictus* tadpoles at a wide range of water pO_2 (from acute hypoxia to moderate hyperoxia) without air phase so that lungs cannot function as respiratory organs. The confining of stage XVIII tadpoles to an absolutely aquatic medium must not be very stressing since at this stage aquatic O_2 uptake (skin plus gills) still supposes around 90 % of total $\dot{V}O_2$ in *R. catesbeiana* tadpoles with access to air (6). Experiments were performed in active and in anesthetized animals as a first approach to the undestanding of the reflexes involved in oxygen regulation in amphibian larvae. *D. pictus* was the species selected as it normally lives in chronically hypoxic (70-30 mmHg) natural ponds (2).

Materials and Methods

Animals. — Experiments were performed in tadpoles from the same offspring obtained in the laboratory in March. Parental *Discoglossus pictus* adults were captured around Madrid and injected with 300 units of human chorionic gonadotropin (Physex-Leo) to induce ovulation, spawning and mating behaviour. The eggs were developed in dechlorinated water at 20 ± 2 °C in natural photoperiod and fed *ad libitum* with boiled spinach. Food was supplied every day between 10 and 11 hours a.m. Developmental stages were determined according to the series of TAYLOR and KOLLROS (13) for *Rana pipiens.*

$\dot{V}O_2$ measurements. — Aquatic routine aerobic metabolism of *D. pictus* stage XVIII tadpoles was measured in undisturbed spontaneously active animals. Aquatic oxygen consumption ($\dot{V}O_2$) was measured in 300 ml glass respirometers completely filled with water so that bobbing behaviour and therefore lung ventilation was completely avoided. The respirometers were round-bottomed flasks fitted with rubber stoppers. The aquatic $\dot{V}O_2$ of active tadpoles was measured after transferring the animals to the respirometers previously filled with water of a given O_2 tension which was attained by bubbling the water with N_2. The number of tadpoles per respirometer was always three. All the experiments were initiated 30 min after the transference to let the larvae become accustomed to the respirometers. The temperature was held constant at 20 °C by immersing the flasks in a constant temperature water bath. The tadpoles were not fed for 24 hours prior to the measurement in order to minimize the effect of the specific dynamic action (SDA) of food upon $\dot{V}O_2$.

At the beginning of each measurement the oxygen tension was tested with an EIL electrode and O_2 meter and the respirometers were closed with the stoppers and sealed with paraffin. One hour later the respirometers were opened and the O_2 tension of the water was measured again. The height of the respirometers was similar to that of the O_2 electrode in orden to avoid erroneous measurements due to O_2 stratification. This is specially important as our method for measuring $\dot{V}O_2$ does not include shaking the water inside the respirometers which would disturb the animals and would increase $\dot{V}O_2$ very sig-

nificantly. The maximal increase in CO_2 tension observed did not exceed 1.2 mmHg. Control respirometers without tadpoles did not show significant changes in O_2 tension throughout the experiments. The O_2 concentrations were calculated from oxygen tensions using the oxygen absorption coefficient at 20 °C - 31 ml/l. The values were expressed at STP. The aquatic $\dot{V}O_2$ was calculated from the decrease of O_2 concentration inside the respirometers, and it was considered to be representative of a routine metabolism since the tadpoles had space enough to swim spontaneously. The $\dot{V}O_2$ values were expressed in µl of O_2 (STP) per larva, per mg of wet weight or per mg of dry weight, per hour. Each measurement was repeated three times for every group of three tadpoles. The arithmetic mean of O_2 tension between the beginning and the end of each measurement was computed and taken as the mean oxygen tension for that measurement. The measurements were thus performed at 247, 150, 119, 90, 52 and 35 mmHg of mean oxygen tension. A total of 72 tadpoles were used and the number of tadpoles in each group of environmental oxygen tension was 12. Different animals were used to measure the $\dot{V}O_2$ at the various aquatic pO_2 in order to assure that the obtained values would represent truly acute (non-chronic) responses to the environmental O_2 tension. Thus, all the tadpoles respond to a direct change from chronic normoxia (151 mmHg O_2) to the pO_2 of exposition without previous experience of other O_2 tensions. The size of the tadpoles was statistically similar among the six groups of tadpoles.

When the $\dot{V}O_2$ measurements in active animals finished, the same six groups of 12 tadpoles were anesthetized with 1/10.000 MS-222 (ethyl 3-aminobenzoate methanosulfonate) and the measurements were repeated again at 20 °C. The anesthetic was also present at the same concentration in the water that filled the respirometers during the measurements. Each group of animals was always acutely exposed to the same aquatic pO_2 for the measurement of $\dot{V}O_2$ values when both active and anesthetized. Measurements were performed as for active animals except that the time of measurement was 2 hours instead of 1 hour. The mean water pO_2 at which $\dot{V}O_2$ was measured were 249, 146, 122, 93, 63, and 41 mmHg O_2 on this occasion. These values are similar to those obtained for active $\dot{V}O_2$ measurements. At the end of the measurements the animals were transferred to anesthetic-free fresh water and 100 % of them resumed normal activity in 10-30 min. When this had occurred they were weighed (wet weight) and placed on Petri dishes inside a stove in order to measure the dry weight by total dehydration.

Statistical analyses. — Results were computed as means ± SD. Data from anesthetized tadpoles were subjected to least-squares linear regressions, and correlation coefficients (r) and their levels of statistical significance were calculated. Variance analyses were computed for variations of $\dot{V}O_2$ values measured at different water pO_2. The test of Sheffé was used for comparisons between $\dot{V}O_2$ means measured at contiguous pO_2 values. The 0.05 level was chosen as the point of statistical significance throughout.

Results

Active animals. — The variations of aquatic $\dot{V}O_2$ values at 20 °C (standard routine metabolisms) were similar when expressed per larva, per mg of wet weight or per mg of dry weight (table I). The $\dot{V}O_2$ values per unit weight did not differ between moderate hyperoxia and normoxia. A very significant ($P < 0.01$) reduction of $\dot{V}O_2$ values was found when the oxygen tension fell from normoxia to moderate

Table I. *Effect of water oxygen tension on active routine metabolism of stage tadpoles.* Values are means ± S.D. n = 12 animals per mean. ** Significant difference ($p < 0.01$) between two means vertically contiguous in the table. T = 20 °C.

O_2 tension (mmHg)	μlO_2/tadpole·h	μlO_2/mg wet weight·h	μlO_2/mg dry weight·h
247	132.5 ± 10.2*	0.38 ± 0.02	4.08 ± 0.43
150	115.9 ± 3.0**	0.33 ± 0.04**	3.98 ± 0.36**
119	55.8 ± 3.2	0.13 ± 0.01	1.87 ± 0.12
90	58.0 ± 6.5	0.14 ± 0.03	1.81 ± 0.23
52	57.8 ± 5.4**	0.16 ± 0.01**	1.85 ± 0.08
35	25.0 ± 4.3	0.07 ± 0.01	0.81 ± 0.07

hypoxia (119 mmHg O_2) (the $\dot{V}O_2$ values in normoxia duplicated those of moderate hypoxia). Nevertheless, further reduction of the water oxygen tension did not cause additional decreases of $\dot{V}O_2$ values which remained constant from 119 mmHg pO_2 to values of water O_2 tension as low as 52 mmHg. When the water pO_2 was additionally reduced from 52 to 35 mmHg O_2 (acute hypoxia) a very significant 50 % decrease in all three $\dot{V}O_2$ values was detected. Thus, it can be summarized that the active routine metabolism of *D. pictus* stage XVIII tadpoles is independent of the water pO_2 in the range of moderate hyperoxia (150-247 mmHg O_2) and moderate hypoxia (119-52 mmHg) whereas decreases of water pO_2 from normoxia to moderate hypoxia and from moderate hypoxia to acute hypoxia cause drastic reductions of $\dot{V}O_2$.

Anesthetized animals. — When the above mentioned experiments were concluded, the tadpoles used were anesthetized with MS-222 and the $\dot{V}O_2$ was measured again. The results obtained for $\dot{V}O_2$ per tadpole, per mg both of wet weight and dry weight were absolutely different from those of active routine metabolism (table II). Now all the three $\dot{V}O_2$ values linearly decreased as water pO_2 was reduced except in acute hypoxia where no significant changes were detected between 63 and 41 mmHg for any of the three $\dot{V}O_2$ values. These decreases were very significant and led to $\dot{V}O_2$ values around 15 times greater at 249 than at 63 mmHg O_2. This linear dependence allowed to subject the $\dot{V}O_2$ data measured between 249 and 63 mmHg O_2 to least-squares analysis in order to obtain the corresponding regression lines. The equations and correlation

Table II. *Effect of water oxygen tension on the metabolism of anesthetized (MS-222) stage XVIII tadpoles.* Values are means ± S.D. n = 12 animals per mean. Significant differences between two means vertically contiguous in the table: * $p < 0.05$; ** $P < 0.01$; T = 20 °C.

O_2 tension (mmHg)	μlO_2/tadpole·h	μlO_2/mg wet weight·h	μlO_2/mg dry weight·h
249	59.5 ± 4.5**	0.169 ± 0.017**	1.68 ± 0.10**
146	24.2 ± 3.2*	0.069 ± 0.004**	0.84 ± 0.04**
122	17.8 ± 1.2*	0.041 ± 0.003**	0.60 ± 0.07**
93	10.7 ± 1.6*	0.027 ± 0.004**	0.33 ± 0.04**
63	4.0 ± 0.6	0.011 ± 0.001	0.13 ± 0.01
41	3.2 ± 0.9	0.009 ± 0.002	0.10 ± 0.02

coefficients (r) obtained were: $\dot{V}O_2$ per larva = -17.14 ± 0.3 pO_2 ($r=0.99\pm0.09$); $\dot{V}O_2$ per mg wet weight = $-0.055+8.8\ 10^{-4}$ pO_2 ($r=0.99\pm0.07$); $\dot{V}O_2$ per mg dry weight = $-0.43\pm8.5\ 10^{-3}$ pO_2 ($r=0.99\pm0.02$), when $\dot{V}O_2$ is expressed in $\mu l O_2$ and pO_2 in mmHg O_2. The correlation coefficients r where always very significant ($P < 0.01$). Since the three $\dot{V}O_2$ values at 41 mmHg O_2 were not statistically different from $\dot{V}O_2$ values at 63 mmHg O_2, they were not included in the calculation of the three regression lines referred to above.

When the three $\dot{V}O_2$ parameters measured at similar water pO_2 were compared between active and anesthetized animals, values from two to 14 times greater were found for active metabolism. The three values of active routine metabolism measured in acute hypoxia are statistically similar to those obtained in normoxia for anesthetized animals.

Discussion

In this study, active stage XVIII *D. pictus* tadpoles were capable of oxygen regulation down to pO_2 a low as 52 mmHg O_2. This regulation was not perfect since a significant decrease of $\dot{V}O_2$ was detected from normoxia to 119 mmHg. The presence of an area of O_2 conformism preceding the region of O_2 regulation in hypoxia has not been described in amphibian tadpoles previously. Nevertheless, this has been found in some fish species such as *Pimephales promelas* (14) even though the authors gave no explanation for this phenomenon. This first transitory range of O_2 conformism in *D. pictus* larvae might be due to the fact that tadpoles, on visual observation, showed a marked decline in spontaneous activity as a first response to moderate hypoxia. This response can be adaptive since it saves the energy which would otherwise be probably invested in increasing gill ventilation for O_2 regulation. The possibility that water O_2 tension is limiting aerobic metabolism already at the relatively high pO_2 of moderate hypoxia (119-150 mmHg O_2) is not probable, especially if the very high O_2 affinity tipically showed by the hemoglobin of amphibian larvae (11, 12) is taken into account.

The range of oxygen regulation of active *D. pictus* tadpoles exclusively breathing from water was extremely large. Thus, the critical O_2 pressure (Pc) for the change from oxygen regulation to strict conformism was at least as low as 52 mmHg. This is in contrast to Pc values of around 75 mmHg O_2 described for *Ambystoma punctatum* (3) and *Ambystoma maculatum* (4). Furthermore, when obliged to respire only in water, *Rana berlandieri* (8) and *Xenopus laevis* (9) tadpoles show Pc values higher than 100 mmHg O_2. The same occurs in lungless tadpoles of *Bufo woodhousei* (9). Even when given free access to air, *R. berlanderi* tadpoles are very poor O_2 regulators (8) and stage XVIII *R. catesbeiana* tadpoles show decreases of gill $\dot{V}O_2$ at pO_2 smaller than 96 mmHg (15). The high capacity for O_2 regulation of *D. pictus* tadpoles is probably an adaptation to the strongly hypoxic habitats in which these larvae develop normally (2). The O_2 regulation of *D. pictus* tadpoles is probably produced by an increase in the frequency of gill ventilation, a common response to hypoxia at least in premetamorphic (before stage XVIII) larvae (5, 8) although the involvement of a skin vasodilation cannot be ruled out withouth further experimentation. Nevertheless *R. catesbeiana* tadpoles at stage XVIII have been shown to respond to hypoxia with lung ventilation increases without changing the frecuency of gill ventilation and decreasing the gill $\dot{V}O_2$ (5, 15). However, those experiments, contrary to the present ones, were performed on tadpoles with free access to air and their conclusion, that in amphibian tadpoles in hypoxia «acute adjustements in gill ventilation frequency

dominate early larval development, while it is primarily lung ventilation frequency that is modified during late larval development» is correct for animals with free access to air but not so for tadpoles which respire only in water. This might be very important for the compensation of acute aquatic hypoxia in nature. Thus, if lung ventilation is dangerous because of the presence of predators at the water-air interphase, tadpoles can stay inside the water and still regulate their $\dot{V}O_2$. On the other hand, it is not very surprising to find aquatic oxygen regulation in hypoxia at stage XVIII when skin and gills have been shown to account for almost 90 % of total oxygen uptake (6).

When water pO_2 was further reduced to 35 mmHg active tadpoles failed to regulate their $\dot{V}O_2$ which strongly decreased. This is in agreement with previous observations which showed that *D. pictus* tadpoles definitively adapt to 37 mmHg O_2 only after progressive and slow decreases of water pO_2 during chronic acclimation whereas they die in 4-5 hours if they are suddenly exposed to 37-46 mmHg O_2 (1, 2). Thus, the O_2 conformism detected in this research at 35 mmHg O_2 must not be regarded as an adaptative response, but rather as a state which can be maintained only for a short period of time during which partial reliance on anaerobic metabolism can be expected. This also agrees with the fact that active $\dot{V}O_2$ at 35 mmHg pO_2 is so small that it is similar to the $\dot{V}O_2$ of normoxic anesthetized animals.

When the *D. pictus* tadpoles were anesthetized O_2 regulation completely disappeared between 249 and 63 mmHg. Similarly, in *Salmo gairdneri*, also anesthetized with MS-222, arterial pO_2 linearly followed water pO_2 whereas non-anesthetized animals regulated arterial pO_2 (10). It is then likely that in our animals MS-222, a lipid-soluble substance that readily equilibrates with many tissues including brain and cerebrospinal fluid, abolished O_2 regulation by depressing nervous or muscular components of the reflexes which modulate gill ventilation frequency. Nevertheless further research is needed as almost nothing is known about the afferent arm of the respiratory reflexes arising from the gills in amphibian tadpoles.

In anesthetized stage XVIII *D. pictus* tadpoles $\dot{V}O_2$ and water pO_2 are linearly related for very large ranges of O_2 tension (249-63 mmHg). This suggests that in the anesthetized animal the rate of water flow over the gills and cardiac output do probably not increase as water pO_2 falls and their values become low enough to make aerobic metabolism O_2 dependent in spite of the usually very high O_2 affinity of tadpole hemoglobins. Finally, it should be stated that O_2 conformism in anesthetized animals was not extended in the range of acute hypoxia. Thus, at these very low values of water pO_2 regulation appeared again and similar $\dot{V}O_2$ was found at 63 and 41 mmHg O_2. This suggests that when hypoxia is very acute, its stimulatory activity on aquatic respiratory reflexes is high enough to overcome the inhibition of such responses caused by anesthesia. This is specially plausible in this work where relatively low MS-222 concentrations (1/10.000) where used, but which nevertheless were high enough to prevent spontaneous swimming behaviour.

Acknowledgements

The authors are especially grateful to A. Núñez for typing the manuscript. This work was partially supported by FISS (Spanish Social Security Health Research Fund, n.° 88/1030).

Resumen

Se estudian las variaciones del consumo de oxígeno en agua, en larvas activas y anestesiadas de *Discoglossus pictus* (Anfibio, Anuro) en el estado XVIII, en función de la presión parcial de O_2 ambiente. Las larvas activas son buenas reguladoras frente al O_2 en hiperoxia e hipoxia moderada, pero son O_2-confor-

mistas en hipoxia aguda (la presión crítica para el O_2 tiene un valor de 52 mmHg). La anestesia transforma a las larvas en O_2-conformistas absolutas entre hiperoxia e hipoxia moderada (249-63 mmHg O_2). Estos resultados indican que, en el estado XVIII los órganos de respiración acuática de los animales activos aún conservan capacidad reguladora frente al O_2, si se impide su acceso a la fase aérea. Esta capacidad concuerda con el ambiente fuertemente hipóxico en el que estos animales viven frecuentemente en la naturaleza.

Palabras clave: Consumo de oxígeno, Hipoxia, Hiperoxia.

References

1. Barja de Quiroga, G., Alonso-Bedate, M. and Fraile, A.: *Mol. Physiol.*, 2, 251-262, 1982.
2. Barja de Quiroga, G., Rojo, S., Gutiérrez, P. and Alonso-Bedate, M.: *Comp. Biochem. Physiol.*, 74B, 579-586, 1983.
3. Boell, E. J., Greenfield, P. and Hille, B.: *Develop. Biol.*, 7, 420-431, 1963.
4. Branch, L. C. and Taylor, D. H.: *Comp. Biochem. Physiol.*, 58A, 269-274, 1977.
5. Burggren, W. W. and Doyle, M.: *Resp. Physiol.*, 66, 279-291, 1986.
6. Burggren, W. W. and West, N. H.: *Resp. Physiol.*, 47, 151-164, 1982.
7. Dejours, P.: In «Respiration of Amphibious Vertebrates» (Hughes, G. M., ed.). Academic Press, New York, 1976, pp. 1-15.
8. Feder, M. E.: *J. Exp. Biol.*, 104, 79-95, 1983.
9. Feder, M. E.: *J. Exp. Zool.*, 228, 11-19, 1983.
10. Laurent, P.: *J. Physiol., Paris*, 70, 571-581, 1975.
11. Pinder, A. and Burggren, W.: *J. Exp. Biol.*, 105, 205-213, 1983.
12. Riggs, A.: *J. Gen. Physiol.*, 35, 23-44, 1951.
13. Taylor, A. C. and Kollros, J. J.: *Anat. Rec.*, 94, 7-23, 1946.
14. Wares, W. D. and Igram, R.: *Comp. Biochem. Physiol.*, 62A, 351-356, 1979.
15. West, N. H. and Burggren, W. W.: *Resp. Physiol.*, 47, 165-176, 1982.