Mar Biol (2009) 156:515–519
DOI 10.1007/s00227-008-1096-7

SHORT COMMUNICATION

Abiotic conditions in cephalopod (*Sepia officinalis*) eggs: embryonic development at low pH and high pCO_2

Magdalena A. Gutowska · Frank Melzner

Received: 18 September 2008 / Accepted: 18 November 2008 / Published online: 9 December 2008

Abstract Low pO_2 values have been measured in the perivitelline fluids (PVF) of marine animal eggs on several occasions, especially towards the end of development, when embryonic oxygen consumption is at its peak and the egg case acts as a massive barrier to diffusion. Several authors have therefore suggested that oxygen availability is the key factor leading to hatching. However, there have been no measurements of PVF pCO_2 so far. This is surprising, as elevated pCO_2 could also constitute a major abiotic stressor for the developing embryo. As a first attempt to fill this gap in knowledge, we measured pO_2, pCO_2 and pH in the PVF of late cephalopod (*Sepia officinalis*) eggs. We found linear relationships between embryo wet mass and pO_2, pCO_2 and pH. pO_2 declined from >12 kPa to less than 5 kPa, while pCO_2 increased from 0.13 to 0.41 kPa. In the absence of active accumulation of bicarbonate in the PVF, pH decreased from 7.7 to 7.2. Our study supports the idea that oxygen becomes limiting in cephalopod eggs towards the end of development; however, pCO_2 and pH shift to levels that have caused significant physiological disturbances in other marine ectothermic animals. Future research needs to address the physiological adaptations that enable the embryo to cope with the adverse abiotic conditions in their egg environment.

Communicated by U. Sommer.

Electronic supplementary material The online version of this article (doi:10.1007/s00227-008-1096-7) contains supplementary material, which is available to authorized users.

M. A. Gutowska (✉)
Marine Animal Physiology,
Alfred-Wegener-Institute for Polar- and Marine Research,
Am Handelshafen 12, 27570 Bremerhaven, Germany
e-mail: magdalena.gutowska@awi.de

F. Melzner
Biological Oceanography, IFM-GEOMAR Leibniz Institute
of Marine Sciences, Hohenbergstrasse 2, 24105 Kiel, Germany

Introduction

Designed to protect embryonic stages from predation, egg capsules also can provide severe physiological challenges to their inhabitants, as the egg wall represents a barrier to diffusion of gases. Previous work has demonstrated that oxygen diffusion coefficients (K_{O_2}) of marine animal egg capsules are typically 10–20% that of pure water (e.g., Brante 2006). In molluscs (as in all other developing embryos), oxygen consumption rates rise dramatically during development (e.g., Cronin and Seymour 2000; Brante 2006). Thus, in order to enable rising oxygen fluxes by means of diffusion, many molluscan eggs swell during development, leading to enhanced surface areas and reduced egg wall thicknesses (e.g., Kress 1972; Cronin and Seymour 2000), and consequently increased oxygen conductances (Seymour 1994). In addition, embryos inhabiting fluid filled capsules often produce convective currents that prevent the formation of pO_2 gradients within the egg fluid (amphibians: Burggren 1985, fish: Rombough 1988, molluscs: Cronin and Seymour 2000). While thinning of egg capsule walls has been shown to enable consistently high pO_2 values in eggs of the marine gastropod *Fusitriton oregonensis* (ca. 12 kPa; Brante 2006), the combination of egg swelling and convection does not prevent pO_2 from consistently falling during embryonic development of the cephalopod *Sepia apama*, from initial values of 14 kPa down to 5–6 kPa close to hatching (Cronin and Seymour

2000). It has been hypothesized that this might eventually trigger hatching once critical pO_2 values (pO_{2crit}) are reached within the egg (pO_{2crit} = 5–8 kPa for *S. apama* and *S. officinalis* late embryonic stages (DeWachter et al. 1988; Cronin and Seymour 2000). While low pO_2 values in eggs also have been proposed to stimulate hatching in vertebrates (reptiles and birds: Vleck and Hoyt 1991, amphibians: Seymour and Bradford 1995), little attention has been devoted to the flip side of the coin: egg pCO_2 and pH, especially in marine animals. In order to excrete metabolically produced CO_2 at a rate that almost equals that of oxygen consumed (of course, depending on the respective respiratory quotient), a pCO_2 gradient has to be maintained in the opposite direction, with high values inside the egg. To our best knowledge, there are currently no published egg fluid pCO_2 values available in the literature for any marine animal. This is quite surprising, as high pCO_2 values most likely go along with low pH values and potentially constitute another stressor that may significantly affect embryonic physiology. Using late embryos of the cephalopod *Sepia officinalis* as a model system, we aimed at characterizing in more detail the abiotic conditions within the perivitelline fluid (PVF), with an emphasis on the pCO_2/pH gradient between the egg and the environment.

Materials and methods

Laboratory laid *Sepia officinalis* eggs were obtained from the Biological Station in Luc-sur-Mer, Université de Caen (Normandy, France) and transported to Germany at the age of 7–8 days. They were then incubated in a recirculating aquarium system (200 l volume) at the AWI Bremerhaven at 17°C (±0.2°C) for approximately four weeks. A nitrification filter (Eheim Professional 2, Eheim, Deizisau, Germany), a protein skimmer (AquaCare 2000, AquaCare, Germany) and a 36 Watt UV sterilizer (Rebie, Bielefeld, Germany), in combination with frequent water changes, aided in maintaining a high water quality within the system (pH > 8.1, pCO_2 < 0.042 kPa, pO_2 > 20 kPa, $[NH_4^+]$ < 0.1 mg/l, $[NO_2^{2-}]$ < 0.1 mg/l, $[NO_3^-]$ < 5 mg/l, S > 32 ppt). pH, pO_2 and salinity were checked daily using a WTW multimeter (WTW, Weilheim, Germany), nitrogenous waste products were assessed bi-weekly using photometric test kits (Merck, Darmstadt, Germany). Carbonate system parameters were calculated from pH_{NBS} and weekly determinations of total dissolved inorganic carbon (C_T) (see below).

Eggs were placed individually on the bottom of the incubation tank until close to hatching (stages 29–30). All (n = 13) eggs of the present study were sampled on the same day. Eggs were gently lifted out of the tank and immediately sampled for PVF. All PVF samples were taken within 15 s, thus minimizing the chance of artificially increased pCO_2 values caused by disturbed embryos. To measure pH and pO_2, a 1 ml plastic syringe was equipped with miniature fiber optic sensors (optodes, tip diameter 140 µm, Presens GmbH, Regensburg, Germany) and filled with 200–300 µl PVF, (previously described in Melzner 2005). Stable pH values were obtained after 10 min, pO_2 values after 10 s. During the measurement period, the syringe and sensors were placed in a thermostatted water bath at 17°C. The oxygen optodes were calibrated according to the manufacturer's instructions with water vapor saturated air and a Na_2SO_3 solution. The pH optodes were calibrated using five seawater standards (North Sea seawater, 31 psu, 0.2 µm filtered) adjusted to pH values between 7 and 8 with 1 M HCl. A pH electrode (WTW sentix81 and pH340i pH meter, WTW, Weilheim, Germany), calibrated with Radiometer precision buffers, was used to prepare the seawater buffers. Calibration of the pH optodes with seawater buffers was found necessary as these sensors are sensitive to the ionic strength of the measurement medium.

Another 350 µl of PVF was sampled with a gas-tight glas syringe for the determination of total dissolved inorganic carbon (C_T). C_T was measured in triplicate (100 µl each) using a gas chromatographic method (Lenfant and Aucutt 1966, modified after Pörtner et al. 1990) on an Agilent 6890 N gas chromatograph. Carbonate system speciation (i.e., pCO_2, $[HCO_3^-]$) was calculated from C_T and pH_{NBS} using CO2SYS software (Lewis and Wallace 1998), with dissociation constants from Mehrbach et al. (1973) as refitted by Dickson and Millero (1987).

Following PVF sampling, eggs were dissected and embryo and yolk wet mass was determined using a Sartorius precision balance (Sartorius, Göttingen, Germany). Embryonic stages were determined according to Lemaire (1970).

PVF pO_2, pCO_2, $[HCO_3^-]$ and pH were graphed against embryo wet mass. Subsequent regression analyses were performed using GraphPad lnStat 3.0 software (GraphPad Software, San Diego, USA). A Runs test was used to test for deviations from linearity, an ANOVA was used to assess whether slopes differed from zero.

Results and discussion

The eggs of *S. officinalis* are particularly suited for the investigations of abiotic parameters in the PVF due to their large size. At hatching, the eggs contain >1 ml of PVF and have a diameter between 1.5 and 2 cm (see supplementary movie, S1). Schematic drawings of a freshly laid egg, as well as of late stage embryos, are nicely illustrated in

Wolf et al. (1985) (Figs. 1 and 2). All of the cuttlefish eggs we investigated contained living embryos, ranging in wet mass between 134 and 310 mg (mean 238 mg, SD 44 mg). Organogenesis was completed in all embryos (stages 29–30, Lemaire 1970), and embryos had mostly absorbed their external yolk (mean wet mass of external yolk 18 mg, SD 20 mg). The larger embryos (>250 mg) were probably <1 week away from hatching, especially the two largest embryos, who had consumed all external yolk reserves (Lemaire 1970). Measured PVF pO_2, pH and pCO_2 values were tightly linked to embryo wet mass, increasing (pCO_2) or decreasing (pO_2, pH) in a linear fashion (Fig. 1a, b, c). The results of all regression analyses are depicted in Table 1.

PVF Oxygen partial pressures

Sepia officinalis PVF oxygen partial pressures varied between 12.8 kPa (ca. 61% air saturation) and 4.6 kPa (ca. 22% air saturation). Wolf et al. (1985) found *S. officinalis* PVF pO_2 values to rise with embryonic development, increasing from 12 kPa (stage 24) to 15.5 kPa (stage 29). They concluded that improved diffusion properties of the swelling and thinning egg case would aid in the maintenance of high PVF pO_2. Unfortunately, Wolf et al. did not provide embryo masses, making it difficult to draw meaningful conclusions between our studies. However, the smallest of our embryos was also classified as stage 29 and its PVF pO_2 value was high (>12 kPa, see Fig. 1a). It is thus not unlikely that Wolf et al. missed the final growth phase in their study, as this is when PVF pO_2 rapidly declines. Support comes from the study by Cronin and Seymour (2000), who also demonstrated an inverse correlation between embryo mass and pO_2 in Australian giant cuttlefish eggs (*Sepia apama*, 12°C). In *S. apama*, pO_2 declined to values of about 6 kPa during the last 50 days of development. As Cronin and Seymour determined a pO_{2crit} of 8 kPa for hatchling animals, they suggested that late embryos probably experience diffusion limitation close to hatching. pO_{2crit} for 200–300 mg *S. officinalis* incubated at 18–19°C has been shown to be in the 4.5–5 kPa range (DeWachter et al. 1988), thus in the range of our lowest PVF values (4.6 kPa). These findings support the idea of oxygen diffusion limitation being one critical factor in *S. officinalis* late embryonic development.

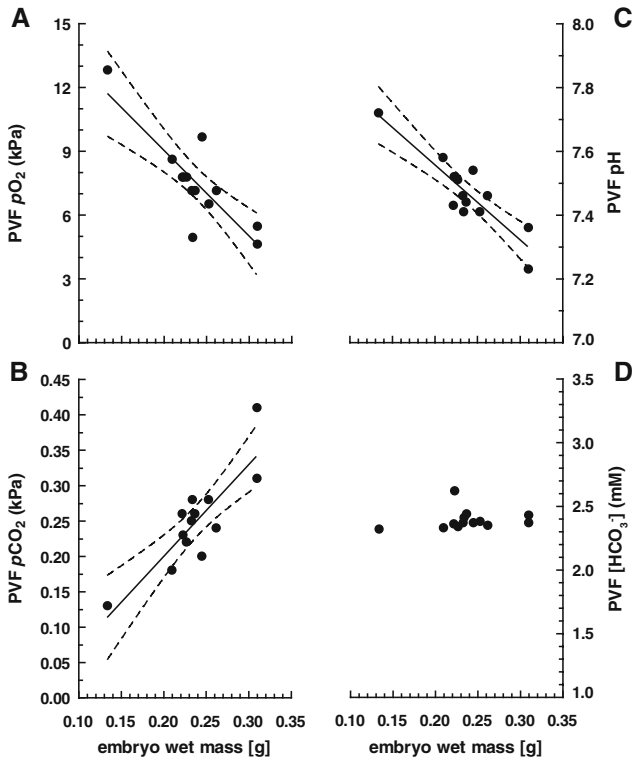

Fig. 1 pO_2 (**a**), pCO_2 (**b**), pH (**c**) and [HCO_3^-] in perivitelline fluid (*PVF*) of *Sepia officinalis* eggs (stages 29–30), displayed against embryo wet mass (excluding the external yolk sac). pCO_2 and [HCO_3^-] were calculated from PVF C_T and pH. See Table 1 for regression analyses and equations

PVF pCO_2 and pH

As expected, PVF pCO_2 values rose with increasing embryo mass, from 0.13 kPa in the smallest up to 0.41 kPa in the largest embryos (Fig 1b). Thus, cuttlefish embryos are surrounded by tenfold higher pCO_2 values than those of ambient sea water (ca. 0.04 kPa) at the end of their embryonic development. High pCO_2 values in the PVF also imply that blood pCO_2 values must be even higher in order to maintain CO_2 excretion rates across the gill/skin epithelia by means of diffusion. Typically, pCO_2 values in extracellular fluids of high-power animals such as fish or cephalopods are 0.2–0.4 kPa above those of the ambient seawater (e.g., Heisler 1986; Johansen et al. 1982; Pörtner et al. 1991). Therefore, cuttlefish embryos are probably exposed to blood pCO_2 values of 0.6–0.8 kPa at the end of their development. It would be quite rewarding to study blood pH regulation in embryos under such conditions, as cephalopods are known for the high pH sensitivity of their extracellular respiratory pigment hemocyanin (Melzner et al. 2007). The occurrence of special embryonic hemocyanins (Decleir et al. 1971) may be one adaptation to the high pCO_2 values encountered during late embryogenesis.

The CO_2 gradient between the PVF and seawater was much shallower than that of oxygen, almost by a factor of 70 ($\Delta pO_2/\Delta pCO_2 = 66.6$, SD 11.3). This ratio is higher than expected from Henry's and Graham's laws, which suggest an approximately 26-fold higher partial pressure gradient of O_2 versus CO_2 across the egg envelope (for seawater at 17°C, Dejours 1975). These results can only be

Table 1 Linear regression analyses. $N = 13$ eggs were analyzed, pCO_2 and $[HCO_3^-]$ are derived parameters (calculated from pH and C_T, see text), pCO_2 and pO_2 in kPa

Regression	ANOVA	Runs test	R^2	Equation
pO_2 vs. pCO_2	$F_{(1,11)} = 36.5$, $P < 0.001$	0.88, NS	0.77	$y = -27.9x + 14.5$
pO_2 vs. mass	$F_{(1,11)} = 25.1$, $P < 0.001$	0.15, NS	0.70	$y = -40.3x + 17$
pCO_2 vs. mass	$F_{(1,11)} = 31.9$, $P < 0.001$	0.98, NS	0.74	$y = 1.31x - 0.06$
pH vs. mass	$F_{(1,11)} = 42.6$, $P < 0.001$	0.88, NS	0.80	$y = -2.32x + 8.02$
$[HCO_3^-]$ vs. mass	$F_{(1,11)} = 0.3$, $P > 0.59$	0.47, NS	0.03	NS

See also Fig. 1

explained if either a significant portion of the excretory CO_2 is retained within the embryo to aid in the formation of the internal $CaCO_3$ shell, or if Krogh's diffusion coefficient for CO_2 (K_{CO_2}) is much higher than K_{O_2} for *S. officinalis* egg capsules. It has been demonstrated for sea urchin larvae and corals that significant portions of inorganic carbon for calcification are derived from metabolic CO_2 (>50%, Sikes et al. 1981; Furla et al. 2000). However, a more extensive follow-up study that will focus on the determination of oxygen consumption and CO_2 excretion, as well as egg capsule surface area and thickness, will enable us to give estimates for both, K_{O_2} and K_{CO_2}.

As pCO_2 increased, pH strongly decreased, from 7.72 to 7.23 (Fig. 1c). Again, there are no pH measurements for marine animal eggs at present that our data could be compared with. In freshwater fish (salmonids), 0.3–1.0 unit lower pH values have been recorded in egg PVF at ambient pH between 7 and 8 (Kugel and Peterson 1989). No accumulation of HCO_3^- was visible in *S. officinalis* PVF in order to actively buffer pH (Fig. 1d), a mechanism that is used by many marine organisms to compensate for extra- and intracellular acidification (e.g., Heisler 1986; Cameron 1986). $[HCO_3^-]$ fluctuated around 2.39 mM (SD 0.08 mM) in all eggs.

It is noteworthy that cuttlefish embryos are able to form an internal $CaCO_3$ (aragonite) shell under the low pH and high pCO_2 prevailing in their egg environment. We recently demonstrated, that calcification rate in juvenile cuttlefish is not impaired at external pCO_2 values of 0.4 and 0.6 kPa (Gutowska et al. 2008). This sets *S. officinalis* apart from other marine invertebrates studied so far, as the majority show a decrease in calcification under comparable conditions (see Fabry et al. 2008 for a review). It is tempting to propose that this capacity is causally linked to an embryo that is already adapted to cope with relatively high pCO_2/low pH values.

Perspectives

As mentioned, no data on egg fluid pCO_2 and pH is available for other marine organisms; however, judging from the pO_2 versus pCO_2 ratios obtained in our study, in comparison to pO_2 values in- and around eggs or egg masses of other marine animal taxa, it seems likely that many embryos will also be surrounded by fluids of high pCO_2 and low pH: For example, Diez and Davenport (1987) showed that pO_2 values in shark eggs decrease from ca. 18 kPa in early embryos to ca. 10 kPa in late embryos, Fernandez et al. (2000, 2002) measured water pO_2 values <5 kPa in decapod crustacean egg masses, Cohen and Strathmann (1996) found pO_2 values of less than 6 kPa in egg masses of opisthobranch gastropods and those of a polychaete worm.

Delayed development of embryos in central positions of egg masses has usually been causally linked to reduced metabolic rates due to low ambient pO_2 (e.g., Chaffee and Strathmann 1984). However, high pCO_2/low pH may be important factors as well, as they could also elicit reductions in respiration rates: Metabolic depression, in combination with reduced growth and calcification performance, has recently been observed in mussels (*Mytilus galloprovincialis*) exposed to sea water of similar pCO_2 (ca. 0.5 kPa, Michaelidis et al. 2005).

Quite clearly, PVF pCO_2 and pH represent important abiotic factors that might influence the physiological performance of marine animal embryos to a large degree. To date, these factors have been thoroughly neglected. We hope that this study sparks some interest in studying CO_2 excretion and pH homeostasis in eggs of marine animals.

Acknowledgments We thank M.P. and R. Chichery, Universite de Caen, France, for provision of cuttlefish eggs. M.A.G. was supported by the German Academic Exchange Service (DAAD) and the AWI MARCOPOLI Program. F.M. was supported by the DFG Excellence Cluster 'Future Ocean'.

References

Brante A (2006) An alternative mechanism to reduce intracapsular hypoxia in ovicapsules of *Fusitriton oregonensis* (Gastropoda). Mar Biol (Berl) 149:269–274. doi:10.1007/s00227-005-0199-7

Burggren W (1985) Gas-exchange, metabolis, and ventilation in gelatinous frog egg masses. Physiol Zool 58:503–514

Cameron JN (1986) Acid–base equilibria in invertebrates. In: Heisler N (ed) Acid-base regulation in animals. Elsevier, Amsterdam, pp 357–394

Chaffe C, Strathmann RR (1984) Constraints on egg mass. 1. Retarded development within thick egg masses. J Exp Mar Biol Ecol 84:73–83

Cohen CS, Strathmann RR (1996) Embryos at the edge of tolerance: effects of environment and structure of egg masses on supply of oxygen to embryos. Biol Bull 190:8–15. doi:10.2307/1542671

Cronin ER, Seymour RS (2000) Respiration of the eggs of the giant cuttlefish *Sepia apama*. Mar Biol (Berl) 136:863–870. doi:10.1007/s002270000274

Decleir W, Lemaire J, Richard A (1971) The differentiation of blood proteins during ontogeny in *Sepia officinalis* L. J Comp Biochem Physiol 40B:923–930. doi:10.1016/0305-0491(71)90038-1

Dejours P (1975) Principles of comparative respiratory physiology. North. Holl. Publ. Comp, Amsterdam

Dewachter B, Wolf G, Richard A, Decleir W (1988) Regulation of respiration during juvenile development of *Sepia officinalis* (Mollusca, Cephalopoda). Mar Biol (Berl) 97:365–371. doi:10.1007/BF00397767

Dickson AG, Millero FJ (1987) A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep Sea Res Part A 34:1733–1743. doi:10.1016/0198-0149(87)90021-5

Diez JM, Davenport J (1987) Embryonic respiration in the spiny dogfish (*Scyliorhinus canicula* L.). J Mar Biol Assoc UK 67:249–261

Fabry VJ, Seibel BA, Feely RA, Orr JC (2008) Impacts of ocean acidification on marine fauna and ecosystem processes. ICES J Mar Sci 65:414–432. doi:10.1093/icesjms/fsn048

Fernandez M, Bock C, Portner HO (2000) The cost of being a caring mother: the ignored factor in the reproduction of marine invertebrates. Ecol Lett 3:487–494. doi:10.1046/j.1461-0248.2000.00172.x

Fernandez M, Pardo LM, Baeza JA (2002) Patterns of oxygen supply in embryo masses of brachyuran crabs throughout development: the effect of oxygen availability and chemical cues in determining female brooding behavior. Mar Ecol Prog Ser 245:181–190. doi:10.3354/meps245181

Furla P, Galgani I, Durand I, Allemand D (2000) Sources and mechanisms of inorganic carbon transport for coral calcification and photosynthesis. J Exp Biol 203:3445–3457

Gutowska MA, Pörtner HO, Melzner F (2008). Growth and calcification in the cephalopod *Sepia officinalis* under elevated seawater pCO_2. Mar Ecol Progress Ser (accepted)

Heisler N (1986) Acid–base regulation in fishes. In: Heisler N (ed) Acid–base regulation in animals. Elsevier, Amsterdam, pp 309–356

Johansen K, Brix O, Lykkeboe G (1982) Blood gas transport in the cephalopod *Sepia officinalis*. J Exp Biol 99:331–338

Kress A (1972) Changes in egg-capsule volumes during development of different opisthobranch species (Mollusca, Gastropoda). Mar Biol 16:236

Kugel B, Peterson RH (1989) Perivitelline fluid of rainbow trout (*Oncorhynchus mykiss*) eggs in relation to ambient pH. Can J Fish Aquat Sci 46:2070–2073

Lemaire J (1970) Table de développement embryonnaire de *Sepia officinalis* L. (Mollusque Céphalopode). Bull Soc Zool Fr 95:773–782

Lenfant C, Aucutt C (1966) Measurement of blood gases by gas chromatography. Respir Physiol 1(4):398–407. doi:10.1016/0034-5687(66)90007-7

Lewis E, Wallace DWR (1998) Program developed for CO_2 system calculations. ORNL/CDIAC-105. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory. U.S. Department of Energy, Oak Ridge

Mehrbach C, Culberso Ch, Hawley JE, Pytkowic Rm (1973) Measurement of apparent dissociation constants of carbonic acid in seawter at atmospheric pressure. Limnol Oceanogr 18:897–907

Melzner F (2005) Systemic investigations on the physiology of temperature tolerance in the common cuttlefish *Sepia officinalis*. PhD Thesis, University of Bremen, Bremen, Germany. urn:nbn:de:gbv:46-diss000106137

Melzner F, Mark FC, Pörtner HO (2007) Role of blood-oxygen transport in thermal tolerance of the cuttlefish, *Sepia officinalis*. Integr Comp Biol 47:645–655. doi:10.1093/icb/icm074

Michaelidis B, Ouzounis C, Paleras A, Portner HO (2005) Effects of long-term moderate hypercapnia on acid–base balance and growth rate in marine mussels *Mytilus galloprovincialis*. Mar Ecol Prog Ser 293:109–118. doi:10.3354/meps293109

Pörtner HO, Boutilier RG, Tang Y, Toews DP (1990) Determination of intracellular pH and pCO_2 after metabolic inhibition by fluoride and nitrilotriacetic acid. Respir Physiol 81:255–274. doi:10.1016/0034-5687(90)90050-9

Pörtner HO, Webber DM, Boutilier RG, Odor RK (1991) Acid–base regulation in exercising squid (*Illex illecebrosus, Loligo pealei*). Am J Physiol 261:R239–R246

Rombough PJ (1988) Growth, aerobic metabolism, and dissolved oxygen requirements of embryos and alevins of steelhead, *Salmo gairnderi*. Can J Zool Revue Can De Zool 66:651–660. doi:10.1139/z88-097

Seymour RS (1994) Oxygen diffusion through the jelly capsules of amphibian eggs. Isr J Zool 40:493–506

Seymour RS, Bradford DF (1995) Respiration of amphibian eggs. Physiol Zool 68:1–25

Sikes CS, Okazaki K, Fink RD (1981) Respiratory CO_2 and the supply of inorganic carbon for calcification of sea urchin embryos. Comp Biochem Physiol Physiol 70:285–291. doi:10.1016/0300-9629(81)90181-X

Wolf G, Verheyen E, Vlaeminck A, Lemaire J, Decleir W (1985) Respiration of *Sepia officinalis* during embryonic and early juvenile life. Mar Biol (Berl) 90:35–39. doi:10.1007/BF00428212

Vleck CM, Hoyt DF (1991) Metabolism and energetics of reptilian and avian embryos. In: Ferguson MWJ, Deeming DC (eds) Egg incubation its effects in embryonic development in birds, reptiles. Cambridge Univ Press, Cambridge, pp 285–306