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Prenatal catch-up growth: A study in avian embryos

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ABSTRACT

Whether the growth of embryos after a period of stunt becomes accelerated (Catch-Up Growth, CUGr), as it occurs postnatally, has rarely been examined experimentally in any class of animals. Here, hypoxia or cold of different degrees and durations caused growth retardation in chicken embryos during the first or second week of incubation. On average, on the day of removal of the growth-inhibition, the weight of the experimental groups was 73% (wet) and 61% (dry) of control embryos, while near end-incubation (embryonic day E18) their weight averaged significantly more, respectively, 80% and 84% of controls ($P < 0.001$). When compared as function of developmental time, the post-intervention growth of experimental embryos was faster than that of controls. The faster growth was fully accounted for by their smaller weight at end-intervention, because embryonic growth is higher the smaller the weight. Hence, their growth was appropriate for their weight, rather than for their age. In fact, out of eight different models of growth based on age and weight (wet or dry) in various combination, the model based on embryonic wet weight at end-intervention, and weight alone, was the best predictor of the embryo's post-intervention growth. The oxygen consumption of the experimental embryos during CUGr was appropriate for their weight. In conclusion, in this experimental model of CUGr, the embryo's weight at the end of a stunt could fully predict and explain the rate of growth during the post-intervention recovery period.

1. Introduction

Many situations can decrease or halt the growth of a developing organism. In infants and children food deprivation, malnutrition or malabsorption, growth hormone deficiency or chronic hypoxia by congenital cardiac defects are some of the causes of stunted growth that can be corrected by medical interventions. Upon removal of the causative insult, growth resumes at a pace faster than in normal children of the same age, a 'catch-up growth' (CUGr) recognised long ago in humans (Prader et al., 1963) and probably much earlier in animals (Boersma and Wit, 1997, for references). Although shown to occur in animal models after hypoxia or starvation (e.g., Okubo and Mortola, 1988; Sant'Anna and Mortola, 2003; Chin et al., 2013) and in the pediatric clinical setting, such as after correction of a cyanotic heart disease or upon restoration of normal caloric intake (Garrow and Pike, 1967; Cheung et al., 2003), the mechanisms involved in CUGr remain purely speculative. In contrast to the compensatory growth after loss of an existing mass, like the post-pneumonectomy lung growth (Brody et al., 1978; Faridy et al., 1988), the liver regeneration after resection (Michalopoulos and DeFrances, 1997; Chen et al., 1991) or the regrowth of limbs and appendices in some vertebrates (Scadding, 1981), CUGr aims to a *potential* mass that represents the norm in a given species at any point in time. The distinction from compensatory growth is important because it suggests that CUGr cannot be accounted for by a simple feedback mechanism (Williams, 1981). Williams (1981) remarked that, while "compensatory growth depends on an alteration in spatial parameters", CUGr "is a response to an alteration in temporal parameter". Hence, time or age are variables that would seem to be an essential component of CUGr, while absent in compensatory growth.

How does the body estimate what the appropriate size should be at any point in time is an open question, the answer of which remains hypothetical. In fact, it is not clear how body mass could sense, even indirectly, the passing of time (Schilder, 2016), although the existence of circadian, ultradian and circannual patterns may give the impression that organisms can perceive and respond to chronological time. Some authors raised the possibility that CUGr reflects a central regulation of body growth, possibly via the neuroendocrine system ("neuroendocrine hypothesis", Tanner, 1963) or the somatotrophic axis (Boersma et al., 2002). Another line of thoughts is that tissues and organs control their own development, such as the continued endochondral ossification in cases of delay in the normal process of epiphyseal senescence ("growth plate hypothesis") (Baron et al., 1994; Gat-Yablonski et al., 2008). It is also possible that organs somehow relate to each other through biochemical or physical interaction (Eder et al., 2017); in which case CUGr may depend on the degree of inter-organ unevenness produced by the growth retardation (Sant'Anna and Mortola, 2003).

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Studies at the cellular level are also pertinent to the mechanisms of CUGr. Hypoxia slowed down or arrested cells replication (e.g., Born et al., 1976; Shrieve and Begg, 1985) especially during the synthesis phase of the cycle (Pettersen and Lindmo, 1983). Upon reaeration, cell cycle resumed at low pace (Shrieve and Begg, 1985). Other DNA synthesis-inhibitors caused an over-replication of DNA (Schimke, 1984), an amplification phenomenon that may be relevant to the recovery upon removal of the cell-cycling inhibition.

One major obstacle in formulating any hypothesis regarding the possible mechanisms of CUGr is the lack of information regarding the precise growth trajectory that follows removal of a growth perturbation. This deficiency is particularly apparent for the prenatal period, when experimental data on CUGr are very rare for any vertebrate class. Many reasons contribute to the lack of quantitative data on the CUGr of embryos and fetuses. In infants, it is almost impossible to obtain a large population of cases that are homogeneous with respect to age and severity of growth retardation, because of the large variety of clinical cases and patterns of growth (Tanner, 1981; Boersma and Wit, 1997; de Wit et al., 2013). In fact, the growth-trajectories adequate for quantitative analysis of CUGr require a large number of measurements at various time points during gestation, and meaningful conclusions require analysis of growth inhibitions of different magnitudes, durations and age at onset. Prenatally, the growth of a fetus before, during and after the stunt can vary greatly according to the responses of the pregnant mother, uterine and placental circulation. Despite the paucity of data, it seems clear that, as is the case postnatally, embryo's growth is faster upon termination of the growth-inhibiting condition. For example, after re-oxygenation, zebrafish embryos recovered the growth lost during a period of hypoxia (Kamei et al., 2011). Similarly, the neural structures of embryonic mice recovered their morphological appearance after removal of a teratogenic agent that caused developmental disruption (Shum and Sadler, 1988; Shum and Sadler, 1991). In some crustaceans, the maternal switching from aquatic to air conditions permitted the brood to recover body growth, which was limited by the low oxygen availability of the aqueous environment during the earlier phase of development (Horváthová et al., 2017).

The current study was performed on chicken embryos, which are easily available and can be manipulated at will at any time of incubation. In eggs, nutrients availability and the embryo's responses and recovery are not confounded by the maternal and placental factors characteristic of mammalian experimental models. The embryonic growth, which normally follows a trajectory convex with respect to the time axis, can be altered at various times by a decrease in incubation temperature (Mortola, 2006) or by hypoxia (Azzam et al., 2007).

First, the weight (wet and dry) and growth of normothermic-normoxic embryos were established until embryonic age E18, to construct the control age-weight and age-growth time trajectories; from these functions the weight-growth relationship was also obtained. Then, in many experimental groups growth was blunted either by cold or by hypoxia, for various durations during the first or the second week of incubation. Upon removal of the growth-inhibitory factor, the weight that the experimental embryos attained at E18 was compared to that of controls and to that predicted by growth trajectories modelled according to different possible patterns. These models allowed to establish whether CUGr had any relation with the age of the embryo during or immediately after the intervention, or with its weight (wet or dry), or with a combination of weight and age. Finally, measurements of oxygen consumption in groups of control and experimental embryos have quantified the energetics involved in the process of CUGr.

2. Materials and methods

Experiments were performed on embryos of the domestic chicken (*Gallus gallus*), White Leghorn variety. The fertile eggs (fresh weight of ~60 g) were obtained from a local producer, who fed the hens a diet of standard chicken feed complemented by other protein sources, vitamin and mineral supplements. Some 1800 eggs were used for these experiments over a period of six years. After a storage time at 15 °C for no more than 6 days from laying, the fresh weight was noted and, at midday (embryonic age E0), the eggs were placed in incubators set at the temperature of 38 °C and 60% relative humidity, with automatic rotation four times a day. Incubation temperature and relative humidity were monitored by a data logger and a hygrometer placed inside the incubator; the former collected the temperature-data every 10 min, while humidity was read daily.

2.1. Control growth curves

The control age-weight relationship was constructed from 906 eggs, opened at various days throughout incubation from embryonic day 5 (E5) to embryonic day 19 (E19). On the day of the measurement, after weighing the egg, the embryo was killed by exposure to CO_2 and cold. The egg was cut open, the embryo dissected free from adjacent tissues, gently blotted on absorbing paper and weighed on a digital scale accurate to 10^{-4} g. Then, the specimens were placed in a still-air oven set at 70 °C; the dry weights were noted after no less than 4 days, most commonly after 6–7 days. Preliminary daily measurements of the samples indicated that the values of dry weights remained stable during 3 to 9 days of the drying process.

The age-weight relationship was adequately fitted by a power function of the type $Y = a \cdot X^b$ ($r = 0.999$, see Results). From this function, the percent daily rate of growth over two successive days n and n + 1 was computed as the percent difference in weight,

$$\text{Growth} = \{[\text{Weight}(n+1) - \text{Weight}(n)]/\text{Weight}(n)\} \cdot 100$$

from which the growth at each day was calculated. From these two curves (Age-Weight and Age-Growth), the Weight-Growth relationship was also produced. To verify the appropriateness of the curve fitting over the E5-E18 range, the daily weight calculated from these data-fitting functions was compared to the original raw data set (see Results). The same approach was adopted to construct the Age-Weight, Age-Growth and Weight-Growth curves from the embryos' dry weights.

2.2. General experimental approach

The general protocol consisted, first, in blunting embryonic growth either by cold exposure ($N = 579$) or by hypoxia ($N = 199$) of various degrees and durations, with onset and end at different times throughout incubation. At the end of the cold or hypoxia, half of the eggs were opened, to measure the degree of the embryo's blunted growth by comparison to the normal growth curve. The remaining half was returned to the original (normothermic and normoxic) incubation conditions until E18. Then, at E18, embryo's weight was compared to that of controls and to that predicted by various models of body growth (Section 2.4.), to see which model best predicted the post-intervention growth. E18 was chosen because it is the last day before the onset of the hatching process, when the intra-abdominal inclusion of the yolk would have complicated the interpretation of the embryo's weight and its CUGr (Mortola, 2009).

2.3. Interventions

Fourteen cold (Table 1) and six hypoxic (Table 2) exposures were tested, with onset at the first or second week of incubation (between E5 and E14), each lasting between 1 and 10 days, in a variety of combinations. In the case of the cold exposures, the eggs were transferred from the normothermic incubator (37.5 °C) to an incubator set at 35 °C. In the case of hypoxia the eggs were transferred from the normoxic incubator (21% O_2) to an incubator made hypoxic (15% O_2) by bleeding humidified N_2 under the control of a flowmeter, with the O_2 concentration recorded at all times by an O_2 analyser and displayed on a computer monitor. These degrees of cold or hypoxia were known to

Table 1
Cold exposure: egg numbers, weight and embryo's outcome.

Protocol #	Fresh egg weight at E0 (g)	age of onset-end intervention (days)	N of Exp. eggs (at end-intervention and at E18)	wet or dry weight	Body weight: Exp/Controls	
					at end of the growth-stunting period	at E18
1	60.7 (± 0.3)	E6-E9	24-24	wet	0.79 (± 0.01)	0.89 (± 0.01)
				dry	0.70 (± 0.01)	0.97 (± 0.01)
2	62.0 (± 0.5)	E6-E9	25-28	wet	0.65 (± 0.01)	0.85 (± 0.02)
				dry	0.53 (± 0.02)	0.90 (± 0.02)
3	62.3 (± 1.0)	E6-E13	14-12	wet	0.45 (± 0.02)	0.54 (± 0.02)
				dry	0.24 (± 0.01)	0.46 (± 0.03)
4	61.7 (± 0.9)	E11-E15	10-12	wet	0.79 (± 0.01)	0.79 (± 0.02)
				dry	0.71 (± 0.03)	0.84 (± 0.03)
5	61.8 (± 0.6)	E14-E15	20-19	wet	0.87 (± 0.03)	0.86 (± 0.01)
				dry	0.81 (± 0.04)	0.91 (± 0.02)
6	61.0 (± 0.7)	E11-E15	25-25	wet	0.71 (± 0.02)	0.69 (± 0.02)
				dry	0.53 (± 0.03)	0.71 (± 0.03)
7	58.8 (± 0.3)	E10-E13	23-21	wet	0.57 (± 0.02)	0.67 (± 0.01)
				dry	0.41 (± 0.01)	0.66 (± 0.01)
8	59.3 (± 0.4)	E11-E14	20-21	wet	0.73 (± 0.02)	0.77 (± 0.02)
				dry	0.53 (± 0.02)	0.76 (± 0.02)
9	60.2 (± 0.5)	E5-E12	26-22	wet	0.63 (± 0.01)	0.79 (± 0.02)
				dry	0.45 (± 0.01)	0.86 (± 0.05)
10	60.6 (± 0.4)	E5-E10	21-20	wet	0.72 (± 0.02)	0.85 (± 0.02)
				dry	0.55 (± 0.02)	0.90 (± 0.02)
11	59.4 (± 0.9)	E6-E13	20-20	wet	0.50 (± 0.01)	0.70 (± 0.01)
				dry	0.31 (± 0.01)	0.71 (± 0.02)
12	60.1 (± 0.4)	E8-E11	20-18	wet	0.55 (± 0.02)	0.71 (± 0.02)
				dry	0.36 (± 0.01)	0.70 (± 0.02)
13	61.0 (± 0.4)	E7-E14	25-21	wet	0.53 (± 0.02)	0.64 (± 0.02)
				dry	0.34 (± 0.02)	0.62 (± 0.03)
14	59.3 (± 0.4)	E14-E17	20-23	wet	0.98 (± 0.02)	0.96 (± 0.01)
				dry	1.10 (± 0.02)	0.97 (± 0.02)

Values are means (± 1 SEM). Exp: embryos subjected to growth retardation for the days indicated in 3rd column.

blunt embryonic growth without compromising successful hatching (Mortola, 2006; Azzam et al., 2007).

Each exposure started with about 40 eggs, half of which was opened at the end of the intervention and the remaining half was returned to normal incubation conditions (normothermia and normoxia), to be opened at day E18. At end-intervention and at E18 both wet and dry embryo's weights were measured. The ratio between the average embryo's weight at the end of the intervention and that of the same-age controls represented the degree of growth blunting caused by the cold or hypoxic exposure. The same ratio was obtained at E18 and compared to that at end-intervention to evaluate the extent of the growth recovery.

2.4. Models of CUGr

Four models were employed to anticipate the growth trajectories of the embryos following each intervention. Two of the models examined the possibility that the post-intervention growth depended solely on the age of the embryo at end-intervention or on the average embryonic age during the intervention. A third model assumed that post-intervention growth depended solely on the embryo's weight at end-intervention, irrespective of its age. The fourth model examined the possibility that growth depended both on embryonic weight and age. Each model was applied to the embryo's wet and dry weight. Fig. 1, A-to-D, is a graphical example of these models and their implications on the post-intervention growth trajectories.

Table 2
Hypoxic exposure: egg numbers, weight and embryo's outcome.

Protocol #	Fresh egg weight at E0 (g)	age of onset-end intervention (days)	N of Exp. eggs (at end-intervention and at E18)	wet or dry weight	Body weight: Exp/Controls	
					at end of the growth-stunting period	at E18
1	59.7 (± 0.8)	E6-E13	13-10	wet	0.89 (± 0.02)	0.90 (± 0.04)
				dry	0.75 (± 0.02)	0.94 (± 0.06)
2	57.3 (± 0.6)	E7-E11	20-20	wet	0.82 (± 0.05)	0.85 (± 0.04)
				dry	0.67 (± 0.04)	0.87 (± 0.05)
3	59.4 (± 0.7)	E9-E13	17-15	wet	0.78 (± 0.02)	0.81 (± 0.02)
				dry	0.64 (± 0.02)	0.86 (± 0.03)
4	59.5 (± 0.9)	E7-E14	10-11	wet	0.89 (± 0.05)	0.93 (± 0.01)
				dry	0.85 (± 0.08)	1.00 (± 0.02)
5	59.1 (± 0.8)	E4-E11	19-19	wet	0.84 (± 0.02)	0.94 (± 0.02)
				dry	0.70 (± 0.02)	1.09 (± 0.04)
6	58.2 (± 0.6)	E4-E14	22-23	wet	0.86 (± 0.03)	0.92 (± 0.01)
				dry	1.07 (± 0.14)	0.97 (± 0.01)

Values are means (± 1 SEM). Exp: embryos subjected to growth retardation for the days indicated in 3rd column.

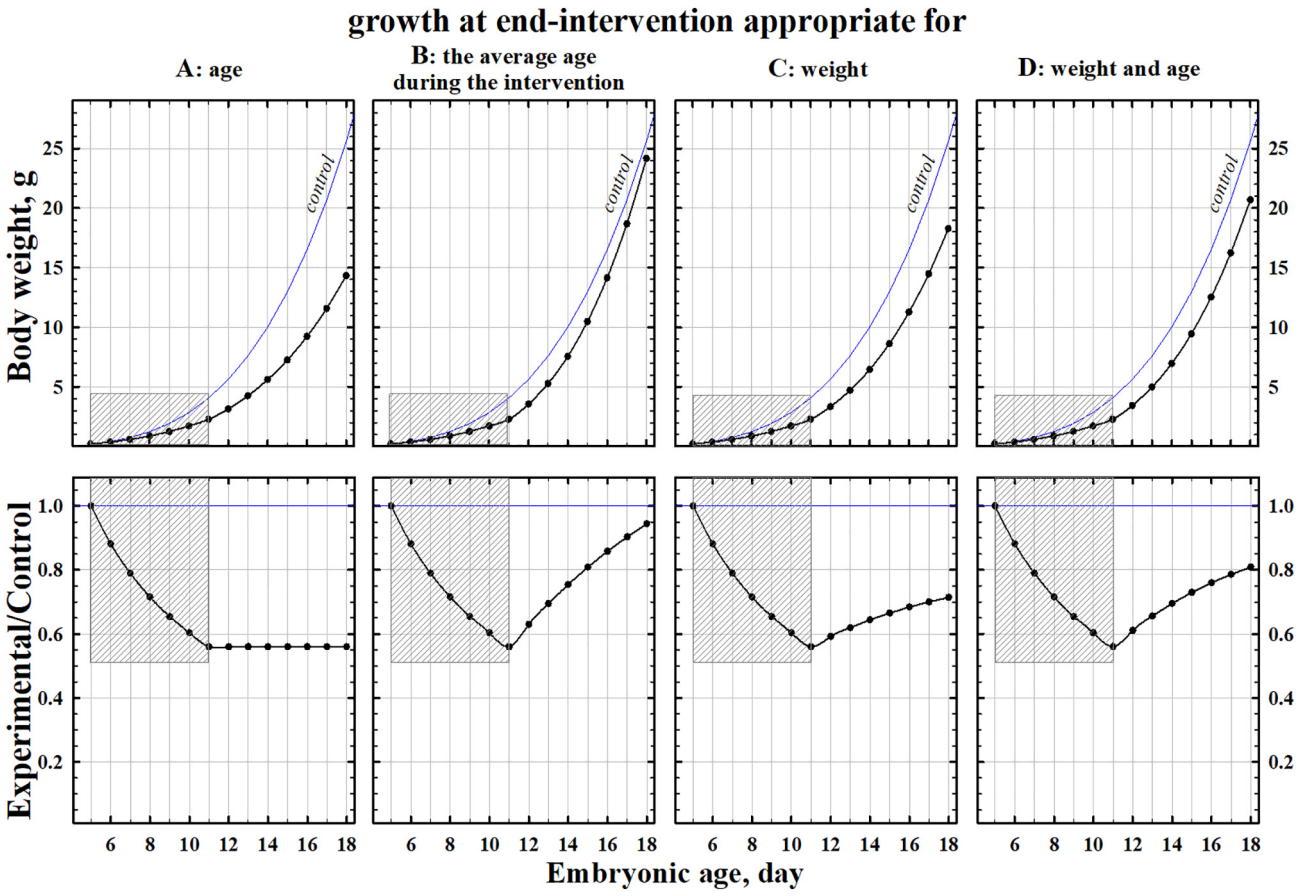

Fig. 1. Examples of growth trajectories generated by the four models. "Control" refers to the normal trajectory of undisturbed embryos. The models permitted to change onset, duration and severity of the intervention. The examples here represented refer to a perturbation that lasted six days, from E5 to E11 (width of hatched box), eventually resulting in a body weight 55% of controls (bottom panel, depth of hatch box). The pattern of recovery following the intervention varied depending on the criteria of each model. For further explanations, see text.

2.4.1. A- "Growth appropriate for age"

The post-intervention growth-trajectory of this model assumes that growth following the intervention resumes at the rate appropriate for the age of the embryo at end-intervention (Fig. 1A). Hence, the model was based on the control Age-Growth relationship (Results, *3.1.*). According to this model, the intervention carries no consequences on later growth, which, upon return to control conditions, resumes as appropriate for the actual age of the embryo. Therefore, during the post-intervention period the weight of the experimental embryos will increase in absolute values (Fig. 1A, top) while it will remain unaltered when expressed as fraction of control (Fig. 1A, bottom).

2.4.2. B- "Growth appropriate for the average age during the intervention"

According to this model, upon return to control conditions growth resumes with the average growth of the control embryos over the age-period of the intervention (Fig. 1B). Hence, this model assumes that the intervention acted as a developmental delay in the growth process. Because during normal embryonic development growth progressively declines with age (Results, *3.1.*), the average growth over the period of the intervention must be higher than the growth at end-intervention. Therefore, at any age during recovery, growth of the experimental embryos exceeds the growth of same-age controls. Hence, the post-intervention embryo's weight will increase both in absolute (Fig. 1B, top) and in relative terms (Fig. 1B, bottom).

2.4.3. C- "Growth appropriate for current weight"

By this model, the growth at the end of the intervention resumes according to the weight of the embryo (Fig. 1C). The post-intervention growth, because it is solely determined by the current weight of the embryo, is computed according to the Weight-Growth relationship (Results, *3.1.*). Since growth is inversely related to weight, during the post-intervention period the growth of the experimentally treated embryos must exceed that of same-age controls, with a progressive increase both in absolute (Fig. 1C, top) and in relative weight (Fig. 1C, bottom).

2.4.4. D- "Growth according to age and weight"

This model considers the possibility that the post-intervention growth depends on the embryo's weight (like in model *C*) and on the average control growth over the period of the intervention (like in model *B*). As a result, the post intervention growth is higher than in model C, because the average growth during the intervention is higher than at end-intervention. Therefore, the growth upon removal of the growth inhibition will describe a trajectory approximately intermediate between that of models *B* and that of model *C,* and the weight of the experimental group will be intermediate both in absolute (Fig. 1D, top) and in relative terms (Fig. 1D, bottom).

These four models A, B, C, D were constructed for both wet and dry weights. The degree of blunting (quantified as the ratio of the weight of experimental embryos over that of controls for wet and dry weights) was measured at the end of each intervention and entered in the models to forecast the corresponding outcomes at E18. Then, the experimentally observed wet and dry weight ratios at E18 were compared to the ratios anticipated by each of the eight models, to evaluate which of them most closely represented the experimental results.

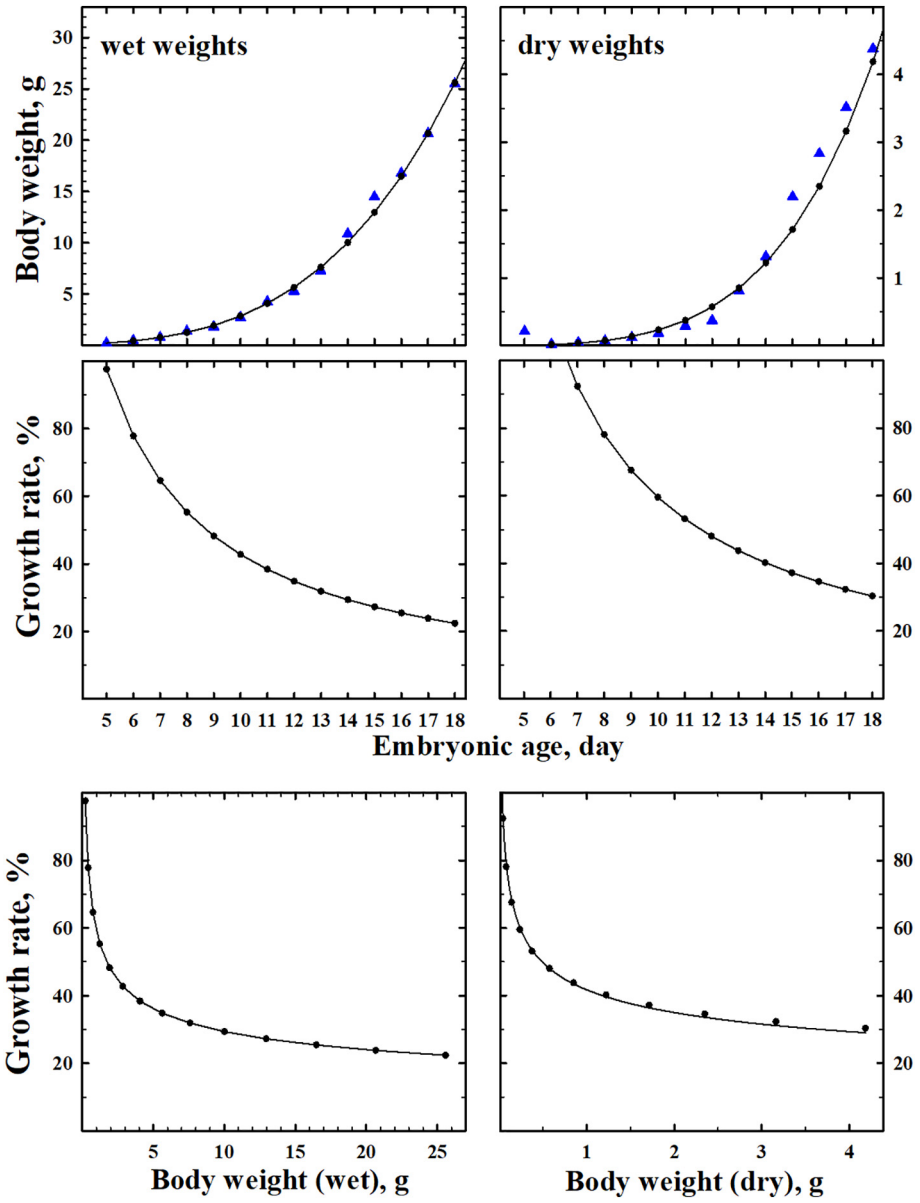

Fig. 2. Top: Body weight (wet weights at left, dry weights at right) as function of embryonic age. Middle: daily growth computed from the age-weight power functions of the top panels. Bottom: weight-growth relationship calculated from the top and middle panels. In the top panel, the experimental averages are the filled triangles, from a total of 906 eggs, opened at various days between E5 and E19. The experimental data corresponded well to the values (black dots) computed from the power function of weight and growth, meaning that the fitting functions adequately described the data.

2.5. Energetics of CUGr

The cost of CUGr was computed following the concept that the total oxygen consumed by an embryo ($\dot{V}_{O_2}$) can be considered the sum of a component related to its weight and one related to its growth, as

$$\dot{V}_{O_2} = a \cdot \text{Weight} + b \cdot \text{Growth} \quad (1)$$

where a·Weight and b·Growth represent, respectively, the 'maintenance' and 'growth-related' components of $\dot{V}_{O_2}$ (Vleck et al., 1980; Rombough, 2006; Mortola and Cooney, 2008). To obtain the coefficients of maintenance and growth (a and b in Eq. (1)) during CUGr, two sets of eggs were studied. One acted as controls ($N = 60$), the other one was subjected to cold (35 °C, between E7 and E10, $N = 63$) and to normothermia thereafter. By end-exposure (E10) the weight and $\dot{V}_{O_2}$ of cold-treated embryos were, respectively, 69% and 60% of controls. Then, control and cold-treated eggs were divided into subsets, for measurements of $\dot{V}_{O_2}$ and weight at embryonic ages E12, E14, E16 and E18.

Measurements of $\dot{V}_{O_2}$ were obtained by an open-flow methodology (Frappell et al., 1992) with a setup like that described previously (Mortola and Labbè, 2005). Essentially, the egg was positioned in a respirometer, the temperature of which was maintained at 37.5 °C by a water-bath. A flowmeter controlled the air-flow through the respirometer. The O_2 and CO_2 concentration of the inflowing and outflowing gases were monitored by appropriate gas analyzers, in dry conditions. All data were continuously acquired on-line and displayed on a computer monitor. Further details, including the correction factors for a Respiratory Exchange Ratio < 1, are in Mortola and Besterman (2007).

Growth was computed as the difference in weight between two neighbouring measures divided by two (the time interval in days); hence, four data points each were obtained for controls and cold-treated embryos. From the values of Growth, Weight and $\dot{V}_{O_2}$ Eq. (1) was solved for a and b using the linear regressions

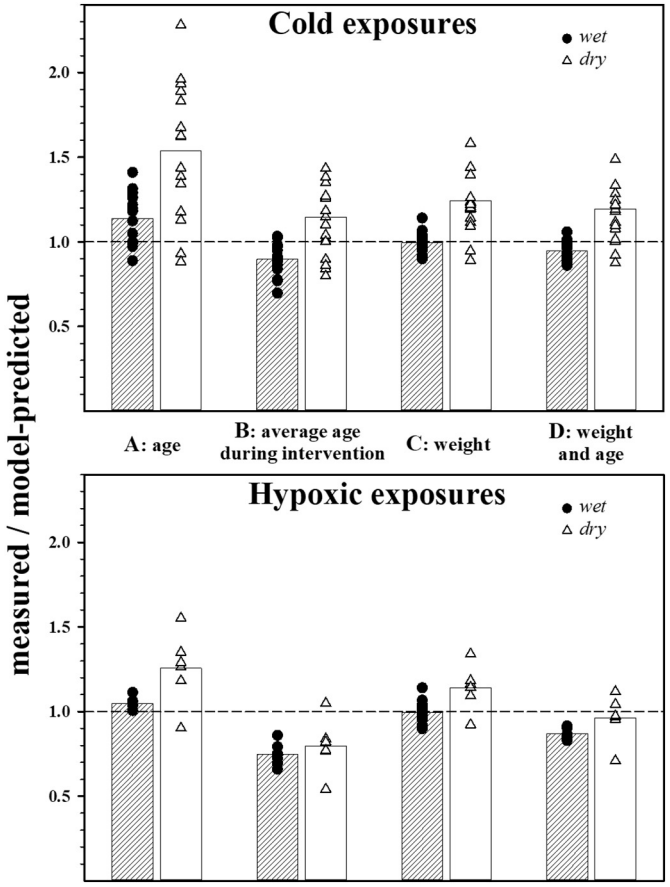

Fig. 3. Ratio between the body weight (wet, filled circles, or dry, open triangles) actually measured and that expected at E18 according to the four models (A-to-D) for interventions that caused blunted growth with cold (top) or with hypoxia (15% O_2) (bottom). Each symbol refers to a different protocol (see Tables 1 and 2), columns indicate mean values. The dashed horizontal line at 1 indicates perfect agreement between experimental and prediction values, which was the case for model "C: weight" (for wet weight).

$$\dot{V}_{O_2}/\text{Weight} = a + b \cdot \text{Growth}/\text{Weight} \quad (2)$$

and

$$\dot{V}_{O_2}/\text{Growth} = b + a \cdot \text{Weight}/\text{Growth} \quad (3)$$

where a and b represent the daily average cost of, respectively, maintaining 1 g of tissue and growing 1 g/day over the E10-E18 period. Although either Eq. (2) or Eq. (3) would be sufficient, the averaging of the coefficients between the two equations eliminated the risk that the highest X-data points may skew the regression lines (Mortola and Cooney, 2008).

2.6. Data analysis

The number and fresh weight of the eggs used for each protocol are given in Tables 1 and 2, after exclusion of sterile eggs and of eggs with dead or malformed embryos. Throughout the manuscript, data are presented as means ± 1 SEM.

For each of the 20 protocols (14 with cold, 6 with hypoxia), the average ratio between the weight of experimentally treated embryos and that of controls at E18 was compared to the ratio predicted by each of the models. The closer the results were to the value of 1.0 the higher the goodness of fit for the model. Then, one way-ANOVA followed by post-hoc limitations was adopted to evaluate whether the top-ranking model differed statistically from any of the remaining seven lower-ranking models. Differences among data sets and appropriateness of the correlation coefficients of the linear regressions were considered statistically significant at $P < 0.05$.

3. Results

3.1. Control weight- and growth-trajectories

The embryonic body weight at the various ages is presented in Fig. 2 (top panel at left). The power function weight (wet, g) = 0.000526·age (day)$^{3.734}$ ($r = 0.999$) was the best fit for the data, from which the age (day)-growth (%) function was Growth = 600.01 · Age$^{-1.143}$ ($r > 0.999$) (left middle panel) and the weight (wet, g)-growth (%) function was Growth = 59.5·Weight$^{-0.306}$ (left bottom panel). The difference between the daily weight recalculated from these functions (filled circles in Fig. 2, left top panel) and the original raw data (filled triangles) was quite small, indicating that the two power functions adequately described the weight and growth values over the E5-E18 period.

The corresponding relationships for the embryo's dry weights are presented in the right panels of the figure. The power function weight (dry, g) = 0.000003·age (day)$^{4.899}$ ($r = 0.985$) was the best fit for the data, from which the age (day)-growth (%) function was Growth = 974.89 · Age$^{-1.207}$ ($r > 0.999$) (left middle panel) and the weight (dry, g)-growth (%) function was Growth = 42.4·Weight$^{-0.246}$ (left bottom panel). The coefficients of the power functions differed from those of the wet weights because dry and wet weights are not proportional to each other (Azzam and Mortola, 2007); in fact, during embryogenesis the progressive loss of tissue water increased the dry/wet weight ratio, from 6.4% at E5 to 17.1% at E18.

3.2. Stunted growth

The degree of stunt in body weight varied greatly among interventions, owing to the differences in type of perturbation (cold or hypoxia), duration (1 to 10 days) and the age at onset (E5-E17) (Tables 1 and 2). On average, by the end of the intervention, weight of the experimentally-treated embryos was 73% ± 3 (wet weight) and 61% ± 5 (dry weight) of controls, the effect on dry weight significantly larger than that on wet weight ($P < 0.001$). This was the case for either mode of exposure, cold (experimental embryos = 68% of control for wet weight and 54% for dry weight) or hypoxia (respectively, 85% and 78%).

By E18, the average weight of the experimental embryos for all twenty interventions was 14.4 ± 1 g (wet) or 2.8 ± 0.2 g (dry) heavier than at end-intervention; over the same age-interval, controls grew slightly more, respectively, 17.5 ± 1 g ($P < 0.001$) and 3.2 ± 0.2 g ($P < 0.005$). When considered relative to controls, by E18 the experimental embryos averaged 80% ± 3 and 84% ± 3 for wet and dry weight respectively, both percentages significantly higher than at end-intervention ($P < 0.001$). Hence, in absolute values the growth of the experimentally-treated embryos during the post-intervention recovery period averaged slightly less than that of controls over the same time interval, while in relative terms their weight became closer to that of controls than it was at end-intervention.

3.3. CUGr: experimental versus model-predicted weight

The top panel of Fig. 3 gives the ratio between the experimental results at E18 and the model-predicted results for each trial. Of the eight models, the closest to the perfect score of 1.0 (dashed line) was model C applied to wet weight; in fact, the average ratio between experimental and model-expected values for all the fourteen protocols of cold-stunted growth was 1.00 (± 0.01). This model was statistically superior to model D (wet weight) which, with an average of 0.95 (± 0.1), ranked second ($P < 0.01$). Models based on dry weight ranked the lowest.

Very similar results were obtained with the hypoxic interventions (Fig. 3, bottom panel), although the predictions of the various models did not differ as much as for the cold exposures. As was the case for the cold exposures, the results for the hypoxic interventions were best predicted by model C (wet weight), with an experimental-predicted ratio averaging 1.00 (± 0.01). The same result was obtained after combining all the twenty-one interventions (cold and hypoxic exposures), with model C superior to all others, whether based on wet or dry weight ($P < 0.01$).

Even though model C (wet weight) on average resulted in the experimental-predicted ratio of 1.00, the results of the individual fifteen cold interventions ranged from 1.14 to 0.90 and those of the six hypoxic interventions varied between 0.96 and 1.03. These values did not bear any statistically significant relation with either the degree of blunting during the intervention or the duration of the intervention; however, they correlated significantly with the embryonic age at the onset of the intervention. In fact, the experimental/predicted ratios for this model C (wet weight) differed significantly between those interventions that started earlier in incubation (≤E8, $N = 13$) and those that started later (> E8, $N = 8$), respectively, 1.02 (± 0.01) and 0.96 (± 0.01) ($P < 0.01$) (Fig. 4).

3.4. Energetics

Oxygen consumption was measured in embryos which experienced a cold-stunt between E7 and E10. At end-intervention (E10) both weight and $\dot{V}_{O_2}$ of these embryos were lower than in controls; however, these and the data points of the post-intervention period fell on the control trajectory, so that their weight- $\dot{V}_{O_2}$ relationship was virtually identical to that of controls (Fig. 5), meaning that at all ages $\dot{V}_{O_2}$ during CUGr was typical for weight. The 'maintenance' coefficient a (Eq. 1) of the cold-treated embryos was 22.4 ± 1.3 ml O_2/g and the 'growth' coefficient b was 43.8 ± 4.6 ml O_2/g/day; either value did not differ significantly from the corresponding one in controls (respectively, 19.0 ± 1.2 ml O_2/g and 49.8 ± 4.8 ml O_2/g/day).

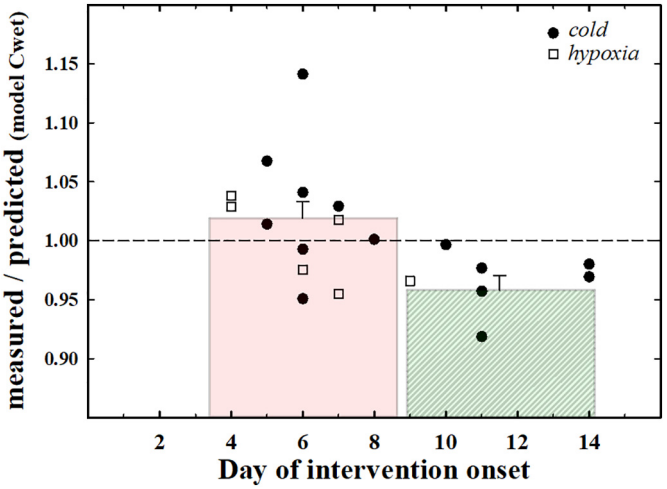

Fig. 4. Ratio between measured and predicted embryo's weight at E18 according to model "C: weight" (for wet weight) after cold (filled circles) or hypoxic interventions (open squares), as function of the embryonic age at the onset of the intervention. On average, for this model the ratio was one (horizontal dashed line), whether the two types of interventions were considered separately or combined. However, the interventions that started at, or before, embryonic age E8 averaged a ratio slightly higher than unity, while the opposite occurred for interventions that started later in incubation ($P < 0.01$). Columns and vertical bars indicate, respectively, group mean values and 1SEM.

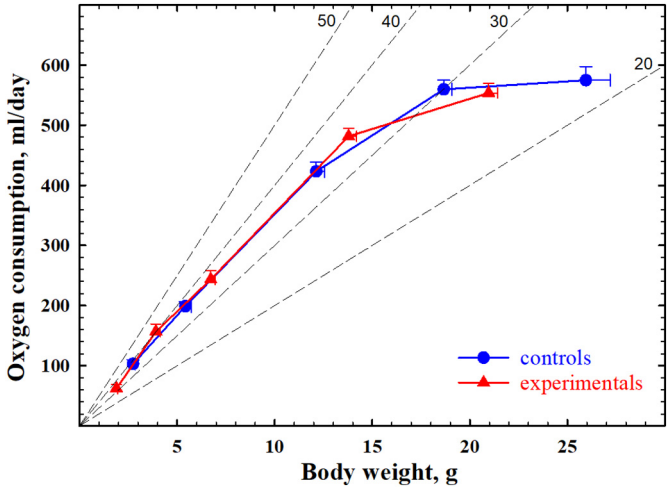

Fig. 5. Body weight-oxygen consumption ($\dot{V}_{O_2}$) relationship from data collected every other day between E10 to E18 in control embryos (- circles) and in embryos that had cold-induced stunt between E7 and E10 (experimentals, - triangles). Oblique dashed lines are isoplets of$\dot{V}_{O_2}$-weight ratios. Each symbol represents mean values of 8–18 embryos, bars represent 1 SEM.

4. Discussion

As the mechanisms responsible for the perception and control of body weight are unknown, so too the mechanisms responsible for CUGr remain hypothetical. It would be important to know whether the body can adjust its weight in relation to its age, since this property would seem to be an essential necessity for CUGr. During embryogenesis, age and weight increase together but they mismatch in conditions that stunt growth. Hence, upon termination of the growth-stunted period, it is possible to compare the experimental observations of body growth to those predicted by models that link growth either to age, or to weight or to various combinations of the two. This comparison was the approach used in the current study and the results showed that age had no bearing on the embryo's growth during CUGr, which depended entirely on weight.

4.1. Growth retardation and CUGr

Both types of interventions (cold or hypoxia) caused growth retardation, which varied with the duration and the age at onset. Upon termination of the growth-inhibiting condition, growth resumed. The magnitude of the post-intervention recovery depended on how growth was quantified. In fact, the mean weight gain of the experimentally-treated embryos from the end of the intervention to E18 averaged 14.4 g (wet) and 2.8 g (dry); because both values were lower than the corresponding weight-gain of controls (respectively, 17.5 and 3.2), one could conclude that the post-intervention growth of the experimental groups was less than in controls. However, a different conclusion can be reached when weight is considered relative to control, because the average weight of the experimental embryos at end-intervention was 73% (wet) and 61% (dry) of controls and increased to, respectively, 80% and 84% at E18. In fact, a constant absolute difference between experimental and control embryos with age must imply a progressive increase in the weight of the experimental group when expressed in percent of control. Hence, whether the experimental-control comparison is made on absolute or on relative terms leads to different conclusions regarding the extent of CUGr. Postnatally, because the normal growth trajectory is concave relative to the time axis, even minimal growth permits a growth-retarded organism to reduce the gap from controls (Azzam and Mortola, 2007). Prenatally, because of the convexity of the growth curve with respect to the time-axis (Fig. 2, top panel), even substantial growth may not be enough for the

experimentally-treated embryos to approach the control values. Commonly, growth curves are compared over the same age-interval (Boersma and Wit, 1997; de Wit et al., 2013); equally, age intervals are the base for the assessment of postnatal CUGr in animals (e.g., Brown and Guthrie, 1968; Williams and Hughes, 1975; Okubo and Mortola, 1988; Sant'Anna and Mortola, 2003) and in children (e.g., Prader et al., 1963; Tanner, 1963; Garrow and Pike, 1967). In keeping with this approach, we conclude that the embryos of the present study experienced CUGr. As discussed below, though, the usage of time (X-axis) as the independent variable is a convenient and time-honored approach, but should not distract from the fact that time per se has no causative or mechanistic link with the embryonic growth during CUGr.

4.2. Embryonic CUGr: age versus weight

Four models were adopted to investigate whether embryonic age or weight may have a role in the post-intervention CUGr; two considered embryonic age at end-intervention (model A) or the average age during the intervention (model B) as determinants of post-intervention growth, one considered solely embryo's weight (model C) and the fourth one combined both age and weight. Each model separately considered the wet and dry weight, because body water content drops, and the dry-wet weight ratio rises, during embryogenesis (Azzam and Mortola, 2007). The results were unequivocal in indicating that wet weight was the best descriptor of the embryo's growth during CUGr; in fact, this model produced the perfect score, with an average measured/predicted ratio of 1.0 (Fig. 3). The addition of age as a parameter possibly influencing post-intervention growth (model D) decreased the predictive accuracy of the model; age alone had the lowest predictive value.

Despite its perfect average score, model C (wet) included some variability among the various interventions. Specifically, the CUGr following interventions in the second week of incubation was slightly lower than predicted by the model (Fig. 4). One reason could be some time lag in the onset of CUGr, because control conditions most likely were not re-established as soon as the intervention was removed. This was definitively the case for the cold interventions, because the thermal time constant of a 60-g egg is approximately 50 min (Mortola and Gaonac'h-Lovejoy, 2016), meaning that thermal equilibrium was not reached before 3–4 h of normothermia. The time lag of re-establishing normal oxygenation cannot be easily estimated. Previously, it was shown that cells arrested by hypoxia, upon aeration resumed their cycle at low pace (Shrieve and Begg, 1985). In the postnatal period, some data have shown that the extent of recovery varied with the age of the growth stunt. For example, the CUGr of quails which underwent food restriction during the first days after hatching was less complete than those with food-restriction at a later age (Chin et al., 2013). In rats, food restriction at early stages of development reduced cell numbers and permanently compromised growth, while at later stages the reduction in cell size, rather than number, permitted growth recovery (Winick and Noble, 1966; Winick et al., 1968). In children, the final height is more likely to be compromised when CUGr coincides with pubertal growth rather than at an earlier age (Boersma and Wit, 1997).

4.3. How does the body estimate its weight?

The present results support the view that embryonic weight, and weight alone, is relevant for the growth during CUGr. However, how weight is regulated during CUGr remains hypothetical, as hypothetical are the current theories on weight detection and regulation (Wood, 2018).

With respect to CUGr, Tanner (1963) postulated the existence of circulating inhibitory factors produced by growing tissues and of a central set-point represented by the appropriate concentration of growth factors at various ages; the higher the concentration of the circulating factors, the closer the organism would be to its weight-'target'. From this hypothesis, CUGr would result from the mismatch between the peripheral circulating factors and the central target. The existence of circulatory factors inhibitory on tissue growth was hypothesized previously, but their direct demonstration remains hypothetical even at present times. Baron et al. (1994), based on experiments on rabbits, noted that after inhibition of growth of a single growth plate, recovery was restricted to the affected plate, which led them to propose that CUGr depended on local, rather than systemic, factors. This hypothesis is conceptually similar to what proposed earlier by Williams and Hughes, 1975; Williams, 1981, who noted that CUGr of the whole body resulted from organ-specific responses of variable magnitude. More recently, Sant'Anna and Mortola (2003), in juvenile rats with stunted growth because of hypoxia or undernutrition, quantified the unevenness in growth retardation among organs and found that CUGr continued for as long as some degree of inter-organ unevenness remained present. Indeed, inter-organ communication through biochemical interaction and mechanical forces is recognised as a mechanism to regulate the organ final size (Eder et al., 2017).

As is the case postnatally, in embryos hypoxia causes inter-organ unevenness because of the differences in hypoxia-sensitivity and hypometabolism among organs (Azzam and Mortola, 2007). These differences were fully accounted for by the decrease in growth. In fact, because during embryogenesis organs grow at different rates, the mere fact of a stunt in body growth must introduce developmental differences among organs; then, these differences decrease as growth resumes. Therefore, because growth is higher the lower the weight (weight-growth relationship, Fig. 2, bottom panel), the comparison of growth curves of experimentally-treated and control embryos as function of developmental age would give the impression of faster growth in the former group when, in fact, the growth of the experimentally-treated embryos is totally appropriate for their weight.

In support of the conclusion that growth of the experimental embryos is only apparently faster than that of controls are the data on the energetics. In fact, the weight- $\dot{V}_{O_2}$ curves of experimental and control embryos were almost identical (Fig. 5), with no significant differences in the maintenance and growth coefficients (a and b in Eq. 1), meaning that the $\dot{V}_{O_2}$ values of the experimental embryos were appropriate for their weight. These results are not compatible with the idea of "faster" growth in the experimental embryos, which would have required higher $\dot{V}_{O_2}$, especially because the cost of growth is a major component of the embryo's total energy budget (Mortola and Cooney, 2008). There are no previous studies on CUGr of embryos or fetuses of any class of vertebrates that examined the possibility of weight as the causative variable responsible for the post-stunt growth. With respect to the development of insects, it was proposed long ago that body weight was the critical variable to initiate the process of larval metamorphosis (Nijhout and Williams, 1974). How this critical size is assessed, though, remains still unresolved in insects as it is in vertebrates (Nijhout, 2003).

4.4. Conclusions

Chicken embryos, after periods of growth retardation caused by hypoxia or cold, resumed their growth. When compared to control as function of developmental time, the post-intervention growth of the experimentally-treated embryos appeared to be faster than that of controls, or CUGr. This was because, being growth greater the smaller the weight, their growth was appropriate for their weight, rather than for their age. In fact, models of post-intervention growth indicated that embryonic weight, and weight alone, was the best predictor of growth, a result confirmed by data on the $\dot{V}_{O_2}$ of experimental embryos following a period of growth retardation.

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Declaration of interests

The author declares no competing interests.

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