World Rabbit Science

World Rabbit Sci. 2010, 18: 229 - 236
doi:10.4995/wrs.2010.780

METABOLIC ADAPTATIONS IN NEONATAL MOTHER-DEPRIVED RABBITS

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Abstract: In order to study the metabolic adaptation in response to 48 h transient doe-litter separation (DLS) in young rabbits (5 rabbits/d group) between postnatal 9 and 11 d, plasma concentrations of thyroid hormones T3 and T4, insulin, leptin, glucose, triglycerides (TG), and free fatty acids (FFA) were examined before (6-8 d), during (9-11 d), and after separation (12-16 d). T3 concentrations in newborn control rabbits gradually increased from 0.6 ng/mL at postnatal 6 d to 1.0 ng/mL at postnatal 16 d, whereas those of T4 remained fairly constant (25 ng/mL) up to postnatal 14 d, when T4 gradually declined to 8 ng/mL. T3 values of DLS newborn rabbits did not differ from those of controls at postnatal 10 and 11 d, but were lower (P<0.05) at postnatal 12 d, while T4 concentrations in DLS animals increased, although not significantly, between postnatal 10 and 12 d compared to controls. Insulin concentrations in young control rabbits ranged between 0.6 and 1.0 mg/L in the early postnatal days, whereas those for leptin averaged 2-3 ng/mL. Insulin and leptin values in DLS newborn rabbits were lower (P<0.05) at postnatal 10 and 11 d, but thereafter rebounded to levels close to those of controls. Glycaemia showed a comparable trend in both groups, ranging between 170 and 190 mg/dL up to postnatal 14 d, but thereafter decreased (P<0.05) to values of 120-130 mg/dL independently of treatment. Concentrations of TG varied greatly from day to day around a mean value of 300 mg/dL, whereas those of FFA remained at approximately the same steady-state levels from postnatal 6 to 16 d, averaging 0.8 mM without any significant differences between groups. In conclusion, these findings confirm that newborn rabbits can cope with the metabolic stress of starvation associated with DLS by lowering insulin and leptin concentrations while maintaining those of thyroid hormones, an overall endocrine response which, together with temporary increase of glucorticoids, successfully maintains an adequate energy balance.

Key Words: maternal separation, thyroid hormones, insulin, leptin, glucose, rabbits.

INTRODUCTION

Transient doe-litter separation (DLS) is a bio-stimulatory technique that improves sexual receptivity and fertility of lactating rabbit does (Theau-Clément *et al*., 1998; Bonanno *et al*., 1999, 2002; Theau-Clément 2007). In newborn rabbits, it was recently observed that a 48 h DLS between postnatal 9 and 11 d modified their hypothalamic-pituitary adrenal axis (HPA) reactivity later in life (Brecchia *et al*., 2009). In that case, however, the stress-dependent activation of the HPA axis was likely carried by metabolic signals due to forced starvation rather than to temporary interruption of the natural mother-offspring contact, normally

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Received *December 2009* - Accepted *November 2010*

limited to a few minutes a day during the single suckling episode (Escobar *et al*., 2000). Additionally, as a consequence of the DLS-provoked imbalance between energy intake and expenditure, kits lost 20% of their body weight, which was only partially recovered at weaning (Bonanno *et al*., 2004; Brecchia *et al*., 2009).

The effect of feed restriction on the regulatory mechanisms of metabolism has been investigated in several animal species (Booth *et al*., 1996; Buyse *et al*., 2002; Cassar-Malek *et al*., 2001; Ferguson *et al*., 2003), and only recently in rabbits. In this species, long-term energy intake deficiency during development as well as short-term fasting have major neuroendocrine consequences evoking prominent homeostatic reactions of the corticotropic, leptinergic, thyrotropic, and ovarian axes (Rommers *et al*., 2004; Brecchia *et al*., 2006; Boiti *et al*., 2008). To date, however, no experiments have been conducted to assess whether similar endocrine and metabolic responses are activated in neonate rabbits to minimise tissue loss from the negative energy balance caused by DLS-associated forced starvation. There is compelling evidence, in fact, that adverse environmental conditions during the prenatal and/or neonatal period can lead to long-term negative effects in the lifetime of an exposed individual (Breton *et al*., 2009; Forhead and Fowden, 2009).

Therefore, in this study the peripheral blood profiles of key metabolic hormones (thyroid hormones T3 and T4, insulin, and leptin) and metabolites (glucose, fatty acids and triglycerides) were examined before, during, and after a 48-h neonatal maternal separation, at postnatal 9 to 11 d, in order to better understand the underlying hormonal and metabolic adaptation mechanisms induced by DLS in neonate rabbits. Data on corticosterone and individual body weight of kits as affected by DLS were presented in a previous study (Brecchia *et al*., 2009).

MATERIALS AND METHODS

Animals

New Zealand White (NZW) lactating does of different parities (n=20), aged 5-12 months and weighing 3.8-4.1 kg, were kept in indoor brick shed facilities under a constant 16:8 h light-dark cycle photoperiod. Ambient temperature was maintained between 22-24 °C. The animals were raised individually in standard metal cages equipped with an outside placed feeder and nest-box. Water and feed (a commercial rabbit feed containing, as provided by the feed company, 11.0 MJ digestible energy/kg dry matter, 17.2% crude protein, and 15.8% crude fibre) were provided *ad libitum*. The does were artificially inseminated (AI) following a 42 d reproduction rhythm with semen collected from NZW rabbit males. Each litter size was equalised to 8-9 kits by cross fostering within 2 d after parturition. The day of birth was designated as postnatal 0 d. At 9 d post partum, does of the same parity were randomly assigned to either control or DLS group. Control does had free access to nest boxes for nursing, whereas DLS does were physically separated from their litters by a metal screen for 48 h, from 9 d (11:00 a.m.) to 11 d (11:00 a.m.), when the nest boxes were reopened to allow nursing of litters. However, to guarantee a post-suckling interval of at least 3-4 h before blood sampling, all the nest boxes were closed at 07:00 a.m. and then reopened soon after the bleeding procedure was concluded.

Blood sampling experimental design

Blood samples (1 mL) were drawn daily from both control and DLS rabbit kits (n=130) of different litters from postnatal 6 to 9 d (5 rabbits/d) and from postnatal 10 to 16 d (5 rabbits/d group) by cardiac puncture, using either an insulin or a 2.5 mL syringe provided with a 23 gauge needle. The bleeding procedure took a maximum of 30 sec from the moment the kit was removed from the nest-box. Whenever the bleeding procedure exceeded this period, the sample was discarded. Casualties due to blood sampling were 1.5%.

Upon successful or unsuccessful blood collection, rabbits were marked with an indelible pen before being returned to their nest boxes. For each day, a different set of neonate rabbits was used so that each animal underwent only one bleeding throughout the experiment. The blood samples, placed in pre-cooled plastic tubes containing EDTA, were immediately centrifuged at 3000 g for 15 min and the plasma was stored frozen until assayed for hormones and metabolites. The bleeding sessions were carried out between 10:00 and 11:00 a.m.

Hormone assays

Total triiodothyronine (T3), insulin, and leptin concentrations were determined by RIA, as reported elsewhere (Rommers *et al*., 2004). Thyroxine (T4) was assayed by RIA according to the procedure provided by the manufacturer (Ortho-Clinical Diagnostic, Amersham, UK). The assay sensitivity was 3.1 ng/mL. Intra- and inter-assay coefficients of variations were 2.6 and 3.6%, respectively. All samples were assayed in duplicate within the same run to limit inter-assay variability.

Metabolite assays

Glucose was analysed by the glucose oxidase method using the Glucose Infinity kit obtained from Sigma (Sigma Diagnostic Inc., St. Louis, MO, USA). Triglyceride (TG) concentrations were analysed using an enzymatic colorimetric assay from Sigma (Triglycerides Reagent Infinity). Free fatty acid (FFA) concentrations were measured with an enzymatic colorimetric assay from Wako (Wako Chemicals GmbH, Neuss, Germany) as previously reported (Brecchia *et al*., 2006).

Statistical analysis.

Data relative to overall treatment effects on hormones and metabolites were analysed by ANOVA according to a mixed model which included the fixed effects of treatment group, time period, interaction between group and time period, and the random effect of the young rabbits within treatment group, which was used as the error term. Comparison between effects was performed by Student's t-test. A value of $P<0.05$ was considered significant.

RESULTS

Thyroid hormones

In control rabbits, T3 concentrations gradually increased ($P<0.05$) from 0.57 ± 0.06 ng/mL at postnatal 6 d to 0.97 ± 0.092 ng/mL at postnatal 16 d (Figure 1A). T3 plasma values of DLS rabbits did not differ from those of controls at postnatal 10 and 11 d during the period of mother separation, but were lower ($P<0.05$) at postnatal 12 d (Figure 1A). T3 concentrations tended to be higher in DLS than in control rabbits for 14 and 15 d (Figure 1A), although not significantly. In control rabbits, the mean peripheral plasma T4 concentrations remained fairly constant (25 ng/mL) up to postnatal 14 d (Figure 1B), when T4 gradually declined ($P<0.05$) to lower levels (8 ng/mL). In DLS rabbits, T4 concentrations showed a profile without significant differences from that of the controls (Figure 1B).

Insulin and leptin

In control rabbits, insulin and leptin concentrations did not change throughout the postnatal period examined (Figure 2A and B). In DLS rabbits, insulin and leptin values were lower ($P<0.05$) than those obtained for the controls at postnatal 10 and 11 d during the maternal separation, but thereafter matched those of the controls (Figure 2A and B).

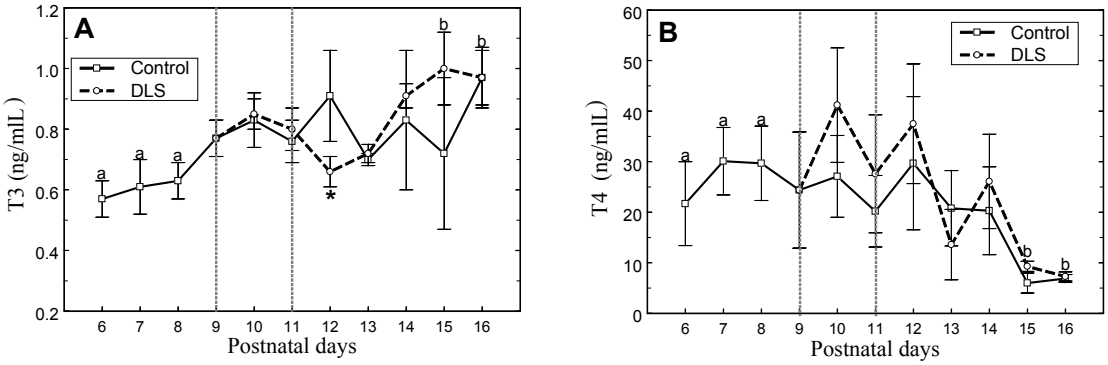

Figure 1: Plasma T3 (panel A) and T4 (panel B) concentrations in newborn rabbits from postnatal 6 to 16 d. The DLS rabbits were separated from their mother for 48 h from postnatal 9 to 11 d (dotted lines); thereafter, the does were allowed the same free access to the nest-box as the control rabbits. Blood samples were taken by cardiac puncture within 30 s from the capture of each newborn rabbit. Each time point represents the mean±standard error of mean of 5 young rabbits. Different letters mark significantly (P<0.05) different values from previous ones, while asterisks indicate different values between groups.

Metabolites

In the early postnatal period up to 14 d, the mean plasma glucose concentrations of control rabbit kits ranged between 170 and 190 mg/dL, but thereafter decreased (P<0.05) to values between 120 and 130 mg/dL (Figure 3A). In DLS young rabbits from postnatal 11 d onward, glucose concentrations overlapped those of controls (Figure 3A). TG plasma concentrations of control rabbits greatly varied from day to day around a mean value of 250 mg/dL (Figure 3B). No difference in TG concentrations between DLS and control rabbits was observed during the 48-h separation, or in the period up to postnatal 16 d (Figure 3B). In control rabbits, FFA concentrations remained at approximately the same steady state levels from

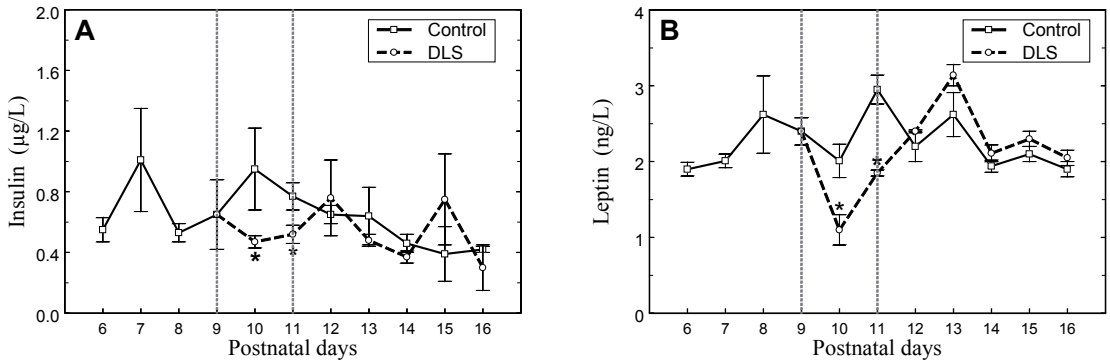

Figure 2: Plasma insulin (panel A) and leptin (panel B) concentrations in young rabbits from postnatal 6 to 16 d. The DLS young rabbits were mother-deprived for 48 h from postnatal 9 to 11 d (dotted lines); thereafter, the does were allowed the same free access to the nest-box as the control rabbits. Blood samples were taken by cardiac puncture within 30 s from the capture of each newborn rabbit. Each time point represents the mean±standard error of mean of 5 young rabbits. Asterisks mark significantly (P<0.05) different values from corresponding controls.

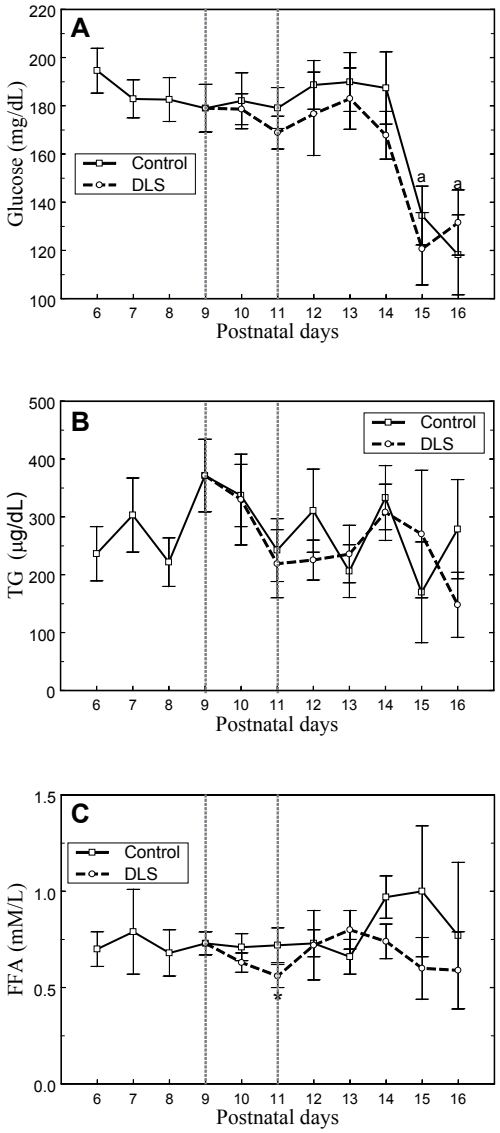

Figure 3: Glucose (panel A), triglycerides (TG; panel B), and free fatty acid (FFA; panel C) profiles in young rabbits from postnatal 6 to 16 d. The DLS young rabbits were mother-deprived for 48 h from postnatal 9 to 11 d (dotted lines); thereafter, the does were allowed the same free access to the nest-box as the control rabbits. Blood samples were taken by cardiac puncture within 30 s from the capture of each newborn rabbit. Each time point represents the mean±standard error of mean of 5 young rabbits. Letters mark significantly (P<0.05) different values from previous ones, while asterisk indicate different values between groups.

postnatal 6 to 16 d, averaging 0.8 mM (Figure 3C). During maternal separation, at postnatal 11 d, FFA levels were lower (P<0.05) in DLS young rabbits but no difference was subsequently found between groups (Figure 3C).

DISCUSSION

To our knowledge, this is the first paper to characterise the metabolic adaptations of newborn rabbits to DLS by examining the dynamic changes of key metabolic hormones and metabolites in the early days of life. Newborn rabbits rely on a daily milk supply from suckling for their postnatal growth (Blumberg and Sokoloff, 1998). Usually, nursing occurs once a night and lasts only a few minutes (Zarrow *et al*., 1965; Hudson and Distel, 1982; González-Mariscal, 2007), but the doe's high milk fat content and the kits' gradual gastric emptying (Escobar *et al*., 2000) allow newborn rabbits to endure fasting between each nursing (Coureaud *et al*., 2000; Rödel *et al*., 2008). In the case of DLS, the two-day separation means that young rabbits miss two successive nursings. This forced starvation, longer than normally experienced fasting, caused around 20% decrease in body weight that was not fully recovered during the subsequent growth up to weaning (Brecchia *et al*., 2009). This weight loss, reported in all other studies to date (Bonanno *et al*., 2002, 2004; Rebollar *et al*., 2004, 2006), clearly indicates degradation of fat tissue and decrease in lean tissues, both compensatory mechanisms to prolonged food deficiency. Nevertheless, the mortality rate and viability of rabbits up to weaning were not affected by maternal separation (Bonanno *et al*., 2002, 2004; Brecchia *et al*., 2009), suggesting that kits can cope with the stressful event of DLS and the associated negative energy balance by adaptations that involve not only the adrenal axis, as already described (Brecchia *et al*., 2009), but also the entire metabolism.

The thyroid gland plays an important role in the regulation of energy homeostasis through the effects of thyroid hormones T4 and T3 on the entire metabolism. Thyroid hormones increase obligatory as well as facultative thermogenesis by

stimulating several metabolic pathways and the sympathetic nervous system (Silva, 1995). During the two-day dam-litter separation, the peripheral plasma concentrations of thyroid hormones were comparable to those of controls. In contrast, at postnatal 12 d, following re-establishment of nutrient supply through regular nursing, the T3 concentrations of DLS rabbits were lower than those of controls, but thereafter both T3 and T4 values of DLS and control rabbits did not differ. These findings prove that the thyroid axis of DLS rabbits was only marginally affected by the abrupt reduction in energy intake and suggest that in the neonatal period compensatory mechanisms other than those involving thyroid hormones come into play to counteract starvation.

Several studies have examined the metabolism of thyroid hormones, which produce either T3 or the metabolically inactive reverse T3 from the deiodination of T4 in the liver. During fasting in both animals and humans (Boelen *et al*., 2008), a decrease in circulating blood T3 and T4 has been observed. A similar down-regulation of thyroidal function was also found in growing rabbits during long-term feed restriction (Rommers *et al*., 2004), in adult rabbits during fasting (Brecchia *et al*., 2006), and also in several other species when undernourished (Cassar-Makel *et al*., 2001; Booth *et al*., 1996; Buyse *et al*., 2002). During fasting or prolonged feed restriction, reduced thyroid function decreases the resting metabolic rate so that animals can save energy and cope with the emergency of food shortage. Some recent studies assert that the drop of thyroid hormones during starvation is mediated by decreased expression and activity of thyrotropin-releasing hormone (TRH) in the hypophysiotropic neurons of the hypothalamic paraventricular nucleus, in response to falling leptin levels and increased sensitivity to the negative feedback inhibition by thyroid hormones (Lechan and Fekete, 2006). Interestingly, TRH not only regulates energy homeostasis through its control of thyroid function, but also has central effects that regulate feeding behaviour, thermogenesis, locomotor activation, and autonomic function. Thus, it is not surprising that in newborn rabbits, born blind and hairless, with a limited ability to regulate body temperature, the function of the thyroid gland and/or the metabolic pathways of thyroid hormones are preserved.

Insulin is a key player in the control of intermediary metabolism by exerting an overall anabolic action. Leptin, a hormone secreted by fat cells, is also involved in the regulation of energy balance during short- and long-term changes of nutritional state. It is well established that circulating insulin and leptin concentrations are proportional to body adiposity, although their secretion and circulating levels are also influenced by energy intake (Jéquier, 2002). Numerous peripheral signals as well as changes in circulating concentrations of glucose and other nutrients (amino acids and fatty acids) together with gastrointestinal (GI) peptide hormones contribute to the short-term regulation of feed intake. All these signals likely interact with long-term regulators such as insulin and leptin for the maintenance of energy homeostasis (Havel, 2001).

In the present study, the marked reduction in insulin and leptin plasma concentrations observed after DLS is probably a response to the reduced carbohydrate intake and/or to energy deficit and mobilisation of fat stores. The reduction of plasma insulin and leptin concentrations as well as the concurrent increase in corticosterone described for the same animals in a previous report (Brecchia *et al*., 2009) were the main components of an integrated endocrine and neuronal response to hypoglycaemia that involves the hypothalamus pituitary adrenal cortex axis, the pancreas, and adipose tissue. Similar dynamic changes have been observed in studies of fasting in adult rabbits (Brecchia *et al*., 2006) and other species (Ferguson *et al*., 2003; Henry *et al*., 2004), demonstrating that adaptive mechanisms are already in place in the early days of life. Similar findings were also observed in growing rabbits as a result of restricted feed intake (Rommers *et al*., 2004).

Since there is evidence that insulin and leptin are transported to the brain (Woods *et al*., 2003, Ziylan *et al*., 2009), these hormones might have a role in signalling the metabolic state of the animal and in modulating the expression of hypothalamic neuropeptides known to regulate feeding behaviour and body

weight. Reduction of the leptin signal induces several neuroendocrine responses that tend to limit weight loss and energy expenditure, such as food-seeking behaviour and suppression of plasma thyroid hormone levels (Lecham and Fekete, 2006). The mechanisms by which mother separation decreases circulating leptin levels are still unclear. However, an increasing body of evidence suggests that in adult animals, leptin secretion is regulated by factors not directly dependent on adipose body mass content (Barb, 1999; Chilliard *et al*., 2001), but rather by the availability of oxidisable nutrients (Obici and Rossetti, 2003). The time course of leptin adaptation to DLS suggests that a similar mechanism is already activated in the early days of life. After DLS, both insulin and leptin concentrations of newborn rabbits matched those of controls even if their body weight was approximately 20% lower than that of normally nursed rabbits (Brecchia *et al*., 2009).

Glycaemia was relatively high in all newborn rabbits during the first 2 wk of age and then decreased by postnatal 15 d in both groups. This finding indicates that metabolic changes in the use and/or active production of oxidisable carbohydrates likely occur at this stage of postnatal development to support the rapid growth rate of this period (Mayor and Cuezva, 1985). Glucose concentrations were not influenced by DLS. FFA plasma concentrations declined during the second day of maternal separation, but when nursing was allowed again at postnatal 11 d, their values matched those of controls up to postnatal 16 d. Free fatty acids are released by the action of hormone sensitive lipase on TG stores in adipose tissue and increased FFA concentrations are indicative of negative energy balance (Emery *et al*., 1992; Rukkwamsuk *et al*., 1999). Hence, decreased circulating FFA may reflect redirection of fat metabolism towards reduced lipolysis due to increased plasma insulin concentrations. In contrast, TG were not affected by DLS. Taken together, these findings suggest that the carbohydrate and fat metabolisms of kits were only marginally influenced by the sudden shortage of energy intake due to DLS.

Adipose tissue, the main place for energy storage and energy balance regulation through insulin, is targeted by thyroid hormones, which act as pleiotropic factors by regulating genes involved in differentiation of adipocytes during development as well as those involved in lipogenesis, lipolysis, and thermogenesis in the brown adipose tissue (Obregon, 2008). Decreased insulin and leptin plasma concentrations during maternal separation together with transitory increased secretion of corticosterone observed at postnatal 11 d in mother-deprived rabbits (Brecchia *et al*., 2009) were important events for metabolic adaptation to DLS.

In conclusion, our findings confirm that kits can cope with the metabolic stress of DLS-associated forced starvation by lowering insulin and leptin concentrations while maintaining those of thyroid hormones, an overall endocrine response which, together with temporary increase of glucorticoids, successfully maintains an adequate energy balance by favouring gluconeogenesis, lipolysis, and protein-sparing to preserve muscle and visceral proteins. Moreover, DLS applied to neonate rabbits could represent an interesting animal model for studying how the risk of developing major chronic diseases such as diabetes, obesity or cancer may be conditioned by the early days of life.

Acknowledgments: This work was partially supported by a grant from the Italian "Ministero Istruzione Università e Ricerca" (Progetto Ricerca Interesse Nazionale – Prot. 2001072484 and 2003074002) and by generous donations from the BC Red-water Trust (Perugia, Italy). Procedures conformed to animal care guidelines and were approved by the Bioethical Committee of the University of Perugia in accordance with the current laws of Italy. The authors gratefully acknowledge the revision of the English text by Sheila Beatty.

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