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Summary

Neonatal mortality in dogs (within the first three weeks after birth) accounts on average for 20% of puppies born alive, being thus responsible for a great economic loss to dog breeders. However, immunological and nutritional determinants of puppies survival are poorly described. This dissertation thus investigated the risk factors of neonatal mortality in dogs, and in particular the importance of colostrum intake for survival. The first part of results revealed a strong association between early growth rate (during the first two days of life) and neonatal losses. As early growth rate reflects the colostrum intake, the role of colostrum was then addressed. Passive immune transfer was shown to affect mortality rate, with serum IgG concentration at two days of age lower than 2.3 g/L being characterized as in deficit of maternal immunoglobulins. Similar lack of immunoprotection was observed for a specific canine pathogen (canine parvovirus type 2), as puppies with low antibody titers at two days of age seroconverted or underwent parvovirus infection significantly earlier than puppies with higher titers. Energy intake, evaluated via blood glucose concentration at 24h of life, was also found associated with survival: puppies with low glucose concentration (≤ 92 mg/dl) were found at higher risk of death. Besides the strong relationship between colostrum ingestion, providing passive immunity and energy, the impact of birth weight and vitality at birth (evaluated via Apgar score) on puppies' survival was also evidenced. Colostrum immune quality (evaluated via IgG concentration), although not directly linked with the risk of neonatal death, was found of great variability, most probably putting some puppies at a risk of passive immune deficit. The present study contributed to the knowledge about the risk factors of mortality to be controlled in breeding kennels. Results provided in this work revealed the crucial role of the fetal growth, course of parturition and intake of the colostrum for the newborn dog. Regular weighing of newborns can be advised as a practical application for dog breeders, as it allows to identify puppies at a higher risk of death and to provide puppies with additional nursing and veterinary care.

Résumé

Environ 20% des chiots nés vivants meurent au cours des trois premières semaines de vie. Cette mortalité néonatale est ainsi responsable de pertes économiques importantes pour les éleveurs. Néanmoins, les déterminants immunitaires et nutritionnels de ces cas de mortalité sont très mal connus dans l'espèce canine. L'objectif de ce travail était donc d'identifier les facteurs de risque de mortalité néonatale chez le chiot, dont en particulier l'importance de la prise colostrale pour la survie. La première partie des résultats a mis en évidence une association forte entre la croissance précoce (sur les deux premiers jours de vie) et les pertes néonatales. Cette croissance précoce reflétant directement la prise colostrale, la suite des travaux s'est ensuite intéressée aux différents apports colostraux. Le transfert d'immunité passive affecte le taux de mortalité, une concentration sérique en immunoglobulines G à l'âge de 2 jours inférieure à 2,3 g/l étant associée à un risque de mortalité néonatale plus élevé et caractérisant un déficit de transfert. Un résultat similaire a été obtenu avec l'étude de l'immunité spécifique dirigée contre le parvovirus canin de type 2 : les chiots ayant acquis les titres en anticorps les plus faibles à deux jours d'âge séroconvertissent et excrètent du virus significativement plus tôt que les chiots avec de plus forts titres d'anticorps maternels. L'apport énergétique colostral était également associé aux chances de survie : les chiots présentant une glycémie à 24 heures de vie inférieure à 0,92 g/l ayant un plus fort risque de mortalité. Outre la forte relation entre survie et prise colostrale (apportant immunoglobulines et énergie), notre travail montre également l'impact du poids et de la vitalité à la naissance (évalué par le score Apgar). Par ailleurs, la qualité immunologique du colostrum (évaluée par sa concentration en IgG), bien que non directement liée aux chances de survie, s'est montrée très variable entre les chiennes, aggravant probablement le risque de déficit de transfert de l'immunité passive chez certians chiots. Tous ces travaux contribuent à une meilleure connaissance des facteurs de risque de mortalité qu'il est important de contrôler en élevage. Ils révèlent le rôle crucial de la croissance fœtale, du déroulement de la mise-bas et de l'ingestion colostrale. Une pesée des chiots au cours des premiers jours de vie peut être a minima conseillée aux éleveurs pour identifier les chiots à risque et auxquels des soins plus attentifs devront être apportés.

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Valorization of research results

Part of studies described in this dissertation was published or is accepted for publication in international journals, and another part was presented at national and international congresses in form of oral and poster presentations. Some of the results were submitted for publications and communication at different congresses. Ten veterinary theses were supervised.

1. Accepted publications

H. Mila, A. Grellet, A. Feugier, S. Chastant-Maillard. Differential impact of birth weight and early growth on neonatal mortality in puppies. *Journal of Animal Science* 2015; 93(9): 4436-4442.

H. Mila, A. Feugier, A. Grellet, J. Anne, M. Gonnier, M. Martin, L. Rossig, S. Chastant-Maillard. Immunoglobulin G concentration in canine colostrum: evaluation and variability. *Journal of Reproductive Immunology* 2015; 112: 24-28.

H. **Mila**, A. Feugier, A. Grellet, J. Anne, M. Gonnier, M. Martin, L. Rossig, S. Chastant-Maillard Inadequate passive immune transfer in puppies: definition, risk factors and prevention in a large multi-breed kennel. *Preventive Veterinary Medicine* 2014; 116: 209-213.

H. **Mila**, A. Grellet, C. Desario, A. Feugier, N. Decaro, C. Buonavoglia, S. Chastant-Maillard Protection against canine parvovirus type 2 infection in puppies by colostrum-derived antibodies. *Journal of Nutritional Science* 2014; 3(e54): 1-4.

2. Submitted publications

H. Mila, A. Grellet, A. Feugier, J. Anne, M. Gonnier, M. Martin, L. Rossig, S. Chastant-Maillard, Le transfert d'immunite passive chez le chiot : importance dans le contrôle de la mortalité néonatale [Transfer of passive immunity in the puppy: importance in the control of neonatal mortality]. *Point Vétérinaire,* submitted February 2015.

3. Patent

H. Mila, A. Grellet , S. Chastant-Maillard, A. Feugier. Canine health product. *Patent number WO/2015/004181. Application number PCT/EP2014/064711*. 15.01.2015.

4. Keynote lecture

H. Mila, S. Chastant-Maillard. The first two days of life of puppies: crucial steps for survival. *18th Congress of European Veterinary Society for Small Animal Reproduction (EVSSAR)*. Wroclaw, Poland, 26th September. 2014. p 127-130.

5. Oral communications

H. Mila, A. Feugier, A. Grellet, B. Carrez, J. Anne, M. Gonnier, M. Martin, L. Rossig, S. Chastant-Maillard. Failure of passive immune transfer in puppies: risk factors and consequences. *17th Congress of European Veterinary Society for Small Animal Reproduction (EVSSAR)*. Toulouse, France. 5th-6th July, 2013. p 115. Award for the best oral presentation.

H. Mila, C. Desario, A. Grellet, A. Feugier, N. Decaro, C. Buonavoglia, S. Chastant- Maillard S. CPV-2 antibody titers from birth to weaning in puppies under natural infection. *4th Symposium on Veterinary Sciences Toulouse - München –Zaragoza*. Toulouse, France. 11th-13th April, 2013. p 28-29.

S. Chastant-Maillard, **H. Mila**, Colostrum chez les chiots [Colostrum in puppies]. *Congress of Association Française des Vétérinaires pour Animaux de Compagnie (AFVAC).* Toulouse, France, 15th November, 2013. p 7-10.

H. Mila, B.C. Guard, C. Mariani, A. Feugier, A. Grellet, S. Chastant-Maillard, J.M. Steiner, J. Suchodolski. Improvement of intestinal microbiota richness in canine neonates after oral hyper-immunized plasma supplementation. *25th Congress of the European College of Veterinary Internal Medicine - companion animals (ECVIM-CA).* Lisbon, Portugal, 10th-12th September, 2015.

D. Broussou, **H. Mila**, A. Grellet, A. Feugier, C. Mariani, JL. Pingret, C. Boucraut-Baralon, S. Chastant-Maillard. Excretion of canine parvovirus type 2 (CPV-2) during gestation and lactation in bitches and puppies. *25th Congress of the European College of Veterinary Internal Medicine - companion animals (ECVIM-CA).* Lisbon, Portugal, 10th-12th September, 2015.

H. Mila, S. Coinus, A. Grellet, A. Feugier, C. Mariani, M. Power, M. Maslanka, S. Chastant-Maillard. Energy or immunity? Nutritional and immunological composition of canine colostrum. *19th Congress of European Veterinary Society for Small Animal Reproduction (EVSSAR).* Hannover, Germany, 11th-12th September, 2015. p 109.

H. Mila, B.C. Guard, C. Mariani, A. Feugier, A. Grellet, J.M. Steiner, J. Suchodolski, S. Chastant-Maillard. Effect of immunoglobulin supplementation on growth and intestinal microbiota in pre-weaning puppies. *18th Congress of the European Society of Veterinary and Comparative Nutrition (ESVCN).* Toulouse, France, 17th-19th September, 2015. p 89.

6. Poster presentation

H. Mila, M. Catteau, A. Grellet, A. Feugier, C. Mariani, S. Chastant-Maillard. Relationship between body temperature, growth rate and neonatal survival in puppies. *19th Congress of European Veterinary Society for Small Animal Reproduction (EVSSAR).* Hannover, Germany, 11th-12th September, 2015. p 185.

H. Mila, M. Catteau, A. Grellet, A. Feugier, C. Mariani, S. Chastant-Maillard. Evaluation of non invasive methods for temperature measurement in neonatal puppies. *19th Congress of European Veterinary Society for Small Animal Reproduction (EVSSAR).* Hannover, Germany, 11th-12th September, 2015. p 186.

H. Mila, A. Feugier, A. Grellet, S. Chastant-Maillard. Variability of mortality risk factors with age in puppies. *Annual Meeting of the Society for Veterinary Epidemiology and Preventive Medicine*. Ghent, Belgium, 25th-27th March, 2015.

H. Mila, A. Grellet, M. Delebarre, A. Feugier, S. Chastant-Maillard. Metabolic status in canine neonates – importance for survival. *18th Congress of European Veterinary Society for Small Animal Reproduction (EVSSAR)*. Wroclaw, Poland, 26th September, 2014. p 197.

H. Mila, G. Elia, A. Grellet, A. Feugier, S. Chastant-Maillard S, N. Decaro, C. Buonavoglia. Kinetics of systemic and intestinal immunity in puppies after natural infection by canine parvovirus type 2. *The WALTHAM® International Nutritional Sciences Symposium (WINSS).* Portland, Oregon, USA, 1st-4th October, 2013. p 123.

H. Mila, C. Desario, A. Grellet, A. Feugier, N. Decaro, C. Buonavoglia, S. Chastant-Maillard Protection against canine parvovirus type 2 in puppies depending on maternally derived antibody titers. *The WALTHAM® International Nutritional Sciences Symposium (WINSS).* Portland, Oregon, USA, 1st-4th October, 2013. p 124.

H. Mila, A. Feugier, A. Grellet, B. Carrez, J. Anne, M. Gonnier, M. Martin, L. Rossig, S. Chastant-Maillard. Variability in immunoglobulin G concentration in dog colostrum. *17th Congress of European Veterinary Society for Small Animal Reproduction (EVSSAR)*. Toulouse, France, 5th-6th July, 2013. p 115. Award for the best poster presentation.

H. Mila, A. Feugier, A. Grellet, B. Carrez, S. Chastant-Maillard. Increased risk of death and low weight gain in puppies with low immunoglobulin G level. *9ème colloque du réseau français d'Immunologie des Animaux Domestiques (IAD)*, Paris, France, 22nd-23rd January 2013. Award for the best poster presentation.

H. Mila, A. Grellet, S. Chastant-Maillard. Prognostic value of birth weight and early weight gain on neonatal and pediatric mortality: a longitudinal study on 870 puppies. *7th International Symposium on Canine and Feline Reproduction (ISCFR)*. Whistler, Canada, 26th-29th July, 2012. p 163-164.

7. Submitted abstracts

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8. Supervised veterinary thesis defended

M. Catteau. Température du chiot en période néonatale et pédiatrique : mesure, variation, intérêt pronostique [Temperature in the puppy during neonatal and pediatric period: measurement, variability and prognostic value]. *Université Paul Sabatier and Ecole Nationale Vétérinaire de Toulouse*, France, 2014.

M. Delebarre. Evaluation de la santé néonatale chez le chiot : identification des facteurs de risque de mortalité néonatale [Evaluation of the neonatal health in the puppy: identification of risk factors for neonatal mortality]. *Université Paul Sabatier and Ecole Nationale Vétérinaire de Toulouse*, France, 2014.

D. Broussou. Etude de l'excrétion du parvovirus canin CPV-2 par la chienne pendant la gestation et la lactation [Study on the excretion of canine parvovirus CPV2 by the bitch during gestation and lactation]. *Université Paul Sabatier and Ecole Nationale Vétérinaire de Toulouse*, France, 2014.

S. Coinus. Composition nutritionnelle et immunologique du colostrum canin [Nutritional and immunological composition of canine colostrum]. *Université Paul Sabatier and Ecole Nationale Vétérinaire de Toulouse*, France, 2014.

C. Olivier. Impact d'une supplémentation précoce en immunoglobulines chez le chiot sur la croissance, la morbidité et la mortalité néonatale et pédiatrique [Impact of an early immunoglobulin supplementation on growth, morbidity and neonatal and pediatric mortality in the puppy]. *Université Paul Sabatier and Ecole Nationale Vétérinaire de Toulouse*, France, 2014.

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M. Gonnier and L. Rossig. Etude du colostrum et du transfert passif de l'immunité dans l'espèce canine [Study on colostrum and passive immune transfer in the canine species]. *Université Paul Sabatier and Ecole Nationale Vétérinaire de Toulouse*, France, 2013.

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S. Jougneau. Impact d'une supplémentation immunitaire en période pédiatrique sur la croissance et la mortalité des chiots [Impact of immune supplementation durig pediatric period on growth and mortality in puppies]. *Université Paul Sabatier and Ecole Nationale Vétérinaire de Toulouse*, France, 2015.

E. Lavergne. Intérêt de la réfractométrie dans l'évaluation de la qualité immunologique du colostrum canin [Usefulness of refractometry in evaluation of immune quality of canine colostrum]. *Université Paul Sabatier and Ecole Nationale Vétérinaire de Toulouse*, France, 2015.

A.L. Erbacher. Inflammation mammaire subclinique chez la chienne : prévalence et impact sur la croissance et la mortalité des chiots [Subclinic inflammation of the mammary galnd in the bitch : prevalence and impact on growth and mortality in puppies]. *Université Paul Sabatier and Ecole Nationale Vétérinaire de Toulouse*, France, 2015.

10. Oral communications for dog and cat breeders

H. Mila, S. Chastant. How to boost immunity in puppies? International Dog Breeders Convention, Royal Canin, La Grande Motte, France, 28th-30th January 2014.

S. Chastant-Maillard, **H. Mila**. Colostrum: an asset for kittens to begin life and stay alive. Royal Canin. Predeal, Roumanie, 13th June 2014.

S. Chastant-Maillard, **H. Mila**. Colostrum: an asset for puppies to begin life and stay alive. Royal Canin. Predeal, Roumanie, 13th June 2014.

S. Chastant-Maillard, **H. Mila**. Colostrum : une clef pour un bon départ dans la vie. Conférence destinée à des éleveurs félins [Colostrum: cornerstone at the early stage of life]. L'élevage félin cette passion contagieuse. MERIAL Laboratory, Lyon, France, 21st June 2014.

11. Other technical communications

S. Chastant-Maillard, **H. Mila**. Colostrum: an asset for puppies to begin life and stay alive. Royal Canin. Pro Launch. International technical directors. Maisons Alfort, France, 24th-25th September 2013.

S. Chastant-Maillard, **H. Mila**. Mortality in puppies: when? Royal Canin. Pro Launch. International technical directors. Maisons Alfort, France, 24th-25th September 2013.

S. Chastant-Maillard, **H. Mila**. Early growth and puppies mortality. Royal Canin. Pro Launch. International technical directors. Maisons Alfort, France, 24th-25th September 2013.

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Abbreviations

CPV2 – canine parvovirus type 2

CV – coefficient of variation

ELISA - enzyme-linked immunosorbent assay

FCI - Fédération Cynologique Internationale

HI - inhibition of haemagglutination test

IgA – immunoglobulin A

IgG – immunoglobulin G

MDA – maternally derived antibodies

SD – standard deviation

General introduction

Dogs (*Canis familiaris*) are the first animals domesticated by human beings. Their skeletons were found near human bones, as far as 11-16,000 years ago, far earlier than small ruminants or cattle (1). Primitive dogs were used for hunting purposes; however, the role of the dog in human society and related with benefits evolved extremely during last decades. Dogs are not only herding our properties, protecting from strangers or predators, but also assisting in lifesaving actions, such as searching for people trapped in avalanches, collapsed buildings or lost in wilderness. Dogs improve also indirectly human health, as dog owners are found more physically active (2), less susceptible to allergies (3) and of better mental health (4). Nowadays, a new role of dogs appears in human medicine, as dogs are able to detect certain diseases earlier than laboratory tests. Thanks to greatly developed odor receptors and olfactory cortex, medical detection dogs can diagnose hypoglycemia, breast, colon or urinary bladder cancers only by sniffing the person or his/her specimens (5–9).

All of the mentioned skills would not be possible without selective breeding. Already in the third millennium BC, two types of dogs were developed: molossoids protecting the livestock from predators, and greyhound type providing assistance during hunting (1). Nowadays, 343 breeds are registered by the Fédération Cynologique Internationale (FCI), with a great variation in phenotypes and behaviors, varying from sledding Siberian Husky, sheep guarding Border Collie to water retrieving Poodle (10). The overall world dog population accounts for 700 millions, and within all European countries, the highest number of dogs are housed in France (7.4 - 8.6 millions) (1,11). Over 30,000 French breeders, occasional, amateurs or professional, produce the average of 1 million puppies per year (12).

Puppies mortality represents a significant source of economic loss for breeders, with 5-40% of puppies dying before weaning (13). Data collected from breeding kennels around the world, with several thousand puppies included in each study, shows on average mortality rate until weaning (8-9 weeks of age) of 20% (14–17) (Table 1). Three periods of mortality can be distinguished in puppies until two months of age (before leaving the breeding kennel): stillbirth, neonatal mortality and pediatric mortality. Mortality due to stillbirth, standing for all deaths occurring in the course of parturition, varies between different breeding kennels from 12 and 43% of all dying puppies (18). Stillborn puppy exits the birth canal already dead, which can be confirmed by a lungs float test at necropsy. Mortality during the first three weeks after birth,

Table 1. Mortality in puppies at different ages (14–17)**.**

Reference	Number of puppies in the study	Mortality % (ni/n)			
		Stillbirth#	1-3rd week#	4-8th week#	Total¤
Potkay and Bacher, 1977, USA	2872	12.0 (63/524)	62.5 (328/524)	25.4 (133/524)	**18.2** (524/2872)
Nielen et al., 1998, Netherlands	2527	31.3 (147/469)	57.3 (269/469)	11.3 (53/469)	**18.6** (469/2527)
Gill, 2001, Australia	2574	34.7 (180/519)	65.3 (339/519)	*	**20.2** (519/2574)
Mila et al., 2015 France	2288	43.1 (226/524)	39.7 (208/524)	17.2 (90/524)	**22.9** (524/2288)

* Data not available
# In proportion of puppies dying between birth and eight weeks of age
¤ In proportion of all puppies born

accounting for 40 to 65%, is considered as neonatal mortality in dogs. Especially, the first week seems to be crucial, as majority of all neonatal losses occur at that period (Fig.1). During the neonatal period, puppies have to cope with two crucial moments steps: adaptation to extrauterine life and colostrum intake. The first one requires from the neonate the onset of efficient breathing and blood oxygenation together with drastic changes in metabolism. Colostrum provides to the newborn nutrients, energy, maternal antibodies and many other bioactive compounds indispensable for basic activities and correct development. Any abnormality of mentioned processes may thus lead to neonatal mortality. During the pediatric period (between 3 weeks and 2 months of age), circulating maternal antibodies absorbed from colostrum during the first day of life have dropped to very low levels, making the young animal susceptible to pathogens (19). This so called “critical period” ends when the animal achieves sufficient own immunity via vaccination or by going through infection. Animal meeting new agents during this immunological gap may either develop specific antibodies without any clinical signs or go through symptomatic infection, with, in some cases, possibly lethal issues (20). Pediatric mortality accounts for 11 to 25% of the total number of puppies dying between birth and eight weeks of age.

Our work focused on the neonatal period, period at the highest risk of mortality for puppies, and especially, to the first days of life. Our study concerns in particular the adaptation process after birth, colostrum intake and their relationship with survival.

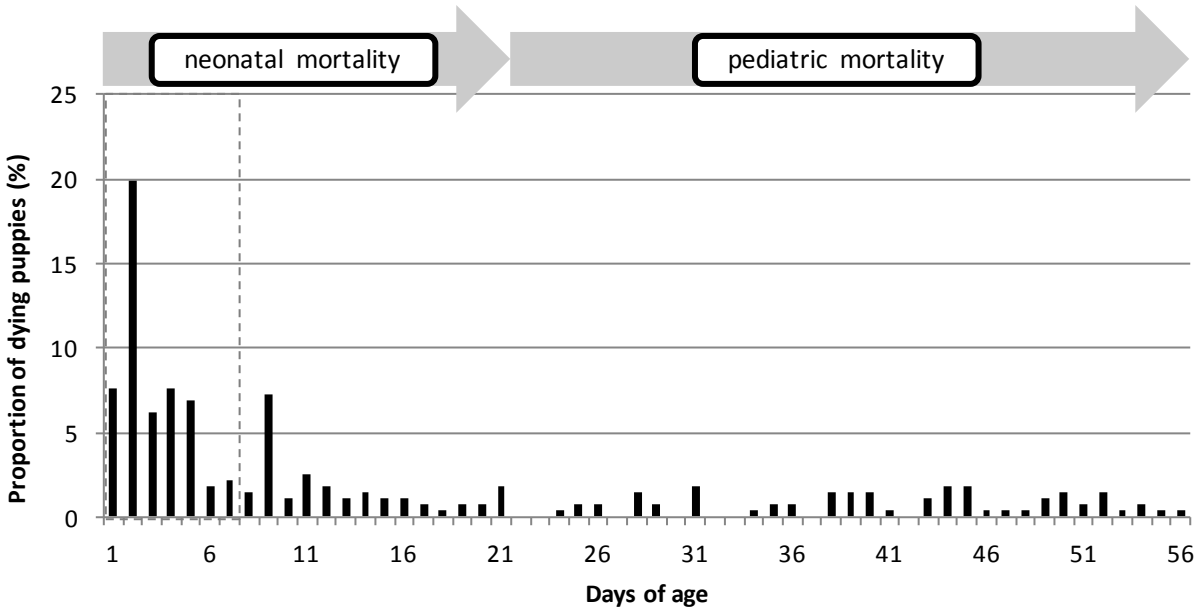

Fig.1. Proportion of puppies dying at different ages among all dying puppies (n=276). Results obtained within one breeding kennel. Author's unpublished data.

In this dissertation, a review of literature concerning crucial steps for survival in neonates will be first presented in form of an article. This comparative study on 12 domestic species (human, horse, cattle, sheep, goat, pig, dog, cat, rabbit, guinea pig, mouse and rat) describes the crucial steps at the very early stage of life. Consequences on newborns of different physiological strategies during intrauterine period (placentation, gestation length, litter size) are discussed. Characteristics regarding birth weight, thermoregulation process and suckling behavior are provided. This review addresses also the importance of colostrum intake during the neonatal period. Finally, interspecies differences regarding the risk of neonatal mortality together with mortality causes are described.

Secondly, the objectives and research strategy of dissertation will be presented, introducing the five experimental studies conducted to evaluate the impact of different factors on neonatal mortality in puppies. In particular, the importance of colostrum intake, in terms of immunity and energy, is investigated. Finally, major findings are discussed. In this part, future work interesting to perform is proposed and practical applications are described.

Bibliography – Article 1

H. Mila, S. Chastant-Maillard

Neonatal period in domestic animals and human – crucial steps for survival

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Neonatal period in domestic animals and human – crucial steps for survival

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Key words: neonate, mammals, passive immune transfer, suckling reflex, litter size, mortality, colostrum

INTRODUCTION

Perinatal period is crucial for all newborn mammals, as its environment shifts dramatically in course of birth from the intra to the extrauterine life. In consequences of the separation from placental supply, not only breathing mechanism begins, but also intermittent feeding becomes and environmental temperature is no more stable. Finally, no barrier exists for pathogens, which requires from the newborn activation of its own defense mechanism. The adaptation process to these dramatic changes differs markedly from one species to another, depending mainly on the level of developmental maturity of the neonate. In precocial species such as domesticated ungulates (horse, pig, ruminants) and some rodents (guinea pig, chinchilla), the newborn is able of an at least partial independency. On the other hand, human infants, mouse and rat pups, as well as canine and feline newborns (classified as altricial) are born completely dependent from the dam and in case of her death, the litter will neither survive. Whatever precocial or altricial, birth and subsequently neonatal period are crucial steps of highly elevated risk of mortality for all species.

The purpose of this review was to compare the different patterns of adaptation to the extra uterine life in the newborn domestic animals, with a focus on interspecies particularities.

DEFINITION OF THE NEONATAL PERIOD

According to World Health Organization, neonatal period in human infants accounts for the first 28 completed days of life (1), during which majority of the extra uterine adaptations are completed (2). In case of death of the fetus at the end of gestation or during parturition, the term “stillbirth” is applied. Stillbirths, together with deaths occurring until postnatal day 7 are considered as perinatal mortality (3). Unlike in human medicine, no official definition of the neonate has been provided in animals. Among species and even within one given species, periods considered as neonatal are very diverse. Mellor and Stafford

(4) proposed the first 7 days of life as the neonatal stage for all farm animals. Indeed, the first week of life is taken into consideration in studies on neonatal mortality in cattle (5), but other definitions encompass much larger periods (e.g. until 45 days of age (6)). Similarly, in small ruminants, the entire period from birth until weaning (at about 30-45 days) maybe be named as neonatal (7–9). Due to legislative reasons, part of neonatal mortality is considered as stillbirth in bovine medicine: since mortality during the first 48h after birth may be related to infection during the fetal life by *Brucella sp.*, calves dying until day two are considered as stillborn (10–12). Foals are considered as neonates either until one or two weeks of age (13,14). Also in carnivores, the end of the neonatal period is not precise, varying between 3 and 4 weeks in puppies (15–17), and from 2 to 8 weeks in kittens (18,19). Finally, in pigs, rather than referring to neonatal period, a pre-weaning period is considered (20–22), lasting until 21 to 35 days after birth. Similar vocabulary (also nest period, suckling period) is used to describe the early stage of life in rabbit and rodent infants (23–25).

INTRAUTERINE PERIOD

Gestation length

Depending on gestation length and intrauterine growth rate, the different species are born at various degrees of maturity, roughly distinguished into two groups: precocial and altricial. The guinea pig newborn, after relatively long pregnancy (median 67-68 days), is able not only to stand up, hear and see, but also to ingest solid food, in a difference with hairless and suckling only rabbit pup (gestation length: 30-31 days). Relatively long pregnancy (if corrected by adult weight) in precocial species allow not only obtaining higher percentage of adult weight (26), but also a more advanced development of the neonate (27).

Despite the median values established for each species (Table 1), the gestation length may vary with the two major variation factors being breed and litter size. In guinea pig females

delivering 1 pup, gestation lasts on average 70.5 days, while if 6 pups are born it is shortened to 66.8 days (28). Similar negative correlation was observed in dogs (29) and in cows (30), but not in pigs (31). This general tendency may suggest that in case of more numerous litters a longer time is needed for fetal maturation. However, gestation length in cows was shorter not only in case of twins, but also in case of birth of heifer calves (32). Sex of the offspring may thus influence gestation length; shorten in case of female fetuses. Nonetheless, it happens sometimes, that delivery starts too early, leading to prematurity with strongly elevated risk of death in preterm infants of all domestic species.

Litter size

The litter size, defined as the total number of animals born within a litter, is the result of both ovarian activity (number of preovulatory follicles per estrus cycle) and embryonic/fetal mortality (30-50% of early embryos are lost in pigs (33)). Although in monotocous species, only a single oocyte per oestrus cycle is selected to be fertilized, a multiple pregnancy occurs as frequently as 1-9% in cows (30,32), 1-4% in horses (34) and 1.3-1.5% in human (35,36). These values however, are inflated due to assisted reproduction techniques routinely used nowadays in all mentioned species. In polytocous domestic animals, the average litter size undergoes large variations between species: 1-2 newborns in sheep and goats, 5 in dogs, 10 in mice and rats and 13 in pigs (Table 1). Except the porcine species, in which selective breeding increased the litter size of 100%, the negative correlation between birth weight, degree of maturity at birth and litter size is evident.

Table 1. *Reproductive characteristics of different domestic species and human.*

Species	Group	Seasonal reproduction	Estrous cycle length	Gestation per year	Placentation	Gestation length*	Litter size*#	References
Human	Altricial	polyestrus	28d	1	discoid, hemochorial	278-281d	monotocous (twins in 1.3-1.5%)	(35–39)
Cattle	Precocial	polyestrus	21d	1	cotyledonary, epitheliochorial	279-280d	monotocous (twins in 1-9%)	(39,12,34,32)
Sheep	Precocial	seasonal polyestrus	17d	1	cotyledonary, epitheliochorial	143-145d	polytocous, 1-2	(30,39–42)
Goat	Precocial	seasonal polyestrus	20-21d	1-2	cotyledonary, epitheliochorial	147-151d	polytocous, 1-2	(30,43,44,39,45)
Horse	Precocial	seasonal polyestrus	21d	1	diffuse, epitheliochorial	340-342d	monotocous (twins in 1-4%)	(34,39,46)
Pig	Precocial	polyestrus	21d	2-3	diffuse, epitheliochorial	114-115d	polytocous, 13-15	(31,34,39,47)
Dog	Altricial	monoestrus	7m	1-2	zonary, endotheliochorial	62-63d	polytocous, 5 small - 3.7 medium - 5.7 large - 7.5 giant - 8.7	(29,48–50)
Cat	Altricial	seasonal polyestrus	14-21d	2-3	zonary, endotheliochorial	65-67d	polytocous, 4-4.7	(19,39,48)
Rabbit	Altricial	no estrus cycle	7-10d of receptivity	8	discoid, hemochorial	30-31d	polytocous, 4-8	(51–53)
Guinea pig	Precocial	polyestrus	15-16d	4-5	discoid, hemochorial	67-70d	polytocous, 3-4	(28,39,51,52,54)
Rat	Altricial	polyestrus	4-5d	every 20-25d	discoid, hemochorial	20-22d	polytocous, 10	(51,53,55)
Mouse	Altricial	polyestrus	4-5d	every 20-25d	discoid, hemochorial	19-20d	polytocous, 10-12	(51,55–57)

* Range of mean values from cited studies.
\# Number of animals born per litter
d - days

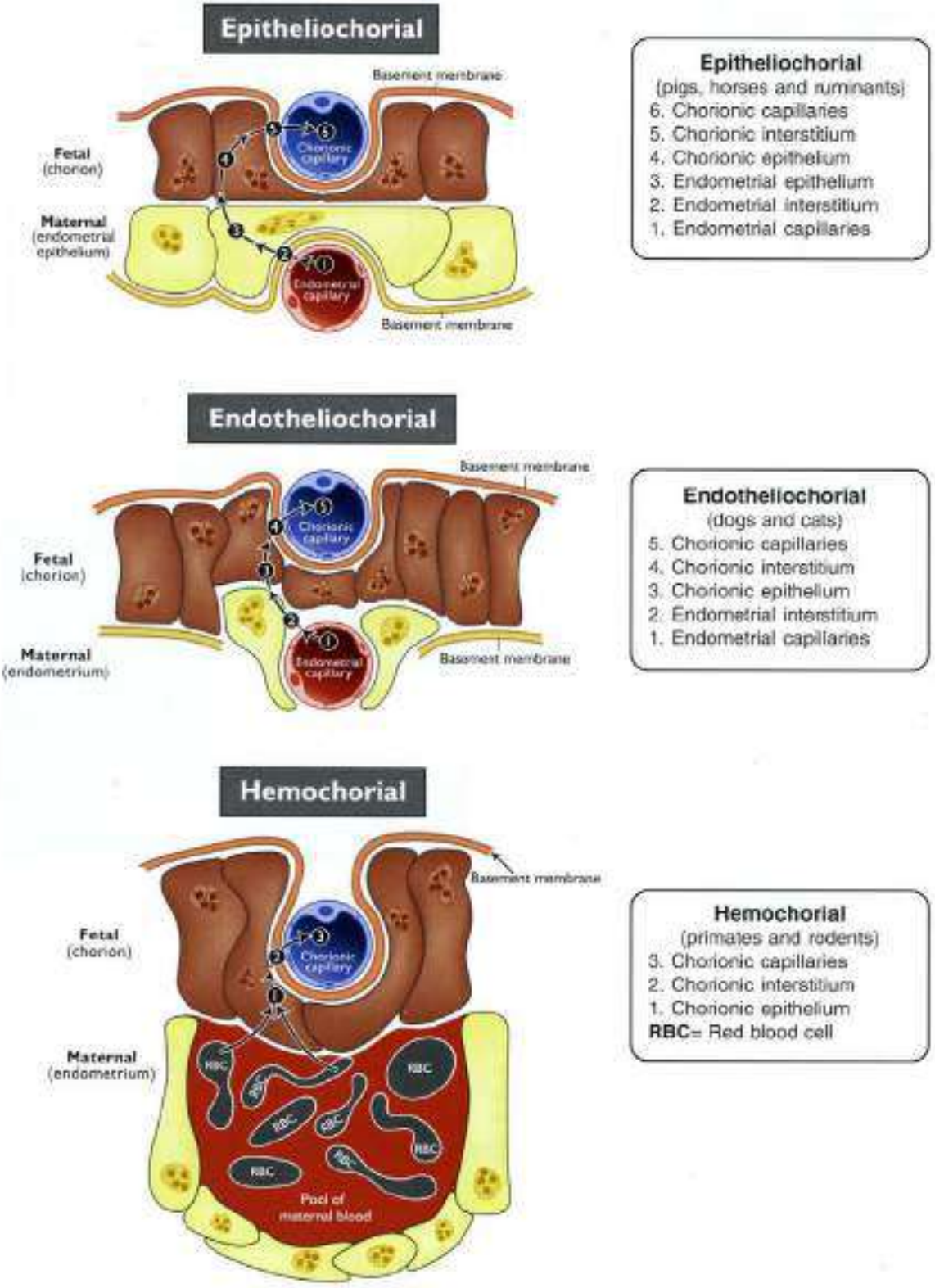

Fig.1. *Placental classification according to histological structure in domestic animals* (39).

Placentation

Many processes adapting the newborn to the external environment take their origin already during the fetal life. Depending on the type of placentation (Fig.1), the transport of oxygen, nutrients, and antibodies varies markedly between species (58,59) (Table 1). In presence of hemochorial placenta, as in human and rodents, maternal and fetal blood come in direct contact, facilitating for example the transfer of maternal antibodies to the fetus (60). Since about 13-16 weeks until the end of gestation, immunoglobulin G (IgG) is transferred to the human fetus via a specific receptor-mediated mechanism (FcRn receptors binding) (61,60). Thanks to this process, not only human infant, but also rat, guinea pig and rabbit pups are born with antibody titers at least as high as those of their mothers ensuring immune protection of the neonate against surrounding pathogens since birth (61). On the contrary, premature delivery in these species (i.e. in human <33 weeks) leads to lower IgG concentration, and thus risk of immune deficiency for the neonate (62). Opposite to human and rodents, in carnivores, presenting endotheliochorial placenta, transplacental immune transfer is very limited, and only 5-10% of circulating antibodies in the newborn's bloodstream are acquired during the fetal life (63,64). The placental barrier is even more tight in case of epitheliochorial placentation (horse, cow, sheep, goat and pig), where no passage of immunoglobulins exists, making the newborn ungulates agammaglobulinemic, and so fully susceptible to infections (34). In both cases (endothelio and epitheliochorial placentations), the transfer of passive immunity to the newborn occurs via colostrum intake after birth.

Birth weight

Birth weight is the final outcome of growth during the fetal life. Birth weight is strongly correlated with the mother's weight in many species, accounting on average for 5-10% of the dam body mass (30) (Fig.2). However, this proportion varies a lot from one species to

another. In horses and cattle, neonates obtain about 10% (30,65) of adult body weight, in dogs 1-3% (66), while in pigs only 0.5% (67,68). Thanks to a higher percentage of the adult body weight in large animal newborns (except pigs), these animals are born more mature compared with small domestic animals. On the other hand, majority of domesticated ungulates are monotocous, whereas, as described above, altricial species use to give numerous litters. Considering the proportion between maternal weight and newborn litter weight, the relationship becomes opposite. Higher the adult body weight of the dam (as a percent of the maternal body weight), greater the weight of her litter, as i.e. in domestic mouse in which litter weighs over 30% of maternal weight vs. only 6.5-7.5% in the cow (56,12).

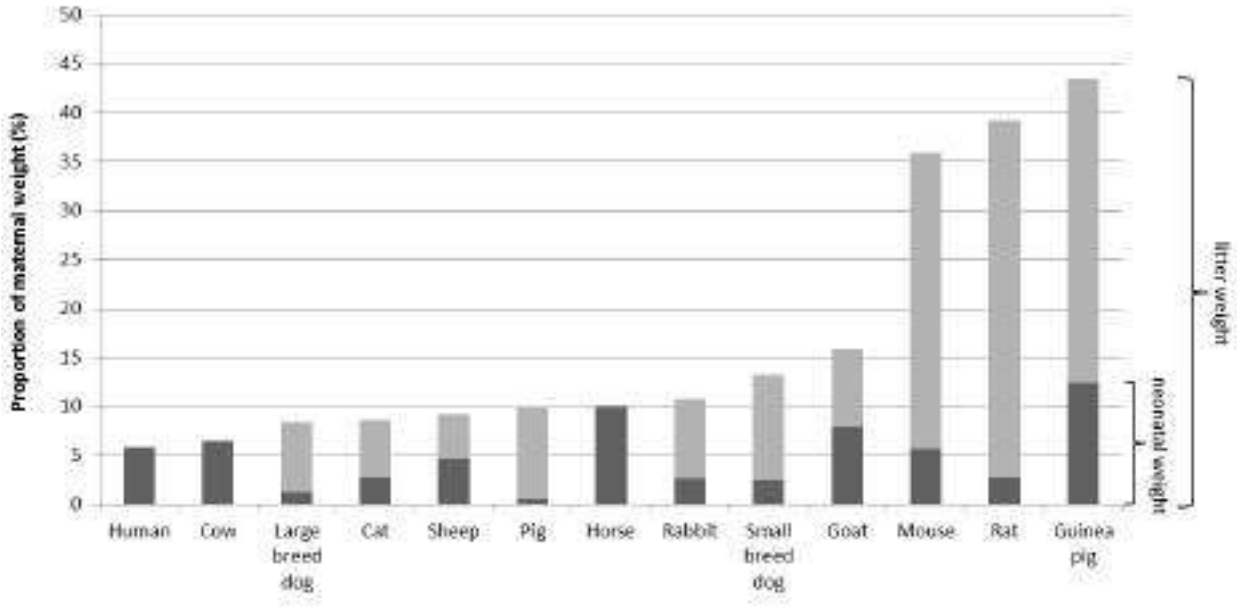

Fig.2. *Relationship between maternal and newborn body weights. Weight of the newborn (black) and that of the litter at birth (black + grey) are expressed as percentage of post partum maternal weight or adult female weight.*

Adapted from: (69,45,70,12,54,71,72,66,53,73,74,57,75).

Birth weight varies not only according to maternal weight, but also to litter size with greater number of animals per litter associated with lower birth weight values. In cats, each additional kitten decreases the mean birth weight by 2.2% (19), in pigs by 2.5% (76), but in mice by as much as 14% (77). However, further decrease in weight at birth may be dangerous for the newborn, as small for gestational age human and porcine infants are the major at-risk group for neonatal mortality (78,79).

BIRTH

Delivery length

Parturition is the moment when the fetus(es) gives a signal about their readiness to end up the fetal life. As a consequence of limited intrauterine space, stress hormones are released and the cascade of events leading to fetus delivery occurs. The duration of delivery (fetal expulsion) is not only species specific, from 20min in the mare to 42h in the queen, but it also depends on the litter size (39,80). Physiologically the second stage of labor in polytocous domestic animals lasts from 5-10min / pup in guinea pig (55), 15-20 min / piglet in the sow (21,68) to 10min-3h / puppy in the bitch (81). In case of delivery prolonged beyond the physiological length, dystocia may occur with increased risk of stillbirth. Prevalence of dystocia, standing for difficult parturition, may be as low as 1-2% in sows and dogs (34,82) to over 10% in cows and horses (13,32). However, due to genetic selection, some breeds are especially predisposed, with over 90% of Belgian Blue-White calves and over 80% of Bulldogs puppies delivered by cesarean section (83,84). Nevertheless, parturition prolonged over the physiological thresholds leads to low vitality, low body temperature and long term neurological disorders in the newborn. Hypoxia due to prolonged parturition is one of the major causes of perinatal mortality in pigs and cows. Difficult parturition in cows, often due to a high birth weight in calves (10,12), leads to either *intra partum* death or early post-

neonatal death within the first 24-48h after birth (85,86). Although in the sow, the incidence of dystocia is low, prolonged interval between piglets or hypoxia lasting 2-3 min, leading to metabolic acidosis, strongly increases the incidence of stillbirth (87).

Sex ratio

Sex ratio at birth (secondary sex ratio), defined as a percentage of males within all born animals, varies between different species, from about 49% in sheep, 49-52% in pigs to 52-55% in dogs (88,89). However, the overall secondary sex ratio in mammalian species is considered in favor of males. In pigs at the early stage of pregnancy the domination of XY embryos is marked even stronger (90). Nevertheless, mortality and morbidity were demonstrated to be significantly higher in males during the perinatal period, in consequence of maternal selection and a greater susceptibility of male newborns to the common mortality factors (91).

Thermoregulation

For the newborn, a stable environmental temperature *in utero* is interrupted at parturition. The just delivered fetus faces a drop of environmental temperature of at least 10°C, eventually much more depending on atmospheric conditions. and it will take various time to the newborn to reach a stable adult level (40,86,92,93). In precocial species, this drop may last from few hours in ruminants, up to 24h in pigs. In altricial species, such as rats and mice, markedly immature at birth, a stabilization and maintenance of body temperature occurs only at about 18 days of age (88). Several methods of heat production are potentially available developed to avoid hypothermia in the neonate: by metabolism of the brown adipose tissue (non-shivering thermogenesis), by muscle contractions (shivering thermogenesis), by physical activity and by food intake (65). Nevertheless, strategies are diversely exploited among species. Indeed, fat body content at birth ranges from 15% in human infant, 4-5% in calves, 2.5% in lambs to only

about 1% in piglets and puppies (65,94,95). While in lambs shivering thermogenesis provides about 46% of heat produced during the first day of life (65), majority of the altricial species present no muscle contractions at this age and the shivering reflex is absent. Late maturation of thermoregulation (rats, rodents, dogs, cats), together with reduced possibilities of heat production in some species (pigs, dogs and cats) put their neonates at high risk of hypothermia and make the energy provision via colostrum intake almost the unique option for thermogenesis.

COLOSTRUM INTAKE

Colostrum is a particular secretion of mammary glands, specific of the first 24 hours *postpartum* (96–99). Highly concentrated in proteins, hormones, vitamins, white blood cells, and many other bioactive factors (100), its definition refers mostly to its richness in milk immunoglobulin content, and especially IgG.

Passive immune transfer

As mentioned previously, hypo and agammaglobulinemic at birth animals acquire maternal antibodies via colostrum intake after birth. Indeed, the colostral IgG concentration in these species is high compared with adult serum level (2-4 fold higher), unlike in humans with low colostral IgG content (Fig.3). Nonetheless, neonates of almost all domestic species achieve higher IgG concentration than the adult at the end of the passive immune transfer (both via placenta and via colostrum intake). An adequate level of maternally derived antibodies seems essential for the newborn animal, as in ruminants, horses and piglets the level of IgG in the newborn bloodstream within the first days of life is strongly associated with risk of neonatal morbidity and mortality (21,101–103). In few species, the threshold of IgG concentration associated with a lower risk for neonatal mortality has been determined and newborns below this limit are considered in deficit of passive immune transfer (Table 2; 109,7,102,113).

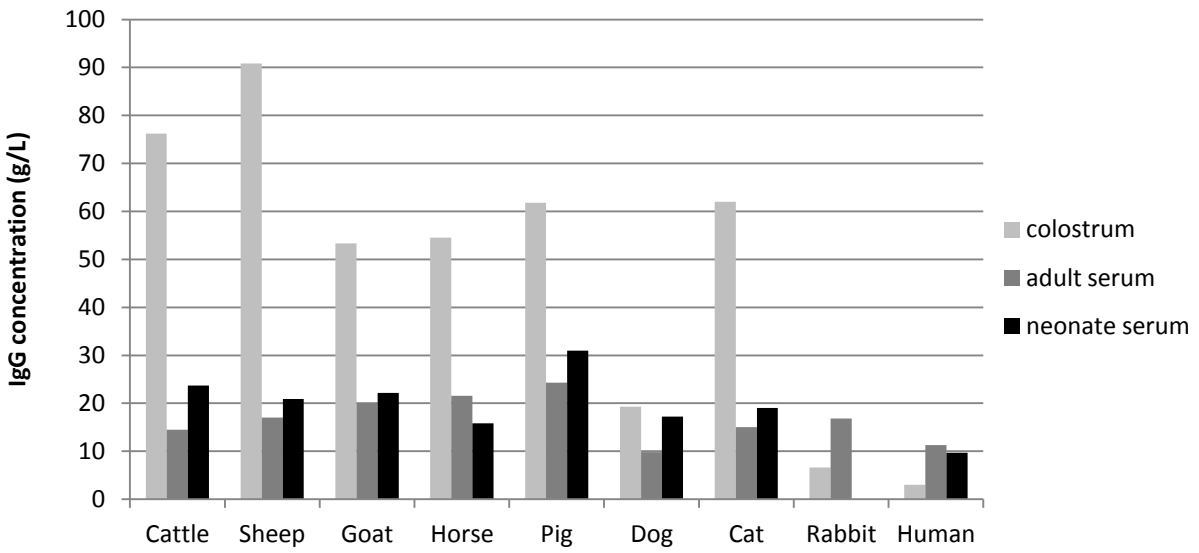

Fig.3. *IgG concentrations in adult serum, colostrum and neonate serum after passive immune transfer (at birth in human or at 24-48h after birth in other species). Adapted from:* (106–118).

The level of passive immunity achieved by the newborn can be evaluated via its serum IgG concentration, but also via serum total protein level (in calves (119), lambs (105), piglets (120)), concentration of γ-glutamyltransferase (in calves (119), lambs (105)), alkaline phosphatase (in kittens (121)) or by refractometry on serum samples (in calves (122), foals (123); Table 2). Compared with γ-glutamyltransferase concentration that only allows to check whether colostrum has been ingested or not, an equation, on the basis of weight gain during the first 25h of life, was developed for porcine neonates, estimating colostrum intake quantitatively (124). Achievement of the minimal protective level depends on the early time of first suckling, the volume and the immune quality of ingested colostrum (119).

Table 2. *Minimal serum IgG concentration of the newborn for decreased risk of neonatal mortality; field methods for passive immune transfer evaluation.*

Species	IgG threshold (g/L)	Technique of evaluation of passive immune transfer	References
Human	> 2.9	-	(125)
Cattle	> 5-12	Serum total protein, GGT, refractometry	(104,119,122,126)
Sheep	> 6-16	Serum total protein, GGT	(105)
Goat	> 8-12	Refractometry, sodium sulfite precipitation test	(7,127,128)
Horse	> 4	Refractometry	(106,123)
Pig	> 15-17	Serum total protein, weighing	(129–131)
Dog	-	GGT¤, ALP¤	(132)
Cat	-	ALP	(121)

- At the authors' best knowledge
GGT - γ-glutamyltransferase
ALP - alkaline phosphatase
¤ Association with colostrum ingestion only

Intestinal barrier closure

The time for absorption of colostral immunoglobulins via digestive tract is limited. In all species, the absorption rate of macromolecules progressively decreases with time elapsed from birth. This phenomenon, known as the intestinal barrier closure (133), is associated with the maturation of intestinal epithelial cells. In majority of domestic species, the cessation of immunoglobulin transfer occurs at 24-36h of age (98,99,134,135), except kittens, in which no more absorption of IgG was evidenced as early as 16h after birth (136). The intestinal barrier closure may be delayed up to 36h or even 86h in case of fasting since birth, as demonstrated in calves (137), lambs and pigs (133). The kinetics of IgG absorption differs among species(Fig.4) (130,138–140). Nonetheless, 70% of the final IgG concentration is obtained during the first 6-12 hours in most of cases.

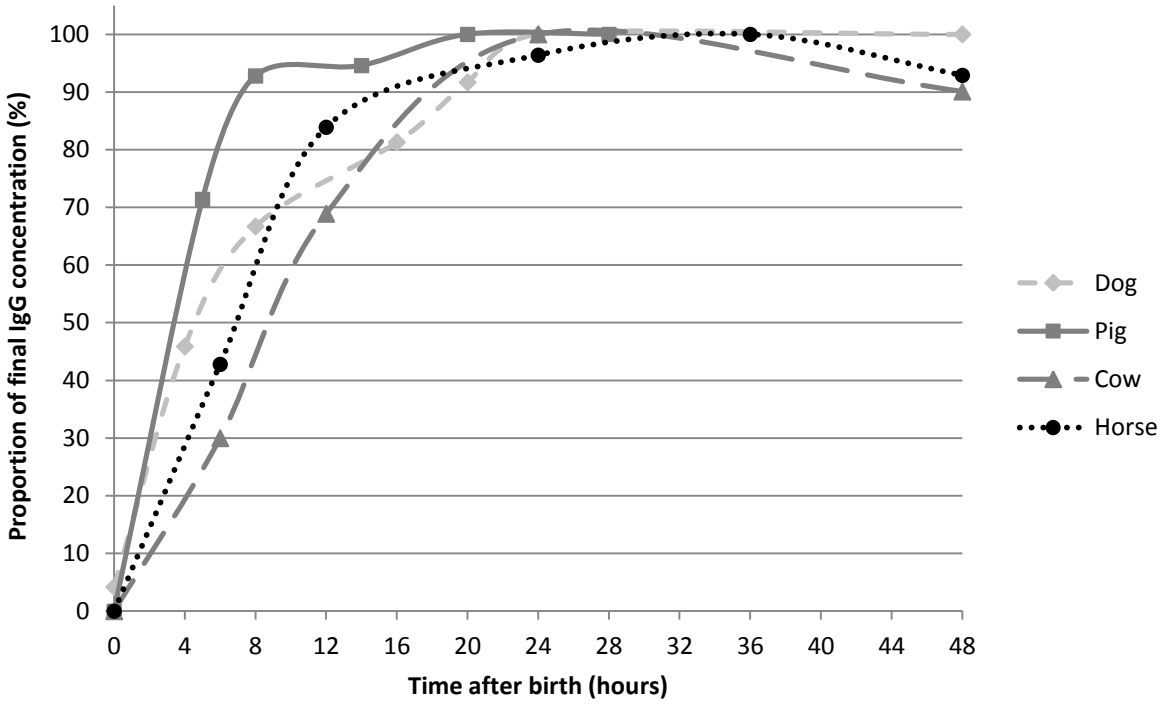

Fig.4*. Kinetics of IgG absorption after birth in 4 species agammaglobulinemic at birth. Data are presented as the proportion of the maximal serum IgG concentration obtained via colostrum intake at different time points*. Adapted from:* (138,141,130,140).

* Data were obtained from studies with different methods of IgG assay and different methods of calculations.

Colostrum yield and quality

The level of passive immune transfer achieved by the newborn is strongly associated with the immune quality of the colostrum (i.e. its concentration of IgG), at least in cattle (142; Table 3). Good quality colostrum, allowing a transfer of the minimal level of immunoglobulins for higher survival rate, has been estimated at 50-60g/L of IgG (98,143) in cows and at 60g/L in mares (99,144). However, colostral IgG concentration undergoes a rapid decline in already few hours *post partum*, most probably linked to a dilution effect of the mammary secretion (145,146,97). Therefore, a minimal total amount of IgG is advised to be ingested soon after

birth, rather than colostral quality *per se*, in order to assure the correct passive immune transfer in calves and foals (129; Table 3). This decline contributes to the effect of birth order on passive immune transfer: the last piglets born within a liter are affected a colostrum less concentrated in IgG and have limited access to teats, undergoing sibling competition with piglets born earlier (21).

Energy intake

In addition to its immunological role, colostrum provides energy to the newborn animal. Fat content is higher in colostrum compared to milk in queens (13% vs. 11%; 147), in does (10.3% vs. 6.1%; 97), but not in sows (5% vs. 6.7%; 145) and even opposite in women (1.2% vs. 5.8%; 149). As previously mentioned, the energy requirements in the newborn are high due to a decrease in environmental temperature after birth and immature thermoregulation, therefore adequate colostrum intake prevent the neonate from hypothermia and thus mortality. In newborn lambs taking longer than 1h to stand up after birth and/or for which the first suckling occurs later than 2h after birth, the rectal temperature is lower than in normally behaving animals (standing and suckling within the first hour of life), decreasing their chances for survival in case of difficult environmental conditions (40). Similarly, a colostrum ingestion below 250g in piglets is associated with lower than in survivals blood glucose and rectal temperature at birth and at 24h of age and at the end higher risk of mortality before weaning (149). Since colostrum yield in the sow is not correlated with litter size (68), colostrum volume ingested by piglets may become insufficient in large litters.

***Table 3**. Characteristics of lactation period in different species.*

Species	Teats pairs	Colostrum yield (1st day)	Colostrum yield /body weight	Colostrum IgG threshold for correct PIT¤	Milk yield/day#	Milk yield/day /body weight#	Lactation length	References
Human	1	36ml; 37g	0.05%*	NA	0.8-1.2kg	1%	up 1 year	(39,148,150,151)
Cattle	2	7-8kg (Holstein)	1.1-1.6% (Holstein)	>100g (IgG1) in 2-4L	29.5kg (Holstein)	4-5% (Holstein)	305d (Holstein)	(152,39,153,146,42)
Sheep	1	2-2.9kg	0.4%	>20g (IgG1) in 280ml*	1.2-1.7L	1.5-2.5%	77-167d	(154,39,155–157,42)
Goat	1	1-2.7kg	3.1-3.5%	>1.7g	1-3kg	3-6%	296-313d	(39,42,97,158)
Horse	1	0.56kg	0.07%	>60g in 1-1.5L*	13-18kg (saddle mares)	2-3.5%	20w	(159–162,13,99)
Pig	5-10	3.2-3.7kg	1.2-1.4%	160-200g of colostrum	7-9kg	2-3%	21-28d	(21,31,67,96,163–165)
Dog	4-6	-	-	-	1kg (medium size)	8%	9w	(48,166)
Cat	4	-	-	-	178g	4-6%	9w	(48,167,168)
Rabbit	4-5	50-100g	-	NA	320g	8%	4-5w	(169,39,170)
Guinea pig	1	-	-	NA	47g	7%	4w	(52,54)
Rat	5-4	-	-	NA	10-30g	9%	3-4w	(171–173)
Mouse	5-4	-	-	NA	3.1g-15ml	35%	3-4w	(56,174,175)

- At the authors' best knowledge
***** Data originating from review articles or calculated
¤ Passive immune transfer
At the peak of lactation
NA - Not applicable

Other roles of colostrum

In addition to a higher concentration of immunoglobulins, colostrum contains other components modulating the immune function, such as hormones, proteins and immune cells (100,176,177). Lactoferrin is characterized by broad-spectrum antimicrobial, antiviral and anti-inflammatory actions (100). This protein is present in higher concentrations in colostrum than in milk in numerous species (women, horses, cattle, goats, pigs, guinea-pigs and mice), but not all (dogs, rabbits or rats; 179). Cells, such as neutrophils, macrophages and lymphocytes, crossing the newborn intestinal wall via bloodstream, kill bacteria directly and enhance the process of opsonization by immunoglobulins (176). The phenomenon of cell migration via intestines was observed in numerous species: mice (179), rats (180), sheep (181), cattle (182) and pigs (183).

Not only thermoregulation and respiration of the newborn animal, but also gastrointestinal (GI) tract requires adaptation to the extrauterine life. Colostrum intake will contribute to the correct maturation and function of the GI tract including an increase of the absorption surface area, a thickening of the epithelial layer together with development of the glands. Such maturation not only improves the food intake, but also its digestion and nutrients absorption. As demonstrated in dogs, pigs and calves (184–186), newborns receiving colostrum during the first day of life presented significantly greater increase in the GI tract weight and size than formula-fed animals. Indeed, numerous growth factors stimulating GI tract development were found at high concentrations in colostrum compared with mature milk, such as Epidermal Growth Factor (EGF) and Insulin-like Growth Factors (IGF-I, IGF-II) (187). In human and porcine colostrum, EGF concentration is 10-fold higher than in milk (188,189), and in cattle and pigs, IGF-I and IGF-II concentrations decrease in the milk by 2 at the second day of lactation (190,191). Newborn's organ maturation and growth relies on several hormones present in the colostrum, such as insulin, cortisol (both suspected involved in intestinal barrier

closure), leptin, thyroxin, Transforming Growth Factor (TGF), Nerve Growth Factor (NGF), lactoferrin and others (100,187).

Suckling behavior

Due to decreased body temperature of the newborn, progressive reduction in intestinal IgG absorption rate, as well as rapid change in colostrum composition, the time of the first suckle is important for neonatal survival. Precocial species, able to stand up soon after birth, start to seek for a teat almost immediately afterwards. In beef cows, teat seeking occurs at about one hour of age with the effective first colostrum ingestion intervening one hour later (192,193). The first suckling is slightly delayed in dairy calves (about 3-4h), most probably due to a pendulous udder, more difficult to achieve for the newborn (193). In the free-ranging cattle, suckling occurs five times per day, for on average 10 min (194), while dairy calves may be allowed to suckle 2-3 times during a day (depending on farm management). Newborn foals take about 1 hour to stand up and the first suckling occurs within the first 2 hours after birth (195,162). The feeding frequency is higher compared to cattle, with a mean of 10 nursing sessions per day (99). In sheep, the first suckle occurs earlier than in cows or horses (at 30-40 min of age (196)) and the dam assists in seeking as well as suckling, i.e. by correction of the newborn position. Lambs usually ingest milk during the first week of life, as frequent as 30 times per day, and no evident suckling pattern exists between twins (twin lamb suckle from both teats) (192).

In piglets, teat seeking occurs immediately after birth, as the newborn piglet stars to localize the teat already at 3min after birth. Since the parturition onset, sow stays in a lying position about 6h, permitting free colostrum ingestion to all newborns (197). However, piglets which failed to suckle during that period miss that irreversible opportunity. Also, contrary to mono or ditocous animals, a high aggression between littermates appears soon after birth as

piglets start to select their preferable teat. The final teat order is defined within the first week after birth (198) and the nursing occurs 20 to 30 times per day after a well-defined signal emitted by the sow (rhythmic grunting) (192). The sibling competition was observed also in kittens, in which the nipple preferences are established already by postnatal day 3 (199). Although cats, as an altricial species, do not stand up before 2-3 weeks of age, crawling movements, occurring immediately after birth, allow kittens to attach to preferable teat. To date, no adaptive explanation was given for the competitive behavior between littermates neither in porcine, nor in feline neonates (197,200). A few studies report suckling behavior in domestic dog, in which fights between siblings remain not clearly evidenced (201). At the early stage of life, newborn puppies present difficulties in finding the teat, as during considerably long time they root over the mother's head, back and legs, as well as over the littermates. During each suckling session, puppies use on average 2.5 nipples and in one fourth of sessions, at least one puppy do not attach at all. The first pair of mammary gland (anterior, out of five) is rarely used and puppies are found attached most often to the second and third pairs. Although essential for adequate passive immune transfer, time of the first suckling remains unknown for both carnivore species.

Sibling competition was described also in guinea pigs, in which females possess only one pair of mammary glands, while litters often account for 3-4 pups. Decreased access to milk is the reason of suckling fights between littermates, with higher weight gain observed in pups getting access to the teat at first (202).

Studies on rodents and rabbits, in which suckling occurs immediately after birth, pointed out that not only presence of the female is essential to initiate the first suckling, but also experience of maternal olfactory signals (203,204). Pheromones, present in milk, but also in amniotic fluid permit the recognition of the maternal odor prior to the first suckling.

Unlike rodents or carnivores, in which the mother spends most of the early lactation period with her offspring, rabbit females leave their newborns almost immediately after birth. Moreover, nursing occurs only once a day, for about 3-4 minutes (except the first nursing of 10-12 min); the attraction of predators is thus limited (203). Rabbit pups do not only compete during suckling, but also benefit to greater milk intake in case of deaths within a litter (205).

In human infants, the access to the nipple, as well as time spent on milk ingestion are unlimited. After birth, babies are immediately in contact with mothers and massage-like hand movements occur on the mother's breast as early as 11 min later (206). After licking and sucking the aerola and nipple, the first successful suckling is observed at 60-80 minutes of age (148,206). The feeding frequency during the first day is about six times, which increases subsequently up to 7-8 times per day (148).

MORTALITY

Mortality rates

As described above, the neonatal period is highly challenging for the newborn animal and in case of any physiological aberration, a risk of morbidity and mortality increases. Pre-weaning mortality rates vary from as low as <1% in human neonates (36,78) until 30% in rabbits and guinea-pigs (53,207). Stillbirths and deaths occurring within the first few days after birth account for the vast majority of the neonatal losses (Fig.5) proving that the adaptation process is a critical moment for all newborns. Survival rate seems to be higher in human, bovine and equine compared with other species investigated in this review (Table 4). This may be due to different reproductive strategies, with greater maternal investment in monotocous species allowing a lower mortality rates than in polytocous animals. However, mortality rates vary not only between species, but also between different breeds, regions or farms of one given species (4).

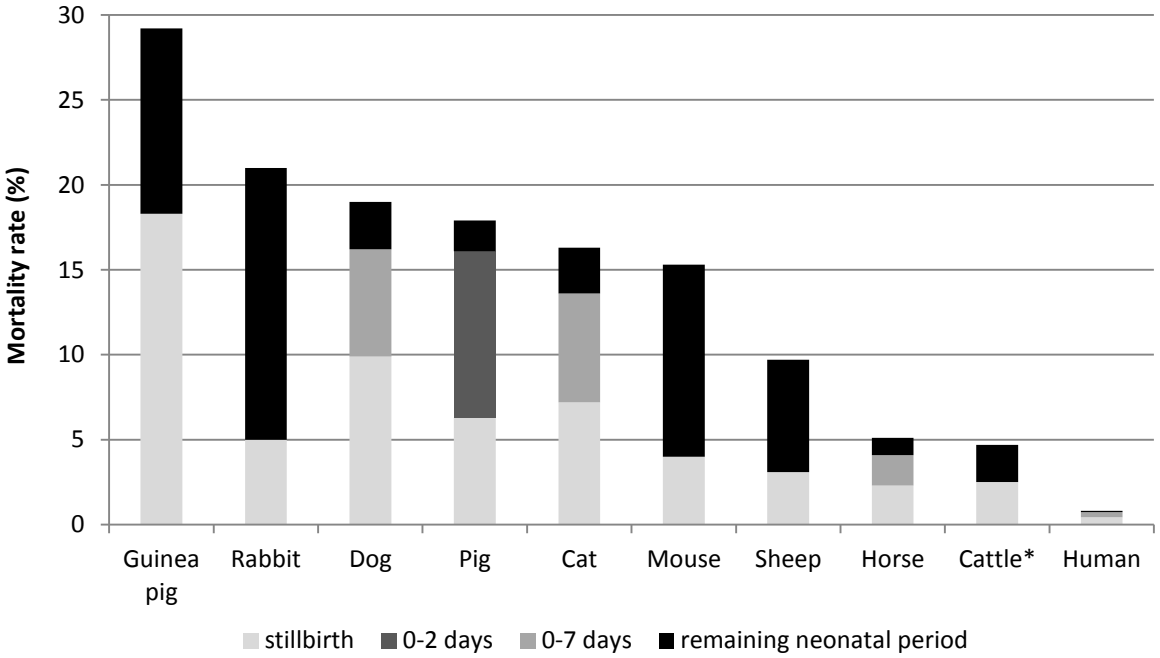

Fig. 5. *Species specific proportion of neonates dying at different age among all newborns. Adapted from:* (207,208,77,36,24,19,156,57,209,21).

*In cattle stillbirth is defined as death occurring either during parturition or during the first 24-48h after birth.

Causes of neonatal death

As already mentioned, conditions such as *intra partum* hypoxia, prematurity, inadequate colostrum intake and mismothering, may lead to neonatal morbidity and mortality (Table 4). In human infants, prematurity is the prime cause of death, accounting for 75% of the overall perinatal mortality and 50% of chronic diseases later in life (210). In cows, neonatal losses are mostly due to *intra partum* hypoxia, with almost a half of deaths occurring due to dystocia (10). The second major cause of high mortality and morbidity rate in young calves is failure of passive immune transfer (consequence of inadequate colostrum intake), with higher risk of infectious diseases in compromised animals until three months of age (101,103).

Table 4. *Pre-weaning mortality: causes and incidence in different domestic animals and human.*

Species	Pre-weaning mortality (%)	Demonstrated most frequent causes of death	References
Human	0.3-0.81	Congenital and hereditary abnormalities, prematurity, low birth weight	(36,78,211)
Cattle	4.7-7.2	Dystocia, passive immune deficit	(6,12,212)
Sheep	0.5-32.1	Mismothering and inadequate colostrum intake	(9,102,156,196,213)
Goat	28-38	Mismothering and inadequate colostrum intake	(7,8,213)
Horse	4.7-22	Infectious diseases	(214–216)
Pig	13.7-24.6	Asphyxia, inadequate colostrum intake	(22,31,217,218)
Dog	13.6-20.2	Infectious diseases, asphyxia*	(15,219–222)
Cat	14.8-16.3	Infectious diseases, mismothering	(223–226)
Rabbit	10-32.4	Mismothering, starvation, cannibalism	(227,24,228,229,23)
Guinea pig	18.1-29.2	-	(207,230,231)
Rat	1.7	-	(232)
Mouse	11.3	-	(57)

* Data obtained from 1 study on 1 brachiocephalic breed (Boxer)
- At the authors' best knowledge

In sheep and goats, not only inadequate colostrum intake was described as a major cause of neonatal mortality, but also poor maternal behavior (7,196,233). In living outdoor natural herds, colostrum intake is indispensable for adequate heat production. Thus in case of mismothering, starvation leads not only to a deficit of maternal antibodies, but also to hypothermia. Neonatal foals are highly susceptible to infectious diseases, with feto-placental bacterial infections and neonatal septicemia most often find at *post mortem* examination (208,215,216). Therefore adequate passive immune transfer, associated with a decreased risk of infection, is essential for equine newborn survival (214). In piglets, mortality due to stillbirth is highly prevalent, and as previously described prolonged parturition is found strongly associated with incidence of perinatal asphyxia (87). Crushing by the dam was reported as the main cause of mortality in live-born piglets (22), but according to Edwards (2002) crushing is only a consequence of inadequate food intake, as starving piglets spend more time in proximity to the dam. High mortality rate in domestic rabbits was found mostly due to maternal causes (23): abandonment of the litter was a cause of over 30% of the pre-weaning mortality, cannibalism of 18% and insufficient milk of another 12% (24). To date, causes of neonatal mortality in dogs and cats are poorly described. Only few studies described

the necropsy findings, with infectious diseases as the major cause of neonatal mortality for both carnivores (15,219,224). However, infectious disease may be only an outcome of another underlying pathology. The relationship between maternal behavior, passive immune transfer and insufficient energy intake with neonatal mortality has never been studied in these species.

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Objectives and research strategy

The comparative study presented above pointed out some crucial steps for the neonatal survival. As shown in Table 4, Article 1, canine species presents one of the highest mortality rates among various domestic animals, with the mean neonatal mortality rate (during the first three weeks of life) of about 20%. In dogs, similarly as in other species, majority of neonatal losses occur during the first week after birth (21; Fig.1). Among principal causes of death, some appear common to the majority of species: mismothering, inadequate colostrum intake and infectious diseases (Table 4, Article 1). Indeed, poor maternal behavior is responsible for starvation, hypothermia or even cannibalism of the offspring. In case of good maternal care, but lack or low colostrum yield, numerous animals within a litter or weak viability of animals at birth, some newborns may undergo inadequate colostrum intake, leading to insufficient energy provision and/or deficit of passive immune protection. Infectious diseases, identified as a major cause of neonatal mortality in horses (22,23), but also in dogs (15,24), may be a consequence either of failure of passive immune transfer or that of hypothermia and starvation. In foals and calves, the first scenario has been demonstrated, as newborns with low serum IgG concentration were found of higher risk of infectious diseases and death (25–27). Studies in these species allowed to define the minimal protective level of IgG for higher survival rate (Table 2, Article 1). In pigs, the passive immune transfer is also ensured by colostrum intake. However, low colostrum volume necessary to obtain adequate IgG concentration is not sufficient to provide the minimal energetic level. Therefore, in porcine species inadequate colostrum intake was demonstrated as limiting factor for minimal energy requirements rather than the immunoglobulin deficit (28).

Physiology of the neonatal period, as well as causes of neonatal morbidity and mortality, has been poorly described in the canine species. Although some studies report the infectious diseases to be the major cause of neonatal losses, the underlying issue has been never explored. Relationship between passive immune transfer and morbidity or mortality was neither established, although it has been demonstrated that canine neonates are born hypogammaglobulinemic (29,30). Also the energetic role of the colostrum on puppies' health has not been studied, while hypothermia and hypoglycemia are often mentioned as major risk factors for neonatal death. Finally, the colostrum itself has been well investigated in many domestic animals, but not in the canine species. The composition and yield of colostrum were demonstrated to influence the health status of the offspring in horses, cows and pigs (31–33). To date, only few data, obtained on limited number of animals report on canine colostrum (34,35). Heddle and Rowley (34) presented the kinetics of milk immunoglobulins over the

entire lactation period on example of 2-4 mongrels (depending on time point). In the second study of Schäfer-Somi et al. (35), the same analysis of immune milk composition was performed on six Rottweiler bitches, with sampling beginning from 24h *post partum*.

Four objectives were addressed in this dissertation:

(1) To evaluate a general role of colostrum intake, on canine neonate survival, and to develop a method for its estimation. If applicable, to establish a threshold value for higher survival rate (Article 2).

In order to explore the global role of the colostrum during the neonatal period, newborn dogs were followed since birth until three weeks of age. Literature display different methods used in order to estimate the colostrum intake in farm animals. However, the technique developed in piglets seems to be the most precise. Pig, similarly as the dog, is a polytocous species, with a low body fat content and limited thermogenesis at the very early stage of life (36). Moreover, porcine neonates are born agammaglobulinemic, with the passive immune transfer acquired via colostrum intake (37). Therefore, piglet was used as a model in order to develop techniques of colostral intake measurement. Devillers and coworkers developed an equation relating colostrum intake to *inter alia* weight gain (38). As birth weight and weight vary between dogs of different breeds, growth rate was used instead of absolute weight gain, to estimate colostrum intake in our study. Subsequently, the relationship between colostrum intake and neonatal survival was tested. As birth weight is associated with higher risk of death in many domestic neonates (39–41), its influence on neonatal survival in puppies was also analyzed. The threshold of early weight gain, reflecting colostrum intake and defining the population at higher risk of death, was estimated based on regression models.

(2) To determine the role of colostrum intake, in terms of energy source on neonatal survival (Article 3).

Canine neonates are born poikilothermic, with absence of shivering thermogenesis and very low fat body content (42,43). Thus, colostrum intake, as the unique source of energy, is almost the only possibility for thermogenesis. Glucose level and body temperature were found positively correlated with colostrum intake in piglets (44). Therefore, blood glucose concentration and rectal temperature were monitored in puppies in order to evaluate the energetic effect of colostrum intake on neonatal survival in dogs. Except hypothermia and hypoglycemia, hypoxia was described as a risk factor for neonatal mortality in dogs (45).

Lactate concentration was measured in newborns in order to control for asphyxia and its consequences on survival in dogs.

(3) To evaluate a role of passive immune transfer, general via IgG absorbed at birth, and specific, via specific antibodies against canine parvovirus, on the risk of mortality and morbidity in puppies (Article 4 and 5).

In all hypo or agammaglobulinemic at birth animals, such as the dog, the acquisition of the passive immune transfer occurs via colostrum ingestion. IgG is the predominant class of immunoglobulin in colostrum of these species and mainly absorbed to the bloodstream of the newborn. IgG is thus routinely used as a marker of passive immune transfer in calves and foals for example (35,46–49). In case of insufficient colostrum intake (due to low volume or low colostral quality) before the intestinal barrier closure, a deficit of passive immune transfer occurs, leading to a higher risk of disease or death. Therefore, we assayed IgG concentration in puppies at two days of age (after intestinal barrier closure) and registered the mortality until three weeks of age. Logistic regression models were used to analyze the relationship between passive immune transfer and risk of neonatal mortality, as well as to define the IgG threshold above which the risk of neonatal death was significantly decreased.

In the second study, instead of approaching puppies' immunity via non specific IgG, passive immunity was evaluated though specific antibodies directed against canine parvovirus (CPV2). The experiment was performed in a breeding kennel undergoing a natural CPV2 circulation. Puppies could suckle freely and remained non-vaccinated until weaning whereas their dams were annually vaccinated. The titers of CPV2-specific antibodies were evaluated (inhibition of haemagglutination test – HI) since ingestion of the colostrum until development of their own immunity at the weaning time. In parallel, the CPV2 excretion was quantified on rectal swabs by PCR. The variability of maternally derived antibodies (MDA) circulating in blood and relationship the between MDA titers and CPV2 infection were analyzed.

(4) To evaluate the immune quality of canine colostrum, via IgG concentration, as well as its variability between bitches and between mammary glands (Article 6).

Immune quality of the dog colostrum was evaluated via assessment of IgG concentration. In order to analyze the variability of IgG concentration, colostrum was collected from bitches of different breeds within one kennel. Samples were collected within the first 24h *post partum*,

separately from each pair of mammary glands, which allowed to evaluate whether all puppies have access to colostrum of similar immune quality.

Article 2

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Differential impact of birth weight and early growth on neonatal mortality in puppies

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The comparative study in domestic animals presented in Article 1 highlighted a lack of principal information regarding the neonatal period in the dog. Although low birth weight was determined as a risk factor in human and other species (39–41), its impact on neonatal health has never been established in dogs, neither was the definition of low birth weight, nor the relationship between colostrum intake and neonatal survival.

In Article 2, the importance of colostrum intake, evaluated via early growth rate, on neonatal health was addressed. The effect of birth weight on neonatal survival was also investigated. Threshold values for both parameters crucial for survival were estimated.

Birth weight and growth between birth and two days of life (reflecting the colostrum intake) were measured. Birth weight was classified into quartiles in order to compare dogs of different breed sizes (small, medium and large). Growth was expressed in percentage of the birth weight (early growth rate). Breed size, sex, litter size (number of puppies per litter), age of the mother were also registered. Puppies were then followed until 21 days of age and deaths were recorded over two periods: from birth until two days; two days until three weeks.

In the first part of the study, factors of variation for both birth weight and early growth rate were analyzed. This part of the study revealed that birth weight in dogs is influenced by the litter size, with greater weights noted in small-sized litters and lesser weights in large-sized litters. On the contrary, none of the factors tested (breed size, sex of the puppy, age of the mother) had any impact on early growth rate. No relationship between birth weight and early growth rate was either established.

In the second part of the study, the relationship between birth weight and mortality within the first two days of life was tested. A major effect of birth weight was demonstrated, with 81.1% of dying puppies presenting low birth weight (quartile 1). When evaluating the effects of both birth weight and early growth rate on mortality between 2 and 21 days of age, only growth rate appeared associated with mortality during this period. The cut-off value of early growth rate defining puppies at higher risk of death was established at -4%. Among all so defined at-risk puppies, 38.5% died within the neonatal period *vs.* 5% in puppies with higher growth rate.

This study thus demonstrated that within the whole neonatal period (0-21 days), two periods have to be distinguished considering risk factors for mortality. Low birth weight is a risk

factor for early neonatal mortality (during the first two days after birth), whereas early growth rate, and not birth weight, is a risk factor of death between two days and three weeks of age.

Colostrum intake, as indirectly evaluated by early growth rate, appears crucial for the survival between two days and 21 days. Among the numerous components of the colostrum, energy and immunoglobulins seem to be essential for the neonate. The relative importance of those two factors is not so clear and may differ among species. Foals, homeothermic at birth, are susceptible rather to infectious diseases than to hypothermia and a high risk of mortality exists in case of inadequate immunoglobulin acquisition (50). Conversely, lambs are initially more susceptible to hypothermia (51), and their survival is first dependent on colostral energy rather than on passive immune transfer. Canine newborns are not only hypogammaglobulinemic at birth, but also poikilothermic during the first week of life (52). The effect of colostrum intake demonstrated in this study needs further investigation in order to determine the relative importance of passive immune transfer and energy intake on neonatal survival in puppies.

Running title: "Birth weight and early growth in puppies"

Differential impact of birth weight and early growth on neonatal mortality in puppies[1,2]

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ABSTRACT

Breeding kennels face a high rate of neonatal mortality, on which the impact of nutrition remains to be determined. This study was designed to evaluate the impact of birth weight (reflecting intrauterine growth) and early growth rate (reflecting colostrum intake) on risk of neonatal mortality in puppies, and to determine the critical thresholds of both parameters. Puppies from various breeds were weighed at birth (n=514) and at two days of age, and the growth rate over that period (early growth rate) was calculated for all survivors (n=477). Linear mixed models evaluated the effect of birth weight on mortality between birth and 2 days of age and the effect of both birth weight and early growth rate on mortality between 2 and 21 days of age. Birth weight was influenced by litter size ($P = 0.003$), with more low-birth-weight puppies (the lightest 25 % within a breed size) in large litters compared with smaller litters. Mortality over the first two days after birth was associated with birth weight ($P < 0.001$), with 81.1% of dying puppies characterized by a low birth weight. Mortality between 2 and 21 days of age was not related to birth weight, but was found to be associated with early growth rate ($P < 0.001$), with higher risk of death in puppies with growth rate at or below -4% after the first two days of life. This study demonstrates the differential effect of intrauterine nutrition, impacting mortality during the first two days of life and that of colostrum intake impacting mortality until 21 days of life. Birth weight and early growth rate thresholds provided in this study allow identification of puppies at risk, whereby provision can be made for adequate nursing to increase their chances to survive.

Key words: birth weight, colostrum intake, litter size, mortality, puppy, weight change

INTRODUCTION

Prevalence of mortality during the first three weeks after birth in the canine species is one of the highest among domestic animals: around 17% of puppies die during this perinatal period (stillbirth and neonatal mortality) (Potkay and Bacher, 1977; Gill, 2001; Nielen et al., 2001; Indrebø et al., 2007). Mortality risk first depends on intrauterine growth, with puppies of the lowest weight at birth being at higher risk of neonatal death compared with littermates (Grundy, 2006), as observed in kittens and piglets (Lawler, 2008; Devillers et al., 2011). In large sized breeds, puppies dying during the first week after birth had over 100g lower birth weight than puppies still alive at 8 weeks (Indrebø et al., 2007). However, birth weight thresholds for the different breed sizes defining puppies at risk of death, requiring more intensive nursing, as well as factors impacting birth weight are not defined to date.

After birth, at an early stage of life, canine newborns depend entirely on colostrum intake. This specific mammary secretion provides puppies not only with nutrients, but also hormones, growth factors and passive immunity. All of mentioned components are indispensable for puppy's life, as hypoglycemia and hypothermia, together with infectious diseases are recognized as the major causes of neonatal mortality in puppies (Indrebø et al., 2007; Münnich and Küchenmeister, 2014). While energy intake covers the basal metabolic needs, ensuring thermoregulation and body growth, immunoglobulin acquisition early after birth provides the only immune protection during the first weeks. In piglets, early weight gain is used to evaluate the amount of colostrum ingested and eventual risk of neonatal death, as piglets dying before weaning gained less weight between birth and 24 hours of life (Devillers et al., 2011). Although a 10% loss of birth weight is commonly considered to be physiological in 2-day-old puppies (Grundy, 2006), the real impact of early weight change on canine neonatal mortality is unknown.

The aim of this study was to evaluate the relationship between neonatal mortality in puppies and both birth weight and growth rate during the first 2 days of life (early growth rate). The critical thresholds of birth weight and early growth rate defining puppies at higher risk of death were also determined.

MATERIALS AND METHODS

The protocol was reviewed and approved by the Royal Canin Internal Ethics Committee (AF/20140704).

Study population

The experiment was carried out on all puppies born alive from 100 bitches within one breeding kennel. Ninety eight bitches were multiparous. Between one week before parturition and the end of lactation bitches were housed in a single box (2-4 square meters) and fed a dry balanced diet for growing dogs (Starter, Royal Canin, Aimargues, France) *ad libitum*. Whelping boxes were heated (under floor heating continuously plus a heat infrared lamp during the first 3-5 days after whelping), so that the stable temperature of 28-30°C at the ground level was assured. None of the whelpings were assisted, no cesarean section was performed and no puppies were hand reared during the experiment. After whelping, the total number of puppies born within a litter (born alive or dead-stillborn-), defining litter size, was recorded. Each puppy was identified by a colored woolen collar and its sex, breed, and the age of its dam were recorded. Within the first 8 hours after birth, puppies were weighed using a calibrated analytical scale in 1g increments (Fisher Scientific International Inc., Hampton, USA). At 48 hours of life, mortality was registered and all surviving puppies were weighed again. Their growth rate over the first two days of life was calculated [(weight at 2 days - weight at birth) ÷ weight at birth × 100%]. Mortality between 2 and 21 days was then registered.

Statistical analyses

Statistical analyses were performed with the Statistical Analysis Systems statistical software package version 9.3 (SAS Institute Inc., Cary, NC, USA). The normality was evaluated with Shapiro-Wilk test. Univariable statistical analyses were performed by Kruskal-Wallis test.

Dams were classified into young (≤ 6 years of age) and old (> 6 years of age). Depending on adult weight, neonates and their dams were classified into small breed dogs (<15 kg), medium breed dogs (15-25 kg) and large breed dogs (>25kg) (Table 1). Since birth weight and litter size vary among breeds (Grundy, 2006), birth weight and litter size values were classified into quartiles, separately for small, medium and large breed puppies (Table 2). The first quartile represents the lowest 25% of registered values, second and third quartiles represent 25% of values below and above the median, and fourth quartile represents the highest 25% of registered values.

First, a generalized linear mixed model (GLIMMIX procedure) with birth weight as an outcome (transformation in ordinal outcome) was used to assess the following fixed effects: sex of the puppy (male/female), litter size and age of the dam (young/old). Subsequently, GLIMMIX procedure with mortality between birth and 2 days of age as a binary outcome (logit transformation) was used to assess the following fixed effects: sex of the puppy, litter size, age of the dam, breed size and birth weight.

Secondly, a linear mixed model (MIXED procedure) was performed to determine the variables affecting growth rate between birth and 2 days of age. As residuals of this multivariable model were not normally distributed, a non-parametric analysis was performed (rank transformation of the outcome). This model included as fixed effects: sex, litter size, age of the dam, breed size (small/medium/large) and birth weight. Subsequently, GLIMMIX procedure with mortality between 2 and 21 days of age as a binary outcome (logit

transformation) was used to assess the following fixed effects: sex, litter size, age of the dam, breed size and birth weight. Moreover, the effect of growth rate was added as a covariate. In all multivariable models, litter was modeled as a random effect.

A receiver operating characteristic curve was drawn based on the result of the logistic model on mortality between 2 and 21 days of age. The Hosmer and Lemeshow Goodness-of-Fit test permitted assessment of the quality of this model. The best cutoff from the model for high and low mortality risk populations was defined based on Youden's index. Differences were considered significant at P value < 0.05. Quantitative data are presented as medians with interquartile range (**IQR**).

RESULTS

The median age of the 100 bitches included in the study was 6 years (IQR: 4 - 7 years) and number of puppies born per litter (**litter size**) was 5 (IQR: 4 - 7). From a total of 532 puppies born, 18 were stillborn (3.4%). The sex ratio in 514 puppies born alive was 1.2 (280 males and 234 females). Birth weight varied from 80 g to 604 g. Both birth weight and litter size were significantly different between small, medium and large breeds (P < 0.001 in both models; Table 2). Weight at birth was significantly influenced by litter size (P = 0.003) and litter effect (P < 0.001) (Fig. 1). Among all puppies belonging to large litters (quartile 4), 37.1% (36/97) were of low birth weight (quartile 1) and 12.4% (12/97) of high birth weight (quartile 4), while proportions in small-sized litters (quartile 1) were 8.6% (7/81) of low birth weight and 50.6% (41/81) of high birth weight puppies. None of the other factors tested in this model (sex of the puppy and age of the dam) has any influence on puppy birth weight.

A total of 20.6% (106/514) of live-born puppies died between birth and 21 days of age, with 34.9% (37/106) of deaths occurring during the first 2 days after birth. Mortality between birth and 2 days of age was influenced by birth weight (P < 0.001; Table 3; Fig. 2)

and tended to be influenced by breed size and litter effect ($P = 0.09$; $P = 0.06$, respectively). Among all puppies dying within the first 48 hours after birth, 81.1% (30/37) were of low birth weight (quartile 1). None of the other factors tested (such as litter size, sex of the puppy, age of the dam) had any influence on mortality between birth and two days of age.

Median growth rate during the first 48 hours of life calculated for 477 puppies still alive at Day2 was 3.3% (IQR: -4.9 - 13.2%). No influence of birth weight on early growth rate was evidenced (Fig. 3). Neither an effect of breed size, litter size, age of the dam or sex of the puppy was observed on growth rate, but an effect of litter as a random term ($P < 0.001$).

Mortality rate between 2 and 21 days of age was influenced by growth rate within the first 2 days of life and litter effect ($P < 0.001$; $P < 0.001$, respectively; Table 3; Fig. 4). Median growth rate in puppies dying between 2 and 21 days of age was -11.3% (IQR: -16.7 - -4%) compared with 5.1% (IQR: -2.2 - 13.2%) in puppies still alive at Day21. The optimal cut-off value of growth rate within the first 48 hours of life to assess predictive likelihood of mortality between 2 and 21 days of age was -4% with a sensitivity of 75.4% and specificity of 79.7%. Among all puppies which survived until Day2, 28.3% (135/477) had an early growth rate below or equal to -4%. Mortality rate was 38.5% (52/135) for puppies with early growth rate below this threshold vs. 5.0% (17/342) for puppies with higher growth rate values ($P < 0.001$). Neither an effect of the birth weight on mortality between 2 and 21 days of age nor of any other factor tested in that model (breed size, litter size, sex of the puppy, age of the dam) was evidenced.

DISCUSSION

Canine neonates are born with very low body fat content (1.3% of the body) (Kienzle et al., 1998) with most energy being provided by glycogenolysis. The decline in muscle and liver

glycogen concentrations after birth is rapid (Kliegman and Morton, 1987), and gluconeogenesis is very limited due to immature liver (Miettinen and Kliegman, 1983). In parallel, shivering thermogenesis is absent up to 6 days (Münnich and Küchenmeister, 2014) which, taken together, make newborn puppies susceptible to hypoglycemia and hypothermia, and as a consequence death. The total perinatal mortality (stillbirths and mortality during the first three weeks of age) in our study was 23.3%, with over one third of live-born puppies dying during the first two days after birth. This mortality rate is higher than those reported in other studies, ranging between 13.6 and 20.2% (Potkay and Bacher, 1977; Gill, 2001; Nielen et al., 2001; Indrebø et al., 2007). No additional nursing or hand rearing was performed in the present study, what could explain a higher mortality rate. The majority of the puppies (81.1%) dying within the first 48 hours after birth were characterized by a low birth weight, previously shown in newborn infants and piglets as a risk factor for hypoglycemia and hypothermia (Williams, 1997; Laptook and Watkinson, 2008; Devillers et al., 2011). Low-birth-weight puppies, with a higher ratio between body surface and body mass than littermates, have a decreased ability to maintain stable blood glucose concentration and body temperature, as well as lower ability to suckle. These factors taken together increase their risk of neonatal death (Grundy, 2006).

In our study, birth weight was negatively affected by litter size. A similar effect has been demonstrated in kittens, in which each additional kitten in a litter decreased mean body weight by 2.2 g (mean kitten birth weight = 100 g) (Sparkes et al., 2006; Gatel et al., 2011). No effect of dam age or parity has been shown in kittens, nor in the puppies in our study, although in foals, birth weight increases by 0.5 kg for every extra year of age of the mare (Elliott et al., 2009). Other effects common for all puppies coming from one litter (litter effect) had an impact on puppy's birth weight in our study. In pigs, intrauterine growth retardation, associated with reduced birth weight, is caused not by a limited uterine space, but

by a smaller size of placenta and so limited transport of *inter alia* amino acids from dam to the fetus (Ashworth et al., 2001). In women, fetal growth retardation is due to insufficient concentration of nutrients in the dam's bloodstream and due to maternal vascular diseases in as many as 35% of the cases (Howie, 1982). To date, the impact of canine placental disorders on birth weight in puppies remains unknown.

To identify puppies at higher risk of death due to a low birth weight, a cut-off value has to be defined differentially according to breed size, since this factor determines weight at birth. Low birth weight values for small, medium and large-sized dogs were provided in this study (Table 2), defined by 25% the lowest birth weights, as puppies from the first quartile were proven to be at significantly higher risk of death. However, birth weights vary not only between different breed sizes (Grundy, 2006, our study), but also between breeds of the same size (Trangerud et al., 2007). Therefore, building a multi-breed database with puppies' birth weights and their mortality could lead to an even better estimate of the chances of a just born puppy to survive and to provide it an adequate care if needed.

In contrast to a dramatic impact on mortality within the first two days after birth, birth weight was not associated with mortality between 2 and 21 days of age. A major risk factor for mortality during that period was growth rate during the first two days of life. This relationship could be explained by colostrum intake and the nutritional and/or immunological value of the colostrum (gross energy at Day 1 of lactation: 548 kJ/100g [authors' unpublished data]; immunoglobulin G [**IgG**]: 19,4 g/L [Mila et al., 2014]). Due to the endotheliochorial placenta, the transfer of immunoglobulins from dam to fetus is very limited in dogs. Puppies acquire 90% of their passive immunity via colostrum ingested within the first 12-16 hours of life (Chastant-Maillard et al., 2012). Indeed, serum IgG concentration at two days of age (as a marker of passive immune transfer) has been demonstrated to be strongly associated with growth rate within the first two days of life as well as with neonatal mortality. Over 44% of

puppies with an IgG concentration at Day2 at or below 2.3 g/L, defined as passive immune deficit in dogs, died during the neonatal period compared with only 5% in puppies with higher IgG concentrations (Mila et al., 2014).

Energy provided by the colostrum can also explain the link between early growth and neonatal mortality. In two-day-old piglets, colostrum intake, evaluated through weight gain after the first 24 hours of life, was positively associated with rectal temperature and glucose concentration, showing the important role of colostrum in thermoregulation and glucose homeostasis (Devillers et al., 2011). The ability to maintain stable body temperature, as well as blood glucose level is very limited in canine newborns, and hypothermia and hypoglycemia may have fatal consequences for puppies (Münnich and Küchenmeister, 2014). Early growth rate threshold, below which risk of mortality is significantly increased, was calculated at or below - 4% in this study. Weight monitoring, together with cut-off value calculated in our study, provide an easy tool to detect and nurse puppies at increased risk of hypoglycemia or hypothermia, and by consequence risk of neonatal mortality.

The positive effect of colostrum on survival might be also related to its bioactive compounds, such as prolactin, steroids, insulin, leptin and many growth factors, essential for correct organ development and maturation (Hamosh, 2001; Farmer et al., 2006). Amino acids together with Insulin-like Growth Factors, highly concentrated in swine colostrum (Donovan et al., 1994), have a stimulatory effect on protein synthesis in the piglet intestinal tract 50-fold stronger than mature milk (Burrin et al., 1992). In puppies, a large increase in intestinal dimensions (i.e. 42% in mucosal weight) occurs within the first 24 hours of life, dramatically improving food intake, digestion and nutrients absorption (Paulsen et al., 2003). Insufficient colostrum intake, as evidenced by reduced growth over the first two days of life, may thus reduce nutrient absorption later in life leading to higher mortality rates in puppies.

Although differences in post-weaning growth curves and adult weights between females and males have been demonstrated in dogs (Helmink et al., 2000) and in cats (Moik and Kienzle, 2011), no sexual dimorphism in birth weights or early growth was evidenced in our study. Interestingly, growth during the first two days of life was not found to be associated with birth weight, whereas in many other species an accelerated growth occurs, compensating the lower weight at birth (Binkin et al., 1988; Moik and Kienzle, 2011). In rabbits and rats, litter size, negatively correlated with pup growth, explains most of the pre-weaning growth variation (Rödel et al., 2008). In our study, not litter size, but litter effect as a random term for all littermates had an influence on early growth rate. Insufficient milk yield, as shown previously in pigs (Marshall et al., 2006), together with poor maternal behavior and other circumstances precluding colostrum intake, could be responsible for decreased growth in some litters.

CONCLUSIONS

This study illustrates the differential impact of birth weight and early growth rate on neonatal mortality, either mortality during the first two days after birth or mortality between two days and three weeks of age. It also provides critical thresholds allowing identification of puppies with particular need of monitoring and nursing during the neonatal period. However, these values remain to be refined for various dog breeds as well as different kennels. This study highlights the need for further investigation on intrauterine growth (to decrease the incidence of low birth weights) and on colostrum intake (to optimize early growth) in order to reduce the high incidence of neonatal mortality in the canine species.

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Table 1. Breed size classification according to the adult body weight and numbers of litters and puppies included in the study.

Breed size	Breed	Number of litters born	Number of live-born puppies
Small, <15 kg	Bichon Frise	4	15
	Bichon Maltese	7	40
	Jack Russell Terrier	4	12
	Lhasa Apso	11	50
	Pomeranian	1	4
	Poodle	8	28
	Shih Tzu	6	32
	German Spitz	3	11
	Scottish Terrier	1	1
	West Highland White Terrier	7	35
	Yorkshire Terrier	2	15
Medium, 15-25 kg	Cocker Spaniel	17	90
Large, >25 kg	Boxer	1	8
	Labrador	11	58
	German Shepherd	2	11
	Golden Retriever	15	104
TOTAL		100	514

Table 2. Classification of puppies according to birth weight and litter size (514 puppies) depending on breed size.

Breed size	Number of puppie	Median birth weight, g	Quartiles of birth weight, g				Median litter size	Quartiles of litter size, number of puppies per litter			
			Q1	Q2	Q3	Q4		Q1	Q2	Q3	Q4
Small	243	185 [a]	< 151	151-185	186-219	> 219	4 [a]	< 4	4	5	> 5
Medium	90	267 [b]	< 225	225-267	268-309	> 309	5 [b]	< 5	5	6	> 6
Large	181	377 [c]	< 330	330-377	378-428	> 428	7 [c]	< 6	6-7	8-9	> 9

[a,b,c] Median values within one column with unlike superscript letters were significantly different ($P < 0.05$).

Table 3. Predictive factors for neonatal mortality in puppies (514 puppies; 100 litters).

Factor	Mortality 0-2 days			Mortality 2-21 days		
	P value	OR[1]	95% CI[2]	*P* value	OR[1]	95% CI[2]
Age of dam	0.15	0.5	0.2, 1.3	0.68	0.8	0.3, 2.3
Breed size	0.09			0.25		
Small		1.0[3]	-		1.0[3]	-
Medium		0.6	0.1, 2.4		0.3	0.1, 1.3
Large		0.3	0.1, 0.9		1.1	0.4, 2.9
Sex	0.75	1.14	0.5, 2.6	0.75	0.9	0.4, 1.8
Litter size	0.65			0.50		
Q1		1.0[3]	-		1.0[3]	-
Q2		0.6	0.1, 2.6		1.0	0.2, 4.0
Q3		0.4	0.1, 1.8		0.5	0.1, 1.7
Q4		0.5	0.1, 2.6		0.6	0.1, 2.8
Birth weight	<0.001			0.53		
Q1		1.0[3]	-		1.0[3]	-
Q2		22.7	5.0, 102.9		1.0	0.4, 2.5
Q3		59.4	7.3, 481.5		1.4	0.5, 4.0
Q4		16.4	4.7, 57.3		2.1	0.7, 6.7
Growth rate 0-2 days	-	-	-	<0.001	0.9	0.8, 0.9
Litter effect	0.06	-	-	<0.001	-	-

[1] Odd ratio
[2] 95% Confidence Interval
[3] Reference category
- Data not available

Figure 1. Correlation between litter size and mean quartile of birth weights in puppies from one given litter (100 litters; 514 puppies). Both variables are expressed in quartiles according to Table 2.

Figure 2. Relationship between mortality from birth until two days of age and birth weight (observations for 514 puppies; $P < 0.001$). Birth weight is expressed in quartiles as defined in Table 2.

Figure 3. Relationship between birth weight and growth rate after the first two days of life (observations for 477 puppies; $P = 0.20$). Birth weight is expressed in quartiles as defined in Table 2. Growth rate is defined as ((weight at 2 days – weight at birth) / weight at birth x 100%).

Figure 4. Correlation between early growth and neonatal mortality at a litter level (observations for 100 litters; $P < 0.001$). Early growth is expressed as the mean growth rate in puppies between 0 and 2 days of life from one litter. Mortality rate is the proportion of puppies dying between 2 and 21 days of age within one litter.

Figure 1.

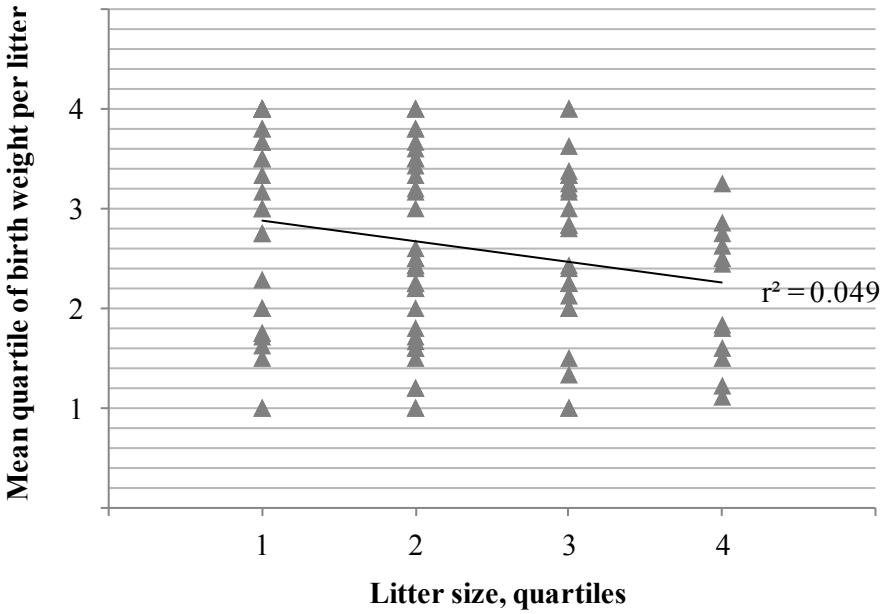

Mean quartile of birth weight per litter
4
3
2
1
r² = 0.049
1
2
3
4
Litter size, quartiles

Figure 2.

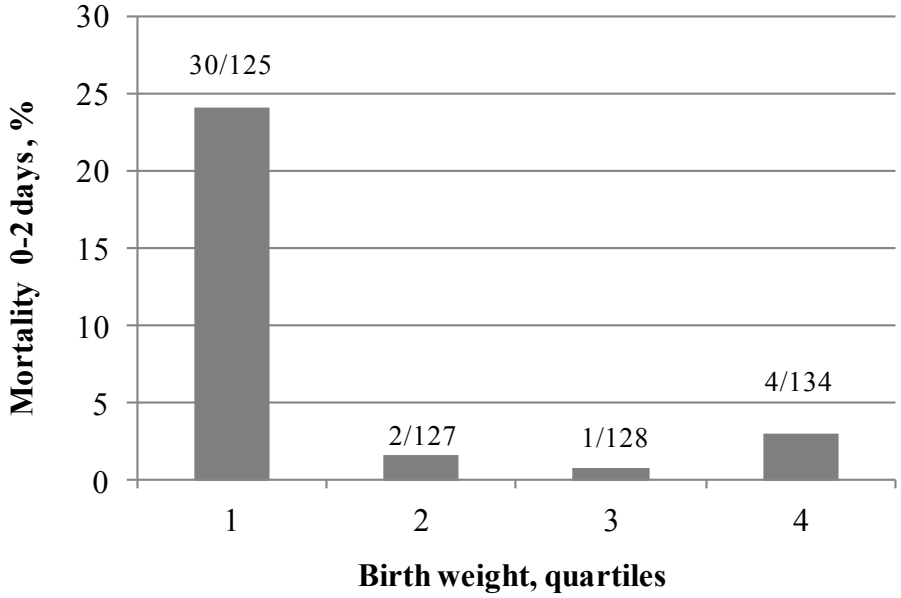

30
25
20
15
10
5
0
Mortality 0-2 days, %
30/125
2/127
1/128
4/134
1
2
3
4
Birth weight, quartiles

Figure 3.

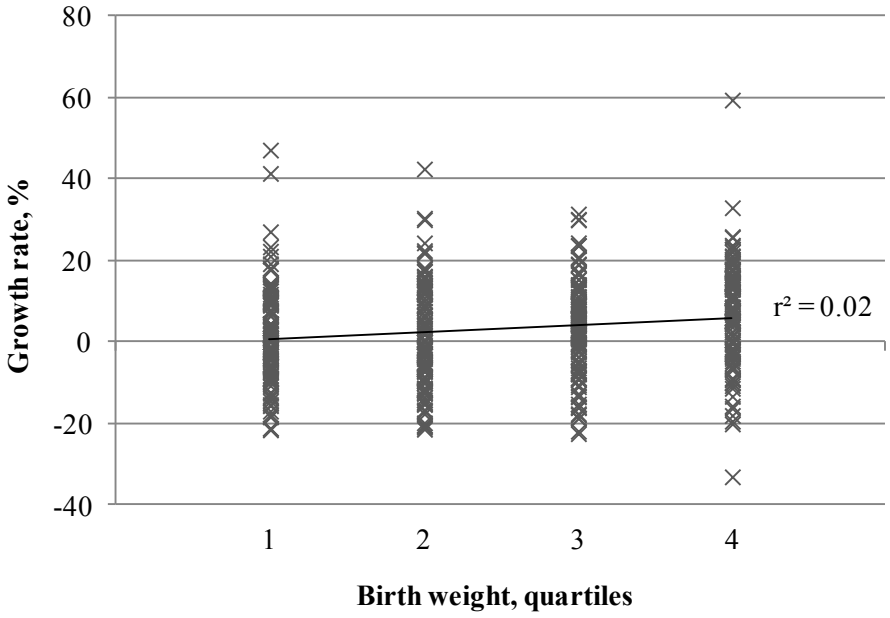
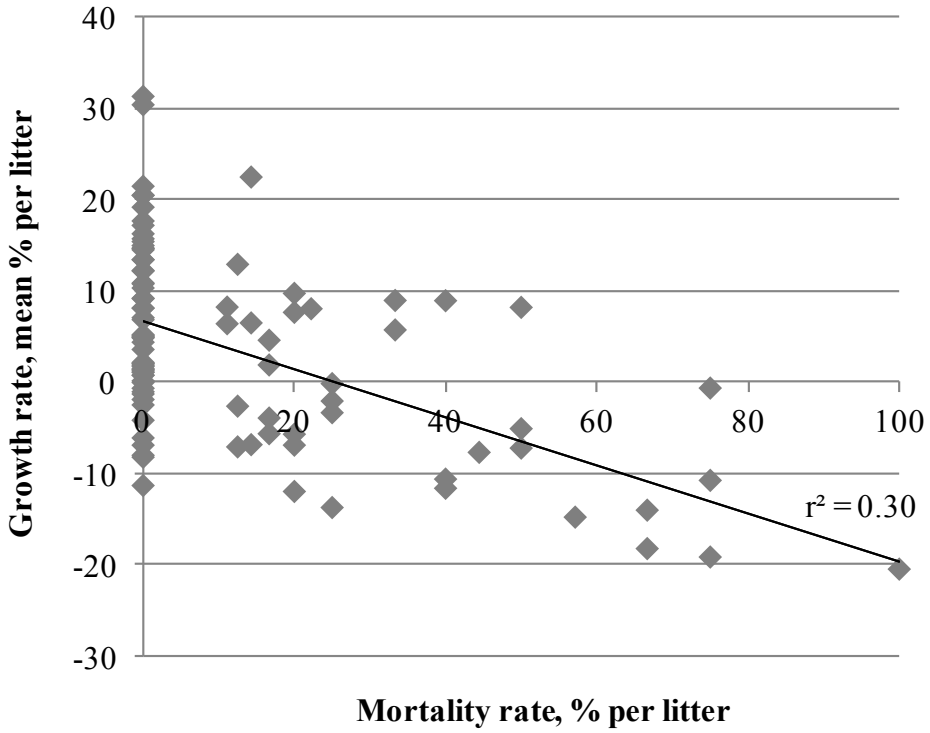

80
60
40
20
0
-20
-40
Growth rate, %
r² = 0.02
1
2
3
4
Birth weight, quartiles

Figure 4.


40
30
20
10
0
-10
-20
-30
0
20
40
60
80
100
Growth rate, mean % per litter
Mortality rate, % per litter
r² = 0.30

Article 3

H. Mila, A. Grellet, A. Feugier, M. Delebarre, S. Chastant-Maillard

Monitoring of the newborn dog and prediction of neonatal mortality

Preventive Veterinary Medicine (submitted May 2015).

In the Article 2, the questions arose on the respective importance of the energetic and the immunologic role of the colostrum for the survival in puppies. The study presented in Article 3 investigates the influence of colostrum intake in terms of energy source on neonatal losses. Adequate energy intake was evaluated via blood glucose concentration and body temperature. Reference values of monitored parameters, as well as a cut-off value for mortality predictor were provided.

The thermoregulation system in the newborn dog remains immature until one week of age (52). In case of low environmental temperature, separation from the dam and lack of nutritional source of energy, hypoglycemia and hypothermia may occur. As shown in lambs and piglets, a strong association exists between colostrum intake, glucose and temperature maintenance and neonatal mortality (44,51). In human neonates, hypoglycemia is accompanied by an increase in β-hydroxybutyrate blood concentration, reflecting the mobilization of the adipose tissue as an alternative source of energy (53). Therefore, this study investigated the impact of the energetic function of colostrum intake, via blood glucose and β-hydroxybutyrate concentrations and rectal temperature on neonatal survival in puppies. Lactate concentration, Apgar score (reflecting the level of hypoxia and vitality) and hydration status (evaluated via urinary density) were also evaluated.

All parameters were evaluated at birth (within the first 8 hours of life) and at 24h of life. Breed size, sex, birth weight, litter size, age of the mother were also recorded. Mortality between birth and 21 days of age was registered.

First, the physiological ranges, as well as factors of variation for each parameter were determined. This part of the study pointed out that birth weight is the predominant factor influencing vitality of the newborn dog (via Apgar score), its rectal temperature (at 24h) and blood glucose concentration (at birth and 24h), with low-birth-weight puppies presenting lower values in all cases.

Finally, impacts of all health parameters evaluated at birth and at 24h were tested on neonatal mortality. None of the parameters evaluated at birth appeared as a mortality predictor. On the contrary, blood glucose concentration at 24h was associated with mortality rate. The threshold for glucose concentration defining at-risk population was established at or below 92 mg/dl. Among all puppies with blood glucose concentration below the cut-off value, 38.6% died before three weeks of age *vs.* 9% in puppies with higher glucose concentration.

As in Article 2, these results confirm the importance of birth weight on health status of the newborn. Mortality was associated with glucose concentration at 24h. Low glucose concentration in dying puppies could thus witness insufficient colostrum intake.

The impact of blood glucose concentration on neonatal mortality highlights the importance of the colostrum intake as a source of energy for the newborn dog. However, the role of colostrum as a source of passive immune transfer remains to be evaluated.

Monitoring of the newborn dog and prediction of neonatal mortality

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Abstract

Despite the high neonatal mortality rate in puppies, pertinent criteria for health evaluation of the newborns are not defined. This study was thus designed to measure six health parameters in dog neonates (and their factors of variation), and to evaluate their value as predictors of neonatal mortality. A total of 347 purebred puppies under identical conditions of housing and management were examined at birth (within the first 8h) and then at 24h of age. The first health evaluation included Apgar score, weight, blood glucose, lactate and β-hydroxybutyrate concentration, rectal temperature and urine specific gravity (SG). The second evaluation at 24 hours included the same parameters, excluding Apgar score and weight. The mortality rate over the first 24h and over 21 days of age was recorded. The early predictors of neonatal mortality in the dog were determined with generalized linear mixed models. An Apgar score at or below 6 evaluated within the first 8 hours after birth was found associated with a higher risk of death during the first 24 hours. A reduced glucose concentration (≤ 92 mg/dl) at 24h of life was found to be associated with higher mortality between 24h and 21 days of age. Low-birth-weight puppies were characterized by both low viability (low Apgar score) and low blood glucose concentration, and thus indirectly at higher risk of neonatal mortality. This study promotes two low cost easy-to-use tests for health evaluation in puppies, i.e. Apgar scoring and blood glucose assay. Further investigation is necessary to establish if the strong relationship between blood glucose and neonatal survival reflects high energy requirements or other benefits from colostrum intake.

Keywords: lactate, glucose, rectal temperature, β-hydroxybutyrate, Apgar score, puppy

Introduction

The mortality rate in puppies until weaning (7-8 weeks of age) is high, estimated to account for 20% [1–5]. The majority of deaths (75-90%) occur during the first 3 weeks corresponding to neonatal mortality [1,2,4,6,7]. The adaptation from the intra to the extra uterine life at birth, as well as colostrum intake during the first 24h are critical steps for the canine newborn, as is true for many mammals [8,9]. Despite this, no validated monitoring system exists to identify puppies at elevated risk of death and so to provide them adequate aid, as practiced in other species. In human medicine, the newborn is systematically evaluated through an Apgar scoring system 5 minutes after birth [10], and a complete physical examination together with blood screening tests are performed before 24h of life in order to estimate the health status [11]. In foals, there is macroscopic examination of placenta and an evaluation of foal vitality (similar to the Apgar score) at birth, followed by the evaluation of passive immune transfer during the first day of life to identify individuals at risk of asphyxia or septicemia [12,13]. In the canine neonate, Apgar score (adapted for puppies) and umbilical lactate concentration at birth, reflecting the level of oxygenation of the newborn, were found to be associated with the risk of mortality during the first 24-48h [14,15]. However, the predictive value of these parameters for canine mortality during the first three weeks after birth has never been tested. Furthermore, even when hypoglycemia and hypothermia are described as major causes of canine neonatal mortality [3,16,17], the published reference values for the canine newborn vary widely (88 mg/dl to 133 mg/dl for glucose concentration) [18–20]. Neither glucose concentration nor rectal temperature critical thresholds, defining the at-risk population, have been established for the newborn puppy.

The objective of this study was to develop new techniques of neonatal monitoring in the dog in order to identify puppies at higher risk of death and requiring specific medical assistance. Moreover, we focused on easy-to-use techniques, readily available to clinicians,

providing immediate results, and minimally invasive for puppies. Six parameters were measured, evaluating general vitality (Apgar score), energetic metabolism (rectal temperature, glucose, β-hydroxybutyrate), oxygenation (lactate) and hydration status (urine specific gravity). After describing the evolution of monitored parameters during the first 24h of life, predictors of neonatal mortality were identified and their cut-off values determined.

Material and Methods

Ethics statement

The protocol has been reviewed and approved by the Royal Canin Internal Ethics Committee (AF/20140704).

Animals

The study was conducted within one breeding kennel on all puppies born from 66 included bitches. Management conditions including diet, feeding system, housing conditions, hygiene protocols and veterinary prophylaxis were identical for all animals included. From one week before parturition until the end of lactation, bitches were housed in a single, heated box (under floor heating continuously plus a heat lamp during the first 3-5 days after whelping; temperature at puppy level between 28 and 31°C). During this period, dams were fed a dry balanced diet for growing dogs (Starter, Royal Canin, Aimargues, France) *ad libitum*. The date and time of whelping, as well as the total number of puppies born per litter (litter size) were registered for each dam. Each puppy was identified by a colored woolen collar, and its sex and breed recorded. Puppies remained with their dams and were allowed to suckle freely until the end of the experiment. Depending on expected adult body weight, puppies belonging to different breeds were classified into small, medium and large breed dogs (Table 1).

Experimental protocol

Within the first 8 hours after birth (defined as at birth), a first evaluation of the neonate was performed including Apgar score, weight, blood glucose, lactate and β-hydroxybutyrate concentrations, rectal temperature and urine specific gravity (SG). A second evaluation, including the same parameters, with the exception of Apgar score and weight, was performed at 24 hours. The mortality between birth and 21 days of age was recorded.

Puppies were weighed using a calibrated analytical scale in 1g increments (Fisher Scientific International Inc., Hampton, USA). Apgar scoring was performed according to Veronesi et al. [14] including: mucous membrane color, heart rate, respiratory rate, irritability reflex and motility evaluation. For each criteria puppies were scored from 0 (the worst note) to 2 points (the best note) and the values obtained for the five criteria were summed, so that the final score ranged from 0 to 10 points. A drop of blood was obtained by pricking the marginal ear vein [21] and the following metabolites assayed in sequence using disposable test strips and portable devices: glucose (Freestyle Optium, Abbott, Illinois, USA), lactate (Lactate Pro, Arkray, Kyoto, Japan) and β-hydroxybutyrate (Freestyle Optium). The measuring range and precision are as follow: glucose 20-500 mg/dl, coefficient of variation (CV) = 2.7-4.0%; β-hydroxybutyrate 0.07-5.2 mmol/L, CV = 3.1-3.8%; lactate 0.8-23.3 mmol/L, CV = 2.6-3.2%. Body temperature was measured via a digital rectal thermometer (Torm 10s mt-403s, Hangzhou Sejoy Electronics & Instruments Co., Hangzhou, China) with the measuring range from 32°C to 42.9°C and precision from ±0.1°C (for temperatures between 35.5°C and 42°C) to ±0.2°C (for temperatures below 35.5°C and above 42°C). Urine SG was measured using a clinical refractometer (Rogosampaic, Wissous, France) with the measuring range from 1.000 to 1.050 and precision of ±0.002. All values below the device detection range were included in the calculations as the lowest detectable value, and all values above as the highest detectable value.

Data analysis

Statistical analyses were performed with the SAS version 9.3 software (SAS Institute Inc., Cary, NC, USA). The normality was evaluated with Shapiro-Wilk test.

The effect of time on each parameter evaluated at birth and at 24h of life was tested with nonparametric ANOVA for repeated measurements (Friedman's ANOVA by Ranks). The correlation matrix was evaluated with Spearman's rho correlation test. Parameters were considered correlated if $\rho > 0.40$ and P value < 0.05.

Linear mixed models (MIXED procedure) with litter modeled as a random effect were performed to determine the variables affecting each monitored parameter, separately at birth and at 24h. These models included as fixed effects: sex of the puppy (female/male), birth weight (g), breed size (small/medium/large), age of the dam (years) and litter size (total number of puppies born within a litter). Since birth weight and litter size vary among breeds [18], birth weight and litter size values were nested within breed size. Residuals of these multivariable models were not normally distributed; therefore, non-parametric analyses were performed (rank transformation of the outcome) (except in the model on body temperature).

Generalized linear mixed models (GLIMMIX procedures) with neonatal mortality as a binary outcome (logit transformation) and litter modeled as a random effect were used to assess the effect of different monitored parameters (fixed effects). As Apgar score is a complex parameter, effect of the Apgar score was tested on mortality between birth and 24h and on mortality between birth and 21 days separately from other parameters. Effect of glucose, lactate, β-hydroxybutyrate, rectal temperature and urine SG evaluated at birth were tested on mortality during the first day of life and on mortality between birth and 21 days. The final GLIMMIX model assessed the effect of above mentioned parameters evaluated at 24h on mortality between 24h and 21 days of age.

Receiver operating characteristic (ROC) curves were drawn based on the results of the logistic models on mortality between birth and 21 days of age. The Hosmer and Lemeshow goodness-of-fit test permitted assessment of the quality of these logistic models. The best cut-off values from the models for high and low mortality risk populations were defined based on Youden's index.

Differences were considered significant at P value < 0.05. Quantitative data are presented as medians with interquartile range (IQR). To express graphically the effect of birth weight and litter size nested within a breed size, both birth weight and litter size were transformed into quartiles separately for small, medium and large breed dogs. The first quartile represents the lowest 25% of registered values, second and third quartiles represent 25% of values below and above the median, and fourth quartile represents the highest 25% of registered values.

Results

Studied population

The ages of the 66 bitches included in the study varied from 1 to 8 years (median = 6 years). A total of 367 puppies were born with a median litter size of 5 (range: 1-13 puppies). Sixteen puppies were stillborn and 4 puppies were euthanized due to congenital abnormalities (cleft palate). Within the remaining 347 puppies which underwent the first examination, 141 belonged to small-sized breeds (40.6%), 72 to medium-sized breeds (20.7%) and 134 to large-sized breed (38.6%, Table 1). Sex ratio in live-born puppies was 0.56 (194 males to 153 females). Median birth weight of small-sized-breed puppies was 177g (range: 80-276g), medium 268g (range: 95-383g) and large 372g (range: 139-604g).

Health evaluation at birth and at 24h of age

Apgar score

Median Apgar score at birth (< 8h of age) was 10 points (range: 2-10 points) (Table 2). In the linear mixed model, Apgar score was influenced by birth weight ($P < 0.001$). In low-birth-weight puppies (quartile 1), median Apgar score was 9 points [IQR: 7-10 points] vs. 10 points [9-10 points] in puppies with higher birth weight values (quartile 2+3+4) (Fig.1).

Glucose

At birth, median glucose concentration was 97 mg/dl [71-132 mg/dl], with a significant influence of birth weight ($P < 0.001$) and age of the dam ($P = 0.026$). A lower puppy weight at birth was associated with a lower glucose concentration at birth, whatever the breed size of the dog (Fig.1). Median glucose concentration at birth was 125 mg/dl [100-145 mg/dl] in puppies born from bitches less than 4-year-old, 97 mg/dl [75-131 mg/dl] when the dam was between 4 and 6 years of age and 82 mg/dl [52-114 mg/dl] for bitches above 6 years of age. Glucose concentration increased at 24h of life, with a median value of 116 mg/dl [89-147 mg/dl] ($P = 0.003$). At 24h, glucose concentration was influenced by birth weight ($P < 0.001$), with significantly lower values in low-birth-weight puppies (quartile 1) compared with littermates (Fig.1).

Lactate

Lactate concentration at birth was 2.1 mmol/L [0.8-4.1 mmol/L], without any association with the fixed effects tested in the linear mixed model. No correlation between blood lactate concentration at birth and Apgar score was evidenced ($\rho = 0.002$; $P = 0.484$). Lactate concentration at 24h was significantly lower than at birth with a median value of 1.4 mmol/L [0.8-2.0 mmol/L] ($P = 0.001$). At 24h, a significant effect of breed size was evidenced ($P = 0.016$) with a median lactate concentration of 1.3 mmol/L [0.8-1.8 mmol/L] in

small, 1.8 mmol/L [1.1-2.2 mmol/L] in medium and 1.4 mmol/L [0.8-2.0 mmol/L] in large breed puppies.

β-hydroxybutyrate

Median concentrations of β-hydroxybutyrate at birth and at 24h were 0.2 mmol/L [0.3-0.4 mmol/L]. However, a significant difference between the two time points was observed ($P = 0.039$). None of the tested factors was found to have an impact at birth, nor at 24h of life.

Rectal temperature

Median rectal temperature within the first 8h after birth was 33.5°C [32.0-35.2°C], with no significant influence of any of the tested fixed effects. At 24h, rectal temperature increased significantly to a median of 36.6°C [35.9-37.2°C] compared with temperature at birth ($P < 0.001$). An increase in birth weight was associated with an increase in rectal temperature at 24h ($P < 0.001$) (Fig.1).

Urine specific gravity

At birth, median urine SG was 1.022 [1.015-1.028]. Urinary density was influenced by litter size ($P = 0.049$): median urine SG in small sized litters (quartile 1) was 1.026 [1.018-1.034] vs. 1.018 [1.012-1.022] in large sized litters (quartile 4). Urine SG at 24h increased (1.024 [1.020-1.028]; $P < 0.001$) and at this time the effect of the litter size, or any other factors was no longer evidenced.

A significant influence of the random litter effect appeared in all linear mixed models tested, both at birth and at 24h ($P < 0.05$). No correlation between the six parameters evaluated was seen at birth or at 24h ($\rho < 0.4$).

Mortality predictors at birth

Among all included puppies, 10 died within the first 24h after birth (2.9% (10/347); Fig.2).

Apgar score was found to be significantly associated with mortality during the first 24 hours after birth ($P < 0.001$; Table 3). The optimal cut-off value of the Apgar score to assess predictive likelihood of mortality during the first 24 hours after birth was 6 (odd ratio = 0.48; 95% confidence interval: 0.33 - 0.69) with a sensitivity of 70.0% and specificity of 87.5%. Six out of 27 (22%) puppies with Apgar score at or below 6 died within the first 24h *versus* 4 out of 319 (1%) in puppies with higher score (Fig.3). Glucose and body temperature evaluated at 8h tended to be associated with mortality during the first 24h of life ($P = 0.076$ and $P = 0.058$, respectively; Table 3). None of other parameters tested had any influence on mortality during this period.

Apgar score was also found to be associated with mortality between birth and 21 days of age ($P = 0.011$; Table 3). The diagnostic value of the Apgar score to assess predictive likelihood of mortality during entire neonatal period was poor (odd ratio = 0.82; 95% confidence interval: 0.70; 0.96; sensitivity: 25.7%; specificity: 88.8%). Nevertheless, more puppies with an Apgar score of 6 points or less died until Day 21 compared with puppies with higher Apgar scores (11/27 (40.7%) vs. 59/319 (18.5%); $P = 0.005$; Fig.3). Among all parameters evaluated at birth, neither glucose, lactate, β-hydroxybutyrate, rectal temperature nor urine SG, demonstrated any influence on mortality between birth and 21 days of age (Table 3).

Mortality predictors at 24h

Sixty puppies died between 1 and 21 days of age (17.3% (60/347)), with the majority of deaths occurring within the first week (61.7% (37/60); Fig.2).

From all parameters evaluated at 24h, only glucose concentration was significantly associated with higher risk of death within the first 21 days (P = 0.011). Median glucose concentration at 24h in puppies subsequently dying was 88 mg/dl [56-128 mg/dl] compared with 120 mg/dl [96-149 mg/dl] in puppies still alive at Day 21 (Table 3). The optimal cut-off value of glucose concentration at 24h of life to assess predictive likelihood of mortality between 1 and 21 days was 92 mg/dl (odd ratio = 0.4; 95% confidence interval: 0.3 - 0.5) with a sensitivity of 65.0% and specificity of 76.4%. A total of 30.1% (101/335) of puppies presented a glucose concentration at 24h equal or below this threshold. Among all puppies with glucose concentration ≤ 92 mg/dl, 38.6% (39/101) died before 21 days of age, compared to 9.0% (21/234) in puppies with higher glucose concentration (P < 0.001; Fig.4).

Discussion

Mortality in puppies

Worldwide, neonatal mortality (within the first three weeks after birth among live-born puppies) ranges from 11 to 13% [1,2,4]. Such a high prevalence makes canine neonatal mortality a major concern, both for an animal welfare and economic perspective. Most of the puppies die during the first week of life: in our study, the mortality until Day 7 accounted for 67% of all dying puppies, a percentage similar to that observed elsewhere [4,6,16,22]. The major causes of neonatal mortality described in the literature are hypoxia, hypoglycemia and hypothermia, often complicated by an infectious disease [3,16]. Our results show that the Apgar scoring system and glucose monitoring are useful tools in risk evaluation for neonatal mortality in puppies.

Survival during the first 24h

Birth is a major physiological challenge for a fetus since dramatic adaptations to aerian life must be made. Failure to clear fetal lung fluid, lack of consistent breathing,

together with inadequate changes in blood flow and energy metabolism are examples of potentially lethal maladaptations [8]. In human medicine, the Apgar scoring system, evaluating a level of viability of the newborn at 5 min after birth, is routinely used as a mortality predictor during the neonatal period (until 28 days of age in this case) [10]. Recently, a similar system was developed for canine medicine and a score at or below 6 obtained within the first 5 minutes after birth was found associated with an increased risk of mortality during the first 24h [14]. In our study, Apgar score was also proven to be predictive for mortality during the first day, even when performed at any time before 8 hours of life. The test fulfils conditions for use by dog breeders in everyday practice to identify newborns requiring specific nursing: no specific skills needed, cost-free, quickly conclusive, with no need to attend birth. Moreover, we demonstrated that Apgar scoring in puppies is predictive for mortality over the entire neonatal period, as in human medicine, although sensitivity of the method is much lower than for mortality during the first 24h after birth.

Hypoxia, occurring in cases of prolonged uterine contractions without fetus expulsion, followed by increased anaerobic glycogenolysis, leading to metabolic acidosis, was demonstrated as a major cause of intra-partum mortality in many species (human, porcine, equine [9,23,24]). Blood lactate, reflecting the level of hypoxia, was suggested as an early mortality predictor in puppies [15]. However, our results, as well as data obtained in foals [25], show no difference in blood lactate concentration at birth between surviving neonates and those dying later on (either during the first 24h or after). In mammals, fetal hypoxia commonly occurs during parturition and numerous adaptive mechanisms allow the fetus/neonate to develop so called “neonatal hypoxia tolerance” [26]. Moreover, blood acidosis was found not associated with the level of viability at birth, as among human neonates with high Apgar score (> 7), 72% presented blood acidosis [27]. Also in our experiment, lactate concentration was not related to Apgar score and significantly higher at

birth compared with concentration at 24h of life or with reference values reported for healthy adult dogs (1.80±0.84 mmol/L [28]). We could hypothesize that puppies, as human neonates, suffer at birth from physiological moderate metabolic acidosis. Therefore, it remains unclear which parameters could be reliably used to assess perinatal distress in puppies. In accordance with our results, the Apgar score was demonstrated in human newborns as a better predictor of neonatal survival than the degree of acidosis [10].

The transition to extra uterine life is challenging for the newborn not only due to perinatal hypoxia, but also glucose supply, permanent via placenta during fetal life and intermittent through milk ingestion after birth. Therefore, in many species, a low glucose concentration is observed during the first hours after birth, which increases, often above the adult range, after the onset of suckling. For instance, in babies, glucose concentration is < 50 mg/dl at birth but increases to 81 mg/dl during the first days of life [29]. Similar sudden glucose increase is observed in foals (75 mg/dl at birth vs. 135 mg/dl at Day 1) [30] and calves (65 mg/dl at birth vs. 120 mg/dl at Day 1) [31]. Also, in healthy puppies from our study, glucose concentration rose from 98 to 120 mg/dl within the first 24h, which is in agreement with previously published data in dogs [20]. The present study showed the predictive value of glucose level for survival, i.e. newborns dying before 24h presented median glucose concentration at birth of 37 mg/dl (compared with 98 mg/dl in surviving puppies). In cases of hypoglycemia, not only neurological issues can appear, but also decreased body temperature, decreased heart and respiratory rates [32], which can be lethal for the newborn. Therefore, development of an algorithm for neonatal hypoglycemia screening in puppies would be desirable. In small-for-gestational-age and preterm babies, recognized to be at high risk of hypoglycemia, the first glucose measurement is advised before 4h after birth and thereafter repeated every 2-3 hours until 24h [33,34]. In the case of symptoms of hypoglycemia (such as cyanosis, apnea or hypothermia) and/or glucose

concentration below <35 mg/dl (<25 mg/dl at 4h of age), 10% dextrose is immediately administered intravenously. If no clinical symptoms develop and/or no hypoglycemia occurs, more frequent feeding alone is advised for at-risk infants.

Survival during neonatal period

In our study, glucose concentration at 24h was found to be a predictive factor for neonatal mortality in puppies. Due to a lack of adipose tissue (only 1.3% of the body), rapid glucagon decline and limited hepatic activity, the glucose and fatty acids obtained thanks to colostrum ingestion represent practically the only source of energy for the newborn dog [35]. At the same time, the energy requirements are high due to large body surface / body mass ratio, no shivering thermogenesis [18] and large brain surface, with glucose being the main fuel for cerebral tissue [36]. Low level of glucose concentration in puppies dying before Day 21 could thus reflect insufficient energy supply from the colostrum, leading to a decrease in body temperature, among other consequences. A vicious cycle results, hypothermia *per se* being responsible for suckling failure, depressed gut motility, reduced milk digestion and thus decreased energy supply, with ultimately an increased risk of septicemia [16]. In the present work, no predictive value of rectal temperature was evidenced. This may suggest that if correct environmental temperature is provided in the whelping area, hypothermia alone becomes a minor cause of mortality and death most probably occurs as a consequence of low glucose level, as indicated by the higher risk of mortality of puppies with lower glucose concentration.

The glucose threshold at 24h associated with increased risk of death in this experiment was surprisingly high - at or below 92 mg/dl, with 30% of puppies being below this threshold. The majority of puppies dying did not present hypoglycemia *sensu stricto* (in puppies defined if blood glucose < 40 mg/dl [3]) at the time of examination, but only decreased glucose

concentration compared with survivors [88 mg/dl vs. 120 mg/dl]. The critical threshold determined in this study thus reflects either elevated energetic requirements in the newborn puppy or inadequate colostrum intake, with glucose only as a biomarker. Indeed, colostrum provides not only energy, but also immunoglobulins and growth factors, mandatory for canine neonate survival [5]. Further investigation is necessary in order to establish which of these factors (energy or immunity) has predominant impact on the canine neonatal mortality.

Elevated concentration of ketone bodies, released from adipose tissue as an alternative fuel, was described as a physiological compensation for hypoglycemia in the human at birth [33]. However, higher β-hydroxybutyrate concentration was observed at 24h of age in normally fed canine newborns, compared with fasted ones [37]. Our results show that β-hydroxybutyrate concentration is not correlated with glucose at birth or at 24h, and not associated with canine neonatal mortality. Adipose tissue in the newborn dog is very limited and liver function not yet fully developed, which probably limits ketone bodies formation. Nonetheless, the increase in ketone bodies in correctly developing puppies may suggest an external source of fat, namely the colostrum which contains 5.8% of fat (authors unpublished data). This phenomenon complicates the interpretation of results and makes ketone bodies assay a useless tool for canine neonate monitoring.

To our knowledge, only one study previously reported the urinary density values in newborn puppies (1.006-1.0017 SG) [38]. Based on its results, a diagnosis of dehydration was suggested in canine neonates with urine SG at or above 1.017 [16]. However, our data based on a relatively large number of animals, show that urine SG at birth and 24h of age are higher than previously described (1.022 and 1.024, respectively), although slightly below the normal adult reference (1.025-1.035 [21]). Nonetheless, in our study, urine refractometry values were not associated with neonatal mortality in puppies.

Intrauterine growth restriction

In a recent study, low-birth-weight neonates were demonstrated to be an at-risk population for neonatal mortality in dogs (Mila et al. 2015 in press). The present findings may provide some explanation for this phenomenon. Low-birth-weight puppies were found to be predisposed to lower viability at birth (lower Apgar score), and decreased glucose concentration and rectal temperature were demonstrated as mortality risk factors. As described in piglets, intrauterine-growth-retarded neonates, clinically manifested by a low birth weight, are deprived of adequate oxygenation and nutrition during the fetal life. In such cases, further reduction of metabolic demands during parturition are not possible, and therefore perinatal asphyxia and hypoglycemia strongly increase risk of death [26,39].

Conclusions

Adaptation to the extra-uterine life and colostrum intake seem to be crucial steps for neonatal survival in dogs, as the level of vitality at birth (evaluated via Apgar score) and energy intake during the first 24h of life (evaluated via blood glucose concentration) were found to be strongly associated with the risk of death in our study. Investigation of canine pregnancy and its impact on vitality of the puppy at birth, together with a parturition monitoring system is necessary to control the vitality of canine newborns. Development of an easy-to-use glucose monitoring system for low-birth-weight and other at-risk puppies (i.e. orphaned, starving), together with an easily applicable energy supplement would be desirable for dog breeders, in order to decrease the high neonatal mortality rate in kennels. Further investigation is necessary to establish if the strong relationship between blood glucose and neonatal survival reflects high energy requirements or other benefits from colostrum intake in both normal and low-birth-weight puppies.

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Author Contribution

Designed the experiment: HM, AG, AF, SCM. Performed the experiment: HM, MD. Analyzed the data: HM, MD, AG, AF, SCM. Wrote the paper: HM, AG, AF, SCM.

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Tables

Table 1. Breeds of puppies included in the study.

Breed size#	Breed	Litters (n)	Puppies born (n)	Puppies examined (n)*	TOTAL (n)*
Small (< 15 kg)	Bichon Frise	2	8	8	
	Bichon Maltese	3	20	19	
	Jack Russell Terrier	3	9	9	
	Lhasa Apso	6	29	28	
	Poodle	4	18	15	
	Scottish Terrier	1	1	1	141 (40.6%)
	Shih Tzu	3	13	13	
	Spitz	3	11	11	
	West Highland White Terrier	5	26	22	
	Yorkshire Terrier	2	17	15	
Medium (15-25 kg)	Cocker Spaniel	14	73	72	72 (20.7%)
Large (> 25 kg)	Boxer	1	8	8	
	German Shepherd	1	5	5	134 (38.6%)
	Golden Retriever	10	74	74	
	Labrador Retriever	8	55	47	
TOTAL		66	367	347	

#According to expected adult body weight
*Puppies not stillborn and not euthanized

Table 2. Health evaluation in puppies at birth and at 24h of life.

Parameter	Age of puppy (hours)	Number of observations	Median	IQR*	Minimal value	Maximal value
Apgar score (points)	< 8	346	10	9-10	2	10
Glucose (mg/dl)	< 8	323	97[a]	71-132	20	287
	24	335	116[b]	89-147	20	228
Lactate (mmol/L)	< 8	273	2.1[a]	0.8-4.1	0.8	16.3
	24	309	1.4[b]	0.8-2.0	0.8	5.9
β-hydroxybutyrate (mmol/L)	< 8	241	0.2[a]	0.3-0.4	0.07	3.1
	24	308	0.2[b]	0.3-0.4	0.1	1.9
Body temperature (°C)	< 8	340	33.5[a]	32.0-35.2	32.0	38.1
	24	335	36.6[b]	35.9-37.2	32.0	38.8
Urine specific gravity	< 8	261	1.022[a]	1.015-1.028	1.001	1.050
	24	325	1.024[b]	1.020-1.028	1.009	1.050

[a,b] Median values for one parameter within one column with unlike superscript letters were significantly different ($P < 0.05$).
* Interquartile range

Table 3. Health parameters at birth and at 24h of life depending on puppies' survival (at 24h and at 21 days).

Predictors	Puppies dying < 24h (median [IQR*])	Puppies dying < Day 21 (median [IQR*])	Puppies alive until Day 21 (median [IQR*])
Parameters measured before 8h			
Apgar score (points)	6 [6-8]	9 [7-10]	10 [9-10]
Glucose (mg/dl)	37 [21-69]	90 [55-138]	98 [75-128]
Lactate (mmol/L)	1.9 [1.4-2.1]	2.1 [1.0-3.2]	2.1 [0.8-4.2]
β-hydroxybutyrate (mmol/L)	0.6 [0.4-0.8]	0.4 [0.2-0.5]	0.3 [0.2-0.4]
Body temperature (°C)	32.0 [32.0-32.8]	33.4 [32.0-34.8]	33.5 [32.0-35.4]
Urine specific gravity	1.019 [1.015-1.022]	1.022 [1.014-1.028]	1.021 [1.015-1.028]
Parameters measured at 24h			
Glucose (mg/dl)	NA	88 [56-128]	120 [96-149]
Lactate (mmol/L)	NA	1.2 [0.8-2.0]	1.6 [0.9-2.0]
β-hydroxybutyrate (mmol/L)	NA	0.3 [0.2-0.4]	0.3 [0.2-0.4]
Body temperature (°C)	NA	36.3 [35.4-36.8]	36.7 [35.9-37.2]
Urine specific gravity	NA	1.022 [1.018-1.025]	1.024 [1.020-1.028]

NA – Not available
* Interquartile range

Figures

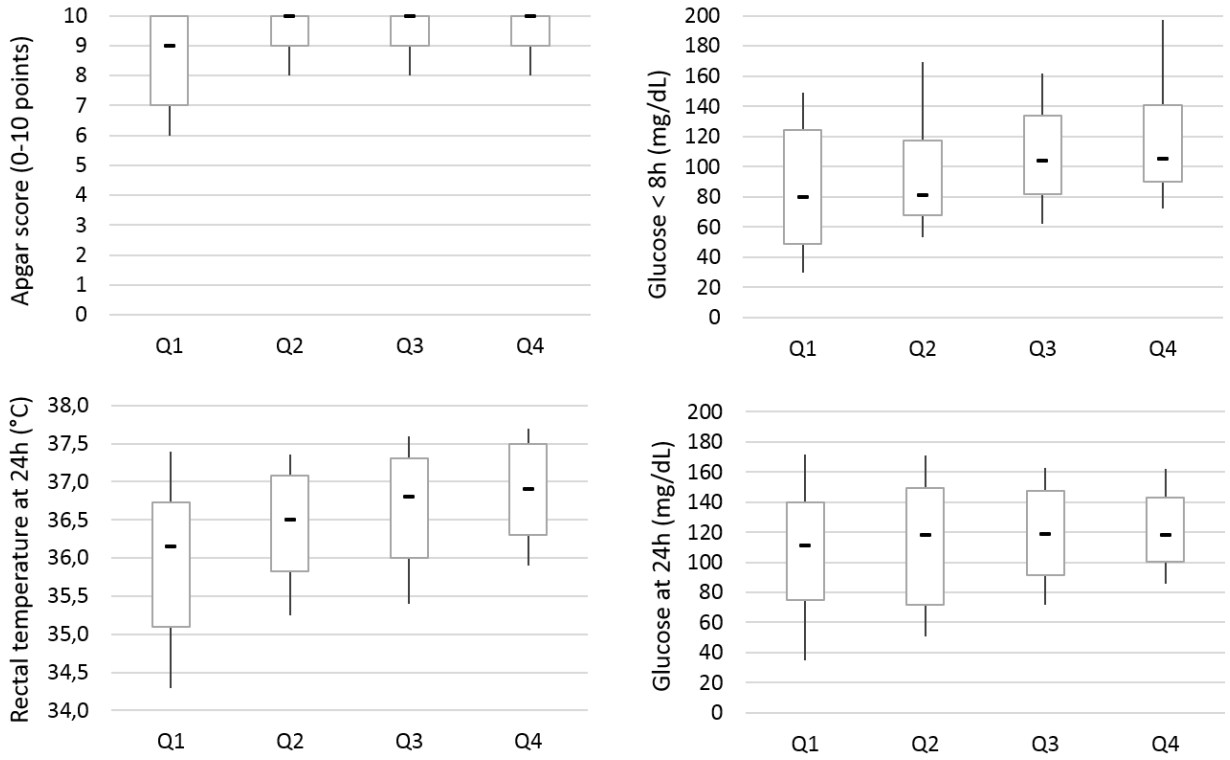


Figure 1. Box-and-whisker plot of Apgar score, glucose concentration at birth and at 24h, and rectal temperature at 24h in puppies with different birth weight (Q1 – puppies with the lowest 25% of registered birth weight values, Q2 and 3 - 25% of values below and above the median, Q4 – puppies with the highest 25% of registered values). Each box represents the first to third quartiles (25th to 75th percentiles), the bar in each box represents the median, and the whiskers represent the first to ninth decile (10th to 90th percentiles).

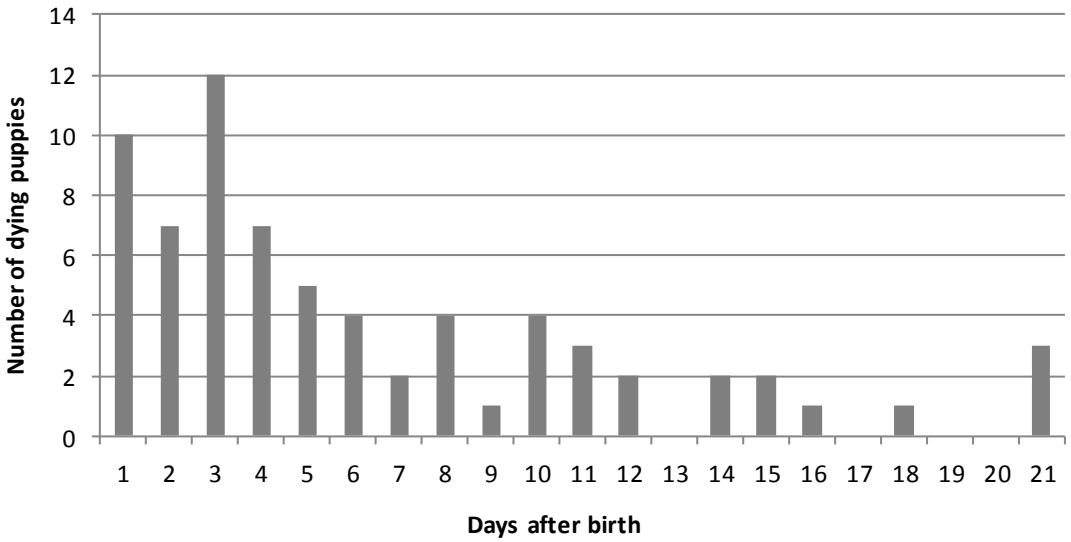
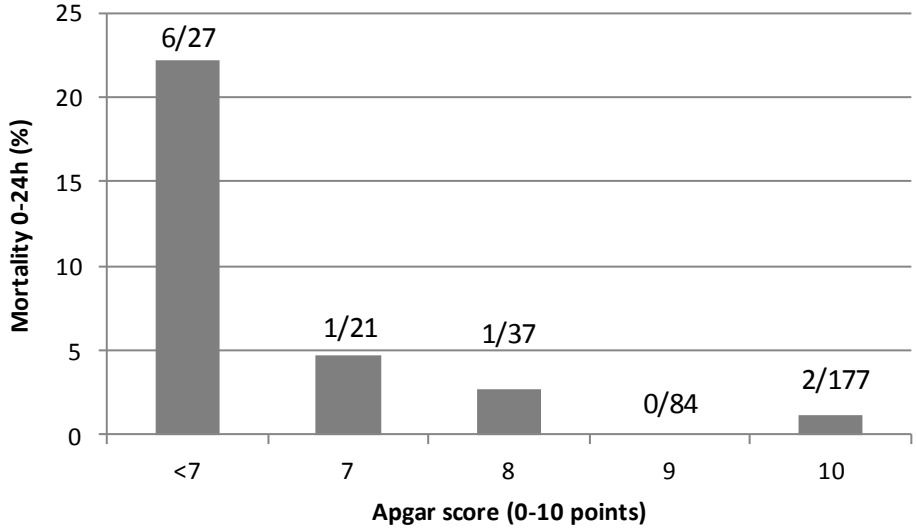


Figure 2. Distribution of the deaths during the neonatal period (n = 70).

A



B

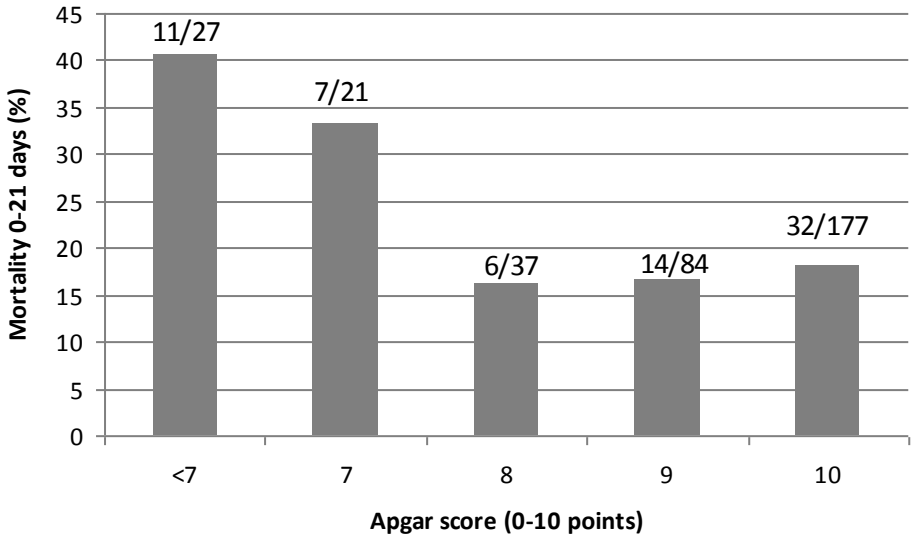
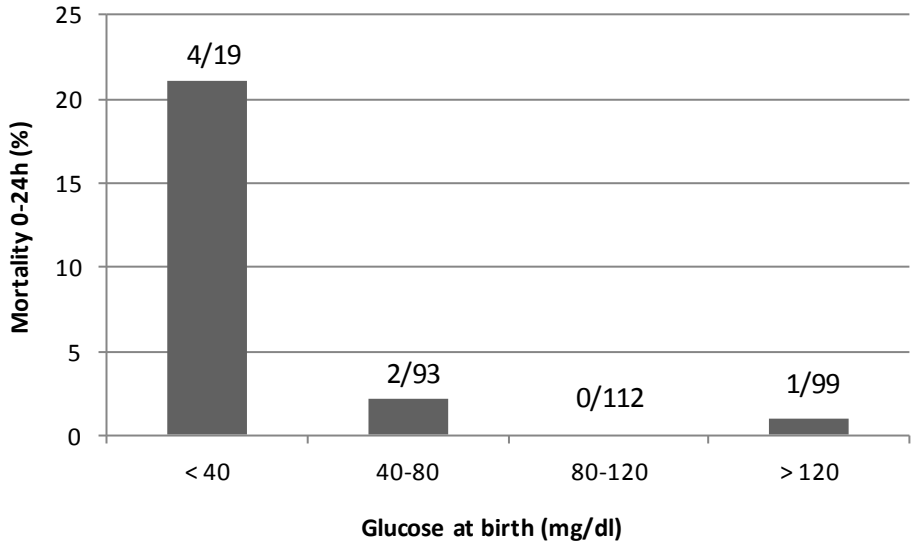


Figure 3. Mortality rate depending on Apgar score evaluated before 8h of life (n=346). Apgar score was associated with higher risk of mortality during the first 24h of age (A; $P < 0.001$), and mortality between birth and 21 days of age (B; $P = 0.011$). Numbers above columns represent number of died puppies / total number of puppies for each value of the Apgar score.

A



B

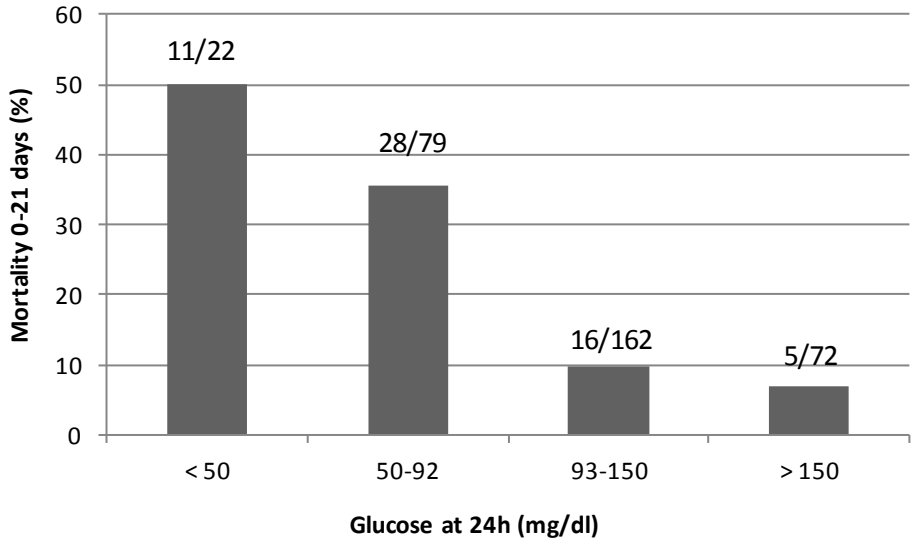


Figure 4. Mortality rate depending on glucose concentration at birth (A; n=323) and at 24h of life (B; n=335). Glucose concentration at birth tended to be associated with higher risk of mortality during the first 24h after birth (P = 0.076). Glucose concentration at or below 92 mg/dl was associated with higher risk of mortality during the entire neonatal period (P=0.001). Numbers above columns represent number of died puppies / total number of puppies for each range of glucose concentration.

Article 4

H. Mila, A. Feugier, A. Grellet, J. Anne, M. Gonnier, M. Martin, L. Rossig, S. Chastant-Maillard

Inadequate passive immune transfer in puppies: definition, risk factors and prevention in a large multi-breed kennel

Preventive Veterinary Medicine 2014; 116: 209-213.

The study described in Article 2 demonstrated that colostrum intake is essential for the newborn dog, and in Article 3, neonatal mortality was found associated with energy provided by the colostrum. Nevertheless, canine newborns acquire maternal immunoglobulins mostly at birth, as only 5-10% of total serum IgG at two days of age derived from transplacental transfer (29). In parallel, few studies describe infectious diseases as the most frequent findings at *post mortem* examination in neonatal dogs (15,24). Author's results confirm the importance of infections in neonatal losses, as 61% of puppies dying within the first three weeks of life presented a positive bacteriological culture on spleen samples (study on 18 puppies). In Article 4, the relationship between passive immune transfer and neonatal mortality was thus evaluated. Factors influencing IgG acquisition were also determined and IgG threshold defining a deficit of passive immune transfer was determined. An oral supplementation with hyper-immunized dog serum was proposed in order to decrease the proportion of puppies in deficit of maternal immunoglobulins.

Puppies were allowed to suckle freely during the entire experiment. Blood samples were collected at two days of age and the serum was assayed for IgG concentration. Mortality was registered since two days until three weeks of age.

In the first part of the work, the effect of passive immune transfer, as evaluated via serum IgG concentration at two days of age, was tested on mortality until three weeks of age. The results demonstrated that puppies with higher IgG concentrations had higher chances to survive compared with other puppies. The minimal serum IgG concentration associated with decreased risk of neonatal mortality was determined at 2.3g/L. Among different parameters (breed size, sex, supplementation, colostrum IgG concentration, litter size), only weight gain within the first two days of life was found positively correlated with serum IgG concentration.

In the second part, the effect of an oral IgG supplementation was tested. Puppies were assigned into two groups. The first one received hyper-immunized canine adult serum within the first 8 hours after birth (before the intestinal barrier closure). The second one remained as a control group (non supplemented). Neither serum concentration of immunoglobulin was increased, nor neonatal mortality reduced in supplemented puppies compared with controls.

This study clearly demonstrated that, as in other agammaglobulinemic newborns (foals, calves, etc.), passive immune transfer in puppies is closely related to mortality. Nevertheless, the concentration of serum IgG at two days of age was found positively correlated with early

weight gain. Serum IgG may thus witness the adequate intake of energy and nutrients, perhaps more important for neonatal survival in dogs than the passive immune transfer.

Dealing with IgG, this study addressed the non specific passive immune acquisition. Transfer of specific antibodies remained to be investigated in order to evaluate the variability in specific antibody titers in the newborn dog, as well as its consequence on natural immunization and morbidity.

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Short communication

Inadequate passive immune transfer in puppies: definition, risk factors and prevention in a large multi-breed kennel

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ABSTRACT

The prevalence of neonatal mortality is high in the canine species and far from well-studied. In most domestic neonates, an appropriate colostrum intake is a key element of the control of neonatal mortality. The aim of this study was to evaluate the impact of passive immune transfer on puppy mortality, assessed through serum immunoglobulin G (IgG) concentration at 2 days of age. Factors impacting passive immune transfer and the value of an oral immunoglobulin supplementation to prevent it were also analyzed. A total of 149 puppies from 34 litters (12 breeds) within one breeding kennel were included. Blood samples were collected at 2 days of age and colostrum was collected from their dams 1 day after whelping to assay IgG concentration. Puppies were weighed at birth and at 2 days of age for calculation of growth rate. Mortality was recorded until 3 weeks of age. Seventy randomly assigned puppies were orally supplemented with hyper-immunized adult plasma twice within the first 8 h of life. IgG concentration at 2 days of age was significantly correlated with weight gain during the first 2 days of life. The multivariable model with litter as a random effect demonstrated that neonatal mortality was not influenced by breed size, sex, supplementation, litter size, nor colostrum IgG concentration, but by puppy IgG concentration at 2 days of age. According to the ROC curve, the minimal IgG concentration at and below which puppies were at higher risk of death was determined at 230 mg/dL. Puppy IgG concentration was significantly associated with growth rate, but not with breed size, sex, supplementation, litter size or colostrum IgG concentration in a multivariable model with litter as a random effect. This study demonstrates that neonatal mortality in puppies is related to the quality of passive immune transfer. The oral supplementation with hyper-immunized canine plasma neither decreased risk of mortality, nor improved serum IgG concentration at 2 days of age in puppies. Attention must thus be paid to early colostrum intake to control the neonatal mortality in puppies.

1. Introduction

Neonatal mortality in the canine species (within the first 3 weeks after birth) is highly prevalent ranging between 17 and 26% (Bowden et al., 1963; Nielen et al., 1998; Indreba et al., 2007). Infectious diseases are described as the primary cause of death in puppies born alive

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0167-5877/

(Nielen et al., 1998; Van der Beek et al., 1999) with *Escherichia coli*, *Staphylococcus* sp., *Streptococcus* sp., canine herpesvirus type 1 most frequently isolated from neonates dying within the first week after birth (Münnich, 2008; Dahlbom et al., 2009).

Puppies, as well as calves, piglets, foals and kittens, are born agammaglobulinemic or hypo-gammaglobulinemic (Bouchard et al., 1992). In many species, it has been demonstrated that adequate transfer of maternal immunity through colostrum is crucial for survival and control of infectious diseases in the newborn (Tyler et al., 1998; Christley et al., 2003; Vallet et al., 2013). Blood immunoglobulin G (IgG) concentration in neonates is the routine criteria used to evaluate the quality of passive immune transfer (Beam et al., 2009). Transfer of immunoglobulins from dam to a newborn is influenced by numerous maternal factors (age of the dam, parity, maternal behavior, quantity and quality of colostrum), together with several neonatal factors (litter size, birth weight, vitality, time and quantity of ingested colostrum). Despite the high prevalence of lethal infections in canine species, the direct link between mortality and passive immune failure has been poorly evaluated in puppies.

The economic importance of correct passive immune transfer in large animals has led to the development of numerous colostrum replacers and immunoglobulin supplements (calves: Vega et al., 2011; piglets: Yokoyama et al., 1992; foals: Franz et al., 1998). In contrast, few studies have investigated IgG supplementation in small animal neonates. In experimental conditions, adult dog serum, administrated either orally or subcutaneously to colostrum-deprived newborn puppies, proved to be an alternative source of immunoglobulins (Bouchard et al., 1992). However, there is no data in the literature regarding the improvement of passive immune transfer in puppies under natural conditions and with unlimited access to colostrum, with the aim to control mortality.

The first objective of this study was to evaluate the impact of passive immune transfer through puppy blood IgG concentration at 2 days of age on mortality within the first 3 weeks after birth; subsequently to determine a critical threshold below which the risk of neonatal mortality is significantly increased. The second objective was to identify factors influencing IgG concentration at 2 days of age. A third objective was to estimate the efficacy of an oral immunoglobulin supplementation within the first hours after birth on passive immune transfer.

2. Materials and methods

2.1. Animals

The experiment was conducted in a French breeding kennel from March to June 2012. All canine neonates born during this period ($n = 195$) and their dams ($n = 39$) were included in the study. All bitches in the kennel were routinely vaccinated annually (EURICAN CHPPi2, Merial, Lyon, France). Puppies were housed with their dam in a single, heated whelping box (2–4 m^2 of surface) from birth until 56 days of age and were allowed to suckle freely. Both bitches and their offspring at weaning were fed a dry balanced diet for growing dogs (Starter, Royal Canin, Aimargues, France) ad libitum. From a total of 195 puppies born in the kennel, 149 puppies from 34 litters which survived with no abnormalities until the time of blood collection were included in the study. Depending on the average adult body weight of their breed, puppies were divided into small breed dogs (body weight < 25 kg: Poodle ($n = 10$), Cocker Spaniel ($n = 16$), Bichon Frise ($n = 9$), Bichon Maltese ($n = 15$), Lhasa Apso ($n = 20$), Shih Tzu ($n = 17$), West Highland White Terrier ($n = 12$), Jack Russell Terrier ($n = 3$), Pomeranian ($n = 4$)) and large breed dogs (body weight ≥ 25 kg: Labrador Retriever ($n = 10$), Golden Retriever ($n = 28$), German Shepherd Dog ($n = 5$)).

2.2. Data collection and immunoglobulin G assay

Immediately after birth, each neonate was identified by a colored woolen collar and its breed and sex were recorded. Puppies were weighed at birth and at 2 days of age to calculate a growth rate over the first 2 days of life ((weight at 2 days − weight at birth)/weight at birth). For each litter, the total number of puppies born was recorded (litter size). Mortality over the first 3 weeks after birth was also recorded. One milliliter of blood was collected into a plain tube from each puppy at 2 days of age (between 36 and 48 h) from the jugular vein. One day after whelping onset, 0.5–1 ml of colostrum was collected from the dams. Milk and blood were stored at −20 °C until IgG assay in duplicate using a previously described and validated ELISA method (Dog IgG-Quantitation Kit, Bethyl Lab, Montgomery, USA) (Schäfer-Somi et al., 2005).

2.3. Immunoglobulin supplementation

Seventeen large breed non pregnant bitches from the same kennel, which were not included in the protocol described above, were vaccinated against canine herpesvirus type 1 (EURICAN Herpes 205, Merial) and *Bordetella bronchiseptica* (PNEUMODOG, Merial). The same vaccination was repeated 2 weeks later combined with a polyvalent vaccine (EURICAN, Merial). Two weeks after the last vaccination, blood (7.5 ml/kg body weight) was collected from each bitch into heparinized containers and centrifuged. Plasma from the 17 bitches was pooled and aliquoted before storage at −20 °C. Plasma IgG concentration was assayed as previously described. Ensuring equal distribution of individuals, in terms of birth weight and breed size, puppies were assigned to 2 different groups within each litter. In the first group (non-supplemented, $n = 79$), puppies did not receive hyper-immunized plasma. In the second group (supplemented, $n = 70$), puppies received 2 doses of hyper-immunized plasma before intestinal barrier closure: the first dose was administrated a maximum of 4 h after birth by a feeding tube (1.5 ml/100 g body weight); the second administration, using the same method and dose, was performed 4 h later, at a maximum of 8 h after birth. Both supplemented and non-supplemented puppies were allowed to suckle their dam during the entire experiment. The dose of hyper-immunized plasma administrated to puppies was chosen as the best compromise between maximal volume for administrated supplement

and maximal volume for ingested colostrum, as volume of stomach in newborn puppy is 5 ml/100 g of body weight.

2.4. Statistical analyses

Statistical analyses were performed with the SAS version 9.3 software (SAS Institute Inc., Cary, NC, USA) to determine the risk factors for mortality and factors influencing puppy IgG concentration. Spearman's rho correlation coefficient was used to evaluate the correlation between puppy IgG concentration at 2 days of age, IgG concentration in colostrum, litter size and growth rate.

Firstly, a generalized linear mixed model (proc GLIMMIX) with mortality as a binary outcome (logit transformation) was used to assess the following fixed effects: sex (male/female), breed size (small/large), supplementation (yes/no), litter size, colostrum IgG and puppy IgG concentrations. Litter was modeled as a random effect. Two way interactions between sex and supplementation were investigated, as it improved the quality of the model. As it was found that growth rate and puppy IgG concentration were correlated; puppy IgG concentration was chosen to be included into described model, as evaluation of its effect on mortality was of primary interest. Subsequently, a receiver operating characteristic (ROC) curve was drawn based on the final logistic model. The Hosmer and Lemeshow Goodness-of-Fit test was used to assess the quality of this model. Youden's index was used to define the best cutoff from the model for high and low mortality risk populations. Finally, a Kaplan–Meier plot and the Log-rank test were used to assess the survival between these two groups.

Secondly, a linear mixed model (proc MIXED) was performed to determine the variables affecting puppy IgG concentration at 2 days of age. As residuals of this multivariable model were not normally distributed, a non-parametric analysis was performed (rank transformation of the outcome). This model included as fixed effects: breed size, sex, supplementation with their related interactions (encoded as described for previous model). Moreover, the effects of litter size, colostrum IgG and growth rate were added as covariates. Litter was modeled as a random effect.

Quantitative data are presented as medians with interquartile range (IQR).

3. Results

3.1. Study population

The total mortality in puppies within the first 3 weeks of life was 31.3% (61/195). Among all puppies 28.7% (56/195) died within the first week after birth. IgG concentration was assayed on sera from 149 puppies which survived until the blood collection (Day 2) and on colostrum from their 34 dams. Eighteen out of 149 puppies died before 3 weeks of age. The IgG concentration in puppies at 2 days of age was 610 mg/dl (360; 975 mg/dl). The colostrum IgG concentration was 1940 mg/dl (1500; 2290 mg/dl). IgG concentration of the pooled hyper-immunized plasma was 1430 mg/dl, i.e. 73.7% of median IgG concentration in colostrums collected in this experiment. The growth rate between birth and 2 days of life was 3.1% (−5.2; 10.9%) and was significantly correlated with puppy IgG concentration ($\rho = 0.7$; $p < 0.001$). The other variables in correlation matrix were not correlated.

Table 1
Risk factor assessment for mortality in puppies between 2 and 21 days after birth evaluated by a generalized linear mixed model ($n = 149$).

Factor	P value	Odd ratio	95% confidence interval	
Breed size	0.386	0.3	0.0	4.8
Sex	0.765	0.8	0.2	3.1
Supplementation	0.851	0.8	0.1	5.0
Litter size	0.382	1.2	0.8	2.0
Colostrum IgG	0.728	1.0	0.9	1.2
Puppy IgG at Day 2	0.018	0.7	0.5	0.9

3.2. Factors influencing mortality

In the generalized linear mixed model, risk of neonatal mortality was influenced by puppy IgG concentration at 2 days of age and litter effect as a random term ($p = 0.018$; $p = 0.003$; respectively). Puppies with lower IgG concentrations presented higher risk of death than other puppies (Table 1). IgG concentration in puppies dying between 2 and 21 days was 172 mg/dl (42; 508 mg/dl) vs. 669 mg/dl (430; 1030 mg/dl) in puppies still alive at Day21. None of the other factors tested (sex, breed size, supplementation, colostrum IgG concentration and litter size) demonstrated any influence on neonatal mortality (Table 1). The optimal cutoff value of IgG concentration to assess predictive likelihood of mortality was 230 mg/dl (odd ratio = 0.7; 95% confidence interval: 0.5; 0.8) with a sensitivity of 66.7% and a specificity of 87.8%. Among the 149 puppies which survived until the blood collection, 18.1% (27/149) presented a blood IgG concentration at 2 days of age below or equal to 230 mg/dl. The survival analysis indicated significant differences in survival kinetics between puppies above or under the cutoff value for IgG concentration ($\chi^2 = 30.33$; $p < 0.001$; Fig. 1). Among puppies with IgG concentration ≤230 mg/dl at 2 days of age, 44.4% (12/27) died, while it

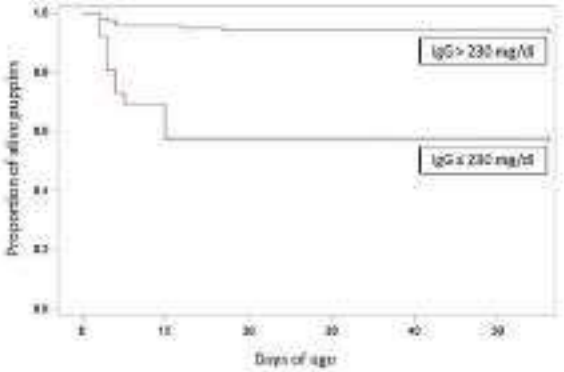

Fig. 1. Kaplan–Meier plot of survival kinetics in puppies above ($n = 123$) and under ($n = 26$) the critical threshold of puppy IgG concentration at 2 days of age (230 mg/dl) for higher risk of death ($\chi^2 = 30.33$; $p < 0.001$).

was the case only for 4.9% (6/122) of puppies with higher IgG concentration.

3.3. Factors influencing blood IgG concentration in puppies at Day 2

Growth rate between 0 and 2 days after birth and the litter effect as a random term had an impact on puppy IgG concentration at 2 days of age ($p < 0.001$; $p = 0.007$, respectively). None of the other factors tested in this linear mixed model (breed size, sex, supplementation, colostrum IgG concentration, litter size) demonstrated any influence on IgG concentration at 2 days of age.

4. Discussion

4.1. IgG concentration and mortality

The percentage of neonatal mortality (over the first 3 weeks of life) was higher in this study (31.3% of all born puppies) compared with other published data (Nielen et al., 1998; Indrebø et al., 2007). Among the puppies that died during the first 3 weeks after birth, 91.8% died within 7 days, similar to other studies (Nielen et al., 1998; Tønnessen et al., 2012). In many species, passive immune transfer through colostrum is known to be crucial for the control of neonatal mortality (i.e. in bovine Tyler et al., 1998; in porcine Vallet et al., 2013).

We evidenced in this study a strong relationship between passive immune transfer (IgG concentration assayed in serum at 2 days of age) and neonatal mortality in puppies. In our experiment puppy IgG concentration below or equal to 230 mg/dl defines the deficit of passive immune transfer in the canine species associated with decreased survival rate. The reliability of this threshold has to be evaluated in other kennels, with different management and infectious conditions. Dam, as one of a random term for all puppies coming from the same litter, displayed a significant impact on canine neonatal mortality in this study. Maternal factors influencing colostrum ingestion in other species are maternal behavior, quantity of colostrum produced and anatomic morphology of teats (more or less easy to be suckled) (Rooke and Bland, 2002). In this study, neither breed size nor litter size had an influence on neonatal mortality, although Tønnessen et al. (2012) previously reported higher mortality rates in puppies from large litters and puppies belonging to large or giant breeds.

4.2. Factors influencing IgG concentration

In our study, growth rate over the first 2 days of life was highly associated with puppy serum IgG concentration. Both reflect colostrum intake, as this secretion plays not only an immune but also nutritional role. In piglets, the concentration of serum immunoglobulins was found to be associated with the quantity of colostrum ingested (Rooke and Bland, 2002). It suggests that routine weighing of newborns can be used to evaluate the colostrum intake and thus, indirectly the passive immune acquisition in puppies. Although colostrum provides energy together with immunological protection, absorption of immunoglobulins, unlike nutrients, depends on time elapsed from birth, because of the progressive intestinal barrier closure. In puppies, IgG absorption rate at 4 h of life is decreased two-fold compared to the level at birth and is almost null at 12–16 h of life (Chastant-Maillard et al., 2012). As observed for mortality, common effect within a litter, such as dam also influenced immunoglobulin transfer in puppies. Dystocia, inappropriate care of newborns or absence of colostrum secretion at whelping onset may decrease the passive immune transfer in puppies before the gut closure. In cattle and pigs, attention is paid to the immune quality of colostrum, since IgG concentration in colostrum is known to be a significant factor in the quality of passive immune transfer to the offspring (Beam et al., 2009; Devillers et al., 2011). In the present study, the IgG concentration in colostrum was significantly associated neither with mortality, nor with serum IgG concentration of puppies. The relationship between IgG level in colostrum and puppy serum could be nonexistent or masked by several factors of larger impact such as timing of colostrum intake and quantity of colostrum ingested by the newborn.

4.3. Hyper-immunized plasma supplementation

Supplementation was designed to optimize IgG absorption and decrease the risk of failure of passive immune transfer: plasma was administrated orally before intestinal barrier closure (within the first 8 h of life; Chastant-Maillard et al., 2012), and plasma immunoglobulins were protected from digestion since puppies were suckling colostrum providing antitrypsin. Nevertheless, in our experiment, no effect of supplementation on neonatal mortality has been observed, but a lack of statistical power cannot be ruled out. Since puppies had free access to the colostrum of their dam, the supplementation may have partially substituted spontaneously ingested colostrum. As the hyper-immunized plasma used in the experiment had an IgG concentration 30.5% lower than the colostrum, supplementation might even have decreased the total quantity of ingested immunoglobulins. In another study, puppies fed milk replacer and adult serum while being colostrum deprived, absorbed significantly less IgG than puppies fed colostrum (Bouchard et al., 1992). The efficacy of supplementation with higher IgG concentration in order to decrease the frequency of passive immune failure needs to be tested.

5. Conclusions

This study clearly demonstrates that neonatal mortality in puppies is related to passive immune transfer. Based on these data, obtained from one kennel, the deficit of immunoglobulin G in puppies, associated with higher risk of death, was defined at 230 mg/dl. More data have to be collected to confirm this cutoff value. However, our results show that attention has to be paid to the colostrum intake during the first 12 h of life in order to diminish the risk of passive immune failure and mortality in canine neonates. To date, at variance with some other species, no efficient alternative source of immunoglobulins is available for puppies. The design of an adequate immune booster

immediately after birth, to decrease high mortality rate in canine neonates remains to be established.

Conflict of interest

No product branded by Royal Canin has been tested in the experiment and authors belonging to Royal Canin staff have no conflict of interest to declare. Other authors also declare no conflict of interest.

Acknowledgments

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Article 5

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Protection against canine parvovirus type 2 infection in puppies by colostrum-derived antibodies

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Canine parvovirus type 2 (CPV2) is a ubiquitous virus, highly pathogenic and highly lethal for young dogs (54,55). Colostrum brings the only protection against CPV2 infection before weaning. As demonstrated in the previous study (Article 4), the risk of neonatal mortality in dogs is strongly associated with the passive immune transfer, evaluated via blood IgG concentration. However, this global maternal immunity provides an approximate evaluation of level of specific antibodies, such as CPV2 antibodies. From our own unpublished data, the correlation between IgG concentration and CPV2 antibody titer, although strong, is not linear (Fig.2).

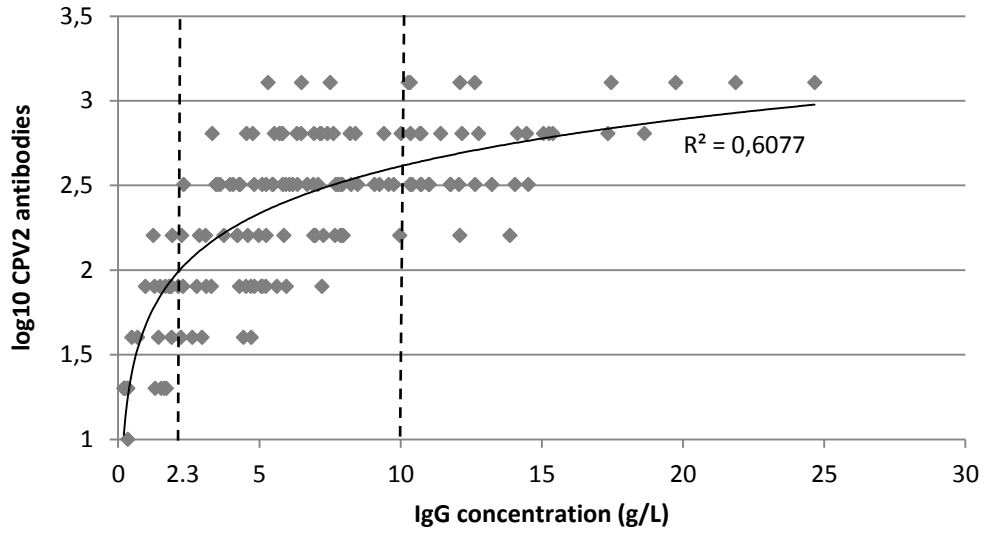

Fig.2. Correlation between blood IgG concentration and CPV2 specific antibody titer (HI titer) in puppies at 2 days of age (n=151; rho=0.7, p<0.001; unpublished data).

Since Decaro et al. (20) reported that CPV2 infection is possible in puppies at antibody titer 1:160 (log10=2.2), the minimal protective level of CPV2 specific antibodies can be set at >1:160. Among all puppies in deficit of passive immune transfer (IgG ≤ 2.3g/L) from our unpublished data, 100% are at CPV2 antibody titer ≤1:160 (Fig.2). Among puppies with IgG between 2.3 and 10 g/L, 40% are at low level of CPV2 antibodies. Among puppies with IgG concentration above 10 g/L, only 5% have CPV2 antibody titer ≤1:160. These data show that serum IgG concentration at 2 days of age cannot be used to predict the level of specific antibodies against CPV2.

The study presented in Article 5 investigated the acquisition of the CPV2 specific antibodies, its kinetics until weaning, as well as the variation factors for CPV2 titers achieved at two days

after birth. The role of the CPV2 specific maternally derived antibodies (MDA) on protection against parvovirosis was also explored under condition of natural infection.

Puppies were housed with their routinely vaccinated dams during entire period of the study. Blood samples were collected from puppies weekly from birth until weaning and the titer of CPV2 specific antibodies was assayed by inhibition of haemagglutination test (HI test). Excretion of the parvovirus was evaluated via RT-PCR, on rectal swabs collected weekly since three weeks of age until weaning.

Depending on CPV2 antibody titer at two days of age, puppies were classified into two groups: group A with low level of maternally derived antibodies (MDA titer ≤1:160) and group B with high level of antibodies (titer >1:160). CPV2 specific MDA level at two days of age was influenced by early growth rate (during the first two days of life) and breed size (MDA greater in puppies with higher growth rate and in large-sized breeds). Although MDA declined with age in both studied groups, the MDA protection against CPV2 infection lasted longer in puppies with higher MDA at two days of age (group B), compared with group A. A total of 96% of puppies underwent CPV2 infection and seroconversion.

The present study evidenced the importance of adequate colostrum intake for a long-term protection against infectious diseases, such as CPV2. As morbidity and mortality due to CPV2 is age dependent (54,56,57), longer protection due to greater antibody titer absorbed at birth increases the chances of the puppy to survive in case of viral contamination. Nevertheless, in natural conditions, with CPV2 circulation in the kennel and no vaccination provided to puppies, majority of them underwent CPV2 infection at similar age (6-7 weeks). It suggests that apart the systemic protection thanks to colostrum intake, other sources of protection exists. Especially, the role of milk antibodies in local immune protection (in digestive tract) against CPV2 needs further investigation.

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WALTHAM SYMPOSIUM

Protection against canine parvovirus type 2 infection in puppies by colostrum-derived antibodies

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Abstract

During the first weeks of life puppies remain protected against canine parvovirus type 2 (CPV2) infection thanks to maternally derived antibodies (MDA) absorbed with colostrum after birth. The objective of the present study was to present the variability in CPV2-specific passive immune transfer and its consequences in puppies naturally exposed to the parvovirus. Seventy-nine puppies from one breeding kennel were included in the study at birth and followed until 56 d of age. Once per week the MDA titre for CPV2 specific antibodies was determined in blood. Viral excretion was also evaluated on a rectal swab by CPV2 PCR assay and puppies were weighed to determine growth rate. At 2 d of age, thirty-four out of seventy-nine puppies (43 %) had MDA <1:160 (designed group A) and forty-five puppies (57 %) had greater MDA titres (designed group B). The level of absorbed maternal antibodies was shown to be associated with breed size and growth rate during the first 48 h of life. The MDA level declined with age in all cases; however, the proportion of puppies with the antibody level considered as protective against CPV2 infection was significantly higher in group B compared with A from day 2 until 42. Among all puppies surviving until 56 d of age, sixty-seven out of seventy (95·7 %) underwent CPV2 infection. However, puppies from group A excreted CPV2 significantly earlier than puppies from group B. The present study demonstrates the link between passive immune transfer, in terms of level of specific MDA absorbed, and length of the protection period against parvovirus infection in weaning puppies.

Key words: **Puppies: Canine parvovirus: Colostrum: Maternally derived antibodies**

The prevalence of canine parvovirus type 2 (CPV2) in diarrhoeic puppies varies from 64 % in North America and 70 % in Europe[1,2]. CPV2 is a ubiquitous enteropathogen that is responsible for outbreaks of acute gastroenteritis, with a high mortality rate[3]. During the first weeks of life, maternally derived antibodies (MDA) provide the only specific systemic protection against CPV2 in puppies. Only 10 % of circulating CPV2 antibodies in the neonate are from transplacental origin[4]. The vast majority is transferred from the dam to puppies through colostral ingestion during the first hours of life. Systemic CPV2 MDA titre decreases with age[5–8]. When the serological titre has fallen under 1:80 (haemagglutination inhibition (HI)), the systemic MDA level seems to be no longer protective against CPV2[4,7]. To date, the variability in maternally derived protection against CPV2 and its consequences in puppies have been studied exclusively under experimental conditions and only on weaned puppies or puppies deprived from maternal milk. Since canine colostrum and milk have been proven to provide significant amounts of CPV2 antibodies[9], these lactogenic MDA could potentially interfere with CPV2 intestinal replication either by coating the enterocytes or trapping the faecal CPV2 particles, preventing their multiplication

Abbreviations: CPV2, canine parvovirus type 2; HI, haemagglutination inhibition; MDA, maternally derived antibodies.

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in the mucosa. Moreover, since viral challenge induces a more rapid decrease in circulating CPV2 antibodies,[8] MDA kinetics may differ depending on environmental infective pressure.

In the present study, we focused on CPV2 MDA in puppies maintained under natural conditions, housed in a breeding kennel with natural CPV2 circulation. The aim of the work was to analyse the variability and kinetics of systemic maternally derived CPV2 antibodies under these field conditions and to evaluate factors influencing MDA. The link between initial MDA level, viral shedding and growth performance was also studied.

Materials and methods

The study protocol was reviewed and approved by the Royal Canin Internal Ethics Committee.

Animals

The experiment was conducted in a commercial breeding kennel over a 4 months period (March–June 2012). Seventy-nine puppies from various breeds (twenty-six litters, ranging from one to eight puppies per litter alive at day 2; day 0 = whelping) were included and followed from birth until 56 d of age. Breeds whose adult weight was less than 25 kg were considered small breeds and dogs with a greater adult weight large breed dogs. All puppies were housed with their dam in heated whelping boxes from birth to 56 d. Puppies were allowed to suckle freely. Lactating bitches and their puppies were fed *ad libitum* with the same diet, a dry expanded complete diet balanced for growing dogs (Starter, Royal Canin).

Blood (1 ml per puppy) was collected from the jugular vein at days 2 and 7 and every week until day 56. Samples were immediately centrifuged (3000 *g*, 15 min) and serum separated. Rectal swabs were performed at day 17 and every week until day 52. Sera and rectal swabs were stored for 4 months at −20°C until assayed.

Canine parvovirus type 2 antibodies assay

Titres of antibodies directed against CPV2 were evaluated on serum by HI test as previously described[7]. Tests were performed at +4°C using ten haemagglutinating units of CPV2 antigen and 1 % pig erythrocytes. All samples from one puppy (from one to nine samples) were tested on one plate with a maximum of ten plates performed at one time. Twofold dilutions in PBS of each serum sample starting from 1:10 were tested and the HI titre was the highest serum dilution completely inhibiting viral haemagglutination. Titres below 1:80 were considered as non-protective against infection[9]. Seroconversion was defined as a minimum 4-fold increase of HI titre. After a seroconversion episode (indicating a viral contamination), puppies were considered as no longer protected by MDA.

Canine parvovirus type 2 faecal excretion

A homogenate (10 %) of the faecal sample was prepared in PBS (pH 7·2) and centrifuged at 1500 *g* for 15 min. The viral DNA was extracted from prepared supernatant by boiling the sample (10 min) and subsequently chilling on ice. To reduce inhibition of DNA polymerase, samples were diluted 1:10 with distilled water. No more than ten extractions were performed at one time. CPV2 real-time PCR assay with the TaqMan probe was conducted on faecal samples as described by Decaro *et al.*[9] with ovine herpesvirus 2 DNA as internal control. A dilution of standard DNA in a CPV-negative faecal suspension was performed (serial log 10 dilutions) and tested to determine the detectability and the linearity of the assay. The following thermal protocol was used: iTaq DNA polymerase was activated at 95°C for 10 min followed by forty cycles consisting of denaturation at 95°C for 15 s, subsequently primer was annealed at 52°C for 30 s and the process was extended at 60°C for 1 min.

Growth

Puppies were weighed at birth, 48 h and every week until 56 d of age using a calibrated analytical scale in 1 g increments (Fisher Scientific International Inc.). Subsequently, growth rate (%) over the first 2 d of life was calculated ((weight at 2 d − weight at birth)/weight at birth × 100), together with growth rate between 21 and 56 d of life ((weight at 56 d − weight at 21 d)/weight at 21 d × 100).

Statistical analysis

Statistical analyses were performed using Tanagra® freeware (Tanagra 1·4, Lyon, France). All datasets were tested for normality by the Shapiro–Wilk test. As data were not normally distributed, they were presented as medians and range. A two-sided Mann–Whitney *U* test or a Kruskal–Wallis test was used according to the number of groups considered. The level of statistical significance was set at $P < 0{\cdot}05$ for all analyses.

Results

Variability in canine parvovirus type 2-specific passive immune transfer

At 2 d of age, MDA titres displayed large variability between puppies, titres ranging from 1:10 to 1:1280 (log10 = 1·3–1; Fig. 1). At that time (day 2), thirty-four out of seventy-nine puppies (43 %) had HI titre <1:160 (log10 < 2·2; group A), among which thirteen (38 % of total population) had not reached the HI titre 1:80 (log10 = 1·9), considered as the minimal protection against CPV2 infection. Only forty-five of seventy-nine animals (57 %) had HI titres > 1:160 (group B) with seven puppies at HI > 1:1280. Mortality rate between 2 and 56 d of age was significantly higher in puppies from group A than group B (9/34; 26 % *v.* 3/45; 7 %; $P = 0{\cdot}022$).

Factors influencing canine parvovirus type 2-specific passive immune transfer

The breed size and growth rate between birth and 48 h of life were associated with CPV2 specific antibody transfer from bitch colostrum to puppies. Large breed puppies had higher

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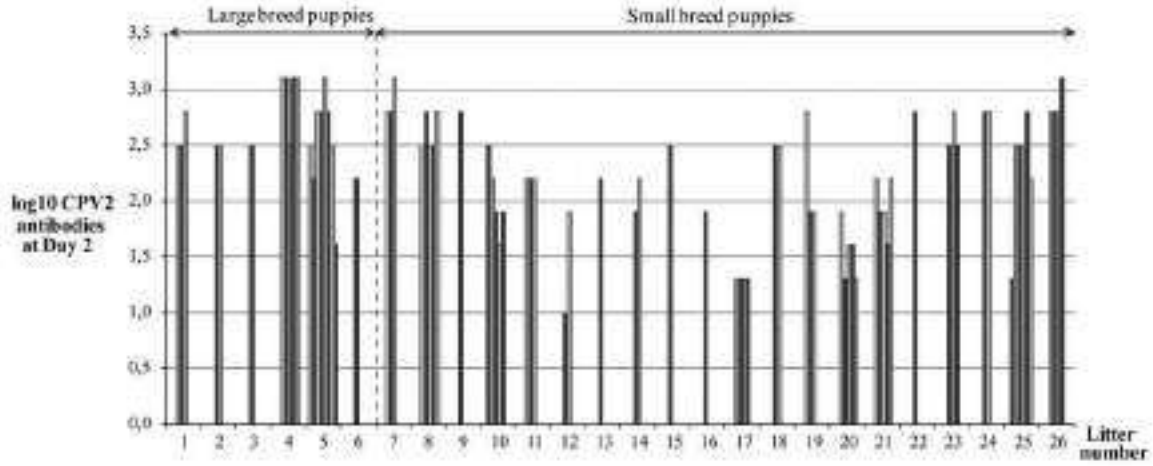

Fig. 1. Variability in maternally derived antibody titres against canine parvovirus type 2 at 2 d of age (*n* 79). Each bar represents one puppy, each group of bars represents one litter (*n* 26), and puppies are divided in small and large breeds (arrows).

MDA titres at day 2 compared with small breed puppies (median HI titre: 1:320 (range: 1:40 1:1280) *v.* 1:160 (1:10 1:1280); $P = 0{\cdot}003$). Puppies which lost weight during the first 48 h of life had lesser MDA titres at day 2 than puppies which gained weight (HI titre: 1:120 (1:10 1:1280) *v.* 1:320 (1:40 1:1280); $P < 0{\cdot}001$).

Maternally derived antibody level and canine parvovirus type 2 infection

Kinetics of canine parvovirus type 2 maternally derived antibodies. The titres of MDA progressively declined with age in both groups. The proportion of puppies with MDA protection against CPV2 infection in group B was significantly higher from day 2 until 42 than in group A. At day 56 none of the 67 surviving puppies displayed MDA titre >1:80 (Table 1).

Sixty-three out of the sixty-seven puppies still alive at the end of the experiment underwent a seroconversion, with sixty-two of them reaching an HI titre of 1:1280. The four puppies which did not seroconvert belonged to group B. Median age at seroconversion was 49 d (28 56) in puppies from group A and 56 d (35 56) in group B ($P < 0{\cdot}001$). Among thirteen puppies with MDA titre at day 2 < 1:80, the first seroconversion appeared at day 42. Half-life of systemic MDA was 13·4 d.

Viral excretion. Among seventy puppies still alive at 17 d of age, sixty-seven (96 %) displayed a significant viral excretion (>1000 copies) at some point during the study period. Puppies from group A excreted CPV2 at significantly earlier age compared with puppies from group B (day 38 (17 52) *v.* day 45 (17 52); $P = 0{\cdot}011$).

At the time of the first significant viral excretion, fifty-two puppies had HI titres less than 1:80, fourteen puppies had HI titres of 1:80 or 1:160 and only three puppies had HI titres > 1:160.

Growth. Growth rates between 21 and 56 d of age were not significantly different between groups A and B (65 % (53 83 %) *v.* 62 % (28 79 %); $P = 0{\cdot}11$).

Discussion

MDA are crucial for the protection of puppies against CPV2 infection since puppies are nearly agammaglobulinemic at birth. Canine neonates acquire systemic antibodies via colostral ingestion within the first hours of life before gut closure[9,10]. In the present study, at 2 d of age, MDA titres displayed large variability between puppies, with titres ranging from 1:10 to 1:1280. The variability in MDA level could be due to unequal colostrum ingestion from maternal or puppy's origin. In the present study, we noted a relationship between the early growth rate and the absorption of specific CPV2 MDA. Both reflect colostrum intake as this secretion plays not only an immune, but also a nutritional role. Systematic weighing of puppies could therefore be performed by breeders in

Table 1. Proportion of puppies protected from CPV2 infection (HI > 1:80) depending on MDA level at 2 d of age

	Age of puppies (weeks)								
	2	7	14	21	28	35	42	49	56
Group A	21/34 (62)	14/30 (47)	6/26 (19)	0/26 (0)	0/26 (0)	0/25 (0)	0/25 (0)	0/25 (0)	0/25 (0)
Group B	45/45 (100)	44/44 (100)	41/44 (93)	34/44 (77)	24/42 (57)	10/44 (23)	9/44 (20)	2/44 (5)	0/43 (0)
P value for each period of time	<0·001	<0·001	<0·001	<0·001	<0·001	0·011	0·021	0·531	–

n/n = number of puppies protected in the category considered/total number of puppies in the category (%).

order to control for correct passive immune transfer and energy intake at the very early stages of life.

After the first 24 h of life, MDA are no longer absorbed and they decline with age[4,6]. Pollock & Carmichael[4] observed a half-life for CPV2 MDA of 9·7 d, with puppies reaching seronegative levels between 10 and 14 weeks of age. Gooding & Robinson[5] observed the HI titres <1:10 after day 49. In the present study, half-life was slightly longer at 13·4 d. From the observation of Macartney *et al.*[8], who described an acceleration in the decline of blood CPV2 titres after viral challenge, one could expect a more rapid MDA decrease in the present study, which was conducted under a high CPV2 environmental pressure. In this situation, systemic MDA may be recruited to limit the multiplication of CPV2 virus, thereby leading to an earlier entry into a susceptibility period for viral infection. Nevertheless, in our conditions of natural infection, this hypothesis was not confirmed.

The early consumption of a sufficient quantity of maternal colostrum to maximise passive immune transfer appears to increase the length of the protective period. Indeed, the proportion of protected puppies was higher in the group with the higher MDA level (group B) until 42 d of age. The study demonstrates thus the importance of optimal colostrum intake in puppies in order to induce a longer immunoprotection during the pediatric period.

The large variation in the CPV2 susceptibility period between puppies observed in the present study underlines that a routine vaccination protocol should be adapted not only to the breeding kennel epidemiologic situation, but also to a puppy's individual needs. Although the early vaccination appears controversial since MDA may interfere with CPV vaccination, decreasing the vaccines response[9,11,12], recently a high antigen titre vaccine administrated as early as 4 weeks of age was demonstrated effective in the reduction of the CPV2 susceptibility window[13].

Over the study period, nearly all puppies (96 %) underwent viral infection and seroconversion. Viral infection appeared in the vast majority of puppies when HI titres were lower or equivalent to 1:80 (90 % of the infected puppies with HI < 1:80), as described for experimental viral challenges[4,7]. In puppies with a MDA level lower than protective against CPV2 infection at birth, seroconversion appeared for the first time only at day 42. Further work would be necessary to assay pathogenic viral loads (by haemagglutination test) in parallel with global viral load (as obtained by PCR), together with copeoantibodies[3,11], to verify the importance of lactogenic MDA before and during natural CPV2 infection episodes.

Conclusions. Based on systemic MDA, optimal passive immune transfer lengthens the protection period against CPV2 infection. Breeders should be encouraged to pay attention to early suckling within the first 12 h after birth. Systematic weighing at early age, evaluating indirectly the passive immune transfer, could indicate puppies at risk and allow adaptation of the vaccination protocol to individual needs. Nevertheless, the potential role of local MDA, as provided by milk, to limit viral replication and its consequences on morbidity and mortality merits further investigation.

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Article 6

H. Mila, A. Feugier, A. Grellet, J. Anne, M. Gonnier, M. Martina, L. Rossig, S. Chastant-Maillard

Immunoglobulin G concentration in canine colostrum: evaluation and variability

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Definition of colostrum relies mainly on its immunoglobulin concentration, and especially IgG. In all farm animals, IgG is the predominant immunoglobulin at the beginning of lactation (58). Due to the significant reduction in IgG concentration within the first 24h since the parturition onset, colostrum is defined in pigs as the secretion of the first day of lactation only (59). Also in dogs, IgG is of high concentration at the first day *post partum*, followed by a significant decline during the next days (Fig.3).

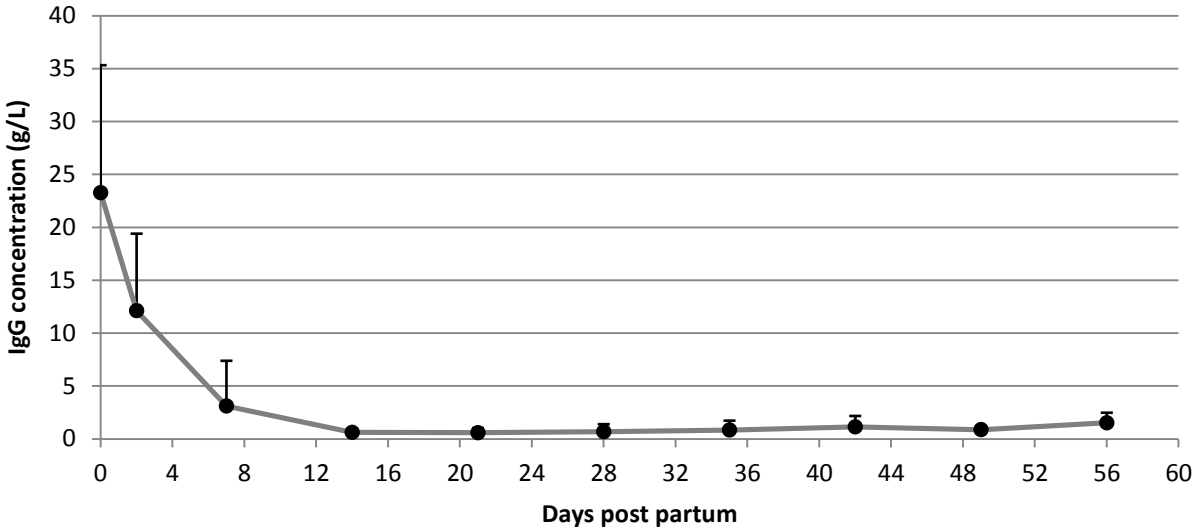

Fig.3. IgG concentration (mean ± SD) in dog mammary secretion since parturition until the end of lactation (study on 11 bitches; Chastant-Maillard et al. unpublished data).

This last study (Article 6) dealt with the immune quality of canine colostrum, evaluated via IgG concentration. The objective was to describe the variability of immune colostrum quality among bitches, but also among different pairs of mammary glands of one given bitch. Secondly, an on-kennel method for colostrum evaluation was tested.

Colostrum was collected separately from each pair of mammary gland within the first 24h from the parturition onset and after expulsion of the last puppy. IgG concentration was evaluated on colostrum samples by ELISA test (gold standard method) and refractometry. Breed size, litter size and age of the bitch were also recorded.

A significant variation was observed among bitches, with coefficient of variation of 39%. An even greater variation in IgG concentration was found among different pairs of mammary glands of one given bitch (mean coefficient of variation of 42%), with no particular teat pair producing systematically a better quality colostrum. Neither breed size, litter size nor age of

the bitch appeared to be associated with the IgG concentration. Relationship between ELISA test and refractometry was investigated and the correlation coefficient was found of moderate strength.

In front of the great variability of colostrum immune quality observed between bitches, the question of the minimal immune quality for adequate passive immune transfer remains to be addressed. Similarly, the variability between mammary glands makes necessary investigation of the suckling behavior of puppies before the intestinal barrier closure. In parallel, neonatal losses in dogs are related to the colostrum intake, not only in terms of source of immunoglobulins, but also energy. Thus the variability in energy content among bitches and among mammary glands remained also to be evaluated in the canine colostrum.

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Immunoglobulin G concentration in canine colostrum: Evaluation and variability

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ABSTRACT

Canine neonates are born hypogammaglobulinemic, and colostrum is their main source of immunoglobulins. The purpose of this study was to evaluate the immune quality of canine colostrum and its variability both among bitches and among mammary glands. The immune quality was estimated from immunoglobulin G (IgG) concentration (ELISA test). The correlation of IgG concentration with refractometry was evaluated. From a total of 44 bitches from 13 different breeds from a single breeding kennel, samples of colostrum and blood were collected one day after the parturition onset. Colostrum was collected separately from each pair of mammary glands (180 pairs). The mean colostrum IgG concentration in our population was 20.8 ± 8.1 g/L (ranging from 8.0 to 41.7 g/L) with no influence of breed size, litter size, age of dam or serum IgG concentration. Colostrum IgG concentration varied widely among pairs of mammary glands within one bitch (variation coefficient: 42 ± 32.1%). Nevertheless, no single pair of mammary glands was found to produce regularly a secretion of higher quality. No difference in IgG concentration was recorded between anterior and posterior pairs either. The BRIX index and the refractive index were significantly, but moderately correlated with colostrum IgG concentration ($r = 0.53$ and 0.42, respectively). This study demonstrates a great variability in immune quality of colostrum among bitches and among mammary glands within one bitch. Further studies on the suckling behavior of puppies and on determination of the minimal immune quality of colostrum are required to evaluate their impact of this high variability on neonatal mortality in dogs.

1. Introduction

The immune status of the newborn puppy depends entirely on colostrum ingestion, since canine neonates are nearly agammaglobulinemic at birth (Rouchard et al., 1992). From all circulating immunoglobulins after closure of the intestinal barrier, 90–95% originate from the colostrum (Chastant-Maillard et al., 2012). Inadequate colostrum intake leads to a deficit in the transfer of passive immunity, associated with higher mortality and morbidity rates in calves, lambs and piglets (Christley et al., 2003; Devillers et al., 2011; Virtala et al., 1999), but also in puppies (Mila et al., 2014). In large animals, apart from quantity and age at ingestion, the concentration of immunoglobulins in the colostrum is one of the limiting factors of adequate passive immune transfer to the newborn (Weaver et al., 2000). In ruminants and foals, the immune quality of colostrum is easily evaluated by refractometry before first suckling (Morrill et al., 2012; Waelchli et al., 1990). This dam-side test indicates refractive or BRIX index values, well correlated with the immunoglobulin G (IgG) concentration (Bielmann et al., 2010; Morrill et al., 2012). Colostral total proteins, of which immunoglobulins account for a large portion, refract light. This property has been used in refractometry in order to estimate the level of proteins, and thus indirectly IgG. To date, only laboratory procedures (ELISA test) allow to determine the IgG concentration in dog colostrum; however, these are time-consuming, expensive and not adapted for in-kennel application.

The amount of IgG in colostrum varies widely between females, ranging from 11.7 to 101.4 g/L in sows, and from 25.7 to 168.7 g/L in cows (Inoue et al., 1980; Quigley et al., 1995). Within one given dam, IgG may vary also between mammary glands, as described

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in pigs and cows (Farmer and Quesnel, 2009; Guatteo et al., 2013). Numerous factors, such as parity, nutrition and genetic selection are known to impact colostrum immunoglobulin concentration (Godden, 2008; Inoue et al., 1980; Quesnel, 2011). The immune quality of the colostrum in the canine species has been poorly explored. IgG concentration values were reported only in a few studies conducted on a few animals from one breed (Heddle and Bowley, 1975; Schäfer-Somi et al., 2005). Variation factors have neither been evaluated.

This study was designed to analyze the variability in IgG concentration in colostrum among bitches and among teats, and to identify some factors influencing the quality of colostrum. The value of refractometry for evaluating IgG concentration in colostrum was also investigated.

2. Materials and methods

The study protocol was reviewed and approved by the Royal Canin Internal Ethics Committee (AE/20140704).

2.1. Animals and data collection

Forty-four bitches from one breeding kennel were included in the study. Starting 2 weeks before parturition, each female was single housed and fed a dry balanced diet for growing dogs (Starter, Royal Canin, Aimargues, France) *ad libitum*. The bitches belonged to 13 different breeds: Bichon Frise ($n=4$), Bichon Maltese ($n=4$), Cocker Spaniel ($n=4$), German Shepherd ($n=1$), Golden Retriever ($n=8$), Jack Russell Terrier ($n=1$), Labrador Retriever ($n=4$), Lhasa Apso ($n=6$), Pomeranian ($n=1$), Poodle ($n=4$), Shih Tzu ($n=3$), West Highland White Terrier ($n=3$), Yorkshire Terrier ($n=1$). The age of each bitch and the total number of puppies born (litter size) were recorded. Samples of blood and colostrum were collected once from each bitch after expulsion of the last puppy (between 8 and 24 h since the onset of parturition). Mammary glands were washed with antimicrobial soap containing chlorhexidine and dried prior to the collection. Each pair of mammary gland was collected separately after intramuscular administration of oxytocin (1–2 UI; Ocytovem®, CEVA, Libourne, France). About 1 ml colostrum samples were obtained by a gentle massage of the mammary gland and subsequently manual milking. Puppies had no access to their dams only during the duration of samples collection (15–20 min), and no anesthetics were administrated neither to bitches nor to puppies. Blood, collected from the jugular vein into a plain tube, was centrifuged (15 min, 1500 × *g*). Serum and colostrum samples were stored at −20 °C until analysis.

2.2. Immunoglobulin G assay

IgG concentration in serum and colostrum were evaluated by a commercial ELISA test following the manufacturer's instructions (Dog IgG-Quantitation Kit, Bethyl Lab, Montgomery, USA; Mila et al., 2014). Colostrum samples were first thawed at room temperature and centrifuged (30 min, 2000 × *g*, 4 °C). Fat free whey was diluted 1:100,000; 1:400,000 and 1:600,000. Serum was thawed and diluted 1:50,000 and 1:100,000. Repeatability of the colostrum assay within one plate (intra-assay coefficient of variation) was 4.7% and 2.8% for the serum assay. Repeatability of the colostrum assay between plates (inter-assay coefficient of variation) was 5.4%. All serum samples were analyzed within a single plate.

2.3. Refractometry

The BRIX and refractive indexes were measured in thawed colostrum at room temperature (21 °C), on non-diluted samples (EkoTonick, Roubaix, France; BRIX scale: from 0 to 40%) and samples diluted 1:2 in distilled water (Rogosampaic, Wissous, France, refractive scale: from 1.333 to 1.360). The refractive index, as defined by Morrill et al. (2012), is an index of refraction of a solution measured at the wavelength of the sodium D line (589.3 nm) at 20 °C. BRIX refractometer is a modified method of refractive index evaluation. As not only proteins, but all total solids may reflect light, BRIX scale was developed to measure sugar content in no or low protein food products (jus, honey, etc.). In this study, BRIX refractometer was used due to larger measurement range (if converted to refractive index), and thus probability of higher precision of the measurement. The units of BRIX refractometer (%) remain not converted to refractive index in order to differentiate the two different devices used. All samples were analyzed within one session.

2.4. Statistical analyses

Statistical analyses were performed using the SAS software (version 9.3; SAS Institute Inc., Cary, NC, USA). The age of bitch was encoded as young (<3 years), middle-aged (3–6 years) and old (>6 years). The breed size was encoded as small (bitches <25 kg of body weight) or large (≥25 kg of body weight). The litter size was encoded separately for each breed size (Bloge et al., 2011) as small (<4 puppies for small breed dogs; <5 puppies for large breed dogs), medium (4–5 puppies for small breeds; 5–6 puppies for large breeds) or large (>5 puppies for small breeds, >6 puppies for large breeds). The normality on colostrum IgG concentration per teat and mean colostrum IgG concentration per bitch (mean of IgG concentrations from all pairs of mammary glands within one bitch) were tested with Shapiro–Wilk test. The percentage of coefficient of variation (CV) was calculated to express the variation of the IgG concentrations among different mammary glands within one bitch. Either the average IgG concentration per pair of mammary glands or IgG concentration per bitch were used in multivariable statistical analyses and variance analyses. The effect of teat pair number, encoded respectively as M1, M2, M3, M4, M5 (with the most anterior pair as M1) on the colostrum IgG concentrations was evaluated using a linear mixed model (PROC MIXED), with a fixed effect of breed size and individual number of the bitch as a random term. Mammary glands were then classified according to the anatomical localization as anterior (three cranial pairs: M1, M2, M3) or posterior (two caudal pairs: M4, M5). The relationship between teat position (anterior or posterior) and the colostrum IgG concentration was evaluated using a linear mixed model (PROC MIXED), with a fixed effect of the breed size and individual number of the bitch as a random term. The relationship between dam serum IgG concentration, age of the bitch, breed size, litter size and mean colostrum IgG concentration were evaluated using generalized linear model (PROC GLM). Since residuals of all multivariable models were not normally distributed, non-parametric analyses were performed (rank transformation of the outcomes). The correlations between IgG concentration, BRIX index and refractive index in colostrum were evaluated by Spearman's rho correlation coefficient. The results are presented as means ± SD.

3. Results

3.1. Population

The average age of the 44 bitches included in the study was 5.1 ± 1.6 years, ranging between 2 and 8 years (4.5% young; 68.2% middle-aged; 20.5% old; 6.8% unknown); with 70.5% (31/44) of them belonging to small breed dogs. The average litter size was 5.0 ± 2.4 puppies (from 1 to 10). Twenty-five percents (11/44) of

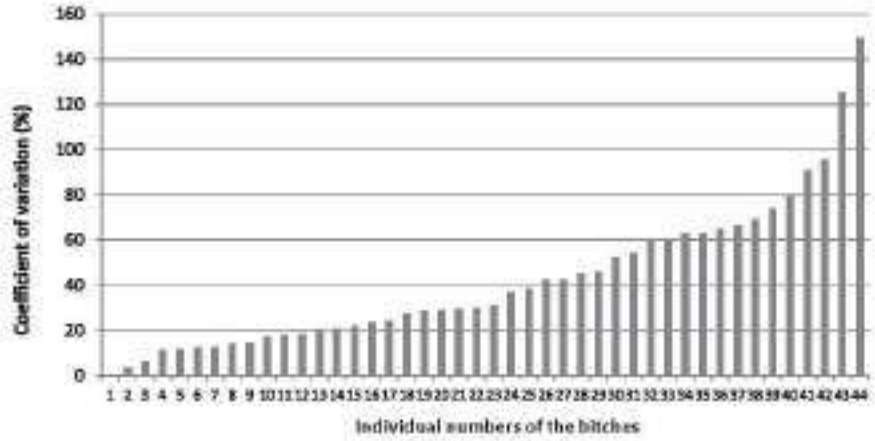

Fig. 1. Intra-individual coefficients of variation of colostrum IgG concentrations from different pairs of mammary glands (n = 44). Each bitch was identified by one unique number and each bar displays the coefficient of variation for colostrum samples from one given bitch.

dams delivered small sized litters, 34% (15/44) medium sized litters and 41% (18/44) large sized litters.

3.2. Variation in IgG concentration

In 180 pairs of mammary glands (from 3 to 5 samples per bitch) colostrum IgG concentration ranged between 0.8 and 61.4 g/L. The mean coefficient of variation between IgG concentrations from different mammary glands in a given dam was 42.0 ± 32.1% (Fig. 1).

The mean IgG concentration considered per pair of mammary gland ranged from 17.9 g/L in M1 to 21.7 g/L in M2 (Fig. 2). The IgG concentration in colostrum was not significantly different between M1, M2, M3, M4 and M5 whatever the breed size of the bitch ($p = 0.752$) but it was influenced by the dam as a random term ($p = 0.001$).

The mean IgG concentration in colostrum from anterior mammary glands (n = 99) was not significantly different from that from posterior mammary glands (n = 81), whatever the breed size of the dog (anterior 20.7 ± 12.3 g/L vs. posterior 20.3 ± 10.1 g/L; $p = 0.390$).

The mean IgG concentration in colostrum (value per bitch) was 20.8 ± 8.1 g/L, ranging between 8.0 and 41.7 g/L (Fig. 3). The IgG concentration in serum was 8.1 ± 4.3 g/L, ranging between 4.3 and 30.9 g/L (Fig. 3). Colostrum appeared 2.8-fold more concentrated in IgG than serum, and this ratio ranged among bitches from 0.89 to 6.3-fold. The mean IgG concentration in colostrum was not significantly associated with breed size ($p = 0.858$), litter size ($p = 0.777$), age of the bitch ($p = 0.797$) or serum IgG concentration ($p = 0.937$).

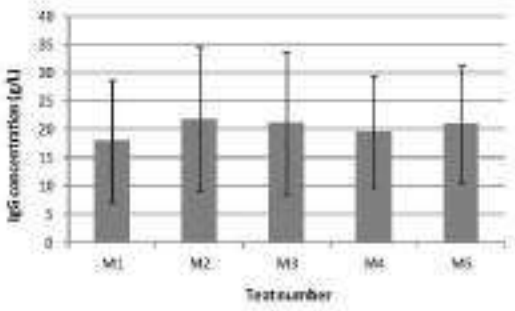

Fig. 2. Variability of colostrum IgG concentration depending on test number (180 samples. M1 n = 20; M2 n = 41; M3 n = 38; M4 n = 41; M5 n = 40). Results are presented as mean ± SD.

3.3. Refractometry

The average BRIX index value was 18.2 ± 3.9% (145 colostrum samples) and refractive index value was 1.343 ± 0.003 (131 samples), with a correlation coefficient between the two indexes amounting to 0.77 ($p < 0.001$). IgG concentration was significantly, but moderately correlated with BRIX index values ($r = 0.53$; $p < 0.001$) and refractive index values ($r = 0.42$, $p < 0.001$) (Fig. 4).

4. Discussion

Colostrum plays a crucial role in the survival of canine neonates. It is indeed the only source of immunoglobulins for hypogammaglobulinemic newborn puppies (Chastant-Maillard et al., 2012). Immunoglobulin G, in this mammary secretion in the first two days *post partum*, originates almost entirely from the female bloodstream (Bourne and Curtis, 1973; Godden, 2008). During gestation, specific receptors (FcRn) develop on the alveolar epithelial cells and trap circulating IgG. These immunoglobulins are then transported into mammary secretion, making colostrum markedly more concentrated than serum. In this study, the IgG concentration was on average 2.8-fold higher in colostrum than in the serum. This ratio differs greatly from that recorded in other species (cat: 4.1-fold (Claus et al., 2006); pig: 5.4-fold (Foisnet et al., 2010); cow: 28.7-fold (Morin et al., 1997)). Similarly like in sows (Foisnet et al., 2010), but not in queens (Claus et al., 2006), no link between colostrum and serum IgG concentrations was found in the bitch in our study.

The concentration of maternally derived IgG absorbed after birth varies considerably among puppies and among litters, with 18% of neonates suffering from poor passive immune transfer (Mila et al., 2014). One of the reasons for this deficit in IgG might be the ingestion of low immune quality colostrum. In this study, the average IgG concentration in colostrum, partially reflecting its immune quality, was 20.8 g/L, with a huge variation among bitches (range from 8.0 until 41.7 g/L). Large difference in colostrum IgG concentration, recorded also in sows and cows (Inoue et al., 1980; Quesnel, 2011; Quigley et al., 1995), may put some litters or some puppies within a litter at a risk of death. None of the bitch characteristics, such as breed, age of the dam or litter size could account for this variability in our study population, although a breed effect was described in other species. Large White sows present higher colostrum IgG levels than Landrace × Large White crossbred sows (Quesnel, 2011) and beef cows present higher IgG colostrum levels than dairy cows (Guy et al., 1994).

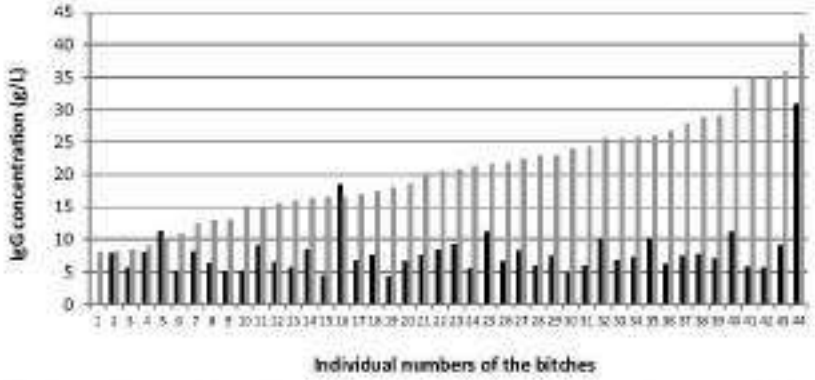

Fig. 3. Mean IgG concentrations in colostrum (gray bars, n = 44 bitches) and in serum (black bars, n = 43 bitches).

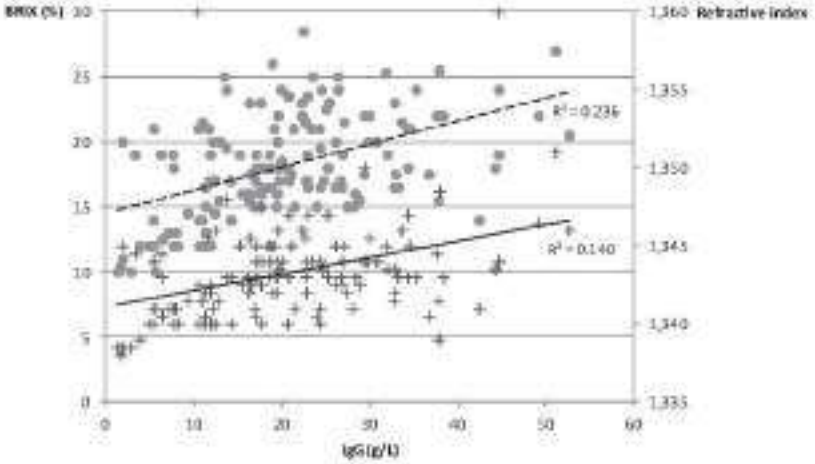

Fig. 4. Correlation between IgG concentration and BRIX index (dots and interrupted line, n = 146; r = 0.53; p < 0.001) and IgG concentration and refractive index (crosses and continuous line, n = 131; r = 0.42; p < 0.001) in colostrum samples.

We demonstrated not only an important variability in colostrum IgG concentration between bitches, but also between different mammary glands within one bitch (mean CV of 42%). A few phenomena could explain such differences. As described above, IgG is not produced in the mammary gland tissue, but only stored until the end of gestation. At the beginning of lactation, milk yield is low and it increases greatly few hours after parturition. Hence, colostrum initially rich in immunoglobulins is gradually diluted in increasing mammary secretion (Levieux and Ollier, 1998; Morin et al., 2010). Drop of IgG concentration occurs shortly after the lactation onset and a decrease of 72% in goats and 32% in pigs is observed as early as 6 h later (Klobasa et al., 1987; Moreno-Indias et al., 2012). At the same time, duration of parturition may vary greatly between bitches depending on litter size or individual characteristics of the bitch. Therefore, puppies born from bitches with long delivery may experience colostrum of reduced immunoglobulin concentration. In polytocous dogs, a birth order is formulated on course of parturition and puppies born as first most probably start to suckle also as first. Thus some teats may be already drained (and of reduced IgG content), while others remain not sucked when new puppies arrive. Birth order and parturition length, associated with the quality of ingested colostrum, could be the reason for great intra and inter-individual variability in IgG concentration determined in our study.

Nevertheless, no particular pair of mammary glands appeared to be better in terms of higher IgG concentration. Posterior pair of mammary glands did not secrete colostrum of better immune quality than anterior ones, or vice versa. The concentration of IgG in colostrum related to mammary gland position has been studied intensively in sows, with controversial findings. From one study to another, either anterior, posterior or even middle teats have been found producing secretion of higher immunoglobulin content (Foisnet et al., 2010). Slightly higher IgG concentrations were found in colostrum from posterior quarters in dairy cows (4.7% higher than from anterior quarters) (Guatteo et al., 2013). In the absence of studies describing the early suckling behavior of puppies and measuring the immune quality of colostrum, the impact of such wide variations on passive immune transfer to the newborns remain to be established in dogs. In piglets and kittens, a teat order is early formulated, determining rather a constant position of each neonate at an occupied teat (Hudson et al., 2009; Skok and Škorjanc, 2013). To date, it is unknown whether each puppy suckles constantly a particular mammary gland or several of them between birth and closure of the intestinal barrier.

As the level of immunoglobulins in colostrum is highly variable, both among dams and among mammary glands, an easy-to-use test would be desirable for dog breeders to evaluate the immune quality of colostrum before the intestinal barrier closure. Refractometry

is routinely used in bovine and equine neonatology to evaluate the immune quality of colostrum before pooling, freezing (banking) and administration to the newborns (Bielmann et al., 2010; Cash, 1999). The present work evidenced a statistical correlation between colostrum IgG concentration and two refractometry indexes also in the canine species; although, the correlation strength was moderate. Our results remain to be confirmed on fresh colostrum samples, as in cows, freezing–thawing cycles were demonstrated to influence the relationship between IgG concentration and refractive index (Morrill et al., 2012). Rather than evaluating IgG concentration *per se*, refractometry would be expected to distinguish high from low quality colostrum. A conclusion regarding the benefit of this technique would thus require defining the minimal IgG colostrum concentration ensuring protective passive immune transfer. To date, in contrast with bovine and equine species (Bielmann et al., 2010; Pritchett et al., 1994), the cut-off value defining low and high immune quality colostrum remains to be determined in dogs.

5. Conclusions

Large variability in immunoglobulin content of colostrum among bitches and among teats of one bitch evidenced in this study could be a reason for inadequate passive immune transfer in some puppies and thus a higher risk of neonatal mortality. Defining the threshold for good colostrum immune quality would be a prerequisite for efficient colostrum banking, as performed in farm animals. Development of colostrum replacer or immunoglobulin supplement, designed for puppies, could be used to compensate for insufficient immune quality of colostrum and thus to decrease the risk for passive immune deficit in canine neonates.

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General discussion and perspectives

Facing the lack of the information in scientific literature on neonatal period in puppies (Article 1), this dissertation aimed to better identify the risk factors of neonatal mortality in dogs, and particularly the role of the colostrum intake. Risk factors of several kinds were studied (Fig.4):

- Colostrum intake was evaluated via early growth rate (reflecting the global benefits of colostrum), via glucose and β-hydroxybutyrate concentration and rectal temperature at 24h (reflecting colostrum as a source of energy) and via blood IgG concentration and CPV2 specific antibody titer (reflecting the immune role of colostrum);
- Consequences of intrauterine growth and delivery were evaluated via birth weight, blood lactate concentration (reflecting the degree of hypoxia) and Apgar score (reflecting general vitality of the newborn);
- Importance of the maternal factors was taken into account by analyzing breed size (with all its particularities), age of the bitch (reflecting partially maternal behavoir, development of the mammary glands, etc.), and other factors considered as a random term for all puppies coming from one litter;
- Colostrum quality, as another maternal factor, was evaluated via its IgG concentration (considered as a marker of its immune quality).

All samples were collected within one commercial breeding kennel during 8 months in total. Experimental manipulations of animals (puppies and bitches) were conducted in the respect of animal welfare. Fourteen veterinary students participated in the sampling part of this work. All laboratory work was performed by one person – the author of this dissertation. All animals belonging to a single kennel, were submitted to similar management and housing conditions: food, temperature in the whelping box, sanitary protocols, vaccination and antiparasitic prophylaxis. This allowed to analyze the influence of selected risk factors on mortality, with limited factors of confounding. For example, similar environmental pressure (microbes present in the kennel), and thus common causes of infections, allowed the definition of IgG concentrations characterizing deficit in passive immune transfer. Maternal nutrition during gestation and lactation is known to affect fetal growth and maturation, together with colostrum and milk composition. Thus similar nutrition system, and once again environmental pressure equal for all animals included, permitted the evaluation of colostrum immune quality and its factors of variation. Finally, working in a kennel with natural CPV2 circulation allowed the evaluation of specific passive immune transfer and its role on

morbidity in weaning puppies. However, the generalization of here-obtained results should be done carefully. All presented data remain to be confirmed in other kennels, facing other causes of mortality and managed under different environmental conditions. Minimal IgG concentration necessary for puppy's survival may differ depending on microbes present in the environment and the cut-off value for blood glucose concentration may be different in kennels with different sources of heat, and thus different ambient temperatures provided in whelping boxes.

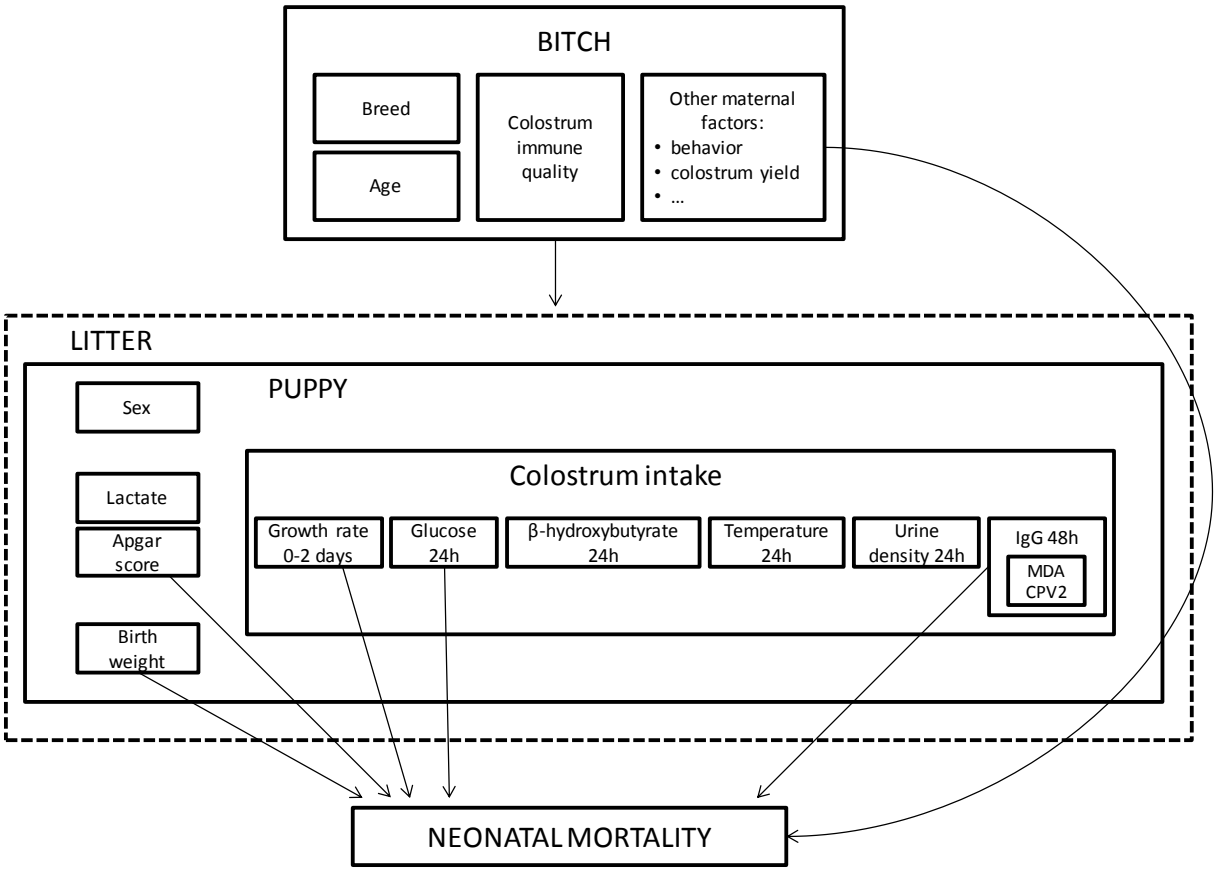

Fig.4. Risk factors for neonatal mortality in dogs analyzed in this dissertation. Arrows indicate confirmed significant associations.

Experimental protocols presented in this work were conducted on relatively large numbers of individuals (between 79 and 514 puppies depending on protocols). Such a large population could be obtained thanks to the large size of the kennel in which we performed the sampling part of the protocols. Only purebred puppies of different breeds were included in the studies. One of the particularities of the canine species is a great anatomical variation among breeds. Especially, traits may vary strongly, from about 2 kg of adult body weight and 20 cm height in Chihuahua to 60 kg and 70 cm in Newfoundland. Such important differences in size

impact some physiological features, as digestive physiology (60,61), body temperature (62,63) and growth (62,64). We thus classified adult females and puppies into small (adult weight <15kg), medium (15-25kg) and large breeds (>25kg). Also, not a weight gain (in g as absolute values), but growth rate (% of birth weight) was used in our analyses in order to allow comparisons between breeds.

The present study contributed to the knowledge about the risk factors of mortality in breeding kennels. Some practical applications for dog breeders can thus be drawn from our findings:

- Assistance on parturition should be performed and puppies should be examined at birth via Apgar scoring in order to identify those of them with low vitality, being at risk of rapid death.
- Puppies should be weighed at birth and low-birth-weight puppies should rest under special care. Especially, adequate nursing should be ensured, as regarding our results, low-birth-weight puppies are at higher risk of hypoglycemia and hypothermia.
- All newborns should be weighed daily and the growth rate over the first two days of life should be calculated, as growth rate value provides breeders with much of important information. It indicates not only a correct food intake, but also an adequate transfer of immunoglobulins, as both were strongly associated with growth in the present work. The threshold for correct growth rate during the first two days was defined at -4%, thus breeders can consider a null growth rate as the alter limit.
- Blood glucose could be assayed at 24h also to ensure colostrum intake, as well as to indicate puppies requiring additional nursing. Such assay is rapid and easy to perform: blood droplet can be obtained via auricular puncture and the device (human glucometer) is available in veterinary warehouses. Minimal threshold can be considered at 90 mg/dl.

This work revealed a strong association between early growth rate (within the first two days of life) and risk of neonatal death, with an 8-fold higher mortality rate within the first 21 days in puppies losing weight (Article 2). Weighing was chosen as an indirect criterion for the colostrum intake, but also for the global health of the newborn. Moreover, weight change was strongly correlated with both global and specific immune transfer (IgG and CPV2 specific

MDA) measured in blood. Thus early growth allowed to estimate the overall role of the colostrum intake for the newborn dog. The determination of the exact amount of ingested colostrum could have been performed via weight-suckle-weight method. However, this technique, although precise, requires separation of newborn dogs from mothers in order to ensure defecation and urination before weighing the puppy. As our protocols were conducted in commercial kennel and not the experimental one, the separation of puppies was not conceivable.

The parameters evaluated in this dissertation allowed to approach intraunterine growth, course of parturition and finally the neonatal period with a specific focus on colostrum. Birth weight, as an outcome of the fetal growth, was related to mortality during the first two days, with low-birth-weight puppies defined as at-risk population (Article 2). The impact of the course of parturition and of the intrauterine growth on puppies' neonatal survival was studied respectively through Apgar score and blood lactate concentration (Article 3). Low vitality at birth (Apgar score), but not hypoxia (blood lactate concentration) was determined as a risk factor for canine newborns within the first 24h of life only. No more association between Apgar score, neither lactate level was found in older puppies. Hence, our findings suggest that except the first 24-48h, survival in dogs during the neonatal period is determined by colostrum intake rather than intrauterine life or course of parturition.

Our work clearly demonstrated that both the immune and the nutritional role of the colostrum strongly related with puppy's chances to survive. Puppies with low serum IgG concentration and with low glucose level were at higher risk of death. Both global immunity (blood IgG concentration) and the specific one (CPV2 specific MDA) were greatly variable among puppies, with some of them in deficit of passive immune transfer. The threshold value for the adequate immunoglobulin acquisition was determined in this dissertation, with the minimal blood IgG concentration at two days of age estimated at 2.3 g/L (Article 4). However, as mentioned above, our study was conducted within one breeding kennel only, with all included puppies being exposed to the same infectious pressure. Such conditions allowed to evidence the potential role of immunoglobulins in puppies being at the similar risk of morbidity and mortality. On the other hand, the defined IgG threshold needs to be validated in other kennels, with different epidemiologic situation and management.

In calves and foals, immune quality of colostrum was associated with risk of passive immune failure (31,32). In puppies, as demonstrated by Bouchard et al. (29) and Chastant-

Maillard et al. (65), only a very limited amount of immunoglobulins are obtained during the fetal life, and colostrum is also the main source of passive immunity. A great variability in colostral immunoglobulin content among different mammary glands of one given bitch, demonstrated in the Article 6, could explain the absence of direct relationship with serum IgG level achieved by puppies, and subsequently with mortality (Article 4). In our statistical model a mean value of colostral IgG per bitch was used, masking the great difference between mammary glands (mean CV of 42%). Thus puppies suckling from teats with higher IgG concentration might absorb a greater quantity of maternal immunoglobulins, and conversely. What is more, a rapid drop of colostral IgG concentration after the onset of partition was described in other species (66,67). Depending on litter size and duration of expulsion phase, puppies are born with a different delay since the beginning of parturition. Therefore, the quality of colostrum within one teat may vary depending on suckling time for puppies with different birth order.

In addition to the immune provision, colostrum represents a major source of energy. As demonstrated in foals, piglets and lambs, feeding frequency is higher at the early stage of lactation (Article 1). Therefore, it is not surprising that glucose concentration in suckling animals (also in dog) is higher than in adults (68–70). In piglets, lambs and infants, colostral fat and carbohydrates were demonstrated to be the unique source of energy metabolized by newborns (36). On the contrary, in case of insufficient energy intake, glycogenolysis and protein catabolism occur in order to maintain homeostasis (71,72). However, glycogen reserves in the newborn dog are very limited. Our results demonstrated that puppies with low glucose concentration at 24h (estimated at 92 mg/dl, Article 3) are at higher risk of neonatal mortality. It is noteworthy that this threshold is twice higher than that usually defining hypoglycemia in puppies (<40 mg/dl (73)). Low glucose level at 24h of life in puppies dying before 21 days of age indicates thus inadequate colostrum intake, and especially insufficient energy intake directly link with glucose maintenance. Increased concentration of blood ketone bodies released from fat tissue accompanied hypoglycemia in newborn infants (53). Nevertheless, β-hydroxybutyrate was associated neither with glycemia nor with neonatal losses in our study (Article 3). As, the fat body content is very low in the newborn dog (1.3% (43)), alternative fuels originating from fat metabolism are almost nonexistent. Thus other metabolites need to be evaluated in puppies in order to determine the main energy sources in colostrum fed and starving dog, as well as eventual shift in nutrients use, as demonstrated in piglets (71). Nonetheless, our work does not allow to distinguish which of the two roles of

colostrum is of higher importance for canine newborn survival: as a source of immunity or energy.

Apart from immune and energetic quality of the colostrum, age of the bitch and her breed, many other maternal factors (colostral or not) may influence puppy's health. Maternal behavior, colostrum and milk yield and maternal health status are only some examples of factors common for all puppies coming from one given litter. The common effect of the dam for all littermates was thus included in all statistical analyses (random effect models). The results showed the significant effect of the random term almost in all models analyzed in this dissertation. The strong relationship between the dam and her litter needs thus further investigation in order to define essential elements for the newborn.

New original data concerning the neonatal period in dogs were provided in this dissertation. However, many questions remain to be addressed, in order to understand the epidemiology of canine neonatal losses, as well as to provide some practical solutions to dog breeders (Fig.5).

Techniques of newborn assessment

Our study demonstrated an impact of IgG concentration absorbed at birth on chances to survive for the newborn dog. The information on the immune status of the newborn puppy could indicate to the breeder an animal at higher risk of death, and thus special care to such a puppy could be provided to increase its chances to survive. To date, the assay of canine IgG concentration is time-consuming, expensive and possible only in laboratory conditions. In farm animals, especially the total protein level, evaluated by refractometry on serum samples, is used as a marker of passive immune transfer (74). Thus development of an easy-to-use test for passive immune transfer, such as refractometry, would be desirable in dogs for an on-kennel application. Since, in newborn dogs, the glomerular filtration rate is low and both immunoglobulins and glucose are easily detected in urines (75,42), one can imagine the development of non-invasive methods of IgG and glucose assessment via urine evaluation. Such methods could facilitate monitoring of the newborn dog in the kennel conditions and in the respect of animal welfare.

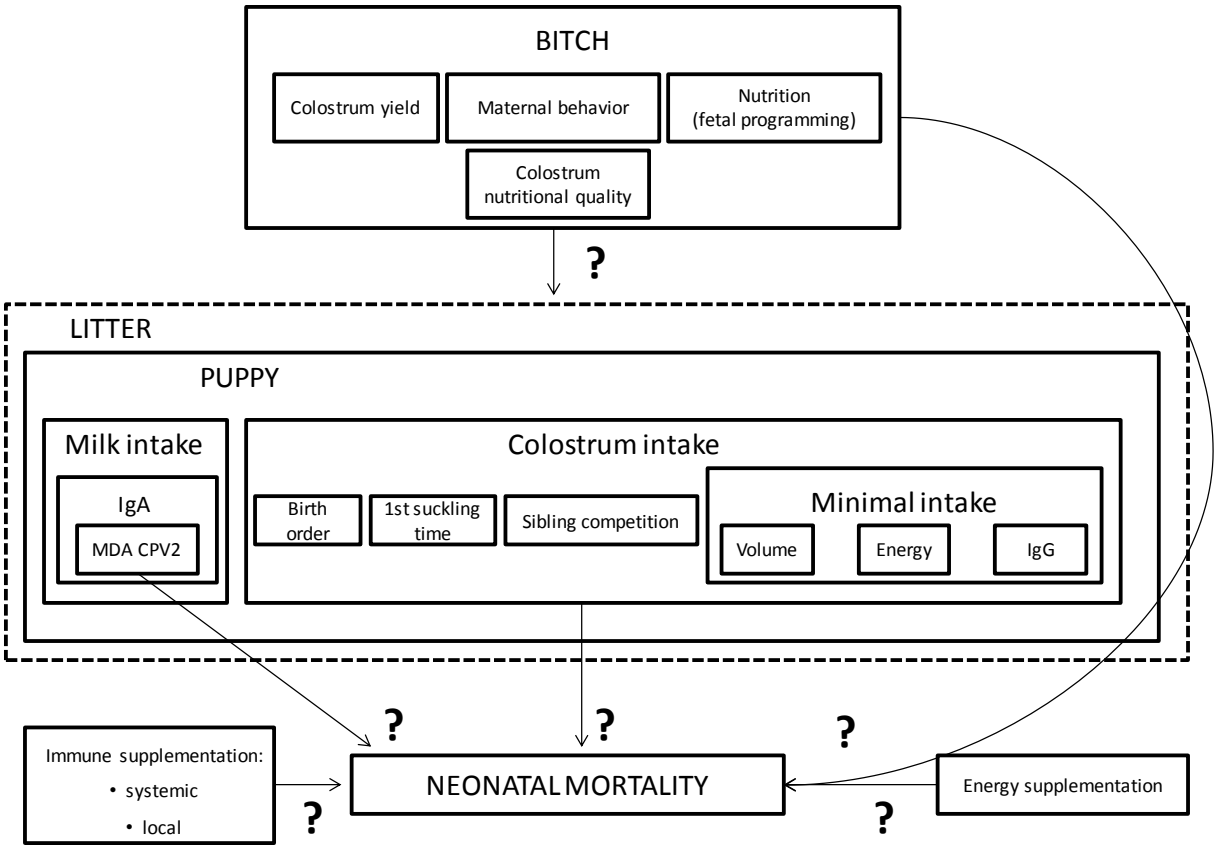

Fig.5. Factors of risk for neonatal mortality in dogs remaining to be analyzed. Arrows indicate associations to be tested.

Colostrum assessment

The great variation among bitches, but also among pairs of mammary glands, may put some puppies at risk of maternal immunoglobulin deficit, and thus of neonatal death. As not only passive immunity, but also energy intake was demonstrated as essential for puppies' health, evaluation of nutritional composition of canine colostrum and its variability remain to be investigated. If no great variation in nutritional quality exists, selection of the colostrum of the highest IgG concentration could be performed to maximize the chances of newborns. However, if the content of colostral nutrients and energy varies a lot, the correlation between energetic value and colostral IgG concentration would need to be explored. If negative, the predominant factor between immunity and energy for puppy survival would need to be established.

Colostrum intake

We demonstrated the global, immune and/or nutritional importance of colostrum intake on survival in puppies. Energetic requirements for newborn puppies are estimated at 250 kcal/kg body weight per day (76) and minimal IgG at two days of age at 2.3 g/L (Article 4). However, it remains unknown which quantity of colostrum needs to be ingested, especially in order to achieve the IgG and glucose thresholds determined in this work. Thus the minimal volumes of colostrum providing adequate energy and IgG for higher survival remain to be established. Moreover, facing a great variability in colostral IgG concentration, development of a score for colostrum immune quality is needed. It would allow to adapt the minimal volume administrated to puppy depending on colostrum IgG content.

According to the author's data, variability in IgG concentration between puppies within a litter is high (Fig.6) In piglets, such a variability was also evidenced and explained, at least partially, by birth order and time of the first suckling (71,77). As the concentration of IgG in colostrum drops rapidly after the onset of parturition, birth order could be strongly associated with the immune quality of colostrum ingested by puppies, with most probably puppies being born first ingesting colostrum of the highest immunoglobulin concentration. Both phenomenons, a drop of colostral IgG and effect of the birth order, although well described in pigs, remain hypothetical for puppies. As recently described, the absorption of IgG is strongly limited already after 8-12h of life and null at 24h (30). However, the average time of the first colostrum ingestion, as another factor influencing the level of absorbed IgG, is also unknown for canine newborns. The variability of immune quality of colostrum from different teats, as demonstrated in our study (Article 6), may also contribute to such important differences in level of passive immune transfer among littermates.

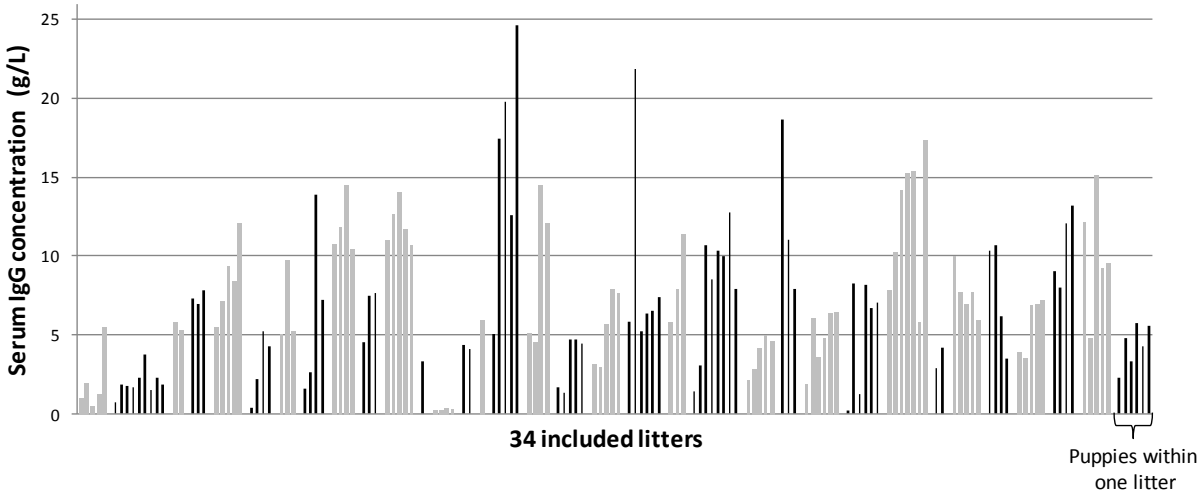

Fig.6. Variability in serum IgG concentration at 2 days of age among puppies and among litters (n=149; unpublished data).

Local immunity provided by milk

Transfer of specific antibodies, such as antibodies against CPV2, were demonstrated to play an important role on the immune protection, with longer protective period in puppies of higher antibody titer obtained at birth. On the other hand, majority of puppies with low level of MDA in our study underwent CPV2 infection only at 42 days of age (Article 5). If the CPV2 circulation is permanent in the studied kennel, why does the infection occur only at the weaning age in moderately protected puppies? In breastfed babies, an important role of locally acting milk immunoglobulins was demonstrated (78). Babies receiving breast milk until 6 months of age had lower incidence of gastrointestinal infection of various origins than those fed milk formula (79). Most probably, also in puppies, mature milk of high IgA content, provides a strong local protection against gastrointestinal pathogens, such as parvovirus, as higher fecal IgA concentration were demonstrated in suckling *vs.* weaned puppies (80). Thus the role of colostral/milk immunoglobulins in puppies with completed intestinal barrier closure requires further studies.

Maternal and suckling behavior

As in many domestic animals, the role of the dam on chances to survive seems to be crucial for canine newborns (significant "litter effect" in every statistical model; Article 2-4). In altricial species, such as dogs, newborns are unable not only to see, hear or stand-up (until 10-14 days), but also to urinate and defecate on their own (until 7-10 days) (81,82). Thus maternal behavior such as lying during nursing, assistance on nursing, licking the newborn in

order to stimulate micturition and defecation, are indispensable for correct development and growth of puppy. The maternal care can be complicated in case of numerous litters. As the number of mammary glands is limited, one could expect fights between canine littermates, as described in kittens and piglets (83,84). To date, only few studies describe maternal and suckling behavior in dogs (85,86). However, these data were obtained on a very limited population (24 and 47 included puppies from 5 and 10 bitches, respectively), and on puppies of few days of age, and not at birth. Such behavioral studies are challenging, as a permanent observation within 24h period is necessary in order to establish daily duration of the direct contact between mother and offspring, number of nursing session or frequency of use of particular teats. Moreover, canine newborns are very small, regarding the size of the mother, and thus often hide under limbs during suckling, making difficult the observation. To date, suckling behavior at the very early stage of life, as well as the maternal behavior remains unknown in dogs.

Immune and/or nutritional supplementation

High variability in colostrum immune quality, as well as in the level of immunoglobulins acquired from colostrum by newborn dogs was shown in the present dissertation, with almost 20% of puppies suffering from the deficit of passive immune transfer (Article 4). A strategy to limit the incidence of passive immune deficit and to optimize the level of passive immunity could be the administration of a hyper-immune solution providing puppies with higher systemic immunoglobulins. Indeed, such immune therapy, with antibodies specific for the species is utilized in babies, but also foals, calves and small ruminants, and commercial products are easily accessible on the veterinary market (87–89). None of those exists for canine newborns, although infectious diseases are admitted as a frequent cause of neonatal death. In dogs, the immunoglobulin absorption rate decreases rapidly after birth, thus immunoglobulin supplement should be used as prevention, rather than a treatment. Moreover, development of an immune solution easy to use for dog breeders, such as orally administrated solutions, would be of interest in order to supplement puppies as soon as possible after birth.

One of here-presented studies tested the effect of adult serum administrated to canine newborns orally at birth on the level of total serum IgG concentration at 2 days age and on neonatal mortality. Unfortunately, no difference between supplemented and control puppies was demonstrated. On the other hand, mortality in puppies was found associated not only with

the level of absorbed IgG, but also blood glucose. As adult serum is a poor source of energy, a supplementation of both immunoglobulins and energy could be tested.

If further studies confirm the protective role of milk immunoglobulins, locally acting hyper-immune solution could be developed. Such a dietary supplement could be provided either to at-risk puppies (i.e. with the deficit of passive immune transfer) or as a systematic prevention.

Conclusions

The presented work evidenced the impact of the fetal growth, parturition and colostrum on neonatal survival in the dog. The role of the dam at all mentioned steps of puppies' life is unquestionable. Thus future work on improvement of canine neonatal survival should include not only puppies, but also the bitch. In pigs, less piglets of low birth weight were born from sows supplemented with dextrose during the week before insemination (90) and higher vitality in porcine newborns was observed after fish oil administration to sows at the end of gestation (91,92). Also colostrum quality can be altered through female nutrition, as a drastic reduction in feed allowance in sows at the end of gestation increased the fat content in the colostrum from 6 to 7.3% (93). Not only nutritional content, but also immune content of colostrum can be altered by maternal nutrition, as dietary conjugated linoleic acid increased both fatty acids and IgG concentration of sow colostrum (94). Such a fetal programming via female nutrition is a promising research field also in the canine species.

Annex

Other publications and communications, not directly linked to the subject of this dissertation, were written during the period of PhD thesis.

1. Other peer reviewed publications in international journals

A. Grellet, **H. Mila**, R.M. Heilmann, A. Feugier, N. Gruetzner, J.S. Suchodolski, J.M. Steiner, S. Chastant-Maillard. Effect of age, gestation and lactation on faecal IgA and calprotectin concentrations in dogs. *Journal of Nutritional Science* 2014; 3(e41): 1-5.

W. Niżański, A. Partyka, M. Gotowiecka, A. Antończyk, **H. Mila**, R. Strzeżek, M. Koziorowska-Gilun. The influence of seminal plasma and two antioxidants: Catalase and N-acetyl-l-cysteine on the extent of apoptotic-like changes in canine spermatozoa. *Reproductive Biology* 2013; 13:27.

A. Antończyk, W. Niżański, A. Partyka, M. Ochota, **H. Mila.** The usefulness of Real Time Morphology software in semen assessment of teratozoospermic boars. *Systems Biology in Reproductive Medicine* 2012; 58(6): 362-368.

2. Articles in peer reviewed veterinary journals

N. Mikołajewska, **H. Mila**, E Stańczyk. Zaburzenia okresu poporodowego u suk: diagnostyka różnicowa i metody leczenia [Pathology of the perinatal period in bitches: differential diagnostics and methods of treatment]. *Weterynaria w Praktyce*, 2015, in press.

S. Chastant-Maillard, **H. Mila**, C. Boucher, P. Bergamo, A. Grellet. Situation des élevages canins et félins en 2014 [Situation of breeding kennels and cattery in 2014]. *Point Vétérinaire*, 2014, 351: 16-19.

H. Mila, S. Chastant-Maillard, C. Boucher, P. Bergamo, A. Grellet. Comment réaliser une visite d'élevage canin ou félin ? [How to conduct a visit at the breeding kennel or cattery?] *Point Vétérinaire*, 2014, 351: 20-23.

C. Boucher, P. Bergamo, **H. Mila**, S. Chastant-Maillard, A. Grellet. Evaluation des animaux, de leur alimentation et de leur bien-être [Evaluation of animals, their alimentation and welfare]. *Point Vétérinaire*, 2014, 351: 24-29.

P. Bergamo, S. Chastant-Maillard S, **H. Mila**, C. Boucher, A. Grellet. Evaluation des méthodes d'élevage, des locaux et du personnel [Evaluation of management, housing and staff at the breeding kennel]. *Point Vétérinaire*, 2014, 351: 30-36.

H. Mila, S. Chastant-Maillard, C. Boucher, P. Bergamo, A. Grellet. Visite d'un élevage canin pas à pas [Visit of the breeding kennel step by step]. *Point Vétérinaire*, 2014, 351: 37-39.

C. Boucher, S. Chastant-Maillard, **H. Mila**, P. Bergamo, A. Grellet. Visite d'un élevage félin pas à pas [Visit of the cattery step by step]. *Point Vétérinaire*, 2014, 351: 40-42.

3. Keynote lecture

A. Grellet, **H. Mila**, E. Fontaine, S. Chastant. Use of deslorelin implants to schedule canine breeding activity: a study on 442 bitches. *7th International Symposium on Canine and Feline Reproduction (ISCFR)*. Whistler, Canada, 2012. p 77-78.

4. Other oral communications

A. Grellet, **H. Mila**, M.A. Debouchaud, A. Feugier, S. Chastant-Maillard. Risk factors of Giardia infection and pathogenicity in weaning puppies. *24th Congress of the European College of Veterinary Internal Medicine - companion animals (ECVIM-CA)*. Mainz, Germany, 3rd-6th September, 2014.

A. Grellet, R.M. Heilmann, **H. Mila**, A. Feugier, N. Gruetzner, J.S. Suchodolski, J.M. Steiner, S. Chastant-Maillard. Effect of age, gestation and lactation on fecal immunoglobulin A and calprotectin concentrations in dogs. *The WALTHAM® International Nutritional Sciences Symposium (WINSS)*. Portland, United States, 1st-4th October, 2013. p 36.

5. Other posters

J. Anne, **H. Mila**, M-O. Semin, C. Bleuart, S. Chastant-Maillard, I. Raymond-Letron. Histological features of puppies' eyes. *4th Symposium on Veterinary Sciences Toulouse-München-Zaragoza.* Toulouse, France, 11th-13th April, 2013. p 23.

6. Book translation

G. England, A. von Heimendahl. BSAVA Położnictwo i neonatologia psa i kota [BSAVA Manual of Canine and Feline Reproduction and Neonatology]. 2014. ISBN: 978-83-7609-938-5.

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