ROYAL CANIN®

SPECIAL EDITION

NEWS from RESEARCH by ROYAL CANIN®

NeoCare Center

Toulouse National Veterinary School (ENVT)

5 years of science to improve the health of newborn puppies

NeoCare center - Toulouse National Veterinary School (ENVT): 5 years of science to improve the health of newborn puppies

INCREDIBLE IN EVERY DETAIL

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Introduction

ROYAL CANIN® was founded in 1967 by a veterinarian: Dr Jean Cathary who, frustrated to see dogs he was treating for eczema coming back as soon as the course of the treatment was finished, concentrated his studies on Health Nutrition with the aim of providing more precise nutritional answers, adapted to the specific needs of cats and dogs.

Since then, ROYAL CANIN® has remained loyal to this philosophy: placing the animal needs at the very heart of all innovations. Knowledge is clearly the only possible route to understand the specific requirements of cats and dogs.

This is why science feeds our knowledge on a daily basis and why we continually expand our knowledge about cats and dogs.

Birth and growth stages, in particular, have always been a major focus for ROYAL CANIN®, as they are critical times, determining the health and well-being of cats and dogs throughout their lives.

Indeed, the neonatal period is a crucial stage for all newborns, with many adaptations from the intra to the extra uterine life. The newborn must not only start to oxygenate by itself, but also maintain blood glucose and stable body temperature. Finally, the immune system is mature but naive and of slow reactivity, making the neonate fully susceptible to infections. Managing the first days and weeks of life is vital for survival and later health of the animal. In addition to animal welfare, health issues that may occur to puppies or kittens are responsible for important economic losses for breeders, who are looking for healthy and balanced pets. The knowledge on neonatal period in dogs and cats is thus key. Neglected for too long in carnivores, this area has been explored only recently. With this magazine, ROYAL CANIN® aims to share with veterinarians, breeders and any other enthusiasts of dogs and cats, both scientific and practical knowledge in canine and feline neonatology that the NeoCare Center has acquired this past 5 years focusing on research on the health of newborn puppies, with the objective to contribute to improve health of the newborns.

Claire Mariani

DVM, phD

Claire graduated from the French Vet School of Lyon and did her phD about the microbiota of cheese in human food. After being in charge of food safety at the French Technical Institute of Milk and Milk products, she joined Royal Canin's Research & Development in 2009 where she is in charge of research in dentistry and microbiology.

Laure Boutigny

DVM

Graduating from the Alfort Veterinary School in 2005, Laure Boutigny specialized in canine and feline breeding by continuing her studies as trainee Vet in the Sport & Breeding Medecine unit (UMES). Laure joined the International Marketing at Royal Canin in 2008 as international product manager for dog & cat breeders and professionals. In 2014, Laure joined the Royal Canin Research & Development department.

I

Fighting against puppy neonatal mortality is crucial

Mortality rates during the first weeks of life in the canine species are amongst the highest in domestic animals. Data collected from around the world, with several thousand puppies included in each study, show that average mortality before weaning (8-9 weeks of age) reaches 20%, ranging between 5% and 35% (figure 1).

Key fact

In average **one out of five** puppies dies before reaching 2 months of age.

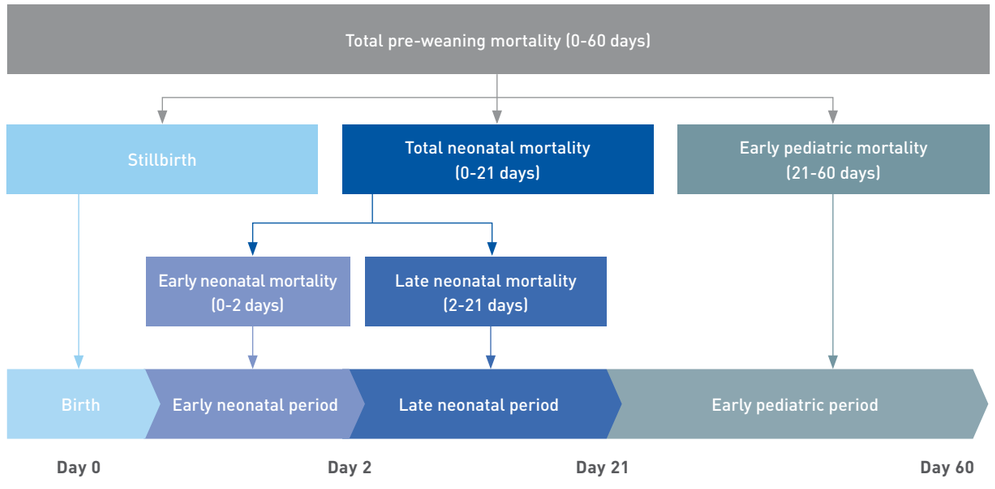
DEFINITIONS

Puppy neonatal mortality:

Neonatal mortality is defined as the death of a neonate born alive, between 0 and 21 days after birth. One can distinguish between "early neonatal mortality" (0-2 days) and "late neonatal mortality" (2-21 days).

Puppy early pediatric mortality:

Early pediatric mortality is defined as the death of puppy between 21 and 60 days of age.



$$\text{STILLBIRTH} = \frac{\text{Total of stillborn puppies}}{\text{Total number of puppies born (stillborn+ born alive)}}$$

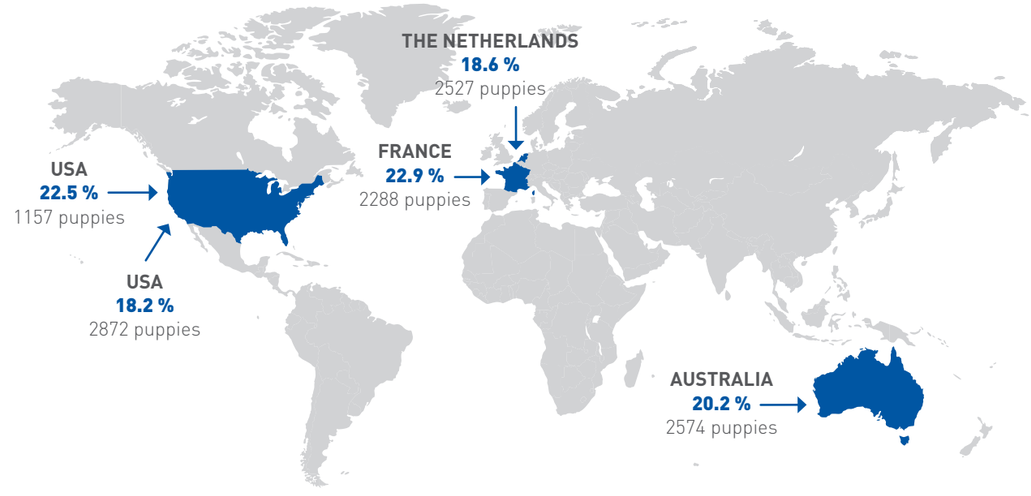
$$\text{EARLY NEONATAL MORTALITY} = \frac{\text{Total number of puppies dying between birth and 2 days of age}}{\text{Total number of puppies born alive}}$$

$$\text{LATE NEONATAL MORTALITY} = \frac{\text{Total number of puppies dying between 2 and 21 days of age}}{\text{Total number of puppies alive at 2 days of age}}$$

$$\text{EARLY PEDIATRIC MORTALITY} = \frac{\text{Total number of puppies dying between 21 and 60 days of life}}{\text{Total number of puppies alive at 21 days of age}}$$

$$\text{TOTAL PRE-WEANING MORTALITY} = \frac{\text{Total number of puppies dying between birth and 60 days (stillborn+ dying during the 60 first days)}}{\text{Total number of puppies born (stillborn and born alive)}}$$

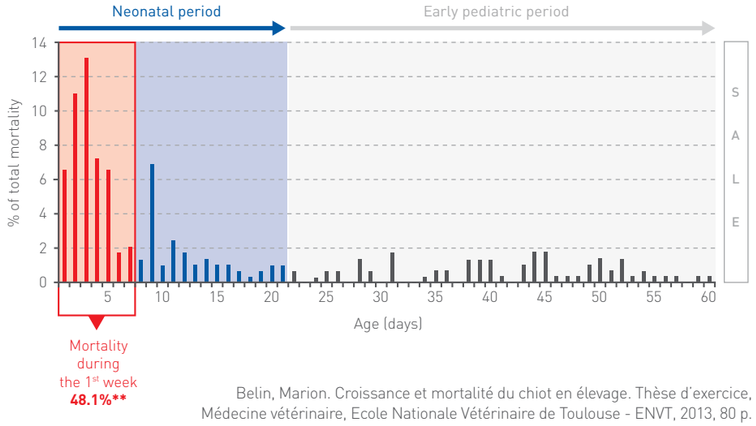
Figure 1. Prevalence of mortality in puppies between 0 and 2 months of age.



COUNTRY
Total mortality (%)
Number of puppies in the study

From Australia: Gill (2001); France: Mila et al (2015); The Netherlands: Nielen et al (1998); USA: Smith et al (1968) and Poktay et al (1977)

Figure 2. Proportion of live-born puppies dying at different ages *(n=294)**



Belin, Marion. Croissance et mortalité du chiot en élevage. Thèse d'exercice, Médecine vétérinaire, Ecole Nationale Vétérinaire de Toulouse - ENVT, 2013, 80 p.

Indeed, puppy mortality occurs very early, i.e. during the neonatal period (0-21 days of life) and most deaths even occur during the first week of life (figure 2).

Such a high rate of puppy mortality occurring very early on, and in most of the cases within hours after the appearance of symptoms (if any), stresses **the importance of early detection of puppies at higher risk of neonatal death**. Once identified, specific care and nursing of these animals can be implemented. This is why in 2010, ROYAL CANIN®, in partnership with NeoCare Center (Toulouse National Veterinary School), decided to explore canine neonatology. The specific aims of the project were:

- **To better understand the physiology of the neonatal period in dogs**, especially any factors affecting survival, such as passive immune transfer and energy intake
- **To develop non-invasive tools for puppy health monitoring** during the first days of life
- **To set up an innovative nutritional solution** to boost puppy immunity and energy metabolism, and to increase immune protection around weaning. ■

Key fact

Majority of neonatal deaths occur during the first days after birth.

*Study on 294 puppies followed in 1 kennel
**% of total mortality in puppies born alive

II

ROYAL CANIN® partners with the NeoCare Center in the Toulouse National Veterinary School

Toulouse National Veterinary School (France)

Founded in 1928, Toulouse National Veterinary School (ENVT) is located in the Southwest of France on a 53-hectare site. It is one of France's four veterinary schools. Toulouse has a very active university campus and the ENVT is part of the Institut National Polytechnique, which includes 7 schools in various fields of expertise (agri-food, chemistry, engineering, climatology, etc.) with a total of 100,000 students. ■

INP ECOLE NATIONALE VETERINAIRE

Key numbers

700 vet students

140 for each year of the curriculum

with **5** years spent at the vet school, preceded by **2** years of preparation for the entrance exams

280 persons working on the campus,

including **120** research engineers and technicians

and **76** teachers, involved in both pedagogical and scientific activities

24 PhD students

12 residents

12 000 canine consultations per year

8000 feline consultations per year

125 papers published each year in peer-reviewed international journals

26 American and European board-certified specialists

Staff at NeoCare Center

Five veterinarians are currently working in NeoCare Center.

Prof. Sylvie Chastant-Maillard

DVM, PhD, Dip ECAR, Hab

Sylvie Chastant-Maillard graduated from Alfort National Veterinary School (Paris, France) with a veterinary diploma in 1990 and taught reproduction there until 2010. After a PhD (on mammalian pre-implantation embryos) in 1995, she funded a scientific laboratory at Alfort dedicated to canine and feline reproductive biotechnology (especially in vitro maturation and fertilization). Sylvie Chastant-Maillard is a Diplomate of the European College of Animal Reproduction. She is currently a full Professor in Reproduction at Toulouse National Veterinary School, where she teaches small animal reproduction and develops research on canine neonatology and pediatrics.

Dr. Hanna Mila

DVM, PhD, ECAR resident

Hanna Mila graduated from Wrocław Veterinary Faculty (Poland, 2009), where she worked for two years at the Clinic for Small Animal Reproduction. In 2012, she joined NeoCare Center. In September 2015, she obtained her PhD degree with a research project on immunological and nutritional determinants of survival in puppies. Today, Hanna Mila is following a residency program at the European College of Animal Reproduction, with a sub-specialty in small animal reproduction at the ENVT. Her research focuses on canine neonatology and pediatrics, and in particular on the influence of growth during the fetal period and the first weeks of life on puppy health, as well as the influence of colostrum and milk quality and intake on health.

Dr. Patricia Ronsin

DVM

Patricia Ronsin obtained a veterinary diploma at the Toulouse National Veterinary School in 1984. Since 1990, she has worked in the Reproduction Unit and now at NeoCare Center. She is especially interested in semen evaluation for various species (dog and cat, but also ruminants). She is also passionate about feline reproduction, as she was also a breeder of Birmans.

Dr. Aurélie Fournier

DVM, PhD student

Aurélie Fournier graduated from Alfort National Veterinary School (Paris, France) in 2011, and specialized in small animal medicine and surgery during a one-year internship at Lyon Veterinary School (France). After 3 years in private practice (mainly the Veterinary Hospital of Reims, France), she joined NeoCare Center as a PhD student to investigate kitten infectiology and metabolism. Her research interests are feline neonatology and pediatrics, with a focus on the influence of colostrum and milk quality on kitten health.

Dr. Aurélien Grellet

DVM, PhD

Aurélien Grellet graduated with a veterinary diploma from Liege Veterinary faculty (Belgium). After his internship at the same vet faculty, he spent five years at Alfort Veterinary School (Paris, France), where he was in charge first of clinical work in reproduction and then of the canine and feline breeding sector. In 2012, he obtained his PhD degree, with a project on the risk factors for weaning diarrhea in puppies. Between 2011 and 2016, he worked for ROYAL CANIN® in Research and Development (scientific communication). Aurelien Grellet joined NeoCare Center in 2016. His research focuses are canine and feline pediatrics, and in particular the influence of infectious diseases on digestive health during the weaning period. ■

Main activities

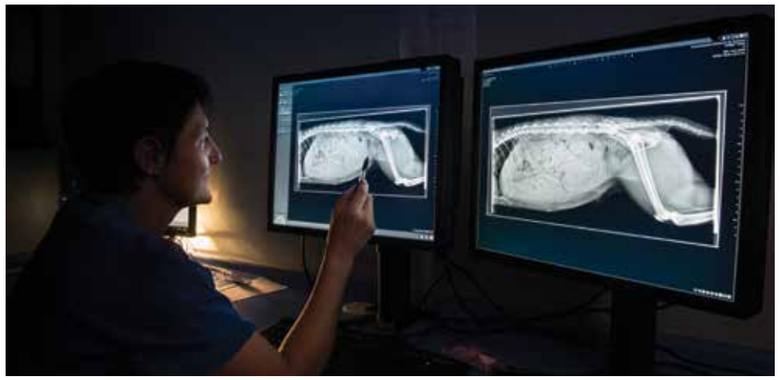
The NeoCare Center (Director: Prof. Sylvie Chastant-Maillard), houses services which include research and teaching activities, as well as clinical work.

Teaching

The teaching curriculum includes specific courses on animal reproduction for veterinary students and postgraduates. On top of this, lectures and practical training sessions are offered to veterinarians, specialists, breeders, as well as various professionals involved in canine and feline breeding and management. The center also participates in the residency program of the 'European College of Animal Reproduction' (ECAR).

Clinical activity

NeoCare Center offers a wide variety of services related to all aspects of small animal reproduction (assessment and treatment of male and female infertility, estrus monitoring and ovulation timing, vaginal and endoscopic artificial insemination, pregnancy monitoring, parturition monitoring: assisted-labor, neonatal medicine, treatment of dystocia, C-sections; semen evaluation and shipping, male and female reproductive pathologies).



"After arriving at NeoCare Center, one of my first clinical cases was in the field of canine neonatology. An owner of an 8-day-old German Shepherd puppy came into our clinic. He was very worried, because two out of the nine puppies born had died. It is a frequently observed problem with puppies. And very often there are no clinical signs, or signs which appear just a few hours before death. Breeders with similar issues often come to our clinic too, or we visit them at their kennel for a global diagnosis. Our aim, as a unit specialized in canine and feline neonatology, is to find the cause of the problem and try to treat or prevent the underlying pathology."

Dr Hanna Mila

Research activity

The research performed in NeoCare Center focuses on relevant fundamental and applied questions in the fields of canine and feline neonatology, with special emphasis on:

- Puppy and kitten **immunity**
- Factors **improving survival** of neonates up to weaning
- Factors influencing **colostrum and milk quality**, both nutritional and immunity-linked
- **Early growth** of puppies and kittens and the link to weight and health in later life

This activity is made possible thanks to PhD programs conducted in collaboration with ROYAL CANIN®, but also with breeders, veterinarians, other units within the Toulouse National Veterinary School, and several research centers abroad. Over the last 5 years, international research projects were, for example, established between the NeoCare Center and the Gastrointestinal Laboratory (GI lab, Texas A&M University, Tamu, USA), the Smithsonian Conservation Biology Institute (National Zoological Park, Washington, USA), and the Faculty of Veterinary Medicine of Bari University (Department of Animal Health and Well-Being, Bari, Italy). ■

Despite the importance of the subject for small animal reproduction, few research centers show interest in canine and feline neonatology. There is a very limited knowledge available, making it difficult today for breeders and veterinarians to progress in this field. So, many questions remain unanswered: how to help puppies and kittens to acquire the correct level of immunity? How to protect neonates from major infectious diseases? To what extent is energy a limiting factor for survival? Which pathogens are involved in puppy and kitten diseases (we are convinced that a lot of agents are still to be identified)? How to optimize environment for harmonious development and well-being of the puppy and its further health? These questions remain largely unanswered, and from what is already known, little information is communicated to vets and breeders.

Prof. Sylvie Chastant-Maillard

Between 2012 and 2015, I performed my PhD project, "The canine neonatal period: immunological and nutritional determinants for survival". This was made possible by working very closely with breeders. And I actually spent ten months of my PhD thesis time within breeding kennels, in order to better understand the factors influencing neonatal health.
More than 18 veterinary students have helped me to monitor puppy development over the last 3 years.

Dr Hanna Mila

Key figures
on studies performed by NeoCare Center between 2010 and 2015

10 months spent in breeding kennels

726 puppies monitored **216** dams monitored

20 breeds represented

4,801 weight measurements taken

4,448 clinical exams performed

15,674 clinical parameters recorded

477 milk samples collected

10 diagnostic tools assessed

The team has been working on kitten and puppy neonatal growth for 5 years. Most of the information was collected in breeding kennels by the team. Currently, we are launching large-scale studies relying on data provided by breeders. For example, we are calculating the mean reproductive performance and puppy mortality rates from data collected on more than 27,000 bitches and 205,000 puppies. This will allow us to provide the reference figures that are currently lacking, with figures calculated by breed. In the very near future, we will be able to provide the same analysis for the feline species, from data collected from 5500 females and 29000 kittens. We are still collecting information, in order to provide reference figures for birth weight and early growth per breed. Dog and cat breeders or veterinarians wishing to share data on puppy and kitten growth, are warmly requested to contact us by email at the following address: repro@envt.fr

Prof. Sylvie Chastant-Maillard

Key figures
on scientific communication between 2010 and 2015

26 research abstracts

5 articles published in peer-reviewed journals

1 patent

23 vocational training lectures

6 articles for vocational training

18 veterinary theses presented

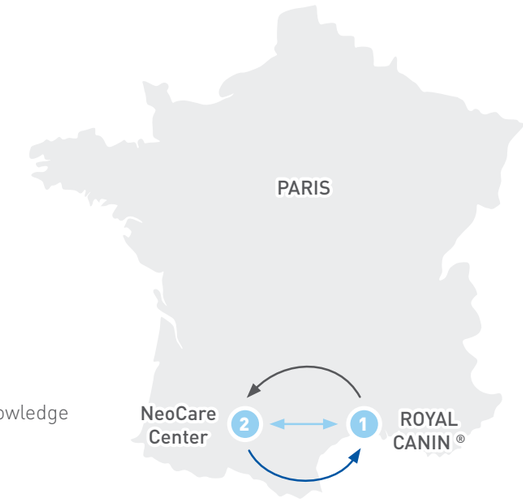
Interactions
between NeoCare and Royal Canin

1. ROYAL CANIN® Research & Development Center
2. NeoCare Center

→ Provides nutritional solutions/Supports PhD students and research programs

↔ Research advice

→ Data collection/Provides clinical research/Increases knowledge



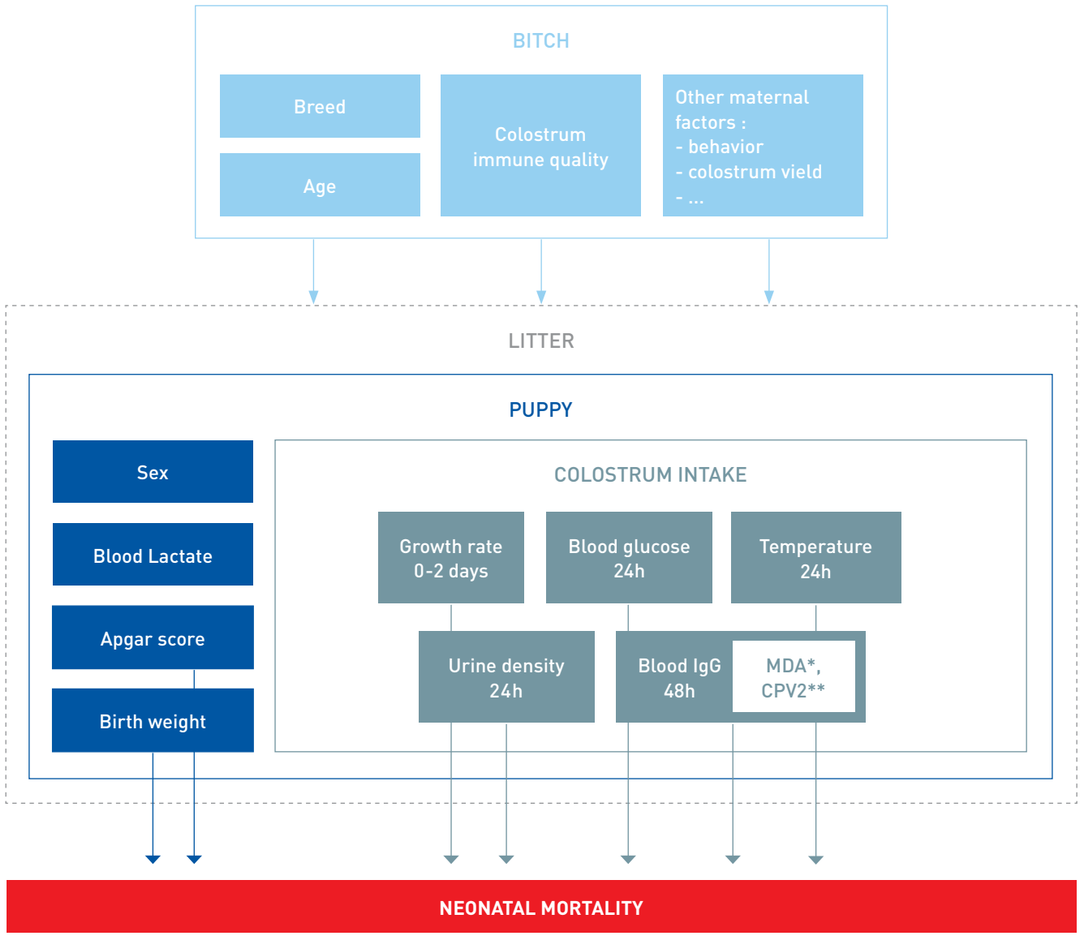
III

Risk factors for neonatal mortality

Due to a real lack of information in scientific literature on the puppy neonatal period, this project aimed to better identify risk factors of canine neonatal mortality (figure 3), with a focus on the role of the colostrum intake:

- **Colostrum intake** was indirectly assessed via early growth rate (reflecting the global benefits of colostrum), via puppy blood glucose concentration and rectal temperature at 24 hours after birth (colostrum as a source of energy), and via puppy blood immunoglobulin (IgG) concentration and titer in anti-parvovirus CPV2 antibodies (the role of colostrum in immunity);
- Consequences of **intrauterine growth and delivery** were assessed through birth weight, puppy blood lactate concentration (reflecting the degree of hypoxia) and Apgar score (scoring the global vitality of the newborn);
- The importance of **maternal factors** were taken into account, by analyzing breed size (including all specificities), or the age of the bitch (reflecting partially maternal behavior, development of the mammary glands, etc.).
- **Colostrum quality**, as another maternal factor, was assessed through its IgG concentration (considered as a marker of immunity quality). ■

Figure 3. Risk factors for neonatal mortality in dogs



*MDA: Maternally Derived Antibodies
**CPV2: Canine Parvovirus Type 2

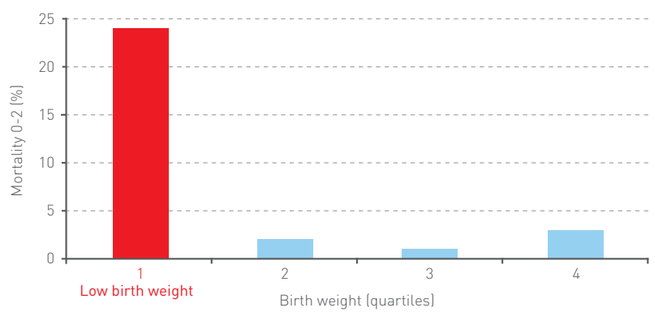
Birth weight and Apgar score can be used to assess the adaptation of neonates to extra-uterine life

Neonatal mortality is not only affected by environmental factors after birth, but also by fetal life during gestation and by the course of delivery. Adequate growth during fetal life ensures the maturity of the fetus at birth, and its ability to cope with extra-uterine life. Immediately after birth, the newborn must adapt to many drastic changes, the main ones being interrupted oxygen and nutrient supply, due to separation from the placenta. The quality of this adaptation process is reflected by the vitality of the newborn during the first few hours after birth. In human, as in veterinary medicine, birth weight is used to assess intrauterine growth, and the Apgar score the level of vitality. Both parameters can be used to identify which puppies are at higher risk of neonatal mortality.

Birth weight

Birth weight is of major importance in predicting mortality between birth and 2 days of age, since according to our results, over 80% of puppies which die during this period are of low birth weight (figure 4). The risk for mortality during the 2 first days of life is increased if the puppy belongs to the lightest 25% of its breed.

Figure 4. Effect of birth weight on neonatal mortality between birth and 2 days of age (n=514 puppies)



Key fact

Puppies with a low birth weight are at higher risk of death, between birth and 2 days: the risk is multiplied by 13

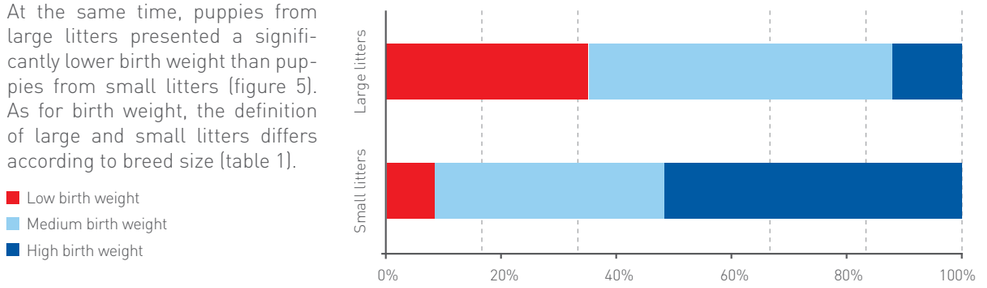
Table 1. Definition of a low birth weight in puppies linked to breed size

BREED SIZE	THRESHOLD DEFINING A LOW BIRTH WEIGHT	LITTER SIZE (number of puppies per litter)
Small (adult body weight <15 kg)	**< 151 g**	**< 4** (small litter) **4-5** (medium litter) **> 5** (large litter)
Medium (adult body weight between 15 and 25 kg)	**< 225 g**	**< 5** (small litter) **5-6** (medium litter) **6-9** (large litter)
Large (adult body weight >25 kg)	**< 330 g**	**< 6** (small litter) **6-9** (medium litter) **> 9** (large litter)

Our studies have showed the **two main factors** influencing birth weight: **breed size and litter size**. Indeed, higher the adult body weight of the bitch, higher the birth weight of the puppy. Since birth weight varies amongst breed sizes, low birth weight was defined independently for each breed size (table 1).

Figure 5. Influence of litter size on birth weight *(n=514)*

At the same time, puppies from large litters presented a significantly lower birth weight than puppies from small litters (figure 5). As for birth weight, the definition of large and small litters differs according to breed size (table 1).



Key fact

At birth, puppies weigh around 1-3% of the adult weight of their breed, and kittens are born at around 2-3% of their mother's weight. This is very similar to premature babies, born at 1kg rather than the usual 3 to 4kgs.

Key fact

The risk of having a small birth weight puppy is four times higher in large than small litters

Apgar score

The Apgar score, widely used on human babies in the few minutes following birth, has proven **a useful way to detect puppies at risk of death between birth and 24 hours of age**. Puppies with an Apgar score at or under 6, are at higher risk of death during this period (figure 6). The Apgar score is calculated from the result of the clinical examination on the puppy (table 2). The Apgar score is cost-free, very easy to perform, and the results are immediate. No **information available** about Apgar scoring for kittens. ■

Key fact

Puppies with an Apgar score ≤ 6: mortality risk during the first 24 hours is multiplied by 22

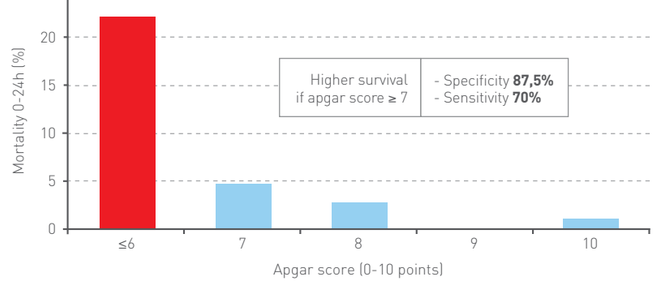
Table 2. Parameters for the assessment of an Apgar score in puppies.

Each parameter is scored between 0 and 2. The scores obtained for the 5 parameters are added, providing the "Apgar score". Bpm: beats per minute. rr: respiratory rate

PARAMETER	APGAR SCORE		
	0	1	2
Heart rate	<180 bpm	180 - 220 bpm	>220 bpm
Respiration	No crying <6 rr	Mild crying 6-15 rr	Crying < 15 rr
Irritability reflex	Absent	Grimace	Vigorous
Mobility	Flaccid	Some flexions	Active motion
Mucous color	Cyanotic	Pale	Pink

Adapted from Veronesi et al. An Apgar scoring system for routine assessment of newborn puppy viability and short-term survival prognosis. Theriogenology. 2009;72(3):401-7.

Figure 6. Effect of Apgar score on neonatal mortality between birth and 24 hours of age *(n=346 puppies)*



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. 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.

DEFINITIONS

Sensitivity of a clinical test:

The sensitivity of a clinical test refers to the ability of the test to correctly identify patients with the disease. A test with 100% sensitivity correctly identifies all patients with the disease. A test with 80% sensitivity detects 80% of patients with the disease (true positives), but 20% with the disease go undetected (false negatives).

$$\text{SENSITIVITY} = \frac{\text{True positives}}{\text{True positives + False negatives}}$$

$$\text{SPECIFICITY} = \frac{\text{True negatives}}{\text{True negatives + False positives}}$$

Specificity of a clinical test:

The specificity of a clinical test refers to the ability of the test to correctly identify patients without the disease. Therefore, a test with 100% specificity correctly identifies all patients without the disease. A test with 80% specificity, correctly identifies 80% of patients without the disease as test negative (true negatives) but 20% of patients without the disease are incorrectly identified as test positive (false positives).

Immediately after birth: the importance of colostrum intake

Immediately after birth, neonates enter a non-sterile environment. At the same time, nutrients are no longer supplied by the placenta and energy requirements are high. **Infectious diseases are indeed recognized as the most frequent cause of neonatal mortality in puppies and kittens**. Immunity and energy are closely linked in neonates, and those suffering from hypothermia or hypoglycemia are predisposed to septicemia. Both passive immunity and energy are supplied to neonates via a very specific mammary secretion, colostrum.

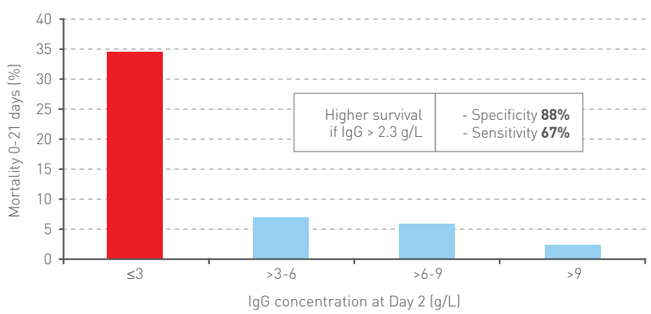
Key fact

Puppies with a blood IgG concentration below 2.3 g/L at 2 days of age, have a risk of mortality during the neonatal period (0-21 days) which is multiplied by 9

Colostrum is the mammary secretion of the two first days of lactation, and is characterized both by high energy value and high concentration in immunoglobulin, especially in class G immunoglobulin (IgG), on which the newborn's immune defense relies. The colostral richness of IgG is vital to puppies and kittens since they are almost agammaglobulinemic at birth, due to their placenta, which is not permeable to macromolecules. Transplacental transfer of Ig is thus very limited during gestation, accounting for only 10% of circulating Ig in the puppy's bloodstream at 2 days of age. **Kittens and puppies thus acquire the largest part of their Ig after birth through colostrum**. Colostrum will also provide other factors which are positive for neonatal immunity, such as antimicrobial factors (lactoferrin), antiproteases (antitrypsin limiting Ig destruction within the digestive tract) and polymorphonuclear cells.

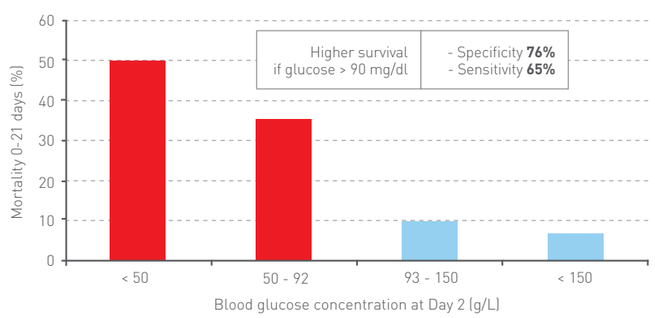
Acquisition of passive immunity after birth thus depends on three factors: the quantity of colostrum ingested, the immune quality of the colostrum (i.e. its concentration in IgG) and the ability of the neonatal digestive tract to absorb the Ig ingested. **The quality of passive immune transfer is generally assessed through IgG concentration in neonate blood at 2 days of age**. In puppies, the threshold for IgG concentration has been set at 2.3 g/L: puppies with blood IgG concentrations lower than 2.3 g/L are more at risk of death during the neonatal period than those with higher IgG concentrations. About 40% of puppies with IgG concentration below the threshold (≤ 2.3 g/L), will die during the neonatal period, versus only 5% of puppies with adequate passive immune transfer (figure 7). The IgG threshold, defining the quality of passive immune transfer is currently not defined in kittens.

Figure 7. Importance of passive immune transfer for puppy survival. Influence of blood IgG concentration at 2 days of age on neonatal mortality *(n=149)*



In addition to IgG, colostrum also provides the newborn with energy, vital in the early stages of life to combat the decrease in environmental temperature after birth (compared to constant intrauterine temperature), and the immature thermoregulation of the newborn. **Inadequate colostrum intake may thus lead to hypothermia and mortality**. Glycemia at 24 hours of life can be used to detect puppies with insufficient energy intake, and thus at higher risk of death during the neonatal period: glucose concentration under 90 mg/dl in puppy blood at 24 hours of life, is associated with higher mortality during the neonatal period (figure 8). ■

Figure 8. Importance of puppy blood glucose concentration for survival.



Key fact

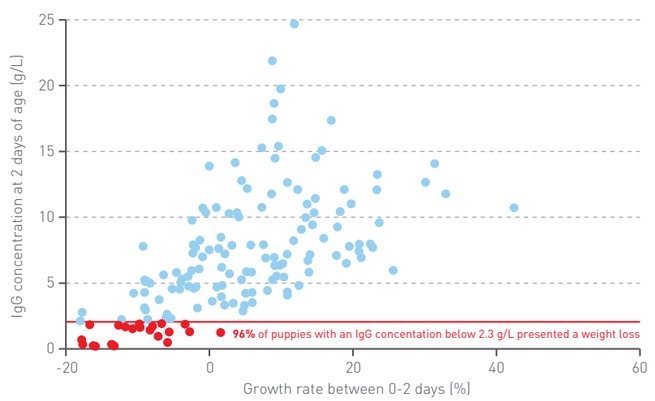
Mortality rates are four times greater for puppies with a glycemia level under 90 mg/dl at 24 hours of age

Early growth as a marker of colostrum intake

In addition to considering the impact of colostrum intake on passive immune transfer or on glycemia separately, colostrum intake can be assessed globally through the early growth of the neonate. Our results show that growth rate over the first 2 days of life is highly correlated with puppy serum IgG concentration (figure 9). Systematic weighing of newborns can be carried out to assess colostrum intake and thus, indirectly, the quality of passive immune transfer.

Although a 10% loss of birth weight is commonly considered to be physiological and tolerable in 2-day-old puppies, our results seem to dispel that myth. Indeed, we demonstrated that negative growth rates during the first two days of life are linked to a higher risk of neonatal mortality (during the first 21 days of life). The cut-off value of early growth rate (over the first two days of life) defining puppies at risk is - 4%. In the population studied, almost 40% of puppies with retarded growth died during the neonatal period, versus only 5% of puppies with normal growth rates (figure 10). Negative growth may suggest inadequate intake of colostrum, which is pivotal for newborn puppies, both for energy supply and passive immune transfer.

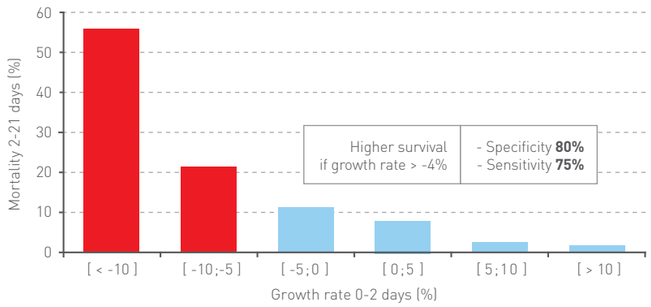
Figure 9. Relationship between growth rate between 0 and 2 days of age and IgG concentration at 2 days of age *(n=149 puppies).*
Growth rate has been calculated as weight at (Day 2 – weight at birth)/weight at birth x 100.



Key fact

Early growth rate can be used to assess the quality of passive immune transfer

Figure 10. Relationship between growth rate between 0 and 2 days of age and neonatal mortality *(n=149 puppies).*
Growth rate has been calculated as (weight at Day 2 – weight at birth)/weight at birth x 100.



Early growth rate is a good indicator of colostrum intake. It is easy to measure, but it monitors colostrum intake afterwards only, indicating which puppies require specific care after 2 days of age. As a preventive attitude, attention must be paid to ensuring adequate colostrum ingestion, in order to limit neonatal mortality in puppies. Maternal behavior, the quantity of colostrum produced and the anatomic morphology of teats (more or less easy suckling), are factors which should be assessed in the bitch.

Key fact

Puppies with a weight loss > 4 % between 0 and 2 days: mortality risk multiplied by 8

As well as the availability of colostrum, the time at which colostrum is ingested after birth is vitally important, due to the progressive "intestinal barrier closure". At birth, macromolecules can cross the digestive wall from the lumen through the lymph, and finally enter the bloodstream. Despite relative permeability, only 40% of the Ig ingested as early as 0-4 hours after birth, is finally transferred into the puppy bloodstream. The situation worsens with time after birth: maturation of the digestive tract is associated with the development of tight junctions between cells and the brush border on epithelial cells, rendering the digestive wall progressively non-permeable to macromolecules (figure 11). In puppies and kittens, the intestinal barrier is closed at 12-16 hours after birth. **Attention must thus be paid, not only to the quantity of colostrum ingested, but also to starting suckling as early as possible after birth**, in order to take advantage of maximal digestive absorption capacity. ■

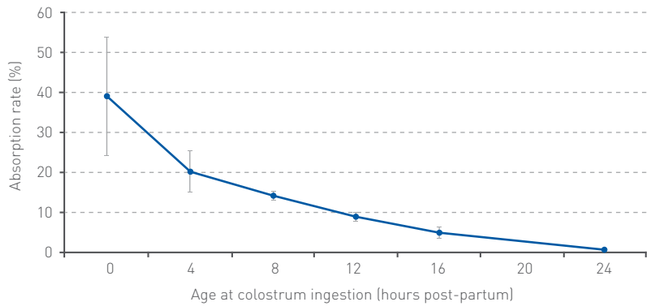
H. Mila, A. Feugier, A. Grellet, J. Anne, M. Gonnier, M. Martin, L. Rossig, S. Chastant-Maillard Inadequate passive immune transfer in puppies: definition, risk factors and prevention in a large multi-breed kennel. Preventive Veterinary Medicine 2014; 116: 209-213.

H. Mila, A. Grellet, 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. 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.

S. Chastant, L. Freyburger, E. Marcheteau, S. Thoumire, JF Ravier, K. Reynaud. Timing of the intestinal barrier closure in puppies. Reprod Domest Anim. 2012 Dec;47 Suppl 6:190-3.

Figure 11. Immunoglobulin absorption rate in the newborn dog *(n=21 puppies)*



Risk factors for puppy mortality before 2 months of age

Early identification (i.e. during the two first days of life) of factors associated with increased risk of neonatal mortality means that we can provide targeted puppies with special nursing and care (figure 13-14). **Systematic and regular weighing after birth, is the first easy-to-implement tool, completed by Apgar scoring performed during the first 8 hours after birth**. Unlike with human babies, (for whom the Apgar score must be taken within the 10 first minutes after birth, in order to be of high predictive value), breeders can score after the birth of an entire litter, with no need to be physically present at time of birth. **Glycemia measurement** is non-invasive (a simple prick in the ear, as with diabetic patients), and the result can be obtained within seconds, but does require some specific material (glucometer, although inexpensive). To date, IgG cannot be tested in the field, even at veterinary clinics, but **calculation of early growth rate** over the two first days of life can be suggested as an interesting alternative. As shown in figure 13, the proportion of puppies found at higher risk of neonatal death via four tests is quite high, from 8 to 30%. ■

figure 12. Risk factors of neonatal mortality in puppies between birth and 2 months of age

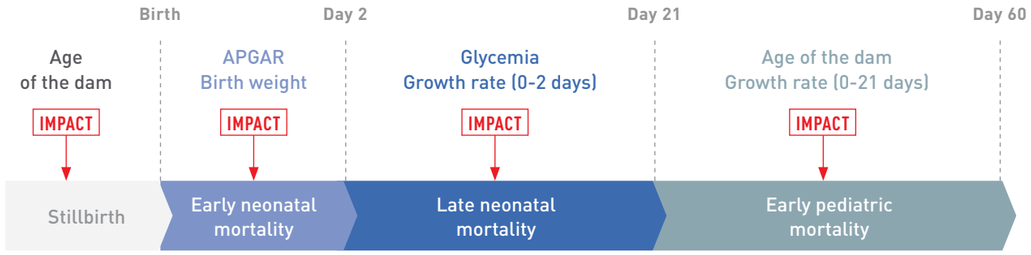


Figure 13. Defining a puppy at risk during the neonatal period

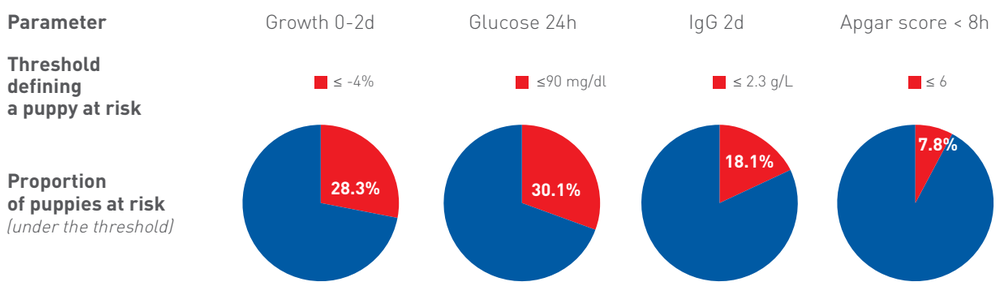
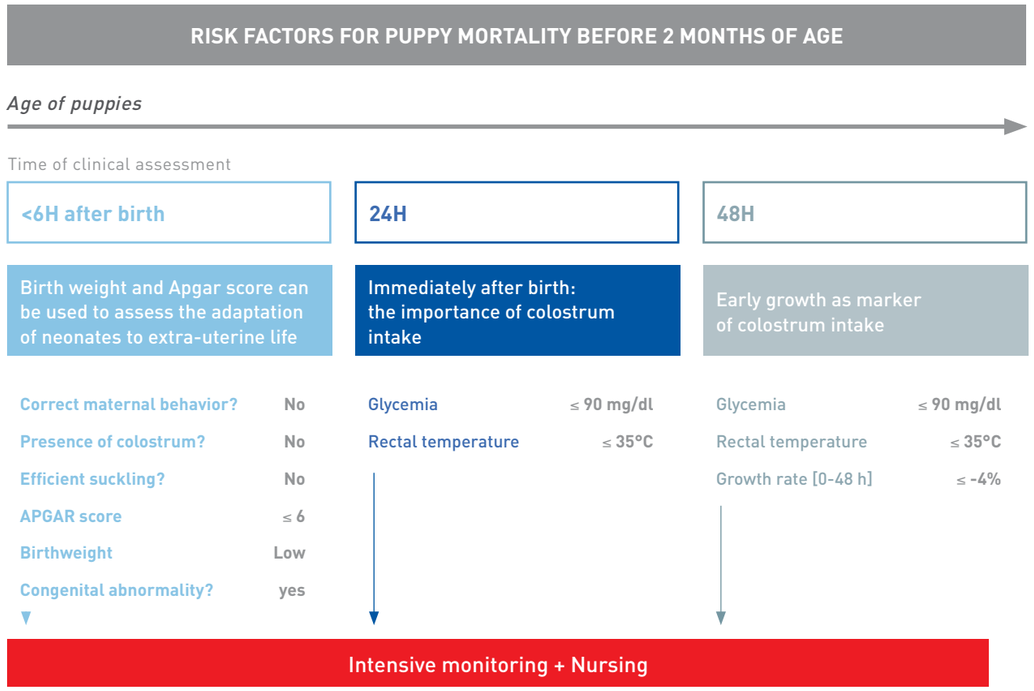


Figure 14. Risk factors for neonatal mortality in puppies between birth and 2 months of age



Key fact

Each puppy needs to be identified (using colored collar for example) for an accurate follow-up

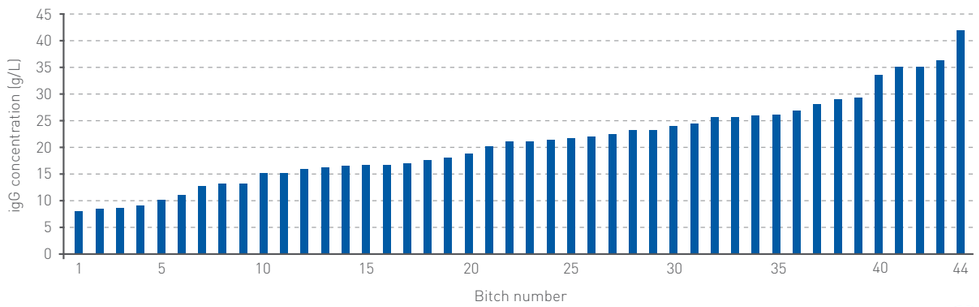
IV

Colostrum in bitches: immune quality and composition

Colostrum immune quality varies greatly between bitches and even between teats

The immune status of the newborn puppy depends entirely on colostrum ingestion, since canine neonates are almost agammaglobulinemic at birth. On top of quantity and age at ingestion, the concentration of immunoglobulin in colostrum is one of the limiting factors of adequate passive immune transfer to the newborn. For pigs and cows, IgG concentration rates may vary depending on parity, nutrition, genetic selection and mammary glands within one given dam. When studying the immune quality of canine colostrum, we did not see evidence of any of these factors. However, a **great variation in colostral IgG concentration was found among bitches** (from a ratio 1:5; figure 15), and even, within one bitch, among her different pairs of mammary glands (from a ratio 1:2 between best and worst quality colostrum). Nevertheless, no particular teat pair systematically produces colostrum of higher immune quality. ■

Figure 15. Variability of immune quality of colostrum between bitches. Mean IgG concentrations in colostrum from 44 bitches.



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.

Key fact

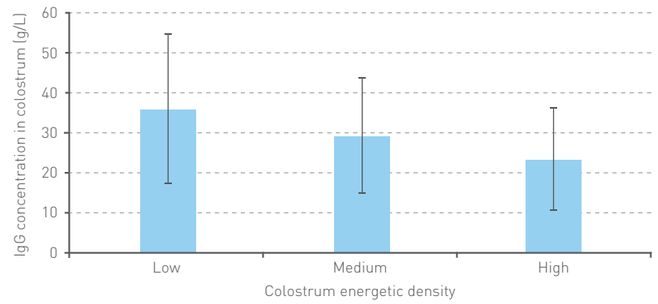
High variability of immune quality of colostrum is observed between bitches

Colostrum immune quality is unrelated to nutritional composition

Dogs are born poikilothermic (no temperature regulation) and hypogammaglobulinemic (almost no immunoglobulin at birth). Colostrum ingested during the first day of life provides them with both a high level of energy and immunoglobulin. The danger, in case of ingestion of low quality colostrum, is that passive immune transfer does not occur, and there is a risk of hypothermia and inadequate growth. Unfortunately, our results demonstrate that no positive correlation exists between IgG concentration and gross energy content in colostrums. On the contrary, according to our preliminary results, a negative correlation could be suspected (figure 16), meaning that **ingestion of colostrum rich in immunoglobulin may not provide sufficient energy to the newborn and vice versa**. ■

Figure 16. Composition of canine colostrum: no relationship between energy and immune quality (IgG concentration).

Colostrums were separated into three groups depending on energy level. (Results presented as mean ± SD, n=21 bitches)



Key fact

Posterior pairs of mammary glands do not secrete colostrum of better immune quality than anterior ones, or vice versa

V

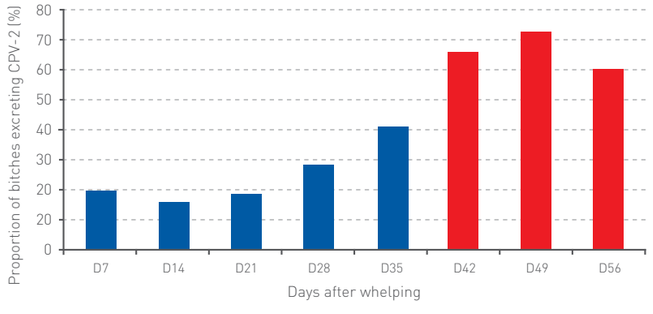
Canine parvovirus type 2: risk of infection and immune protection

Canine parvovirus type 2: risk of infection and immune protection

Adult bitches can excrete canine parvovirus during gestation and lactation without any clinical signs of disease

Canine parvovirus type 2 (CPV-2) is a ubiquitous enteropathogen, responsible for outbreaks of acute gastroenteritis, with a high mortality rate for puppies. Controlling the spreading of infection within kennels via disinfection and isolation of patients, is of limited efficiency, thus raising questions about the sources of contagion. We have studied the epidemiological role of dams in viral circulation during the reproductive period, and have found that gestating and lactating females could be a major source of contamination for growing puppies (figure 17).
Bitches, correctly vaccinated against CPV2 since primo-vaccination, were sampled once a week during gestation and lactation. During gestation, 80% of all bitches included in our study excreted CPV2 at least once, but only one sample was above the quantification threshold (2.10^5 copies/g feces). During lactation, all bitches tested positive at least once (3 times on average), and over 60% went over the quantification threshold at least once. High viral loads were excreted at later stages of lactation (between the 6th and 8th week). Despite testing higher than the threshold of 5.10^8 copies/g feces (associated with clinical parvovirosis in puppies), none of the bitches expressed any symptoms.

Figure 17. Proportion of bitches excreting canine parvovirus after whelping in a breeding kennel undergoing a CPV2 circulation *(n=32 bitches)*



Key fact

Bitches, even annually vaccinated, can excrete high viral load of canine parvovirus type 2 but without clinical signs

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.

CONCLUSION

Due to the high quantity of CPV-2 excreted during lactation, females probably represent a major source of contamination for their puppies. Viral excretion by bitches after lactation and until the next breeding period, and by males, should be studied in order to better understand the role of adults in CPV2 circulation.

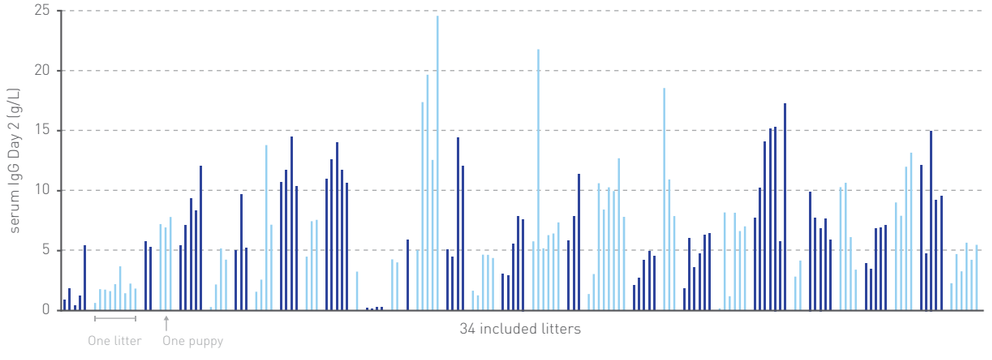
Protection against parvovirus infection in puppies depends on colostrum intake

During the first weeks of life, maternally derived antibodies (MDA) provide the only specific systemic protection against canine parvovirus infection in puppies. The vast majority is transferred from the dam to puppies through colostral ingestion during the first hours of life. As for the immunity passively transferred from dam to puppy, the level of anti CPV2 maternally derived antibodies (MDA) declines with age. When the serological titer falls under 1:80, maternally derived antibodies no longer confer protection against parvovirus. Our work has shown that as early as 2 days of age, there is great variability in specific antibody levels between puppies, with antibody titers ranging from 1:10 to 1:1280. In our study, **38% of puppies had not reached the antibody titer of 1:80, considered as the minimal protective level against infection**. The same variability of passive immune transfer was shown when we tested IgG blood concentration in puppies: 18% of puppies did not reach the minimal protective level (2.3 g/L), and there were obvious differences between puppies, both between litters and within litters (figure 18).

Key fact

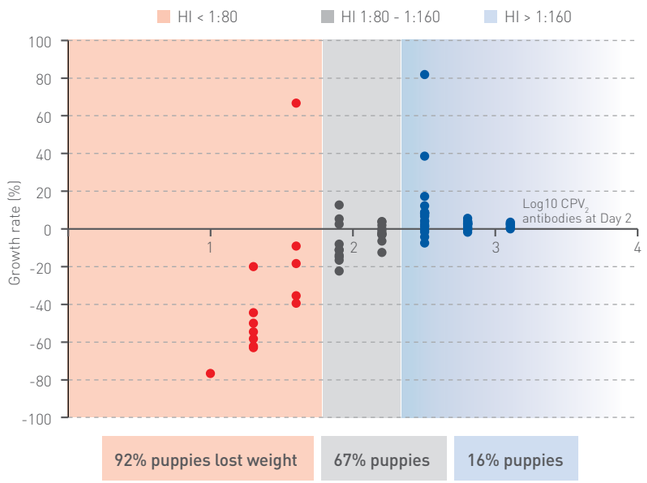
38 % of puppies did not have minimal protection against canine parvovirus type 2 at 2 days of age.
18 % of puppies did not absorb the minimal IgG quantity to reach minimal level of passive immune transfer

Figure 18. Passive immune transfer in 149 puppies from 34 litters



As for IgG concentration, the transfer of anti CPV2 specific maternal antibodies is associated with growth rate between birth and 48 hours of life. According to our data, **puppies which lose weight during this period, have lower MDA titers at day 2 than puppies which gain weight** (figure 19). Parvovirus specific antibodies acquired from colostrum declined progressively with age, until finally becoming null. However, puppies with a high antibody titer at two days of age (puppies with adequate colostrum intake), are protected longer against CPV2 infection than other puppies (figure 20). ■

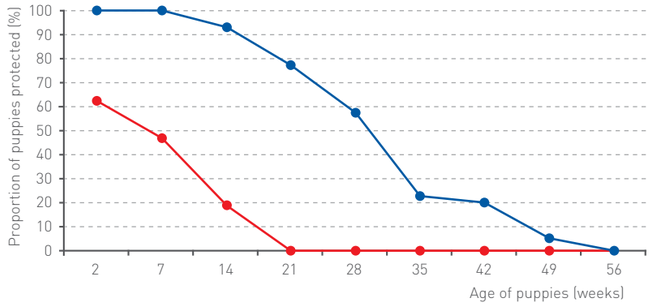
Figure 19. Relationship between growth rate (0-2 days) and parvovirus antibody titers at 2 days of age *(n=79 puppies)*



CONCLUSION

Our study underlines that early consumption of sufficient colostrum to maximize passive immune transfer, increases the length of the protective period against parvovirus infection. Thus, breeders should be encouraged to pay attention to early suckling within the first 12 hours after birth. The large variation in the parvovirus susceptibility period among puppies highlights the fact that vaccination protocol should be adapted to the epidemiological situation of each breeding kennel.

Figure 20. Proportion of puppies protected from CPV2 infection (HI ≥ 1:80) depending on MDA* level at 2 days of age *(n=79 puppies)*



*MDA= Maternally Derived Antibodies
** HI= Haemagglutination Inhibition

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.

H. Mila, A. Grellet, A. Feugier, C. Mariani, C. Desario, N. Decaro, C. Buonavoglia, S. Chastant-Maillard. Relationship between general and pathogen-specific passive immune transfer in puppies on example of canine parvovirus antibodies. 10ème colloque du réseau français d'Immunologie des Animaux Domestiques (IAD). Ploufragan, France, 17-18 Mars, 2016.

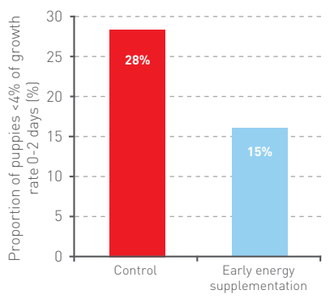
VI

Hyper-immune solutions for the improvement of neonatal health

Effect of early energy supplementation in puppies to control risk of neonatal mortality

Energy intake is one of the factors which impacts puppy survival. Puppies with a growth rate under -4% during the two first days of life, have a higher risk of neonatal mortality. For human beings, formulas for premature infants are composed of lactose and glucose polymers, in order to reduce lactose content, and to assist neonates whose lactase activity is not yet fully active. In our study, supplementation of puppies every 6 hours during the first two days of life with a milk replacer PUPPY PRO TECH, ROYAL CANIN®, (59 % of energy from fat, 22 % from protein and 19 % from carbohydrates), improved early growth, helped to maintain body temperature, and reduced the number of puppies at risk of neonatal mortality (growth rate 0-2 days <-4%; figure 21) ■

Figure 21. Effect of an early energy supplementation on proportion of puppies at risk of death *(growth rate 0-2 days ≤ -4%)*



A. Le Gal, H. Mila, A. Grellet, S. Chastant-Maillard. Interest of early energy supplementation in puppies to control the risk of neonatal mortality. 8th International Symposium on Canine and Feline Reproduction (ISCFR). Paris, France, 26th-30th June, 2016.

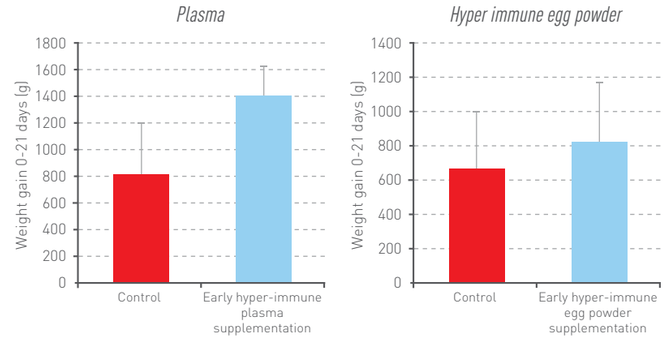
Key fact

Early energy supplementation during the first two days of life decreases risk of neonatal mortality

Improvement of weight gain and fecal microbiota in puppies supplemented with adult plasma

Microbiota undergoes massive changes during the early stages of life, and is increasingly seen as critical to understanding the immune system and metabolic function in neonates of various species, including human beings. Given the highly dynamic and unique interaction between the immune system and the intestinal microbiome, strategies for conditioning and maintaining a healthy gut may be useful in the prevention of neonatal morbidity and mortality. **Our results show that administration of plasma from hyper-immunized adult dogs to newborn puppies influences the intestinal microbiome during the early stages of life**. In our study, large breed puppies, which received hyper-immunized plasma within the first 8 hours of life, and then every two days until weaning, had not only increased microbial diversity and altered microbial communities, but also increased weight gain (figure 22). Improved parameters in supplemented puppies show that they are in better health, and thus potentially are less at risk of mortality. ■

Figure 22. Effect of hyper-immune solutions on growth in large breed puppies



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.

Key fact

Oral supplementation with plasma of puppies during the first two months of life improves neonatal health

Effect of hyper-immune egg yolk supplementation on neonatal health

Colostrum provides puppies with most of the essential passive immune transfer, and inadequate colostrum intake during the first days of life will deprive puppies, not only of immunoglobulins, but also of many hormones, growth factors and nutrients. Hence, the risk of neonatal morbidity and mortality is increased. Supplementation during the first hours of life with canine adult plasma increased puppy growth during the entire neonatal period (0-3 weeks). A similar clinical effect has been achieved with the supplementation by exogenous specific antibodies against canine parvovirus type 2 and *E. coli*. These specific antibodies were obtained from eggs laid by hens vaccinated against one of the mentioned agents. In our study, **large-breed puppies supplemented at birth with the hyper-immune egg yolk powder showed greater weight gain during the entire neonatal period**, compared to the placebo group. As retarded growth in the early stages of life increases the risk of morbidity and mortality in puppies, it could be said that sound growth in supplemented puppies reflects a better health status. ■

H. Mila, C. Oliver, A. Feugier, C. Mariani, A. Grellet, S. Chastant-Maillard. Effect of the hyper-immune egg yolk supplementation on weight gain in neonate puppies. Congress of the American College of Veterinary Internal Medicine (ACVIM). Denver, Colorado, United States, 8th-11th June, 2016.

Key fact

Supplementation during the first hours of life with hyper-immune egg yolk solution improves neonatal health

VII

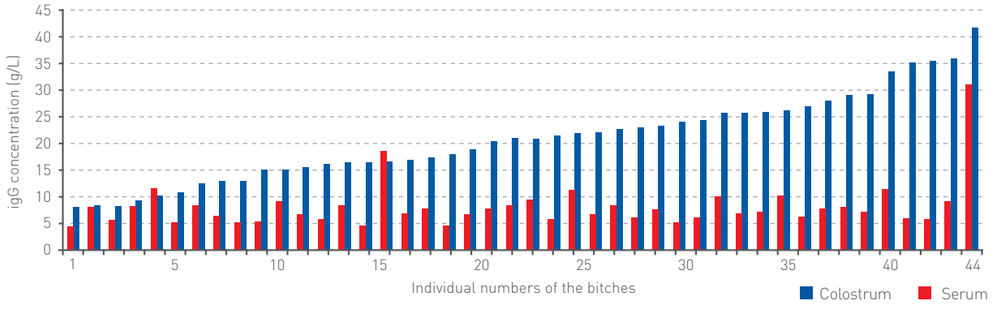
Truth and fallacy in canine neonatology

Plasma has higher IgG concentration than colostrum

False!

A study was carried out on forty-four bitches in order to assess IgG concentration in serum and colostrum. Leaving aside the variation of IgG concentration between bitches, their colostrum IgG concentration was on average 2.8 times higher than their serum IgG concentration (figure 23). The average IgG concentration in colostrum (mean value of the 5 mammary pairs per bitch), was 20.8 ± 8.1 g/L, ranging between 8.0 and 41.7 g/L, whereas the IgG concentration in serum was 8.1 ± 4.3 g/L, ranging between 4.3 and 30.9 g/L. ■

Figure 23. Relationship between serum and colostrum IgG concentration. Mean IgG concentration in colostrum and in serum *(n=43 bitches)*



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.

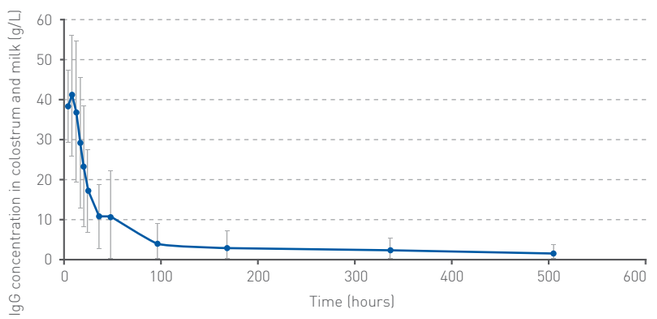
In hand-reared puppies, colostrum can be replaced by mature milk from another bitch

False!

A definition of colostrum mainly concerns immunoglobulin concentration, and especially IgG. IgG is the predominant immunoglobulin at the beginning of lactation. However, its concentration rapidly decreases soon after birth, to reach only 10% of the initial IgG level after one week of lactation. Hence, colostrum is defined as the secretion of the two first days of lactation only, and mature milk (after the first week postpartum) cannot be used as an alternative source for passive immune transfer because of insufficient IgG concentration (figure 24). However, the interest of administering transitional milk (between days 1 and 7 of lactation), with an IgG concentration of around 15% that in immediate post-partum, to ensure sufficient passive immune transfer in hand-reared puppies, must be further investigated. ■

Mila H. Neonatal period in the dog: immunological and nutritional determinants for survival. PhD Thesis, 2015; 176 p.

Figure 24. Pattern of IgG concentration (mean ± SD) in canine mammary secretions, from parturition and during the 21 first days of lactation *(study on 58 bitches)*



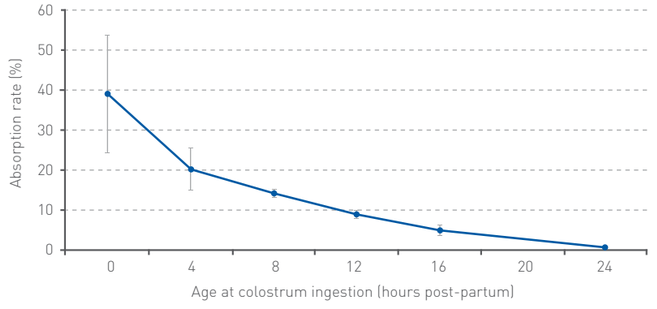
Puppies can acquire passive immunity until 24 hours after birth

False!

A recent study on the timing of intestinal barrier closure in newborn puppies shows that IgG absorption from the gut to the bloodstream is significantly affected by the time of colostrum ingestion. In this study, puppies which received colostrum between birth and four hours of life, had the highest immunoglobulin absorption rate (40%). Subsequently after birth, the absorption rate dropped through time, becoming almost null sixteen hours after birth. In one-day old puppies, there was no further immunoglobulin transfer. Thus, in the canine species, gut closure seems to begin as early as 4–8 hours after birth, and is complete at 16–24 hours (figure 25). Colostrum should thus be ingested as soon as possible after birth in order to achieve optimal passive immune transfer and limit the risk of neonatal mortality. ■

S. Chastant-Maillard, L. Freyburger, E. Marcheteau, S. Thoumire, JF Ravier and K. Reynaud Timing of the intestinal barrier closure in puppies. Reproduction in Domestic Animals, 2012; 47 (Suppl. 6), 190–193.

Figure 25. Immunoglobulin absorption rate in the newborn dog *(n=21 puppies)*



VIII

Scientific references of research performed at NeoCare Center

2016

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H. Mila, J. Bourcier, A. Grellet, S. Chastant-Maillard. Energy balance in the bitch – effect on birth weight and survival in puppies. 8th International Symposium on Canine and Feline Reproduction (ISCFR). Paris, France, 26th-30th June, 2016.

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A. Albaret, H. Mila, A. Grellet, S. Chastant-Maillard. Pattern of immunoglobulin G concentration in canine colostrum and milk during the lactation. 8th International Symposium on Canine and Feline Reproduction (ISCFR). Paris, France, 26th-30th June, 2016.

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