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New advances in estimating the age of dog puppies for medico-legal purposes

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ABSTRACT

Age determination of dog puppies represents a significant issue of animal welfare and forensic medicine. Despite the legislation in force, the illegal trade of puppies that are too young is expanding. To date, none of the available age estimation methods can be credited with a degree of accuracy sufficient for medical-legal purposes due to the many variability factors affecting the measured biological phenomena, such as teeth and skeletal development, and the lack of standardisation.

This work aimed at quantifying the degree of correlation between the chronological age of puppies and the biological age that can be estimated by visual teeth examination and the radiographic examination of limb ossification centres (OCs), and combining the information in a predictive model to obtain greater accuracy in estimating the age.

The study included 93 puppies of 10 different breeds, which were examined on a bi-weekly basis from 4 to 20 weeks of age and radiographed from 6 to 16 weeks, when not sold earlier.

Teeth eruption and development was affected by a wide degree of variability, in line with the information found in the Literature. On the basis of the number of OCs present, the breeds included in the sample could be divided in “early” and “late” breeds, and no correlation between this variability and size or morphological type was observed. However, the radiographic examination of limb ossification centres allowed to determine, with a fair degree of accuracy, whether a puppy was younger or older than 12 weeks old. Contrarily, at eight weeks, it was not possible to determine the age with a good degree of precision, as various degrees of variability were observed. A model to predict if a puppy was 6 or 8 weeks old was constructed using random forests, which proved to be a powerful tool to be further developed.

1. INTRODUCTION

1.1 THE IMPORTANCE OF AGE DETERMINATION IN DOG PUPPIES

Age determination in dogs is of great importance both in clinical veterinary practice and in veterinary forensic medicine. The age affects the decision-making processes concerning many aspects of veterinary medicine, including differential diagnoses, treatment modalities, anaesthetics and drugs to be used, life expectancy and euthanasia decisions. Furthermore, the dog's age has to be considered in the purchase of animals as well as for their movement for non-commercial or trading purposes and this is especially the case for young animals. In this respect, the trade and movement of pet animals, namely dogs and cats, have to comply with clearly defined rules that prevent them from being sold or transported before they have reached a certain age.

According to a study commissioned by the European Commission on the welfare of dogs and cats involved in commercial practices, the annual value of cat and dog sales in the EU is estimated at 1.3 billion euro and generates direct employment of 300 thousand people (DG SANCO, 2015). As emerged from the European Conference on the welfare of dogs and cats in the EU (held in Brussels on the 28th October 2013), the rise in the trade of dogs and cats has caused several problems, including excessive genetic selection, mutilations and puppy farming, which have consequences for animal welfare.

The key EU objectives include public health, animal health and welfare, protection of the consumer and the proper functioning of the internal market, by ensuring the free movement of goods, people and animals. In the view to the completion of the internal market, Directive 90/425/EEC[1] (implemented in Italy by Legislative Decree No 28/1993[2]) abolished veterinary and zootechnical checks at the Union's internal borders and placed emphasis on those carried out at the place of origin, during transit and at

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[1] Council Directive 90/425/EEC of 26 June 1990 concerning veterinary and zootechnical checks applicable in intra-Community trade in certain live animals and products with a view to the completion of the internal market. *Off J,* **L 224**, 18/08/1990, 29-41

[2] Legislative Decree 30 January 1993, No. 28 "Attuazione delle direttive 89/662/CEE e 90/425/CEE relative ai controlli veterinari e zootecnici di taluni animali vivi e su prodotti di origine animale applicabili negli scambi intracomunitari". *Off J*, **28**, 04/02/1993, Ordinary Supplement No. 12

the place of destination. Hence, the Member State of dispatch was made primarily responsible for ensuring compliance with the legislation requirements. On the other side, as regards the non-commercial movements of pet animals, the so-called Pet Travel Scheme (PETS), introduced by Regulation (EC) No 998/2003[3], allowed animals to travel more easily between Member Countries, without undergoing quarantine and customs controls. Consequently, the fact that systematic checks were no longer carried out at EU-internal borders resulted in a challenge for the authorities responsible to enforce animal health requirements for trade and non-commercial movement of dogs between Member States. According to DEFRA (Department for the Environment, Food and Rural Affairs) the number of dogs entering Great Britain via PETS rose by 61% in the first year when controls were relaxed (Dogs Trust, 2014). As regards the commercial movements, it is estimated that every month around 46,000 dogs are traded between EU Member States. However, this is not reflected in the registrations in the Commission's TRACES system, which registered a total of 20,779 dogs involved in intra-EU trade throughout 2014 (DG SANCO, 2015). This marked difference rises a concern, especially if considering the rapid rise of internet trading, which is even more difficult to trace. The impact of these changes and the possibility of an increased number of puppies being imported illegally have worrying implications for the control of infectious diseases, including zoonoses like rabies and parasitic diseases, particularly by toxascaris, dipylidium and echinococcus species.

In addition to the risk of spreading diseases transmissible to both animals and humans, the illegal pet trade can also affect the welfare of the animals involved. This is not only related to transport conditions; inappropriate housing and management conditions in rearing and sale sites indeed increase the likelihood of health and behavioural problems (Hird et al., 1992; Hubrecht et al., 1992; Scarlett et al., 1994; McMillan et al., 2013).

[3] Regulation (EC) No. 998/2003 of the European Parliament and of the Council of 26 May 2003 on the animal health requirements applicable to the non-commercial movement of pet animals and amending Council Directive 92/65/EEC. *Off J*, **L 146**, 13/06/2013, 1-9

The only EU legislation directly targeting the welfare of dogs and cats relates to their transport for commercial reasons[4]. However, the level of implementation of the legislation in force is not homogeneous between the Member States. In order to investigate the commercial movement of dogs and cats and its compliance with the EU legislation, the Italian Ministry of Health funded the research project “Movement of pet animals: impact on public health and animal welfare”, which was conducted by the Istituto Zooprofilattico Sperimentale of Abruzzo and Molise (IZSAM). The collected data revealed that the number of inspections carried out at national level is low; only two of the interviewed local health authorities declared to have performed, during years 2011-2013, inspections aimed at ensuring the enforcement of Regulation (EC) No. 1/2005 for the movement of dogs and cats and only one irregularity was reported, which concerned the non-correspondence between the real age of the animals and the age declared in the transport papers (D’Intino et al., 2015).
Conversely, animal welfare standards for dog keeping and breeding is the sole responsibility of Member States. However, national standards are not equally stringent and the level of compliance with national laws varies between Member States (DG SANCO, 2010). Some breeders’ organisations have developed guidelines[5], but the adherence to these policies is on a voluntary basis and it just represents a pre-requisite for the registration of pedigree dogs on the breeding books. Moreover, there are large differences between Member States in the requirements for the registration or licence conditions for pet breeders. Registration is required for professional dog breeders in all Countries except Poland and Slovakia, and a legal framework establishing requirements for housing, licensing, training and other conditions is present in all Countries except Poland and Romania. Registration of hobby breeders is required only in Belgium. As resulted from the study commissioned by the European Commission, 87% of 2,020 surveyed breeders were hobby breeders and only 13% were professional breeders, thus confirming that most of the breeders have no official licence (DG SANCO, 2015).

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[4] Council Regulation (EC) No. 1/2005 of 22 December 2004 on the protection of animals during transport and related operations and amending Directives 64/432/EEC and 93/119/EC and Regulation (EC) No. 1255/97. *Off J*, **L 3**, 05/01/2005, 1-44

[5] http://www.enci.it/media/2115/f-7249_01.pdf

An important step in the fight against illegal puppy trade was recently made by the United Kingdom with the enactment of the “Animal Welfare (Licensing of Activities Involving Animals) (England) Regulations 2018”. According to this measure, anyone who raises three or more litters per year must hold an official licence, including anyone breeding dogs and advertising a business of selling dogs. Moreover, requirements concerning staffing, housing environment, diet, monitoring of behaviour, training, animal handling and animal welfare are provided in this legislation. Puppies may be shown to a prospective purchaser only if they are together with their biological mothers; the licenced breeders can only sell the dogs they raised themselves, after they have reached 8 weeks of age.

The lack of systematic registration of dog breeders and of harmonised legislation that addresses the welfare issues of companion animals results in different standards and rules between Member States, which cause distortions in the internal market due to unfair competition and which could lead to adverse consequences for animal welfare, for animal and human health and for consumer protection. High standards of pet welfare in some Member States increase prices and lead to a competitive advantage for those businesses operating in countries with lower standards. Furthermore, the low prices undercut legitimate licenced breeders, who incur expenses for housing, health checks, vaccinations, certificates and taxes. Consumers find low prices attractive and they appear not to be fully aware of the risks associated with the purchase of a pet from a non-reliable breeder.

Despite the legislation currently in force, the growing demand of purebred puppies at low prices has indeed been fostering the illegal puppy trade from Eastern European Countries to Western Europe. Puppies are bred in extremely poor conditions in so-called “puppy mills”, where low enforcement of transport, health and welfare legislation allows the cheap prices; later they are transported to the distribution Countries, often when they are too young to be moved.

Puppy smuggling has been primarily investigated by Non-Governmental Organisations, but there are no official data providing reliable information on this issue. According to the FOUR PAWS International’s report on puppy trade in Europe, dog trafficking in Italy and France is estimated at 43 million euro (FOUR PAWS International, 2013). In a more recent report, puppy smuggling is estimated at 300 million euro, with 8,000 puppies imported illegally in Italy every week and sold at prices ranging between 60 and 1,200 euro, for an average commercial value of more than 5 million (Coldiretti et al., 2019).

The trafficking of puppies from Eastern Europe is thus proving to be one of the most profitable businesses which involves thousands of animals every year and which also sees the involvement of actual criminal organisations. The most frequently reported offences are cruelty to animals, transport and keeping of animals in conditions incompatible with their nature, commercial fraud, handling stolen goods, unlawful impersonation of a person or an authority, criminal association, forgery, false misrepresentation and fraud (Troiano, 2018).

A survey on the movement of pet animals carried out by the Istituto Zooprofilattico Sperimentale of Abruzzo and Molise showed that 86% of private veterinary practitioners reported to have visited, in 2014, imported puppies which were too young to be moved, while 48% of them observed irregularities in the accompanying documents. The most frequently observed anomaly (more than 90% of cases) was the non-correspondence between the real age of the animals and the age declared in the documents (Arena et al., 2015).

Consequently, determining the exact age of animals becomes legally crucial.

The difficulty in assessing the age of dog puppies, in case of lack of transport papers or in order to verify their regularity, is undoubtedly the most critical factor in legal disputes. As a result, the offences alleged against traders are rarely followed up in court.

The method used to assess age in these scenarios needs to be rapid, non-invasive, reproducible, reliable, precise and accurate.

These are the underlying grounds that led to this research, which was aimed at quantifying the degree of correlation between the chronological age of dog puppies and the biological age that can be estimated by visual teeth examination and the radiographic examination of limb ossification centres (OCs), and then combining the gathered information in a predictive model in order to obtain greater accuracy in estimating the age.

In this introductory part the legal, health and welfare implications of illegal puppy trade and movement will be presented, followed by an overview of the currently available methods for assessing the age in dogs. A more in-depth analysis will be carried out on teeth and limb development, with a brief description of the underlying physiology and a review of the existing literature regarding these phenomena.

1.2 LEGAL IMPLICATIONS

The laws that imply the knowledge of the dog's age essentially concern the identification, the trade and the movement of animals for non-commercial and trading purposes.

In Italy, Law No 281/1991[6] established the obligation to identify and register dogs in a national database; the definition of modalities and timing was left to the individual Regions. The identification requirements were subsequently harmonised by the State-Regional Agreement of 24 January 2013, according to which dogs must be identified and registered within two months and can be sold only after they have reached that age; finally, the sale or transfer of unidentified dogs is forbidden.

Despite what was established by the State-Regional Agreement, only in the late 2017 the Marche[7] and Lombardia[8] Regions amended their regional laws (which prohibited the sale of dog puppies before 90 days of age) to bring them into line with the Agreement.

In order to be moved within the European Union, either for non-commercial or trading purposes, dogs must first be identified by the implantation of a transponder or by a clearly readable tattoo (if applied before July 2011) and then vaccinated against rabies by an authorised veterinarian. The anti-rabies vaccine must be an inactivated or recombinant vaccine that has been granted a marketing authorization. Vaccination can be administered no earlier than 12 weeks of age and the period of validity of the vaccination starts not less than 21 days from the completion of the vaccination protocol for the primary vaccination, in order to allow protective immunity to be established. Dogs must also comply with any preventive health measures for diseases or infections other than rabies required by the receiving country, such as treatment against the parasite *Echinococcus multilocularis*. Finally, the pet animal must be accompanied by a passport completed and issued by an authorised veterinarian, which includes several

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[6] Law 14 August 1991, No. 281. "Legge quadro in materia di animali di affezione e prevenzione del randagismo". *Off J*, **203**, 30/08/1991, 3-5

[7] Regional Law 15 December 2016, No. 31 "Modifiche alla Legge regionale 20 gennaio 1997, n. 10 "Norme in materia di animali da affezione e prevenzione del randagismo. B.U.R., **138**, 22/12/2016, Art. 14 quinquies (e)

[8] Regional Regulation 13 April 2017, No. 2 "Regolamento di attuazione delle disposizioni di cui al Titolo VIII, Capo II, della L.R. 33/2009 recante norme relative alla tutela degli animali di affezione e prevenzione del randagismo", B.U.R.L., **15**, suppl., 14/04/2017, Art. 6 (7)

information, such as: the alpha-numeric code displayed by the transponder, its location and the date of application, the signalment of the dog (date of birth, breed, sex, colour), the name and contact information of the owner and of the authorised veterinarian issuing the document, the details of the anti-rabies vaccination, and where applicable, the details of the treatment against *E. multilocularis*. In lieu of the passport, a dog coming from a third country must be accompanied by an animal health certificate completed and issued by an official veterinarian, or by an authorised veterinarian and subsequently endorsed by the competent authority, valid for 10 days from the date of issue until the date of the documentary and identity checks at the travellers' points of entry designated by Member States. The import of a dog from a third country also requires a rabies antibody titration test to be carried out at least 30 days after the date of vaccination and not less than three months before the date of the movement by an approved laboratory. The test is considered satisfactory if the neutralizing antibody level is equal to or greater than 0.5 IU/ml.

For what concerns the non-commercial movement of dogs within the EU, the relevant legislation is Regulation (EU) No 576/2013[9], together with Commission Implementing Regulation (EU) No 577/2013[10]. According to this regulation, the maximum number of pet animals which may accompany the owner or an authorised person during a single non-commercial movement cannot exceed five, with the exception of movement of pet animals older than six months for the purpose of participating in competitions, exhibitions or sporting events or in training for such events.

The intra-Union pet trade is regulated under the Directive 92/65/EEC[11] (implemented in Italy by Legislative Decree No 633/1996[12]), according to which the animals must

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[9] Regulation (EU) No. 576/2013 of the European Parliament and of the Council of 12 June 2013 on the non-commercial movement of pet animals and repealing Regulation (EC) No. 998/2003. *Off J*, **L 178**, 28/06/2013, 1-26

[10] Commission Implementing Regulation (EU) No. 577/2013 of 28 June 2013 on the model identification documents for the non-commercial movement of dogs, cats and ferrets, the establishment of lists of territories and third countries and the format, layout and language requirements of the declarations attesting compliance with certain conditions provided for in Regulation (EU) No. 576/2013 of the European Parliament and of the Council. *Off J*, **L 178**, 28/06/2013, 109-148

[11] Council Directive 92/65/EEC of 13 July 1992 laying down animal health requirements governing trade in and imports into the Community of Animals, semen, ova and embryos not subject to animal health requirements laid down in specific Community rules referred to in Annex A (I) to Directive 90/425/EEC. *Off J*, **L 268**, 14/09/1992, 54-72

[12] Legislative Decree 12 November 1996, No. 633. "Attuazione della direttiva 92/65/CEE che stabilisce norme sanitarie per gli scambi e le importazioni nella Comunità di animali, sperma, ovuli ed embrioni

come from registered establishments. As for non-commercial movements, dogs must be identified, vaccinated against rabies, eventually treated against *E. multilocularis*, and accompanied by a passport. Dogs must undergo a clinical examination in order to verify that they show no signs of diseases and are fit to be transported for the intended journey, in accordance with Regulation (EC) No 1/2005[13] on the protection of animals during transport. Regulation (EC) No 1/2005 also states that puppies younger that 8 weeks of age are unfit for transport unless accompanied by their mother. Following the clinical examination, the official veterinarian issues a health certificate and notifies the movement to the competent authorities of the destination country through the Community Trade Control and Expert System (TRACES).

On the basis of the over mentioned laws, the movement of dogs, either for non-commercial or trading purposes, is strictly dependent on the completion of the rabies vaccination protocol, which basically prevents puppies from being moved before 15 weeks of age. However, it must be noted that EU countries have discretion whether or not to allow the introduction onto their territory of "young dogs", i.e. dogs which are less than 12 weeks old and have not received an anti-rabies vaccination, or dogs which are between 12 or 16 weeks old and have received an anti-rabies vaccination but are not yet fully protected. This derogation is possible for non-commercial movements from another EU country or a third country and for trade purposes within EU countries. Nevertheless, countries like Italy, Germany, France, United Kingdom, Spain, The Netherlands do not admit this exception. On the contrary, imports from non-EU countries of young dogs which are not vaccinated against rabies are not allowed under any circumstances.

From the information available on pet movement and puppy smuggling, it is clear that EU legislation and TRACES guidelines are often violated (FOUR PAWS International, 2013; Dogs Trust, 2014; Arena et al., 2015; DG SANCO, 2015; Troiano, 2018; Coldiretti et al., 2019). Puppies are transported when they are still too young, with no

non soggetti, per quanto riguarda le condizioni di polizia sanitaria, alle normative comunitarie specifiche di cui all'Allegato A, sezione I, della direttiva 90/425, CEE". *Off J*, **296**, 18/12/1996, Ordinary Supplement No. 222

[13] Council Regulation (EC) No. 1/2005 of 22 December 2004 on the protection of animals during transport and related operations and amending Directives 64/432/EEC and 93/119/EC and Regulation (EC) No. 1255/97. *Off J*, **L 3**, 05/01/2005, 1-44

identification, without or with incorrect vaccinations, with no or counterfeit passports and transport papers, in unsanitary conditions and with little regard for their wellbeing.
In Italy, the offence of illegal pet trade was introduced by Law No 201/2010[14], art. 4: *<<Anyone who, in order to provide themselves or others with a profit, repeatedly or through organised activities, introduces in the national territory pets without individual identification, the necessary certifications and without an individual passport, is liable to imprisonment for a period of between three months and one year and a fine of between 3,000 to 15,000 euro. […] The punishment is increased if the animals are less than 12 weeks old. […] In case of conviction… the confiscation of the animal is always ordered... It is also ordered the suspension from three months to three years of the activity of transport, trade or breeding animals... In case of recidivism, the prohibition from the exercise of the same activities is ordered.>>*
The illegal introduction of pet animals is also punished (art. 5): *<<Unless the act constitutes a crime, anyone who introduces in the national territory pets… without individual identification is punished with an administrative sanction of between 100 to 1,000 euro. […] The sanction is increased to a sum from 1,000 to 2,000 euro for each animal younger than 12 weeks old introduced…>>*
The measure of punishment for illegal puppy trafficking is therefore severe enough on paper but is in reality rarely prosecuted in court. According to the data provided by the 82% of the national public prosecutor's offices, 58 proceedings for the crime of illegal pet trade were recorded in 2017, i.e. only the 0.68% of the total number of proceedings (Troiano, 2018). Therefore, the deterring function of the punishment is close to nil.
The difficulty in determining the exact age of the animals, in case of lack of transport papers or in order to verify their regularity, is undoubtedly the most critical element that arises in this kind of judicial proceedings.

[14] Law 4 November 2001, No. 201. "Ratifica ed esecuzione della Convenzione europea per la protezione degli animali da compagnia, fatta a Strasburgo il 13 novembre 1987, nonché norme di adeguamento dell'ordinamento interno". *Off J*, **283**, 03/12/2010, 1-27

1.3 Health and welfare implications

The trade and movement of dogs that are too young have not only legal implications, but also consequences on human health, animal health and welfare.

Animal movement is one of the main risk factors for the spread of diseases and the illegal trade of pet animals is one of the most important routes of transmission. Illegally imported animals may in fact come from infected areas and not be checked from a health point of view, posing a serious threat to human and animal health, as evidenced by cases of infection in non-endemic regions (Fèvre et al., 2006). The risk of infectious disease spread becomes critical when zoonoses, such as rabies, are involved. In order to move pets within the EU only vaccination is required, as the risk of moving a rabid pet within the EU is considered negligible (EFSA, 2006). Instead, pets entering the EU from a third country must be tested for a satisfactory virus-neutralizing antibody level $\geq$ 0.5 IU/ml (OIE, 2018). However, the current epidemiological situation shows an uneven distribution of the disease, especially in the eastern countries (see Figure 1 and Table 1). Therefore, no European country can afford to lower its guard.

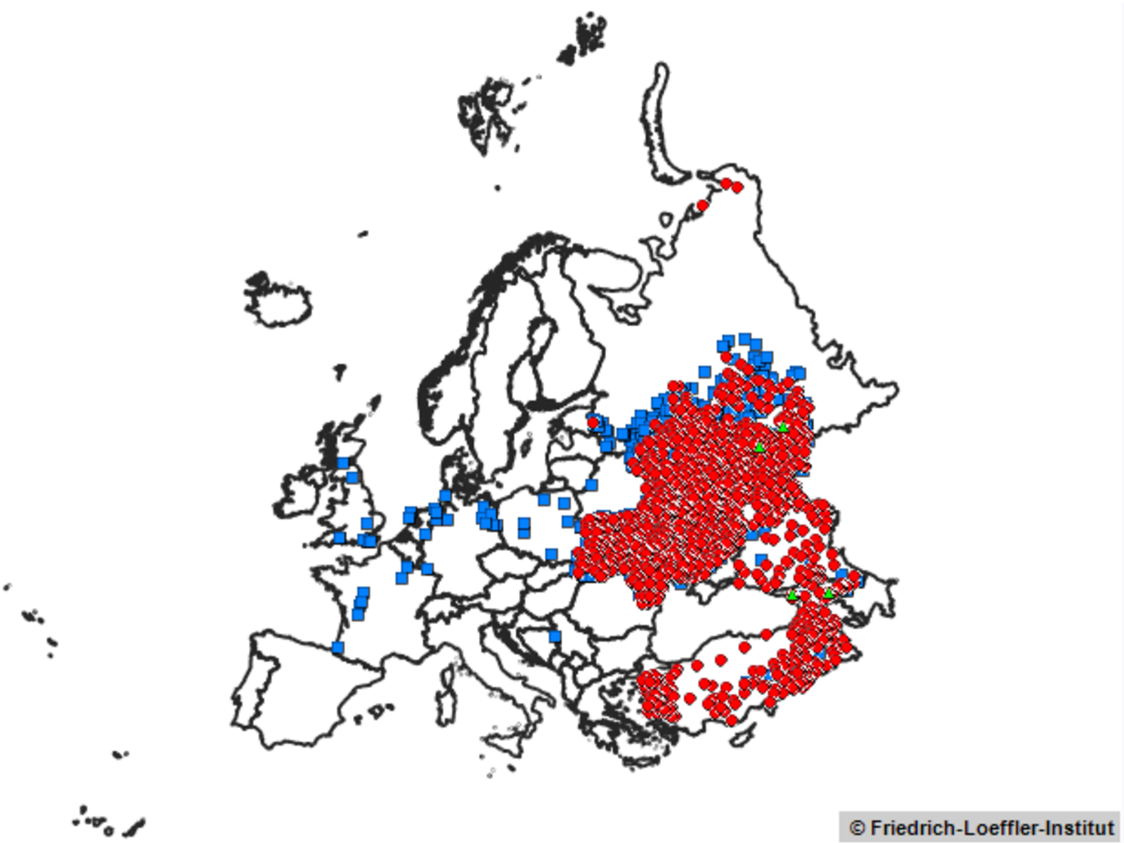

Figure 1. *Rabies cases reported in Europe in 2018. Red dots: domestic animals; blue squares: wildlife (including bats); green triangles: human cases (https://www.who-rabies-bulletin.org/site-page/queries)*

Table 1. *Rabies cases reported in Europe in 2018 (https://www.who-rabies-bulletin.org/site-page/queries)*

Country	**Domestic animals**	**Wildlife**	**Bats**	**Human cases**	**Total**
Austria	0	0	0	0	0
Belgium	0	0	0	0	0
Bosnia-Hercegovina	0	0	0	0	0
Croatia	0	0	0	0	0
Czech Republic	0	0	0	0	0
Denmark	0	0	0	0	0
Estonia	0	0	0	0	0
Finland	0	0	0	0	0
France	0	0	7	0	7
Georgia	42	5	0	2	49
Germany	0	0	17	0	17
Greece	0	0	0	0	0
Hungary	0	0	0	0	0
Ireland	0	0	0	0	0
Italy	0	0	0	0	0
Kosovo	0	0	0	0	0
Latvia	0	0	0	0	0
Liechtenstein	0	0	0	0	0
Lithuania	0	1	0	0	1
Moldova	59	19	0	0	78
Poland	0	4	5	0	9
Portugal	0	0	0	0	0
Romania	3	1	0	0	4
Russian Federation	1303	722	0	2	2027
Serbia	0	1	0	0	1
Slovak Republic	0	0	0	0	0
Slovenia	0	0	0	0	0
Spain	0	0	0	0	0
Switzerland	0	0	0	0	0
The Netherlands	0	0	2	0	2
Turkey	328	11	0	0	339
Ukraine	1081	833	2	0	1916
United Kingdom	0	0	10	1	11
Total	**2816**	**1597**	**43**	**5**	**4461**
%	**63.1**	**35.8**	**1.0**	**0.1**	**100**

Rabies vaccination of puppies can be regularly performed only from 12 weeks of age on and becomes effective 21 days after the completion of the vaccination protocol. However, it has been demonstrated that the likelihood of an animal achieving a protective serological level can be influenced by various factors, including the vaccine used, the size, breed and age of the dog (Mansfield et al., 2004; Kennedy et al., 2007; Berndtsson et al., 2011; Rota Nodari et al., 2017). Of note, a higher rate of vaccine failure has been observed in puppies imported from Eastern Europe (Klevar et al., 2015; Rota Nodari et al., 2017; Kaila et al., 2019). The failure in vaccine response may be imputed to the vaccination of puppies prior to the recommended age and/or the counterfeiting of vaccine certificates, as frequently reported (Arena et al., 2015).

A poorer vaccination response could also be attributed to transport-related stress. Poor management during transport can, in fact, have a negative effect on dogs' clinical status and well-being. Different studies report travel-related problems, such as excessive barking, drooling, panting, restlessness, overexcitement, phobia, motion sickness and vomiting (Gandia Estellés and Mills, 2006; Wells, 2006; Cannas et al., 2010; Mariti et al., 2012), the latter being most frequent in young animals (Benchaoui et al., 2007). Furthermore, transport and promiscuity lead to an increased health risk, latent infections may become reactivated and therefore puppies often become ill and die during the transport or soon after (Englund and Pringle, 2003). An inquiry carried out by the Italian Veterinary Councils Federation on the control of pet import revealed that 52% of dog puppies were found to be sick: 34% were infested with endoparasites, 23% were infected with parvovirus, 17% had fungal infections, 16% had scab and 10% were carriers of distemper (Benini, 2008).

In addition to the risk of spreading infectious diseases, the illegal pet trade may compromise the genetic heritage of purebred dogs. Illegally-trafficked animals are not tested for genetic diseases (such as hip dysplasia, heart disease, congenital deafness) for obvious economic reasons. However, as they are often accompanied by forged documents and pedigrees, they can enter the breeding programmes in the countries where they are traded, thus causing significant damage to the genetic breed heritage and nullifying the efforts to breed healthy dogs.

Other welfare issues include the inadequate socialisation of puppies in their country of origin before being sold and the early maternal separation, which may play a role in the development of behavioural disorders. Early life experiences have indeed great consequences on the development of dogs' temperament and behaviour (Serpell and Jagoe, 1995).

During the neonatal period (from birth to approximately two weeks) and the transition period (at three weeks) puppies are strictly dependent on their mother. Following the neonatal and the transition period, during which the mother-pup relationship is of outmost importance, the early socialisation period begins, when the experiences with the social and non-social stimuli received have long-term effects on the dog's behaviour as an adult. This developmental stage goes from 3 to approximately 12 weeks of age, but breed-specific variations have been observed (Scott and Fuller, 1965; Morrow et al., 2015).

The quality and quantity of maternal care appear to affect the behavioural development of puppies. It has been observed that removal from the litter prior to eight weeks of age may cause severe distress (Serpell and Jagoe, 1995). Compared to puppies separated from their mother and littermates at two months of age, puppies separated at 30 to 40 days of age are more likely to develop a variety of behavioural problems as adults, including excessive barking, destructiveness, attention-seeking, fearfulness on walks, noise reactivity and aversion to strangers (Pierantoni et al., 2011). Moreover, the separation of dog puppies from their mother at six weeks of age impairs their physical condition and weight gain, with an increase of disease susceptibility and mortality (Slabbert and Rasa, 1993).

Puppies that experience greater maternal care during the first three weeks of age show increased engagement with the environment and reduced signs of distress, while a lower level of maternal care is associated with distress vocalisations, increased locomotion and destructive behaviours during isolation at 8 weeks of age (Guardini et al., 2016). Likewise, in German Shepherd dogs maternal care seems to affect the behaviour and temperament of the offspring: puppies that received more maternal care scored higher for engagement with humans, objects and aggression at 18 months of age (Foyer et al., 2016). Furthermore, a questionnaire-based study showed that fearful behaviour is associated with lower quality of maternal care and less socialisation experiences (Tiira and Lohi, 2015).

Interactions with the mother and littermates also play an important role in the development of social behaviour, by introducing the puppies to submissive, dominant, agonistic and appeasement behaviours. Therefore, it is important for the puppy to stay with its mother at least until weaning, around 7-8 weeks (Case, 2005).

Appropriate stimulation during the socialisation period allows the pup to build adaptive capacity in order to cope with novelty and to build relationships with humans and conspecifics. Puppies spend most of the sensitive period at their breeder's, who therefore has the primary responsibility to provide the animals with a stimulating and variable environment (Howell et al., 2015). However, the puppies' behavioural needs during the socialisation period are often not considered in puppy farms (FOUR PAWS International, 2013).

The rearing environment influences the probability of developing behavioural disorders such as fearfulness, aggression and separation anxiety (Serpell and Jagoe, 1995). A retrospective study showed that puppies raised in a domestic environment (i.e. the breeder's home) are less likely to develop avoidance behaviour and aggression towards unfamiliar people compared to dogs coming from non-domestic environments (i.e. kennel, garage, barn or shed) (Appleby et al., 2002). A survey carried out on Belgian breeders revealed that the early environment in which puppies are raised is often inadequate: in many kennels weaning occurred when the puppies were too young, puppies did not have sufficient contact with adult dogs other than the mother, unfamiliar humans, other non-canine animals and unfamiliar locations, and insufficient visual, auditory and olfactory stimuli or toys were provided (De Meester et al., 2005). More recently, McMillan (2017) reviewed various studies involving dogs reared in commercial breeding establishments and/or sold through pet stores, which highlighted an increased incidence of behavioural problems, such as aggression, most commonly directed toward owners and family members, and increased fear in response to strangers, children, other dogs and non-social stimuli, compared with dogs coming from other sources, particularly non-commercial breeders. These behavioural disorders are the main cause for sheltering and euthanasia of dogs (Reisner et al., 1994; Overall and Love, 2001; Lambert et al., 2015).

The increased incidence of health and behavioural problems deriving from irresponsible breeding practices, coupled with the owners' lack of knowledge and awareness of these risks, contributes to poor companion dog welfare. Puppy smuggling entails social costs deriving from new owners needing to treat their sick animals and to manage potential behavioural problems, such as aggression, which also has a significant impact on public health (Sacks et al., 1996; Méndez Gallart et al., 2002; Langley, 2009; Rosado et al., 2009).

2. METHODS FOR ASSESSING THE AGE IN DOGS

The methods available for assessing the age in dogs are various. They differ in precision and accuracy, execution times, equipment needed, costs, invasiveness, speed in obtaining results, applicability to young or adult dogs. However, to date none of the available methods can be considered valid for medico-legal purposes, due to the numerous variability factors (breed, sex, blood line, diet, environment, health status) of the measured biological phenomena (teeth development, bone development, development of the eye structures) and the lack of standardisation.

The assessment of age through the observation of deciduous and permanent teeth eruption and succession has been used for a long time in veterinary practice, starting with production animals. This non-invasive method requires no special equipment and can be performed both in young and adult animals, either living or dead. Dogs, like other mammals, have a diphyodont dentition. At birth, a puppy is toothless; within a few weeks, deciduous teeth, also known as “milk teeth”, develop, which are later replaced by permanent teeth. The permanent set is deemed to be complete within seven months of age (see paragraph 2.1).

Once the eruption of permanent teeth is completed, in order to estimate the age of the adult dog an assessment of the grade of dental abrasion and tartar, which are increasingly frequent with increasing age, can be performed. However, dental wear and tartar accumulation are strongly influenced by many factors, such as the dog’s size (small breeds are more affected), diet (soft versus dry food or bones), habits (e.g., excessive chewing) and dental care. Therefore, this method is subject to a high degree of variability (Harvey and Emily, 1993a).

Another way to estimate the age of the adult and aged dog is the evaluation of the ocular lens reflections and appearance. As the dog gets older, nuclear sclerosis increases the refractivity of the crystalline lens, which causes the two pinpoint lens reflections to increase in size. The reflections are produced by placing the animal in a dark room and shining a penlight into its eye and then measured using a reference scale. Starting at 4 years of age, changes in the appearance of the lens nucleus also occur as it develops a faint blue-grey appearance that becomes increasingly intense.

These changes have been divided into five grades, from clear to severe opacity (cataract). This technique does not require special equipment or training, and appears to be more than twice as accurate as teeth examination in dogs older than 4 years of age. The age of the dog can be determined to within ± 1.7 years with a 75% degree of confidence, whereas at a confidence level of 95%, the method can predict the age to within ± 2.8 years (Tobias et al., 1998; Tobias et al., 2000).

Table 2. *Accuracy of estimating age by dental versus ocular methods in dogs (Tobias et al., 2000).*

	Percentage of correct age estimations	
Age range (years)	**Dental method**	**Ocular method**
0 – 4	78.3	43.2
0 – 15	39.5	49.6
4 – 15	22.9	51

Gesierich and coll. developed a linear regression model for age determination using ocular lens reflection, dental abrasion and tartar:

$$Estimated\ age\ [months] = 13.954 + 33.400 \times lens\ reflection\ [mm] + 8.406 \times dental\ abrasion\ [grade] + 8.871 \times tartar\ [grade]$$

with, however, a standard error of estimation of 2.26 years (Gesierich et al., 2015).

Instrumental analyses such as the radiographic evaluation of limbs' ossification centres and dental radiography to assess pulp cavity/tooth width ratio have been developed. Dental radiography can be performed by obtaining intraoral or extraoral radiographs and using different positionings and techniques depending on the tooth to be examined. Age determination by measuring pulp cavity/tooth width ratio of the canine teeth has proven to be a valuable method in wild canids and cats, but it only allows the animals to be divided into large age groups (e.g. juvenile, yearling, adult) and is therefore of little use in a forensic scenario (Tumlison and McDaniel, 1984; Knowlton and Whittemore, 2001; Kershaw et al., 2005; Park et al., 2014; Mbizah et al., 2016).

X-ray evaluation of limb bones is often used as an alternative or complementary method to teeth examination for determining the age of dog puppies. It is based on the evaluation of the appearance and fusion of the bone secondary ossification centres

(OCs). This technique can be applied until the fusion of all the limb ossification centres is completed, which generally takes place around 12 months of age (von Pfeil and DeCamp, 2009) (see paragraph 2.2).

Radiographic methods previously needed the animal to be transferred to a facility equipped to take radiographs. However, current advances in technology have led to the development of portable X-ray units and computed radiography systems, thus making it possible to take radiographs in the field. Nevertheless, radiographic investigations have not yet found wide application outside the research field due to the cost of the required equipment, the exposure of the practitioner and the animal to X-rays, the eventual need for sedation and the lack of a standardised protocol.

Recently, cerebellar histomorphometry has been investigated for its suitability for age determination of dog puppies in veterinary forensic pathology. A significant correlation between age and the thickness of the external granular layer and between age and the thickness of the external granular layer and thickness of the molecular layer ratio (EGLT/MLT) was observed in puppies up to 75 days of age (Bianco et al., 2017).

Lastly, the histologic investigation of dental cementum deposition, which counts the primary cementum lines, is widely used in wild animals (Linhart and Knowlton, 1967; Grue and Jensen, 1976; Grue and Jensen, 1979; Landon et al., 1998). However, it has been observed that in domestic dogs the specific pattern of the primary cementum lines is not evident since they are not exposed to seasonal variations in food availability and environmental temperature. Therefore, this method proved to be unreliable for age determination in dogs (Van Lancker et al., 2005).

2.1 TEETH DEVELOPMENT IN DOGS: GENERAL FEATURES AND LITERATURE REVIEW

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doi: 10.12834/VetIt.1876.9968.2

Teeth examination has long been used to determine the age in man and domestic animals, it is non-invasive and requires no special equipment. Clinical examination of teeth includes the assessment of number, integrity, shape and colour of the teeth, as well as the potential presence of tartar, halitosis and bleeding. Therefore, it requires a thorough knowledge of the anatomy of the teeth and the physiology of teeth development.

Dogs, like other mammals, are characterised by the possession of a diphyodont dentition. At birth, a puppy is toothless; within a few weeks, deciduous teeth, also known as "milk teeth", develop, which are later replaced by permanent teeth. Teeth in the upper dental arcade normally erupt a few days earlier than the ones in the lower arcade (Girard, 1845; Balasini, 1995). The permanent set is deemed to be complete by the seventh month. The most accredited canine dental formulas are, for the deciduous dentition Id3/3, Cd1/1, Pd3/3 and for the permanent dentition I3/3, C1/1, P4/4, M2/3; where "I" stands for incisor, "C" for canine, "P" for premolar, and "M" for molar (Nickel et al., 1979; Evans and de Lahunta, 2013; Dyce et al., 2018a). There are no deciduous precursors for the first premolar or the molar teeth in dogs.

The canine deciduous and permanent dentitions are illustrated in Figures 2 and 3 respectively.

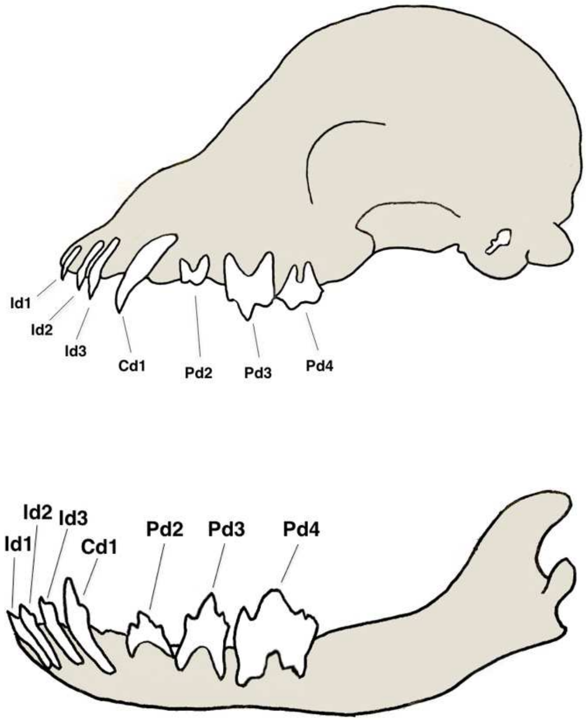


Figure 2. *Canine deciduous dentition.*

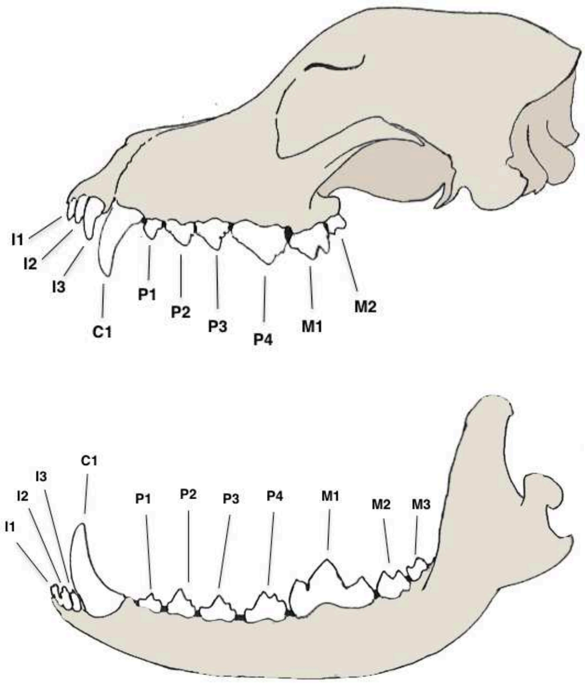


Figure 3. *Canine permanent dentition.*

On the basis of the changes that take place in the evolution and alteration of the teeth, the life of an animal can be divided in four periods: eruption of the deciduous teeth, wearing of the deciduous teeth, eruption of the permanent teeth and wearing of the permanent teeth.

There is a large amount of material on teeth eruption and development, although most of it is dated and sometimes hard to trace. The first documents on age assessment by teeth date back to the late 1800s (Girard, 1845; Liautard, 1885; Huidekoper, 1891). However, these sources were excluded from the present work because both of them state that, in puppies, the incisors are already present at birth. Presumably, considering the early timing of these works, the evolution and selection processes have led to the creation of dogs that are substantially different from those of two centuries ago.

The timing of teeth eruption has been reported in numerous Anatomy, Dentistry, Paediatrics and Zoognostic textbooks (Cornevin and Lesbre, 1894; Miller, 1952; Bourdelle and Bressou, 1953; Silver, 1963; Ferrara, 1965; Nickel et al., 1979; Barone, 2006a; Harvey and Emily, 1993b; Balasini, 1995; Bonetti, 1995b; Hoskins, 2001a; Vaissaire, 2001; Squarzoni, 2003; Reece, 2009; Veggetti and Falaschini, 2009; Van de Wetering, 2011; Evans and de Lahunta, 2013; Gorrel, 2013; Veronesi et al., 2013;

Dyce et al., 2018a) on the basis of previous published data or presumably from direct observations on the animals, but often there is no reference to the original data or sources. Some journal articles merely cite data published in books (Barton, 1939; Piérard, 1967; Hale, 2005; Fulton et al., 2014), while research papers that explicitly declare the number of dogs used in the study, as well as the observed animals, are very few. Mellanby (1929) reported the eruption times for deciduous and permanent teeth through the observation of 17 and 4 puppies respectively, of different types of dog. Deciduous teeth erupted 3 days earlier in larger dogs compared to the smaller ones, while permanent teeth erupted one week in advance.

In a later study, carried out by Arnall (1960), the sample included a Bull Terrier dam and her litter of 7 puppies, but during the period of eruption only 5 puppies were actually available. Data on eruptive and extrusive times for upper and lower teeth were calculated by detailed observations and measurement of extruded crown lengths through plaster casts on a weekly basis, in order to determine the age at which each tooth erupted and the time it took to reach its full stature. Nevertheless, an accurate measurement was not possible for teeth of small dimensions also because of the interference of gingival oedema. Eruption times for deciduous teeth fell between 20-35 days of age, while permanent teeth eruption began at 105-125 days. However, as the Author himself admits, such a limited study can only allow to draw general principles of eruption and any detailed conclusions can only apply to the breed of dog observed.

Regarding the sample size, an exception is represented by the works of Shabestari and coll. (1967) and Kremenak (1969). In order to study dental eruption chronology of Beagles, Shabestari and coll. used 106 closely related purebred Beagle puppies; observations were made three times per week from birth until the eruption of permanent teeth. Mean and standard deviation of the eruption age for each deciduous and permanent tooth (upper and lower, left and right) were obtained. Based on previously published data, the Authors conclude that dental eruption times in dogs vary more between breeds than among individuals of the same breed. Kremenak's study on deciduous teeth eruption was based on the daily observation of 32 purebred (16 Beagles, 10 Labradors, 6 Pointers) and 48 mixed-breed puppies of known age, for a total of 40 male and 40 female puppies. On the average, all teeth erupted from 22 to 34 days of age, and this is quite in accordance with Arnall's results (Arnall, 1960).

Males preceded females in the eruption of 21 of the 28 teeth, but this sex difference was not found to be statistically significant. When comparing puppies of different breeds, results supported the view that deciduous teeth eruption in Beagles occurs later than in strains of larger dog breeds. Interestingly, additional data from the observation of eight female mixed-breed puppies from the same litter allowed the Author to notice a rather wide variability among individuals sharing the same sex and bloodline, with ranges of up to nine days.

It is widely recognised that teeth eruption is affected by several factors, such as general health state, diet, sex, breed and body size. Several Authors agree that teeth erupt earlier in the larger dog strains (Girard ,1845; Mellanby, 1929; Piérard, 1967; Kremenak, 1969; Barone, 2006a; Sisson and Daniels Grossman, 1982; Evans and de Lahunta, 2013; Dyce et al., 2018a). Breed as well influences teeth eruption, development and wear. There are in fact significant differences among dog breeds in terms of head size and shape: brachycephalic and dolichocephalic breeds represent the two extremes of such variability. For this reason, Nickel and coll. (1979) explicitly refer to the German Shepherd’s dentition as a prototype, as it is the closest to the original wild ancestor. Moreover, some breeds are known to be predisposed to dental anomalies, which can make age assessment by teeth examination even more difficult. Hypodontia (missing teeth) is most common in small-breed dogs (Van de Wetering, 2011; Lobprise, 2012), but it also occurs in brachycephalic breeds (Akers and Denbow, 2008; Dyce et al., 2018a) and in large breeds such as Dobermann pinscher, Rottweiler and German Shepherd (Van de Wetering, 2011). The premolars are the most commonly missing teeth. Additional teeth are common in brachycephalic dogs (Boxer, Bulldog) and Mastiff (Van de Wetering, 2011; Lobprise, 2012). Delayed eruption has been observed in Tibetan Terrier, Irish Soft Coat Wheaten Terrier, Portuguese Water Dog and Chinese Crested Dog (Hoskins, 2001a; Lobprise, 2012). For what concerns sex, contrarily to Kremenak's (1969) findings, according to Harvey and Emily (1993) teeth erupt earlier in female dogs. Moreover, they state that season also affects the time of teeth eruption, and teeth erupt earlier in dogs born in the summer. In addition to these factors, the dog’s diet and eating habits determine significant variations in tooth wear, therefore affecting the estimated age (Girard, 1845; Piérard, 1967; Barone, 2006a; Balasini, 1995; Veggetti and Falaschini, 2009; Liebich et al., 2014).

The analysis of the available sources on teeth eruption and development revealed, in the first place, terminological discrepancies in the described phenomena; for example, some Authors include the monophyodont first premolar among the deciduous teeth even if it does not shed, others number the deciduous premolars 1, 2 and 3, which can be confusing. Differences also exist in the degree of detail of the provided data, both in relation to the timing (days vs. weeks) and the tooth classes (time ranges available for each single tooth or, more generally, for all incisors, premolars, etc.). Very few Authors give separate timing for upper and lower teeth (Mellanby, 1929; Arnall, 1960; Shabestari et al., 1967; Kremenak, 1969; Bonetti, 1995).

More importantly, it became evident that there is a wide disagreement in the chronology of dental development among the Authors. In order to highlight this variability, a diagram illustrating the timing of deciduous and permanent teeth eruption according to the different Authors is provided (see Annex 1). Data were derived from 8 manuscripts and 21/24 of the consulted textbooks, which were selected for providing separate information for at least each tooth class.

Diagrams summarizing the earliest and latest ages of deciduous and permanent teeth eruption are given in Table 3 and 4, respectively. In this case, only sources indicating a time range for each single tooth were selected, otherwise the resulting ranges would have been wide to the point of losing usefulness.

Table 3. *The interval between the earliest and the latest age of deciduous teeth eruption. From: Mellanby, 1929; Bourdelle and Bressou, 1953; Ferrara, 1965; Shabestari et al., 1967; Kremenak, 1969; Balasini, 1995; Vaissaire, 2001; Veronesi et al., 2013.*

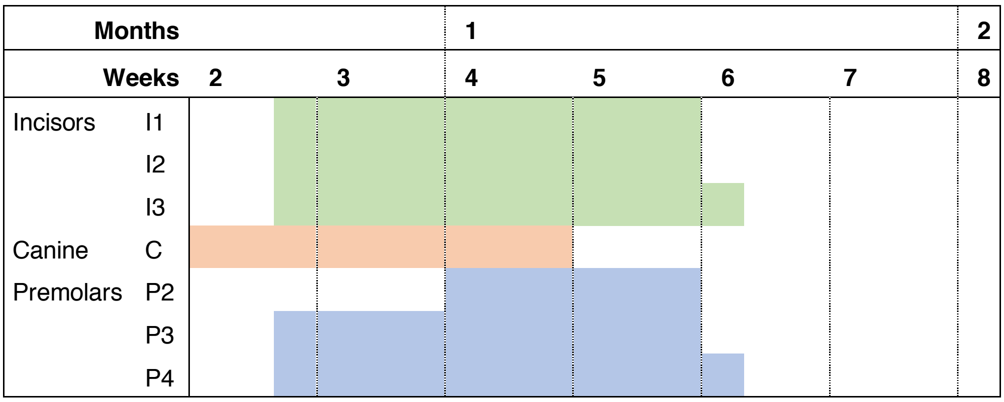
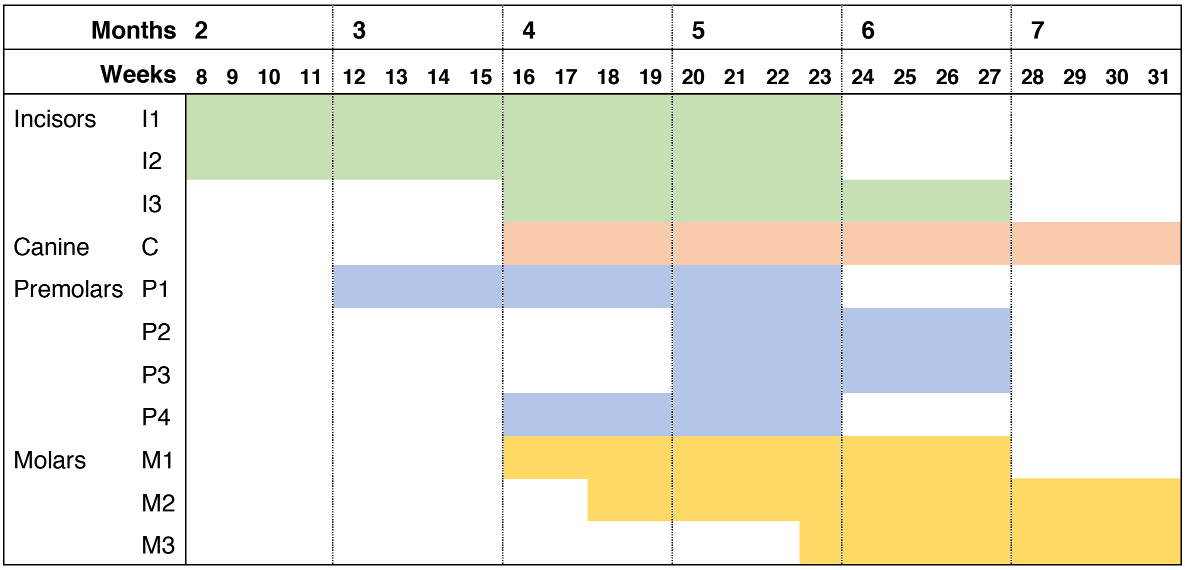


Table 4. *The interval between the earliest and the latest age of permanent teeth eruption. From: Mellanby, 1929; Miller, 1952; Bourdelle and Bressou, 1953; Arnall, 1960; Silver, 1963; Nickel et al., 1979; Shabestari et al., 1967; Barone, 2006a; Balasini, 1995; Vaissaire, 2001; Reece, 2009; Evans and de Lahunta, 2013; Dyce et al., 2018.*



The first deciduous teeth to erupt are the canines. Some Authors place their appearance at 15-20 days (Miller, 1952; Ferrara, 1965; Balasini, 1995), while for the majority of the sources they do not erupt before 3 or 4 weeks of age. Incisors follow immediately after, usually starting from corner incisors (I3), then intermediate (I2) and lastly central incisors (I1). Their eruption window is quite variable, ranging from 3-5 days for some Authors (Ferrara, 1965; Barone, 2006a; Balasini, 1995) up to 15-20 days (Miller, 1952; Arnall, 1960; Kremenak, 1969; Veronesi et al., 2013). The deciduous premolars begin to erupt at the turn of the second and third week of age, normally in this order: P3, P4, P2. For most Authors the eruption of the deciduous dentition is complete by 6 weeks of age, but for others it extends up to 8 (Miller, 1952; Silver, 1963; Evans and de Lahunta, 2013), 10 (Van de Wetering, 2011) or even 12 weeks (Harvey and Emily, 1993b; Squarzoni, 2003; Hale, 2005; Fulton et al., 2014).

Time ranges for permanent teeth eruption are even wider. The substitution process starts with incisors, this time in the opposite order (I1, I2, I3). Data on incisor eruption times are quite divergent. According to Miller (1952) and Evans and de Lahunta (2013) incisors begin to erupt at 2 months of age, but other Authors state that the eruption window starts at 3 months (Silver, 1963; Nickel et al., 1979; Harvey and Emily, 1993b; Hale, 2005; Reece, 2009; Fulton et al., 2014), and the remaining sources place their appearance at 4 months. For most Authors, canines erupt at 5-6 months, whereas for some of them they can appear one or two months earlier (Arnall, 1960; Harvey and Emily, 1993b; Squarzoni, 2003; Gorrel, 2013; Fulton et al., 2014). Premolars erupt between 4-6 months of age, starting from P1, according to almost all the Authors. Only Balasini (1995) and Vaissaire (2001) place the eruption of the first premolar at 3 months. According to half of the consulted sources, the first molar erupts at 4 months, while for the remaining half one month later. The other molars normally follow with a gap of one month between each one. At 7 months, the permanent set is deemed to be complete.

Dental anomalies such as hypodontia or supernumerary teeth are relatively common, especially in purebred dogs, as a consequence of the genetic defect being perpetuated (Hoskins, 2001a; Akers and Denbow, 2008; Van de Wetering, 2011; Lobprise, 2012). Furthermore, individual variations in subjects of the same sex and bloodline have been observed (Kremenak, 1969).

On the basis of the available information, the assessment of a puppy's age by teeth examination is subject to a degree of uncertainty of no less than 2 weeks during the first 2-3 months of age, which is mainly due to the wide genetic variability among breeds. This uncertainty increases hand in hand with the dog's growth as a result of the intervention of other factors, such as the environment and individual habits.

2.2 LIMB DEVELOPMENT IN DOGS: GENERAL FEATURES AND LITERATURE REVIEW

Limb bones are formed by endochondral ossification, where cartilage is gradually replaced by bone tissue. Ossification of cartilage begins in the last third of foetal development and continues until well after puberty. Endochondral ossification generally starts in the middle region of the cartilage shaft, called primary ossification centre, and spreads towards the ends of the bone, thus generating the bone diaphysis, which is composed of dense, compact bone. Later, secondary ossification centres appear at the bone ends, which will form the epiphyses. The diaphyseal and epiphyseal regions are separated by the metaphysis, which is an area of spongy bone, and by a transverse cartilage region called epiphyseal plate or growth plate, which is also responsible for the longitudinal growth of the bone until it becomes completely ossified (Sjaastad et al., 2010).

In dogs, some limb bones derive from a single ossification centre, such as most carpal and tarsal bones, sesamoid bones and distal phalanges, while other bones have several primary ossification centres, like the radial carpal bone and the hip bone. Also, many limb bones have one or more secondary ossification centres (Barone, 2006b).

In young animals the epiphyseal plate is seen radiographically as a radiolucent band or line separating the epiphysis from the metaphysis, which may resemble a fracture. When growth ceases, the epiphysis fuses with the metaphysis and the physis disappears.

It is therefore important to know the location of the ossification centres and the timing of physeal closure in order not to misinterpret what is normal from abnormality. Moreover, knowledge of the timing of appearance and fusion of the ossification centres (OCs) allows to estimate the age of young dogs. However, variations have been observed in the timing of appearance and closure of the physes, even in animals of the same breed (Kealy and McAllister, 2011).

Forelimb and hindlimb ossification centres of dogs are listed in Table 5.

Table 5. *Forelimb and hindlimb ossification centres. From: Burk and Feeney (2003).*

FORELIMB	HINDLIMB
Scapula	**Hip bone (*Os coxae*)**
Body	Ilium
Supraglenoid tubercle	Ischium
Humerus	Pubis
Proximal epiphysis	Acetabular bone
Diaphysis	Iliac crest
Distal epiphysis	Ischial tuber
Medial condyle	Ischial arch
Lateral condyle	Caudal symphysis pubis
Medial epicondyle	Symphysis pubis
Radius	**Femur**
Proximal epiphysis	Proximal epiphysis (head)
Diaphysis	Lesser trochanter
Distal epiphysis	Greater trochanter
Ulna	Diaphysis
Olecranon	Distal epiphysis
Anconeal process	Medial condyle
Diaphysis	Lateral condyle
Distal epiphysis	Trochlea
Carpus	**Sesamoid bones (stifle joint)**
Radial carpal bone	Patella
Radial carpal bone	Muscle Gastrocnemius (Fabellae)
Central carpal bone	Muscle Popliteus
Intermediate carpal bone	**Tibia**
Ulnar carpal bone	Proximal epiphysis
Accessory carpal bone	Medial condyle
Body	Lateral condyle
Epiphysis	Tibial tuberosity
Carpal bone I	Diaphysis
Carpal bone II	Distal epiphysis
Carpal bone III	Medial malleolus
Carpal bone IV	**Fibula**
Sesamoid bone m. abductor pollicis longus	Proximal epiphysis
Metacarpus	Diaphysis
Metacarpal bone I	Distal epiphysis
Proximal epiphysis	**Tarsus**
Diaphysis	Talus
Metacarpal bone II – V	Calcaneus
Diaphysis	Body
Distal epiphysis	Calcanean tuber
Phalanges	Central tarsal bone
Proximal phalanx I – V	Tarsal bone I
Diaphysis	Tarsal bone II
Distal epiphysis	Tarsal bone III
Middle phalanx I – V	Tarsal bone IV
Proximal epiphysis	**Metatarsus**
Diaphysis	(same as forelimb)
Distal phalanx I – V (one centre)	**Phalanges** (same as forelimb)
Sesamoid bones	**Sesamoid bones**
Palmar sesamoid bones	Plantar sesamoid bones
Dorsal sesamoid bones	Dorsal sesamoid bones

Most of the studies concerning the skeletal development of canine limbs have been published in the 50-60's (Seoudi, 1948; Pomriaskinsky-Kobozieff and Kobozieff, 1954; Bressou at al., 1957; Hare, 1959; Hare 1960; Smith, 1960; Smith and Allcock, 1960; Hare, 1961; Chapman, 1965; Sumner-Smith, 1966; Riser, 1973; Gustaffson et al., 1975; Yonamine et al., 1980). In more recent times, Authors have focused their attention on comparative studies (Fukuda and Matsuoka, 1980; Zoetis et al., 2003; Geiger et al., 2016) or developmental anomalies (Breit et al., 2004; Frazho et al., 2010).

Seoudi (1948) published the first radiographic study on the epiphyseal union of the dog's limbs as an aid in estimating the age of dogs. His research included eight Egyptian Armant dogs but the age of the animals and the observation protocols were not specified. From the analysis of 313 radiographs, the timing of epiphyseal union for the secondary ossification centres of the limbs were reported and compared with data from Sisson's "The anatomy of the domesticated animals" (1927).

Pomriaskinsky-Kobozieff and Kobozieff (1954) and Bressou (1957) investigated the skeletal development of the dog's manus and pes, respectively, from birth to the adult age. Their sample included one Cocker Spaniel, radiographed weekly from birth to one hundred and forty-six days, seven German Shepherds, examined from birth to eight months of age (two of which radiographed daily until forty-two days of age, then at regular intervals, as the others), and two crossbreds (Irish Setter x Épagneul Breton) examined at regular intervals from six to forty-two days, on the basis of the previous observations. In addition, the studies also included <<a great number of X-rays of dogs of all ages and all breeds>>. The timing of appearance and closure of the manus and pes ossification centres (including the distal epiphysis of radius, ulna, tibia and fibula, carpus, tarsus, metacarpus, metatarsus and phalanges) in the German Shepherd were given and any differences between breeds were highlighted. The Authors declared that the sample size was insufficient to determine the influence of breed and sex on the development of the examined bones.

The postnatal ossification process of the canine pectoral and pelvic limb was later investigated by Hare (1959; 1960). Seventeen dogs from four breeds were radiographed from birth at regular intervals to study the appearance of the ossification centres, while eight of them (three German Shepherds and five Collies) were used to observe the epiphyseal union. Given the small sample size, the Author himself states that the information given should be considered approximate. Moreover, the presented data are based on his own observations and those of previous works, making it impossible to deduce his own findings.

The appearance of the limb ossification centres was further described in a later study which included dogs from four breeds (German Shepherd, Collie, Bulldog and Beagle): twenty-four puppies were radiographed from birth to three weeks of age and fourteen of them were examined up to eight weeks. Observations were carried out at variable time intervals, which increased as the dogs grew older. Variation in the appearance of the ossification centres was observed in animals from different breeds and, to a lesser extent, among dogs from the same breed or litter. In particular, the ossification centres appeared earlier in Collies and German Shepherds than they did in Bulldogs and Beagles. However, the order of appearance of the OCs remained fairly constant in the sample population (Hare, 1961).

Following up on these studies, the epiphyseal fusion in the limbs of Greyhounds was investigated by examining twenty-eight puppies from six different litters. The observations started at thirteen weeks of age and continued approximately every fortnight. Towards the end of the study (60 weeks) the interval was extended to four weeks (Smith and Allcock, 1960; Smith, 1960). The results were quite in accordance with those given by Seoudi (1948) Pomriaskinsky-Kobozieff and Kobozieff (1954), Bressou et al. (1957) and Hare (1959; 1960), except for the proximal end of the radius and the distal tibial and fibular epiphyses.

The appearance and closure of the ossification centres of the pelvis were further examined by Smith (1964). Nearly four hundred dogs of different breeds were used in this study. The ossification centres of the pelvis were described and ten developmental stages were characterised.

In 1965 Chapman published the times of appearance and fusion of the ossification centres of the pectoral and pelvic limbs, based on the observations made on seven Beagles from two litters. Radiographs were made every three days from one day to two weeks of age, then once a week until 194 days, then every two weeks until the end of the study. The observations made from the 130th day of the experiment were based on only three dogs. A slight variation in the appearance of the OCs was observed, which however never exceeded ten days.

Another study on the epiphyseal fusion of the canine appendicular skeleton was carried out by Sumner-Smith (1966). Repeated observations of the same animals at different times were made on twenty-two Greyhound puppies from twelve to fifty weeks of age, but the time intervals were not specified. Another two hundred and fifty-nine dogs of different breeds and crossbreds of known age were radiographed on one occasion. A common sequence in the fusion of the secondary OCs was observed and variations in the time of fusion occurred even among siblings. Size didn't seem to be a determining factor of union times. The Author concludes that *<<the presence or absence of union of any centre is not a good method of estimating the age of a puppy, other than by very crude standards>>.*

Riser (1973) investigated the development of the normal canine pelvis, hip joints and femur in the Greyhound by using a sample of four female puppies, one of which was radiographed twice a day for the first four weeks of life and then daily until seven months, and a 1-year-old male. Weight gain, growth and shape of hips and femur and their histological development were recorded.

The development of the hip joints and the elbow was further investigated by Gustaffson and coll. (1975) in German Shepherds (28), Greyhounds (16) and their crossbred offspring (11). The pelvis and femur were radiographed every second day from day six until the appearance of both femoral heads, the elbow every week from week three until the olecranon and the anconeal process were observed. The pelvis of 11 Greyhounds and 21 German Shepherds was further radiographed once a week until the appearance of the acetabular bone. The OC of the anconeal process appeared first in German Shepherds, while the femoral head appeared first in the Greyhounds. The OC of the olecranon appeared at about the same age in all puppies. Closure of the growth plates occurred earlier in German Shepherds than in Greyhounds.

A broader sample population was examined by Yonamine and coll. (1980), who studied the skeletal development of the forelimb of two hundred and twenty-two Beagles from birth to fourteen months of age. Changes in body weight, the development of epiphyseal and diaphyseal ossification centres and the increase in length of bones were recorded. A grading system developed for assessing the skeletal age of children was used to describe the development of the forelimb.

The key features of the overmentioned studies are summarised in Table 6.

The appearance of the limb ossification centres was recently investigated in small-sized dogs. The study enrolled 27 puppies spontaneously dead at up to 28 days of age, which were subject to radiological, histological and morphometric investigations (Modina et al., 2017).

The timing of OC appearance and closure is also reported in textbooks of veterinary radiology (Ticer, 1984; Schebitz and Wilkens, 1989; Burk and Feeney, 2003; Dennis et al., 2010; Kealy and McAllister, 2011; Thrall and Robertson, 2011), anatomy (Dyce et al., 2018b), orthopaedics (Newton and Nunamaker, 1985) and paediatrics (Hoskins, 2001b; Peterson and Kutzler, 2011), based on the previously published studies or presumably on direct observations, although sometimes data do not correspond to the original reference – several mistakes were detected in Kealy and McAllister's (2011) textbook compared to the original reference from Ticer (1984) – or the source of information is not specified (Dennis et al., 2010).

The timing of appearance and closure of the limb OCs given in the consulted textbooks is reported in Annex 2. The same data are summarised in Tables 7 and 8.

Table 6. Published researches on limb ossification centres appearance and fusion.

Reference	Aim of the study	Sample size and breeds	Observation intervals
Seoudi, 1948	Epiphyseal union of the limbs	8 Egyptian Armant dogs	Observation intervals not specified
Pomriaskinsky-Kobozieff and Kobozieff, 1954	Appearance and fusion of the OCs of the dog's manus	7 German Shepherds 1 Cocker Spaniel 2 mongrels (Irish Setter x Épagneul Breton)	From birth to 8 months of age at regular intervals (2 German Shepherds radiographed daily until 42 days)
Bressou, 1957	Appearance and fusion of the OCs of the dog's pes	7 German Shepherds 1 Cocker Spaniel 2 mongrels (Irish Setter x Épagneul Breton)	From birth to 8 months of age at regular intervals (2 German Shepherds radiographed daily until 42 days)
Hare, 1959	Appearance and fusion of the OCs in the dog's forelimb	17 dogs from 4 breeds (appearance) 3 German Shepherds and 5 Collies (fusion)	Observation intervals not specified
Hare, 1960	Appearance and fusion of the OCs in the dog's hindlimb	17 dogs from 4 breeds (appearance) 3 German Shepherds and 5 Collies (fusion)	Observation intervals not specified
Hare, 1961	Appearance of the OCs in the dog's limbs	24 dogs from 4 breeds: German Shepherd, Collie (2 litters), Bulldog, Beagle	24 dogs from birth to 3 weeks, 14 dogs up to 8 weeks. Observation at 2 to 15 days intervals, at different times between breeds

Table 7. Published researches on limb ossification centres appearance and fusion (continued).

Smith and Allcock, 1960; Smith, 1961	Epiphyseal fusion in the Greyhound	28 Greyhounds from 6 litters	From 13 to 59 week approximately every fortnight (4 weeks interval towards the end of the series)
Smith, 1964	Appearance and fusion of the OCs in the dog's pelvis	Nearly 400 dog carcasses (breeds not specified) and a radiograph collection	One observation per dog (age not specified)
Chapman, 1965	Appearance and fusion of the OCs in the dog's limbs	7 Beagles from 2 litters (appearance) 3 Beagles (fusion)	Every 3 days from 1 day to 2 weeks of age; then every week until 194 days; then every 2 weeks until the end of the study
Sumner-Smith, 1966	Epiphyseal union of the dog's limbs	Longitudinal data from 22 Greyhounds from 4 litters Latitudinal data from 259 dogs from 29 breeds and crossbreds	Greyhounds radiographed repeatedly from 12 to 50 weeks; observation intervals not specified
Riser, 1973	Growth and development of canine pelvis, hip joints and femurs	4 female Greyhounds from 1 litter (1 dog actually radiographed) and a 1-year-old male	Twice daily for the first 4 weeks of life, daily for the next 26 weeks
Gustaffson et al., 1975	Appearance of some OCs in the dog's limbs	16 Greyhounds from 4 litters 28 German Shepherds from 4 litters 11 Crossbreds from 3 litters	Hip joints every second day from day 6. Elbow joint every week from week 3.
Yonamine et al., 1980	Appearance and fusion of the OCs in the forelimb of the Beagle	222 Beagles	1 day of age, 1 and 2 weeks, 1 to 6, 8, 10, 12 and 14 months of age

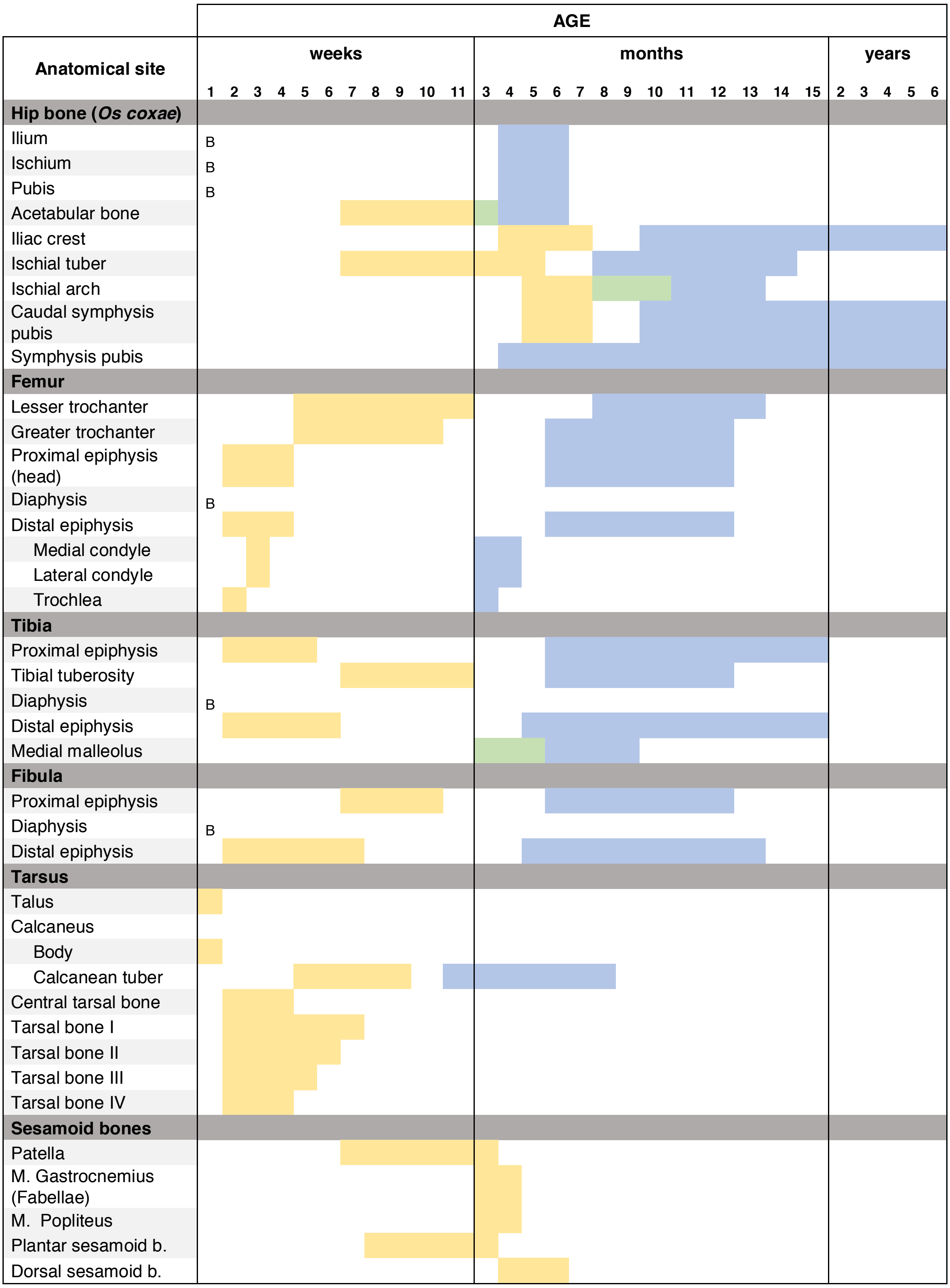
__Table 8.__ The interval between the earliest and the latest timing of OC appearance and closure in the forelimb. From: Ticer, 1984; Schebitz and Wilkens, 1989; Hoskins, 2001b; Dennis et al., 2010; Peterson and Kutzler, 2011; Thrall and Robertson, 2011; Dyce et al., 2018.

AGE

Anatomical site	**weeks**	**months**	**years**
	1 2 3 4 5 6 7 8 9 10 11	3 4 5 6 7 8 9 10 11 12 13 14 15	2 3 4 5 6
Scapula			
Body	B		
Supraglenoid tubercle			
Humerus			
Proximal epiphysis			
Diaphysis	B		
Distal epiphysis			
Medial condyle			
Lateral condyle			
Medial epicondyle			
Radius			
Proximal epiphysis			
Diaphysis	B		
Distal epiphysis			
Ulna			
Olecranon			
Anconeal process			
Diaphysis	B		
Distal epiphysis			
Carpus			
Intermedioradial carpal bone			
Radial carpal bone			
Central carpal bone			
Intermediate carpal b.			
Ulnar carpal bone			
Accessory carpal bone			
Body			
Epiphysis			
Carpal bone I			
Carpal bone II			
Carpal bone III			
Carpal bone IV			
Sesamoid bone m. abductor pollicis longus			
Metacarpus/Metatarsus			
Metacarpal bone I			
Proximal epiphysis			
Diaphysis	B		
Metacarpal bone II – V			
Diaphysis	B		
Distal epiphysis			
Phalanges (forelimb and hindlimb)			
Proximal phalanx I – V			
Proximal epiphysis			
Diaphysis	B		
Middle phalanx I – V			
Proximal epiphysis			
Diaphysis	B		
Distal phalanx I – V	B		
Sesamoid bones			
Palmar sesamoid bones			
Dorsal sesamoid bones			

**Yellow= appearance; Blue= closure; Green= overlap between appearance and closure.*

Table 9. *The interval between the earliest and the latest timing of OC appearance and closure in the hindlimb. From: Ticer, 1984; Schebitz and Wilkens, 1989; Hoskins, 2001b; Dennis et al., 2010; Peterson and Kutzler, 2011; Thrall and Robertson, 2011; Dyce et al., 2018.*



**Yellow= appearance; Blue= closure; Green= overlap between appearance and closure.*

3. AIM OF THE STUDY

In light of the arguments presented in the introduction, it is clear that the difficulty in determining the exact age of dog puppies is the most critical element that arises in judicial proceedings when trying to counteract the illegal puppy trade. To date, none of the methods used to estimate the age of puppies can be credited with a degree of accuracy and precision sufficient for medical-legal purposes due to both the many factors of variability of the measured biological phenomena (breed, sex, bloodline, diet, breeding environment, health status) and the lack of standardisation of the age estimation methods, such as visual teeth examination and skeletal development through radiographic examination.

In this study, the data obtained from repeated observations performed on a high number of dog puppies were compared with the information reported in the Literature, thus helping to update and broaden the specific knowledge regarding these biological phenomena and to identify breed-specific differences.

The main objective of this study was therefore to quantify the degree of correlation between the chronological age of dog puppies and the biological age that can be estimated by visual teeth examination and the radiographic examination of skeletal development. The secondary objective was to combine the information deriving from classical techniques in a predictive model in order to obtain greater accuracy in estimating the biological age of dog puppies.

4. MATERIALS AND METHODS

This study is part of the Project “Nuovi strumenti con finalità medico-legali per la valutazione dell’età dei cuccioli di cane” (IZSLER 12/15 RC), funded by the Italian Ministry of Health.
The protocol was evaluated by the animal-welfare body of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna “Bruno Ubertini” on 14th March 2017 (Prot. No. 7390 of 16/03/2017).
The dogs included in this study were all owned by private breeders, who were recruited according to their geographical proximity and availability.
After a first contact via phone call, a meeting with the breeder was arranged in order to clearly explain the aims and procedures of the research and to acquire their informed consent signature. It is worth mentioning that all the breeders contacted were enthusiastic about being included in the project, given the great relevance of this topic for their business activity.

4.1 ANIMALS

Eighteen litters of 10 different breeds were included, for a total of 93 puppies, 40 males and 53 females, as listed in Table 9. All puppies were born by healthy bitches, regularly dewormed and vaccinated before mating, with normal gestation, parturition and post-partum course. In French Bulldogs C-section was required, but in this breed it is considered a routine procedure.
When recruiting the dogs, an attempt was made to include breeds of different size and morphological type.
Size was determined on the basis of the adult weight indicated in the FCI (Fédération cynologique internationale) standard according to the following classification:

- Extra-small (XS): up to 4 kg;
- Small (S): 5 – 10 kg;
- Medium (M): 11 – 25 kg;
- Large (L): 26 – 44 kg;
- Giant (XL): more than 44 kg.

With regard to morphological type, Megnin's classification, reported by Bonetti (1995a), was taken as reference:

- Molossoid (MOL): voluminous head, round or cuboid; small and dropped ears; short muzzle; level bite or reverse scissors bite (undershot); long and thick lips; massive body of great structure.
- Lupoid (LUP): pyramid-shaped head; generally straight ears; elongated, narrow snout; scissors bite; small tight lips, the upper lips do not go beyond the base of the lower gums.
- Braccoid (BRA): prismatic head; big dropped ears; wide muzzle, separated from the front by a well-marked depression; scissors bite; long and hanging lips, the upper lips go beyond the lower jaw.
- Graioid (GRA): head shape of an elongated cone; narrow skull; small ears, lying backwards or straight; long and thin snout, in a straight line with the forehead; scissors bite; small, short and tight lips; slender body, tucked up belly.

Size and type distributions are reported in Tables 10 and 11.

Table 10. *Description of the sample examined: breed, size, morphological type, number of litters and puppies for each breed, sex distribution.*

Breed	***Size***	***Morphological type***	***No. of litters***	***No. of puppies***	***No. of males***	***No. of females***
Australian Shepherd (AUS)	L	LUP	1	8	8	0
Berger Blanc Suisse (PS)	L	LUP	2	11	4	7
Boxer (BOX)	L	MOL	2	10	2	8
French bulldog (BF)	M	MOL	3	10	5	5
German Shepherd (PT)	L	LUP	2	7	6	1
Labrador Retriever (LAB)	L	BRA	2	15	3	12
Nova Scotia Duck Tolling Retriever (NOV)	M	BRA	2	10	2	8
Pomeranian (POM)	XS	LUP	2	8	5	3
Saarloos Wolfdog (CLS)	L	LUP	1	12	4	8
Toy Poodle (BAR)	XS	BRA	1	2	1	1
TOTAL			**18**	**93**	**40**	**53**

Table 11. Sample distribution according to size.

Size	No. of puppies
Extra-Small	10
Medium	20
Large	63

Table 12. Sample distribution according to morphological type.

Morphological type	No. of puppies
Braccoid	27
Lupoid	46
Molossoid	20

Data collection started in May 2017 and ended in October 2018.
Litters were examined from 4 weeks to 20 weeks of age on a bi-weekly basis. However, depending on the breeder's availability, it was not always possible to carry out all the planned visits and to examine all of the puppies up to 20 weeks of age because they could be sold from the eighth week of age on.

4.2 CLINICAL AND TEETH EXAMINATION

Each puppy was subject to clinical and teeth examination on a bi-weekly basis from 4 weeks to 20 weeks of age, when not previously sold. The weight and clinical status of the puppies were checked in order to eventually exclude non-healthy animals. Subsequently, the development of deciduous and permanent dentition was assessed. Teeth examination was always performed by the same observer in order to avoid inter-observer variability. All the observations were noted on a specifically prepared record sheet (see Annex 3).
To describe the chronology of teeth development, a scoring system was adopted and a different number was assigned to each tooth according to its developmental stage:

Score	Developmental stage
0	Deciduous tooth – not yet erupted
1	Deciduous tooth – eruption just started
2	Deciduous tooth – eruption in progress
3	Deciduous tooth – eruption complete
4	Deciduous tooth – wearing started
5	Deciduous tooth – considerable wearing, thinning out
6	Deciduous tooth – shed
7	Permanent tooth – erupted

Any dental and occlusion anomalies were recorded.

Teeth development was also documented through photographs of the oral cavity in order to assure the possibility to double-check the results of the assessment at a later time.

4.3 Radiographic examination

From 6 to 16 weeks of age, on a bi-weekly basis, radiographic investigations were carried out to evaluate the skeletal development of the limbs.

In order to minimize the exposure of puppies and personnel to X-rays, only the lateral view of each limb was performed, which allows to examine most of secondary ossification centres of the appendicular skeleton. The puppy was placed in right lateral recumbency. To alleviate any superimposition of structures, the right forelimb was extended cranially and ventrally to the sternum, the opposite limb was pulled in a caudo-dorsal direction and the neck was extended dorsally; the right hindlimb was extended ventrally and the opposite limb was drawn caudally. Care was taken not to over-rotate the limb.

The correct positioning, aimed at restraining the animal without resorting to pharmacological sedation and at avoiding the overlapping of the anatomical structures, was made possible by the use of sandbags and the application of gauze ties on the extremities of the limbs (see Figures 4 and 5).

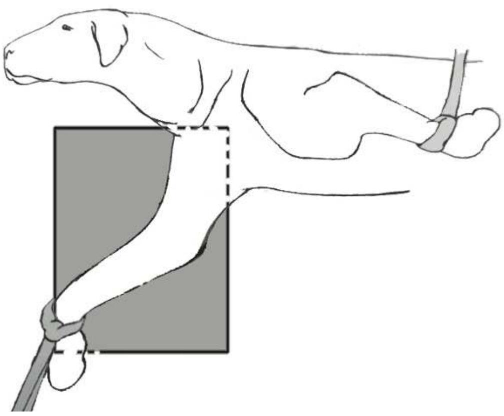


Figure 4. *Positioning for the lateral view of the forelimb OCs*

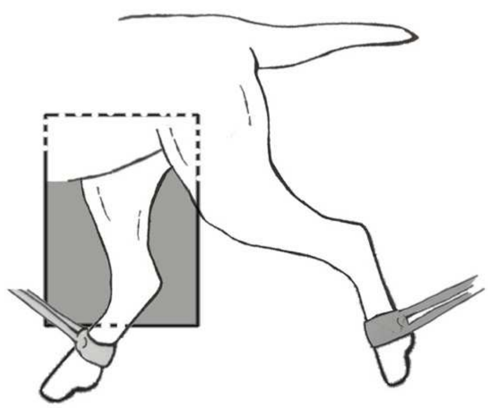


Figure 5. *Positioning for the lateral view of the hindlimb OCs*

The personnel involved wore protective equipment (lead aprons, gloves, thyroid collar) and a dosimeter.

Radiographic images were acquired with the portable X-ray unit Orange 1040HF assembled with the CR system iCR3600 or the Carestream Vita Flex.

The X-ray machine was set on 2.5 mAs, while kilovoltage was determined by following Sante's rule (2 x thickness + 40), according to the puppy's size and age.

For each puppy and at each observation time, forelimb and hindlimb ossification centres (OCs) were evaluated on medio-lateral view by two different operators. The OC was identified as a radiopaque area at the level of the future corresponding bone.

A scoring system was adopted as follows:

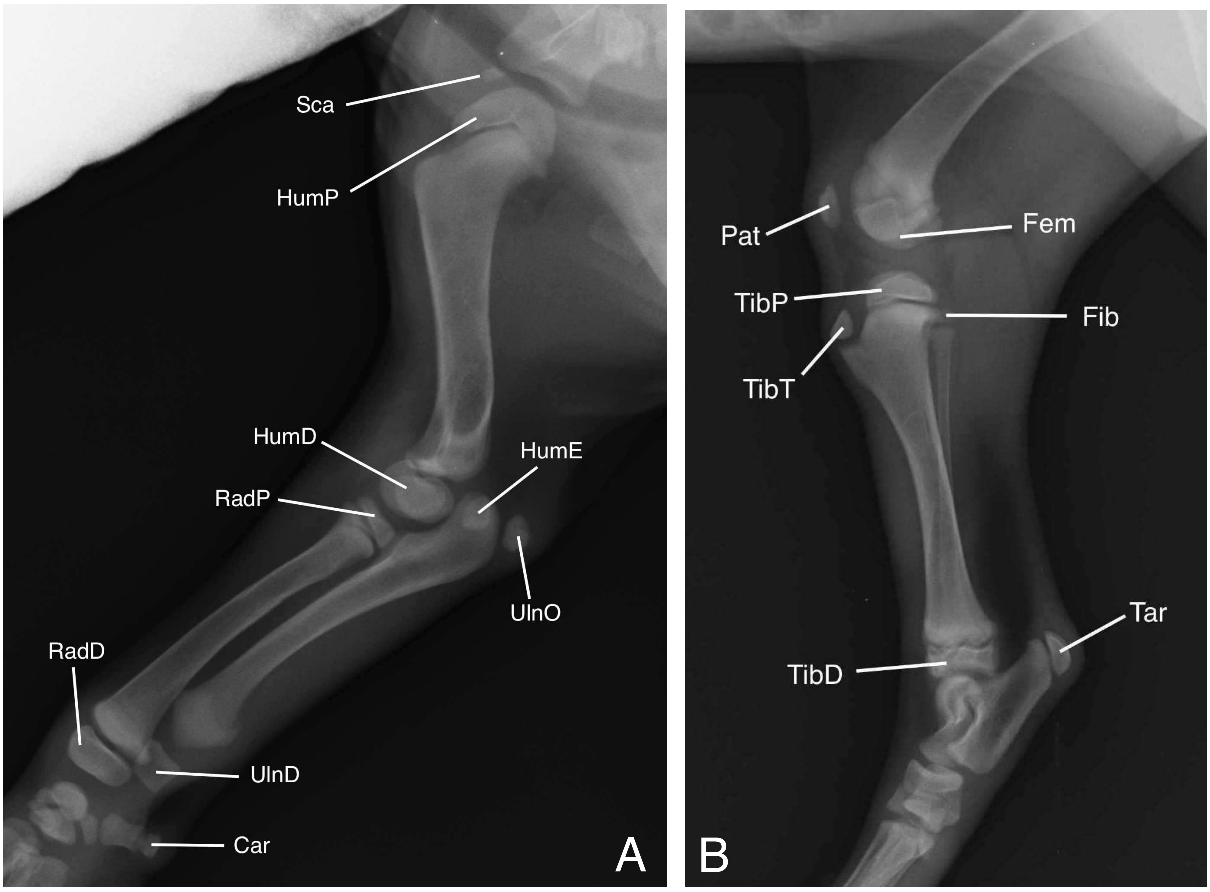
- 0= OC not developed;
- 1= OC present.

The following OCs were examined (see Fig. 6):

FORELIMB		**HINDLIMB**	
Scapula	*Supraglenoid tubercule (Sca)*	***Femur***	*Distal epiphysis (Fem)*
Humerus	*Proximal epiphysis (HumP)*	***Patella***	*(Pat)*
	Distal epiphysis (HumD)	***Fabellae***	*(Fab)*
	Ep. of medial epicondyle (HumE)	***Popliteal***	*(Pop)*
Radius	*Proximal epiphysis (RadP)*	***Tibia***	*Proximal ep. – tuberosity (TibT)*
	Distal epiphysis (RadD)		*Proximal ep. – condyles (TibP)*
Ulna	*Olecranon tuber (UlnO)*		*Distal epiphysis (TibD)*
	Distal epiphysis (UlnD)	***Fibula***	*Proximal epiphysis (Fib)*
Carpus	*Accessory carpal bone (Car)*	***Tarsus***	*Calcaneal tuber (Tar)*

Morphometry was assessed by radiographic measurements. OsiriX Lite (Version 11, Pixmeo SARL, 2019) was used to process DICOM images, while Digimizer (Version 5.3.5; MedCalc Software bv, 2019) was used to analyse TIFF images.

Diaphyseal length measurement was performed by drawing a perpendicular line to the distal diaphyseal end of the bone and the maximum length of the ossified diaphysis was measured along this line. The OC area was measured by drawing freehand the outline of the corresponding radiopaque area.



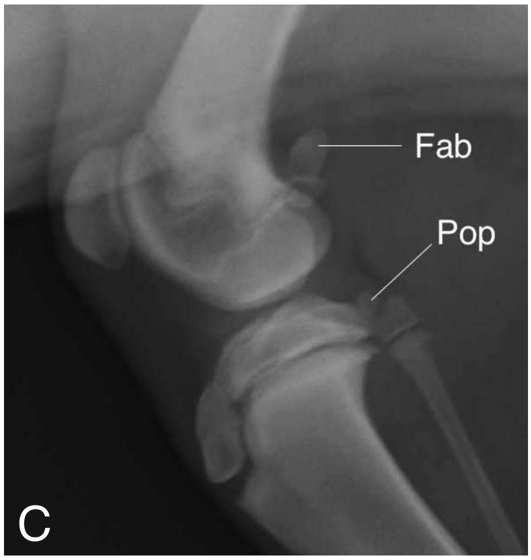


Figure 6.

- ***A.*** *Ossification centres examined in the forelimb (Saarloos Wolfdog, F, 6 weeks; medio-lateral view of the right forelimb);*
- ***B.*** *Ossification centres examined in the hindlimb (Saarloos Wolfdog, F, 8 weeks; medio-lateral view of the right hindlimb);*
- ***C.*** *Ossification centres for sesamoid bones of muscle Gastrocnemius (Fab) and muscle Popliteus (Pop) (Saarloos Wolfdog, F, 16 weeks; medio-lateral view of the right stifle joint).*

Abbreviations:
Fig. 6.A*, Sca= supraglenoid tubercule; HumP= proximal epiphysis of the humerus; HumE= epiphysis of medial epicondyle of the humerus; HumD= distal epiphysis of the humerus; RadP= proximal epiphysis of the radius; RadD= distal epiphysis of the radius; UlnO= olecranon; UlnD= distal epiphysis of the ulna; Car= epiphysis of the accessory carpal bone.*
Fig. 6.B*, Fem= distal epiphysis of the femur; Pat= patella; TibP= proximal epiphysis of the tibia; TibT= tibial tuberosity; TibD= distal epiphysis of the tibia; Fib= proximal epiphysis of the fibula; Tar= epiphysis of the calcaneal tuber.*
Fig. 6.C*, Fab= fabellae; Pop= popliteal.*

The following radiographic measurements were performed (see Fig. 7):

FORELIMB		abbr.	HINDLIMB		abbr.
Scapula	*Supraglenoid tubercule (area)*	aSca	***Patella***	*(area)*	aPat
Humerus	*Diaphysis (length)*	lHum	***Fabellae***	*(area)*	aFab
	Proximal ep. (area)	aHumP	***Popliteal***	*(area)*	aPop
Radius	*Diaphysis (length)*	lRad	***Tibia***	*Diaphysis (length)*	lTib
	Distal ep. (area)	aRadD		*Prox. ep. - tuberosity (area)*	aTibT
Ulna	*Diaphysis (length)*	lUln	***Fibula***	*Proximal ep. (area)*	aFib
	Olecranon tuber (area)	aUlnO	***Tarsus***	*Calcaneal tuber (area)*	aTar

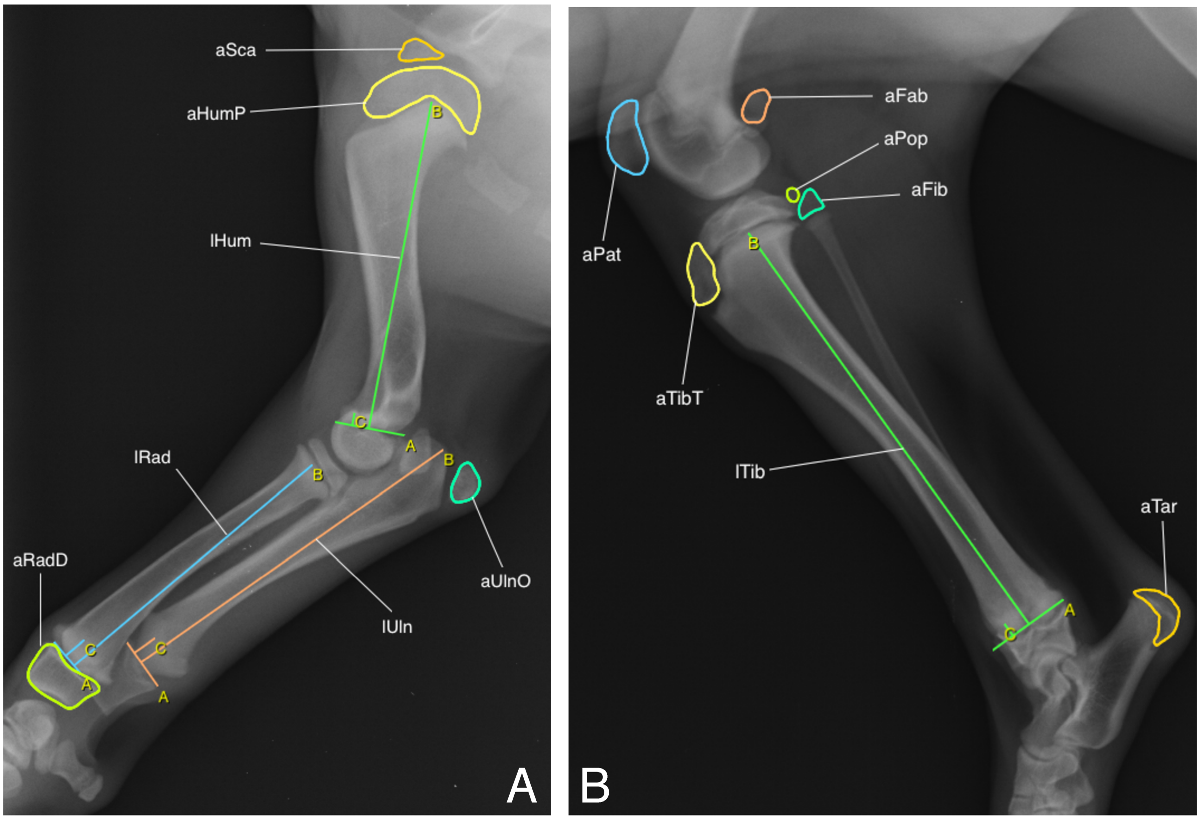


Figure 7.
A. *Radiographic measurements performed in the forelimb (Berger Blanc Suisse, F, 10 weeks; medio-lateral view of the right forelimb);*
B. *Radiographic measurements performed in the hindlimb (Saarloos Wolfdog, F, 16 weeks; medio-lateral view of the right hindlimb).*

4.4 Statistical analysis

Statistical analysis was performed using Stata Statistical Software (Release 15; StataCorp, 2017).

A teeth development score and a skeletal development score were calculated by counting respectively the number of deciduous teeth and ossification centres present at each observation point and in each breed.

The development of deciduous teeth during the observation interval was analysed and any differences among breeds were highlighted.

The appearance of the selected ossification centres was evaluated at each observation point; data were analysed both as a whole and then grouped by breed. A more in-depth analysis on the 6 to 8 weeks interval was carried out.

The average percentage increase in the radiographic measurements of the diaphyseal lengths and OC areas per each breed was calculated.

Finally, an attempt was made to set up a predictive model to determine the age of puppies. In a first exploratory phase, a classification tree was used.

Classification trees are powerful instruments for multiple variable analysis, where the outcome is the result of the combined effects of multiple input variables or factors, as it can be the case of biological phenomena such as teeth and skeletal development. Classification trees can accommodate continuous, ordinal and categorical variables as inputs and maintain accuracy even with many missing data and in presence of nonlinear relationships between variables. Moreover, they are quite straightforward to understand, interpret and visualize.

Each box in the tree represents a node. The top node is called *root node* and contains all the instances in the training dataset. Each node splits into new nodes (*children nodes*), connected by branches. The splitting process is continued until a user-defined stopping criterion is reached, e.g. the minimum number of observations per node. The maximum depth of the tree, which is used to control over-fitting, is also user-defined.

Terminal nodes, also called *leaf nodes*, represent classifications and are then very important when the tree is used for prediction.

Non-binary classification trees, i.e. trees where more than two branches can attach to a single root or node, are built with the CHAID algorithm. The acronym CHAID stands

for Chi-squared Automatic Interaction Detector; in these trees the Chi-square test is used to determine the best next split at each step.
Classification trees are very consistent with a given dataset (training dataset), but the challenge arises when the tree is tested with new data. The classification of new cases (testing dataset) allows to assess the predictive validity of the model. However, even small variations in the data may result in a different tree and this makes it unstable and therefore unsuitable for prediction. Variance can be reduced by increasing the sample size or growing random forests, which result in a more stable classification and variable importance measure. A random forest fits many classification trees to a dataset and then combines the predictions from all trees. It then gives an estimate of which variables are important in the classification.
Each classification tree in the random forest is built on a small number of variables, randomly and independently selected for each node (*mtry*). This number is usually the square root of the total number of variables. Unlike single classification trees, each tree in the forest is grown to the largest extent possible and there is no pruning.
Each tree is built using a different bootstrap sample from the entire dataset, which includes about two-thirds of the observations; the remaining one-third (the so-called out-of-bag data) is used to perform an estimate of the classification error and variable importance. In this way, there is no need for a separate testing dataset to get an estimate of the error as it is estimated internally during the run.

5. RESULTS

5.1 CLINICAL AND TEETH EXAMINATION

The puppies were in good health and made a steady weight gain through the observation period. The growth curves for each breed are illustrated in Fig. 8; the mean weight and standard deviation for each breed are reported in Annex 4, Table 4.1.

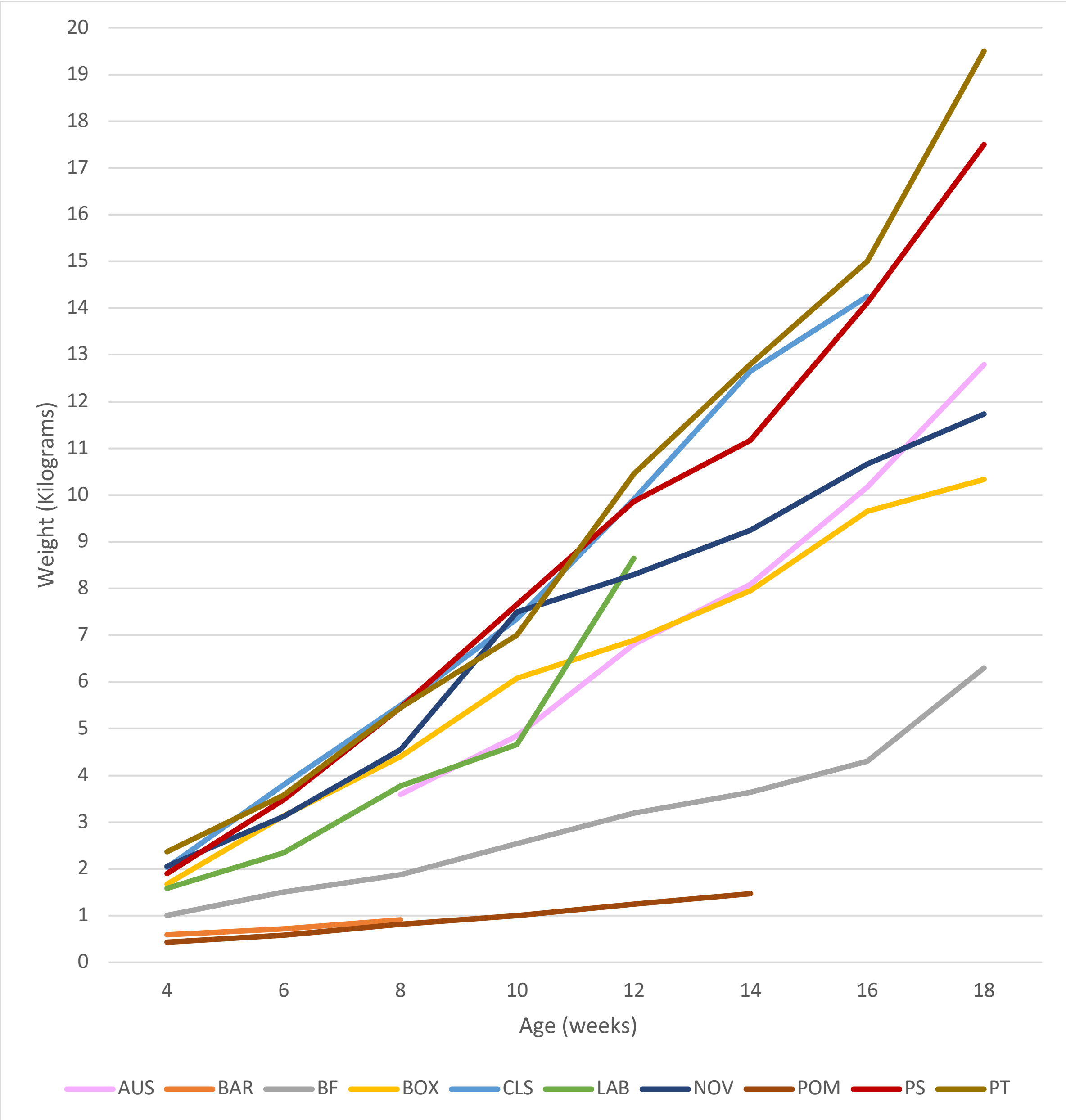


Figure 8. *Mean growth curves for 10 breeds of dogs (SD bars are excluded for clarity). AUS= Australian Shepherd, BAR= Toy Poodle; BF= French Bulldog; BOX= Boxer; CLS= Saarloos Wolfdog; LAB= Labrador Retriever; NOV= Nova Scotia Duck Tolling Retriever; POM= Pomeranian; PS= Berger Blanc Suisse; PT= German Shepherd.*

Table 12 illustrates in detail how many puppies for each breed were subjected to clinical and teeth examination at each timepoint. A total of 443 teeth examinations were performed, documented by more than 3,000 pictures. Reference pictures for the various breeds at each observation point are reported in Annex 4, Figures 4.1 – 4.10.

Table 13. *Number of puppies examined at each timepoint*

Breed	No. of puppies	No. of puppies examined at:								
		4 w	**6 w**	**8 w**	**10 w**	**12 w**	**14 w**	**16 w**	**18 w**	**20 w**
Australian Shepherd	8	8	8	8	8	0	7	3	2	2
Berger Blanc Suisse	11	11	11	11	6	6	5	4	3	1
Boxer	10	3	10	10	8	0	5	4	3	3
French Bulldog	10	9	9	9	6	6	1	2	2	0
German Shepherd	7	7	7	7	4	2	1	1	1	0
Labrador Retriever	15	15	15	15	14	7	1	0	0	0
Nova Scotia Duck Tolling Retriever	10	10	10	10	4	4	0	0	3	3
Pomeranian	8	8	7	7	7	7	4	3	0	0
Saarloos Wolfdog	12	12	12	12	8	6	6	2	0	0
Toy Poodle	2	2	2	2	0	0	0	0	0	0
TOTAL	**93**	**85**	**91**	**91**	**65**	**38**	**30**	**19**	**14**	**9**
PERCENTAGE	**100%**	**91%**	**97%**	**97%**	**69%**	**40%**	**32%**	**20%**	**15%**	**9%**

A dental score was calculated by counting the average number of deciduous teeth in each breed at each observation time. The deciduous teeth (I1, I2, I3, C1, P2, P3, P4) of the upper and lower arch scoring from 1 (eruption just started) to 5 (considerable wearing) were included; the score was calculated on one hemiarch, therefore a puppy could have a maximum score of 14 (Table 13).

The score showed an increasing trend up to 12-14 weeks, when it started to decrease due to the shedding of the deciduous teeth.

Table 14. Breed teeth development score at each timepoint calculated by summing the number of deciduous teeth present out of 14 (maxillary and mandibular I1, I2, I3, C1, P2, P3, P4).
(AUS= Australian Shepherd, BAR= Toy Poodle; BF= French Bulldog; BOX= Boxer; CLS= Saarloos Wolfdog; LAB= Labrador Retriever; NOV= Nova Scotia Duck Tolling Retriever; POM= Pomeranian; PS= Berger Blanc Suisse; PT= German Shepherd)

Breed	Age								
	4 w	**6 w**	**8 w**	**10 w**	**12 w**	**14 w**	**16 w**	**18 w**	**20 w**
AUS	8	12	13	14	-	13	10	6	4
BAR	1	3	9	-	-	-	-	-	-
BF	4	10	11	13	13	13*	10	7	-
BOX	9	13	13	14	-	12	9	4	2
CLS	12	14	14	14	14	14	13	-	-
LAB	9	13	14	14	14	14	-	-	-
NOV	12	13	14	14	14	-	-	8	6
POM	2	10	12	13	13	13	14	-	-
PS	11	13	14	14	14	14	12	9	7*
PT	13	14	14	14	14	14	13*	11*	-

**observations made on one puppy*

The level of dental development among the different breeds at 4 weeks of age was extremely varied: on average, in Toy Poodles only one tooth was erupted, two teeth in Pomeranians and 4 teeth in French Bulldogs. Australian Shepherds had 8 teeth, Boxers and Labradors 9. Berger Blanc Suisse puppies showed 11 teeth, Nova Scotia Duck Tolling Retrievers and Saarloos Wolfdogs had on average 12 teeth while German Shepherds had already 13 out of 14 teeth (Annex 4, Fig. 4.1).

At 6 weeks the number of erupted teeth increased to 10 both in Pomeranians and French Bulldogs, while in Toy Poodles it was only 3. Saarloos Wolfdogs and German Shepherds had all the deciduous teeth. Labradors, Nova Scotia Duck Tolling Retrievers and Berger Blanc Suisse puppies still missed one tooth and at 8 weeks their deciduous dentition was complete (Annex 4, Fig. 4.2).

At 8 weeks, Toy Poodles still had only 9 teeth; they were sold right after that age so it was not possible to examine them any further (Annex 4, Fig. 4.3).

In Australian Shepherds and Boxers deciduous teeth eruption was complete at 10 weeks (Annex 4, Fig. 4.4).

At 12 weeks, some French Bulldogs and Pomeranians were still missing one tooth, which was observed at 14 weeks in the former and only at 16 weeks in the latter (Annex 4, Fig. 4.6).

Between 14 and 16 weeks, deciduous teeth began to shed in all the other breeds and puppies showed a mixed dentition (Annex 4, Figs. 4.7, 4.8).

A variable number of deciduous teeth was still present at 20 weeks in the four breeds that it was possible to examine: Australian Shepherd, Boxer, Nova Scotia Duck Tolling Retriever and Berger Blanc Suisse (Annex 4, Fig. 4.10).

In order to better interpret the variability observed among the different breeds, the development of each deciduous tooth in the examined breeds was investigated individually.

The following graphs illustrate the trend of the average score per breed of each single tooth during the observation period.

Figure 9A. *Mean score of the maxillary (graph on the left) and mandibular (graph on the right) first incisor (I1) at the different observation points. The black vertical lines represent the critical ages according to the legal framework. Each coloured line represents a breed (colour legend at the bottom).*

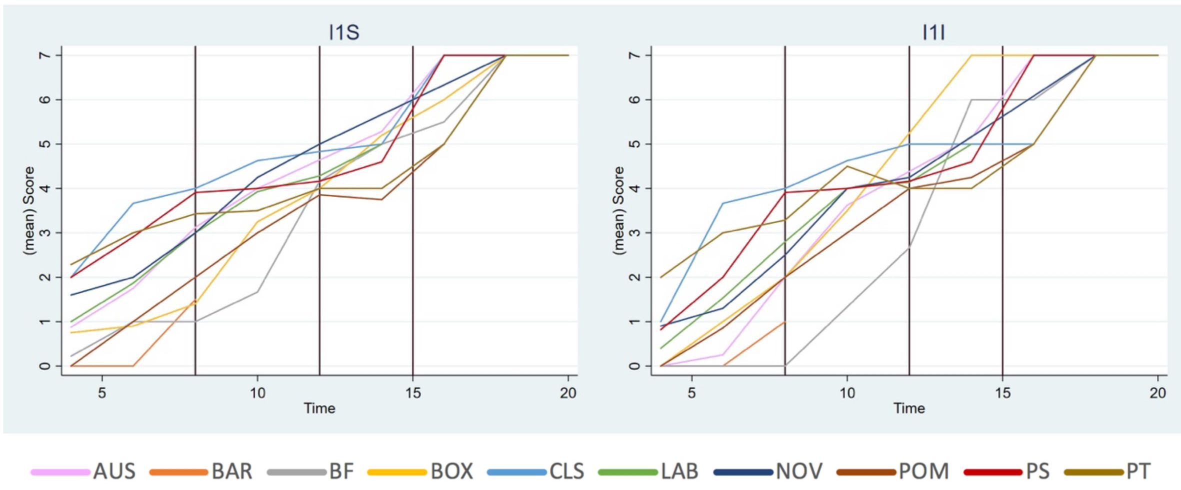


Figure 9B. *Mean score of the maxillary (graph on the left) and mandibular (graph on the right) second incisor (I2) at the different observation points. The black vertical lines represent the critical ages according to the legal framework. Each coloured line represents a breed (colour legend at the bottom of the page).*

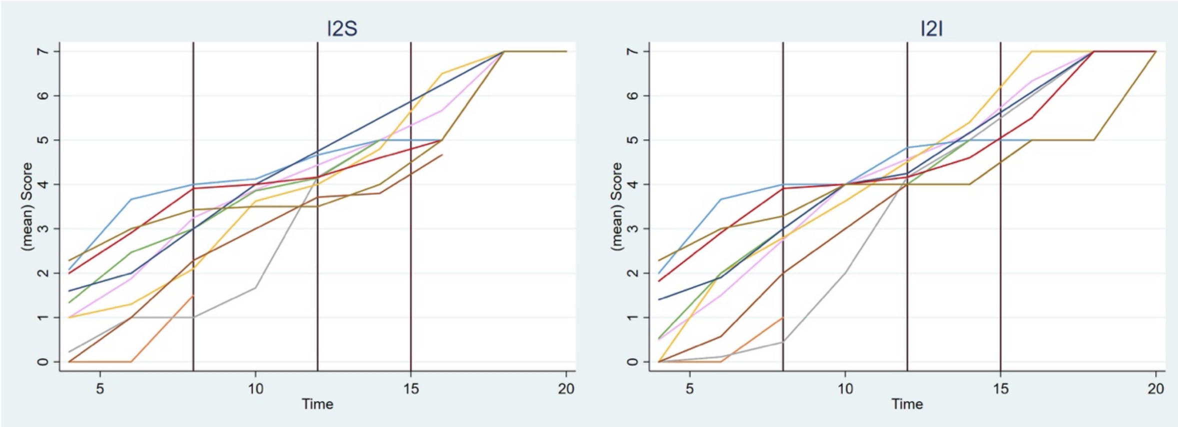


Figure 9C. *Mean score of the maxillary (graph on the left) and mandibular (graph on the right) third incisor (I3) at the different observation points. The black vertical lines represent the critical ages according to the legal framework. Each coloured line represents a breed (colour legend at the bottom of the page).*

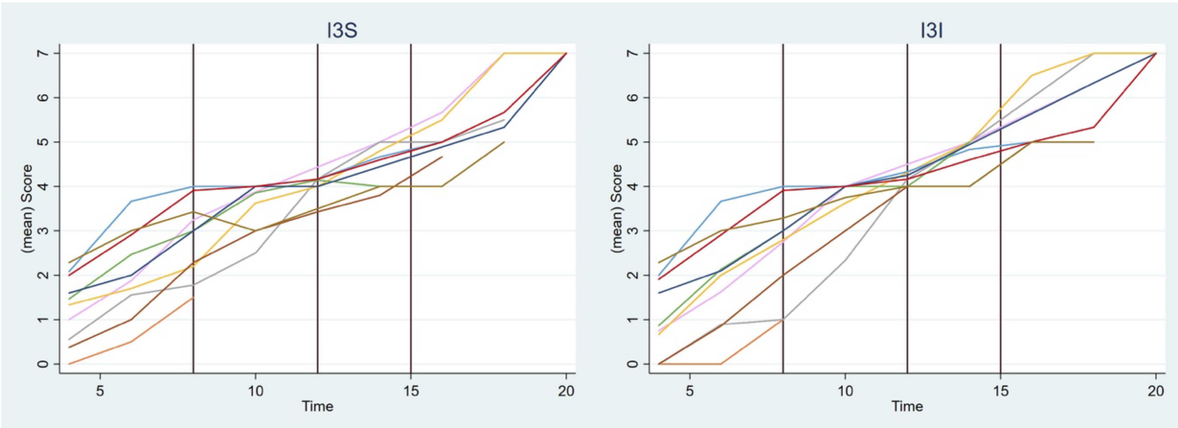


Figure 9D. *Mean score of the maxillary (graph on the left) and mandibular (graph on the right) canine tooth (C1) at the different observation points. The black vertical lines represent the critical ages according to the legal framework. Each coloured line represents a breed (colour legend at the bottom of the page).*

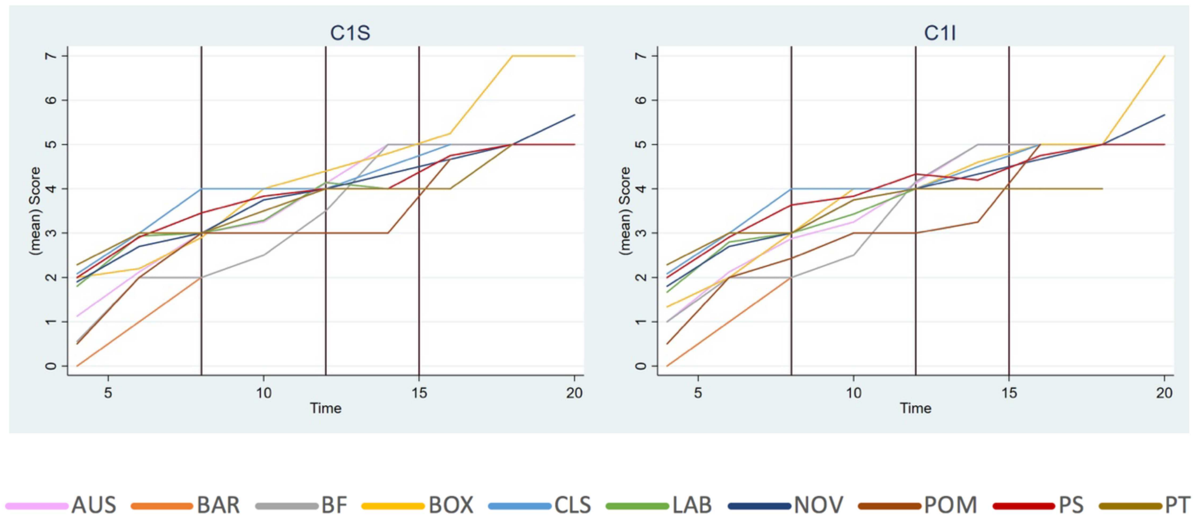


Figure 9E. *Mean score of the maxillary (graph on the left) and mandibular (graph on the right) second premolar (P2) at the different observation points. The black vertical lines represent the critical ages according to the legal framework. Each coloured line represents a breed (colour legend at the bottom of the page).*

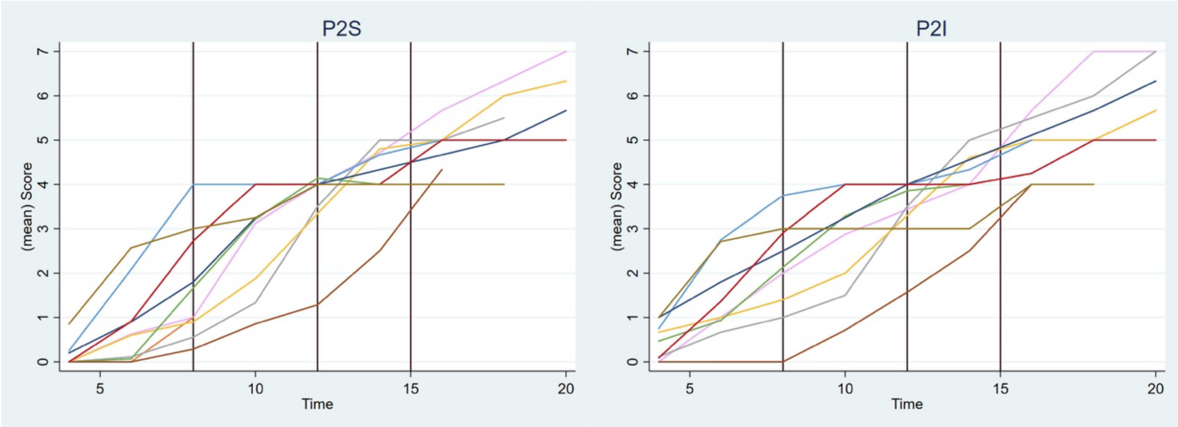


Figure 9F. *Mean score of the maxillary (graph on the left) and mandibular (graph on the right) third premolar (P3) at the different observation points. The black vertical lines represent the critical ages according to the legal framework. Each coloured line represents a breed (colour legend at the bottom of the page).*

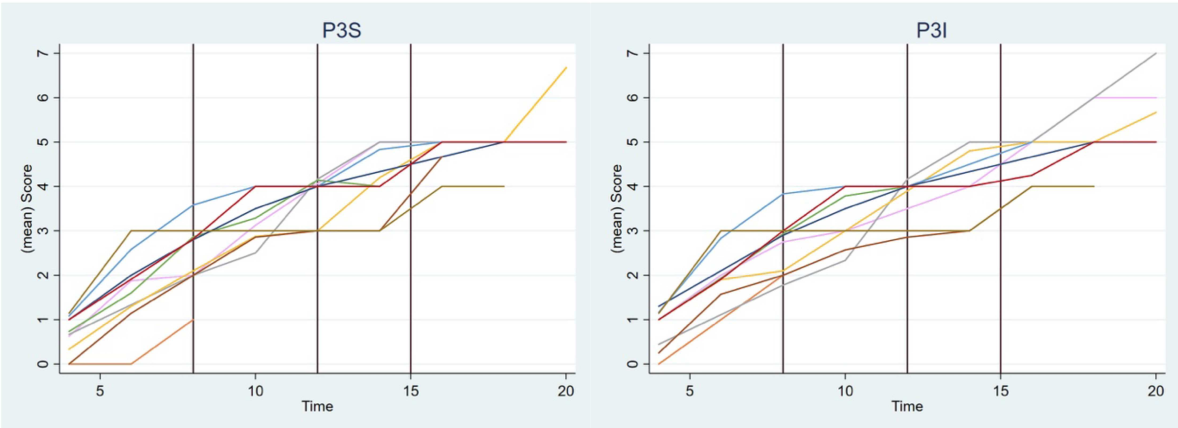
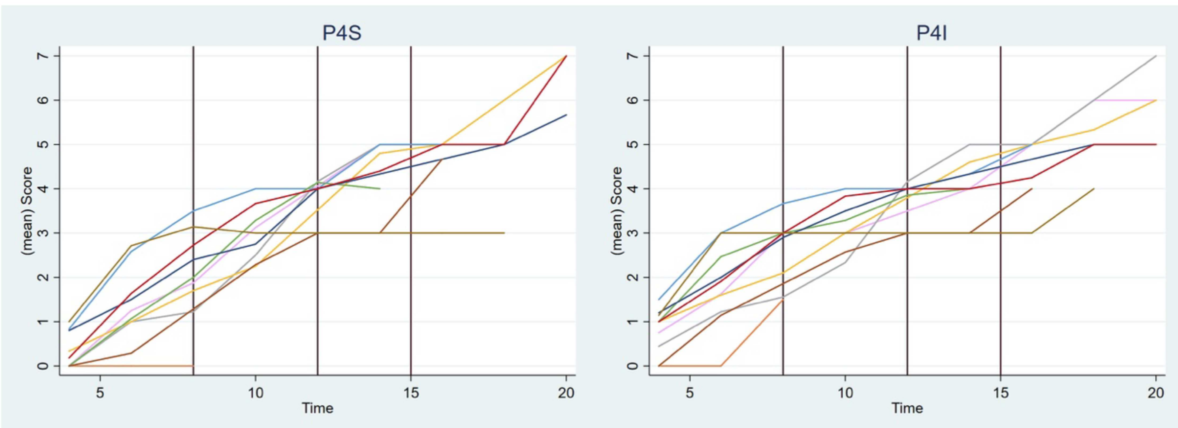


Figure 9G. *Mean score of the maxillary (graph on the left) and mandibular (graph on the right) fourth premolar (P4) at the different observation points. The black vertical lines represent the critical ages according to the legal framework. Each coloured line represents a breed (colour legend at the bottom of the page).*



AUS BAR BF BOX CLS LAB NOV POM PS PT

In general, maxillary teeth erupted earlier than mandibular teeth and a common sequence of eruption was observed: the first to appear were the canine teeth, followed by the incisors, from the corners to the nippers, then the third and fourth premolars and lastly the second premolar.
The eruption of incisor teeth was much slower in Toy Poodles and French Bulldogs. Indeed, in these breeds incisors had just started to erupt at 8 weeks of age (see for example the mandibular third incisor, Fig. 9C) or were even absent, as in the case of the mandibular first incisor in all the French Bulldog puppies (Fig. 9A). At 8 weeks the eruption of the incisors was in progress in Pomeranian and Boxers, while it was complete in Labrador Retrievers, Nova Scotia Duck Tolling Retrievers, Australian Shepherds (with the exception of the mandibular first incisor), German Shepherds, Berger Blanc Suisses and Saarloos Wolfdog, where in some cases wearing had already started. At 12 weeks incisors were completely erupted in all breeds and showed a certain degree of wearing. At 15 weeks the situation was again variable, with teeth shedding already starting in some breeds, this time from nippers to corners. The remaining deciduous teeth showed an increased degree of wearing and thinning out.
The canine teeth were already erupted at 4 weeks in all breeds except for some Pomeranians, French Bulldogs and all Toy Poodles (Fig. 9D). In these puppies the eruption was yet to be completed even at 8 weeks of age. Between 12 and 15 weeks canines continued to wear out but no shedding was observed until 18 weeks of age and only in Boxers. In Pomeranians, the wearing of canine teeth followed a much slower course compared to the other breeds.
At 4 weeks, premolars were not present in all breeds (Fig. 9E-G). At 8 weeks the premolars could be observed in all puppies except from Toy Poodles, which lacked the maxillary P4 and the mandibular P2. At the same age the maxillary second premolar was also absent in some French Bulldogs and Pomeranians, which completely lacked the mandibular P2 as well. In Pomeranians the second premolar reached the complete development at only 15 weeks. In the other breeds the completion of the eruption occurred at variable times, but in any case, within 12 weeks. Saarloos Wolfdogs and German Shepherds were the most precocious puppies; in these breeds the eruption of all premolars was already complete at around 6 weeks. At 15 weeks premolars were still in place in all puppies, showing variable degrees of wearing.

All Toy Poodles were sold at 8 weeks of age, Labradors at 14 weeks and Saarloos Wolfdogs at 16 weeks. Nova Scotia Duck Tolling Retriever could not be examined at 14 and 16 weeks of age. Therefore, exhaustive considerations on the eruption of the permanent teeth could not be made.

The appearance of the permanent dentition could be observed at 14 weeks in Australian Shepherds and Boxers, where permanent incisors and the first premolar began to erupt. At 16 weeks Australian Shepherds also showed the permanent second premolar, while in Saarloos Wolfdogs, German Shepherds and Berger Blanc Suisse puppies the first permanent incisors and the first premolar appeared. The permanent canines could be observed at 18 weeks in Boxers. The first molar began to be visible at 14 weeks in Boxers, at 16 weeks in Australian Shepherds, at 18 weeks in French Bulldogs and in Berger Blanc Suisse puppies.

Dental and occlusion anomalies were observed in some puppies.

All of the French Bulldogs and Boxers showed marked brachygnathism, while one Labrador had a class II malocclusion (short mandible) which resulted in the lower canine tooth traumatizing the maxillary gingiva (Fig. 10A).

As regards abnormalities in the number of teeth, polydontia was observed in three breeds: 2/10 French Bulldogs and 7/10 Boxers had from 1 to 4 supernumerary incisors (Fig. 10B), 1/15 Labrador had an extra maxillary second premolar.

The absence of one tooth was instead observed in 1/11 Berger Blanc Suisse puppy, which missed a maxillary first incisor both in the deciduous and in the permanent dentition (Fig. 10C).

Figure 10. *Developmental anomalies:* ***A.*** *10-weeks-old Labrador with mandibular micrognathia;* ***B.*** *4-months-old Boxer with polydontia;* ***C.*** *6-weeks-old Berger Blanc Suisse with a missing tooth.*

5.2 RADIOGRAPHIC EXAMINATION

Radiographic investigations to assess limb development produced a total of 536 X-rays. Representative X-rays for each observation point are reported in Annex 5, Figures 5.8 – 5.27.

Table 14 illustrates in detail how many puppies for each breed were subjected to radiographic examination at each timepoint. Australian Shepherds were excluded because they proved to be particularly stressed and agitated even during normal handling procedures.

Table 15. *Number of puppies radiographed at each timepoint.*

Breed	Total no. of puppies	No. of puppies examined at:					
		6 w	8 w	10 w	12 w	14 w	16 w
Berger Blanc Suisse	11	4	10	6	6	5	4
Boxer	10	10	10	8	0	5	3
French Bulldog	10	9	5	5	6	1	2
German Shepherd	7	7	7	4	1	1	1
Labrador Retriever	15	15	15	14	3	0	0
Nova Scotia Duck Tolling Retriever	10	0	10	4	4	0	0
Pomeranian	8	7	6	7	7	4	3
Saarloos Wolfdog	12	12	12	8	6	5	2
Toy Poodle	2	0	2	0	0	0	0
TOTAL	**85**	**64**	**77**	**56**	**33**	**21**	**15**
PERCENTAGE	**100%**	**75%**	**90%**	**65%**	**38%**	**24%**	**17%**

The appearance of the selected ossification centres was evaluated at each observation point; data were analysed both as a whole and grouped by breed.

At 6 weeks, i.e. the first observation point, some ossification centres were already present in all the puppies: the proximal and distal epiphyses of the humerus (HumP, HumD) in the forelimb, the distal epiphysis of the femur (Fem) and the proximal and distal epiphyses of the tibia (TibP, TibD) in the hindlimb. The remaining OCs were observed in a variable number of puppies. The appearance of the OCs proceeded gradually until, at 12 weeks, 100% of the puppies presented all the examined OCs, except for the fabellae and popliteus. The former was observed only in 19% of puppies at 12 weeks and in 82% of puppies at 16 weeks; the latter was observed for the first time at 16 weeks in only one Saarloos Wolfdog puppy (Fig. 11).

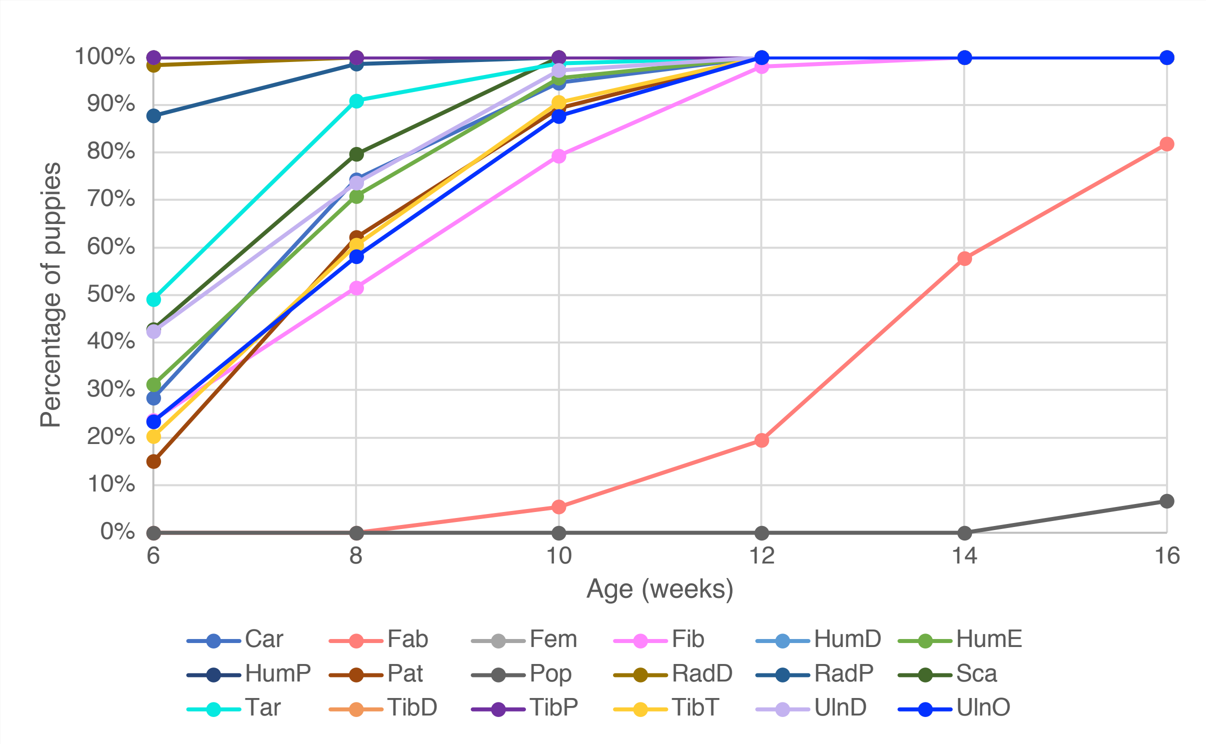


Figure 11. *Appearance of the limb ossification centres in the entire sample.*

The results were then grouped by breed. For each ossification centre, tables reporting the percentage of puppies of each breed in which the OC was observed at each time point are reported in Annex 5. Toy poodles (BAR) were excluded since only 2 puppies were radiographed one single time at 8 weeks.

The appearance of the single OCs in each breed was further investigated in the 6 to 8 weeks interval. Only data available at both observation times were selected. Toy poodles (BAR) and Nova Scotia Duck Tolling Retrievers (NOV) were excluded from the analysis because they were not radiographed at 6 weeks.

The following graphs illustrate the percentage of puppies for each breed in which the single OCs were observed at 6 vs 8 weeks. Graphs for the proximal and distal epiphyses of the humerus (HumP, HumD), the distal epiphysis of the radius (RadD), the distal epiphysis of the femur (Fem) and the proximal and distal epiphyses of the tibia (TibP, TibD) are not shown since these OCs were already present in all puppies at 6 weeks.

At 6 weeks, the supraglenoid tubercule (Sca) was absent in French Bulldogs and Pomeranians, while it was present in 11% of Labradors and 37% of Boxers. It was instead visible in all Saarloos Wolfdogs and German Shepherds. At 8 weeks, the OC appearance reached 100% only in Boxers, whereas it increased by only 1% in Labradors and by 50% in French Bulldogs (Fig. 12). Data regarding Berger Blanc Suisse and Nova Scotia Duck Tolling Retriever breeds are not shown because observations at 6 weeks were not available; however, at 8 weeks the OC was observed in 100% of puppies.

At 6 weeks, the epiphysis of medial epicondyle of the humerus (HumE) was absent in French Bulldog, Boxer, Labrador, Pomeranian and Berger Blanc Suisse puppies, while it was present in all Saarloos Wolfdogs and German Shepherds. At 8 weeks, the OC appeared in all Berger Blanc Suisse puppies, whereas it was observed only in 2/14 Labradors, 1/3 Pomeranians, 1/2 French Bulldogs and 7/10 Boxers (Fig. 13). At 8 weeks, the OC was present in 100% of Nova Scotia Duck Tolling Retriever puppies (data not shown).

The proximal epiphysis of the radius (RadP) was observed in all 6-weeks-old puppies except from Pomeranians, whereas at 8 weeks it was visible only in half of them (Fig. 14). At 8 weeks, the OC was present in 100% of Nova Scotia Duck Tolling Retrievers as well (data not shown).

At 6 weeks, the olecranon (UlnO) was absent in French Bulldog, Boxer, Labrador, Pomeranian and Berger Blanc Suisse puppies, while it was present in 43% of German Shepherds and in all Saarloos Wolfdogs. At 8 weeks, the OC was still absent in French Bulldogs (5), whereas it was observed in 6% of Labradors, 25% of Pomeranians, 30% of Boxers, 85% of German Shepherds and in all Berger Blanc Suisse (Fig. 15) and Nova Scotia Duck Tolling Retriever puppies (data not shown).

At 6 weeks, the distal epiphysis of the ulna (UlnD) was absent in French Bulldogs, Labradors and Pomeranians, whereas it was observed in half of the Boxer and Berger Blanc Suisse puppies and in all Saarloos Wolfdogs and German Shepherds. At 8 weeks, the OC was still absent in French Bulldogs and Pomeranians, while it appeared in 5/14 Labradors and 7/8 Boxers and in all Berger Blanc Suisse (Fig. 16). At 8 weeks, all Nova Scotia Duck Tolling Retriever puppies presented this OC as well (data not shown).

At 6 weeks, the epiphysis of the accessory carpal bone (Car) was absent in French Bulldog, Boxer, Labrador, Pomeranian and Berger Blanc Suisse puppies, while it was present in 5/6 German Shepherds and in all Saarloos Wolfdogs. At 8 weeks, the OC appeared in 7% of Labradors, 60% of French Bulldogs, 70% of Boxers, and in all Pomeranian, German Shepherd and Berger Blanc Suisse puppies (Fig. 17). The OC was also present in all the 8-weeks-old Nova Scotia Duck Tolling Retriever puppies (data not shown).

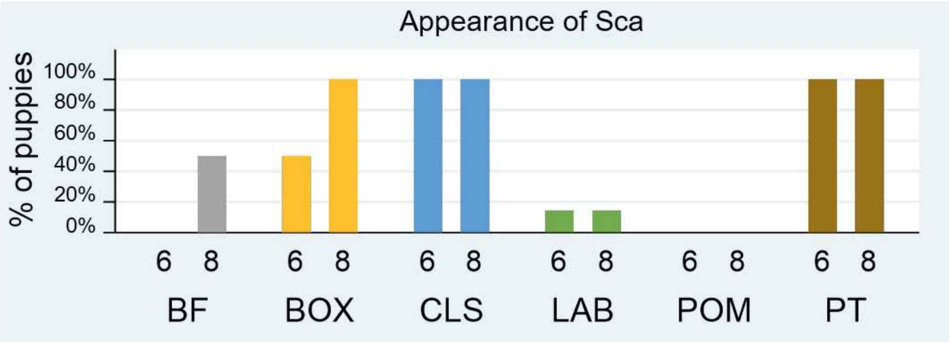


__Figure 12.__ Appearance of the supraglenoid tubercule (Sca) in each breed during the 6-8 weeks interval.

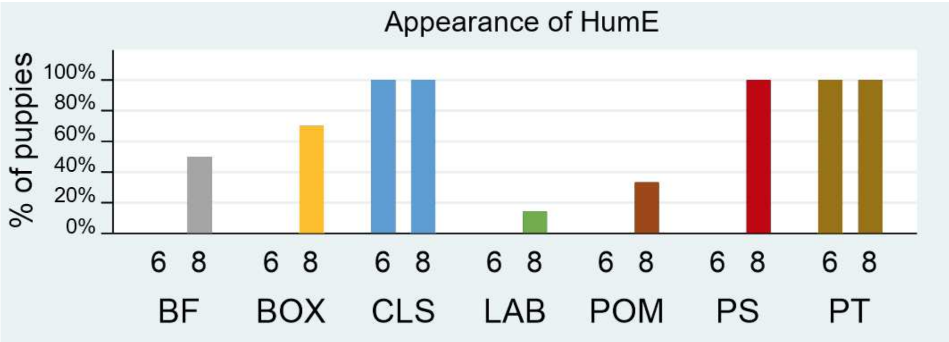


__Figure 13.__ Appearance of the epiphysis of medial epicondyle of the humerus (HumE) in each breed during the 6-8 weeks interval.

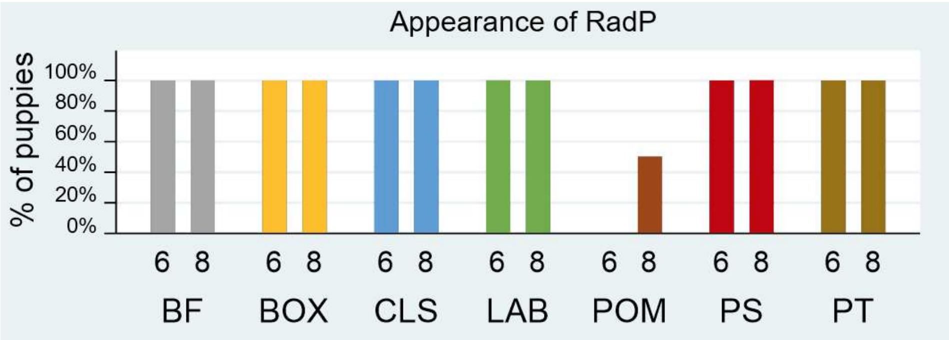


__Figure 14.__ Appearance of the proximal epiphysis of the radius (RadP) in each breed during the 6-8 weeks interval.

BF= French Bulldog; BOX= Boxer; CLS= Saarloos Wolfdog; LAB= Labrador Retriever; POM= Pomeranian; PS= Berger Blanc Suisse; PT= German Shepherd.

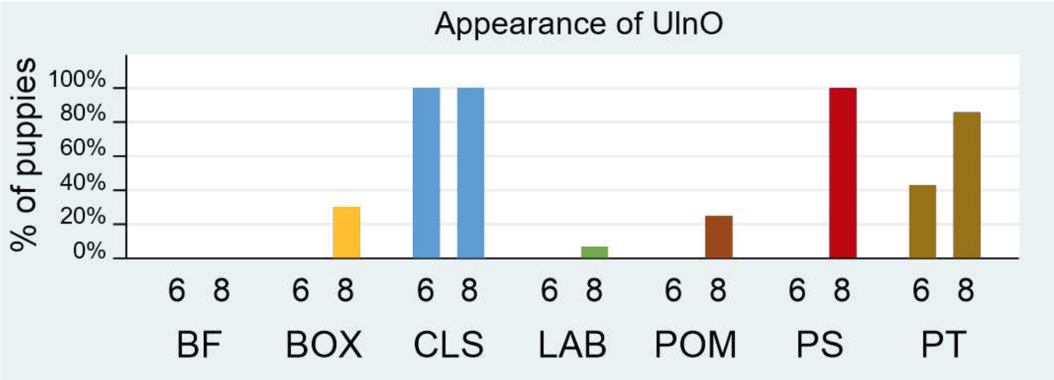


Figure 15. *Appearance of the epiphysis of the olecranon (UlnO) in each breed during the 6-8 weeks interval.*

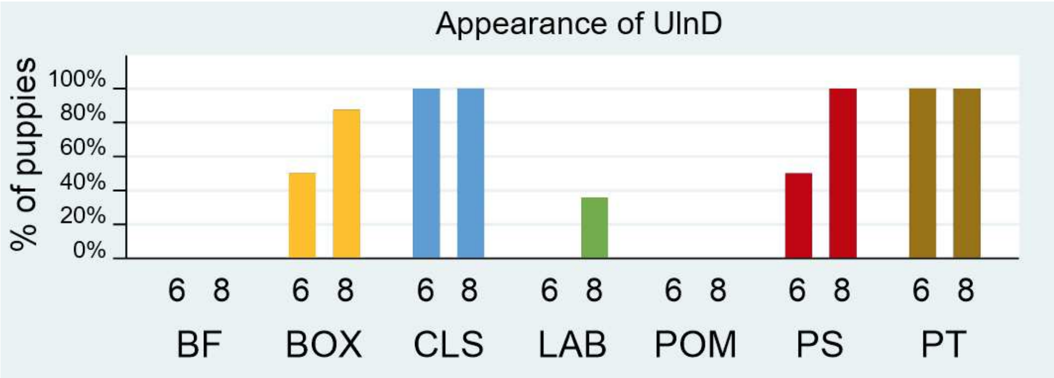


Figure 16. *Appearance of the distal epiphysis of the ulna (UlnD) in each breed during the 6-8 weeks interval.*

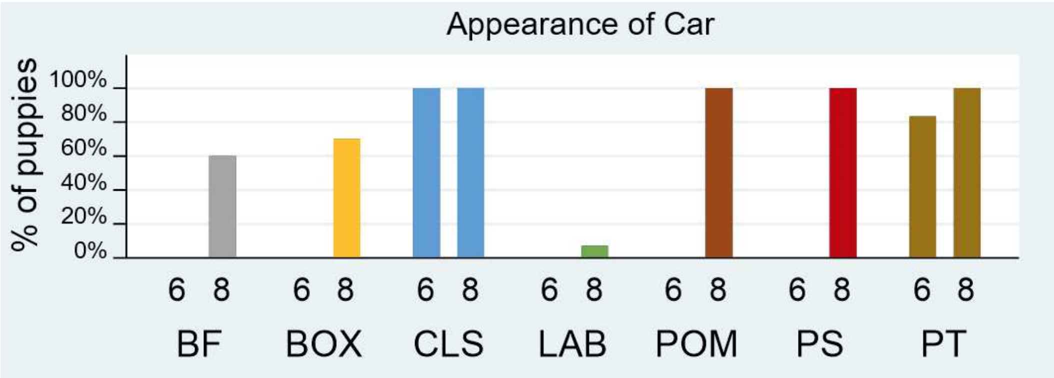


Figure 17. *Appearance of the epiphysis of the accessory carpal bone (Car) in each breed during the 6-8 weeks interval.*

BF= French Bulldog; BOX= Boxer; CLS= Saarloos Wolfdog; LAB= Labrador Retriever; POM= Pomeranian; PS= Berger Blanc Suisse; PT= German Shepherd.

At 6 weeks, the ossification centre of the patella (Pat) was absent in French Bulldog, Boxer, Labrador, Pomeranian and Berger Blanc Suisse puppies, while it was present in 43% of German Shepherds and 66% of Saarloos Wolfdogs. At 8 weeks, the OC was still absent in French Bulldogs, while it appeared in 2/14 Labradors, 1/5 Pomeranian, 4/9 Boxers, 6/7 German Shepherds and in all Saarloos Wolfdog and Berger Blanc Suisse puppies (Fig. 18). The OC was also present in all the 8-weeks-old Nova Scotia Duck Tolling Retriever puppies (data not shown).

The appearance of the tibial tuberosity (TibT) showed a similar pattern to the patella. At 6 weeks, the OC was absent in French Bulldog, Boxer, Labrador, Pomeranian and Berger Blanc Suisse puppies, whereas it was observed in 28% of German Shepherds and 91% of Saarloos Wolfdogs. At 8 weeks, the OC was still absent in French Bulldogs, while it appeared in 1/15 Labradors, 2/6 Pomeranian, 6/10 Boxers, 5/7 German Shepherds and in all Saarloos Wolfdog and Berger Blanc Suisse puppies (Fig. 19). The OC was present in all the 8-weeks-old Nova Scotia Duck Tolling Retriever puppies as well (data not shown).

The proximal epiphysis of the fibula (Fib) was visible at 6 weeks only in Saarloos Wolfdogs and in 40% of German Shepherds. It appeared at 8 weeks only in 1/9 Boxers and 1/10 Labradors, whereas it was observed in 80% of German Shepherds (Fig. 20). Data regarding Berger Blanc Suisse and Nova Scotia Duck Tolling Retriever breeds are not shown because observations at 6 weeks were not available; however, at 8 weeks the OC was observed in 100% of puppies.

Lastly, the calcaneal tuber (Tar) was visible at 6 weeks in all breeds except for Pomeranians (6% of Labradors, 33% of French Bulldogs, 50% of Berger Blanc Suisse puppies, 60% of Boxers, 100% of German Shepherds and Saarloos Wolfdogs). At 8 weeks it reached 100% in French Bulldog, Pomeranian and Berger Blanc Suisse puppies, whereas it was present in 60% of Labradors and 90% of Boxers (Fig. 21). The OC was present in all the 8-weeks-old Nova Scotia Duck Tolling Retriever puppies as well (data not shown).

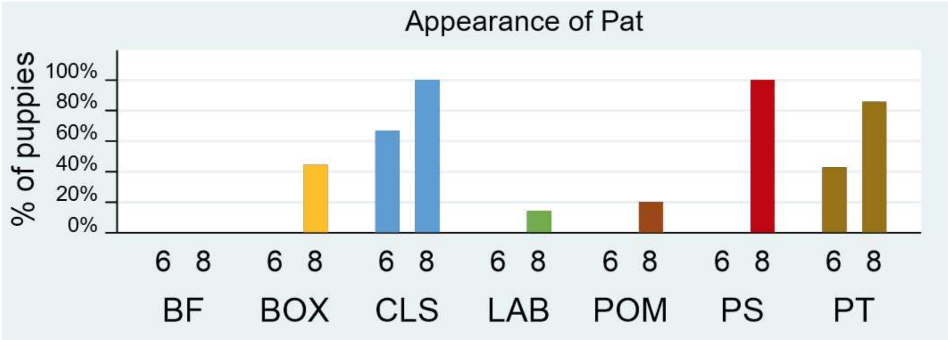


Figure 18. *Appearance of the epiphysis of the patella (Pat) in each breed during the 6-8 weeks interval.*

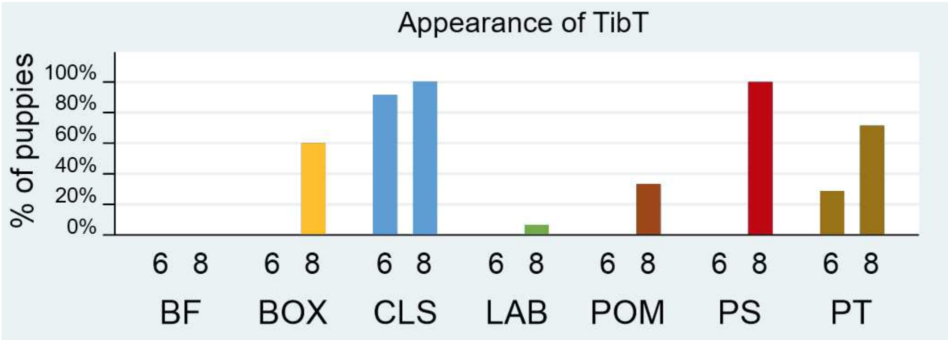


Figure 19. *Appearance of the epiphysis of the tibial tuberosity (TibT) in each breed during the 6-8 weeks interval.*

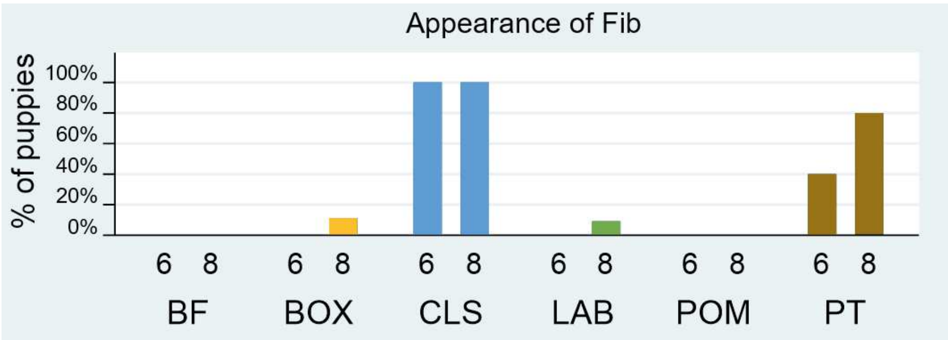


Figure 20. *Appearance of the proximal epiphysis of the fibula (Fib) in each breed during the 6-8 weeks interval.*

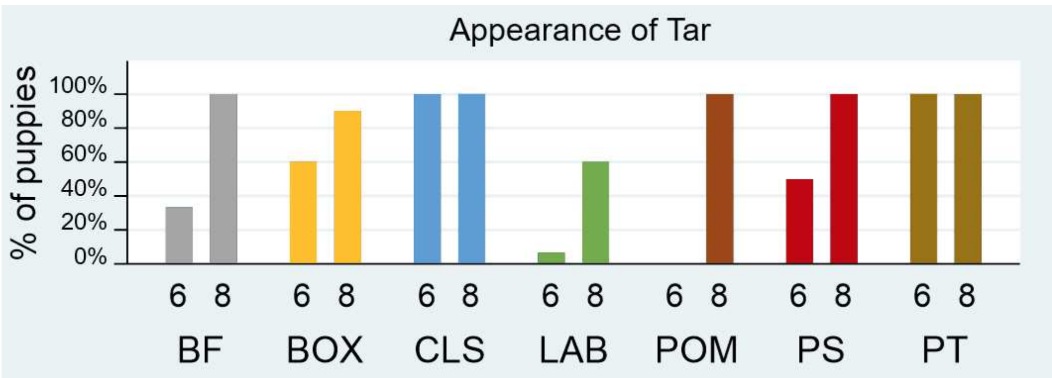


Figure 21. *Appearance of the epiphysis of the calcaneal tuber (Tar) in each breed during the 6-8 weeks interval.*

BF= French Bulldog; BOX= Boxer; CLS= Saarloos Wolfdog; LAB= Labrador Retriever; POM= Pomeranian; PS= Berger Blanc Suisse; PT= German Shepherd.

In order to obtain more concise information, a skeletal development score was calculated by summing the number of OCs present for each breed at each observation point (Table 15). Puppies with missing observations were excluded from the analysis. Therefore, in order to minimise the loss of information, the score was calculated on a total of 14 OCs, with the exclusion of the supraglenoid tubercule (Sca) and the proximal epiphysis of the fibula (Fib), which were often not viewable, and sesamoid bones fabellae (Fab) and popliteal (Pop), which appeared at a much later stage.

Table 16. *Breed skeletal development score at each timepoint calculated by summing the number of OCs present out of 14 (HumP, HumD, HumE, RadP, RadD, UlnO, UlnD, Car, Fem, Pat, TibP, TibT, TibD, Tar).*

Breed	**Age**					
	6 w	**8 w**	**10 w**	**12 w**	**14 w**	**16 w**
BAR	-	9	-	-	-	-
BF	7	9	13	14	14	14
BOX	8	11	13	14	14	14
CLS	13	14	14	14	14	14
LAB	7	8	12	14	14	14
NOV	-	14	14	14	14	14
POM	6	9	14	14	14	14
PS	8	14	14	14	14	14
PT	11	13	14	14	14	14

BAR= Toy Poodle; BF= French Bulldog; BOX= Boxer; CLS= Saarloos Wolfdog; LAB= Labrador Retriever; NOV= Nova Scotia Duck Tolling Retriever; POM= Pomeranian; PS= Berger Blanc Suisse; PT= German Shepherd.

At 6 weeks, in Pomeranian, French Bulldog, Boxer, Labrador and Berger Blanc Suisse puppies only 6 to 8 OCs out of 14 were present, while 11 were visible in the German Shepherd and 13 in the Saarloos Wolfdog (some puppies lacked the patella or the tibial tuberosity) (Fig. 22; Annex 5, Figs. 5.8-5.11).

At 8 weeks, French Bulldogs, Boxers, Labradors, Pomeranians and Toy Poodles still lacked several OCs, while Berger Blanc Suisse jumped to full score, together with Nova Scotia Duck Tolling Retrievers and Saarloos Wolfdogs. In German Shepherds the development was not yet complete, with some puppies missing the olecranon, the patella or the tibial tuberosity (Fig. 23; Annex 5, Figs. 5.12-5.15).

Graphs illustrating the “breed portrait” of the OCs present at 6 weeks and at 8 weeks of age are reported in Annex 5, Figs. 5.1-5.7.

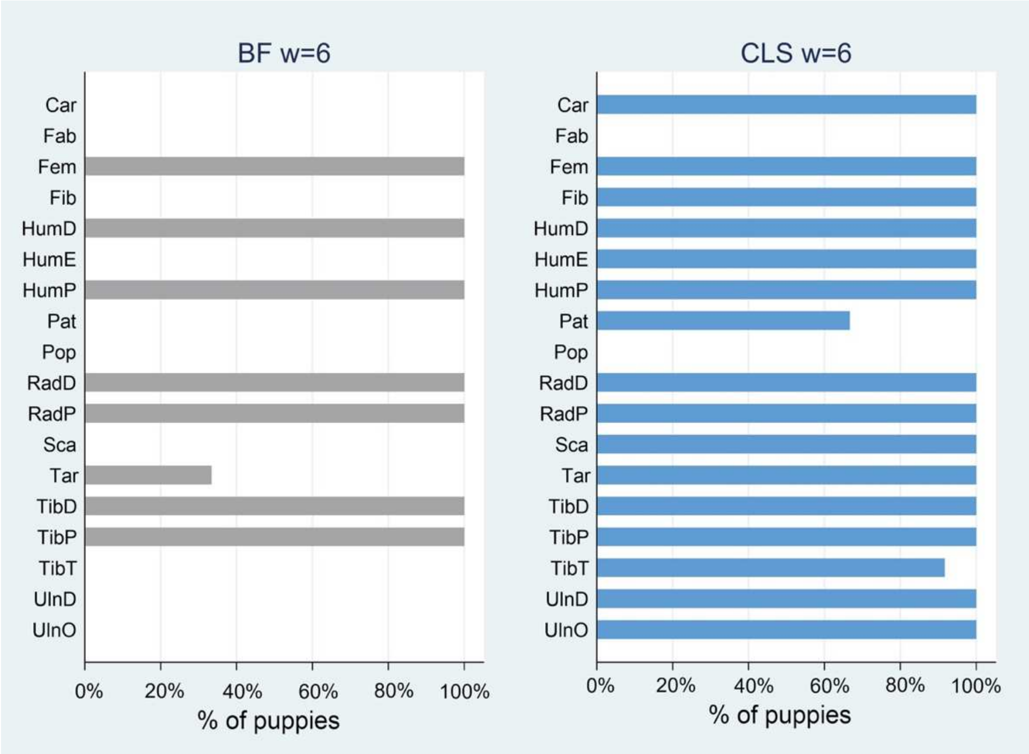


Figure 22. *Ossification centres present at 6 weeks in French Bulldogs (on the left) vs. Saarloos Wolfdogs (on the right).*

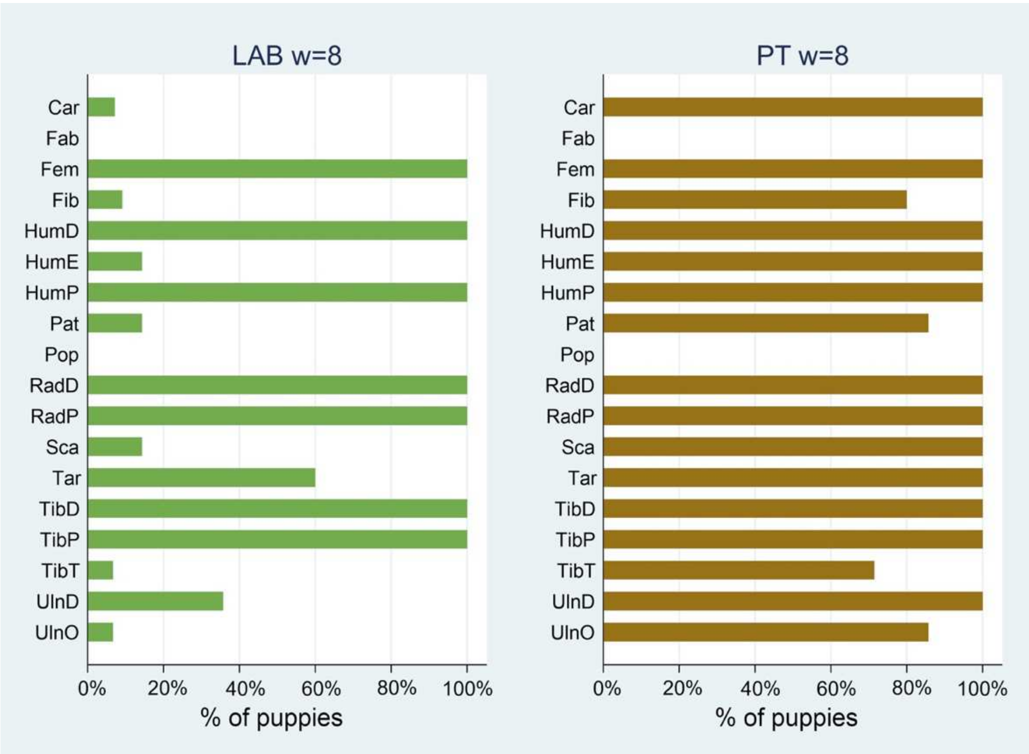


Figure 23. *Ossification centres present at 8 weeks in Labrador Retrievers (on the left) vs. German Shepherds (on the right).*

At 10 weeks, Labradors had an average score of 12, but the missing OCs could be various: the medial epicondyle of the humerus, the olecranon, the distal epiphysis of the ulna, the epiphysis of the accessory carpal bone, the patella, the tibial tuberosity or the calcaneal tuber. Some Boxers could be missing the olecranon or the calcaneal tuber, while in some French Bulldogs the tibial tuberosity was absent (Annex 5, Figs. 5.16-5.19).
At 12 weeks, as already shown in Figure 11, all of the selected OCs (excluding fabellae and popliteus) were present in all puppies (Annex 5, Figs. 5.20-5.23).
The appearance of the ossification centre of the fabellae (Fab) in the different breeds was also analysed (Fig. 24). Before 14 weeks of age, this OC could be observed only in Saarloos Wolfdogs, specifically in almost 40% of 10-weeks-old puppies and in 100% of 12-weeks-old puppies. At 14 weeks of age it was present in 80% of Boxer and Berger Blanc Suisse puppies (Annex 5, Figs. 5.25, 5.27). In these breeds it reached 100% at 16 weeks, as in German Shepherds. At the same age, the fabellae could be observed only in 1/2 French Bulldogs. None of the 16-weeks-old Pomeranians showed the fabellae. Data regarding Labrador Retrievers and Nova Scotia Duck Tolling Retrievers beyond 12 weeks of age are not available.

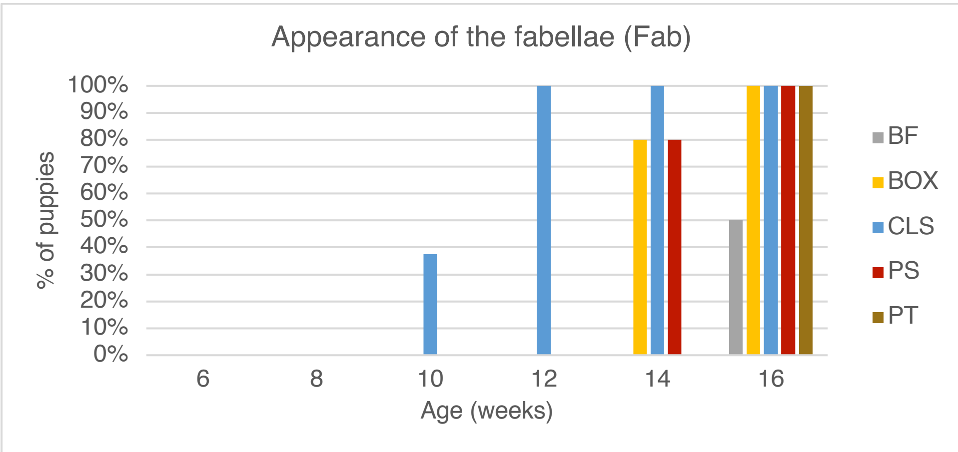


Figure 24. *Appearance of the fabellae (Fab) in the different breeds.*

Radiographic measurements of several diaphyseal lengths and OC areas were also performed. Data are still under analysis and will therefore not be reported here. However, the relative increase in these measures was calculated and the average percentage increase in radiographic measures per each breed is illustrated in Annex 6. As regards area measurements, the increase was calculated on the square root in order to obtain a linear trend. By convention, the eight-week measure was considered to be 100% as it included the highest number of observations. In case the OC was still not present at 8 weeks (olecranon, patella, tibial tuberosity), 100% was moved to 10 weeks.

Standard deviation bars were excluded for clarity. However, it must be noted that standard deviations of area measurements were quite high.

A marked difference in the OC measurements was noted in Labrador Retrievers, where the area of the supraglenoid tubercule (Annex 6, Fig. 6.1), the patella (Annex 6, Fig. 6.9) and the calcaneal tuber (Annex 6, Fig. 6.11) increased much more rapidly than the other breeds.

Similarly, the olecranon (Annex 6, Fig. 6.4) showed a more rapid growth in Labrador Retrievers, Pomeranians and Boxers.

5.3 Building of a prediction model

Initially, a CHAID classification tree was built in order to predict if a puppy is 6 or 8 weeks old (Fig. 25). The input variables were breed, sex, size and morphological type of the puppies, the teeth and the examined ossification centres.

The maximum depth of the tree was set to 3, the minimum number of observations per parent node was set to 20, while the minimum number per children node was set to 10.

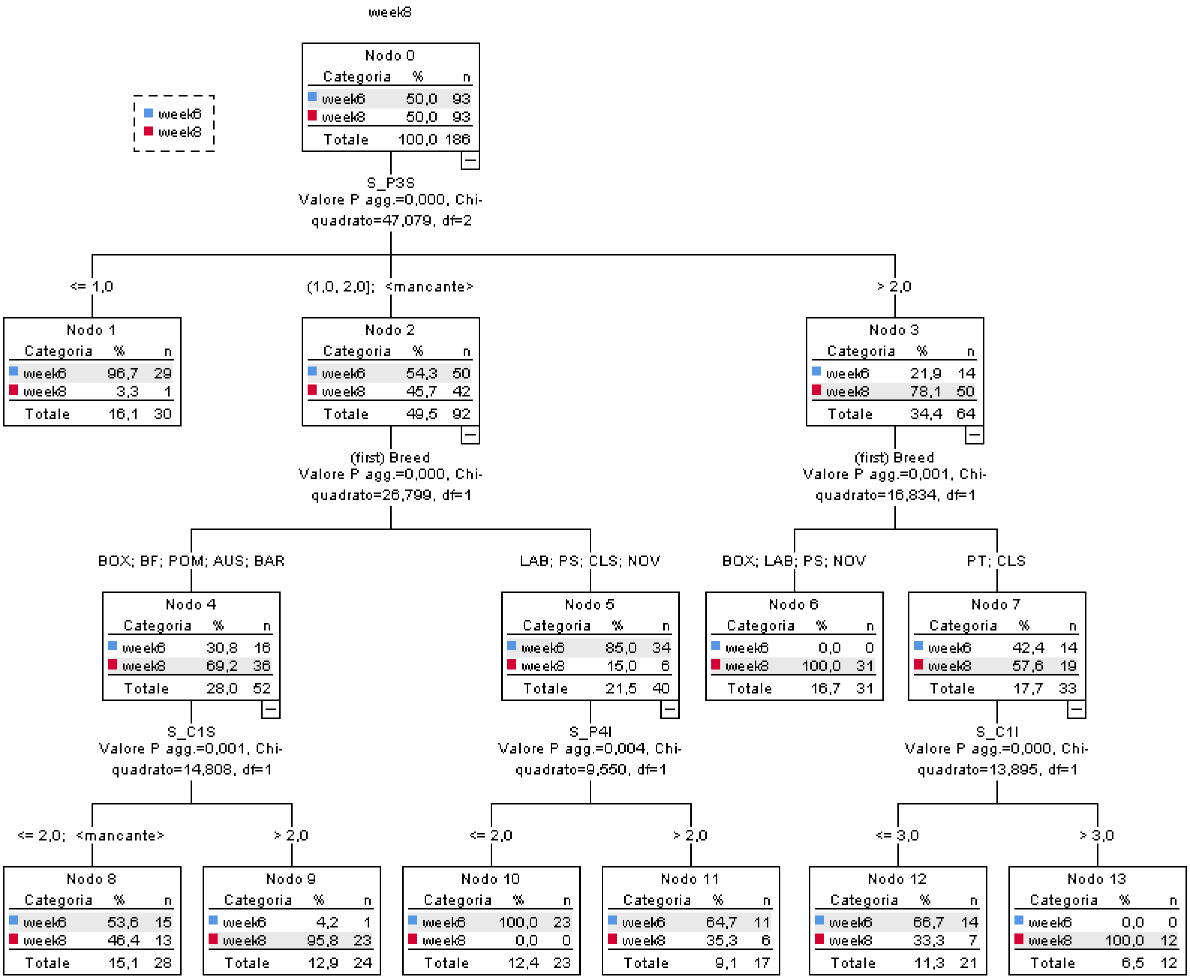


Figure 25. Classification tree built to predict if a puppy is 6 or 8 weeks old.

The resulting tree included five variables: the maxillary and mandibular canine teeth (C1S, C1I), the maxillary third premolar (P3S), the mandibular fourth premolar (P4I) and the dog's breed. The total number of nodes was 14, 8 of which were leaf nodes.

As illustrated in Fig. 25, by following the path from root node to the first terminal node (Node 1), it can be said that if the maxillary deciduous third premolar has scored 0 or 1 (tooth not erupted or eruption just started), then the puppy is, in all likelihood, 6 weeks old. If we analyse Node 6, we can state that a puppy is 8 weeks old if the maxillary third premolar scored more than 2 and if it is a Boxer, Labrador, Nova Scotia Duck Tolling Retriever or a Berger Blanc Suisse. Similarly, the other branches can be analysed and further conclusions can be drawn.

The model could correctly predict whether the puppy was 6 or 8 weeks old with an overall accuracy of 84.9%. However, given the high dependence of classification trees on the training dataset, random forests were grown in order to strengthen and stabilise the prediction model.

A total of two forests, of 500 trees each, were grown: one was built with a mtry of 6, and the other with a mtry of 8 (mtry is the number of variables randomly selected at each split). The resulting error, variable importance estimation and accuracy of the two forests, both during training and testing, are compared below.

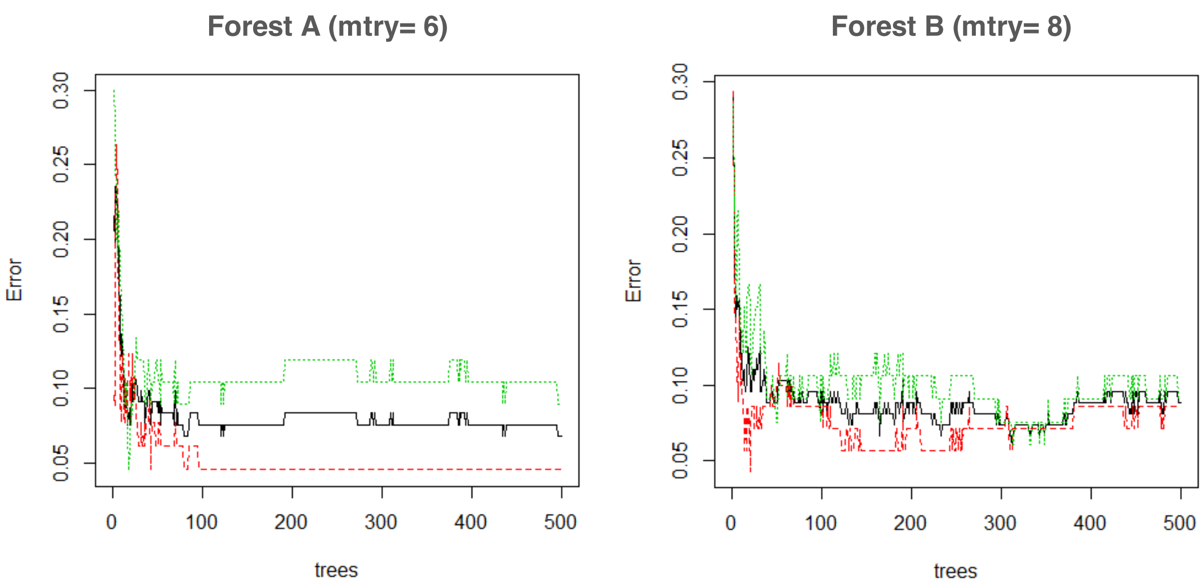


Figure 26. *Graphs illustrating the error rate (black line) for Forest A (mtry= 6) and Forest B (mtry= 8). The green and red lines delimit the confidence intervals.*

The resulting error rate of the random forests appeared not to be sensitive to the value of mtry and stabilised around 7% (Fig. 26).

Similarly, the estimation of variable importance handed back the same variables as the more important ones: breed, above all, followed by the maxillary third premolar (P3S), the mandibular first and second incisor (I1I, I2I) and the ossification centre of the patella (Pat) (Fig. 27).

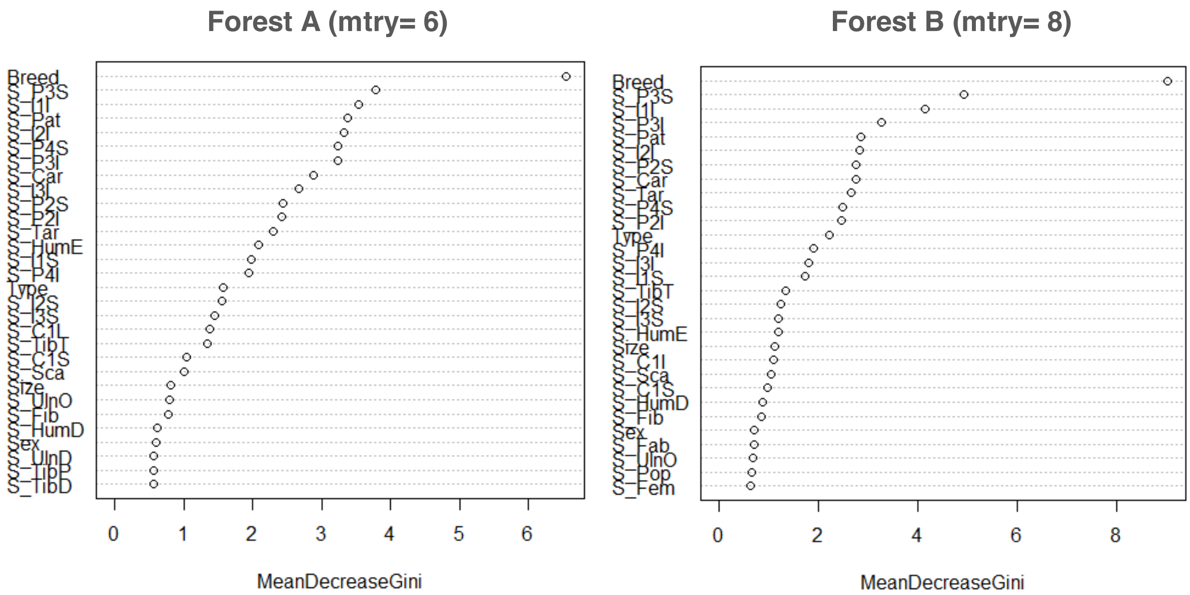


Figure 27. Graphs illustrating the variable importance estimation in Forest A and Forest B. Gini impurity represents the probability that a randomly selected sample from a node will be incorrectly classified. Important variables are chosen as the one that lead to the greatest reduction in Gini impurity.

Finally, the accuracy of the prediction model was very high in both forests, either with the training dataset and the out-of-bag data:

	Forest A	**Forest B**
Accuracy (training)	0.9924%	0.9926%
Accuracy (testing)	0.8519%	0.9000%
95% CI (training)	(0.9585, 0.9998)	(0.9597, 0.9998)
95% CI (testing)	(0.7288, 0.9338)	(0.7819, 0.9667)

6. DISCUSSION

Age determination of dog puppies represents a significant issue of animal welfare and forensic medicine, particularly regarding trade and import of dogs, which, according to the legislation currently in force, cannot be sold or moved before they have reached a certain age. The difficulty in determining the real age of the animals makes it hard to prosecute puppy smugglers in court. Therefore, law enforcement is poorly implemented and the occurrence of illegal puppy trafficking is not uncommon.

The uncertainty of age estimation is the result of several factors: first of all, the uncertainty of the method referable to the biological variability occurring between subjects of similar living and health conditions and belonging to the same breed and morphological type; second, the systemic distortion deriving from the assumption that the observed biological phenomena, developed on a given population, occur in an identical manner in subjects of different breed, morphological type, health conditions and living environments; third, the imprecision lying in the experience of the assessor and in the subjectivity of their judgement, which is inbuilt in any visual assessment that do not provide for objective and comparable measurements, as can be the examination of the teeth.

To date, the most widely used method to assess the age of young dogs is indeed teeth examination, which is quick to perform, non-invasive, and is considered rather easy. However, its validity in the forensic context is rather limited, as teeth examination often results in a wide disagreement between evaluations made by different experts, thus making it arduous to stand up in court. It should also be pointed out that teeth examination is not as simple as it is commonly thought: not all puppies willingly accept to have their mouths opened and inspected, the procedure is more difficult in younger and small-sized puppies and a thorough examination is not always possible. When performing an age estimation by teeth examination for medico-legal purposes, photographs of the oral cavity should always be taken in order to double check the evaluation at a later time and to keep official records of what has been observed.

The information on teeth eruption and development provided by the consulted papers and textbooks are often quite general, with wide time ranges frequently referred to the entire tooth class rather than the single tooth. Research studies on teeth eruption have been performed on medium and large dog breeds (Mellanby, 1929; Arnall, 1960; Shabestari et al., 1967; Kremenak, 1969) and in most cases they include a small sample size. No specific data are available for small-sized dog breeds, which are known to have a delayed teeth eruption (Hoskins, 2001a; Lobprise, 2012).

The analysis of the Literature revealed that teeth eruption and development in dogs is far from being a uniform process. Deciduous teeth eruption should be completed by the sixth week of age, but for some Authors it can last up to 10 or even 12 weeks (Harvey and Emily, 1993b; Hoskins, 2001a; Squarzoni, 2003; Hale, 2005; Van de Wetering, 2011). At 15-16 weeks, the age at which a puppy can be legally moved within EU countries, according to some Authors the eruption of I1 (Miller, 1952; Silver, 1963; Nickel et al., 1979; Harvey and Emily, 1993b; Hale, 2005; Reece, 2009; Evans and de Lahunta, 2013; Dyce et al., 2018a), C (Squarzoni, 2003; Gorrel, 2013; Fulton et al., 2014) and P1 (Balasini, 1995; Vaissaire, 2001) should be already in place, while for others it is just about to start (Mellanby, 1929; Barton, 1939; Bourdelle and Bressou, 1953; Arnall, 1960; Ferrara, 1965; Piérard, 1967; Shabestari et al., 1967; Barone, 2006a; Veggetti and Falaschini, 2009; Veronesi et al., 2013). The eruption window extends up to 5-6 months for incisors, premolars and the first molar, up to 7 months for canines and the remaining molars.

In the present work, we focused on the deciduous teeth eruption and development in a population that included 10 different breeds of different size and morphological type, for a total of 93 puppies. The same puppies were examined at different ages; therefore, unlike most of the published studies, longitudinal data were collected.

A wide degree of variability was observed, which was in line with the variety of information found in the Literature. The eruption of the deciduous dentition was complete at 8 weeks only in Saarloos Wolfdogs and in German Shepherds. At 10 weeks all the deciduous teeth could be observed in Australian Shepherds, Boxers, Labrador Retrievers, Nova Scotia Duck Tolling Retrievers and in Berger Blanc Suisse puppies, while Toy Poodles still had only 9/14 teeth in the hemiarch. In French Bulldogs the eruption was complete at 14 weeks of age, with the appearance of the second premolar and the concurrent shedding of the first incisors. In Pomeranians the completion of the deciduous dentition could only be observed at 16 weeks.

Once the eruption of the deciduous teeth was complete, dental wearing and thinning began. The extent and speed of this process was very variable due to the interplay of external factors such as the eating and playing habits of the puppies.

Between 14 and 16 weeks, the appearance of the first permanent incisors and the first premolar could be observed in all breeds except for the smaller ones, that is French Bulldogs and Pomeranians (data on Toy Poodles and Nova Scotia Duck Tolling Retrievers are not available).

In Italy, puppies cannot be sold or moved before they have reached 8 weeks of age. On the basis of our results, the eruption of all the deciduous teeth is not a suitable criterion to determine if a dog is at least 8-weeks old, since puppies could be still lacking from one up to four teeth in the hemiarch. The smaller the breed size, the more teeth that might be missing.

According to the European legislation, dogs cannot be vaccinated against rabies before 12 weeks of age and be moved among EU countries or imported from third countries before 15 weeks of age. In our sample, at 12 weeks of age the eruption of the deciduous dentition was complete in most breeds and various degrees of dental wearing could be observed. Contrarily, French Bulldogs and Pomeranians still lacked one tooth, which appeared at 14 and 16 weeks respectively, while at the same time tooth shedding was already starting in the other breeds.

Our results confirm that variability due to breed and body size cannot be overlooked and shall be taken into consideration when assessing the dog's age.

In light of these data, it is not surprising that teeth examination cannot be considered a reliable method to determine the exact age of a dog, but at most to estimate it, as agreed by all the consulted Authors. The correspondence between the real age and its assessment by teeth examination is at most 41% (Nickel et al., 1979).

In human forensic science, the main technique for age determination is the radiographic evaluation of ossification centre appearance and closure rates, which is useful until the final completion of the maturation processes occurs, at approximately 18 years. In particular, the hand-wrist region is examined. The most popular methods are the Greulich and Pyle atlas and the Tanner-Whitehouse method. The former is a comparative method between the X-ray of the subject of unknown age and the standards provided in the atlas (Greulich and Pyle, 1959); the latter is based on a scoring system which evaluates the ossification degree and morphological appearance of the ossification centres and bones of the hand and wrist (Tanner et al., 1975). However, questions about the applicability of these methods to different populations have been raised, since ethnic differences in growth patterns have been observed and the need to standardise the methods according to the specific population emerged (Cunha et al., 2009; Zhang et al., 2009).

These issues are even more important in veterinary medicine and in particular in dogs, which are the most morphologically variable domesticated mammals, displaying extreme variations in terms of size, body weight, skull shape, etc.

In dogs, breed-specific differences in growth patterns have been observed, with toy, small and medium sized breeds reaching their adult weight at around 9-10 months, which in larger breeds is not achieved until 15 months of age (Hawthorne et al., 2004). The radiographic evaluation of the appearance and fusion of the ossification centres (OCs) of limb bones is often used as an alternative or complementary method to teeth examination for determining the age of dog puppies.

Several studies documenting the normal development of the limb ossification centres of dogs have been published (Seoudi, 1948; Pomriaskinsky-Kobozieff and Kobozieff, 1954; Bressou et al., 1957; Hare, 1959; Hare, 1960; Smith, 1960; Smith and Allcock, 1960; Hare, 1961; Chapman, 1965; Sumner-Smith, 1966; Riser, 1973; Gustaffson et al., 1975; Yonamine et al., 1980). However, most of these works are dated. Moreover, despite the amount of information provided, these studies often include a small sample of dogs, the observation protocols are not always specified, the timeframe measurements and the anatomical landmarks are not homogeneous and therefore difficult to compare. Medium and large sized breeds like German Shepherd, Greyhound and Beagle are the most studied.

Furthermore, the timing of OC appearance and closure reported in veterinary textbooks is mostly based on the aforementioned studies, although sometimes data do not correspond to the original reference (Ticer, 1984; Newton and Nunamaker, 1985; Schebitz and Wilkens, 1989; Hoskins, 2001b; Burk and Feeney, 2003; Dennis et al., 2010; Kealy and McAllister, 2011; Peterson and Kutzler, 2011; Thrall and Robertson 2011; Dyce et al., 2018).

Information deriving from the analysis of the Literature indicates that the appearance of the limb ossification centres is usually complete at 12 weeks of age, with the exception of the tibial medial malleolus, sesamoid bones and hip bones. The time window of OC closure is much wider, ranging from 3 to 15 months of age for most limb bones and until up to 6 years for the pelvis.

In the present work, we focused on the appearance of the limb ossification centres in a population that included 9 different breeds of different size and morphological type. The same puppies were radiographed at different ages; therefore, unlike most of the published studies, longitudinal data were collected. The lateral view of the right limbs was chosen as it allowed to examine most of the ossification centres of the appendicular skeleton and to minimise the exposure of both puppies and personnel to X-rays. All the puppies underwent the procedure without any problems and pharmacological sedation was never necessary.

At 6 weeks, i.e. the first observation point, the ossification centres present in all the puppies were the proximal and distal epiphysis of the humerus in the forelimb, the distal epiphysis of the femur and the proximal and distal epiphyses of the tibia in the hindlimb. These data are in line with the Literature.

The appearance of the other ossification centres was less homogeneous in the different breeds.

The supraglenoid tubercule was visible at 6 weeks in 100% of Saarloos Wolfdogs and German Shepherds, 37% of Boxers, 11% of Labradors, whereas at 8 weeks of age it was still absent in all Pomeranians, in 88% of Labradors and in 50% of French Bulldogs. Most Authors place the appearance of the supraglenoid tubercule at around 6-7 weeks of age (Ticer, 1984; Burk and Feeney, 2003; Dennis et al. 2010; Kealy and McAllister, 2011; Thrall and Robertson, 2011; Dyce, 2018), while for others the timing for appearance extends up to 8-9 weeks (Hare, 1959; Shebitz and Wilkens, 1989; Hoskins, 2001b; Peterson and Kutzler, 2001).

According to the Literature, the epiphysis of medial epicondyle of the humerus appears at 6 to 9 weeks of age. In our sample, this OC was observed in all Saarloos Wolfdogs and German Shepherds at 6 weeks of age, but it was completely lacking in the other breeds. At 8 weeks the appearance was complete in Nova Scotia Duck Tolling Retriever and Berger Blanc Suisse. The OC was also present in the majority of Boxers and in half of the French Bulldogs, whereas it was lacking in most of Pomeranians and Labradors. At 10 weeks 23% of Labradors still did not show the epiphysis of medial epicondyle of the humerus.

The proximal epiphysis of the radius was present at 6 weeks in all breeds but Pomeranians and half of the Bulldogs. This is in contrast with the Literature, which locates the appearance of this OC at 3-6 weeks. Similarly, the distal epiphysis of the radius, which should already be viewable at 2-4 weeks, was still lacking in 25% of 6-weeks-old Pomeranians.

The timing for the appearance of the olecranon is different among the Authors: some indicate an interval ranging from 6 to 8 weeks (Thrall and Robertson, 2011; Dyce et al., 2018), others state exactly 8 weeks (Ticer, 1984; Burk and Feeney, 2003; Dennis et al., 2010; Kealy and McAllister, 2011) or move the interval up to 9 weeks (Hoskins, 2001b) or 10 weeks (Peterson and Kutzler, 2011). The appearance of the olecranon showed a heterogeneous pattern in our sample as well. At 6 weeks it was present only in Saarloos Wolfdogs and in 43% of German Shepherds. At 8 weeks it could be observed in Nova Scotia Duck Tolling Retrievers, Berger Blanc Suisse puppies and in a small percentage of Labrador, Pomeranian and Boxers, while it was still absent in French Bulldogs. In Boxers and Labradors, the appearance was complete at only 12 weeks of age.

Contrarily to what was indicated by most Authors, who place the appearance of the distal epiphysis of the ulna between 5 and 8 weeks of age or, by the latest, at 9 weeks, in our sample 13% of 10-weeks-old Labradors still lacked the OC. The distal epiphysis of the ulna was also absent in all Pomeranians and French Bulldogs at 8 weeks.

At 6 weeks the epiphysis of the accessory carpal bone was observed only in Saarloos Wolfdogs and German Shepherds; at 8 weeks it appeared in 7% of Labradors, 60% of Bulldogs and 70% of Boxers, while at 10 weeks 29% of Labradors still lacked the OC. These data are in line with the Literature.

The appearance of the patella is placed by most Authors between 6 and 9 weeks of age, while Peterson and Kutzler (2011) argue that it can extend up to 12 weeks. According to our observations, at 6 weeks the appearance of the patella was complete in none of the examined breeds. At 8 weeks, the percentage of puppies for each breed showing the patella was quite various, whereas at 10 weeks 57% of Labradors were still lacking this OC.

Similarly, the appearance of the tibial tuberosity was heterogeneous between breeds, with the Saarloos Wolfdogs being again the most precocious and the Labradors and, to a lesser extent, the French Bulldogs being the later ones. Once again, only Peterson and Kutzler (2011) indicated a wider interval for the appearance of this OC, ranging from 7 to 11 weeks, while for the rest of the Authors the tibial tuberosity should appear by 8-9 weeks of age.

According to the Literature, the proximal epiphysis of the fibula should appear between 7 and 10 weeks. Our data are not in complete agreement with the Literature. In fact, this OC appears at 6 weeks in German Shepherds and Saarloos Wolfdogs, at 8 weeks in Boxers, Labradors and Berger Blanc Suisses, and at only 12 weeks in French Bulldogs and Pomeranians.

The appearance of the calcaneal tuber is placed by most Authors at 6 weeks of age, while according to Peterson and Kutzler (2011) it can extend up to 9 weeks. Our observations only partially agree with the Literature. The calcaneal tuber was present at 6 weeks in all Saarloos Wolfdogs and German Shepherds, and at various percentages in other breeds except from Pomeranians. In this breed and in Boxers, the appearance was complete at 10 weeks of age, while in Labradors it did not happen before 12 weeks.

The ossification centre of the fabellae appeared at a much later stage. The consulted Authors agree in placing its appearance at 3 months of age. According to our observations, the fabellae was already visible in 37% of 10-weeks-old Saarloos Wolfdogs and in 100% of 12-weeks-old puppies of the same breed. At 14 weeks it was also present in Boxers and in Berger Blanc Suisses, while at 16 weeks it appeared in French Bulldogs and German Shepherds.

In order to obtain more concise information, a skeletal development score was calculated by summing the number of OCs present for each breed at each observation point on a total of 14 OCs, with the exclusion of the supraglenoid tubercule, the proximal epiphysis of the fibula, which were often not viewable, and fabellae and popliteal sesamoid bones.

On the basis of the number of OCs present, the breeds included in the sample can be easily divided in an “early” and a “late” group.
The “early” group includes large- and medium-sized breeds: the Saarloos Wolfdog, the Nova Scotia Duck Tolling Retriever, the German Shepherd and the Berger Blanc Suisse. The “late” group is heterogeneous per size and morphological type, and includes the Pomeranian, the French Bulldog, the Boxer and the Labrador Retriever.
It can be inferred from these observations that size does not appear to be a clear dividing line between early and late behaviour in the appearance of the ossification centres, but breed-specific variations exist. The most precocious breed has been found to be the Saarloos Wolfdog, while the Labrador puppies were the furthest behind in the appearance of all the ossification centres, despite being a large-size breed.
With regard to shepherd dogs, German Shepherds appeared to be more precocious at 6 weeks of age compared to Berger Blanc Suisses, while at 8 weeks the situation was reversed. At 8 weeks, Nova Scotia Duck Tolling Retrievers showed more advanced skeletal development than German Shepherds.
Leaving aside for a moment these difference between breeds, a very important fact has emerged from our observations: at 12 weeks of age all of the examined limb ossification centres, except for fabellae and popliteus, are present in all puppies, regardless of breed, size and morphological type.
Based on this information, it is therefore possible to establish with a fair degree of certainty if a puppy is older or younger than 12 weeks of age, which is the minimum age at which dogs can be vaccinated against rabies in order to be moved within or into the European Union.
On the other hand, to determine if a puppy is at least 15 weeks old is still a challenge. The bones that can provide us with further information are the fabellae and the popliteal sesamoid bones. However, the development of the fabellae was quite heterogeneous among the examined breeds. In Saarloos Wolfdogs, the fabellae could be observed in some 10-weeks-old puppies and in all 12-weeks-old puppies. In Boxers, Berger Blanc Suisses and German Shepherds, the presence of the fabellae could mean that they were 14 or 16 weeks old, while if observed in other breeds it could be concluded that the puppies were older than 16 weeks. Contrarily, the presence of the popliteal sesamoid bones certainly indicates that the puppy is more than 16-weeks-old.

Information deriving from teeth examination is also limited in significance. Between 14 and 16 weeks, the deciduous teeth present different degrees of wearing, they begin to shed and puppies show a mixed dentition. However, at the same age some deciduous teeth are still missing in smaller breeds such as Pomeranians and French Bulldogs.
Another time interval of particular importance is the period between 6 and 8 weeks, in which a more heterogeneous situation was observed. This interval is also of medico-legal importance because, in Italy, eight weeks correspond to the minimum age for the trade of puppies. At eight weeks, it was not possible to determine the age with a good degree of precision, as some variability was observed both in the eruption of deciduous teeth and in the appearance of the limb ossification centres between the examined breeds. For this reason, a predictive model was constructed which would consider both these biological phenomena.
Classification trees proved to be a suitable model since they can accommodate either continuous, ordinal and categorical variables as inputs and maintain accuracy even with many missing data and in presence of nonlinear relationships between variables, as it can be the case of biological phenomena such as teeth and skeletal development.
After a first exploratory phase that led to the setting up of a 3-level classification tree with an overall accuracy of 84.9% on the training dataset, random forests were grown in order to strengthen and stabilise the prediction model. A random forest does not need a separate test dataset, as the error and variable importance are estimated internally on one-third of the observations.
The estimation of variable importance selected, as the most important ones, breed, above all, followed by the maxillary third premolar, the mandibular first and second incisor and the ossification centre of the patella. The resulting error rate of the random forests stabilised around 7% and the accuracy on the testing dataset was 0.9%.
In the near future it is planned to recruit more animals to further test and refine the predictive model.

Another innovative contribution of this research is the performance of radiographic measurements in order to evaluate the relative increase of the diaphyseal lengths and the areas of some ossification centres and to determine the relationship between these measurements and the biological age of dog puppies.

Morphometry is poorly investigated in the canine species, except from its application aimed at determining gestational age in order to predict the parturition date (Kutzler et al., 2003).
To our knowledge, the one study performing radiographic morphometry dates back to 1982. It included four 13-month-old and 21-month-old Beagles and X-rays were performed on the dissected femur. The width, length and combined cortical thickness of the bone were measured. The total width of the femur increased significantly with age and body weight, but neither its length nor its combined cortical thickness (CCT) did (Delaquerriere-Richardson et al., 1982).
Another morphometric study was recently carried out on 27 spontaneously dead small-sized newborn dogs. Radiographic measurements of limb bone length were positively correlated with body weight and age of the subjects (Modina et al., 2017).
In the present work, the same puppies were radiographed at different ages; therefore, unlike the previously published studies, longitudinal data were collected. The increase in length of the bone diaphysis showed a constant and homogeneous trend among the different breeds, while the increase in the area of the ossification centres showed a higher variability, both between breeds and between subjects of the same breed. By contrast, the number of ossification centres present at a certain age appeared to be less subject to individual variation. The relationship between radiographic measurements and the biological age of the puppies will be further investigated.

7. CONCLUSIONS

Veterinarians are called on daily in private practice or in forensic scenarios to determine the age of dogs with unknown histories or to examine animals in which stated ages are incorrect.

The assessment of age through the observation of deciduous and permanent teeth eruption and succession has been used for a long time in veterinary practice, starting with production animals. However, this assessment does not provide for objective and comparable measurements and it is highly dependent on the observer's experience. X-ray evaluation of limb bones is often used as an alternative or complementary method to teeth examination for determining the age of dog puppies and its use in human forensic medicine is well established. This technique is better suited to the forensic context since the examination produces X-ray images, which are difficult to tamper with. Current advances in technology have developed portable X-ray units and computed radiography systems, thus making it possible to take radiographs in the field. Nonetheless, there is a consensus that, to date, none of the available methods can be considered valid for medico-legal purposes, due to the numerous variability factors (breed, sex, blood line, diet, environment, health status) of the measured biological phenomena and the lack of a standardised method.

Information regarding teeth eruption and skeletal development provided by the Literature is quite dated and does not take into consideration variability due to body size, breed, sex, morphological type. Therefore, it is unlikely to be useful in forensic investigations.

The objective of this study was to quantify the degree of correlation between the chronological age of dog puppies and the biological age that can be evaluated through teeth examination and the radiographic examination of the limb ossification centres.

Data obtained in this study were compared with the information reported in the Literature and breed-specific differences were identified.

Our observations show that, at 12 weeks of age, all of the examined limb ossification centres, except for fabellae and popliteus, are present in all puppies, regardless of breed, size and morphological type. It is therefore possible to establish with a fair degree of certainty if a puppy is older or younger than 12 weeks of age, which is the minimum age at which dogs can be vaccinated against rabies in order to be moved within the European Union.

Contrarily, at 8 weeks, which is the minimum age for puppies to be sold in Italy, it was not possible to determine the age with a good degree of precision, as a wide variability was observed both in the eruption of the deciduous teeth and in the appearance of the limb ossification centres. In particular, as to the appearance of the ossification centres, it was possible to distinguish breeds showing an “early” or “late” attitude, which appeared to be independent of size and morphological type.

Data obtained from teeth examination and the radiographic investigations were combined in order to construct a predictive model that would be able to predict if a puppy was 6 or 8 weeks old. Random forests have been proved to be a valuable model, with an accuracy of 90% at the testing stage.

Further studies will be undertaken in order to strengthen and refine the predictive model and to improve the sample size, especially including a higher number of small-sized dog breeds, which showed delayed teeth eruption and appearance of the limb ossification centres.

APPENDIX

ANNEX 1. TIMING OF DECIDUOUS AND PERMANENT TEETH ERUPTION

Table 1.1. *Timing of deciduous teeth eruption according to the different Authors. The horizontal time axis ranges in weeks from 2 to 12 weeks of age. Green= incisors; Orange: canine; Blue= premolars.*

AGE

AUTHOR	**Months**				**1**		**2**		**3**
	Weeks		**2**	**3**	**4**	**5**	**6 7 8**	**9 10 11**	**12**
Cornevin and Lesbre, 1894	Incisors	I							
	Canine	C							
	Premolars	P2							
		P3							
		P4							
Mellanby, 1929	Incisors	I1	19-31d						
		I2		20-27d					
		I3	19-28d						
	Canine	C		20-28d					
	Premolars	P2			28-39d				
		P3		21-35d					
		P4		24-37d					
Barton, 1939	Incisors	I							
	Canine	C							
Miller, 1952	Incisors	I1							
		I2							
		I3							
	Canine	C							
	Premolars	P							
Bourdelle and Bressou, 1953 Vaissaire, 2001	Incisors	I1			30d				
		I2			28d				
		I3		25d					
	Canine	C		21d					
	Premolars	P2							
		P3							
		P4							
Arnall, 1960	Incisors	I	20-35d						
	Canine	C	20-35d						
	Premolars	P	20-35d						
Silver, 1963	Incisors	I							
	Canine	C							
	Premolars	P							
Ferrara, 1965	Incisors	I1			30-33d				
		I2			28-29d				
		I3		24-28d					
	Canine	C	15-20d						
Piérard, 1967	Incisors	I							
	Canine	C							
Shabestari et al., 1967	Incisors	I1		21-31d					
		I2		21-30d					
		I3	20-28d						
	Canine	C	19-25d						
	Premolars	P2			27-39d				
		P3		22-33d					
		P4		24-38 d					
Kremenak, 1969	Incisors	I1		21-41d					
		I2	19-35d						
		I3	20-37d						
	Canine	C	18-28d						
	Premolars	P2			27-40d				
		P3	20-34d						
		P4		25-41d					
Nickel et al., 1979; Gorrel, 2013; Dyce et al., 2018	Incisors	I							
	Canine	C							
	Premolars	P							

Table 1.1. *Timing of deciduous teeth eruption according to the different Authors (continued). The horizontal time axis ranges in weeks from 2 to 12 weeks of age. Green= incisors; Orange: canine; Blue= premolars.*

AGE

AUTHOR	**Months**				1		2		3
	Weeks		**2**	**3**	**4**	**5**	**6 7 8**	**9 10 11**	**12**
Barone, 2006a	Incisors	I1			30d				
		I2			28d				
		I3		25d					
	Canine	C		21d					
	Premolars	P							
Sisson and Grossman, 1982	Incisors	I1							
		I2							
		I3							
	Canine	C							
	Premolars	P2							
		P3							
		P4							
Harvey and Emily, 1993; Squarzoni, 2003; Hale, 2005; Fulton, 2014	Incisors	I							
	Canine	C							
	Premolars	P							
Balasini, 1995	Incisors	I1			30-33d				
		I2		25-30d					
		I3		21-25d					
	Canine	C	15-20d						
	Premolars	P2			28-34d				
		P3	20-28d						
		P4	20-28d						
Bonetti, 1995	Incisors	I	20-30d						
	Canine	C	20-30d						
Veggetti et al., 2009	Incisors	I							
Peterson and Kutzler, 2011	Incisors	I							
	Canine	C							
	Premolars	P							
Evans and de Lahunta, 2013	Incisors	I1							
		I2							
		I3							
	Canine	C							
	Premolars	P							
Veronesi et al., 2013	Incisors	I1							
		I2							
		I3							
	Canine	C							
	Premolars	P2							
		P3							
		P4							
Liebich et al., 2014	Incisors	I							
	Canine	C							
	Premolars	P							

Table 1.2. *Timing of permanent teeth eruption according to the different Authors.*
The horizontal time axis ranges in weeks from 8 to 31 weeks of age. Green= incisors; Orange: canine; Blue= premolars; Yellow= molars.

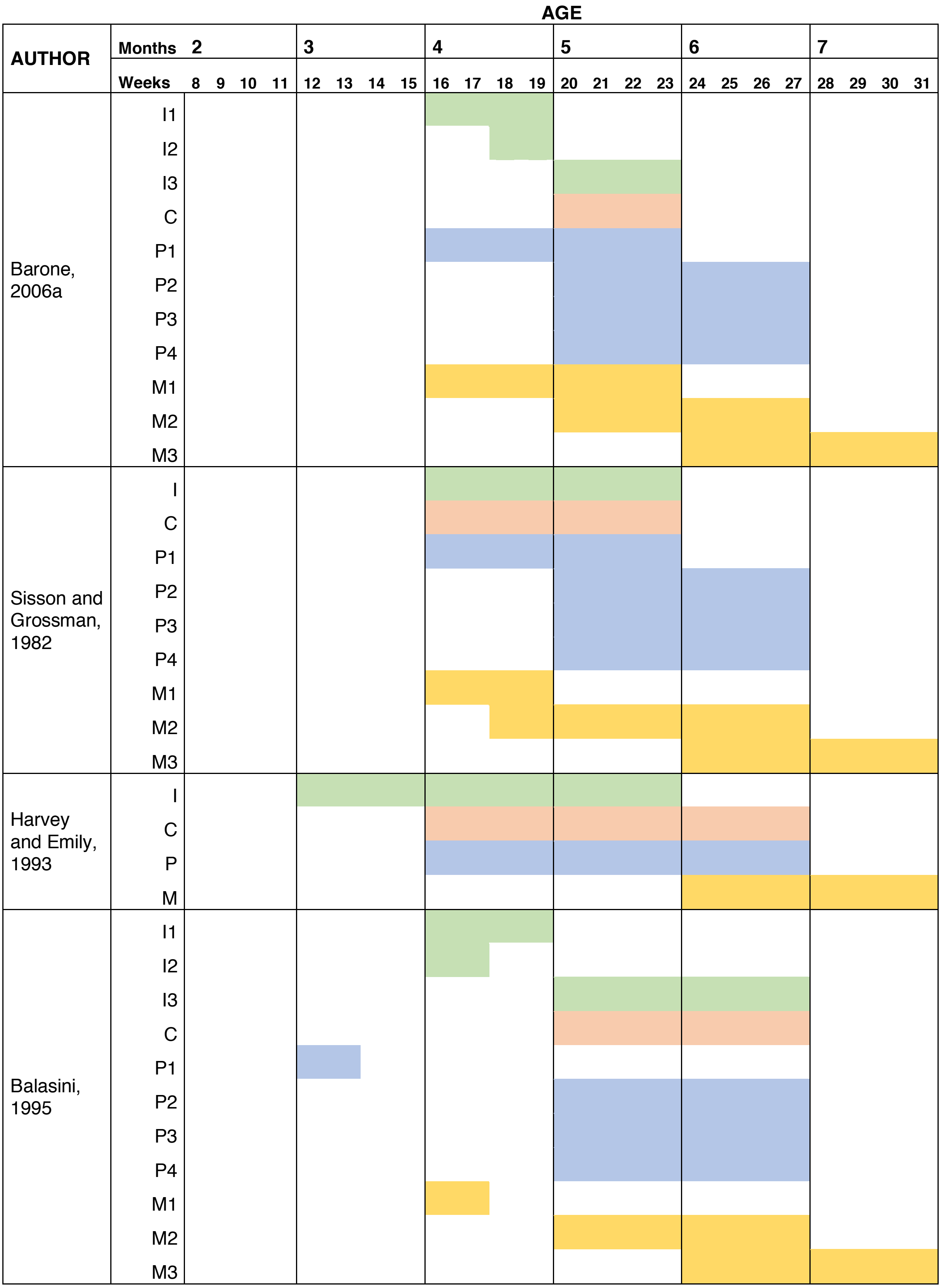


Table 1.2. *Timing of permanent teeth eruption according to the different Authors (continued). The horizontal time axis ranges in weeks from 8 to 31 weeks of age. Green= incisors; Orange: canine; Blue= premolars; Yellow= molars.*

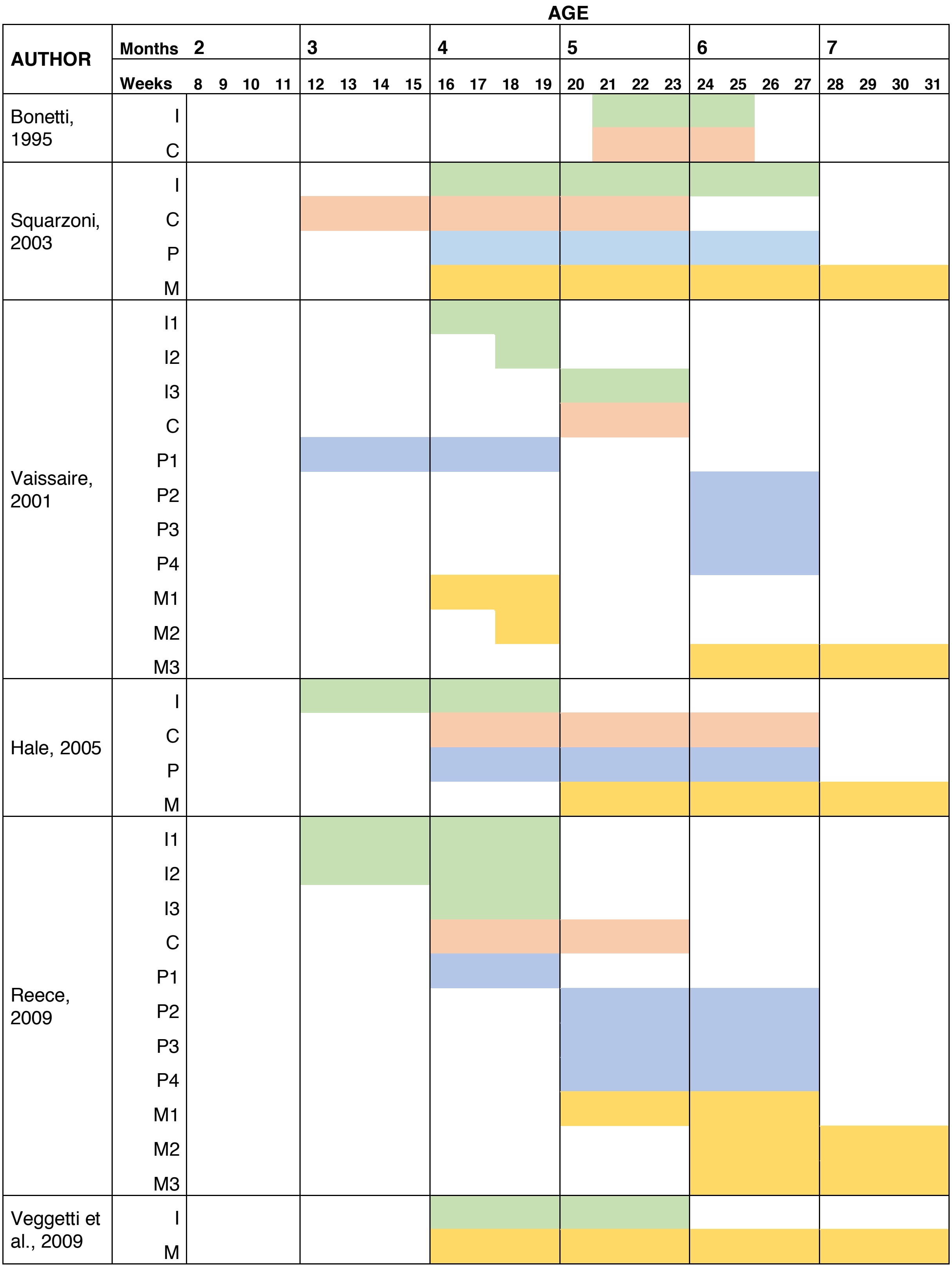
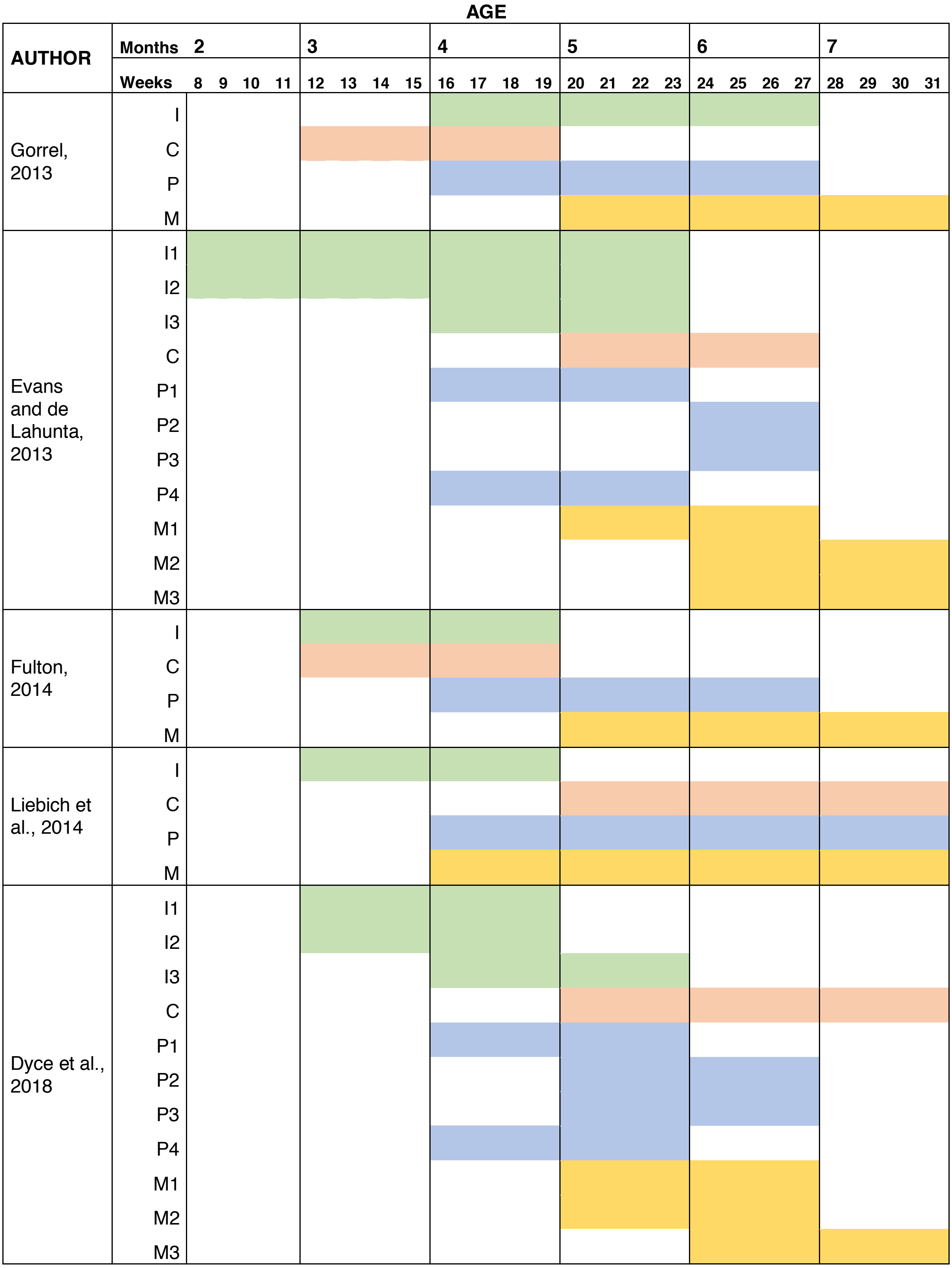


__Table 1.2.__ Timing of permanent teeth eruption according to the different Authors (continued). The horizontal time axis ranges in weeks from 8 to 31 weeks of age. Green= incisors; Orange: canine; Blue= premolars; Yellow= molars.



ANNEX 2. TIMING OF APPEARANCE AND CLOSURE OF THE LIMB OCS GIVEN IN THE CONSULTED TEXTBOOKS

Table 2.1. *Age at appearance of the ossification centres in the dog's forelimb (d= days; w= weeks; m= months).*

Anatomical site	Ticer, 1984; Burk, 2003; Kealy 2011	Shebitz & Wilkens, 1989	Hoskins, 2001b	Dennis, 2010	Peterson & Kutzler, 2011	Thrall, 2011	Dyce, 2018
Scapula							
Body	Birth			Birth	Birth		Birth
Supraglenoid tubercle	7 w	49-65 d	6-9 w	7 w	7-9 w	6-7 w	7 w
Humerus							
Proximal epiphysis	1-2 w	14-16 d	1-2 w	1-2 w	1-2 w	1-2 w	1-2 w
Diaphysis	Birth			Birth	Birth	Birth	Birth
Distal epiphysis							
Medial condyle	2-3 w	21-43 d	2-4 w	2-3 w	2-3 w	2-3 w	2-3 w
Lateral condyle	2-3 w	14-22 d	2-4 w	2-3 w	2-6 w	2-3 w	Birth
Medial epicondyle	6-8 w	49-65 d	6-9 w	6-8 w	6-9 w	6-8 w	6-8 w
Radius							
Proximal epiphysis	3-5 w	28-43 d	3-5 w	3-5 w	3-6 w	3-5 w	3-5 w
Diaphysis	Birth			Birth	Birth		Birth
Distal epiphysis	2-4 w	14-29 d	2-4 w	2-4 w	2-4 w	2-4 w	2-4 w
Ulna							
Olecranon	8 w	49-72 d	7-9 w	8 w	7-10 w	6-8 w	6-8 w
Anconeal process	12 w		-	11-12 w	-	6-8 w	12 w
Diaphysis	Birth			Birth	Birth		Birth
Distal epiphysis	8 w	49-65 d	7-8 w	2-4 w	7-9 w	5-6 w	6-8 w
Carpus							
Radial carpal bone				3-4 w		3-6 w	3-4 w
Radial carpal bone	3-4 w	28-29 d	3 w		3-5 w		
Central carpal bone	4-5 w	28-36 d	4-6 w		3-5 w		
Intermediate carpal bone	3-4 w	16-22 d	2-3 w		3-5 w		
Ulnar carpal bone	4 w	28-36 d	4-6 w	3-4 w	3-5 w	2 w	
Accessory carpal bone							
Body	2 w	14-16 d	2 w	2w	2 w	2 w	3 w
Epiphysis	7 w	49-72 d	7-11 w	7 w	7-10 w	6-7 w	7 w
Carpal bone I	3 w	21-29 d	3 w	3-4 w	3-5 w	2 w	
Carpal bone II	4 w	28-36 d	3-4 w	3-4 w	3-5 w	2 w	
Carpal bone III	4 w	28-36 d	3-4 w	3-4 w	3-5 w	2 w	
Carpal bone IV	3 w	21-29 d	3 w	3-4 w	3-5 w	2 w	
Sesamoid bone m. abductor pollicis longus	4 m	120 d	4 m	-	-	4 m	-
Metacarpus							
Metacarpal bone I							
Proximal epiphysis	5 w	49-57 d	3-5 w	4-6 w	5-8 w	5-7 w	5 w
Diaphysis	Birth				Birth		Birth
Metacarpal bone II – V							
Diaphysis	Birth				Birth		Birth
Distal epiphysis	4 w	28-36 d	3-4 w	4-6 w	4-5 w	3-4 w	4 w

__Table 2.1.__ Age at appearance of the ossification centres in the dog's forelimb (d= days; w= weeks; m= months) (continued).

Anatomical site	Ticer, 1984; Burk, 2003; Kealy 2011	Shebitz & Wilkens, 1989	Hoskins, 2001b	Dennis, 2010	Peterson & Kutzler, 2011	Thrall, 2011	Dyce, 2018
Phalanges							
Proximal phalanx I – V							
Proximal epiphysis	6w (I) 4w (II-V)	28-65 d	5-6 w (I) 4 w (II-V)	4-6 w	4-6 w	5-7 w (I) 4-6 w (II-V)	4-5 w
Diaphysis							Birth
Middle phalanx II – V							
Proximal epiphysis	5 w	28-65 d	5-7 w	4-6 w	4-9 w	4-6 w	4-5 w
Diaphysis							Birth
Distal phalanx I – V	Birth						Birth
Sesamoid bones							
Palmar sesamoid bones	2 m	63-92 d	2 m	2 m	8-13 w	2m	
Dorsal sesamoid bones	4 m	91-141 d	4 m	4-5 m	13-24 w	4m	

Table 2.2. *Age at closure of the ossification centres in the dog's forelimb (w= weeks; m= months).*

Anatomical site	Ticer, 1984; Burk, 2003; Kealy, 2011	Shebitz & Wilkens, 1989	Hoskins, 2001b	Dennis, 2010	Peterson & Kutzler, 2011	Thrall, 2011	Dyce, 2018
Scapula							
Supraglenoid tubercle	4-7 m	5-6 m	4-7 m	4-7 m	4-7 m	4-7 m	3-7 m
Humerus							
Proximal epiphysis	10-13 m	10.5-12 m	10-13 m	10-13 m	10-13 m	10-15 m	10-15 m
Distal epiphysis	6-8 m	5.5-6.5 m	5-8 m	5-8 m	4-6 m	6-8 m	5-8 m
Medial condyle	6 w to lat. cond.		6-9 w to lat. cond.	8-12 w to lat. cond.	6-8 w to lat. cond.	6-10 w to lat. cond.	5 m to lat. cond.
Lateral condyle							
Medial epicondyle	6 m to condyle		4-8 m	6 m to condyle	6 m	6-8 m	5-6 m
Radius							
Proximal epiphysis	6-11 m	9-11 m	6-11 m	5-11 m	6-11 m	7-10 m	5-11 m
Distal epiphysis	8-12 m	9-11 m	8-10 m	6-12 m	8-12 m	10-12 m	6-12 m
Ulna							
Olecranon	6-10 m	6.5-9.5 m	7-9 m	5-10 m	6-10 m	7-10 m	5-10 m
Anconeal process	4-5 m		-	3-5 m	-	< 5 m	3-5 m
Distal epiphysis	8-12 m	9-11 m	8-10 m	6-12 m	8-12 m	9-12 m	6-12 m
Carpus							
Radial carpal bone		3-4 m	3-4 m		3-4 m		3-4 m
Accessory carpal bone, epiphysis	4 m	4-5 m	3-5 m	10 w-5 m	4-5 m	-	3-6 m
Metacarpus							
Metacarpal bone I							
Proximal epiphysis	6 m	5.5-6.5 m	5-6 m	4-7 m	5-6 m	6-7 m	6-7 m
Metacarpal bone II – V							
Distal epiphysis	6 m	6.5-7.5 m	6-7 m	4-7 m	6-7 m	6-7 m	5-7 m
Phalanges							
Proximal phalanx I – V							
Proximal epiphysis	6 m	5.5-6.5 m	4-7 m	4-7 m	6 m	6-7 m	5-7 m
Middle phalanx I – V							
Proximal epiphysis		5.5-6.5 m	4-6 m (II, V) 5-7 m (III, IV)	4-7 m	6 m	6-7 m	5-7 m

Table 2.3. Age at appearance of the ossification centres in the dog's hindlimb (d= days; w= weeks; m= months).

Anatomical site	Ticer, 1975; Burk, 2003; Kealy, 2011	Schebitz & Wilkens, 1989	Hoskins, 2001b	Dennis, 2010	Peterson & Kutzler, 2011	Thrall, 2011	Dyce, 2018
Hip bone (*Os coxae*)							
Ilium	Birth		Birth		Birth	Birth	Birth
Ischium	Birth		Birth		Birth	Birth	Birth
Pubis	Birth		Birth		Birth	Birth	Birth
Acetabular bone	7 w	49-85 d	1-2 m	7 w	7-12 w	2-3 m	7 w
Iliac crest	4 m	120-141 d	4-7 m	4 m	4-5 m	4-5 m	4 m
Ischial tuber	3 m	50-85 d	3-5 m	3 m	7-12 w	3-4 m	3 m
Ischial arch	6 m	141-173 d	6-10 m		5-6 m	6 m	
Caudal symphysis pubis	7 m	147-197 d	-	7 m	7 m		7 m
Symphysis pubis							
Femur							
Lesser trochanter	8 w	35-78 d	-	8 w	5-11 w	7-9 w	8 w
Greater trochanter	8 w	35-50 d	4-10 w	8 w	5-7 w	7-9 w	8 w
Proximal epiphysis (head)	2 w	14-29 d	2-3 w	2 w	2-4 w	1-2 w	2 w
Diaphysis	Birth				Birth		Birth
Distal epiphysis		14-22 d	3-4 w		2-3 w	3-4 w	3 w
Medial condyle	3 w			3 w			
Lateral condyle	3 w			3 w			
Trochlea	2 w						3 w
Tibia							
Proximal epiphysis	3 w	14-22 d	2-5 w	3 w	2-3 w	2-4 w	3 w
Tibial tuberosity	8 w	49-78 d	7-9 w	8 w	7-11 w	7-8	8 w
Diaphysis	Birth	Birth	Birth	Birth	Birth	Birth	Birth
Distal epiphysis	3 w	14-29 d	2-6 w	3 w	2-4 w	2-4 w	3 w
Medial malleolus	3 m	77-92 d	12 w	5 m	11-13 w	3 m	3 m
Fibula							
Proximal epiphysis	9 w	49-72 d	9-10 w	9 w	7-10 w	8-10 w	9 w
Diaphysis	Birth	Birth			Birth		Birth
Distal epiphysis	2-7 w	35-43 d	2-6 w	2-7 w	2-7 w	4-7 w	2-7 w
Tarsus							
Talus	Birth - 1 w		1 w	Birth	Birth - 1 w	Birth	
Calcaneus							
Body	Birth - 1 w		1 w	Birth	Birth - 1 w	Birth	Birth
Calcanean tuber	6 w	49-65 d	5-6 w	6 w	6-9 w	6w	6 w
Central tarsal bone	3 w	14-22 d	2 w	2-4 w	2-3 w	3w	2-4 w
Tarsal bone I	4w	36-49 d	5-6 w	2-4 w	5-7 w	4w	2-4 w
Tarsal bone II	4w	29-36 d	5-6 w	2-4 w	4-5 w	4w	2-4 w
Tarsal bone III	3w	21-35 d	3-4 w	2-4 w	3-5 w	3w	2-4 w
Tarsal bone IV	2w	-	2 w	2-4 w	2 w	2w	2-4 w
Metatarsus							
Metatarsal bone I	See forelimb	49-78 d	4 w	See forelimb	See forelimb	See forelimb	4 w
Metatarsal bone II – V		29-36 d	4 w				4 w
Phalanges							
	See forelimb	35-43 d (I) 35-57 d (II)	4w (I) 6 w (II)	See forelimb	See forelimb	See forelimb	See forel
Sesamoid bones							
Patella	9 w	49-85 d	8-9 w	9 w	7-12 w	6-9	9 w
M. Gastrocnemius (Fabellae)	3 m	91-100 d	12 w	3 m	13-15 w	3 m	3 m
M. Popliteus	3 m	126-169 d	-	3 m	18-24 w	3-4 m	3 m
Plantar sesam. bones	2 m	63-92 d	2 m		See forelimb	See forel	
Dorsal sesam. bones	5 m	126-169 d	5 m		See forelimb	See forel	

Table 2.4. *Age at closure of the ossification centres in the dog's hindlimb (w= weeks; m= months; y= years).*

Anatomical site	Ticer, 1975; Burk, 2003; Kealy, 2011	Schebitz & Wilkens 1989	Hoskins, 2001b	Dennis, 2010	Peterson & Kutzler, 2011	Thrall, 2011	Dyce, 2018
Hip bone (*Os coxae*)							
Ilium	4-6 m	5-6 m	4-6 m	4-6 m	4-6 m		4-6 m
Ischium	4-6 m	5-6 m	4-5 m	4-6 m	4-6 m		4-6 m
Pubis	4-6 m	5-6 m	4-6 m	4-6 m	4-6 m		4-6 m
Acetabular bone	5 m	5-6 m	3-5 m	4-6 m	5-6 m	3-5 m	4-6 m
Iliac crest	1-2 y	10-11 m	12-30 m	1-2 y	1-2 y, sometimes never	24-36 m	15 m-5.5 y
Ischial tuber	8-10 m		8-10 m	8-10 m	8-11 m	10-12 m	8-14 m
Ischial arch	12 m		8-13 m		12 m		
Caudal symphysis pubis	5 y	10-12 m			5 y		15 m-5.5 y
Symphysis pubis	5 y		3-13 y		5y	4-5 m	2.5-6 y
Femur							
Lesser trochanter	8-13 m	11-12 m		8-13 m	8-13 m	9-12 m	8-13 m
Greater trochanter	6-10 m	11 m	8-11 m	6-10 m	6-11 m	9-12 m	6-9 m
Proximal epiphysis (head)	7-11 m	11-12 m	6-10 m	6-11 m	7-12 m	8-11 m	6-9 m
Distal epiphysis	8-11 m	11 m	9-11m	6-11 m	8-11 m	9-12 m	6-12 m
Medial condyle			3-4 m to lat. cond.				
Lateral condyle							
Trochlea	3 m to condyle				3 m to cond.		3 m
Tibia							
Proximal epiphysis	6 w to lat. cond., 6-12 m to diaph.	11-12 m	10-13 m	6-8 m to tuberosity	6 w to lat. cond., 6-12 m to diaph.	9-10 m	6-15 m
Tibial tuberosity	6-8 m to cond., 6-12 m to diaph.	11-12 m	8 m to cond., 6-8 m to diaph.	6-12 m to diaph.	6-8 m to cond., 6-12 m to diaph.	10-12 m	8-10 m
Distal epiphysis	8-11 m	8.5-11 m	8-9 m	5-11 m	8-11 m	12-15 m	5-11 m
Medial malleolus	5 m	4-5 m	8-9 m	5-11 m	4-5 m	3-5 m	4-5 m
Fibula							
Proximal epiphysis	8-12 m	10-12 m	8-11m	6-12 m	8-12 m	10-12 m	6-12 m
Distal epiphysis	7-11 m	10-11 m	8-9 m	5-12 m	7-11 m	12-13 m	5-13 m
Tarsus							
Calcaneus							
Calcanean tuber	3-8 m	6.5-7.5 m	5-7 m	11 w – 8m	3-8 m	6-7 m	3-8 m
Metatarsus							
Metatarsal bone II – V	See forelimb	7-8 m	6 m	See forelimb	See forelimb	See forelimb	5-7 m
Phalanges							
	See forelimb	6.5-7.5 m	6-7 m	See forelimb	See forelimb	See forelimb	See forelimb

ANNEX 3. RECORDING SHEET

LITTER NO. _____ (date of birth __________)

PUPPY ID: ………………………………………………………………

SEX: ……………………………….

Special marks: ……………………………………………………………………………….

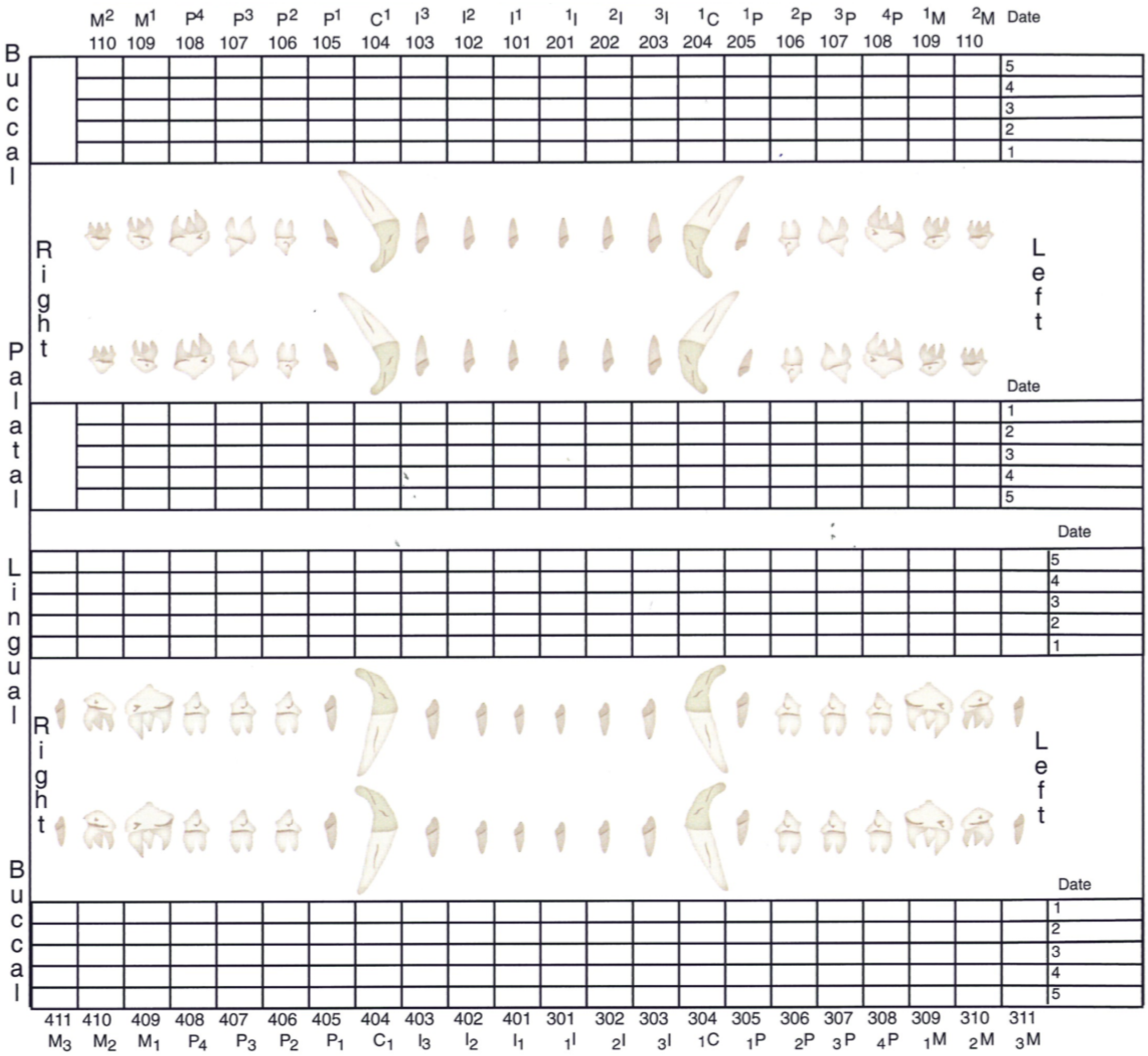
CLINICAL EXAMINATION:

DATE →	4 w	6 w	8 w	10 w	12 w	14 w	16 w	18 w	20 w
Weight									
T (°C)									
PR									
RR									
Mucous membr.									
Skin/coat									
Other									

	6 w	8 w	10 w	12 w	14 w	16 w
RX	○ TB ○ FL ○ HL ○ NO	○ TB ○ FL ○ HL ○ NO	○ TB ○ FL ○ HL ○ NO	○ TB ○ FL ○ HL ○ NO	○ TB ○ FL ○ HL ○ NO	○ TB ○ FL ○ HL ○ NO

TB: total body; FL: frontlimb; HL: hindlimb

TEETH EXAMINATION:



0	Deciduous tooth – not yet erupted	**4**	Deciduous tooth – wearing started
1	Deciduous tooth – eruption just started	**5**	Deciduous tooth – considerable wearing, thinning out
2	Deciduous tooth – eruption in progress	**6**	Deciduous tooth – shed
3	Deciduous tooth – eruption complete	**7**	Permanent tooth – erupted

	4 w	**6 w**	**8 w**	**10 w**	**12 w**	**14 w**	**16 w**	**18 w**	**20 w**
PHOTOS	○ FR ○ LD ○ LS ○ BA	○ FR ○ LD ○ LS ○ BA	○ FR ○ LD ○ LS ○ BA	○ FR ○ LD ○ LS ○ BA	○ FR ○ LD ○ LS ○ BA	○ FR ○ LD ○ LS ○ BA	○ FR ○ LD ○ LS ○ BA	○ FR ○ LD ○ LS ○ BA	○ FR ○ LD ○ LS ○ BA

FR: frontal; LD: right side; LS: left side; BA: open mouth

ANNEX 4. CLINICAL AND TEETH EXAMINATION

Table 4.1. *Mean weight and standard deviation for each of the sampled breeds.*
AUS= Australian Shepherd, BAR= Toy Poodle; BF= French Bulldog; BOX= Boxer; CLS= Saarloos Wolfdog; LAB= Labrador Retriever; NOV= Nova Scotia Duck Tolling Retriever; POM= Pomeranian; PS= Berger Blanc Suisse; PT= German Shepherd.

Breed	**Age (weeks)**							
	4	**6**	**8**	**10**	**12**	**14**	**16**	**18**
AUS	-	-	3.59±0.49	4.85±0.99	-	8.08±0.85	10.17±0.76	12.79±1.12
BAR	0.59±0.13	0.72±0.21	0.91±0.27	-	-	-	-	-
BF	1.01±0.09	1.51±0.60	1.87±0.35	2.54±0.43	3.19±0.73	-	4.30*	6.30*
BOX	1.67±0.09	3.14±0.60	4.41±0.93	6.07±1.43	-	7.96±1.89	9.65±2.43	10.33±2.52
CLS	2.03±0.19	3.80±0.52	5.50±0.61	7.35±0.70	9.91±0.75	12.64±1.51	14.25±1.77	-
LAB	1.58±0.72	2.35±1.24	3.78±2.04	4.66±2.51	8.65±3.69	-	-	-
NOV	2.06±0.16	3.12±0.36	4.55±0.53	7.49±0.91	8.30±1.03	-	-	11.73±1.50
POM	0.43±0.06	0.58±0.08	0.82±0.13	1.00±0.15	1.25±0.21	1.47±0.27	-	-
PS	1.90±0.33	3.48±0.33	5.45±0.54	7.65±0.81	9.87±1.67	11.17±1.28	14.11±1.46	17.50±0
PT	2.37±1.03	3.57±1.03	5.45±2.28	7.00±0.93	10.45±1.34	12.80*	15.00*	19.50*

**observations made on one puppy*

Figure 4.1 *Teeth development at 4 weeks of age.* ***A.*** *Pomeranian;* ***B.*** *Toy poodle;* ***C.*** *French Bulldog;* ***D.*** *Australian Shepherd;* ***E.*** *Boxer;* ***F.*** *Labrador Retriever;* ***G.*** *Saarloos Wolfdog;* ***H.*** *Berger Blanc Suisse;* ***I.*** *German Shepherd.*

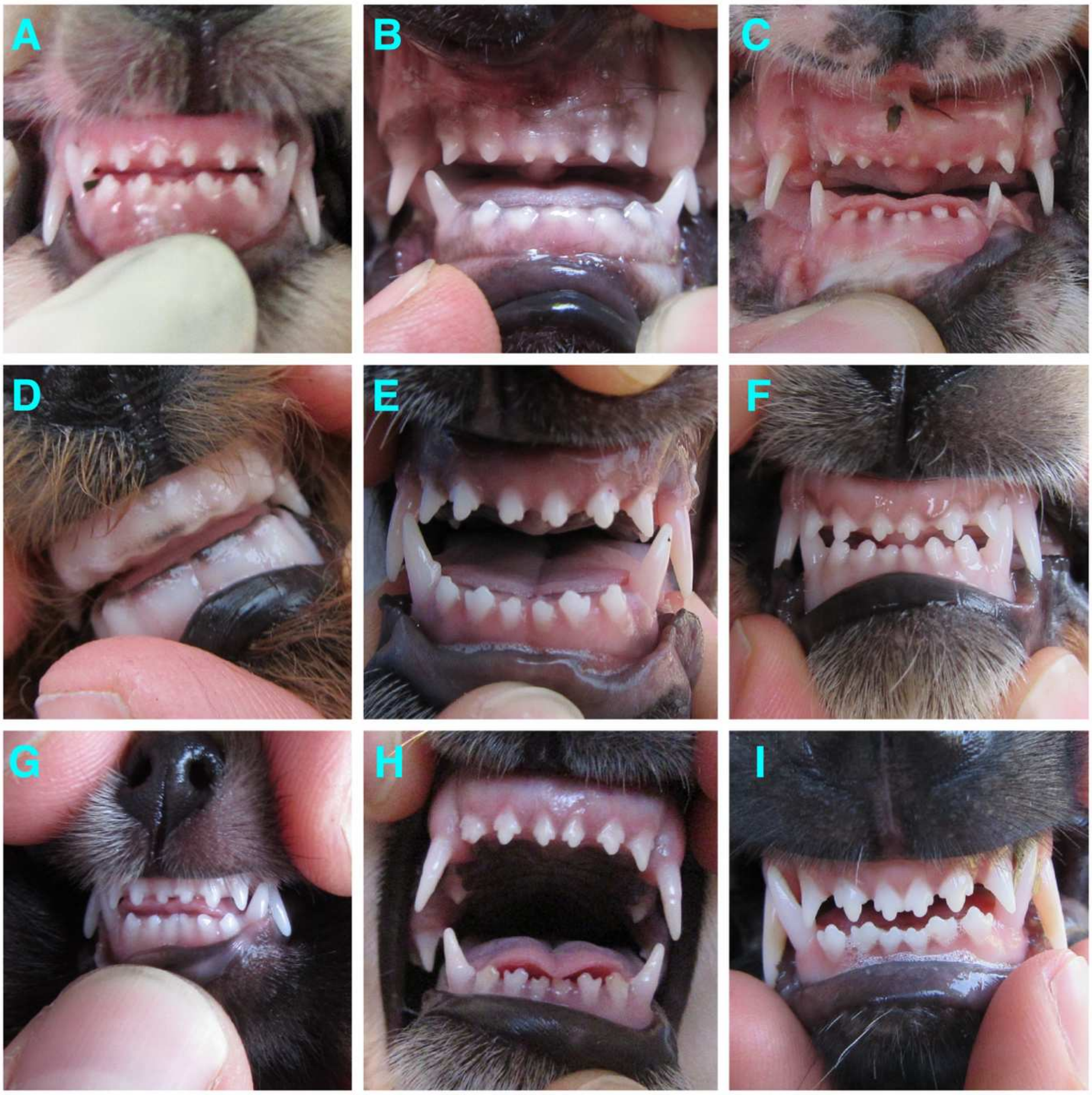


Figure 4.2 *Teeth development at 6 weeks of age.* ***A.*** *Australian Shepherd;* ***B.*** *French Bulldog;* ***C.*** *Boxer;* ***D.*** *Toy Poodle;* ***E.*** *Saarloos Wolfdog;* ***F.*** *Labrador Retriever;* ***G.*** *Pomeranian;* **H.** *Berger Blanc Suisse;* **I.** *German Shepherd.*

Figure 4.3 *Teeth development at 8 weeks of age.* ***A.*** *French Bulldog;* ***B.*** *Boxer;* ***C.*** *Australian Shepherd;* ***D.*** *Toy Poodle;* ***E.*** *Labrador Retriever;* **F.** *Saarloos Wolfdog;* **G.** *Pomeranian;* **H.** *Nova Scotia Duck Tolling Retriever;* **I.** *Berger Blanc Suisse.*

Figure 4.4 *Teeth development at 10 weeks of age.* ***A.*** *Australian Shepherd;* ***B.*** *French Bulldog;* ***C.*** *Boxer;* ***D.*** *Saarloos Wolfdog;* ***E.*** *Labrador Retriever;* ***F.*** *Nova Scotia Duck Tolling Retriever;* **G.** *Pomeranian;* **H.** *Berger Blanc Suisse;* **I.** *German Shepherd.*

Figure 4.5 *4-weeks-old (A) vs 10-weeks-old (B) Labrador Retriever.*

Figure 4.6 *Teeth development at 12 weeks of age.* ***A.*** *French Bulldog (upper teeth);* ***B.*** *Saarloos Wolfdog;* ***C.*** *Nova Scotia Duck Tolling Retriever;* ***D.*** *Berger Blanc Suisse;* ***E.*** *French Bulldog (lower teeth);* ***F.*** *Labrador Retriever;* **G.** *Pomeranian;* **H.** *German Shepherd.*

Figure 4.7 *Teeth development at 14 weeks of age.* ***A.*** *Boxer (upper teeth);* ***B.*** *Saarloos Wolfdog;* ***C.*** *Labrador Retriever;* ***D.*** *Boxer (lower teeth);* ***E.*** *Berger Blanc Suisse;* ***F.*** *German Shepherd.*

Figure 4.8 *Teeth development at 16 weeks of age.* ***A.*** *Boxer (upper teeth);* ***B.*** *Saarloos Wolfdog;* ***C.*** *Labrador Retriever;* ***D.*** *Boxer (lower teeth);* ***E.*** *Berger Blanc Suisse;* ***F.*** *German Shepherd.*

Figure 4.9 *Teeth development at 18 weeks of age.* ***A.*** *French Bulldog (upper teeth);* ***B.*** *Boxer;* ***C.*** *Australian Shepherd;* ***D.*** *French Bulldog (lower teeth);* ***E.*** *Nova Scotia Duck Tolling Retriever;* ***F.*** *Berger Blanc Suisse.*

Figure 4.10 *Teeth development at 20 weeks of age.* ***A.*** *Australian Shepherd;* ***B.*** *Boxer;* ***C.*** *Nova Scotia Duck Tolling Retriever;* ***D.*** *Berger Blanc Suisse.*

ANNEX 5. RADIOGRAPHIC EXAMINATION – OC DETECTION

Table 5.1-5.6. Percentage of puppies of each breed in which the forelimb OCs were observed at each time point. *Sca= supraglenoid tubercule; HumP= proximal epiphysis of the humerus; HumE= epiphysis of medial epicondyle of the humerus; HumD= distal epiphysis of the humerus; RadP= proximal epiphysis of the radius; RadD= distal epiphysis of the radius.*
Colour legend: orange 0-24%; yellow 25-49%; blue 50-74%; green 75-100%.

5.1	Age (weeks)					
Sca	6	8	10	12	14	16
BF	0%	50%	100%	100%	100%	100%
BOX	37.5%	100%	100%	100%	100%	100%
CLS	100%	100%	100%	100%	100%	100%
LAB	11.1%	12.5%	100%	100%	100%	100%
NOV	-	100%	100%	100%	100%	100%
POM	0%	0%	100%	100%	100%	100%
PS	-	100%	100%	100%	100%	100%
PT	100%	100%	100%	100%	100%	100%

5.2	Age (weeks)					
HumP	6	8	10	12	14	16
BF	100%	100%	100%	100%	100%	100%
BOX	100%	100%	100%	100%	100%	100%
CLS	100%	100%	100%	100%	100%	100%
LAB	100%	100%	100%	100%	100%	100%
NOV	-	100%	100%	100%	100%	100%
POM	100%	100%	100%	100%	100%	100%
PS	100%	100%	100%	100%	100%	100%
PT	100%	100%	100%	100%	100%	100%

5.3	Age (weeks)					
HumE	6	8	10	12	14	16
BF	0%	50%	100%	100%	100%	100%
BOX	0%	70%	100%	100%	100%	100%
CLS	100%	100%	100%	100%	100%	100%
LAB	0%	14.3%	76.9%	100%	100%	100%
NOV	-	100%	100%	100%	100%	100%
POM	0%	16.7%	100%	100%	100%	100%
PS	0%	100%	100%	100%	100%	100%
PT	100%	100%	100%	100%	100%	100%

5.4	Age (weeks)					
HumD	6	8	10	12	14	16
BF	100%	100%	100%	100%	100%	100%
BOX	100%	100%	100%	100%	100%	100%
CLS	100%	100%	100%	100%	100%	100%
LAB	100%	100%	100%	100%	100%	100%
NOV	-	100%	100%	100%	100%	100%
POM	100%	100%	100%	100%	100%	100%
PS	100%	100%	100%	100%	100%	100%
PT	100%	100%	100%	100%	100%	100%

5.5	Age (weeks)					
RadP	6	8	10	12	14	16
BF	50%	100%	100%	100%	100%	100%
BOX	100%	100%	100%	100%	100%	100%
CLS	100%	100%	100%	100%	100%	100%
LAB	100%	100%	100%	100%	100%	100%
NOV	-	100%	100%	100%	100%	100%
POM	0%	75%	100%	100%	100%	100%
PS	100%	100%	100%	100%	100%	100%
PT	100%	100%	100%	100%	100%	100%

5.6	Age (weeks)					
RadD	6	8	10	12	14	16
BF	100%	100%	100%	100%	100%	100%
BOX	100%	100%	100%	100%	100%	100%
CLS	100%	100%	100%	100%	100%	100%
LAB	100%	100%	100%	100%	100%	100%
NOV	-	100%	100%	100%	100%	100%
POM	75%	100%	100%	100%	100%	100%
PS	100%	100%	100%	100%	100%	100%
PT	100%	100%	100%	100%	100%	100%

Table 5.7-5.9. Percentage of puppies of each breed in which the forelimb OCs were observed at each time point.
UlnO= olecranon; UlnD= distal epiphysis of the ulna; Car= epiphysis of the accessory carpal bone.
Colour legend: orange 0-24%; yellow 25-49%; blue 50-74%; green 75-100%.

5.7	Age (weeks)					
UlnO	6	8	10	12	14	16
BF	0%	0%	100%	100%	100%	100%
BOX	0%	30%	60%	100%	100%	100%
CLS	100%	100%	100%	100%	100%	100%
LAB	0%	6.7%	64.3%	100%	100%	100%
NOV	-	100%	100%	100%	100%	100%
POM	0%	25%	100%	100%	100%	100%
PS	0%	100%	100%	100%	100%	100%
PT	42.9%	85.7%	100%	100%	100%	100%

5.8	Age (weeks)					
UlnD	6	8	10	12	14	16
BF	0%	0%	100%	100%	100%	100%
BOX	44.4%	88.9%	100%	100%	100%	100%
CLS	100%	100%	100%	100%	100%	100%
LAB	0%	35.7%	86.7%	100%	100%	100%
NOV	-	100%	100%	100%	100%	100%
POM	0%	0%	100%	100%	100%	100%
PS	50%	100%	100%	100%	100%	100%
PT	100%	100%	100%	100%	100%	100%

5.9	Age (weeks)					
Car	6	8	10	12	14	16
BF	0%	60%	100%	100%	100%	100%
BOX	0%	70%	100%	100%	100%	100%
CLS	100%	100%	100%	100%	100%	100%
LAB	0%	7.1%	71.4%	100%	100%	100%
NOV	-	100%	100%	100%	100%	100%
POM	0%	100%	100%	100%	100%	100%
PS	0%	100%	100%	100%	100%	100%
PT	71.4%	100%	100%	100%	100%	100%

Table 5.10-5.11. Percentage of puppies of each breed in which the hindlimb OCs were observed at each time point.
Fem= distal epiphysis of the femur; Pat= patella.
Colour legend: orange 0-24%; yellow 25-49%; blue 50-74%; green 75-100%.

5.10	Age (weeks)					
Fem	6	8	10	12	14	16
BF	100%	100%	100%	100%	100%	100%
BOX	100%	100%	100%	100%	100%	100%
CLS	100%	100%	100%	100%	100%	100%
LAB	100%	100%	100%	100%	100%	100%
NOV	-	100%	100%	100%	100%	100%
POM	100%	100%	100%	100%	100%	100%
PS	100%	100%	100%	100%	100%	100%
PT	100%	100%	100%	100%	100%	100%

5.11	Age (weeks)					
Pat	6	8	10	12	14	16
BF	0%	0%	100%	100%	100%	100%
BOX	0%	44.4%	100%	100%	100%	100%
CLS	66.7%	100%	100%	100%	100%	100%
LAB	0%	15.4%	42.9%	100%	100%	100%
NOV	-	100%	100%	100%	100%	100%
POM	0%	33.3%	100%	100%	100%	100%
PS	0%	100%	100%	100%	100%	100%
PT	42.9%	85.7%	100%	100%	100%	100%

Table 5.12-5.18. Percentage of puppies of each breed in which the hindlimb OCs were observed at each time point. *Fab= fabellae; Pop= popliteal; TibP= proximal epiphysis of the tibia; TibT= tibial tuberosity; TibD= distal epiphysis of the tibia; Fib= proximal epiphysis of the fibula; Tar= epiphysis of the calcaneal tuber.*
Colour legend: orange 0-24%; yellow 25-49%; blue 50-74%; green 75-100%.

5.12	Age (weeks)					
Fab	6	8	10	12	14	16
BF	0%	0%	0%	0%	0%	50%
BOX	0%	0%	0%	0%	80%	100%
CLS	0%	0%	37.5%	100%	100%	100%
LAB	0%	0%	0%	0%	-	-
NOV	-	0%	0%	0%	-	-
POM	0%	0%	0%	0%	0%	0%
PS	0%	0%	0%	0%	80%	100%
PT	0%	0%	0%	0%	0%	100%

5.13	Age (weeks)					
Pop	6	8	10	12	14	16
BF	0%	0%	0%	0%	0%	0%
BOX	0%	0%	0%	0%	0%	0%
CLS	0%	0%	0%	0%	0%	50%
LAB	0%	0%	0%	0%	-	-
NOV	-	0%	0%	0%	-	-
POM	0%	0%	0%	0%	0%	0%
PS	0%	0%	0%	0%	0%	0%
PT	0%	0%	0%	0%	0%	0%

5.14	Age (weeks)					
TibP	6	8	10	12	14	16
BF	100%	100%	100%	100%	100%	100%
BOX	100%	100%	100%	100%	100%	100%
CLS	100%	100%	100%	100%	100%	100%
LAB	100%	100%	100%	100%	100%	100%
NOV	-	100%	100%	100%	100%	100%
POM	100%	100%	100%	100%	100%	100%
PS	100%	100%	100%	100%	100%	100%
PT	100%	100%	100%	100%	100%	100%

5.15	Age (weeks)					
TibT	6	8	10	12	14	16
BF	0%	0%	80%	100%	100%	100%
BOX	0%	60%	100%	100%	100%	100%
CLS	91.7%	100%	100%	100%	100%	100%
LAB	0%	6.7%	53.8%	100%	100%	100%
NOV	-	100%	100%	100%	100%	100%
POM	0%	33.3%	100%	100%	100%	100%
PS	0%	100%	100%	100%	100%	100%
PT	28.6%	71.4%	100%	100%	100%	100%

5.16	Age (weeks)					
TibD	6	8	10	12	14	16
BF	100%	100%	100%	100%	100%	100%
BOX	100%	100%	100%	100%	100%	100%
CLS	100%	100%	100%	100%	100%	100%
LAB	100%	100%	100%	100%	100%	100%
NOV	-	100%	100%	100%	100%	100%
POM	100%	100%	100%	100%	100%	100%
PS	100%	100%	100%	100%	100%	100%
PT	100%	100%	100%	100%	100%	100%

5.17	Age (weeks)					
Fib	6	8	10	12	14	16
BF	0%	0%	0%	100%	100%	100%
BOX	0%	11.1%	57.1%	100%	100%	100%
CLS	100%	100%	100%	100%	100%	100%
LAB	0%	10%	66.7%	100%	100%	100%
NOV	-	83.3%	100%	100%	100%	100%
POM	0%	0%	0%	66.7%	100%	100%
PS	0%	100%	100%	100%	100%	100%
PT	40%	85.7%	100%	100%	100%	100%

5.18	Age (weeks)					
Tar	6	8	10	12	14	16
BF	22.2%	100%	100%	100%	100%	100%
BOX	60%	90%	100%	100%	100%	100%
CLS	100%	100%	100%	100%	100%	100%
LAB	6.7%	60%	93.3%	100%	100%	100%
NOV	-	100%	100%	100%	100%	100%
POM	0%	66.7%	100%	100%	100%	100%
PS	33.3%	100%	100%	100%	100%	100%
PT	100%	100%	100%	100%	100%	100%

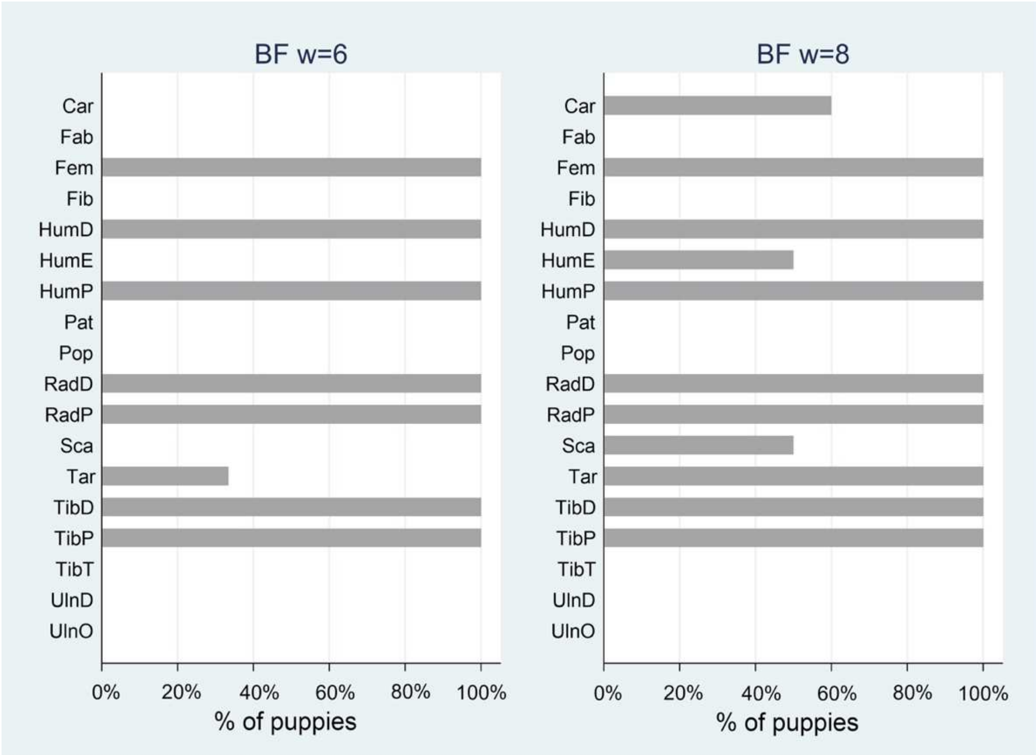


Figure 5.1 *Ossification centres present in French Bulldogs at 6 weeks (on the left) vs. 8 weeks (on the right).*

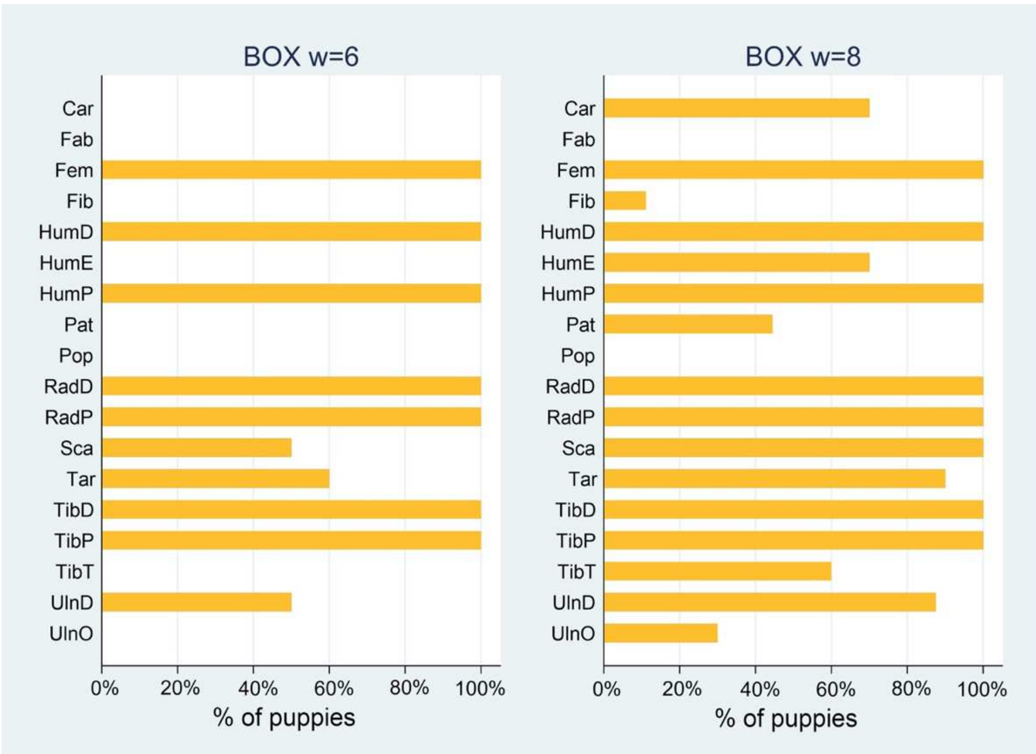


Figure 5.2 *Ossification centres present in Boxers at 6 weeks (on the left) vs. 8 weeks (on the right).*

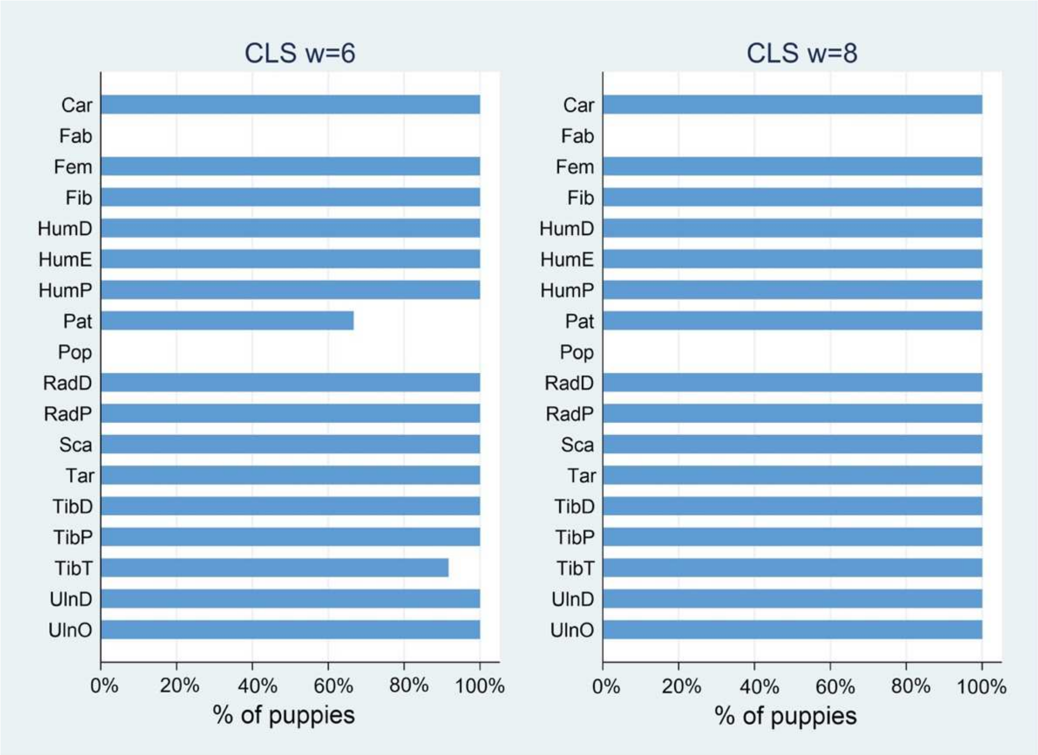


Figure 5.3 *Ossification centres present in Saarloos Wolfdogs at 6 weeks (on the left) vs. 8 weeks (on the right).*

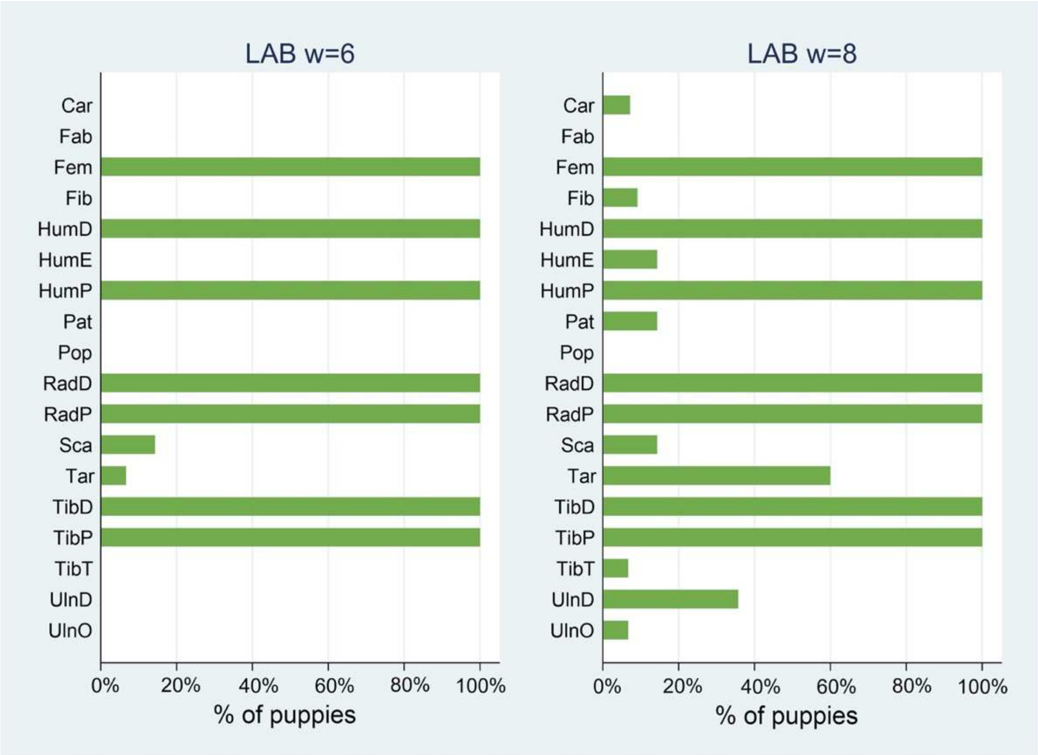


Figure 5.4 *Ossification centres present in Labrador Retrievers at 6 weeks (on the left) vs. 8 weeks (on the right).*

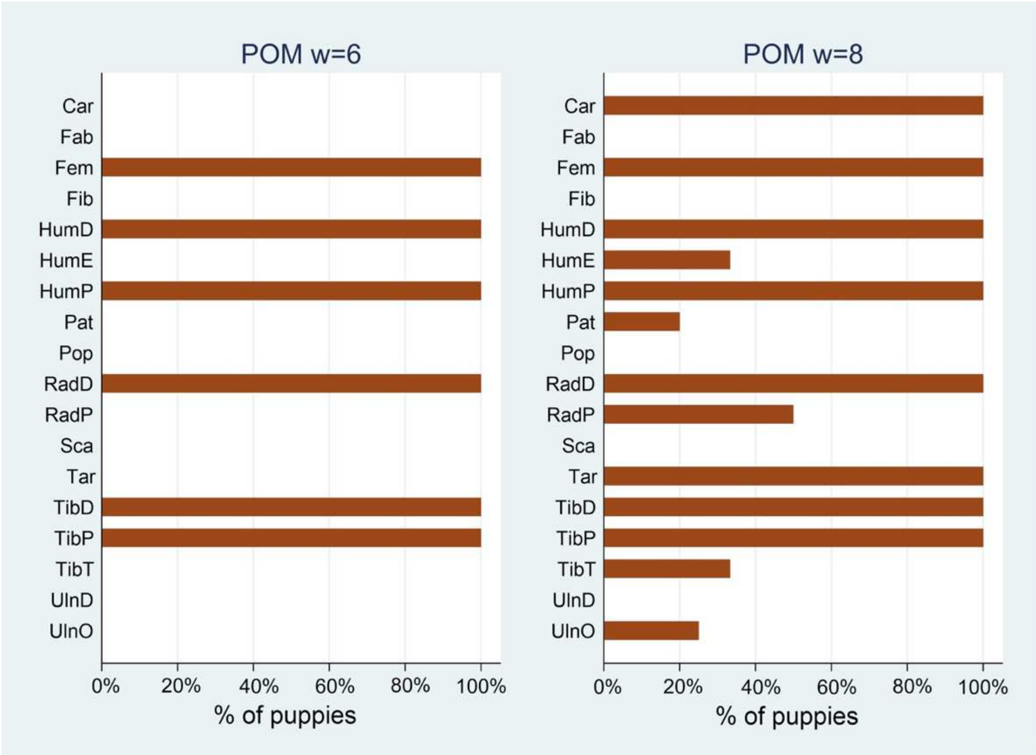


Figure 5.5 *Ossification centres present in Pomeranians at 6 weeks (on the left) vs. 8 weeks (on the right).*

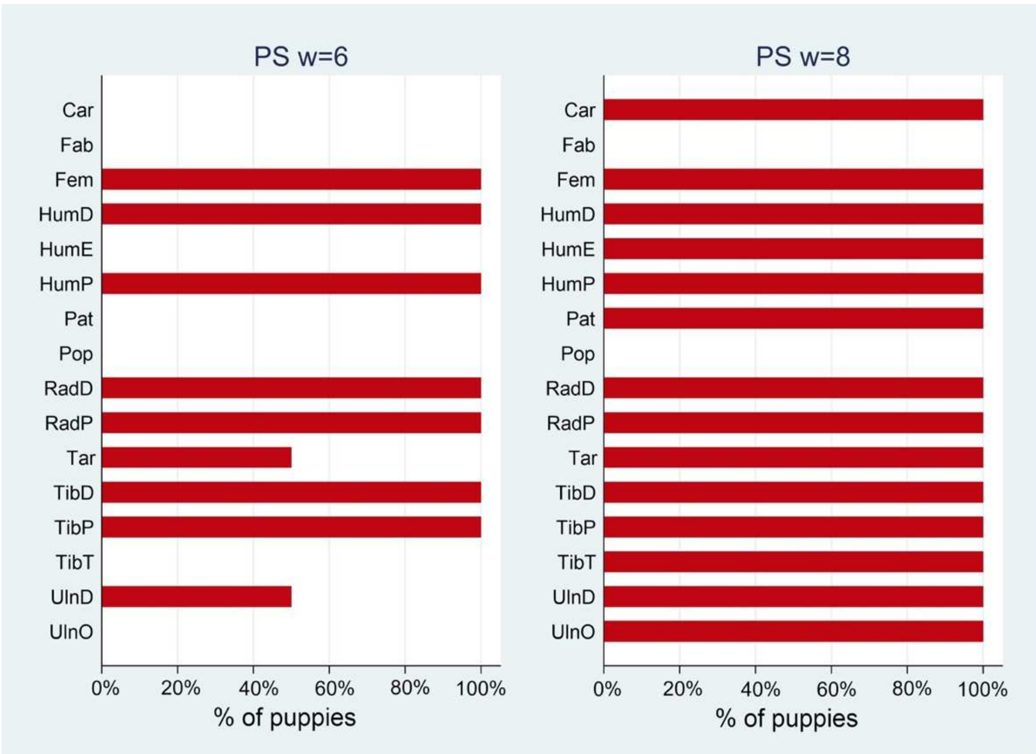


Figure 5.6 *Ossification centres present in Berger Blanc Suisses at 6 weeks (on the left) vs. 8 weeks (on the right).*

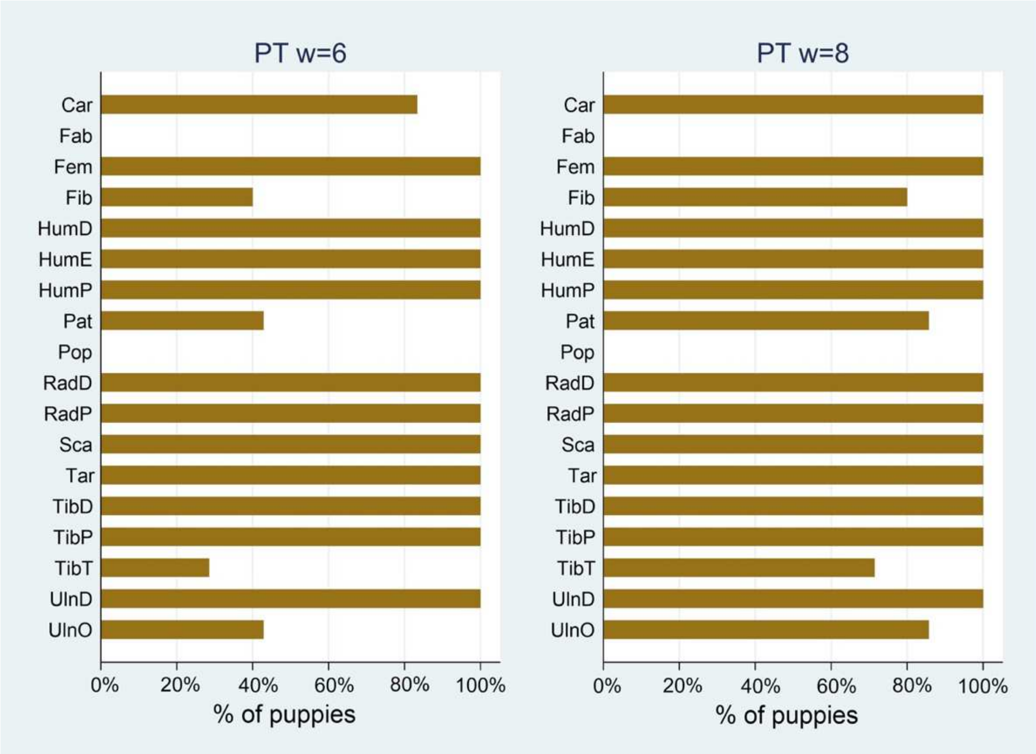


Figure 5.7 *Ossification centres present in German Shepherds at 6 weeks (on the left) vs. 8 weeks (on the right).*

Figure 5.8 *Labrador Retriever, F, 6 weeks; medio-lateral view of the right forelimb.*

Figure 5.9 *Labrador Retriever, M, 6 weeks; medio-lateral view of the right hindlimb.*

Figure 5.10 *German Shepherd, M, 6 weeks; medio-lateral view of the right forelimb.*

Figure 5.11 *German Shepherd, M, 6 weeks; medio-lateral view of the right hindlimb.*

Figure 5.12 *Labrador Retriever, F, 8 weeks; medio-lateral view of the right forelimb.*

Figure 5.13. *Pomeranian, M, 8 weeks; medio-lateral view of the right hindlimb*

Figure 5.14 *Saarloos Wolfdog, F, 8 weeks; medio-lateral view of the right forelimb.*

Figure 5.15 *German Shepherd, M, 8 weeks; medio-lateral view of the right hindlimb.*

Figure 5.16 *Berger Blanc Suisse, F, 10 weeks; medio-lateral view of the right forelimb.*

Figure 5.17 *Boxer, F, 10 weeks; medio-lateral view of the right hindlimb.*

Figure 5.18 *German Shepherd, M, 10 weeks; medio-lateral view of the right forelimb.*

Figure 5.19 *Saarloos Wolfdog, F, 10 weeks; medio-lateral view of the right hindlimb.*

Figure 5.20 *Labrador, M, 12 weeks; medio-lateral view of the right forelimb.*

Figure 5.21 *Pomeranian, M, 12 weeks; medio-lateral view of the right hindlimb.*

Figure 5.22 *Saarloos Wolfdog, F, 12 weeks; medio-lateral view of the right forelimb.*

Figure 5.23 *Nova Scotia Duck Tolling Retriever, M, 12 weeks; medio-lateral view of the right hindlimb.*

Figure 5.24 *Boxer, M, 14 weeks; medio-lateral view of the right forelimb.*

Figure 5.25 *Boxer, M, 14 weeks; medio-lateral view of the right hindlimb.*

Figure 5.26 *Saarloos Wolfdogs, F, 14 weeks; medio-lateral view of the right forelimb.*

Figure 5.27 *Berger Blanc Suisse, F, 14 weeks; medio-lateral view of the right hindlimb.*

ANNEX 6. RADIOGRAPHIC EXAMINATION – MORPHOMETRY

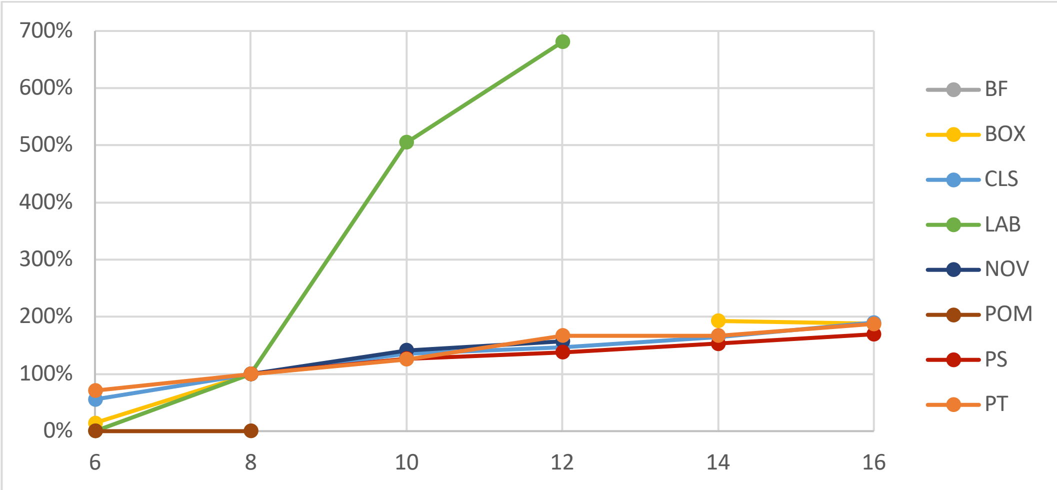


Figure 6.1. *Average percentage increase of the area of the supraglenoid tubercule (aSca) in the different breeds. Increase calculated on the square root. The 8-week measure was considered to be 100%.*

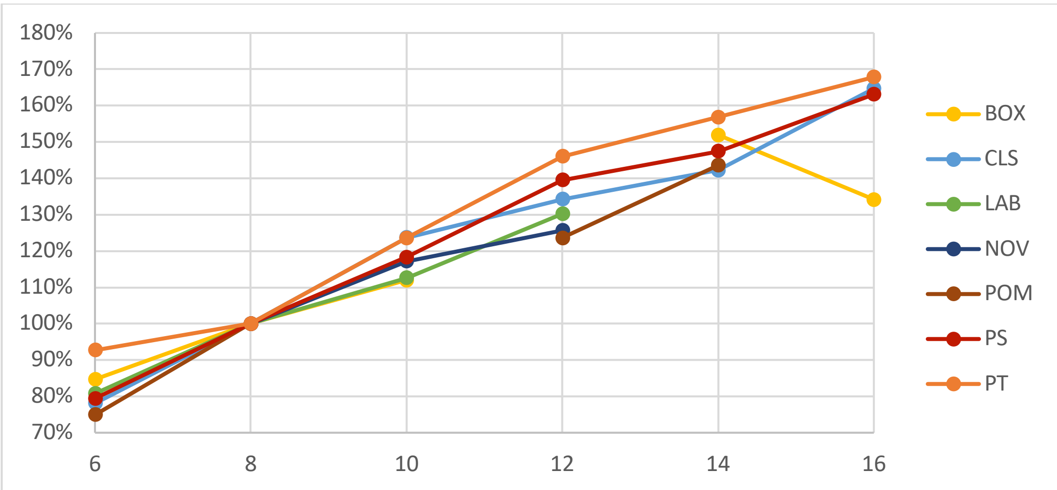


Figure 6.2. *Average percentage increase of the area of the proximal epiphysis of the humerus (aHumP) in the different breeds. Increase calculated on the square root. The 8-week measure was considered to be 100%. At 16 weeks, the measure was performed on only one Boxer.*

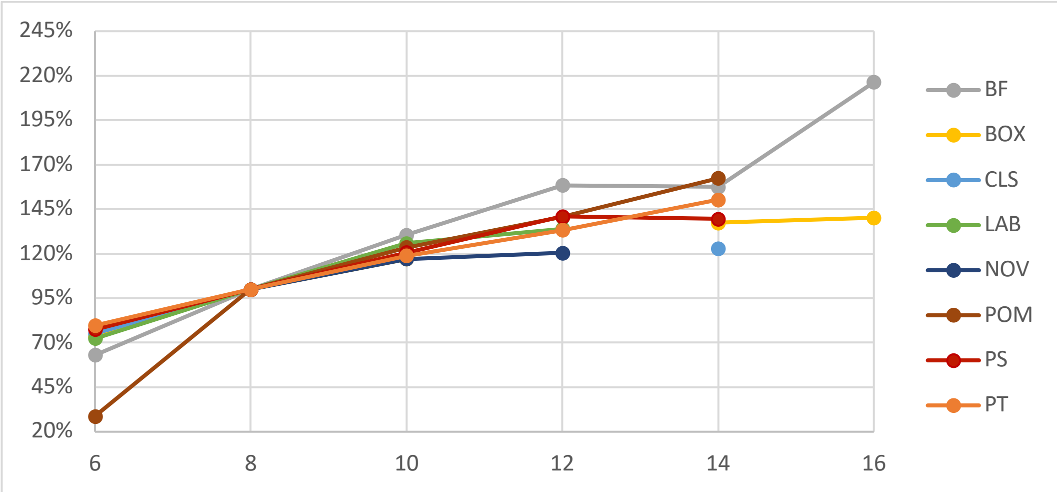


Figure 6.3. *Average percentage increase of the area of the distal epiphysis of the radius (aRadD) in the different breeds. Increase calculated on the square root. The 8-week measure was considered to be 100%.*

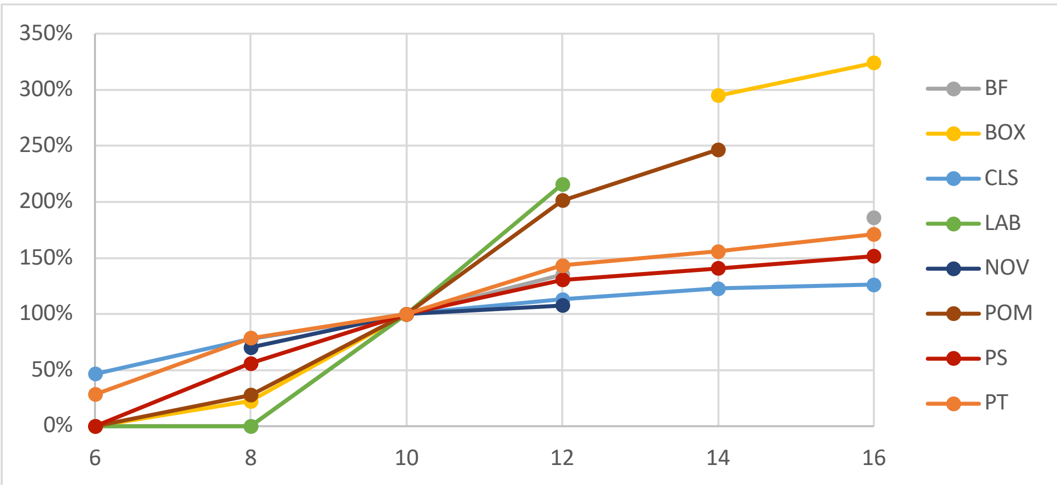


Figure 6.4. *Average percentage increase of the area of the olecranon (aUlnO) in the different breeds. Increase calculated on the square root. The 10-week measure was considered to be 100%.*

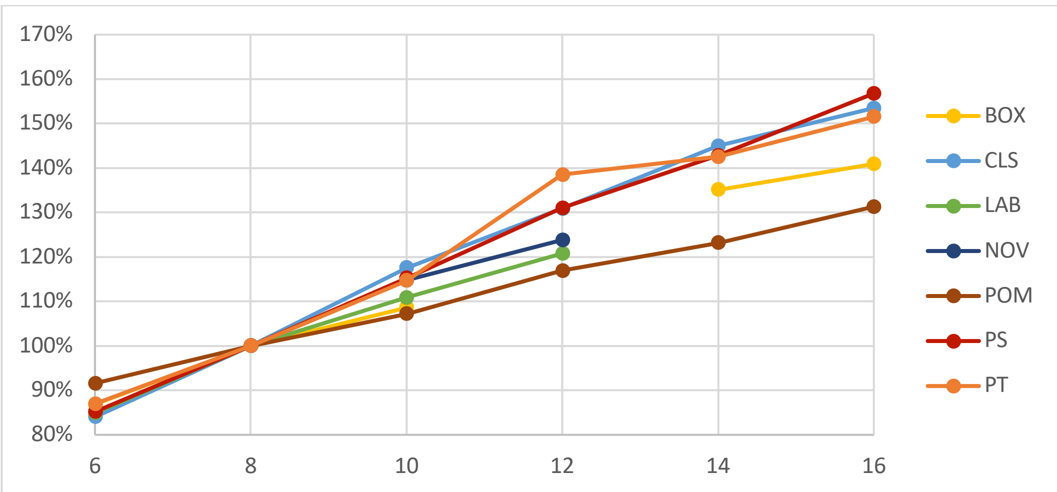


Figure 6.5. *Average percentage increase of the diaphyseal length of the humerus (lHum) in the different breeds. The 8-week measure was considered to be 100%.*

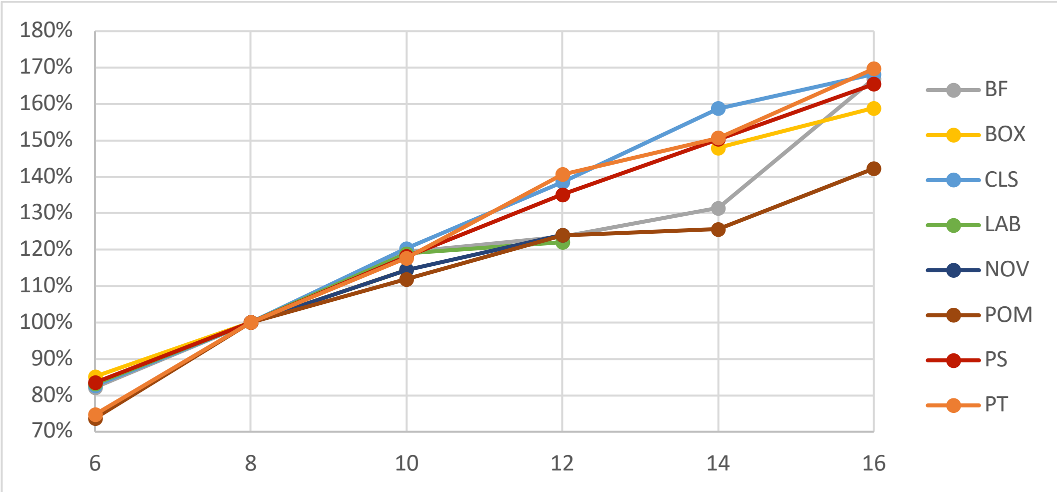


Figure 6.6. *Average percentage increase of the diaphyseal length of the radius (lRad) in the different breeds. The 8-week measure was considered to be 100%.*

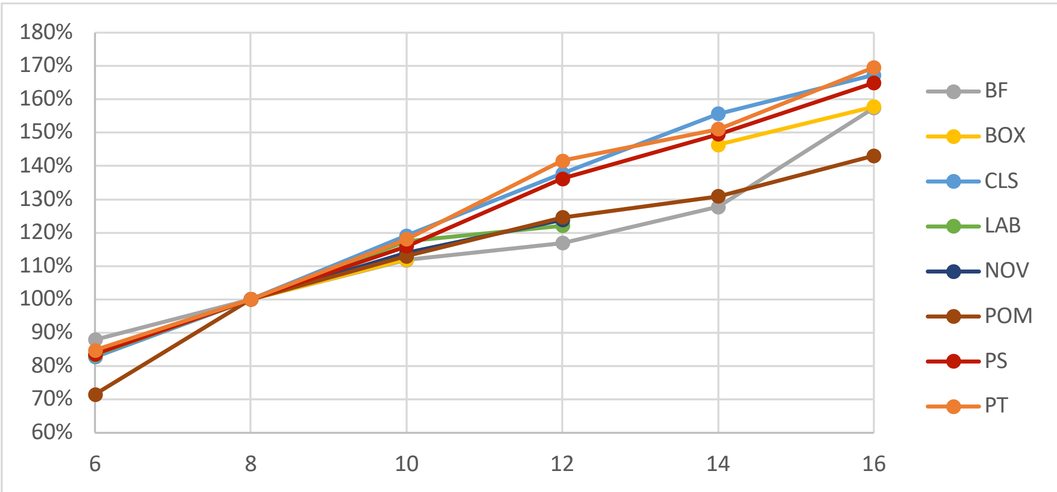


Figure 6.7. *Average percentage increase of the diaphyseal length of the ulna (lUln) in the different breeds. The 8-week measure was considered to be 100%.*

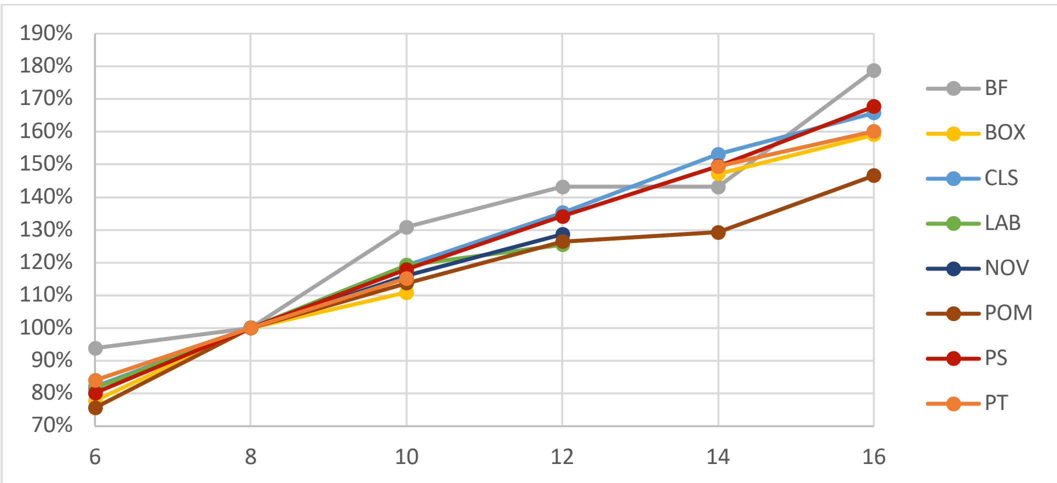


Figure 6.8. *Average percentage increase of the diaphyseal length of the tibia (lTib) in the different breeds. The 8-week measure was considered to be 100%.*

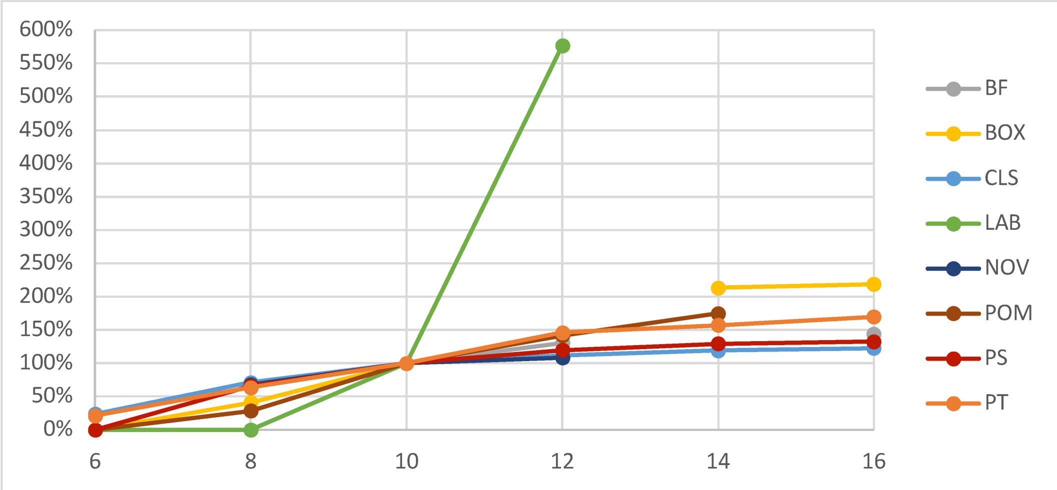


Figure 6.9. *Average percentage increase of the area of the patella (aPat) in the different breeds. Increase calculated on the square root. The 10-week measure was considered to be 100%.*

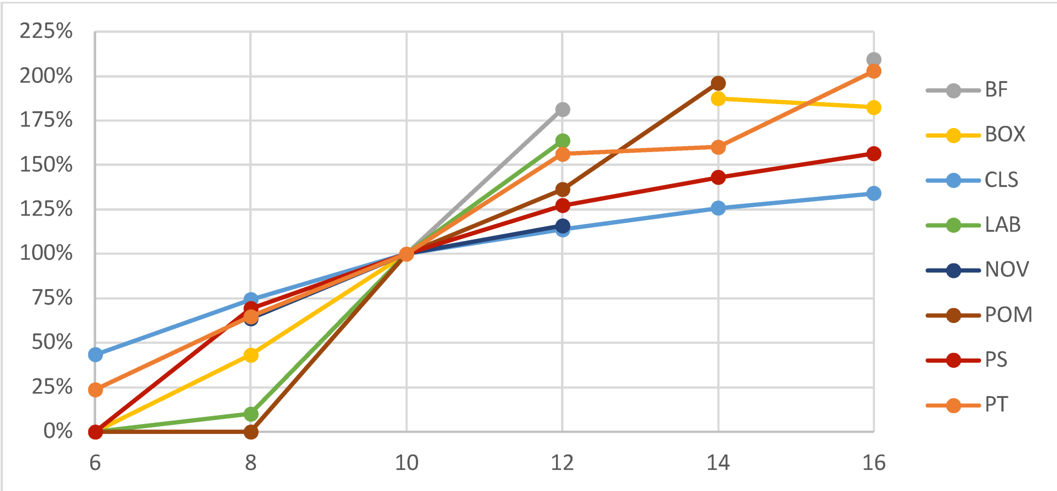


Figure 6.10. *Average percentage increase of the area of the tibial tuberosity (aTibT) in the different breeds. Increase calculated on the square root. The 10-week measure was considered to be 100%.*

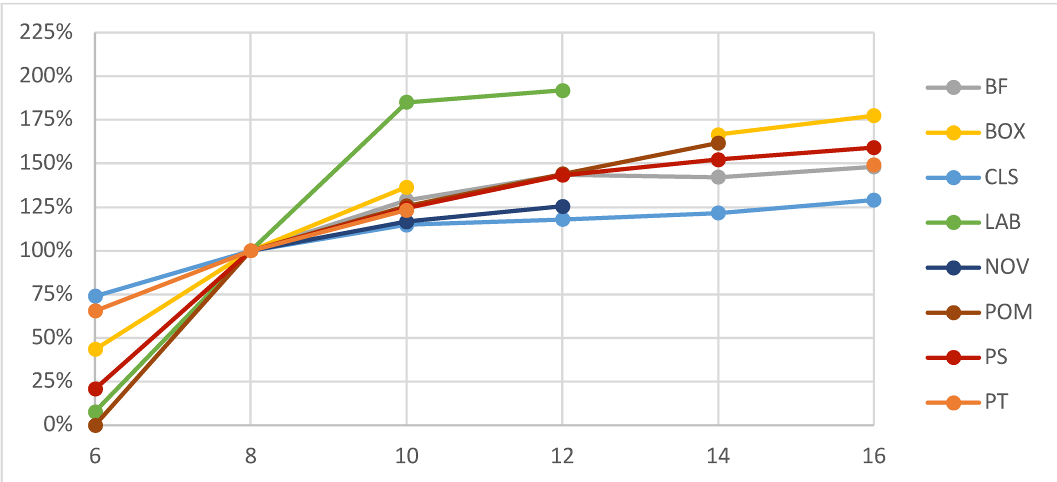


Figure 6.11. *Average percentage increase of the area of the calcaneal tuber (aTar) in the different breeds. Increase calculated on the square root. The 8-week measure was considered to be 100%.*

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