Social behaviour, stress and parasites: comparing free-ranging wolves in Yellowstone (USA), Abruzzo (Italy) and Mercantour (France) national parks

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par

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Neuchâtel, le 20 septembre 2012

Le doyen :
P. Kropf

C'est une triste chose de penser que la nature parle et que le genre humain n'écoute pas.

Victor Hugo

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Mots-clés

Loup, *Canis lupus*, meute, état sauvage, Parc National des Abruzzes, Parc National du Mercantour, Parc National de Yellowstone, parvovirose canine, maladie de Carré, coronavirus canin, helminthe, protozoaire, stress, cortisol, comportement, gestion de conflit.

Résumé

Occupant autrefois la majeure partie de l'hémisphère nord, les populations de loups (*Canis lupus*) ont disparu dans une importante partie de leur aire de distribution initiale, consécutivement à la persécution humaine. Une telle élimination a également eu lieu aux Etats-Unis et dans la majeure partie des pays d'Europe. Suite à la protection de l'espèce dans ces régions, le carnivore a récemment étendu son aire de distribution, par des mouvements de dispersion ou au travers de programmes de réintroduction. Outre les menaces anthropiques, l'infection par des pathogènes et l'exposition à un stress élevé et prolongé peut avoir un impact hautement préjudiciable sur la dynamique des populations de loups évoluant à l'état sauvage. Le suivi de ces variables est ainsi d'intérêt pour la conservation de l'espèce, en particulier pour ce qui est des populations protégées.

Les populations de loups en cours de recolonisation sont soumises à un risque accru en cas d'exposition à des pathogènes canins établis ou émergeants. Les coronavirus canins (CCoV), et en particulier la parvovirose canine de type 2 (CPV-2) et la maladie de Carré (CDV), peuvent chacune induire une importante mortalité chez les louveteaux, et l'infection peut également être létale pour les individus plus âgés. La co-infection par plusieurs de ces virus augmente souvent significativement la sévérité des symptômes. De plus, de nouvelles variantes hautement virulentes de ces virus ont récemment été décrites en Europe. Ainsi, une infection par ces virus pourrait avoir un impact substantiel sur la dynamique des populations, principalement à travers une importante mortalité des louveteaux. L'effet consécutif sur la taille de la population engendrerait une baisse du potentiel de dispersion, diminuant ainsi le taux d'expansion de la population. L'impact d'infections virales peut être plus important sur des populations en cours de recolonisation, plus vulnérables aux perturbations.

Dans cette étude, le terme de "parasite" fait référence aux helminthes et aux protozoaires. L'infection par des helminthes n'est généralement pas fatale chez le loup, mais peut diminuer le fitness et les capacités de chasse des individus. Les louveteaux sont souvent plus vulnérables que les adultes, et peuvent occasionnellement développer des pathologies létales. L'altération de la santé de l'hôte augmente lorsque le parasitisme est accompagné d'une infection virale ou bactérienne, ou encore dans des conditions stressantes.

Un stresseur peut être momentané ou prolongé. Lorsqu'il est prolongé, il induit une réponse chronique, dommageable pour l'organisme. Dans les populations évoluant à

l'état sauvage, un stress soutenu peut être physique, causé par des stresseurs tels que des maladies et/ou la malnutrition, ou psychologique, comme dans le cas d'une instabilité sociale entre groupes ou au sein de ceux-ci. L'effet immunosuppresseur d'un stress soutenu chez les individus peut favoriser les infections virales et parasitaires et ainsi avoir un impact négatif significatif sur la dynamique de populations.
Malgré leur effet potentiellement important sur les populations évoluant à l'état sauvage, ces facteurs fragilisant, soit l'infection par des pathogènes et l'exposition à un stress prolongé, ne sont que rarement pris en considération dans la modélisation de la dynamique de populations ou la planification d'interventions humaines. A ce jour, ces différentes variables ne sont que peu connues dans les populations de loups sauvages, et le suivi entrepris dans la présente étude devrait être poursuivi afin d'évaluer leur évolution à long terme.
Compte-tenu du comportement discret du loup et des dimensions du territoire occupé par les meutes, les interactions sociales au sein de groupes évoluant à l'état sauvage sont difficiles à étudier. Démontrées chez seulement un faible nombre de mammifères différents des primates, des stratégies de gestion de conflits sont prévisibles chez les espèces constituant des unités sociales stables, entretenant des relations individuelles de longue durée, et qui font l'expérience d'interactions agressives au sein d'un groupe et montre de l'hostilité suite à ces conflits. Décrites dans une meute captive, aucune étude n'a précédemment examiné en détails les interactions qui suivent les conflits entre les membres de meutes de loups évoluant en liberté.

Cette étude s'intéresse à différentes variables affectant potentiellement les populations de loups établies dans trois parcs nationaux différents: le Parc National d' Abruzzo, Lazio e Molise (PNALM), en Italie centrale, le Parc National du Mercantour (PNM), dans le sud-est de la France, et le Parc National de Yellowstone (YNP) dans le nord-ouest des Etats-Unis. Nous avons étudié onze meutes de loups évoluant à l'état sauvage, quatre dans chacun des parcs nationaux européens, et trois dans le YNP. Nous avons analysé des échantillons fécaux récoltés dans les régions étudiées pour évaluer l'infection par trois différents virus et relever la présence d'endoparasites. Dans les mêmes échantillons, nous avons mesuré le taux de métabolites du cortisol (CMs) en tant qu'index du stress. Dans le YNP, nous avons étudié le détail des interactions ayant lieu après un conflit dans deux meutes différentes. Les acides nucléiques viraux ont été détectés par *polymerase chain reaction* (PCR). Nous avons employé une technique de concentration suivie d'un examen en microscopie optique pour les investigations parasitologiques, et avons mesurés les taux de CMs par *enzyme immunoassay* (EIA). Pour ce qui est des données comportementales, nous avons réalisé une analyse détaillée d'enregistrements vidéo filmés au YNP, où le suivi télémétrique a aidé à la localisation des meutes étudiées. Des analyses génétiques ou l'observation directe ont permis l'attribution d'une partie des échantillons à des individus spécifiques.

Nous avons comparé les pathogènes détectés et les taux de CMs mesurés entre les parcs nationaux et entre les meutes au sein de chaque parc. Pour les échantillons pour

lesquelles ces données étaient disponibles, nous avons examiné le lien entre les différentes variables d'intérêt et le sexe, l'âge ou le statut social. Nous avons également considéré l'instabilité territoriale et sociale dans l'interprétation des résultats hormonaux. Etant donné que l'infection par des pathogènes peut constituer un stresseur physique soutenu pour l'organisme, nous escomptions une corrélation entre une infection viral et/ou parasitaire et les taux mesurés de CMs.

Nos résultats démontrent l'infection par le CPV-2 dans toutes les meutes de loups de PNALM et dans 50% des meutes de PNM. Notre étude apporte la preuve d'une infection par les CCoV dans 50% et 25% des meutes respectivement dans le PNALM et le PNM. La maladie de Carré n'a pas été détectée, mais est potentiellement présente dans les régions investiguées. Nos résultats suggèrent que la population de loups du PNALM a peut-être développé une immunité à long terme contre le CPV-2, qui apparaît endémique dans la population étudiée. Nos résultats montrent également que, tout comme le CPV-2 et la CDV, les CCoV devraient être largement inclus dans la surveillance épidémiologique des canidés domestiques et sauvages.
En accord avec nos prévisions, les analyses coprologiques ont montré que de nombreux helminthes et protozoaires infectent les loups dans les parcs nationaux investigués. La prévalence parasitaire (défini ici come le nombre d'échantillons fécaux dans lesquelles au moins un parasite du loup a été identifié, divisé par le nombre total d'échantillons analysés) ainsi que la diversité parasitaire sont beaucoup plus élevés dans le PNALM que dans le PNM, tandis que nous avons trouvé des valeurs intermédiaires dans le YNP. L'histoire de l'établissement des trois populations de loups étudiées, divers facteurs écologiques en lien avec la présence de canidés vivant sur le territoire des meutes loups et la variété des espèces proies utilisées par le prédateur sont notamment discutés dans l'interprétation de ces résultats.
L'infection par des espèces additionnelles de parasites pourrait accroître l'impact fragilisant de l'infection sur les individus. Nos résultats montrent que la richesse parasitaire (i.e. le nombre de différents taxa de parasites identifiés dans le même échantillon fécal) est élevé dans le PNALM et bas dans le PNM, tandis que nous avons trouvé des valeurs intermédiaires dans le YNP. La présente étude reporte l'infection par une espèce d'helminthe non encore décrite chez le loup. Dans les trois parcs nationaux investigués, nos résultats montrent la présence de taxa de parasites transmis au travers de la consommation d'espèces proies infectées. Ces parasites sont présents dans un plus grand nombre d'échantillons provenant des populations récemment établies du YNP et du PNM. L'infection du loup par ces parasites dépend du régime alimentaire du carnivore et de l'étendue de l'infection dans les populations d'espèces proies qui sont des hôtes intermédiaires. Au contraire, les parasites les plus fréquemment détectés dans les échantillons du PNALM ont un cycle de vie direct, et l'infection du loup dépend donc de la densité des hôtes potentiels, incluant ainsi les canidés vivant sur le territoire des meutes. Dans la population de loups du PNM, fondée par des individus dispersant depuis l'Italie, la faible diversité parasitaire en comparaison avec le PNALM peut être expliquée

par un phénomène analogue au *founder effect* en génétique des populations. Ceci s'applique également aux virus détectés.

Considérant les résultats hormonaux, l'impact potentiellement stressant de divers facteurs environnementaux ou intrinsèques aux meutes est discuté. Nous n'avons trouvé aucun lien entre les taux de CMs mesurés et le sexe, l'âge ou le statut social des individus. En revanche, comme démontré précédemment chez différentes espèces, nous avons mesuré des taux élevés de CMs dans les meutes de loups dans des périodes d'instabilité territoriale et/ou sociale. De telles variations correspondent à des stresseurs de relativement courte durée agissant sur une partie de la population seulement. Toutefois, dans la population de loups du PNALM, nos résultats suggèrent qu'un stress prolongé affecte les groupes étudiés, puisque nous avons mesurés des taux élevés de CMs dans toutes les meutes considérées au cours des deux hivers de l'étude. Ce type de résultat peut être lié avec l'importante densité de loups dans la région et la présence d'une population de chiens évoluant à l'état sauvage sur le territoire des meutes.
Précédemment décrit dans une meute captive de loups, les interactions de réconciliation, ainsi que de consolation, sollicitée ou non, ont été confirmée dans notre étude de deux meutes de loups à l'état sauvage. La présente investigation est la première à démontrer ce type d'interaction chez le loup évoluant en liberté. En outre, nos résultats suggèrent qu'un type de comportement affiliatif spécifique, "nose touch", est principalement employé dans les interactions qui suivent un conflit entre les membres d'une meute.

Les systèmes étudiés ici sont hautement complexes, comprenant différents effets interactifs entre les pathogènes et les stresseurs physiques et/ou sociaux. Le risque de disparition locale du loup consécutive à une infection par des pathogènes est augmenté par la fragmentation de l'habitat du prédateur. Les canidés vivant sur le territoire des meutes de loups peuvent jouer un rôle important dans la transmission de pathogènes et leur maintenance dans l'environnement. Nos résultats montrent que le loup est infecté par divers pathogènes dans les régions investiguées, et qu'il est parfois exposé à des taux élevés de CMs dont l'impact affaiblissant s'additionne à celui des infections identifiées. L'effet cumulatif de ces facteurs peut avoir un impact important sur la dynamique des populations. Ainsi, la présente étude fournit des information de base importantes à considérer avant toute intervention humaine sur des populations de loups à l'état sauvage.

Key words

Wolf, *Canis lupus*, pack, free-ranging, Abruzzo National Park, Mercantour National Park, Yellowstone National Park, canine parvovirus, canine distemper, canine coronavirus, helminth, protozoan, stress, cortisol, behavior, conflict management.

Abstract

Formerly occupying most of the northern hemisphere, wolf (*Canis lupus*) populations have disappeared from a broad range of their initial distribution range, consecutive to human persecution. Such extirpation also took place in the United States and in most European countries. Following protection of the species in these areas, the carnivore recently expended its distribution range, through dispersal events or helped by reintroduction programs. Besides anthropic threats, infection by pathogens and exposure to elevated and sustained stress can have a highly damaging impact on population dynamics in free-ranging wolves. Monitoring of these variables is therefore of conservation concern, especially in protected populations.

Recovering wolf populations are at enhanced risk when exposed to established and emerging canine pathogens. Canine enteric coronaviruses (CCoV), and in particular canine parvovirus type 2 (CPV-2) and canine distemper (CDV) can each induce important pup mortality, and infection may also be lethal to older individuals. Co-infection often significantly increases severity of symptoms. Additionally, new highly virulent variants of these viruses have recently been described in Europe. Thus, infection by these viruses may have a substantial impact on population dynamics, mainly through important pup mortality. The consequent effect on population size would further impact the number of potential dispersing individuals, and therefore decrease the expansion rate of populations. Impact of viral infections may be stronger on recolonizing populations, more vulnerable to perturbations.

In this study, “parasite” specifically refers to helminths and protozoans. Infection by helminths is generally not lethal to wolves, but can impair fitness and prey-catching ability of individuals. Wolf pups are usually more vulnerable than adults, and can occasionally develop lethal pathologies. Impairment of host’s health increases when parasitism is combined with viral infection, bacterial disease or stressful conditions.

A stressor can be momentary or sustained. When sustained, it induces a chronic, damaging stress response. In free-ranging populations, sustained stress may be physical, caused by stressors such as diseases and/or malnutrition, or psychological, as in case of social instability between or within groups. The immunosuppressive effect of sustained stress in individuals might favor parasitic and viral infections and thus have a significant negative impact on population dynamics.

Despite their potential high impact on free-ranging populations, these weakening factors, namely infection by pathogens and exposure to sustained stress, are seldom taken into account in modeling of population dynamics or planning of human

interventions. To date, little is known about these different variables in free-ranging wolf populations, and the monitoring undertaking in the present study should be pursued to assess their long-term evolution.
Given the secretive behavior of wolves and the size of their home range, social interactions within free-ranging groups are difficult to study. Demonstrated in only few non-primate mammals, conflict management strategies are expected in species living in stable social units, sharing long lasting individualized relationships, and experiencing within-group aggression and post-conflict hostility. Described in a captive wolf pack, no previous study examined in details the interactions that follow conflicts between members of free-ranging wolf packs.

This study investigates different variables potentially affecting wolf populations established in three different national parks: the Abruzzo, Lazio e Molise National Park (PNALM), in Central Italy, the Mercantour National Park (PNM), in southeastern France, and the Yellowstone National Park (YNP), in northwestern United States. We studied a total of eleven free-ranging wolf packs, four in each of the European national parks, and three in YNP. We analyzed fecal samples collected in the study areas to search for three different viruses and record the presence of endoparasites. In the same samples, we measured the level of cortisol metabolites (CMs) as an index of stress. In YNP, we investigated the detail of post-conflict interactions taking place in two different packs. Viral nucleic acids were detected by *polymerase chain reaction* (PCR). We used a concentration technique followed by optic microscopic examination for parasitological investigations, and measured CMs levels by *enzyme immunoassays* (EIA). Regarding behavioral data, we performed detailed analyses of videotapes recorded in YNP, where radio tracking helped locate the studied packs. Genetic analyses or direct observations allowed the attribution of part of the samples to specific individuals.

We compared detected pathogens and measured CMs levels across national parks and across packs within each national park. For the samples for which such data were available, we examined the link between the variables of interest and gender, age or social status. We also considered territorial and social stability in interpreting hormonal results. Since infection by pathogens can be a sustained physical stressor for the organism, we expected a correlation between viral and/or parasitological infection and measured CMs levels.

Our results demonstrate infection by CPV-2 in all wolf packs of PNALM and 50% of packs in PNM. We found evidence for infection by CCoV in 50% and 25% of packs in PNALM and PNM respectively. Canine distemper was not detected but potentially present in the investigated areas. Our findings suggest that the wolf population of PNALM may have developed a long-term immunity against CPV-2, which appears endemic in the studied population. Our results also show that, as CPV-2 and CDV, CCoV should be widely included in the epidemiological monitoring of domestic and free-ranging canids.

Consistent with our expectations, coprological analyses showed that numerous helminths and protozoans infect wolves in the investigated national parks. Parasite prevalence (defined here as the number of fecal samples in which at least one parasite of the wolf was identified, divided by the total number of analyzed samples) and parasite diversity are much higher in PNALM than in PNM, while we found intermediated values in YNP. The history of settlement of the three studied wolf populations, diverse ecological factors linked with the presence of different other canid species living on the territory of wolf packs and the variety of prey species used by the predator are notably discussed in the interpretation of these results.

Infection by additional parasite species may increase the weakening impact of infection on individuals. Our results show that parasite richness (i.e. the number of different parasite taxa identified in the same fecal sample) is elevated in PNALM and low in PNM, while it has an intermediate value in YNP. The present study reports the infection by a helminth species not yet described in wolves. Our results show the presence of parasite taxa transmitted through the consumption of infected prey species in all three national parks, but present in more numerous samples from the newly established populations of YNP and PNM. Infection of wolves by these parasites depends on the diet of the carnivore and the extent of infection in the populations of prey species that are intermediate hosts. On the contrary, the parasites most frequently detected in samples from PNALM have an indirect life cycle, and infection of wolves thus depends on the density of susceptible hosts, including canids living on wolf packs' territory. In the wolf population of PNM, founded by individuals dispersing from Italy, the poor diversity of parasites compared to PNALM can be explained by a phenomenon analogous to the *founder effect* in population genetics. The same also applies to the detected viruses.

Considering hormonal results, the potential stressful impact of diverse environmental factors and factors intrinsic to packs is discussed. We found no link between the measured CMs levels and the gender, age or social status of individuals. On the other hand, consistent with previous findings in different species, we measured elevated CMs levels in wolf packs during territorial and/or social instability. Such variations correspond to relatively short-term stressors acting on part of the population only. In the wolf population of PNALM however, our results suggest that a long-lasting stressor affects the studied groups, as we measured elevated CMs levels in all investigated packs in both winters of the study. Such results may be linked with the high density of wolves in the area, and the presence of a free-ranging population of feral dogs living on wolf packs' territory.

Previously reported in a captive wolf pack, the occurrence of reconciliation, as well as solicited and unsolicited consolation, was confirmed in our study of two free-ranging packs. The present study is the first one demonstrating such interactions in free-ranging wolves. Furthermore, our results suggest that a specific type of affiliative behavior, "nose touch", is mainly used in interactions taking place after a conflict.

The studied systems are highly complex, comprising different interactive effects between infection by pathogens and physical and/or social stressors. The risk of local

extirpation of wolves due to infection by pathogens is enhanced by fragmentation of the predator's habitat. Canids living on wolf packs' territory may play an important role in the transmission of pathogens and their maintenance in the environment. Our results show that wolves are infected by various pathogens in the different investigated areas, and sometimes exposed to additional weakening elevated CMs levels. The cumulative effect of these factors can have an important impact on population dynamics. Thus, the present study provides baseline information of importance to consider prior to any human interventions on free-ranging wolf populations.

Introduction

1. Wolf distribution and ecology

The former distribution range of the wolf (*Canis lupus* L.) covered most of the northern hemisphere. Following extensive hunting, the species went extinct in most parts of Western Europe and the contiguous United-States (Boitani 2003). In the last decades, following full protection of the species, small patches of surviving individuals allowed growth of the present fragmented populations in Western Europe (Lucchini et al. 2002; Boitani 2003; Valière et al. 2003). In northwestern United States, present populations settled subsequently to immigration from Canada and reintroduction programs (Arjo and Pletscher 1999; Bangs and Fritts 1996). The subspecies present in Italy and France is *Canis lupus italicus*, while *Canis lupus occidentalis* is found in the Rocky Mountains of the United States (Nowak 2003).

The wolf is the largest member of the canid family. A top predator in the ecosystem, it feeds mainly on free-ranging ungulates and sometimes carrion and livestock (Peterson and Ciucci 2003). The species typically lives in family-based packs, consisting of a mated pair and their offspring of usually one or two, but up to four generations; composition of unstable groups may however be different (Mech and Boitani 2003; Packard 2003). In this work, we consider two or more individuals travelling together as a pack. Next to African wild dogs (*Lycaon pictus*), wolves are the most social free-ranging canids (Fox 1975), and cooperation is central to the social life of pack members. Wolves are monogamous and usually pair for years, or even for a lifetime (Packard 2003; Vonholdt et al. 2008).

Breeding season takes place from late January through April, depending on the latitude (Mech 1970), so that pups are born in early spring when young ungulates insure sufficient food source to parents (Packard 2003). Most wolves, both males and females, do not reach sexual maturity before 22 months of age (Fuller et al. 2003; Kreeger 2003). Females have a single annual estrous cycle (Seal et al. 1987) and sperm production is cyclic in males, reaching a maximum during the breeding season (Kreeger et al. 2003). In a population, estrous might last at least a month (Kreeger 2003).

An average of 4-6 pups are born after 61-64 days of gestation (Fuller et al. 2003; Packard 2003; Mech 1970). Pups stay in and around the den for approximately 2-2.5 months (Mech 1970). After that, and until late summer, pups mainly remain in above ground rendezvous areas, where adults and yearlings usually take turns in feeding and defending them (Packard 2003). Previous to dispersal, the offspring remain in their natal pack for about 10-54 months, learn social communication, hunting and foraging strategies, and take part in the rearing of pups and territory defense (Packard 2003; Mech and Boitani 2003).

2. Virology

Disease-induced population declines are reported in several large carnivore species, and most epidemic events are caused by viruses (Murray et al. 1999). Small populations of free-ranging carnivores living in sympatry with large, reservoir populations are more at risk when exposed to infection (Murray et al. 1999).

Canine parvovirus type 2 (CPV-2) and canine distemper virus (CVD) are well-known viruses infecting canids and have been reported from several free-ranging wolf (*Canis lupus*) populations. Presence of canine coronavirus (CCoV) in free-ranging canid population is yet poorly documented and exposure to the virus has only been reported from wolves in Alaska (Zarnke et al. 2001). This rapidly evolving virus is, however, described as of growing importance in dogs (*Canis lupus familiaris*), both by its prevalence and by the increasing virulence of the emerging variants of the virus (Evermann et al. 2005; Buonavoglia et al. 2006).

Infection by any of the three viruses induces more severe symptoms in young pups than adults, and can have an important negative impact on pup survival, thus negatively affecting population dynamics. Consequently, CDV and CPV-2 have been associated with important population declines in wolves.

These three viruses are highly contagious and infect a large number of domestic and free-ranging species, allowing for enhanced potential for horizontal transmission. This leads to rapid spread of the viruses in the environment and within susceptible host populations. Thus, high densities of sympatric susceptible hosts favor transmission of these pathogens.

Large carnivores that died of infectious diseases are very rarely recovered, due to their vast home range and secretive behavior (Murray et al. 1999). Thus, disease-induced mortality is probably highly underestimated.

2. 1. Parvovirus

Parvoviruses infecting carnivores belong to the genus *Parvovirus* of the *Parvoviridae* family (Steinel et al. 2001). Canine parvovirus type 2 (CPV-2) is a single-stranded DNA virus (Truyen et al. 1995), which causes systemic infection (Steinel et al. 2001). Epizootic events (outbreak of disease affecting animals) in dogs caused by CPV-2 in the 1970s and early 1980s were characterized by severe gastrointestinal and myocardial symptoms (Kreeger 2003).

CPV-2 probably originated from a wild carnivore, possibly a fox (*Vulpes sp.*; Truyen 1999). Even though CPV-2 was first identified in 1978 (Steinel 2001), exposure to the virus was detected in 1973 in wolves (Mech et al. 2008; Mech and Goyal 2011) and in sera from dogs collected in 1974 in Greece (Koptopoulos et al. 1986 in Steinel et al. 2001). Within a few years, the virus was reported to occur worldwide in domestic and free-ranging carnivores (Steinel et al. 2001). Soon afterwards, two different variants of

CPV-2 emerged, namely CPV-2a and CPV-2b, which spread rapidly around the world (Truyen et al. 1995). While the original CPV-2 virus typically infected Canidae, the two new variants have an extended host range (Steinel et al. 2001; Truyen et al. 1995). Since the emergence of these variants, exposure to CPV-2 has been reported in Canidae, Felidae (Steinel et al. 2001), Mustelidae (Dalerum et al. 2005), Ursidae, (Madić et al. 1993; Marsilio et al. 1997) and Viverridae (Santos et al. 2009). In 2000, a third rapidly spreading CPV-2c variant was identified in Italy (Duonavoglia et al. 2001; Martella et al. 2005). A few years later, the new strain was detected in an increasing number of countries (Decaro et al. 2006; Decaro et al. 2007a). Emergence of these different variants further complicates the disease condition and raises the question of adapted vaccination (Nandi and Kumar 2010; Martella et al. 2005).

Transmission

High titers of CPV-2 are shed in the feces of infected individuals, typically for only 4-5 days, during acute infection (Truyen et al. 1995; Steinel et al. 2001). The virus is also present in saliva and vomitus of severely sick individuals (Kreeger 2003). Transmission of infection occurs by direct or indirect contact with secretions or excretions containing the virus, or by contaminated objects (Kreeger 2003; Steinel et al. 2001; Fico et al. 1996).

CPV-2 is very stable in the environment, highly resistant to heat and desiccation (Steinel et al. 2001; Grinder and Krausman 2001), and can possibly survive for months (Kreeger 2003; Steinel et al. 2001) or even years (Fico et al. 1996) in feces. Therefore, contamination of the environment by CPV-2 can be fast and long lasting, and possibly explains the fast spreading of the virus in susceptible populations.

Numerous close physical contacts with group members are typical of highly social species such as wolves, and within-pack transmission of diseases is probably very important (Johnson et al. 1994). This might explain why, in wolves, a sex-specific difference was not observed regarding exposure to CPV-2 (Johnson et al. 1994; Zarnke et al. 2004).

Transmission from dogs has been suggested as the cause of the CPV-2 outbreaks in free- ranging wolf populations (Peterson et al. 1998). Vaccination of domestic as well as reintroduced free-ranging individuals has been recommended (Steinel et al. 2001).

Infection and clinical signs

First replication of CPV-2 occurs in the pharynx, including lymph nodes and tonsils. The virus then spreads to most lymphoid organs (Steinel et al. 2001) and to the bone marrow. Transplacental transmission occurs, and fetuses of pregnant animals are infected (Steinel et al. 2001). CPV-2 also invades Lieberkühn crypt cells, where the occurrence of numerous mitotic divisions allows the regeneration of epithelial cells in the small intestine (Steinel et al. 2001). Severe destruction of the enteric epithelium resulting in (sometimes hemorrhagic) enteritis is characteristic of the disease (Steinel et al. 2001). CPV-2 only replicates in the nucleus of dividing cells, as it uses host cell DNA

polymerase enzyme for the replication of its genome (Steinel et al. 2001). This further determines the location of the virus in infected individuals. In young animals, dividing cells are numerous and are present in most tissues, while in adults, viral particles will mainly invade the lymphatic system and the epithelium of the digestive tract (Steinel et al. 2005).

Onset of clinical signs appears 5-10 days post infection. Symptoms include severe (hemorrhagic) enteritis, diarrhea, vomiting, dehydration (Kreeger 2003), leukopenia and depression (Zarnke et al. 2004). In fetal or neonatal infection, myocarditis may also occur, leading to death before the age of 12 weeks (Kreeger 2003). Infection can cause high morbidity and moderate mortality (Nandi and Kumar 2010), but infection can also be subclinical, or accompanied by mild symptoms (Steinel et al. 2001). Severity of the infection depends on several factors including the age of the individual, the presence of maternal antibodies, and on co-infection with other viral or parasitic pathogens (Kreeger 2003; Peterson et al. 1998). Poor genetic diversity might also affect resistance of the infected population to the disease (Hedrick et al. 2003; Peterson et al. 1998).

Pups are more frequently and more severely impaired by CPV-2 infection than adults (Nandi and Kumar 2010; Murray et al. 1999), and entire litters can be lost (Decaro et al. 2006; Zarnke et al. 2004). Mortality caused by CPV-2 infection primarily occurs in young pups (< 4 months old) (Nelson et al. 2012). In dogs and coyotes (*Canis latrans*), it is known that maternal antibodies are transferred, through lactation, to only some of the pups (Kreeger 2003), providing them with specific antibodies against infection. Thus, the young, recently weaned, pups show the highest mortality rate (Meunier et al. 1981).

Co-infections of CPV-2 with canine coronavirus (CCoV) are associated with aggravated symptoms of CPV-2 (Decaro et al. 2006). Both viruses settle in the small intestine. CCoV damages the epithelium of intestinal villi, and thus enhances mitotic activity in Lieberkühn crypt cells, which in turn favors CPV-2 replication (Decaro et al. 2006).

Infection by CPV-2 induces long-term, maybe life-long immunity in surviving individuals, with complete eradication of the virus (Steinel et al. 2001). However, it is not known how long seropositivity to CPV-2 may persist in wolves, without re-exposure to the virus (Kreeger 2003). Experimentally infected wolves showed clinical signs and lesions similar to those described in dogs (Zarnke et al. 2004), and the symptoms of the disease are thus probably the same in both canids.

Importance of CPV-2 in wolves and free-ranging populations

CPV-2 can cause high juvenile mortality in naïve populations (Pence 1995). This highly contagious pathogen is therefore of concern for numerous protected or endangered species worldwide. In a free-ranging coyote population, the death of at least 8 of the 14 pups in one year was attributed to CPV-2 (Gese et al. 1997). Longitudinal studies of free-ranging wolves indicate that CPV-2 might have an important demographic impact on small, naïve populations, causing crashes and subsequent

decline of the population, probably in association with other factors (Peterson et al. 1998). Very high (11/12) mortality rate of pups and yearlings attributable to CPV-2 has been reported in captive wolves (Johnson et al. 1994). Infection by CPV-2 has also been associated with high pup mortality in free-ranging wolf populations (Johnson et al. 1994; Mech and Goyal 2011). Thus, CPV-2 can potentially limit the growth of small, recolonizing populations (Mech and Goyal 1993, 2011) by inducing decreased wolf pup survival (Mech and Goyal 1993; Johnson et al. 1994). Therefore, CPV-2 is reported as one of the diseases of major concern in free-ranging wolf populations, and infection could hinder natural recolonization processes by the species (Johnson et al. 1994).

Even though CPV-2 potentially causes high mortality rates in pups, free-ranging wolf populations seem to survive and overcome the presence of CPV-2 in the environment (Mech and Goyal 2011; Almberg 2009). Only one case of CPV-2 causing fatality has been reported in free-ranging wolves, in an 8 months old pup (Mech et al. 1997). However, diseased-caused dead carnivores are rarely recovered (Murray 1999), and morbidity and mortality rates are difficult to evaluate in free-ranging populations (Zarnke et al. 2004). Thus, the mortality of free-ranging carnivores caused by diseases is probably highly underestimated.

Seroprevalence in free-ranging canids

Antibodies to CPV-2 have been reported in numerous free-ranging wolf populations in North America (Kreeger 2003) and in Europe (Fico et al. 1996; Santos et al. 2009; Sobrino et al. 2008; Frölich et al. 2005). Antibody prevalence to CPV-2 in North America is highly variable (Nelson et al. 2012; Mech and Goyal 2011; Zarnke et al. 2004). Some studies indicate that seroprevalence to CPV-2 was significantly higher in adults than in young individuals, less than one (Zarnke et al. 2004) or two years old (Nelson et al. 2012). This difference might be explained by the removal of lethally infected pups from the sampled population or by an enhanced probability of exposure to the virus with age (Zarnke et al. 2004). Seroprevalence rates of 100% (wolves) and 86-100% (coyotes) of antibodies against CPV-2 have been reported in a long-term study on sympatric canid populations, indicating that the virus might be enzootic (animal disease constantly present in a population) in some free-ranging canid communities (Almberg et al. 2009). The survey included individuals of 6 months or older. Outside epizootic events, such high antibody prevalence rates are often representative of a very contagious pathogen inducing low levels of mortality (Pence 1995; Gese et al. 1997), which might be linked to effective host immunity (Murray et al. 1999). The same applies to high prevalence rates of microorganisms recovered from hosts (Murray et al. 1999). However, important mortality among young coyote pups was attributed to CPV-2 in a free-ranging coyote population, despite a seroprevalence rate of 100% against CPV-2 in all older individuals (Gese et al. 1997). Some pups exposed to CPV-2 possibly at a young age, however, survived the infection (Gese et al. 1997). It has been suggested that CPV-2 can be enzootic in coyote populations (Pence 1995) and may cause important periodic mortality in specific conditions, such as high population density, food shortage or co-

infection with parasites (Grinder et Krausman 2001) or other pathogens. CPV-2 appears to be enzootic in different wolf populations as well (Mech and Goyal 2011; Almberg et al. 2009). A long-term survey indicates that CPV-2 had a very important negative impact on pup survival and, consequently, on annual population count for over ten years (Mech and Goyal 2011). However, recruitment settled then back to normal, and the population appeared to be less sensitive to CPV-2 infection, as seroprevalence rates remained high (Mech and Goyal 2011). Developed immunity might explain these observations (Mech and Goyal 2011). However, small populations might be less able to sustain such a long immunization process (Peterson et al. 1998). Therefore, CPV-2 infection can have an important impact on isolated (Peterson et al. 1998) or recolonizing wolf populations (Johnson et al. 1994).

CPV-2 in Western Europe

In Western Europe, CPV-2 is widespread and infecting numerous carnivore species (Santos et al. 2009; Sobrino et al. 2008; Frölich et al. 2005). Reported antibody prevalence rates for wolves are of 32.1% in Portugal (Santos et al. 2009) and 62.2% in Spain (Sobrino et al. 2008). In Italy, 4 wolves (6 to 24 months) captured in 1993 and 1994 all had antibodies against CPV-2, indicative of exposure to the pathogen (Fico et al. 1996). Martinello et al. (1997) first isolated the virus from 3.5% of wolf fecal sample collected in 1995 in north-central Italy. To our knowledge, no additional data are available on the presence of CPV-2 in the Italian wolf population. The occurrence of CPV-2 infection has also been reported in free-ranging Marsican brown bears (*Ursus arctos marsicanus*) in central Italy (Marsilio et al. 1997). A survey of an unvaccinated free-ranging dog population in Southern Italy indicated that 82.9% of the individuals had positive titers for CPV-2, and it is thought that the virus might be enzootic in this area (Corrain et al. 2007). Such high-density free-ranging dog populations might serve as a reservoir for epizootics in pets (Corrain et al. 2007) and wildlife.

Conclusive remarks

Naïve, free-ranging canid populations appear to be very sensitive to exposure to CPV-2, with important impact on population dynamics, principally through increased pup mortality, and consequently decreased population abundance (Pence 1995; Mech and Goyal 2011). In large populations, however, recruitment of young individuals seems to return to previous levels once CPV-2 becomes enzootic (Pence 1995; Mech and Goyal 2011). Nevertheless, CPV-2 might cause a constant, low level mortality (Almberg et al. 2009), thus affecting host demography even if not causing outbreaks (Murray et al. 1999). Alternatively, mortality induced by infection could be compensatory with other mortality factors, and thus not directly affecting local population demography overall (Pence 1995). In this case, however, dispersal events would likely be reduced, and the net effect of infection would therefore be negative within a naturally recolonizing population.

2. 2. Canine distemper

Canine distemper virus (CDV) is a negative-sens, single-stranded RNA virus, which belongs to the genus *Morbillivirus*, of the family *Paramyxoviridae* (Deem et al. 2010), and causes canine distemper (CD) disease. Reported in Europe since the mid-sixteenth century (Kreeger 2003), it has now a worldwide distribution in a wide host range, including numerous terrestrial (Murray et al. 1999) and some aquatic (Stanton et al. 2004; Kennedy et al. 2000) carnivores. Infection by CDV has been reported in all terrestrial carnivore families: Canidae, Felidae, Ursidae, Hyaenids, Mustelidae, Viverridae and Procyonidae (Deem et al. 2000). In aquatic mammals, CD has been described in different species of the Phocidae family (Stanton et al. 2004; Kennedy et al. 2000).

Transmission

The wide range of species susceptible to CDV provides extensive opportunities for horizontal transmission of the disease (Murray et al. 1999). Furthermore, CDV is highly contagious. It is mainly transmitted through aerosolization of nasal or conjunctival exudates, as well as through feces or urine containing the virus (Deem et al. 2000). Transmission can also occur by direct contact with these secretions or excretions (Kreeger 2003) or by fomites (Deem et al. 2000). In highly social carnivores such as coyotes (Almberg 2009) or wolves, intra-pack transmission of CDV might be greatly enhanced by close physical contact between pack members (Johnson et al. 1994). Virus can be shed for 60 to 90 days following infection (Deem et al. 2000), but mean duration of infectiousness is of two weeks (Almberg et al. 2009). CDV is degraded within a few hours at warm temperatures, but can survive for several weeks around freezing point (Almberg et al. 2009; Deem et al. 2000).

Infection and clinical signs

Pathogenesis of CD is well described in dogs, and is thought to be similar in non-domestic species (Deem et al. 2000). CDV causes pneumonia and encephalitis, and infection is considered highly lethal (Murray et al. 1999). After entering the respiratory tract, CDV uses macrophages travelling in local lymphatic vessels to reach the tonsils and bronchial lymph nodes, where it replicates within 2 to 4 days following infection (Deem et al. 2000). Within 4–6 days, the virus proliferates in diverse lymphoid organs. Systemic infection with viremia often occurs (Deem et al. 2000). Hematogen dissemination is probably the way to reach epithelial tissues and the central nervous system (CNS), which are invaded by CDV 8-9 days post-infection (Deem et al. 2000). Virulence of the virus strain, age of the host and various environmental conditions can influence clinical signs and outcome of CD infection (Deem et al. 2000). Pups are more vulnerable to CD than adults, and CDV is suspected as a cause of important pup mortality in free-ranging wolf populations (Almberg 2009; Johnson et al. 1994).

Next to rabies, CD is the disease of greatest concern for carnivores (Murray et al. 1999). CDV can cause severe, systemic, disease (Deem et al. 2000). Infection by CDV leads to high mortality in numerous free-ranging carnivore populations (Kreeger et al. 2003; Deem et al. 2000; Murray et al. 1999). Severity of clinical signs depends on the efficiency of the host's immune system (Deem et al. 2000). Poor immune system is often correlated with a systemic infection, while clinical signs might be absent in hosts with an efficient immune system (Deem et al. 2000). Symptoms of systemic infection include pneumonia, encephalitis, anorexia, ataxia, dyspnea, leukopenia, diarrhea, conjunctivitis, oral ulceration, neurological abnormalities and severe dehydration (Kreeger 2003; Deem et al. 2000; Murray et al. 1999). These can be accompanied by fever and secondary bacterial infections (Deem et al. 2000).

Seroprevalence of antibodies to CDV seems to be age-related in canids, with higher values being reported in adults (Nelson et al. 2012; Zarnke et al. 2004; Gese et al. 1997). This can be explained by the longer exposure time to the virus, and is indicative of the development of adapted immune defense in the host (Gese et al. 1997). Alternatively, high virus-induced pup mortality might withdraw those individuals from a survey before they can be evaluated (Almberg et al. 2009; Zarnke et al. 2004; Grinder and Krausman 2001). Transplacental transmission of CDV has been demonstrated in dogs (Deem et al. 2000). Ten-twelve weeks after birth pups are weaned, and thus lose the protection provided by the antibodies of their mother (Almberg et al. 2009). Young, naïve, weaned pups are therefore the most vulnerable to exposure to CDV. Little (Nelson et al. 2012) or no (Sobrino et al. 2008; Zarnke et al. 2004) sex-specific difference has been found in seroprevalence to CDV in wolves.

CDV in free-ranging canids

CDV is highly contagious, and sudden outbreaks of CD are regularly reported in free-ranging carnivore populations (Monne et al. 2011; Sekulin et al. 2011; Sobrino et al. 2008; Kennedy et al. 2000). The virus is thought to be epizootic, moving rapidly through populations (Almberg et al. 2009). Transmission by domestic dogs is suspected in different cases of epizootic outbreaks of CD in free-ranging populations, leading to major population declines (Kennedy et al. 2000; Murray et al. 1999). Host density may enhance the propagation of CDV (Monne et al. 2011; Almberg et al. 2009). Thus, a large population (Almberg et al. 2009) and a minimum density of susceptible hosts seem to be required to maintain CDV in the environment. This might happen in multi-host susceptible populations (Almberg 2009), in which horizontal transmission of CDV can take place.

CDV is widespread and of concern for numerous free-ranging canid populations around the world, as in Europe (Sekulin et al. 2011; Corrain et al. 2007), North America (Clifford et al. 2006; Gese et al. 1997) or South America (de Oliveira Hübner et al. 2010; Deem et al. 2005). In wolf populations, CDV is considered as one of the main diseases of concern (Almberg et al. 2009; Johnson et al. 1994). However, CD is not seen as an important factor of wolf mortality, as recruitment within different infected populations

was considered good (Kreeger 2003). Furthermore, CDV-caused mortality cases in wolves have only been reported in Canada and Alaska (Stronen et al. 2011; Kreeger 2003). In dogs, up to 70% of infections by CDV may be subclinical (Deem 2000). However, recent investigation attributed important pup mortality to CDV outbreaks in free ranging wolf populations (Almberg et al. 2009).

In wolves, exposure to CDV has mainly been demonstrated by the detection of corresponding antibodies in serological surveys (Nelson et al. 2012; Santos et al. 2009; Zarnke et al. 2004; Kreeger 2003). Reported antibody prevalence rates are of 11.1% in Portugal (Santos et al. 2009), 24.3% in Spain (Sobrino et al. 2008) and an average of 17% in North America (Kreeger et al. 2003). These relatively low rates of seroprevalence to CDV in wolf populations, outside epizootic outbreaks, probably indicate that the pathogen is not enzootic in the investigated wolf populations. This is further supported by a recent study (Almberg et al. 2009) showing that CDV is cycling in Yellowstone canid population, and can cause high pup mortality and thus short-term population decline, through repeated outbreaks.

CDV in Europe

There is no known cure for CD, and vaccination remains the most effective procedure to prevent viral infection (Kreeger 2003; Deem et al. 2000). However, vaccination doesn't prove to be completely efficient (Sekulin et al. 2011), and despite that it is commonly practiced for domestic species in most European countries, CD remains a major threat to susceptible carnivore populations on the continent (Demeter et al. 2007). This ineffectiveness might be explained by recently reported increase in the variety of CDV strains circulating in Europe (Martella et al. 2006), some of which are more closely related to strains from Greenland, North America or China than to the reference European ones (Demeter et al. 2007; Martella et al. 2006). At least three different strains of CDV have been reported in Italian dogs (Martella et al. 2006).

Major epizootic events have recently been reported in Western Europe (Origgi et al. 2012; Monne et al. 2011). A severe CDV outbreak started in 2006 in Northern Italy, and rapidly spreads southwards, possibly helped by a high density of susceptible hosts (Monne et al. 2011). In 2008, an outbreak was observed in diverse free-ranging carnivores in Germany (Sekulin et al. 2011). A year later, a similar epizootic event was first detected in Switzerland and rapidly spread over the country (Origgi et al. 2012). Recently, multiple highly virulent CDV strains were reported, and a wide range of susceptible free-ranging carnivore hosts are concerned by the epizootic (Origgi et al. 2012). Novel genetic variants of CDV seem to be involved in these events (Origgi et al. 2012; Monne et al. 2011; Sekulin et al. 2011), and an increase in pathogenicity of these new strains is suspected, as illustrated by the high morbidity and mortality associated with the infection (Origgi et al. 2012).

In central Italy, serological investigation conducted on 4 wolves captured in 1993 and 1994 indicated that one of them was previously exposed to CDV (Fico et al. 1996). In

the same area, antibodies against CDV were also reported in some free-ranging Marsican brown bears (*Ursus arctos marsicanus*) (Marsilio et al. 1997).

In Italy, a large population of free-ranging dogs (Corrain et al. 2007) provides ground to spread and maintenance of infectious diseases in the environment. A survey conducted between 2001 and 2002 in the south part of the country showed a seroprevalence of 80.3% in unvaccinated dogs, which represent the main fraction of the free-ranging dog population in the area (Corrain et al. 2007). Such a high prevalence rate could suggest that CDV is enzootic in the population (Corrain et al. 2007), the virus being maintained in the environment by an important density of susceptible hosts (Santos et al 2009). Alternatively, the survey might have been conducted during an epizootic outbreak. In any case, infected dogs might play an important role in transmitting CD to free-ranging populations, including wolves (Santos et al. 2009).

Conclusive remarks

Highly contagious pathogens presenting an important horizontal transmission potential, such as CDV, are believed to be of increasing concern for the conservation of susceptible carnivores species (Murray et al. 1999). Characterized by low genetic variability, small or isolated population can present an impaired resistance to diseases, including CD, and thus be of specific concern (Hedrick et al. 2003).

CDV can cause high pup mortality and a consequent short-term population decline, through repeated outbreaks (Almberg et al. 2009). The virus could therefore play a significant role in canids population dynamics, by affecting recruitment. CDV could be especially harmful to recolonizing (Johnson et al. 1994) and small isolated, naïve populations, which are more vulnerable to perturbations. Recent epizootic events in central Europe might have a potentially severe impact on protected wolf population in the Western Alps, the only place inhabited by a specific subspecies of the grey wolf (*Canis lupus italicus*).

2. 3. Canine Coronaviruses

Coronaviruses (CoVs) belong to the family *Coronaviridae* and the order *Nidovirales*. CoVs are large, single-stranded, positive-sense RNA viruses. Three different antigenic groups are distinguished, namely 1, 2 and 3 (Decaro and Buonavoglia 2008), also referred to as genera *Alpha-*, *Beta-* and *Gammacoronaviruses* (Woo et al. 2009). CoVs have been described in a wide array of mammals and birds, causing enteric, hepatic, neurologic and/or respiratory diseases (Decaro and Buonavoglia 2008; Pratelli 2006; Siddel et al. 1983).

In dogs, three distinct CoVs have been identified. These are type I and II canine coronaviruses (CCoVs), also referred to as canine enteric coronaviruses (CECoVs; Priestnall et al. 2007), and are included in the *Alphacoronaviruses* genera. The third CoV is a canine respiratory coronavirus (CRCoV), which is part of *Betacoronaviruses*

(Buonavoglia et al. 2006). CRCoV was first identified in 2003 (Decaro et al. 2007b), and infects the respiratory tract of the host, typically causing mild respiratory symptoms (Decaro et al. 2007b). Severe acute respiratory syndrome CoV (SARS-CoV) of humans also belongs to *Betacoronaviruses* (Decaro and Buonavoglia 2008).

Genetic evolution and pathology

Besides at least 4 different variants of CCoVs I and II (Decaro et al. 2009, 2010; Decaro et Buonavoglia 2008), *Alphacoronaviruses* also comprise variants of feline CoVs (FCoVs). Horizontal transmission of FCoV and CCoV between cats and dogs might be important (Benetka et al. 2006; Pratelli et al. 2002). As an example, it is suggested that CCoV type II strain is a result of a recombination event between FCoV and CCoV (Priestnall et al. 2007), and FCoV type II probably originates from recombination of FCoV I with CCoV (Woo et al. 2009). Emergence of additional recombinant CoVs in both canids and felids is suggested (Benetka et al. 2006). Further recombinant CCoV-II, with porcine *Alphacoronavirus* TGEV, have recently been reported in infected dog pups in Europe (Decaro et al. 2009, 2010).

CCoV was first reported in 1971 in dogs, responsible for mild diarrhea in pups (Decaro and Buonavoglia 2008) and 3 years later, as the cause of acute enteritis in dogs (Evermann et al. 2005). Recently, Pratelli et al. (2003) identified 2 different genotypes of CCoV infecting pups in Italy, namely CCoV type I and type II, which were suggested to be already widespread in the dog population (Pratelli et al. 2003). CCoV infection in dogs is often characterized by the concurrent presence of both CCoV genotypes (Pratelli et al. 2004), although low rates of mixed infections have recently been described (Decaro et al. 2010).

CCoV infection is usually limited to the enteric tract, and infected dogs generally recover rapidly (Pratelli 2007). Therefore, high morbidity and low mortality typically characterize infection by CCoV (Decaro and Buonavoglia 2008; Murray et al. 1999). The virus primarily invades and replicates within enterocytes lining the tip of villi in the small intestine (Pratelli 2006; Evermann et al. 2005). Subsequent lysis during infection causes desquamation and shortening of the villi (Pratelli 2006). As a result, absorption is reduced and digestive enzymes become deficient. Onset of symptoms is usually sudden, and diarrhea can be observed as soon as 18 hours post-infection (Pratelli 2006). Clinical signs may vary between asymptomatic and moderate enteritis (Buonavoglia et al. 2006; Pratelli 2006). The characteristic symptoms are diarrhea, vomiting, loss of appetite, dehydration, depression and anorexia (Decaro and Buonavoglia 2008; Pratelli 2006). However, the symptoms are more severe in young pups (Pratelli 2006), and fatal outcome of infection by CCoV has also been reported, associated with severe necrotizing enteritis and diffuse lymphoid depletion, conceivably caused by a new, recombinant strain of CCoV (Evermann et al. 2005). Furthermore, symptoms are enhanced when co-infections with other canine viruses occur (Pratelli 2006). Lethal outcome is usually caused by or concurrent infection of CCoV with canine parvovirus type 2 (CPV-2)

(Decaro et al. 2006), canine distemper virus (CDV) (Decaro and Buonavoglia 2008) or canine adenovirus type 1 (Pratelli et al. 2001).

CCoV also has a relatively high mutation frequency (Pratelli 2006), leading to the emergence of new variants of the virus (Decaro and Buonavoglia 2008). Recent findings indicate that this genetic evolution is accompanied by increased virulence of these new strains of CCoV (Evermann et al. 2005) together with the ability of the virus to disseminate throughout the organism (pantropic infection) (Decaro and Buonavoglia 2008; Buonavoglia et al. 2006).

In 2005, a highly pathogenic variant of CCoV type II was discovered in Italy (Buonavoglia et al. 2006) and caused severe systemic disease with a high mortality rate in infected pups (Decaro et al. 2008; Buonavoglia et al. 2006). Symptoms consisted of fever, lethargy, loss of appetite, vomiting, hemorrhagic diarrhea and neurological signs of ataxia and seizures, leading to death within 48 hours (Buonavoglia et al. 2006). Anorexia, depression and leukopenia were also observed during 8 to 10 days in experimentally infected pups. Viral RNA was detected in lungs, liver, spleen, kidney and brain (Buonavoglia et al. 2006). When experimentally infected, pups 6 months old were more likely to overcome infection by this highly pathogenic CCoV variant than pups less than 3 months old (Decaro et al. 2008; Buonavoglia et al. 2006). Maternal antibody is thought to protect pups from infection until weaning (Zarnke et al. 2001). However, these antibodies could be inefficient against new viral strains. For example, commonly used inactivated vaccines against CCoV proved to be poorly effective in protecting dogs against the new virulent strains of CCoV (Decaro and Buonavoglia 2008). In the same manner, even strong immunity to enteric CCoV failed to protect pups against infection by the new strain of CCoV inducing pantropic infection (Decaro and Buonavoglia 2008). All naïve individuals are at risk when exposed to CCoV, but young, weaned pups seem to be the most vulnerable.

Transmission

CCoV is highly contagious and the spread of the virus is difficult to control (Pratelli 2008). Typical transmission route of CCoV is fecal-oral (Decaro and Buonavoglia 2008; Pratelli 2006) and oro-nasal (Pratelli 2008). The virus present in the feces is the main source of infection (Pratelli 2006). Infected dogs shed high titers of CCoV in their feces (Decaro and Buonavoglia 2008), usually for 6-9 days, but some individuals might shed the virus for up to 6 months in naturally infected pups (Pratelli 2006), enhancing occasions of transmission and settlement of the virus in the environment. High density of susceptible host populations (Zarnke et al. 2001) as well as increased number of social interactions with conspecifics (Priestnall et al. 2007) further enhances exposure and transmission opportunities of CCoV.

Persistence of CCoV in the environment and host immunity

CoVs are inactivated in a few days at 37°C but remain infective for months at 4°C (Pratelli 2008). In the same way, CCoV has a low stability in normal environmental conditions (Pratelli 2006), but the virus shed in feces of infected dogs survive longer in wintertime (Zarnke et al. 2001). The virus remains infective for at least two years when kept at -20°C (Pratelli 2008). Colder temperatures might thus favor survival of CCoV in the environment and transmission to susceptible hosts. Surveys conducted on free-ranging wolves further support this idea (Zarnke et al 2001). Serological investigations indicated that serum antibody prevalence follows a seasonal pattern, with an average of 25% in fall and 75% in spring, suggesting that CCoV is mainly transmitted in wintertime (Zarnke et al. 2001). This is also supported by higher antibody titers in the spring than in the fall, and by an increase from 0 to 60% of seroprevalence in pups (4-5 to 9-10 months of age) (Zarnke et al. 2001). Immunity seems to be very short lived, as high seroprevalence in spring dramatically decreased each fall, further indicating that re-exposure to CCoV is limited in summertime (Zarnke et al. 2001). Repeated monitoring of seroprevalence within specific individuals (n=14) indicated only short-lasting immunity against CCoV (Zarnke et al. 2001).

Detection of CoVs

Ante mortem diagnosis can be made through serological investigations, which enable to detect the presence of antibodies against CoV, or by fecal examination, detecting viral RNA. The later technique is of obvious convenience in studying endangered, protected or rarely seen species like numerous large carnivores with the advantage of being non invasive. Real time polymerase chain reaction (RT-PCR) is now a widespread tool for nucleic acid screening of viral RNA (Pratelli 2006; Gut et al. 1999).

CCoV in canids

CCoV is well documented in dogs and appears to be widespread worldwide (Priestnall et al. 2007; Pratelli 2006) with a high prevalence reported in Europe (Decaro et al. 2010; Benetka et al. 2006). Serological investigations showed high exposure rate to CCoVs in the dog population of Southern Italy (Priestnall et al. 2007). Several outbreaks have been reported worldwide (Decaro and Buonavoglia 2008), including in Italy (Decaro and Buonavoglia 2008; Decaro et al. 2008).

To date, few studies included CCoVs in investigations of diseases in free-ranging canid populations. It has been reported in maned wolves (*Chrysocyon brachyurus*) in Bolivia (Deem et al. 2005), in island foxes (*Urocyon littoralis*) in the USA (Clifford et al. 2006), and recently in pampas foxes (*Pseudalopex gymnocercus*) and crab-eating foxes (*Cerdocyon thous*) in Southern Brazil (de Oliveira Hübner et al. 2010). To date, the presence of CCoV in wolves has only been demonstrated in Alaska (Zarnke et al. 2001).

In central Italy, no antibodies to CCoV were found in the serological investigation conducted on 4 wolves recovered in 1993 and 1994 (Fico et al. 1996).

Conclusive remarks

Highly contagious pathogens with important potential for horizontal transmission will be of increasing concern for the conservation of susceptible carnivores (Murray et a. 1999). Not only do CCoVs present these characteristics, but the viruses also rapidly mutate (Decaro and Buonavoglia 2008) and undergo recombination events with other *Alphacoronaviruses*, as repeatedly reported in Europe (Decaro et al. 2009). Recent genetic evolution of CCoV lead to the emergence of new viral strains, with increasing virulence, some of which are highly pathogenic for dogs. Spread of these new variants enhances exposure risks of domestic and free-ranging canids to a greater diversity of CCoV infections, and poses the problem of accurate vaccination of animals (Decaro et Buonavoglia 2008). These findings suggest that CCoV should be widely included in epidemiological surveys in domestic and free-ranging canids.

Recovering wolf populations are at high risk from established and emerging canine diseases. The wolf and the dog being subspecies of *Canis lupus* (Wilson and Reeder 2005), few genetic differences exist between them (Vilà et al. 1997; Miklósi 2007), so that one may expect that the characteristic symptoms, the morbidity and the mortality rates observed in dogs might be similar in wolves. In Italy, the large population of free-ranging dogs (Corrain et al. 2007) increases the density of hosts susceptible to CCoVs infection, further enhancing the risk of exposure of the protected wolf population to novel CCoV strains. The important impact of CCoV on pups might notably diminish recruitment in established populations, and seriously hinder recovery of naïve populations, especially when these are in a recolonizing process.

3. Parasitology

3. 1. Helminths and specific protozoans in wolves

Wolves are hosts of numerous endoparasites (protozoans, viruses, bacteria, fungi and helminths) and are infested by different ectoparasites (fleas, ticks, lice and mites) (Kloch et al. 2005; Kreeger 2003; Ratti et al. 1999; Brand et al. 1995; Guberti et al. 1993; Archer et al. 1986). Numerous helminth species have been described in wolves, including trematodes (flukes), cestodes (tapeworms), nematodes (roundworms) and acanthocephalans (thorny-headed worms) (Craig and Craig 2005; Kreeger 2003; Brand et al. 1995), mixed infections being more frequent than single ones (Segovia et al. 2003; Segovia et al. 2001; Custer and Pence 1981). Some of these helminth species are canid specialists, while others are generalists (Segovia et al. 2003) and can also, in some cases, infect humans. In free-ranging wolf populations, investigation through necropsy and fecal sample examination usually reported very high prevalence of helminths (% of individual/fecal sample infected by helminths). In continental Europe, this prevalence reaches 100% in Italy (Guberti et al. 1993), Estonia (Moks et al. 2006) and Latvia (Bagrade et al. 2009), while an average prevalence of 95% have been reported in the United States (Custer and Pence 1981). Diversity of helminths infecting wolf populations is equal or higher in Europe than in North America (Craig and Craig 2005).

Infection by intestinal parasites is not generally lethal to the wolf, but can impair fitness and prey-catching ability of the predator (Kreeger 2003; Brand et al. 1995). Furthermore, when the parasite load is high, infection by helminths can be more harmful to wolves, and even lead to death when combined with viral or bacterial disease, or other weakening factors such as malnutrition (Brand et al. 1995; Mech 1977). Pups are usually more vulnerable than adults and occasionally develop pathologies that can have a lethal outcome (Kreeger 2003). Furthermore, some helminth species are transmitted from an infected mother to the foetus *in utero*, as for example *Toxocara canis* (Kloch et al. 2005; Kreeger 2003; Kazacos and Dougherty 1979).

In vertebrates, higher rates of parasites are reported in males compared to females (Hoby et al. 2006). However, this male-biased parasitism probably applies more to polygynous species (Hoby et al. 2006), where males roam large areas and are thus more exposed to infection by parasites. In the wolf, a typically monogamous species, little evidence exists of an influence of host sex on helminth prevalence or mean intensity of infection (Bagrade et al. 2009; Moks et al. 2006; Guberti et al. 1993; Custer and Pence 1981). Based on necropsies, some host age-related differences in prevalence and density of specific helminth species have been described in wolves (Bagrade et al. 2009; Rajković-Janje et al. 2004; Segovia et al. 2001; Guberti et al. 1993). Investigations through necropsy indicate that, in the same individual, co-infection by several helminth species is usually more frequent than single infection (Bagrade et al. 2009; Segovia et al. 2003; Segovia et al. 2001; Custer and Pence 1981). However, coprological examination often only detects one helminth species per sample (Popiołek et al. 2007).

Increased wolf density implies a higher probability of encounter with potential intermediate hosts (wild ungulates and livestock) and final hosts (free-ranging canids and domestic dogs (*Canis lupus familiaris*)) of parasites infecting wolves (Guberti et al. 1993). In the investigated areas, canid species living in sympatry with wolves are red foxes (*Vuples vulpes*) and feral dogs in Europe, and red foxes and coyotes (*Canis latrans*) in North America (Peterson and Ciucci 2003; Switalski 2003; Arjo and Pletscher 1999). Wild boars (*Sus scrofa*) are also major scavengers of wolf kills in many European countries, as well as eagles (*Haliaeetus leucocephalus*, *Aquila chrysaetos*) magpies (*Pica hudsonica*) raven (*Corvus corax*) and bears (*Ursus* spp.) in North America (Peterson and Ciucci 2003; Wilmers et al. 2003; Stahler et al. 2002), but these species are not known as definitive host of helminth parasites of wolves.

Protozoans infecting wolves comprise *Isospora* spp. and *Sarcocystis* spp. Several species of *Isospora* have been described in dogs, foxes and coyotes (Martínez-Carrasco et al. 2007; Kahn and Line 2005; Murray et al. 1999; Ewing 1986; Arther and Post 1977), but little is known about their presence in wolves (Mech and Kurtz 1999). In the same way, species of *Sarcocystis* are known to infect dogs, foxes and coyotes (Rajković-Janje et al. 2004; Ewing 1986; Fayer and Johnson 1975). This protozoan has been reported in wolves in Minnesota, United States (Emnett 1986) and in Russia (Zasukhin et al. 1979, in Kreeger 2003).

3. 2. Life-cycle of helminths and specific protozoans, and the diet of wolves

The diet of wolves is highly variable, depending on the size, vulnerability and abundance of the available prey species (Peterson and Ciucci 2003). Wolves are opportunistic predators and sometimes scavengers, or can subsist on garbage (Peterson and Ciucci 2003). The gut of the wolf is relatively short, coherent with the diet of the carnivore, which is principally constituted of highly digestible material (Peterson and Ciucci 2003). Passage time of about 8-12 hours depends on the quality of the ingested meal (Peterson and Ciucci 2003; Floyd et al. 1978).

The helminth fauna in wolves is partly acquired through feeding on various prey species (Bagrade et al. 2009; Moks et al. 2006; Craig and Craig 2005; Segovia et al. 2001; Ratti et al. 1999; Brand et al. 1995; Guberti et al. 1993). Transmission of trematodes, cestodes and some nematode species are favored through predator-prey interactions and scavenging (Craig and Craig 2005; Kreeger 2003; Segovia et al. 2001; Guberti et al. 1993; Messier et al. 1989; Holmes and Podesta 1968). When swallowed by the canine predator, the parasites pursue their life cycle in the carnivore, producing eggs that will be eliminated in the feces. Other nematodes infecting wolves, as hookworms, have direct life cycles and their maintenance in a population is helped by the fidelity of wolves to their den sites and rendezvous areas (Kreeger 2003; Segovia et al. 2003; Custer and Pence 1981). Regarding protozoans, *Isospora* spp. require only one host to complete

their life cycles, while *Sarcocystis* spp. need an intermediate host (prey) and a final host (predator) (Bowman 2009; Kahn and Line 2005).

The density of the wolf population has been mentioned as possibly influencing prevalence of some helminth burdens in preys, as a high density of the carnivore induces a high contamination of the environment (eg. eggs in feces) (Marquard-Petersen 1997; Messier et al. 1989). In turn, a heavily infected prey becomes more vulnerable to wolf predation, as its escaping abilities are impaired (Messier et al. 1989). This maintains the parasite in the environment through predator-prey transmission cycles, as reported for *Echinococcus granulosus* in North America (Messier et al. 1989; Choquette et al. 1973) or *Taenia multiceps* in Italy (Guberti et al. 1993).

3. 3. Survival of helminths in the environment

Some helminth eggs are very resistant to harsh weather conditions, as freezing or drying, and remain infective in the environment for at least a year (Marquard-Petersen 1997). However, other eggs cannot withstand prolonged high solar radiation, extremely cold environment or low yearly precipitation (Marquard-Petersen 1997). Yet, helminth eggs and larvae may survive when covered with snow, even in areas submitted to below freezing temperatures. Indeed, in the microclimate underneath the snow cover, temperature is close to 0°C, which might be appropriate for the organisms' survival (Marquart-Petersen 1997).

3. 4. Search for helminths and protozoans in wolves

Depending on the organisms searched for, assessment of infection by helminths can be done through necropsy, biopsy, or analysis of feces, urine, or blood sample (Craig and Craig 2005; Kreeger 2003; Segovia et al. 2003; Guberti et al. 1993).

Diagnosis of most helminthiasis and infection by specific protozoans in wolves has been done through microscopic examination of feces for protozoa, protozoan cysts and helminth eggs (Kreeger 2003) and larvae. The use of coprological analyses for the detection of these parasites has the advantage of being non-invasive. However, the examination of fecal samples has two main disadvantages, leading to incomplete results. First, it is technically impossible, through coprological examination, to distinguish several helminths at the species' level, as for members of the Taeniidae family (Bowman 2009; Foreyt 2001). Furthermore, coprological examination has a high specificity (the presence of parasite eggs indicates that the adult parasite is present in the animal), but a very low sensitivity (Torres et al. 2001; Guberti pers. com.), which means that the absence of parasite eggs in feces doesn't indicate that the adult parasite is not present in the animal, as eggs are produced only periodically (Marquard-Petersen 1997; Guberti pers. com.). For Taeniids, including the most prevalent parasites in canids, reported specificity and sensitivity are of 98% and 10% respectively (Torres et al. 2001; Guberti

pers. com.). Thus, the results obtained by microscopic examination of fecal samples always indicate the lower border of infection by the detected parasites species. However, coprological examination allows for an acceptable estimate of intestinal helminth fauna in populations of free-ranging canids (Torres et al. 2001).

Fecal samples from carnivores can further provide interesting information on parasites infecting local prey species, as some endoparasites need both hosts to complete their life-cycle (Bowman 2009).

3. 5. Detection of helminths in fecal samples

Flotation and sedimentation techniques are commonly used to search for helminth eggs and larvae in preserved fecal samples (Kloch et al. 2005; Ash and Orihel 1991), as these concentration procedures allow for the detection of even small numbers of organisms (Ash and Orihel 1991). However, the sedimentation techniques are preferred in diagnostic laboratories, because all of the parasites occurring in the sample are generally present in the sediment (Ash and Orihel 1991).

4. Endocrinology

4. 1. Stress-response

"Stress" is a state in which homeostasis of the organism is lost, as a consequence of a real or perceived threat to the physical or psychological integrity of an individual. The cause of this disturbance in equilibrium is named a "stressor" (Reeder and Kramer 2005), which can be mild or acute, short or sustained (Sapolsky 2004). When confronted with a stressor, an individual responds with numerous physiological and behavioral adaptations to cope with the situation and reestablish homeostasis (Reeder and Kramer 2005; Touma and Palme 2005; Romero 2004; Herman and Cullinan 1997). The collection of these adaptations is named "stress-response" or "General Adaptation Syndrome" (Sapolsky 2004).

Cannon first described the stress-response by the "Fight or Flight Syndrome" (FFS), focusing on the adaptive aspects of the physiological mechanisms involved in the management of stress (Sapolsky 2004). Thereafter, Hans Seyle subdivided the stress-response into three stages: (a) the initial alarm phase, (b) the resistance or adaptation phase and (c) the exhaustion phase (Sapolsky 2004). The resistance phase helps the organism to adapt and recover from the stressor, reestablishing homeostasis. The exhaustion stage takes place when the organism is exposed to a sustained stressor.

In mammals, the stress-response involves the simultaneous activation of two major systems: the sympathetic branch of the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal (HPA) axis (Reeder and Kramer 2005). This activation triggers the mobilization of resources and their selective delivery to the tissues, in order to enable the organism to cope with the stressor. Investments in long-term needs, such as digestion or reproduction, are deferred (Sapolsky 2004).

When an individual is submitted to a stressor, activation of the sympathetic ANS almost instantaneously triggers the release of two main catecholamines: epinephrine (adrenalin) and norepinephrine (noradrenaline). Epinephrine is the major hormone secreted by the adrenal medulla and released into the blood stream. It promotes glycogenolysis and lipolysis, increasing available glucose levels. Norepinephrine is principally released as a neurotransmitter by sympathetic nerve endings (Reeder and Kramer 2005; Sapolsky 2004). A few minutes later, as a result of a hormonal cascade process, glucocorticoid steroid hormones (GCs) are released into the blood stream. These hormones are mainly cortisol and corticosterone, which are produced by adrenal cortical cells. Cortisol is the major glucocorticoid secreted in primates, carnivores, ungulates and fish, whereas corticosterone is the main glucocorticoid in birds, rodents and reptiles (Touma and Palme 2005; Millspaugh and Washburn 2004). Secretion of GCs increases the energy available to the organism by promoting lipolysis and proteolysis. Subsequently, neoglucogenesis (Silbernagl and Despopoulos 2001; Eckert et al. 1999) provides sufficient glucose level in the blood stream during the resistant phase of the

stress-response, when short-term reserves (glycogen) have been consumed in the alarm phase.

Epinephrine acts in the short term, and rapidly disappears from the circulatory system after the stressor vanished. On the contrary, circulating GCs remain elevated for days, allowing the organism to recover from the stressful event.

The activation of these physiological pathways allows the organism to give an adequate response to a stressor, through adaptations such as increased heart rate and blood pressure, selective perfusion (vasoconstriction and vasodilatation), and elevated breathing rate (Silbernagl and Despopoulos 2001; Eckert et al. 1999). These responses provide the organs involved in defense mechanism with sufficient oxygen and glucose, and enhance elimination of carbon dioxide (Silbernagl and Despopoulos 2001; Ganong 1995). In parallel, unnecessary energy costs are prevented by decreased protein, fat and glycogen metabolism. Furthermore, digestion and reproductive functions are inhibited, and energy spent on growth or tissue repair is reduced (Sapolsky 2004).

Activation of the sympathetic ANS allows the immediate response (within seconds) of the animal to the stressor, while the release of GCs consecutive to the activation of the HPA axis is somewhat delayed. Glucocorticoids help the organism to cope with the stressor within minutes and are involved in the resistance phase of the stress-response. They help the organism to manage prolonged stressors and to recover from a stressful event.

In some species, variations in the levels of GCs have been reported as a function of sex, age, reproductive status or body condition, as well as different environmental factors (Reeder and Kramer 2005; Weingrill et al. 2004). Weather conditions (temperature, humidity, depth of the snow cover), availability of food and water, can also influence GCs levels, as shown in various species of birds and mammals (Hoby et al. 2006; Touma and Palme 2005; Lynch et al. 2002). Variation of GCs levels have been reported as following seasonal cycles and circadian rhythms, or as being influenced by changes in diet (Reeder and Kramer 2005; von der Ohe et al. 2004). The peak of GCs secretion is usually found prior to major activity periods, just before arousal from sleep (e.g. at the end of the dark period in diurnal animals and at the end of the light period in primarily nocturnal animals) (Reeder and Kramer 2005; McEwen et al. 1988).

Additional factors modulate measured GCs levels. For example, various hormones including estradiol, progesterone and testosterone interact in different ways with the hypothalamic-pituitary-adrenal (HPA) axis responsible for the release of glucocorticoids (Viau and Meaney 1996; Kudielka and Kirschbaum 2005). Furthermore, stressors are also known to modify neurotransmission of serotonin (Chaouloff 2000), thus affecting the behavior of individuals.

4. 2. Various origins of a stressor

« Stressors are real or perceived challenges to an organism's ability to meet its real or perceived needs » (Greenberg et al. 2002). A stressor can be physical (environmental or physiological) or psychological (Reeder and Kramer 2005; Touma and Palme 2005). Physical stressors include dehydration, injury or infections (Reeder and Kramer 2005; Sapolsky 2004). Psychological stressors comprise, for example, social stress or loss of an attachment figure. These stressors all induce a similar stress-response of the organism, driven by the activation of the sympathetic ANS and the HPA axis (Sapolsky 2004), and psychological stressors cause further emotions such as anger, frustration or anxiety (Reeder and Kramer 2005). Physical and psychological stressors can simultaneously affect an organism and thus interact in complex ways.

4. 3. Social stress: stressors and stress response

In social mammals, interactions with group members can strongly affect the activity of the HPA axis and induce increased or decreased stress levels in individuals (DeVries et al. 2003; Sachser et al. 1998). Studies investigating relationships between cortisol levels and social status lead to variable results. While a correlation between social status and GCs level has been reported in different studies, these report alternatively higher hormonal levels in subordinates, or on the contrary in dominant individuals (Millspaugh and Washburn 2004; Sands and Creel 2004; Creel 2001; McLeod et al. 1996; Sapolsky 1982, 1991, 1994). Meanwhile, Seals et al. (1987) found no relationship between cortisol levels and social status in a captive wolf pack.

Modification of an individual's position in a dominance hierarchy (Creel 2005; Lyons et al. 1994) or instability of this position (McLeod et al. 1996; Ziegler et al. 1995; Sapolsky 1983, 1993) are also reported as stressful. Other important stressors for individuals are unexpected changes in the social environment, such as the loss of an attachment figure (mother, or mate in pair-bonding species) (Mason and Mendoza 1998; Hennessy 1997) or disruption of a relationship with a socially close member of the group (Aureli and Smucny 2000). Separation or social isolation can induce a prolonged elevated stress level in individuals (Levine et al. 1999). Furthermore, within a same social group, individual differences in GCs levels have been reported as possibly attributable to the temperament or personality of the animals (Anestis 2005; Reeder and Kramer 2005; Virgin and Sapolsky 1997; Sapolsky 1993, 1994, 2004). Some studies showed that the perceived control of a stressor is an important modulator of the physiological stress response of an individual (Virgin and Sapolsky 1997; Sapolsky and Ray 1989; Sapolsky 1983, 1994, 2004). Thus, from two individuals engaged in similar number of aggressive conflicts, individuals initiating these interactions (and thus controlling them, usually dominant individuals) perceive them as less stressful as the victims of such aggressions (most often subordinate individuals). An additional difference have been reported in the

stress physiology of dominant and subordinate wild baboons, indicating that feedback regulation of GCs is impaired in subordinate individuals, which thus show a prolonged elevated GCs levels in response to a stressor compared to dominant animals (Sapolsky 1994).
Additionally, the influence of the stability of the dominance hierarchy within a group on the stress level of individuals has also been reported (Ziegler et al. 1995; Sapolsky 1983, 1993, 1994).

4. 4. Negative effect of elevated GCs levels

Long-lasting elevated GCs levels result from a chronic stress response induced by a sustained stressor, be it environmental, physiological or behavioral, and can compromise mammals' health in various ways (Touma and Palme 2005; Silbernagl and Despopoulos 2001; Sapolsky 1992, 2004; Selye 1973). Immune defenses can be weakened, thereby favoring pathogenic infections (Silbernagl and Despopoulos 2001; Ganong 1995). Sustained elevated GCs levels can also induce kidneys or stomach damage, and may have lethal outcomes in extreme cases (Silbernagl and Despopoulos 2001). High levels of GCs can also induce suppression of reproductive functions (Touma and Palme 2005; Creel 2001; Abbott et al. 1999; Bandelow et al. 1997; Sapolsky 1985, 1991), neuronal cell death, muscle and bone atrophy, poor wound healing, growth inhibition and even complete collapse of the immune system (Reeder and Kramer 2005). Glucocorticoids are transmitted to the fetus during pregnancy and through maternal milk during lactation (Reeder and Kramer 2005). Thus, elevated levels of GCs in pregnant or lactating females may negatively impact developmental processes of a fetus and health and survival of newborns (Wadhwa et al. 2001).

The negative effects of sustained elevated GC levels can severely impair individuals and may further affect the health and dynamics of entire populations (Millspaugh and Washburn 2004), notably through decreased reproductive success and survival. In free-ranging populations, sustained stressors are usually associated with a fragmented habitat and barriers to dispersal. Additionally, physical stressors such as malnutrition and infection by pathogens also impact free-ranging wolf packs.

4. 5. Stress in wolves

Psychological stressors may be important in free-ranging wolves. At the population level, encounters with neighboring packs or dispersing conspecifics, or signs of their presence (sent marking, howling) may be stressful (Mech 1970). Stress level in a pack might therefore be dependent on wolf density on the regional scale (Mech 1970).

Low stress levels seem to be correlated with the stability of the social environment and of relationships with conspecifics in various species (Sachser et al. 1998; Sapolsky 1992), which probably also apply to wolves (McLeod 1996). Social instability stressful

for members of free-ranging wolf packs can further be a consequence of territorial shifts or modification in the breeding (alpha) pair. New mating partners are mostly found in a pack following the loss of one or both of the original breeding individuals, or when more than two dispersing individuals found a group.

Within free-ranging wolves, no significant relationship between rates of agonistic encounters and CMs level has been reported (Sands and Creel 2004). On the contrary, in a captive pack, a negative correlation was reported between individuals' stress level and the time devoted to friendly behaviors (Molnar 2005). This result suggest that friendly interactions between pack members can have a relaxing effect on individuals, and may offset the physiological as well as psychological consequences of agonistic interactions within the group (Molnar 2005). A relationship between friendly interactions and relatively low stress levels was also described in different other group-living species (DeVries et al. 2003; Judge 2000; Sapolsky 1994).

The link between social status and stress level remains unclear in wolves, as different studies lead to contradictory results (Creel 2005; Sands and Creel 2004; McLeod et al. 1996). Higher levels of cortisol were reported in dominant than in subordinate individuals in a study of free-ranging animals (Sands and Creel 2004), while no such correlation was observed in a captive pack (McLeod et al. 1996).

In wolves, no seasonal variation in cortisol level was found in a long-term study of captive individuals (Seal et al. 1987). Wolves are well adapted to snow conditions and they are believed to suffer less from an important snow cover than do their ungulate prey species (Smith and Ferguson 2005). The major activity periods of wolves are usually shortly before sunrise until part of the morning, and from the end of the afternoon until part of the night (Vilà et al. 1995; pers. obs.). However, this activity pattern is variable, and is mainly adapted to avoid encounter with humans in exploited areas (Ciucci et al. 1997). This "fractionated" activity pattern throughout a period of 24 hours might explain the lack of significant influence of seasonal and circadian variations on cortisol levels in wolves, as shown by studies on captive individuals (McLeod et al. 1996; Seal et al. 1987). As the defecation rate is usually low in most carnivores, it is possible that diurnal changes of circulating GCs levels cannot be detected from fecal samples (Touma and Palme 2005). Interestingly, gender differences in basal levels of fecal CMs were demonstrated in dogs (*Canis lupus familiaris*) (Schatz and Palme 2001) but not in wolves (Sands and Creel 2004; McLeod et al. 1996).

4. 6. Measurement

Cortisol or cortisol metabolites (CMs) are measurable in blood, saliva, urine and feces, and blood samples are often used in the study of various species (Abbott et al. 1999; Baker et al. 1999; Virgin and Sapolsky 1997; de Villiers et al. 1995; Seal et al.

1987; Sapolsky 1982, 1983). However, results obtained in this invasive way may be skewed by the release of CMs due to stressful capture and handling of animals (Reeder and Kramer 2005; Touma and Palme 2005).

Glucocorticoids are secreted by the adrenal cortex and released into the vascular system within minutes in response to a stressor, and are later metabolized by the liver and in the gut (Möstl et al. 2005; Palme et al. 2005; Touma and Palme 2005; Möstl and Palme 2002). After a delay, which is mainly dependent on the intestinal transit time, specific metabolites (GCMs) of GCs present in the plasma can be detected in the feces (Möstl et al. 2005; Palme et al. 2005; Touma and Palme 2005). Taking the corresponding intestinal transit time into account, the fecal GCMs and GCs measured in the plasma follow a similar pattern (Goymann 2005; Palme 2005; Palme et al. 2005; Touma and Palme 2005; Möstl et Palme 2002). Thus, GCMs are widely used as a measure of stress in mammals (Touma and Palme 2005), and species differences in baseline levels of GCMs have been reported (Palme 2005; Reeder and Kramer 2005; Touma and Palme 2005). Reliable results have been obtained by analysis of urine (Anestis 2005; Ginther et al. 2001; Bahr 1998; Clarke and Faulkes 1997; McLeod et al. 1996) or fecal samples (Morato et al. 2004; Sands and Creel 2004; von der Ohe et al. 2004; Waiblinger and König 2004; Young et al. 2004; Schatz and Palme 2001). To date, these non-invasive techniques remain the only possible way to collect data in numerous species (Reeder and Kramer 2005). This particularly applies to the study of free-ranging animals (Touma and Palme 2005). Furthermore, levels of fecal GCMs reflect an average level of circulating GCs over a time period rather than an immediate value (Reeder and Kramer 2005). This is due to pooling effect in the gut, which smoothens the episodic fluctuations of GCs levels detectable in blood samples (Touma and Palme 2005). Thus, levels of fecal GCMs represent an integrated measure of adrenocortical activity reflecting the production rate of the metabolites over several hours (Touma and Palme 2005). Consequently, they may provide a more accurate assessment of the hormonal status of an individual than would be measured through a single plasma sample, if long-term stress is investigated (Touma and Palme 2005; Harper and Austad 2000). However, pulsatile secretion of GCMs in response to a stressor results in uneven distribution of the metabolites within a fecal sample (Palme 2005; Touma and Palme 2005; Millspaugh and Washburn 2004). To avoid bias introduced by this factor, each sample has to be homogenized before the analyses are undertaken (Palme 2005; Wasser et al. 1996).

Cortisol has been used to measure stress levels in different packs and wolf populations (Sands and Creel 2004; Creel et al. 2002; McLeod et al. 1996). In dogs, further enzyme immunoassay (EIA) showed the best results in measuring CMs levels, even when only low amounts of the metabolites were present (Schatz and Palme 2001).

4. 7. Validation

In order to account for the species-specificity of secreted GCMs, as well as for possible differences between males and females, adapted validation of the techniques

has to be made for each newly investigated species (Palme 2005; Touma and Palme 2005; Millspaugh and Washburn 2004). This ensures that the assays performed on samples collected in the field detect biologically meaningful changes in GCs levels (Touma and Palme 2005). Validation can be done either physiologically, through an adrenocorticotrophic hormone (ACTH) challenge test (injection), or biologically using external stressors (Palme 2005; Touma and Palme 2005). Events like capture, immobilization, transportation, confinement or human disturbances are stressful for individuals and can thus be used for biological validation (Touma and Palme 2005).

5. Conflict management in social mammals

5. 1. Group living in mammals: benefits and costs

Group living provides individuals with numerous advantages including cooperative foraging or hunting, defense of a territory and related food sources, and sometimes cooperative breeding. Furthermore, individual protection from neighboring groups is gained from living in a social unit. Benefits of group living are, however, almost invariably accompanied by costs related to sociality. These costs are the consequence of disagreement between group members on goals or interests (Aureli et al. 2002). Such a situation is referred to as a "conflict", and may or may not lead to an aggressive encounter (Aureli et al. 2002). In most species studied so far, researchers found that conflicts damage established relationships and may jeopardize further cooperation and associated benefits (Kutsukake and Clutton-Brock 2008; Aureli et al. 2002). The loser of an aggressive encounter might also leave the group, which may have costly consequences for the emigrant as well as for the group (Aureli et al. 2002; Schino 2000). Conflict management is therefore of critical importance in the social life of numerous species living in stable social units (Aureli and de Waal 2000) and thus an essential component of social systems (Aureli et al. 2002). Conflict resolution refers to a restoration (at least partial) of the usual pattern of interaction between former opponents (Aureli et al. 2002). This restoration is often achieved through post-conflict friendly interaction between former opponents, which is referred to as "reconciliation" or "friendly post-conflict reunion" (Aureli et al. 2002; de Waal 2000). Reconciliation typically takes place within a few minutes following the end of the conflict (Cords and Aureli 2000) and leads to conflict resolution (Aureli. et al. 2002). Natural conflict resolution is a relatively new field of study (Aureli and de Waal 2000), and little is known about mechanisms of conflict management in species other than primates.

5. 2. Aggression and conflict management

Agonistic (i.e. aggressive and submissive) interactions have long been the main research interest of most ethological studies, in which aggression, competition and dominance were the key topics of interest (Aureli et al. 2002; Aureli and de Waal 2000). Primatologists first dedicated important effort to the study of affiliative (i. e. friendly) interactions between animals. Among others, cooperation and friendly interactions arose as central topics in behavioral research, and understanding conflict management became of more interest than the study of the conflict itself (Aureli et al. 2002; Aureli and de Waal 2000).

In group-living species, agonistic interactions are deleterious for group members, and mechanisms evolved that minimize costs related to such encounters and control

aggression, thus preserving benefits of group-living (Aureli et al. 2002; Aureli and de Waal 2000). Communicative visual and acoustic displays are largely used to avoid escalated aggression (Aureli et al. 2002), and various forms of appeasing signals may also be used in daily communication to lessen aggression (Aureli and de Waal 2000). Moreover, when a dominant-subordinate relationship is established between individuals, signals mostly replace aggressive encounters in potentially competitive situations (Preuschoft and van Schaik 2000). This helps to prevent escalation of conflicts and subsequent damaging consequences on relationships among group members (Preuschoft and van Schaik 2000). Access to mating partners is an important cause of disagreements reported in group-living mammals (Leone and Palagi 2010; Aureli et al. 2002). Thus, a reduced number of mating partners within a group, as a consequence of (physiological or behavioral) sexual inhibition, might also lessen average conflict rates (Schaffner and Cain 2000).

5. 3. What are the functions of reconciliation?

Following a conflict between group members, reconciliation is the most commonly used mechanism to restore social cohesiveness (Leone and Palagi 2010), and thus relieve the negative consequences of a conflict (Aureli et al 2002). Reconciliation may serve a short-term goal in signaling the end of hostilities (Aureli et al. 2002; Silk 2000) and reduce the likelihood of renewed aggression (Norscia and Palagi 2011; Leone and Palagi 2010; Aureli et al. 2002; Cords and Aureli 2000). In a long-term perspective, reconciliation restores, at least partially, the relationship and associated level of tolerance between former opponents (Aureli et al. 2002; Cords and Aureli 2000; de Waal 2000). Furthermore, reconciliation reduces the negative physiological and psychological consequences of the conflict, namely stress and anxiety (Aureli and Smucny 2000; Cheney and Seyfarth 2000). Both the potential damage to the relationship (i. e. the uncertainty about the future of the relationship) and the perceived risk of physical injury can trigger stressful emotions in opponents engaged in an aggressive encounter (Aureli et al. 2002; de Waal 2000). This is further supported by various studies on primates, in which an increase in post-conflict anxiety levels in both the aggressor and the victim of the aggression was reported (Aureli and Smucny 2000). Following the conflict, risks of renewed aggression further enhance anxiety for the victim of the encounter (Aureli and Smucny 2000). After a conflict that escalated into aggression, precedent opponents can temporarily increase the distance between each other (Aureli et al. 2002), which preserves the victim of the former agonistic encounter from costly renewed aggression. In case the probability of renewed aggression is high, low frequencies of reconciliation are expected (Aureli et al. 2002). Although remaining distant is the only option in some species (Kutsukake and Clutton-Brock 2008), it is not a long-term viable one in various group-living animals (Watts et al. 2000).

Prolonged activation of the stress response consecutive to a conflict has harmful effects on the organism (Sapolsky 2004). To maintain social cohesion, such deleterious

consequences must be minimized. Reconciliation through friendly behaviors induces reassurance and appeasement in previous opponents (Aureli et al. 2002; Aureli and Smucny 2000). A return to baseline levels of anxiety is reported following such affiliative interactions (Aureli and Smucny 2000). The relaxing effect of friendly interaction has been demonstrated in different contexts and species, comprising rodents, horses, monkeys and humans (Sapolsky 2004; Aureli and Smucny 2000; de Waal 2000; Uvnäs-Moberg 1999), and both the receiver and the provider of the affiliative behaviors seem to benefit from these interactions. Following a conflict, a decrease in stress level is most efficient when reconciliation occurs. Post-conflict affiliative contact with third-parties have a slower and less pronounced effect than reconciliation in reducing anxiety levels in former opponents (Aureli et al. 2002; Aureli and Smucny 2000).

The perception of the consequences of an aggressive encounter may depend on the context, and various studies on free-ranging and captive primates have shown that conflict over food are mostly not followed by reconciliation (Aureli et al. 2002). This suggests that conflicts over food are perceived more clearly and cause less uncertainty about the future of the relationship (Aureli et al. 2002). Similarly, reconciliation is a flexible behavioral pattern, the expression of which depends on the context and on individual experiences (Aureli et al. 2002).

5. 4. The valuable relationship hypothesis

Reconciliatory behaviors seem to repair relationships disturbed by conflicts (Aureli et al. 2002). This function is probably a good predictor of the occurrence and of the frequency of reconciliation (Aureli et al. 2002). The "valuable relationship hypothesis" (de Waal and Aureli 1997) predicts that reconciliation is more likely between individuals sharing a relationship that is particularly valuable to them, such as between a mother and her offspring or generally between relatives, or individuals that engage in alliances against opponents (Aureli et al. 2002; Cords and Aureli 2000; de Waal 2000; van Schaik and Aureli 2000). Conscious knowledge of relationship value is not necessary (Aureli and Smucny 2000). In cooperative breeding species, mating partners are likely to be the most valuable individuals to each other (Aureli et al. 2002; Schaffner and Caine 2000), and reconciliation rate following a conflict is expected to be important between mates (Cords and Aureli 2000; van Schaik and Aureli 2000). Thus, the social relationship between former opponents likely influences post-conflict anxiety and the occurrence of reconciliation, as reported in different species (Aureli et al. 2002). In monogamous species, individuals pairing for life probably develop even stronger affiliative bonds and adapted conciliatory behaviors. The level of anxiety associated with a conflict increases with the value of the relationship shared by the opponents (Aureli and Smucny 2000).

Valuable relationships are very probably found in all group-living animals with stable membership, allowing the needed time for these relationships to build up (van Schaik and Aureli 2000). As dyadic relationships rarely are symmetric in terms of importance (i. e. value) to social partners (van Schaik and Aureli 2000), differences are

expected in the motivation of each former opponent to resolve a conflict (Aureli and Smucny 2000). However, if the net benefice of such post-conflict friendly reunion only profits one of the former opponents, the frequency of reconciliatory behaviors is expected to be low (Aureli et al. 2002).

Furthermore, post-conflict anxiety level and conflict management processes are probably also influenced by the temperament of individuals (Aureli et al. 2002; Aureli and Smucny 2000).

5. 5. How to demonstrate the occurrence of reconciliation?

Following conflicts, an increase in affiliative contact is reported in numerous primate species, although usually these behaviors are not displayed at an elevated rate (Leone and Palagi 2010; Thierry 2000; Cheney and Seyfarth 1989). Matched control periods (MC) (de Waal and Yoshihara 1983) allow for comparing the pattern of post-conflict friendly interactions with a baseline. In MC, former opponents are physically close enough to potentially interact. To compare a postconflict period (PC) with MC allows evaluating the influence of a conflict on ensuing interactions between former opponents (Veenema 2000). The timing of the first affiliative interaction between former opponents is compared between PC and MC periods (Veenema 2000). A PC-MC pair is said to be "attracted" if the first affiliative contact takes place earlier or only in the PC period. If such a friendly interaction occurs earlier or only in the MC period, the PC-MC pair is considered "dispersed". If an affiliative interaction takes place at the same time in PC and MC periods, or if no interaction occurs, the PC-MC pair is considered "neutral". Veenema et al. (1994) defined the "conciliatory tendency" as the number of attracted minus dispersed pairs divided by the total number of PC-MC pairs. The conciliatory tendency evaluates the frequency of reconciliation between former opponents and allows for the comparison of different dyads, groups or species (Veenema 2000).

5. 6. Involvement of third parties

In the case of aggressive encounters, conflict management can also involve third parties who did not take part in the initial agonistic encounter (Watts et al. 2000). The victim or the aggressor of the former agonistic interaction can initiate post-conflict friendly interactions, or a third party can do so (de Waal 2000; Watts et al. 2000). When a third party initiates the affiliative interaction with the victim, the friendly reunion is named "consolation", while a "solicited consolation" refers to an affiliative interaction initiated by the victim towards a third-party (Watts et al. 2000). The function of both consolation and solicited consolation may be to reduce conflict-induced stress level and protect against further aggression (Watts et al. 2000). The supportive intervention of third parties is more likely when they share a valuable relationship with the former

opponent (Seed et al. 2007; Watts et al. 2000). All post-conflict affiliative behaviors between former opponents and possible third parties likely reduce general social tension, and thus benefit each member of the group (Watts et al. 2000).

Finally, the victim of an aggressive interaction sometimes redirects aggression by threatening or attacking a third party (Watts et al. 2000; Aureli et al. 1993). Redirection may reduce post-conflict stress level in the victim of the first aggressive encounter and limit further aggression by the initial aggressor or third parties (Watts et al. 2000). Reconciliation between the initial opponents may also be facilitated by redirection if the initial aggressor joins in the redirected aggression towards a third party (Watts et al. 2000).

5. 7. In what species have post-conflict reunions been described?

Most studies have been carried out on captive animals (Aureli et al. 2002). The first systematic study of post-conflict friendly interaction involving former opponents was conducted on chimpanzees (*Pan troglodytes*) (de Waal and van Roosmalen 1979). Since then, reconciliation has been reported in numerous gregarious primates, and it was suggested that this behavioral pattern must also be widespread in various non-primate group-living species (Aureli et al. 2002; Aureli and de Waal 2000; Schino 2000). In fact, such post-conflict interaction may take place in most animals living in stable social units, sharing long-lasting individualized relationships, and experiencing within-group aggression and post-conflict hostility (Aureli et al. 2002; Cords and Aureli 2000). Requirements for reconciliation to happen are individual recognition and a good memory of past social events (de Waal and Yoshihara 1983). Furthermore, when valuable relationships are disturbed, reconciliation is highly probable in these group-living species (Aureli et al. 2002; Cords and Aureli 2000; de Waal 2000; van Schaik and Aureli 2000).

In non-primate mammalian taxa, friendly post-conflict reunions were first described and reported in group-living species in the 1960s, but systematic data were only provided thirty years later (Aureli et al. 2002; Schino 2000). To date, even though data regarding non-primate species remain scarce (Schino 2000), reconciliatory behaviors have been reported in various species including the mouflon (*Ovis ammon musimon*), domestic goat (*Capra hircus*), spotted hyena (*Crocuta crocuta*), lion (*Panthera leo*), and bottlenose dolphin (*Tursiops truncatus*) (Schino 2000; Samuels and Flaherty 2000). However, only a few of these studies rely on statistically tested data. Interspecific reconciliation has been reported only once, in cleaner fish (*Labroides dimidiatus*) providing tactile stimulation to "client" fish (Bshary and Würth 2001). Reports of reconciliatory behaviors in species that are phylogenetically so distant suggests independent evolution of post-conflict management in different taxa (Schino 2000) and further underlines the key role of such mechanisms in group-living species. However, no reconciliatory behavior was reported in meerkats (*Suricata suricatta*) or red-bellied tamarin (*Saguinus labiatus*), suggesting that this behavioral pattern is not shared by all

species living in social units (Kutsukake and Clutton-Brock 2008; Schaffner and Cain 2000). Absence of reconciliation may be the consequence of an important risk of renewed aggression, as is the case in meerkats (Kutsukake and Clutton-Brock 2008). When cooperative behavior is resumed immediately after the end of a conflict, as in red-bellied tamarins, absence of reconciliation may alternatively suggest that conflicts do not alter relationships (Schaffner and Cain 2000).

5. 8. Conflict management in canids

In species defending a territory and related food sources, as carnivores, between-group competition is likely important (Schino 2000) or may be strong in periods of food scarcity. Thus, conciliatory mechanisms are expected in these species in order to promote group cohesion (Schino 2000).

Next to African wild dogs (*Lycaon pictus*), wolves (*Canis lupus*) are the most social free-ranging canids (Fox 1975), and cooperation is central to the social life of pack members. A stable pack is family-based, and strong bonds likely connect parents and offspring of sometimes several generations. As in highly cooperative and socially peaceful callitrichids (tamarins and marmosets) (Schaffner and Cain 2000), frequent affiliative interactions are characteristic of the social life of free-ranging wolves (Packard 2003; Mech 1999). In a stable wolf pack, referred to as a nuclear family (Packard 2003), only one pair reproduces, while offspring of previous years that did not disperse help their parents feed, defend and rear the pups of the year. Such reproductive inhibition is also characteristic of callitrichids, and probably decreases arousal of conflicts through reduced mating competition (Schaffner and Cain 2000).

In stable, undisturbed wolf packs, dominant-subordinate dyadic relationships are established and are primarily based on the age of individuals. The parents are thus naturally the dominant individuals in the pack (Packard 2003). Between siblings, personality of individuals may play a role in the progressive establishment of a dominant-subordinate relationship. Dominance hierarchy is often considered linear, although more complex organizations have been reported (Packard 2003; Lockwood 1976; Zimen 1975). Agonistic interactions are mainly observed between individuals of the same sex, and various authors reported two dominance hierarchies in wolf packs, one for each sex (Mech 1999; Zimen 1975; Schenkel 1947). Older, sexually mature, offspring usually disperse (Packard 2003; Gese and Mech 1991), so that pups and yearlings are most often the only members of the pack besides the parents. Such dispersal decreases within-pack conflicts over access to mates.

Apart from increased social tension during the annual mating season, conflicts are mainly food-related in a stable wolf pack (Packard 2003), and often prevented by dominance-submissive displays. In “disrupted families” or “step-families”, in which one of the original parents is missing or replaced by an immigrant individual, sexual competition is likely to occur (Packard 2003). Aggression involving physical contact is rare, and most agonistic encounters consist of signals transmitted though body posture,

facial mimics and vocalizations (Schenkel 1947). These behavioral characteristics might explain the uncommon occurrence of severe aggressive interactions between pack members in free-ranging wolves (Packard 2003; Mech 1999).

Recent studies on domestic dogs (*Canis lupus familiaris*) (Cools et al. 2008) and captive grey wolves (Palagi and Cordoni 2009; Cordoni and Palagi 2008) reported the occurrence of post-conflict affiliation. In dogs and wolves, these studies not only reported evidence of reconciliatory behaviors, but also of true consolation, initiated by third parties towards the victim of the former conflict. To date, evidence of consolation has been shown in apes and humans, but not in monkeys (Palagi and Cordoni 2009).

6. Objectives of the present study

6. 1. Virology

Given the potential important negative impact that infection by CPV-2, CDV and CCoV can have on canids, it is important to conduct surveys of these pathogens in free-ranging susceptible populations. Furthermore, the impact of infection on recolonizing populations might be enhanced compared to large, well-established populations. In France, no conclusive large-scale investigation has been conducted on the presence of these viruses in the recolonizing wolf population. In central Italy, case-reports on 4 wolves captured in 1993 and 1994 gave first important information on the presence or absence of these viruses in the free-ranging population. An extended study conducted in 1994-1995 in Northern Italy investigated the presence of CPV-2 in wolves. To our knowledge, no survey has been conducted in the wolf population of the country since then. Evaluating disease ecology in these wolf populations allows a better understanding of population dynamics and potential limiting factors.

The presence of harmful pathogens in populations of large carnivores is of increasing conservation concern. The studied wolf subspecies (*Canis lupus italicus*) is protected in Europe, and only present in Italy, and recently recolonized areas of neighboring countries. Because of the potential damaging effect of these highly contagious viruses on wolf populations, our study aimed to evaluate the presence of CPV-2, CDV and CCoV in wolves of 2 national parks: Abruzzo, Lazio e Molise National Park (PNALM, Italy) and Mercantour National Park (PNM, France). We were interested in comparing the long-settled, high-density wolf population of PNALM with the recently recolonizing, low-density population of PNM. Density of other susceptible host populations is also different in the 2 national parks. Thus, investigating these 2 distinct populations further aimed to understand how these ecological factors possibly influence the impact of the viruses on the studied wolf populations.

6. 2. Parasitology

Parasites can have devastating effects on their host and thus be important factors regulating host population dynamics (Holt and Dobson 2007; Tompkins et al. 2002). When parasite species are shared by sympatric host species, entire communities might be affected in their structure and biodiversity (Holt and Dobson 2007). Investigating parasitic infections should thus be part of conservation strategies, and surveys should be conducted on protected host species. For these reasons and this purpose, this study aims to provide comprehensive data (PNM and YNP) and a new evaluation (PNALM) of helminths and specific protozoans infecting wolves in the investigated protected areas. In this study, fecal sample examination was used to detect parasitic organisms.

The wolf populations in the three investigated areas differ by various geographical and ecological factors, allowing us to examine the possible influence of some of these factors on endoparasites in wolves. If these different factors have no influence, we should observe similar parasite communities and prevalence rates in fecal samples from each of these wolf populations (Guberti et al. 1993).

Long-established wolf populations might harbor helminths different from that of newly settled populations (Guberti et al. 1993). Furthermore, part of the helminth community in wolves is consistent with the diet of the predator (Ripple et al. 2001; Marquard-Petersen 1997). The wolf populations of the present study not only are from two continents, but also differ by various ecological factors, including main prey species, wolf density, and presence of a free-ranging dog population in PNALM. Furthermore, the life history of these populations is very different: long-settled (PNALM), naturally recolonizing (PNM) or recently reintroduced (YNP). To investigate any potential inter-pack variations in helminth diversity and prevalence, samples from three to four packs were compared in each studied population.

6. 3. Endocrinology

Numerous studies investigated stress levels in different mammalian species but this is an emerging field of research in free-ranging individuals (Reeder and Kramer 2005).

The consequences of persistent stress are harmful not only for individuals (Touma and Palme 2005; Sapolsky 2004), but can directly or indirectly affect the health and dynamics of a whole population (Millspaugh and Washburn 2004). The present study assessed stress levels in wolves from October/December to March, and evaluated the possible influence of different extrinsic and intrinsic factors on stress levels in free-ranging wolf packs. We studied eleven wolf packs in three different national parks for two consecutive winters. Direct observation and genetic analyses made possible the attribution of part of the collected samples to specific individuals. This information was used to investigate the effect of gender, age, and social status on stress level. Further information on the stability of some packs is used to compare measured hormonal levels in the light of this factor. The time scale of the study encompasses the mating season, allowing us to evaluate the stress level in wolves during that specific period in wolf social life. The possible stressful impact of various environmental factors is also discussed. These included the density of wolves and the presence on wolf territory of a population of free-ranging dogs, as these variables affect the defense of packs' territory and related resources. We further investigated the possible relationship of viral or parasitic infection to stress level.

6. 4. Conflict management

The present study aimed to provide a first study of conflict management strategies used in free-ranging wolves, through detailed behavioral investigations. Reconciliation and post-conflict reunions involving third parties have been studied in two wolf packs: a well established group structured as a nuclear family, and a group recently founded by adult and juvenile dispersers. Detailed video analyses allowed precise examination of the specific behaviors displayed by former opponents following a conflict.

Survey of canine parvovirus, canine distemper virus and canine coronaviruses infections in free-ranging wolves from central Italy and southeastern France

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Introduction

Infectious diseases can have a devastating effect on free-ranging large carnivore populations, and thus hinder conservation efforts devoted to these species (Murray et al. 1999). Disease-induced population decline has been reported in several large carnivore species, and most epidemic events appear to be caused by viruses (Murray et al. 1999). Spread of existing and emerging pathogens in free-ranging animals can cause rapid changes in the abundance and genetic diversity of susceptible populations (Altizer et al. 2003). Even when the effects of diseases are not epizootic and are apparently sublethal, pathogens may affect the size or resilience of infected host populations and increase the probability of decline caused by other factors (Cleaveland et al. 2002; Murray et al. 1999). Detecting the presence of potentially harmful pathogens in large carnivore populations is therefore of increasing conservation concern.

Transmission of numerous viruses is highly influenced by local carnivore densities (Murray et al. 1999). In the future, the contact of large carnivores with domestic animals will probably increase as the consequence of range restriction (Murray et al. 1999) and habitat fragmentation (Cleaveland et al. 2002) caused by human expansion, enhancing risks of infectious disease transmission to free-ranging wildlife (Cleaveland et al. 2002). Thus, small populations of free-ranging carnivores living in sympatry with large, reservoir populations are at a higher risk when exposed to infectious diseases (Murray et al. 1999). Identifying potentially harmful pathogens and their epidemiological characteristics in susceptible populations is important in order to evaluate and mitigate their potential impact on population dynamics (Murray et al. 1999). Understanding which ecological factors are associated with various infections, disease severity and virulence of pathogens can further help control the impact of these infections on host populations (Murray et al. 1999).

Most disease surveys aim to detect antibodies against specific pathogens. Detected specific antibodies indicate previous exposure to the infectious agent but don't provide information on current infection, as the virus disappears from fecal material after active infection. This is in agreement with previous investigations showing 100%

seroprevalence of antibodies against canine parvovirus type 2 (CPV-2) in sampled wolves (n=18), but absence of the virus in all fecal samples collected from the population (Stronen et al. 2011). Search for CPV-2 DNA in tissue samples also led to negative results in a large-scale survey of free-ranging carnivores, even though detected antibodies proved previous exposure to the virus in part of the animals (Frölich et al. 2005).

The wolf subspecies, *Canis lupus italicus*, which is the subject of this study, is protected in Europe. It is only present in Italy and in recently recolonized areas of neighboring countries (Nowak 2003), and is thus of particular conservation interest. The wolf never disappeared completely from Italy, where small patches of individuals survived the large-scale extermination of the species in Western Europe (Boitani 2003). Following protection of the species in the 1970's (Boitani 2003), the wolf population of central Italy grew and the species started to recolonize part of its previous home range in the country (Boitani 2003). Over twenty years ago, the population reached the Alps and started to extend in neighboring countries (Boitani 2003). In France, the first wolf pack settled in Mercantour National Park (PNM), twenty years ago (Houard and Lequette 1993).

Canine parvovirus type 2 and canine distemper virus (CVD) are well-known pathogens of canids and were reported to occur in several free-ranging wolf populations around the world (Almberg et al. 2009; Santos et al. 2009; Sobrino et al. 2008; Frölich et al. 2005; Zarnke et al. 2004; Kreeger 2003). Infection by *Alphacoronaviruses* (ACVs) is poorly documented in free-ranging canid populations. The variants of ACVs infecting canids are canine enteric corona viruses type I and II (CCoV). CCoV has been detected in wolves only in Alaska (Zarnke et al. 2001). Nevertheless, this genetically rapidly evolving virus appears to be enzootic worldwide in dogs (Pratelli 2006) and recently received increased attention in Europe (Decaro and Buonavoglia 2008; Decaro et al. 2008; Benetka et al. 2006; Buonavoglia et al. 2006).

Canine parvovirus type 2, CDV and CCoV can cause morbidity and important mortality in infected animals, with more severe symptoms in pups (Nandi and Kumar 2010; Almberg et al. 2009; Pratelli 2006). CDV infection typically causes pneumonia, encephalitis and/or diarrhea (Kreeger 2003; Deem et al. 2000; Murray et al. 1999). CPV-2 and CCoV primarily affect the small intestine, causing sometimes severe enteritis and consequent dehydration (Decaro and Buonavoglia 2008; Pratelli 2006; Kreeger 2003). Foetal or neonatal infection by CPV-2 can additionally trigger severe myocarditis (Kreeger 2003). Systemic infection by CDV or CCoV can also cause various neurological manifestations (Buonavoglia et al. 2006; Kreeger 2003; Deem et al. 2000).

Each of these viruses is highly contagious and can infect a wide range of domestic and free-ranging species (de Oliveira Hübner et al. 2010; Nandi and Kumar 2010; Buonavoglia et al. 2006; Deem et al. 2000). Furthermore, new variants of each of these viruses have recently been identified in Western Europe, some of which are highly contagious and show increased virulence and increased capacity of horizontal

transmission (Origgi et al. 2012; Le Poder 2011; Monne et al. 2011; Decaro and Buonavoglia 2008; Evermann et al. 2005; Martella et al. 2005; Buonavoglia et al. 2001, 2006). These variants result from mutations and recombination events in local viruses and possibly also from importation of infected animals from other countries (Allison et al. 2012; Origgi et al. 2012; Demeter et al. 2007; Benetka et al. 2006; Buonavoglia et al. 2006).

These shared biological characteristics of CPV-2, CDV and CCoV make them of important concern for the conservation of susceptible host species, which include the wolf. CPV-2 and CDV have been associated with high pup and juvenile mortality in free-ranging carnivore populations (Mech and Goyal 2011; Almberg et al. 2009; Kreeger 2003; Gese et al. 1997; Pence 1995; Johnson et al. 1994), and co-infection of CPV-2 and CCoV is associated with even aggravated symptoms (Decaro et al. 2006; Pratelli 2006). Furthermore, the impact of infection on recolonizing wolf populations might be enhanced compared to large, well-established populations (Johnson et al. 1994).

High seroprevalence rates of these three viruses were recently reported in domestic (Priestnall et al. 2007) and free-ranging (Corrain et al. 2007) dog populations in Italy. Furthermore, a severe CDV outbreak recently spread through part of Europe (Origgi et al. 2012; Sekulin et al. 2011). In Italy, the outbreak started north of the country in 2006, and rapidly spread southwards (Monne et al. 2011). The large population of free-ranging dogs in the country (Corrain et al. 2007) provides ground to spread and maintain infectious diseases in the environment and might serve as a reservoir for infection of wildlife.

Serological investigation of four wolves captured in central Italy in 1993 and 1994 showed exposure of four and one individual to CPV and CDV respectively (Fico et al. 1996). No previous exposure to CCoV has been detected in these four individuals. Similar results were obtained on captive and free-ranging bears in PNALM between 1991 and 1995 (Marsilio et al. 1997). An extended study conducted in Northern Italy in 1994 and 1995 reported the presence of CPV-2 in wolf scats (Martinello et al. 1997). To our knowledge, since then, no large-scale viral disease survey has been conducted on the Italian wolf population. A precedent study was not able to give conclusive evidence of the presence of CPV-2 in the wolf population of PNM. Apart from necropsies of animals sporadically found dead, the occurrence of CPV, CDV or CCoV has not been investigated in the recolonizing wolf population of France, to our knowledge.

Aims of the study

Given the potentially important negative impact of CPV-2, CDV and CCoV infections on canids, it is primordial to conduct disease surveys on these pathogens in protected free-ranging populations. Emergence of very contagious and highly virulent variants of these viruses was recently reported in Western Europe, further strengthening the crucial role of prospective surveys of protected susceptible populations.

In the framework of this view, this study aimed to evaluate the presence of CPV-2, CDV and CCoV in two populations of free-ranging wolves: in Abruzzo, Lazio e Molise

National Park (PNALM) in central Italy, and in PNM, in southwestern France. The wolf populations of these two areas are connected through migratory corridors. PNM is an area recently recolonized by wolves, where the density of the population is relatively low compared to PNALM, where a source, saturated population is present. These ecological variables might influence the impact of CPV-2, CDV and CCoV infections on population dynamics of free-ranging wolves, and hinder conservation efforts dedicated to the species. Our comparative study aimed to add some insight on the possible influence of these variables on the presence of CDV, CPV-2 and CCoV. Spatial distribution of the viruses was examined through sampling of four different packs in each National Park. To assess effective presence of the viruses, and not only exposure of the individuals, we screened collected scats for specific sequences of viral nucleic acids. We predicted that infection rates would be lower in PNM wolf population due to its more recent origin, its lower density, and reduced density of other susceptible hosts.

Material and Methods

Study sites

We conducted this survey in wolf populations in PNALM and PNM. Both study sites are mountainous, partly forested, and at similar latitudes (Table 1). Livestock and different wild ungulates species are present year-round in both national parks. The main prey species of wolves vary with the abundance and accessibility of the ungulates species present in the territory of the packs.

Table 1. Geographic and ecological characteristics of the two national parks.

Study area	**Creation year** [a]	**Location and coordinates** [a]	**Mountain range**	**Size** (km^2) [a]	**Wolf presence / return** [b]	**Wolf density** [c] (ind./1000km^2)
PNALM (included buffer zone)	1923	Central Italy (41°76' N; 13°84' E)	Apennines	1'297 (800)	Always present	57
PNM (included buffer zone)	1979	Southeastern France (44°18' N; 7°05' E)	Alps	2'835 (2'150)	Since 1992	11

PNALM: Abruzzo, Lazio e Molise National Park; PNM: Mercantour National Park.
[a] Source: World database on protected areas, http://www.wdpa.org/
[b] Houard and Lequette 1993; Boitani 2003.
[c] Wolf density was calculated as the mean number of wolves per pack, divided by the mean pack size territory (PNALM: packs' territory: 100-120km^2; Grottoli 2011. PNM: packs' territory: 260-350km^2, www.oncfs.gouv.fr; Duchamp pers. com.).

Investigated packs

We considered $\geq$ 2 wolves travelling together as a pack. When fieldwork was conducted, in both PNM and PNALM, 7 packs were known to have part or the entire of their home range within the boundaries of the parks' buffer zone (Grottoli 2011; Réseau

Loup/Lynx 2005, 2006a, 2006b, 2007). Based on the quality and quantity of collected fecal samples, four packs have been retained for this survey in each of these national parks (Table 2). The number of animals present in each pack was assessed by snow-tracking sessions conducted in winter. In summer, wolf-howling sessions inform on the success of reproduction in these packs (Grottoli 2011; Ciucci and Boitani 2009; Réseau Loup/Lynx 2005, 2006a).

Table 2. Ecological characteristics of investigated packs in PNALM (Abruzzo, Lazio e Molise National Park) and PNM (Mercantour National Park).

Park and winter of data collection	Packs and dispersing / dead individuals	Number of individuals / pack [a]	Successful reproduction [b]	Number of individuals / pack in sub-sequent winter [a]	Successful reproduction [b]
PNALM					
2006-2007	Iorio	6	Yes	4	Yes
	Mainarde	9	Yes	5	Yes
	Orsara	3	Yes	6	Yes
	Villavalelonga	7	Yes	6	Yes
	Total	**25**		**21**	
PNM					
2005-2006	Moyenne Tinée	2-3	Yes	2	No
	Haute Tinée	3-4	No	2-3	?
	Vésubie-Tinée	3-5	?	3-5	No
	Vésubie-Roya	3-4	Yes	4	Yes
	Dispersed/died [c]	5	NA	6	NA
	Total	**16-21**		**17-20**	

NA: not applicable; ?: undetermined (no member of the pack answered).
[a] Pack-size estimates are based on snow-tracking sessions in PNALM, and on snow-tracking sessions and genetic analyses performed on fecal samples in PNM (Réseau Loup/Lynx 2006a, 2007).
[b] Presence of pups during the summer preceding sample collection.
[c] Only one sample per individual, as revealed by genetic analyses (Duchamp pers. com.).

Sample collection

In PNM and PNALM, wolf scats are collected year round by scientists, local co-workers and rangers (Grottoli 2011; Ciucci & Boitani 2009; Duchamp and Marboutin 2007; Marboutin and Duchamp 2005) for the purpose of other studies. In the present survey, we considered samples collected between the 1st of October 2005 and the 31st of March 2006 in PNM, and between the 1st of October 2006 and the 31st of March 2007 in PNALM. In both national parks, most wolf scats were collected while snow-tracking the studied packs, thus directly identifying the contributing pack. In PNALM, additional criteria were used to conservatively discriminate wolf scats from those of other species, including diameter ≥ 2,5-3,0 cm and estimated volume ≥100 cc (Grottoli 2011; Ciucci and Boitani 1998). A sample of 107 scats, collected along wolf snow trajectories from December 2005 to March 2006, was used for sequencing a 600 bp portion of the control region of the mtDNA, providing a direct validation of the selection criteria adopted, as 98% of the scats showed the unique Italian wolf haplotype (W14; Boggiano et al. 2010). Genetic analyses performed on the samples from PNM were used to

discriminate wolf scats from scats from those of other species (Miquel et al. 2006; Valière et al. 2003). These analyses further enabled determination of the identity of the contributing animals (Duchamp and Marboutin 2007). In lack of snow, samples were collected at known scent posts and at exploited carcasses (Grottoli 2011; Harrington and Asa 2003).

Only well preserved samples were retained for analysis. Fecal samples that were partly consumed by birds, dried out, or exposed to rain or to temperatures obviously above freezing point were not retained for analysis. Samples composed mostly of hair (estimated as > 90% of the scat volume) were excluded. Scats over-marked with urine, or lying less than 50 cm away from another scat were not retained for the study. On the day of collection, all samples were stored at -20°C in labeled plastic bags and kept frozen until analysis.

Nucleic acid screening

Before extraction of nucleic acids, samples were vortexed for 1 minute, and centrifuged at 4,000 g for 10 minutes; 140 µl served as template using a commercially available kit (QIAamp viral RNA Kit, Qiagen, Hilden, Germany, suitable to extract viral RNA as well as DNA) following manufacturer's instructions. Extracts were stored at -80°C.

Detection of CDV specific RNA was carried out with the primers PP-I p1 and p2 described by Frisk et al. (1999). RT-PCR assays were run in a volume of 20 µl (18.4 µl reaction mixture, OneStep RT-PCR Kit, Qiagen; 1.6 µl template) and a primer concentration of 0.4 µM. The thermocycler scheme consisted of two pre-PCR steps of 50°C, 30 min and 94°C, 15 min followed by 40 cycles of denaturation (94°C, 30 sec), annealing (58°C, 30 sec) and extension (72°, 1 min) and a final extension (72°C for 10 min).

For the detection of CCoV specific nucleic acids (member of genus *Alphacoronavirus*) realtime-PCR was performed using the primers and probe described by Gut et al. (1999), who indicate a high cross-reactivity among *Alphacoronaviruses*.

CPV-2 specific nucleic acids were detected by realtime-PCR with primers and probe described by Decaro et al. (2005).

Negative controls, consisting only of the components of the kit, were run together with the samples through all procedure steps.

Prevalence refers to the number of wolf scats in which the viral nucleic acid (CPV-2, CDV or CCoV) was detected, divided by the total number of scats analyzed.

Sequence analysis

Amplified DNA was extracted using a commercially available kit (QIAquick® PCR purification kit) following the instructions of manufacturer and served as template for sequencing PCR, which was carried out in a volume of 20 µl with a ready to use sequencing PCR mixture (DNA Sequencing Kit). Forward and reverse sequences of PCR products were analyzed using ABI Prism 310 Genetic Analyser.

Results

We analyzed 79 wolf fecal samples from PNALM and 66 from PNM. We identified CPV-2 in all four packs and 15.2-19.0% (n=12) of the samples in PNALM and in two different packs and 12.1% (n=8) of the samples in PNM (Table 3). We did not detect CDV in any sample. We found CCoV in 2 packs and 8.9-11.4% of the samples in PNALM, and one pack and three dispersers in MNP, with 6.1-7.6% of the samples testing positive in the area.

In order to confirm specificity we analyzed randomly selected sequences of five CPV-2 and two CCoV positive samples. The homology between the five CPV-2 sequences was 99.5 – 100 % and 99 to 99.3 % to CPV-2 strain C-780916 (American Type Culture Collection ATCC VR-953). The sequences of the two CCoV positive samples were up to 98-99 % homolog to various CCoV sequences available in GenBank.

Table 3. Presence of CPV-2, and CCoV in wolf scats in investigated packs in PNALM (Abruzzo, Lazio e Molise National Park) and PNM (Mercantour National Park).

National parks and investigated packs	CPV-2				CCoV				
	Equ.	Pos.	Prev. (%)	CI (%)	Equ.	Pos.	Prev. (%)	CI (%)	N
PNALM (2006-2007)	**3**	**12**	**15.2-19.0**	**(8.4-25.4)-(11.4-29.7)**	**2**	**7**	**8.9-11.4**	**(3.9-18.0)-(5.7-21.0)**	**79**
Iorio		3			1	0			13
Mainarde		4				3			27
Orsara		2				0			19
Villavalelonga	3	3			1	4			20
PNM (2005-2006)		**8**	**12.1**	**(5.7-23.0)**	**1**	**4**	**6.1-7.6**	**(2.0-15.6)-(2.8-17.5)**	**66**
Haute Tinée		7				1			18
Moyenne Tinée		0				0			12
Vésubie-Roya		0			1	0			10
Vésubie-Tinée		1				0			21
Dispersing/dead*		0				3			5
Total	**3**	**20**			**3**	**11**			**145**

*Individuals for which only one sample was found, as revealed be genetic analyses of mtDNA.
Equivocal (Equ.) and positive (Pos.) results are illustrated, together with the prevalence (P) of each virus (number of positive wolf scat divided by the total number of scats analyzed in the National Park) and the corresponding confidence intervals (CI). P and CI are expressed as percentages (%). n: total number of faecal samples analyzed.

We detected co-infection by CPV-2 and CCoV only in samples from the Villavalelonga pack (PNALM). We found two samples collected in January and March positive for both viruses. In the same pack, another sample collected in March was positive for CCoV and equivocal for CPV-2.

Samples positive for CPV-2 were mainly collected in January (PNALM: n=4; PNM, n=4) and October (PNALM: n=5; PNM, n=1), while three, two and one positive sample were collected in February, March and December, respectively. We found no positive

sample from November. Samples with equivocal presence of CPV-2 were all collected in March (n=3).

With respect to CCoV samples with positive PCR were mainly collected in March (PNALM: n=2; PNM, n=4). In PNALM, other samples that were also positive for CCoV were collected in November (n=2), December (n=1) and January (n=2). One equivocal sample was detected per month in November, January and March.

In PNM, genetic analyses of the tested samples allowed us to attribute some of them to specific individuals. At least 2 individuals tested positive for CCoV: a dispersing male (2 positive samples out of 5) and a member of the Haute Tinée pack (n=1). Regarding CPV-2, all positive samples but one were from the Haute Tinée pack, where the two females and the one male all shed CPV-2 DNA in their feces. Another positive sample was from a male in the Vésubie-Tinée pack.

Discussion

To our knowledge, this is the first conclusive demonstration of the large-scale occurrence of CPV-2 and CCoV and in wolves in France, and the first report in 15 years for the Italian wolf population (Martinello et al. 1997; Fico et al. 1996). To our knowledge, this is also the first confirmation of the occurrence of CCoV in European wolves.

Negative impact of diseases on population dynamics is underestimated, as morbidity and mortality are difficult to estimate in free-ranging populations (Zarnke et al. 2004). In this survey, molecular investigations allowed the detection of viral nucleic acids in fecal samples from PNM and PNALM. While this technique is unable to detect previous exposure as indicated by specific anti-viral antibodies, the detection of viral nucleic acids by PCR in fecal samples proves that recent infection of the individual occurred (Murray et al. 1999; Martinello et al. 1997).

Close physical contact between group members is characteristic of social canids such as wolves and greatly enhances within-pack transmission of pathogens (Johnson et al. 1994). Pack members regularly use urine and feces to mark their territory (Harrington and Asa 2003) and inspection of fecal markings is frequent along territory edges. Smell is a crucial sense in wolves, and much information is transmitted through odors (Harrington and Asa 2003). Investigation of the ano-genital area of conspecifics is part of common social interactions (Harrington and Asa 2003). These behavioral characteristics of wolves further enhance oro-fecal transmission of pathogens between individuals. Therefore, the detection of CPV-2 or CCoV in one or more samples from a pack suggests that several members of that pack were infected.

Canine Distemper Virus

None of the samples analyzed tested positive for CDV. That the infected individuals typically shed the virus only for four to five days in feces minimizes the chance to detect the virus, even from sick individuals. However, some infected dogs may shed the virus

for several months (Kahn and Line 2005). The virus rapidly degrades in the environment, but it is known to survive for weeks at freezing point (Deem et al. 2000). Our survey was conducted in winter, and only well-preserved samples were considered for this study, so that virus degradation is not likely to explain the total absence of CDV in the analyzed samples. Thus, our results suggest the absence of the virus in the investigated populations at the time of sample collection. This is in accordance with the fact that distemper infection usually spreads rapidly through populations (Almberg et al. 2009). However, the absence of CDV from the analyzed samples can also be due to the high mortality rate usually associated with infection by this virus (Kreeger 2003; Deem et al. 2000; Murray et al. 1999). In fact, a recent large epizootic CDV infection in Europe probably reached the populations investigated here (Origgi et al. 2012; Monne et al. 2011; Demeter et al. 2007).

More than fifteen years ago, serological surveys reported exposure to CDV in three of nine free-ranging Marsican brown bears (*Ursus arctos marsicanus*) in PNALM (Marsilio et al. 1997), and in one of four wolves in a neighboring geographical area (Fico et al. 1996). Since then, the expending wolf population and the high density of susceptible (unvaccinated) free-ranging dogs present in Italy (Corrain et al. 2007) favor the maintenance of CDV in the environment. Furthermore, the occurrence of different strains of the virus has been recently reported in Italy (Martella et al. 2006) and in Hungary (Demeter et al. 2007). In conclusion, the presence of CDV in the investigated wolf populations cannot be ruled out even if it remained undetected in the present study. An undetected CDV outbreak can, for example, be the cause of the observed shrink in the number of individuals in the Moyenne Tinée pack, down to its reproductive pair in 2006-2007, as revealed by genetic analyses (Table 2), as no CPV-2 or CCoV infection was detected in that pack (Table 3). In PNALM, available information from the last decades suggests that the virus probably cycles through the wolf population.

Canine Parvovirus

Identification of CPV-2 in all investigated packs of PNALM suggests that the virus is enzootic in that wolf population (Mech and Goyal 2011; Almberg et al. 2009). To the contrary, the virus was only identified in two packs out of four in PNM, which indicates that the virus is not yet established in the whole population. Alternatively, a smaller sample size could also explain these negative results, although three positive samples were detected in the Iorio pack of PNALM, where the sample size was similarly low. The low density of susceptible canid hosts could also explain a lower rate of infection in the PNM wolf population. Red fox (*Vulpes vulpes*) and free-ranging dog populations of PNALM might serve as a reservoir for CPV-2 infection, and the presence of infected bears has also been reported in that area. These susceptible populations are both absent from PNM. The wolf population of Italy is expanding and recolonizing the French Alps; it can therefore be expected that similar pathogens of wolves are present in both areas. However, infection can be partially lost during emigration, in a process similar to founder effect (Templeton 1980). Even though CPV-2 is highly resistant in the

environment (Steinel et al. 2001), it is possible that dissemination of CPV-2 in PNM is limited because of a lower density of susceptible hosts as compared to PNALM.

Canine Enteric Coronaviruses

The present study shows for the first time the presence of CCoV in wolves in Europe. CCoV are part of the *Alphacoronaviruses* (ACVs). ACVs are rapidly evolving viruses (Le Poder 2011), and recent mutations and recombination events within this group are reported in dogs in Western Europe (Le Poder 2011; Benetka et al. 2006; Pratelli et al. 2003). Viruses of the ACV group comprise feline, canine and porcine enteric coronaviruses (Decaro and Buonavoglia 2008) as well as human and bat coronaviruses (Le Poder 2011; Woo et al. 2009). Sequencing of randomly selected positive samples from PNALM and PNM indicate specificity for CCoVs.

Detection of ACV from infected wild boars consumed by wolves cannot be excluded, as these are prey species of the carnivore in PNALM (Grottoli 2011) and in PNM (Duchamp pers. com.). However, only very low prevalence of ACV infection is reported in wild boars in Europe (Kaden et al. 2009; Ruiz-Fons et al. 2008; Sedlak et al. 2008; Vengust et al. 2006), and dilution of viral particles in wolf feces would further decrease chances of detection.

CCoV was identified in two packs in PNALM, and one pack in PNM, and was equivocal in an additional pack in each national park. That CCoV was not detected in all packs suggests that the virus is not enzootic in these wolf populations. This could be explained by the low resistance of the virus to elevated summer temperature, leading to low infection rate in summer, which would partly clear the virus from the environment. It has been suggested that CCoV is mainly transmitted in winter (Zarnke et al. 2001). Consistent with this, all positive samples in PNM were from scats collected in March. However, such seasonal variation was not detected in PNALM, where almost equal number of fecal samples tested positive for CCoV between November and January as well as in March. These results suggest that CCoV is established in some wolf packs in PNALM or that the virus is recurrently introduced in the population.

Three out of four samples that tested positive for CCoV in MNP were from individuals that either dispersed or died. Only one sample from these individuals was found in the area, as indicated by genetic analyses (Duchamp pers. com.). Infection by a recently emerging highly virulent strain of CCoV (Buonavoglia et al. 2006) could be the cause of the disappearance of these individuals.

Co-infection by CPV-2 and CCoV

In PNALM, CCoV and CPV-2 were both detected in two packs and concurrent infection by both viruses was found in sample collected in one of them. In PNM, only samples from the Haute Tinée pack showed the presence of both CCoV and CPV-2, with no sample containing the two viruses.

Co-infection by CPV-2 and CCoV is known to enhance the severity of symptoms (Decaro et al. 2006; Pratelli 2006; Evermann et al. 2005), and fatal outcomes have been

reported in dog pups (Decaro et al. 2006). The size of the Haute Tinée pack of PNM, in which both viruses were detected in 2005-2006, shrunk by next winter. Reproduction could not be certified in the pack during both summers 2006 and 2007 (Table 2). Fatal co-infection with CPV-2 and CCoV can be a determinant factor explaining the repeated non-detection of surviving pups and the decrease in pack size of the Haute Tinée pack (Table 2).

No genetic information is available regarding the identity of wolves in PNALM. Young pup mortality typically caused by CPV-2 and CCoV co-infection did not result in early loss of entire litters (Table 2). Once CPV-2 becomes enzootic in a population, its negative impact on pup survival and recruitment seems to be decreased (Mech an Goyal 2011). Possible acquired long-term immunity against CPV-2 (Mech and Goyal 2011; Steinel et al. 2001) in the wolf population of PNALM can correspond to decreased severity of the symptoms of co-infection by CPV-2 and CCoV. This would explain reproductive success in all four packs (Table 2), despite the detection of CPV-2 and CCoV in two of them. However, some, or even most pups might also have been mortally infected but still alive in packs in which both viruses were detected, if detection of reproduction is based on wolf-howling sessions (Grottoli 2011) that took place before pups' weaning.

Infection by either one of the investigated viruses can cause important morbidity and mortality in infected populations. CPV-2, CCoV and CDV all result in highly contagious infection, with important potential for horizontal transmission, and spreading of these viruses to connected wolf sub-populations is to be expected. The presence of both CPV-2 and CCoV in the studied populations could enhance virulence of possible CDV outbreaks.

The impact of viruses on the saturated wolf population of PNALM might be compensatory, and thus harder to detect. In addition to red foxes, the large population of susceptible, unvaccinated, free-ranging dogs may serve as reservoir for these viruses (Corrain et al. 2007; Murray et al. 1999). Transmission of pathogens, including emerging highly virulent viral strains, is increased in such high-density multi-host populations.

Furthermore, even if not detected, demography of large carnivores is likely affected by exposure to pathogens (Murray et al. 1999). Disease-induced mortality and morbidity in the long-established and saturated population of PNALM can have larger scale consequences. A lowered number of dispersing individuals would result in decline of natural recolonizing processes and in decrease of genetic diversity in all connected expending wolf population.

Direct negative impact of these viral infections could be even more important in the recolonizing wolf population of PNM. Low confirmed pup recruitment in PNM as compared to PNALM supports this idea (Table 2). Young, weaned pups are the most vulnerable to viral infections, as they are no longer protected by maternal antibodies and highly at risk to develop disease due to their young age. Co-infection by CPV-2 and CCoV can be the cause of possible important pup mortality.

The weakening effect of CPV-2 infection can prevent infected animals from long distance dispersal, so that possible life-term immunity against CPV-2 (Steinel et al. 2001) could have been lost in the recolonizing population of PNM through founder effect.

As CPV-2 was not detected in two of the four investigated packs, and in none of the dispersing individuals, the virus might not be enzootic in this population. Immunity against CCoV is short-lived and disappears rapidly without re-exposure to the virus (Zarnke et al. 2001). These observations suggest that the wolf population of PNM is more vulnerable to CPV-2 or CCoV infection compared to PNALM. On the other hand, a lower density of wolves and other susceptible species in PNM than in PNALM induces a lower risk of exposure of the French wolf population. Thus, large-scale infections by new viral strains are less likely in PNM than in PNALM.

The wolf is a protected species in France and in Italy. Individuals present in the study area belong to a subspecies only present in Italy (*Canis lupus italicus*) and in the recolonizing alpine population (Nowak 2003). This underlines the importance and necessity of further studies in closely monitoring these wolf populations for possible viral threats and other impairing infections.

Further infectious disease surveys monitoring potentially harmful pathogens in protected wolf populations and in susceptible animal communities living in the same geographical area would be important. Single or mixed infection by CPV-2, CDV or by CCoV can all have a negative impact on population dynamics and should be included in future surveys. The new, sometimes highly virulent, and rapidly spreading viral strains recently reported in Europe (Monne et al. 2011; Nandi and Kumar 2010; Buonavoglia et al. 2006) represent a net increase in the threat to wolf populations. Thus, a prospective longitudinal survey would be important to monitor and evaluate the spread of these new strains and their impact on free-ranging populations. Parallel investigations of specific acquired immunity and detection of virus specific nucleic acid sequences is recommended. Such sequencing would also give further insight on the importance of free-ranging dog populations in the maintenance of these viruses in the environment, and their transmission to wolf populations.

Finally, viral and other infectious diseases should be considered in management strategies (Pence 1995), reintroduction programs or demographic modeling regarding populations of wolf and other susceptible species. A survey of CPV-2, CCoV and CDV should be regularly repeated in PNALM and PNM in order to evaluate the impact of these viruses on the population dynamics of this protected and specific subspecies of the wolf. A continued survey is even more crucial since the emergence of new viral strains in dogs in Western Europe, and as co-infections usually are responsible of increased severity of the symptoms.

Real impact of diseases on a free-ranging population is difficult to evaluate as lethally infected animals are rarely found (Murray et al. 1999). This applies even more to large carnivores, owing to their secretive behavior (Murray et al. 1999).

Highly contagious pathogens with important potential for horizontal transmission will be of increasing concern for the conservation of susceptible carnivores (Murray et

al. 1999). Increased attention should be given to this topic, because of the potential high impact of diseases on population dynamics of free-ranging carnivores which still remains poorly understood (Murray et al. 1999). Density of susceptible host can be determinant in the maintenance of various viruses and other pathogens in the environment.

Our results suggest that CPV-2 is enzootic in the wolf population of PNALM, but not in PNM, and that CCoV might be epizootic in both areas. Future longitudinal surveys are however necessary to bring conclusive evidence regarding these questions. An important epizootic CDV infection was recently reported in Europe (Origgi et al. 2012; Monne et al. 2011; Demeter et al. 2007), which probably reached the investigated wolf populations. Therefore, that we didn't detect CDV in the analyzed samples does not mean the virus is absent from the environment, and a high mortality rate in infected individuals can also be an important factor explaining our results (Kreeger 2003; Deem et al. 2000; Murray et al. 1999).

Recent emergence of highly virulent strains can have important demographic impact on wolf populations. High-density wolf populations living in sympatry with large populations of susceptible species are at higher risk of exposure to these new viral strains.

Our results suggest that the recolonizing wolf population of PNM may be more vulnerable to viral infections and less resilient to epizootic events than the long-established population of PNALM. Viral infections could thus hinder the development of this Alpine wolf population. On the other hand, despite putative decreased vulnerability to enzootic viruses, the wolf population of PNALM is at increased risk of exposure to the emerging strains of highly virulent viruses, as transmission is more likely in high density susceptible multi-host populations as present in the Italian national park. Infection in such a long-established saturated wolf population can have a direct negative impact on connected recolonizing populations, through decreased emigration and consequent limitation of genetic diversity. Vaccination of large carnivores and reservoir hosts is advised, for control of most important diseases and mitigate their impact on free-ranging populations (Murray et al. 1999).

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Geographical and biological factors influencing the helminth parasite fauna in wolves (*Canis lupus*)

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Introduction

The crucial importance of large carnivores to the stability of most ecosystems is acknowledged (Murray et al. 1999) and led to the protection of different endangered species. Following protection, and allowing suitable ecological conditions that include migratory corridors, large predators started recolonizing formerly occupied areas (Boitani 2003; Valière et al. 2003; Lucchini et al. 2002) and, in some cases, were the subject of reintroduction programs (e.g. Bangs and Fritts 1996). When moving or displaced, animals carry along pathogens infecting them. Although habitat loss and fragmentation are primary threats to large carnivores' conservation, the potentially highly damaging impact of infectious diseases on free-ranging populations received increasing attention by conservationists in the past fifteen years (Murray et al. 1999; Macdonald 1996).

In this chapter, parasite specifically refers to helminths and protozoans. Parasites can have devastating effects on their host and may be important factors regulating wildlife population dynamics (Holt and Dobson 2007; Tompkins et al. 2002). When parasite species are shared by sympatric host species, entire communities might be affected in their structure and biodiversity (Holt and Dobson 2007).

In the future, range restriction (Murray et al. 1999) and habitat fragmentation (Cleaveland et al. 2002) are expected to increase contact of large carnivores with domestic animals and human populations, enhancing the risks of transmission of infectious agents to free-ranging wildlife including carnivores (Cleaveland et al. 2002; Murray et al. 1999). As predators, wild canids are exposed to infection by parasites transmitted indirectly through prey consumption, or by direct infection transmitted through contact with feces or interaction with conspecifics and other sympatric canid species (marking, killing). In this respect, free-ranging canid populations are of specific concern, considering the extremely wide distribution of dogs, which are host of numerous parasite species also infecting wild canids. Given their close genetic relatedness with dogs (Wilson and Reeder 2005), assessment of parasites infecting

protected wolf populations is an important conservation issue. No such published data exist on helminthiasis in free-ranging wolves in France, or in the wolf population of Yellowstone National Park. In Italy, a study was published almost 20 years ago (Guberti et al. 1993).

Wolf populations have three different origins: (1) They grew from reintroduced animals (Bangs and Fritts 1996), (2) from naturally dispersing individuals that recolonized a geographical area (Ciucci et al. 2009; Fabbri et al. 2007; Valière et al. 2003) or (3) belong to a population, which never went extinct (Boitani 2003). These different life histories might have shaped the parasite community infecting wolf populations (Roberts et al. 2002). Further ecological factors, such as geographic distribution or local prey species, are also known to influence the variety of parasites present in wolf populations (Bryan et al. 2012; Craig and Craig 2005). In some mammalian species, individual factors such as sex, age or genetics may also influence host susceptibility to parasitism and explain heterogeneities of infections within a population (Wilson et al. 2002). To evaluate the possible influence of these different factors on the parasite community infecting wolf populations might also bring important information for a better conservation of the carnivore.

Infection by helminths is not generally lethal to the wolf, but can impair fitness and prey-catching ability of individuals (Kreeger 2003; Brand et al. 1995). However, given the secretive behavior of wolves and the large size of their home range, animals may die from infections and never be recovered, so that little is known of the real impact of many diseases on population dynamics (Brand et al. 1995). Wolf pups are usually more vulnerable than adults to infection by parasites, and can occasionally develop lethal pathologies (Kreeger 2003).

Even though most helminths cause host morbidity rather than host mortality (Tompkins et al. 2002), impairment of host health increases with parasite load, and also when parasitism is combined with viral infection, stressful conditions (Holt and Dobson 2007), bacterial disease or malnutrition (Kreeger 2003; Brand et al. 1995). As for protozoans, infection by *Sarcocystis* sp. is usually considered asymptomatic in canids, while *Isospora* sp. may cause hemorrhagic enteritis, diarrhea and poor growth (Foreyt 2001), and lethal outcome of infection has been reported in wolf pups (Mech and Kurtz 1999). Thus, information about helminths and protozoans infecting susceptible free-ranging populations is of important conservation concern, especially in the case of protected host populations. Identifying factors that prevail in structuring parasite communities in carnivores is of importance in estimating health and population dynamics of predators and sometimes also prey species. Understanding the impact of the life history of a population on the richness of the parasite species hosted by individuals may further provide useful information on parasite dynamics.

In this study, we considered eleven wolf packs belonging to three populations in Italy (Abruzzo, Lazio e Molise National Park –PNALM–), France (Mercantour National Park –PNM–) and the USA (Yellowstone National Park –YNP–). We choose protected wolf populations of temperate areas, at similar latitude, and settled in similar habitat to

minimize potential variations due to environmental factors as seasonality, humidity or harvesting of wolves, that may impact wolf parasite fauna. The wolf population of PNALM is long settled, and acting as a source for the recolonization of PNM by the carnivore. Wolves in YNP were brought in from Canada, and deworming was part of the reintroduction program. Wolf parasite fauna is described as similar along its distribution range, in Italy or North America (Brand et al. 1995; Guberti et al. 1993). Therefore, we did not expect any differences in parasite richness or prevalence between neighboring packs in the investigated areas. Some differences in species composition of wolf helminth fauna have been reported between Europe and North America (Craig and Craig 2005) and we expected to find such differences. We compared our results with selected data available from previous studies.

Fecal samples are commonly used in investigations of endoparasitic infections (Bryan et al. 2012). This non-invasive technic is particularly valuable in studying endangered or protected free-ranging populations. Fecal sample examination allows satisfactory estimate of helminth fauna in free-ranging canid populations (Torres et al. 2001), and is the most frequent diagnostic tool used to determine the intestinal parasites infecting wolves (Kreeger 2003). Analysis of fecal samples further allows large-scale investigation regarding the presence of helminths and protozoans in free-ranging populations.

In the present study, investigations of selected wolf populations aimed to provide information on the yet not described helminth parasites of wolves in Yellowstone National Park and in France, and provide a new evaluation after 20 years in Italy. The results obtained also allowed to evaluate the effect of different ecological factors on the helminth fauna of these various wolf populations. Furthermore, the selected study areas allowed investigating the putative effect of distinct origins of wolf populations on helminth and protozoan parasites. We further report information on parasites infecting prey species of wolves, as indicated by direct (recovery of helminth eggs infecting prey species) and indirect information (presence of parasites with an indirect life-cycle using a prey species as intermediate host).

Material and methods

Study sites

The packs selected in this study belong to wolf populations that were fully protected at the time of data collection, and whose home range was mainly within the borders of three different national parks: Abruzzo, Lazio e Molise National Park (PNALM) and Mercantour National Park (PNM), in Western Europe, and Yellowstone National Park (YNP), in North America (Table 1). In YNP, investigations took place on the northern Yellowstone winter range (Smith et al. 2003). All three national parks are government-

managed protected areas. In this study, at least two individuals travelling together are considered as forming a pack.

Table 1. Characteristics of the three national parks.

Study area	Creation year [a]	Location and coordinates [a]	Mountain range	Size (km^2) [a]	Wolf presence/ return [b]	Wolf density (ind./1000km^2)
PNALM (included buffer zone)	1923	Central Italy (41°76' N; 13°84' E)	Apennines	1'297 (800)	Always present	57 [c]
PNM (included buffer zone)	1979	Southeastern France (44°18' N; 7°05' E)	Alps	2'835 (2'150)	Since 1992	11 [c]
YNP (included northern range)	1872	Northwestern USA (44°60' N; 110°55' O)	Rocky Mountains	8'890 (1'530)	Since 1995	50 [d]

[a] Source : World database on protected areas, http://www.wdpa.org/
[b] Berger and Smith 2005; Boitani 2003; Smith et al. 2003; Ciucci and Boitani 1998b; Bangs and Fritts 1996; Houard and Lequette 1993.
[c] Wolf density was calculated as the mean number of wolves per pack, divided by the mean size of packs' territory (PNALM: packs' territory: 100-120km^2, Grottoli 2011; PNM: packs' territory: 260-350km^2, www.oncfs.gouv.fr, Duchamp pers. com.)
[d] Information for the northern range of YNP (Berger and Smith 2005; Smith et al. 2003).

The three study sites harbor a mountainous backcountry, with partly forested and partly open areas, and are located at similar latitudes. In PNM and PNALM, livestock was present year-round on packs' territory. A summary of different ecological parameters of the study areas is presented in Table 2.

Table 2. Different ecological characteristics related to the investigated wolf packs.

Study area	Other canids [a]	Wild ungulates	Livestock	Prey species of wolves in winter [c]
PNALM	Foxes, dogs	Chamois [b], roe deer, red deer and wild boar	Sheep, horses, cattle, few goats	Mainly wild boar; roe deer, red deer, domestic ungulates
PNM	Foxes, dogs	Chamois, European mouflon, roe deer, red deer, wild boar, ibex	Sheep, few goats and cattle	Mainly chamois and roe deer; some red deer, ibex, European mouflon, wild boar and few domestic ungulates (sheep and goats)
YNP	Foxes, coyotes, dogs	Red deer, bison, mule deer, white-tailed deer, moose, pronghorn antelope, big horn sheep, mountain goat	None	Mainly red deer; few bison, deer and moose

Source: Grottoli 2011; Smith et al. 2008; Espuno et al. 2004; Smith et al. 2003; Poulle et al. 1998; Houard and Lequette 1993.
[a] Red fox (*Vulpes vulpes*), dog (*Canis lupus familiaris*), coyotes (*Canis latrans*). In PNM, dogs present on the studied packs' territory are working dogs (Shepard dogs and livestock-guarding dogs). In PNALM, dogs belonging to local owners, working dogs as well as feral dogs are all present. Dogs travelling with tourists are prohibited in PNALM, allowed in the buffer zone but excluded from the core area of PNM (Duchamp pers. com.), and restricted to a range of 100 yards off roads and parking lots in YNP.
[b] Chamois (*Rupicapra rupicapra*), roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), wild boar (*Sus scrofa*), european mouflon (Ovis orientalis), alpine ibex (*Capra ibex*), bison (*Bison bison*), mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), moose (*Alces alces*), pronghorn antelope (*Antilocapra americana*), big horn sheep (*Ovis canadensis*), and mountain goat (*Oreamnos americanus*).
[c] Fecal samples collected in PNALM and PNM were submitted to dietary analyses (performed by Ciucci Paolo and collaborators in Italy, Ciucci and Boitani 2009; performed by Duchamp Christophe and collaborators in France; Plan d'action national sur le loup 2008-2012). In YNP, main prey species are assessed through close monitoring of wolves (Smith et al. 2008, 2009, 2010).

Investigated packs

All three populations were protected at the time of sample collection, so that no legal harvesting of individuals occurred. In Yellowstone National Park, wolves were brought in from Canada in the frame of a reintroduction program and received deworming medication prior to release.

Over the duration of the study, 7 wolf packs were known to have part or the entire of their home range within the boundaries of each of the two European parks' buffer zone (PNM and PNALM) (Grottoli 2011; Réseau Loup/Lynx 2006a, 2006b, 2007). In YNP, 8 wolf packs' home range was partly or entirely included in the park's boundaries, on the northern range of the park (Smith et al. 2008, 2009). In each of the two European National Parks, we focused on 4 packs based on the quality and quantity of available fecal samples (Table 3). In PNM, some samples belonging to dispersing individuals in PNM have also been analyzed. In YNP, we collected samples from 3 different packs, based on the animals' visibility and accessibility of collection sites. In PNM and PNALM, the number of animals present in each pack was determined by snow-tracking sessions in the winter (Grottoli 2011; Ciucci and Boitani 2009; Réseau Loup/Lynx ONCFS 2007). In YNP, some individuals in each pack are equipped by radio-collars (Smith et al. 2008, 2009). The 3 packs this study focuses on were daily monitored, so that genealogy is well known and each individual can be identified. Pups were absent from the newly founded Blacktail Deer Plateau pack at the time of sample collection, composed by yearlings (>1 and < 2 years old) and adults (>2 years; n=3).

Table 3. Characteristics of investigated wolf packs

Park	Packs and dispersing individuals	Number of individuals/pack [a]	Total number of individuals
Europe			**42-45**
PNM 2006-2007			**17-20**
	Moyenne Tinée	2	
	Haute Tinée	2-3	
	Vésubie-Tinée	3-5	
	Vésubie-Roya	4	
	Dispersed/died [b]	6	
PNALM 2006-2007			**25**
	Iorio	6	
	Orsara	3	
	Villavalelonga	7	
	Mainarde	9	
United States			**36-39** [c]
YNP 2007-2008			30
	Slough Creek	14	
	Druid Peak	16	
	Blacktail Deer Plateau	(not constituted yet)	
YNP 2008-2009			25
	Slough Creek	(disappeared)	
	Druid Peak	16	
	Blacktail Deer Plateau	6-9	

[a] In Europe, pack-size estimates are based on: in PNALM, snow-tracking sessions (Ciucci and Boitani 2009; Grottoli 2011), in PNM, snow-tracking sessions and genetic analyses performed on fecal samples (Réseau Loup/Lynx 2007). In YNP pack size is known from direct observations; variations are due to dispersal and death.
[b] One sample only per individual, as revealed by genetic analyses (Duchamp pers. com.); a sample from an unidentified individual that was present in the area but could not be assigned to a pack is also included in this category.
[c] Members of the Druid Peak pack present in both 2007-2008 and 2008-2009 are only counted once (6 males from the Druid Peak pack in 2007-2008 joined females from a neighboring pack to found the Blacktail Deer Plateau pack in November 2008).

Fecal sampling

In PNALM and in PNM, analyzed wolf scats were collected by scientists, local co-workers and rangers (Grottoli 2011; Ciucci and Boitani 2009; Duchamp and Marboutin 2007; Marboutin and Duchamp 2005), between the 1st of October 2006 and the 31st of March 2007. In YNP, sample collection took place from the 1st of December to the 31st of March in 2007/2008 and 2008/2009. Investigations were carried on for two consecutive winters in YNP as concerning different wolf packs.

In PNALM and PNM, snow-tracking session allowing sample collection mainly took place as soon as possible after snowfalls. In YNP, the collected samples remained at or below freezing point from defecation to collection time, as attested by daily direct observation. In all three National Parks, only well preserved samples were retained for analyses. Fecal samples that were partly consumed by birds, dried out, crusted following exposure to the sun, washed out by the rain, or mostly composed of hair (estimated as >

90% of the scat volume) were not analyzed. Scats over-marked with urine or less than 50 cm away from another scat were not retained for analyses. On the day of collection, all samples were stored at -20°C in labeled plastic bags and kept frozen until analysis. Contamination of the collected fecal samples by free-living helminths (Marquard-Petersen 1997) was prevented at best by sampling in wintertime only.

Characterization of fecal samples

Genetic analyses of the samples from PNM were used to discriminate wolf scats from the scats of other species (Miquel et al. 2006; Valière et al. 2003). Furthermore, these analyses provided information on the sex, and sometimes the identity of the contributing animals (Duchamp and Marboutin 2007). In PNALM, in wintertime, most of the samples were collected while snow-tracking the studied packs and thus directly identifying the contributing packs (Ciucci 1994). In the absence of snow cover, samples were collected at known scent posts and at exploited carcasses (Grottoli 2011; Harrington and Asa 2003; Vilà et al. 1994). Additional criteria were used to conservatively discriminate wolf scats from those of other species (Ciucci and Boitani 1998b), including a diameter ≥ 2.5-3.0 cm and an estimated volume ≥100 cc. A sample of 107 scats, collected along wolf snow trajectories from December 2005 to March 2006, was sequenced at a 600 bp portion of the control region of the mtDNA, providing a direct validation of the selection criteria adopted, as 98% of the scats showed the unique Italian wolf haplotype (W14; Boggiano et al. 2010). Genetic analyses performed on the samples from PNM were used to discriminate wolf scats from scats of other species (Miquel et al. 2006; Valière et al. 2003). In YNP, fecal samples were collected following direct observation and filming of contributing individuals. When an individual was observed defecating, the animal and the surrounding landscape were filmed meticulously (Canon XL-H1 camcorder, Canon EF Adapter XL, Canon EF 100-400mm f/4.5-5.6L IS USM photo lens, Canon Extender EF 2x II). Later that day, the recorded sequence was visualized on a computer and relevant pictures of the screen were taken using a digital still camera. The next day, or as soon as possible, fecal samples were collected using the following procedure. One person set a spotting scope at the exact place from where the camera filmed the defecating individual. Using walkie-talkies, and with the help of the pictures taken with the still camera, that person guided a collaborator in the field to the sample. During these field trips, previously localized scats, as well any scats found on wolf tracks were collected, if no tracks of other canids were found less than 1 meter away from the fecal sample. This collection procedure provided information on the sex, age and identity of part of the contributing individuals in the Druid Peak and the Blacktail Deer Plateau packs. Sample collection was abandoned when wolves not belonging to the studied pack were known to have used the area between production and collection time.

Coprology

As soon as thawed enough, fecal samples were vigorously hand-mixed for 1 min through the plastic bag. 1.55 ± 0.05 g of feces was then placed in a plastic container, mixed with 10 ml of sodium acetate - acetic acid - formaldehyde (SAF) fixative (Yang and Scholten 1977) and kept at 4°C until tested. To detect protozoan cysts, helminth eggs and larvae, the following modified SAF concentration technique (Yang and Scholten 1977) was used: The fecal suspension was filtered through a strainer and a 4 ml aliquot was centrifuged with 5 ml of physiologic solution (NaCl 0.85%) for 10 minutes at 500x g. The supernatant was removed, the pellet was resuspended in 6 ml of physiologic solution, and 3 ml of ether was added. The solution was shaken vigorously for 30 seconds, and centrifuged for 10 minutes at 500x g. After removal of the supernatant, the pellet was dissolved in a few drops of saline allowing adequate observation under the microscope. Stained and unstained preparations were both made for each sample (Truant et al. 1981). Two drop of the concentrated solution was deposited on a slide. To one of these, a drop of 5X diluted Lugol solution (Ash and Orihel 1991) was added to obtain a stained preparation. A drop of saline was added to the second drop of concentrated solution for the observation of unstained structures. After mixing with the corner of a coverslip (18 X 18 mm), the latter was used to cover the preparation. Each preparation was examined by systematically scanning the entire coverslip at 100x magnification, using a calibrated Olympus BX50 microscope, followed by confirmation at 400x magnification. Eggs were identified on the basis of their size, color, shape, and appearance of content and structure of the shell surface. Eggs, cysts and larvae were identified to genus or species level (Traversa et al. 2010; Foreyt 2001; Bowman 2000a, 2000b, 2009; Uga et al. 2000; Campbell 1991; Ewing 1986; Bailenger 1982; Garcia and Ash 1979; Thienpont et al. 1979; Leger et al. 1977), except for the Taeniidae family whose eggs cannot be discriminated by microscopic examination (Bowman 2009; Kahn and Line 2005; Foreyt 2001). The preparations were not systematically searched for *Sarcocystis* sp. (Coccidia) cysts, but these were noted whenever observed.

Prevalence refers to number of total samples containing at least one egg or cyst divided by the total number of analysed samples, and is expressed as a percentage. Prevalence including detected of unidentified larvae, or excluding protozoans, is specified.

Statistical analyses

Even if the analyzed samples are not independent, 95% confidence intervals were calculated in the presentation of data (Motulsky 1995) as a precision on how good the estimate is (Bush et al. 1997). Statistical analyses were performed using R statistical software (http://www.R-project.org) and SPSS 20.0 (IBM® SPSS® Statistics). We consider our results as indicatory due to non-independence of fecal samples.

The parasite community in wolves was described using parasite diversity (number of parasites taxa in a given area), parasite richness (number of different parasite taxa found in each wolf scat), and overall parasite prevalence (1 = at least one wolf parasite

found in fecal sample, 0 = no wolf parasite found in fecal sample). We also investigated the prevalence of the five most common helminth taxa (*Taenia/Echinococcus*, *T. leonina*, *C. boehmi, C. aerophila and U. stenocephala*). We used generalized linear models with poisson and binomial errors to fit the parasite richness and parasite prevalence data, respectively. Each variable was modeled as a function of park, or pack and the Akaike information criterion (AIC) was used to rank the models. To test whether there was covariance among parasites, we modeled the prevalence of each common parasite as a function of one of the other five parasites.

For the subset of French and American wolf scats for which we had additional explanatory factors such as age (YNP only), sex, individual, and year of collection (YNP only), we ran additional analyses to test whether prevalence of helminth taxa was linked with these factors. For the French wolf scats (n =47), only the family of Taeniidae parasites (*Taenia/Echinococcus*) was common enough (13/47 infected). For the American wolf scats (n = 58), there were two helminth taxa that were common enough for analysis: Taeniidae (36/58), and *Toxascaris leonina* (6/58). We did not perform these analyses on *Sarcocystis* sp. (19/58), as this protozoan was not specifically searched for but opportunistically noted when observed.

Results

Identified parasite taxa

A total number of 342 wolf scats were analyzed, and 11 different parasite taxa infecting wolves have been identified (Table 4). Parasite diversity varied between parks: all 11 different parasite taxa known as infecting canids were found in PNALM, whereas 3 and 6 parasite taxa were recovered in samples from PNM and YNP, respectively. Nematode larvae were also found but couldn't be identified. Appendix 1 presents photographs of detected wolf parasite taxa. Six different parasite taxa were found that are not known as canid helminths, but as parasites infecting prey species (Appendix 2).

Table 4. Canid helminths and protozoans identified in wolf fecal samples. Total number of tested samples (n), number of samples that tested positive (N), prevalence (P) and confidence intervals (CI) are figured. P and CI are expressed as percentages (%). (PNM: Mercantour National Park; PNALM: Abruzzo, Lazio e Molise National Park; YNP: Yellowstone National Park).

Parasites taxa	PNALM (n=88)			PNM (n=68)			YNP (n=186)			TOTAL (n=342)		
	N	P	CI	N	P	CI	N	P	CI	N	P	CI
Protozoa												
*Isospora**	1	1.1	0.1-7.1	0	0	-	1	0.5	0.0-3.4	2	0.6	0.1-2.3
Sarcocystis sp.	9	10.2	5.1-19.0	3	4.4	1.1-13.2	63	33.9	27.2-41.2	75	21.9	17.7-26.8
Trematoda												
Alaria sp.	2	2.3	0.4-8.7	0	0	-	3	1.6	0.4-5.0	5	1.5	0.5-3.6
Cestoda												
*Taenia/Echinococcus***	14	15.9	9.3-25.6	18	26.5	16.8-38.8	125	67.2	59.9-73.8	157	45.9	40.6-51.3
Nematoda												
*Capillaria aerophila****	16	18.2	11.1-28.1	0	0	-	0	0	-	16	4.7	2.8-7.6
Capillaria boehmi	71	80.7	70.6-88.0	0	0	-	0	0	-	71	20.8	16.7-25.5
Physaloptera sp.	2	2.3	0.4-8.7	0	0	-	0	0	-	2	0.6	0.1-2.3
Toxascaris leonina	1	1.1	0.1-7.1	0	0	-	30	16.1	11.3-22.4	31	9.1	6.3-12.7
Toxocara canis	2	2.3	0.4-8.7	0	0	-	0	0	-	2	0.6	0.1-2.3
Trichuris vulpis	0	0	-	0	0	-	7	3.8	1.7-7.9	7	2.0	0.9-4.4
Uncinaria stenocephala	14	15.9	9.3-25.6	1	1.5	0.1-9.0	0	0	-	15	4.4	2.6-7.3
Unidentified larvae	12	13.6	7.5-23.0	29	42.6	30.9-55.2	11	5.9	3.1-10.6	52	15.2	11.7-19.6
Number of identified parasite taxa	**10**			**3**			**6**			**11**		

* Additional undistinguishable *Isospora* sp./*Eimeria* sp.: PNALM (n=2), PNM (n=5), YNP (n=1).
** Taxa identifiable to Family only (Family: Taeniidae) Bowman 2009; Foreyt 2001)
*** (=*Eucoleus aerophilus*; = *Thominx aerophilum*)

Parts of 4 adult cestode worms were collected in wolf tracks of the Druid Peak pack in YNP.

For the purpose of comparisons with other studies, prevalence is reported for each study area, taking or not into account protozoans and nematode larvae (Appendix 3). Unless specified, our study reports prevalence including helminths and protozoans.

Variation of parasite richness and prevalence as a function of geographical scale

Variation among parks

Parasite richness was highest in PNALM (mean ± standard error = 1.50 ± 0.103 parasites/scat, n = 88), slightly lower in YNP (1.19 ± 0.059 parasite taxa/scat, n = 186), and much lower in PNM (0.32 ± 0.064 parasite taxa/scat, n = 68; Fig. 1). All three national parks statistically differed from each other in parasite richness (chi-square, $p<0.05$, number of parasite taxa = 0, 1, 2 and ≥ 3). Detection of 3 or more parasite taxa

per fecal sample occurred in 13.6% and 4.3% of analyzed samples from PNALM and YNP respectively, and was never found in PNM. The models for pack and park had similar levels of support suggesting that both factors were important in structuring parasite richness in wolves (Table 5).

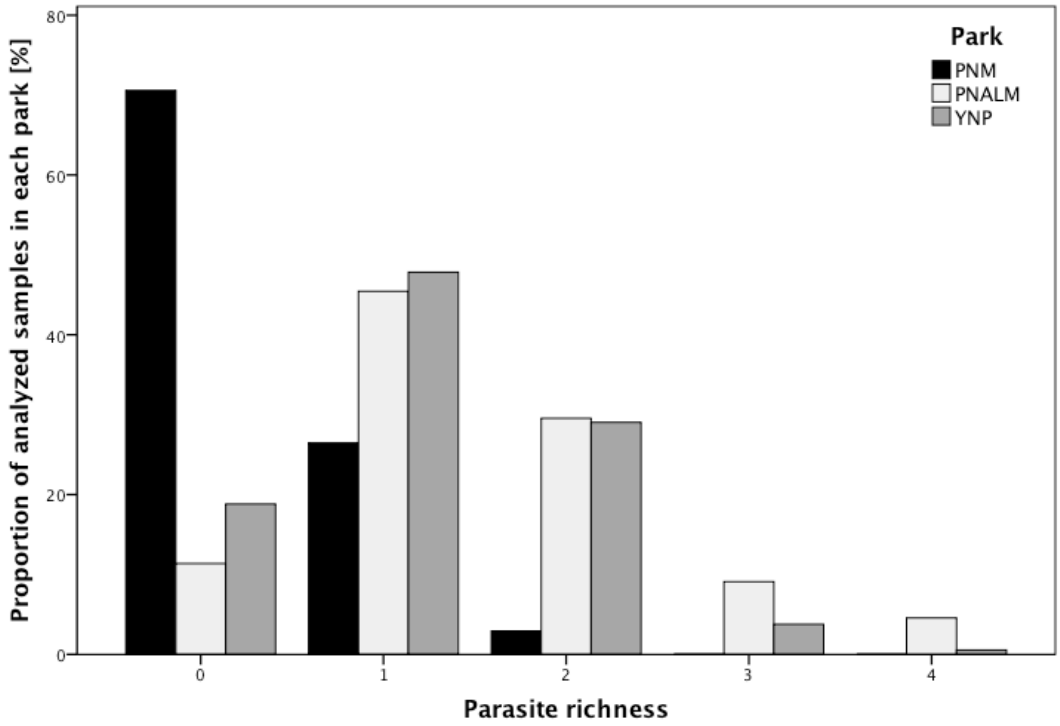


Figure 1. **The distribution of the number of parasite taxa found in wolf scats (parasite richness) differs among the three parks.** PNALM: Abruzzo, Lazio e Molise National Park, PNM: Mercantour National Park, YNP: Yellowstone National Park.

The results for overall parasite prevalence reflected those of parasite richness. Parasite prevalence was highest in PNALM (88.6%), slightly lower in YNP (81.7%), and lowest in PNM (29.4%). The model with park had the highest support from the data (95.5%) suggesting that differences among packs were not important in structuring parasite prevalence (Table 5). Overall prevalence in PNALM and YNP were not statistically different from each other (chi-square, $p=0.120$), but prevalence in both parks differs from that in PNM (chi-square, $p<0.000$). When including unidentified larvae (Appendix A), the same results are found regarding PNM as compared to the two other national parks, while PNALM and YNP slightly differ from each other ($p=0.049$). In further analyses, unidentified larvae are not considered as wolf parasites. As it cannot be excluded that these nematode larvae are wolf parasites, we provide total prevalence details including these larvae in Appendix 3.

For the five most common helminths, we report important differences in prevalence among the three parks (Table 4). *Taenia/Echinococcus* and *T. leonina* were the most common in YNP whereas *U. stenocephala* was most common in PNALM. The two *Capillaria* species were only found in PNALM (Table 4). For these five most common

parasites, park was generally much more important for structuring parasite prevalence than pack, with the exception of *T. leonina* (Table 5), for which pack played a more important role. This result is explained by large differences in *T. leonina* prevalence among packs in YNP: The parasite was not detected in the Blacktail Deer Plateau pack but found in 17.1% and 30.0% of samples from the Druid Peak and the Slough Creek pack respectively. In general, there was no support for models that used other parasites to predict the prevalence of the focal parasite. An exception is *C. aerophila*, which prevalence was best predicted by the presence of *C. boehmi*. *C. aerophila* was only detected in scats containing *C. boehmi* as well.

Table 5. Model selection results for parasite variables. Only the best three models are shown. The number of parameters in the model (k), the AIC difference (ΔAIC) and AIC weight are given for each model. Prevalence refers to total prevalence. A difference of over two units in ΔAIC as compared to the best model indicates that a factor has no statistical influence on the variable. (Taeniidae: *Taenia/Echinococus*).

variable	model	factor	k	AIC	ΔAIC	weight
richness	**1**	**pack**	**12**	**806.4**	**0**	**0.541**
richness	**2**	**park**	**3**	**806.8**	**0.3**	**0.459**
richness	3	1	1	868.1	61.7	0
prevalence	**1**	**park**	**3**	**321**	**0**	**0.955**
prevalence	2	pack	11	327.2	6.1	0.045
prevalence	3	1	1	392.5	71.5	0
C.aerophila	**1**	***C. boehmi***	**2**	**79.8**	**0**	**0.991**
C.aerophila	2	park	3	89.4	9.7	0.008
C.aerophila	3	pack	11	93.1	13.4	0.001
C. boehmi	**1**	**park**	**3**	**92.4**	**0**	**0.982**
C. boehmi	2	pack	11	100.4	8	0.018
C. boehmi	3	*C.aerophila*	2	297.7	205.3	0
Taeniidae	**1**	**park**	**3**	**389.3**	**0**	**0.982**
Taeniidae	2	pack	11	397.3	8	0.018
Taeniidae	3	*C. boehmi*	2	437.6	48.3	0
T. leonina	**1**	**pack**	**11**	**179.3**	**0**	**0.729**
T. leonina	2	park	3	181.3	2	0.271
T. leonina	3	*C. boehmi*	2	201.7	22.4	0
U. stenocephala	**1**	**park**	**3**	**93.4**	**0**	**0.986**
U. stenocephala	2	pack	11	101.8	8.5	0.014
U. stenocephala	3	*C. boehmi*	2	111.3	18	0

Variation among packs

Similar levels of support for models with park and pack as factor (Table 5) suggest that both spatial scales are important in shaping parasite richness in wolves. In YNP, packs did not differ in parasite richness (chi-square, p=0.753, number of parasite taxa=0, 1, 2 or ≥ 3). In PNM, only two samples contained more than one parasite taxa, preventing statistical analyses related to parasite richness. We found substantial variation in the number of parasites detected among packs in PNALM (chi-square, p<0.000; number of parasite taxa ≤ 1, ≥ 2; n=88; Fig. 2), but no difference between Iorio and Mainarde (p=0.392; number of parasite taxa ≤ 1, ≥ 2; n=43) or between Orsara and Villavalelonga packs (p=0.175; nb parasites ≤ 1, ≥ 2; n=45).

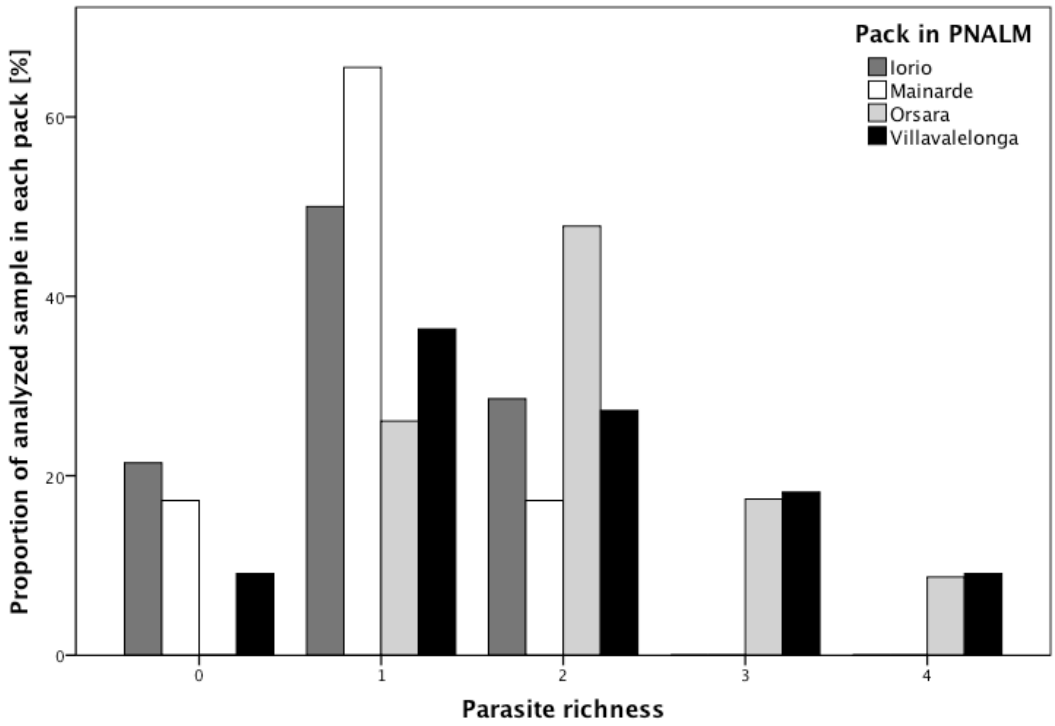


Figure 2. Distribution of the number of parasites found in wolf scats (parasite richness) in the four studied packs of Abruzzo, Lazio e Molise National Park.

Orsara is the only pack of PNALM in which total prevalence was of 100%. In the Iorio and Mainarde packs, most analyzed samples contained no or only one parasite taxa, while in Orsara and Villavalelonga packs, several different parasite taxa per sample were most common (Fig. 2).

As suggested by the model (Table 5), packs do not play an important role in structuring total parasite prevalence. In YNP, we found no difference in total parasite prevalence between packs (chi-square, n=186, p=0.933). The same is true in PNM when excluding the Moyenne Tinée pack for which sample size was too small (chi-square, n=58, p=0.352). No statistical comparison was possible in PNALM due to low number of uninfected samples.

Specific biological factors affecting parasite prevalence and richness

Considering a subset of samples for which complementary information was available in PNM and YNP, we investigated the link between specific biological factors and total prevalence or parasite richness of the detected parasite taxa.

No statistical difference was found in total parasite prevalence between scats collected from males and females, when considering all available data (n=107) and within PNM (n=48) or YNP (n=59) considered separately (chi-square, $p>0.05$). The same results were obtained regarding parasite richness in YNP (chi-square, p=404). No difference in total parasite prevalence or parasite richness was found between adults (> 2 years old) and younger individuals (YNP, chi-square, n=58, $p>0.05$). Low sample size did not allow comparisons of overall parasite richness or prevalence between specific individuals.

In further investigations, we focused on the most common helminths in PNM and YNP: *Taenia/Echinococus* in both parks and *T. leonina* in YNP.

Prevalence of Taeniidae in Mercantour National Park

In sample attributable to specific individuals in PNM (n=48) the prevalence of Taeniidae (*Taenia/Echinococus*) was not explained by individual wolf identity, sex, pack identity, or month of collection. The null model received twice as much support as the next-best model, which included sex as an explanatory variable (Table 6). Prevalence of Taeniidae was higher in scats from males (9/28 = 32.1%) than in scats from females (4/19 = 21.1%) but the sample sizes were too small to give this model more support than the null model.

Table 6. Model selection results testing biological variables on the presence of Taeniidae (*Taenia/Echinococus*) eggs in 47 wolf scats from PNM, France. The number of parameters in the model (k), the AIC difference (ΔAIC) and AIC weight is given for each model. A difference of over two units in ΔAIC as compared to the best model indicates that a factor has no statistical influence on the variable. (Taeniidae: *Taenia/Echinococus*).

variable	model	factor	k	AIC	ΔAIC	weight
Taeniidae	**5**	**null**	**1**	**57.4**	**0.0**	**0.520**
Taeniidae	3	sex	2	58.7	1.3	0.273
Taeniidae	2	pack	5	60.0	2.6	0.144
Taenidae	1	month	6	61.7	4.2	0.063
Taenidae	4	individual	20	71.7	14.3	0.000

Prevalence of Taeniids and T. leonina in Yellowstone National Park

In the 58 wolf scats from YNP that were attributable to specific individuals, prevalence of each of the two most common parasite taxa was explained by different factors (Table 7). For *T. leonina*, the prevalence varied a lot among the 3 age classes: being highest in pups (5/18 = 27.8%), absent in yearlings (0/19) and low in adults

(1/21 = 4.8%). For *T. leonina*, the model with age had 11 times more support than the next best model (Table 7).

Table 7. **Model selection results testing biological variables on the presence of the three most common parasites taxa in 58 wolf scats from YNP, United-States.** Only the best nine models are shown. The number of parameters in the model (k), the AIC difference (ΔAIC) and AIC weight is given for each model. (Taeniidae: *Taenia/Echinococus*).

species	model	factor	k	AIC	ΔAIC	weight
Taeniidae	**1**	**month**	**4**	**62.9**	**0.0**	**0.997**
Taeniidae	2	sex	2	75.7	12.8	0.002
Taeniidae	3	null	1	79.0	16.0	0.000
Taeniidae	4	*Sarcocystis* sp.	2	79.3	16.4	0.000
Taeniidae	5	winter	2	79.3	16.4	0.000
Taeniidae	6	individual	20	80.1	17.2	0.000
Taeniidae	7	pack	2	80.7	17.7	0.000
Taeniidae	8	*T. leonina*	2	80.9	18.0	0.000
Taeniidae	9	age	3	83.0	20.0	0.000
T. leonina	**1**	**age**	**3**	**35.3**	**0.0**	**0.747**
T. leonina	2	pack	2	40.2	4.9	0.066
T. leonina	3	null	1	40.6	5.3	0.054
T. leonina	4	sex	2	40.8	5.5	0.047
T. leonina	5	*Sarcocystis* sp.	2	41.7	6.4	0.031
T. leonina	6	winter	2	42.0	6.7	0.027
T. leonina	7	Taeniidae	2	42.5	7.2	0.020
T. leonina	8	month	4	44.4	9.0	0.008
T. leonina	9	individual	20	51.1	15.8	0.000

For Taeniidae, the prevalence varied a lot among months: being low in December (1/7 = 14.3%) and March (1/7 = 14.3%) and high in January (9/15 = 60.0%) and February (25/29 = 86.2%).

When considering all samples from YNP, similar results are found regarding differences among months ($p<0.000$, n=186). Further investigations show that these results are attributable to the situation in the winter 2007-2008 (n=119), as in 2008-2009 prevalence of Taeniidae was constant in December, January and February (chi-square, p=0.993, n=63) (Fig. 3). Sample size was too low in March (n = 4) to allow comparison.

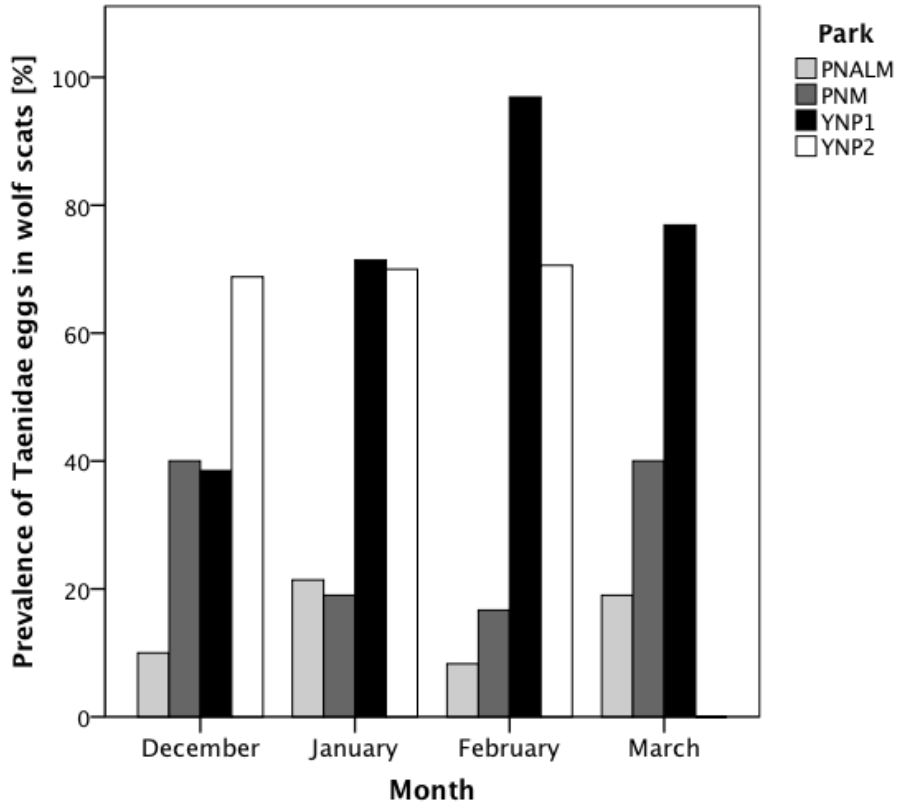


Figure 3. Prevalence of Taeniidae eggs in wolf scats collected from December to March in Abruzzo, Lazio e Molise National Park (PNALM), Mercantour National Park (PNM) and in two consecutive winters in Yellowstone National Park (YNP).

Although data from PNALM, PNM and the second winter in YNP did not allow statistical comparison, prevalence of detected Taeniidae eggs didn't follow a pattern similar to that of the winter 2007-2008 in YNP, as pictured in Fig. 3.

Discussion

In this study, we report a species list of all recovered helminths and two protozoans of wolves in the three investigated areas, obtained by analyses of fecal samples. We also evaluate different biological and geographical variables that can influence the presence of most detected helminths in the studied populations. The results are interpreted reminding the low sensitivity of coprology, as compared to necropsy, and thus represent the lower border of effective infection in samples.

Identified parasite taxa

To our knowledge, this is the first broad investigation on helminths and protozoans in grey wolves of France and the first report on these parasites in the wolf population of northwestern United States. Our results further provide an update on information collected in Italy 20 years ago (Guberti et al. 1993).

In addition, this is the first report on the presence of the nematode *Physaloptera* sp. and the protozoans *Sarcocystis* sp. and *Isospora* sp. in European wolves.

Sarcocystis sp. and the cestode family of Taeniidae are the only two parasite taxa that we found in the three investigated areas. Both parasites have an indirect life cycle involving a predator-prey interaction, and are known to infect a large array of prey as well as carnivore species worldwide. In Canada, high prevalence of these parasites was previously reported in wolves (Bryan et al. 2012; Stronen et al. 2011), and our data show prevalence of *Sarcocystis* sp. similar with those reported in these studies (36.5-43.7%). However, prevalence of Taeniidae in fecal samples from YNP is about twice to three times higher than reported in Canada (Bryan et al. 2012; Stronen et al. 2011), suggesting differences in prevalence of Teaniidae in main prey species of wolves in these areas.

The presence of *Sarcocystis* sp. in wolves has been reported in Russia (Kreeger 2003) and in North America (Bryan et al. 2012; Stronen et al. 2011; Emnett 1986). Numerous species of *Sarcocystis* infect a wide range of domestic and free-ranging ungulates throughout the world (Goldová et al. 2008; Luzón et al. 2008; Kahn and Line 2005; Malakauskas and Grikienienė 2002; Cornaglia et al. 1998; Odening et al. 1996; Nigro et al. 1991; Poli et al. 1988; Ewing 1986; Crum et al. 1981) that are possible preys species of wolves. Despite rarely reported in wolves, the presence of *Sarcocystis* sp. in wolves from all three areas investigated in the present study is therefore not surprising. *Sarcocystis* sp. is well known in dogs, foxes and coyotes (Rajković-Janje et al. 2004; Ewing 1986; Fayer and Johnson 1975), and the only rare reports of this protozoan in wolves may solely be due to low number of investigations searching for this protozoan. The detected cysts can represent various different species, which are hardly distinguishable by micrometry (Bowman 2009). The other protozoan reported in this study is *Isospora* sp., and has been described in wolves in Canada and the United States (Bryan et al. 2012; Mech and Kurtz 1999), where it likely caused the death of three wolf pups (Mech and Kurtz 1999). To our knowledge, this protozoan was never reported in free-ranging wolves outside North America.

The only helminth taxa that we found in all three investigated areas is the Taeniidae family.

Taeniids are known to be among the most frequent intestinal parasite in canids, and very frequent in wolves (Craig and Craig 2005). This family comprises numerous species infecting wolves, including the genus Echinococcus and Taenia. The eggs of these cestodes are very resistant and may remain infective for years in the environment (Marquard-Petersen 1997; Custer and Pence 1981b), probably contributing to the maintenance of the parasites in the ecosystem. The main intermediate hosts of these cestodes are various ungulates, lagomorphs and rodents, depending on the primary prey of the final host (Craig and Craig 2005; Marquard-Petersen 1997; Guberti et al. 1993).

In YNP, detected eggs can correspond to one or several species most frequently reported in North America: *Taenia hydatigena*, *T. krabbei*, *T. pisiformis*, *T. multiceps* and

Echinococcus granulosus (Custer and Pence 1981b). In PNALM and PNM, reported Teaniidae eggs probably correspond to most prevalent species in wolves from Italy: *T. hydatigena* and *E. granulosus* (Guberti et al. 1993). Species less frequently reported in wolves from Italy, i.e. *T. multiceps*, *T. pisiformis*, *T. ovis* (Guberti et al. 1993), may also be present in the investigated packs.

Despite the low sensitivity of coprology (8-9.4%) reported in the literature for the detection of tapeworms (Wilson et al. 2002; Torres et al. 2001), our results show high prevalence of the parasite in the studied populations (15.9-67.2%). Thus, the concentration methods used in the present study might be more efficient in recovering Taeniidae eggs than floatation procedures, which are most frequently used in coprological investigations. Our results suggest that Taeniids are more widespread and available on the northern range of YNP than in the investigated European national parks.

We are not aware of any previous report of *Capillaria boehmi* in wolves. This can be a consequence of this species having long been mistaken for *C. aerophila* (Bowman 2009; Schoning et al. 1993), and further because necropsies did not investigate nasal and paranasal sinuses of carcasses (Bagrade et al. 2009; Moks et al. 2006; Segovia et al. 2001, 2003; Guberti et al. 1993), where the adult worm of *C. boehmi* is found (Bowman 2009). This can explain findings of *Capillaria* sp. eggs in feces but no adult *C. aerophila* (Magi et al. 2009; Nevárez et al. 2005) or Trichuriidae (Torres et al. 2001) in comparative fecal and histopathological examination of fox and wolf carcasses, in Spain, Canada and Italy. Thus, *C. boehmi* can be widespread in other canid populations including wolves, as the parasite is reported for example in 51% and 8% of foxes in Norway (Davidson et al. 2006) and Hungary (Sréter et al. 2003) respectively. *C. boehmi* is the most prevalent parasite detected in PNALM (Table 4), with 80.7% of the sample containing eggs from this parasite. The life cycle of *C. boehmi* is unknown (Bowman 2000a), but may be direct such as that of other related species including *C. aerophila* (Bowman 2000a, 2000b). Transmission via an earthworm, facultative intermediate host for *C. aerophila*, is unlikely to be main infection route as wolves are not known to commonly feed on earthworms. The high prevalence reported in this study suggests very efficient transmission of the parasite and/or a high contamination of the environment in PNALM. Given the location of infected tissues, we suggest that parasitic infection can be acquired through inhalation of infective eggs. As smell is a key sense in canids, sniffing can be an appropriate way for parasites to enter the organism of hosts. Investigation of feces is a common tool used to convey information in canids and contamination of a territory by feces from various host species facilitates transmission of the parasite. A short life span of *C. boehmi* could explain its absence from the wolf population in PNM. Alternatively, the parasite may have just recently spread in the wolf population of PNALM, and is expected to infect wolves of PNM in short delays. Transplacental infection or transmission through lactation would further contribute to high prevalence of the parasite in a population. Detected high prevalence suggests that no immunological mechanism evolved to clear *C. boehmi* from the organism of wolves.

Capillaria aerophila has previously been reported in wolves in Eastern Europe (Szafrańska et al. 2010; Bagrade et al. 2009; Popiołek et al. 2007; Shimalov and Shimalov 2000), Russia and North America (Craig and Craig 2005; Peterson et al. 1998). Range of hosts and geographic distribution of *C. aerophila* in Europe is fragmentary (Traversa et al. 2010). The parasite is however known to infect dogs (*Canis lupus familiaris*) (prevalence ≤2.8%, Traversa et al. 2010) and red foxes (*Vulpes vulpes*) (prevalence=14.5%, Magi et al. 2009) in Italy. The two *Capillaria* species found in PNALM infect the respiratory system of their host. The only precedent study on helminths in wolves in Italy looked for intestinal parasites, investigating helminths present in the gut of wolf carcasses, which explains that no *Capillaria* sp. was reported (Guberti 1993). *Capillaria* species are not common parasites of foxes in central and Northern Italy (Magi et al. 2009; Di Cerbo et al. 2008), suggesting that wolves serve as a reservoir for the parasite in PNALM.

Except for *Capillaria boehmi*, first reported here in the carnivore, all other helminths detected in our study were previously described in free-ranging wolves (Craig and Craig 2005). Our results in PNALM are in accordance with previous findings reporting the presence of *Uncinaria stenocephala* and *Toxocara canis* in Italian wolves (Guberti et al. 1993). However, we found no egg of *Ancylostoma caninum*, previously described in 16% of the wolf carcasses sampled in Italy (Guberti et al. 1993). The destruction of *A. caninum* eggs and larvae by freezing (Bowman 2009) can explain why we did not find them in our samples, all collected in winter. All other helminths previously reported in Italian wolves had a prevalence below 10%, and the lower sensitivity of coprological methods as compared to necropsy used by Guberti et al. (1993) can be the reason why we did not detect previously described *Mesocestoides lineatus*, *Dipylidium caninum* and *Trichuris vulpis*. In a study of red foxes in central Italy, *M. lineatus* and *D. caninum* were frequently found through necropsy but absent in coprological investigations of the same hosts, and 0% sensitivity of coprology was reported for these parasite species. In the same study, prevalence of *T. vulpis* in feces was only of 2.7% (Magi et al. 2009). This information suggests that *T. vulpis* is not widespread in central Italy and our results can therefore accurately indicate the absence of the parasite from the studied packs. Consistent with our results, very low prevalence of *T. leonina* was previously reported in wolves in Italy (Guberti et al. 1993).

Besides the two *Capillaria* species, we found other helminth parasites of wolves that were not previously reported in Italy: the trematode *Alaria* sp. and the nematode *Physaloptera* sp.. *Alaria* sp. was described in wolves worldwide and *Physaloptera* sp. has been found in wolf populations from the United States and in Russia (Craig and Craig 2005). The adult *Physaloptera* lives in the stomach of the carnivorous host, reason for which was not found in previous investigations in Italy that examined guts of wolf carcasses (Guberti et al. 1993). The prevalence of both parasites is however very low in PNALM, and only *Alaria* sp. was found in YNP (Table 4). The indirect life cycle of these two helminths requires the passage in paratenic hosts (host in which immature stages of the parasite survive without developing) that are not common prey species of wolves: a frog, a mouse or a snake in the case of *Alaria* sp., and a cold-blooded vertebrate for

Physaloptera sp. (Bowman 2009). The low occurrence of these helminths in our study is in accordance with these hosts being rarely preyed on by wolves in the study areas (Table 2). Infection by these parasites is further lessened in winter, due to hibernation of most paratenic hosts. Further investigations should evaluate the prevalence of these parasites in wolves during warmer months of the year.

The helminth *T. canis* is more common in young than in older canids (Bowman 2009; Guberti et al. 1993) and can be lethal to pups (Bowman 2009; Foreyt 2001). This parasite was reported in wolves from Italy (Guberti et al. 1993) and in foxes from Northern Italy (Di Cerbo et al. 2008). The absence (PNM, YNP) or very low prevalence (PNALM) of this parasite in our results can relate to timing of sample collection. By winter, infected pups may either have succumbed to infection or overcame it.

Life history of investigated populations and alternative canid hosts

All parasite species found in PNM were also detected in PNALM, and only one helminth, (*T. vulpis*) was found in YNP but absent in the European national parks (Table 4). Detected parasite diversity is different in all three investigated areas, suggesting that different geographical and biological factors structure parasite community of wolves in the three national parks. The important diversity of parasites in PNALM, as compared to PNM and YNP, might reflect a long-term co-evolution between parasites and their hosts, as the wolf never disappeared from central Italy (Boitani 2003; Ciucci and Boitani 1998b). Such important diversity in parasite taxa seems to be characteristic of long established wolf population in temperate areas, and is reported elsewhere in Europe (Bagrade et al. 2009; Moks et al. 2006; Segovia et al. 2001, 2003).

Taxonomically related host species are susceptible to be infected by the same parasite species (Freeland 1983), and part of the helminth and protozoan fauna of wolves is also found in other canids as dogs, red foxes and coyotes (*Canis latrans*) (Bryan et al. 2012; Di Cerbo et al. 2008; Guberti et al. 1993; Campbell 1991; Guberti and Poglayen 1991; Custer and Pence 1981b; Erickson 1944). Helminth transmission rate is usually positively correlated with host population density (Tompkins et al. 2002). Thus, populations of other canid species living in sympatry with wolves have an important impact on the contamination of the environment by helminths and protozoans, and consequent potential infection of wolves. An elevated host density also facilitates the spread of new parasite species in a population (Roberts et al. 2002), and the red fox and free-ranging dog population living in sympatry with wolves in PNALM may have introduced and/or helped maintain some directly transmitted parasite species in the environment. Furthermore, free-ranging dogs might use similar prey species as wolves in central Italy (Ciucci and Boitani 1998a) and therefore contribute to the maintenance, in the environment of PNALM, of parasite species indirectly transmitted through predation.

In YNP, parasite diversity is somewhat lower than previously reported from long established wolf populations in Canada (Bryan et al. 2012; Stronen et al. 2011), but elevated when considering that wolves released in the reintroduction program, in 1995

and 1996, were dewormed (Smith pers. com.). All protozoans and helminths reported in the studied packs of YNP are known coyote parasites (Radomski and Pence 1993; Custer and Pence 1981a; Dubey 1980; Conder et al. 1978; Arther and Post 1977; Hudkins and Kistner 1977; Thornton et al. 1974; Erickson 1944) and some are known to also infect foxes in North America (Erickson 1944). Thus, these parasites where probably maintained in the environment by the canid population of YNP while wolves were absent. Recently, wolves migrating into YNP can also have brought some of these parasites in contact with the wolf population of the park, as the helminth *T. vulpis*, less common parasite of coyotes (Custer and Pence 1981a). Thus, our findings suggest that susceptible, parasite free individuals rapidly acquire parasites from an environment occupied by a sufficient number of alternative hosts.

We found the lowest parasite diversity in wolves from PNM, with only 3 different parasite taxa identified. Observed differences between the two European areas are particularly interesting, as Italian wolf dispersers founded the packs in PNM by a natural recolonization process (Boitani 2003). The poor diversity of parasites found in PNM as compared to PNALM can be explained by a phenomenon analogous to the founder effect in population genetics (Roberts et al. 2002). Small populations of hosts that recolonize new habitats usually harbor a subset of the total variety of parasites present in the source population, and less common parasite species are expected to be absent (Roberts et al. 2002). Thus, the transmission of most prevalent ("core") species of parasites from the source population is likely, if the colonized habitat is suitable for the parasite (Roberts et al. 2002). Considering all parasite taxa present in over 10% of the analyzed samples in PNALM, the two *Capillaria* species are the only ones that were not found in PNM. We are not aware of any meteorological dissimilarity between PNALM and PNM that would have an impact on survival of *Capillaria* eggs in the environment. As both *Capillaria* species infect the respiratory system of the carnivore, heavily infected individuals may be physically less efficient in dispersing to distant areas, thus limiting the transmission of these parasites to naturally recolonized populations.

As for coyotes in North America, foxes are widespread in Europe and harbor numerous parasites species also known to infect wolves (Magi et al. 2009; Di Cerbo et al. 2008). However, *Alaria* sp., *Physaloptera* sp. and *T. leonina* are not reported in foxes from Northern Italy, and *T. vulpis* and *Capillaria* sp. are very uncommon (Di Cerbo et al. 2008). Considering this, and given the absence of a free-ranging dog population in PNM, se suggest that the density of susceptible hosts was insufficient to maintain these parasites in the environment of PNM.

Except for *Physaloptera* sp., and for *C. boehmi* for which this information is unknown, all nematodes reported in our study can be directly transmitted to their carnivorous host. Reinfection is helped by the fidelity of wolves to their den site and rendez-vous area, and is therefore favored in well-established packs (Kreeger 2003; Segovia et al. 2003; Custer and Pence 1981b). During a colonization process, such fidelity might not be immediately established, leading to the incapacity of some parasites to survive in the environment. However, with the expansion of the wolf

population in Northern Italy, dispersers will keep arriving in PNM, and an increase in parasite diversity in the French national park due to transmission by immigrant individuals can be expected.

Variation of parasite richness and prevalence as a function of geographical scale

Variation among parks

Large geographical scales appear to be the best predictor of parasite richness and total parasite prevalence in samples from the investigated areas (Table 2).

Total parasite prevalence in samples from PNALM (88.7%) is in accordance with previous reports in Italy, based on necropsies (100%, Guberti et al. 1993), although explained by different parasite species. The total prevalence observed in YNP (81.7%) is also coherent with previous reports in wolves from North America (95.0%, Custer and Pence 1981b; 91.0%, Rausch and Williamson 1959). This prevalence is higher than reported from analyses of wolf fecal samples in British Columbia (62.6%, Bryan et al. 2012), but show the predominance of the same two parasite taxa: *Sarcocystis* sp. and *Taeniids*.

As availability of directly transmitted parasites in the environment is dependent of susceptible hosts density, this factor is likely a key determinant of both parasite richness and total prevalence in the wolf population. For parasite species with indirect life cycle, density of infected prey species further determines parasite availability. Thus, the important prevalence of Taeniids and *Sarcocystis* sp. in YNP as compared to PNALM and PNM is probably linked with the diet of wolves in the investigated areas, indicating important infection of specific prey species. On the northern range of YNP, the main prey species of wolves is elk (>80%; Smith et al. 2008, 2009, 2010), while the diet of the carnivore is much more varied in the two European national parks (Table 2).

All three national parks differ from each other in parasite richness (number of identified parasite taxa/fecal sample; Fig. 1), and these differences reflect parasite diversity in the three areas. Consistent with our results in PNALM and YNP, parasite richness is usually higher (≥2 parasite species/sample) in long established wolf populations, as reported in Latvia (Bagrade et al. 2009), Spain (Segovia et al. 2001, 2003) and North America (Custer and Pence 1981b; Holmes and Podesta 1968). Lower parasite richness values, similar to those in PNM, are reported in a recently protected and slowly recovering wolf population in western Poland (Popiołek et al. 2007).

Variation among packs

Previous investigation reports no infracommunities of wolf helminth parasites throughout Italy and similar statement has been made in North America (Guberti et al. 1993). Consistent with this, we found no significant differences between packs of the same national park in total parasite prevalence. However, pack seems to be an important factor in structuring parasite richness in PNALM (Fig. 2). Variations between

packs are not explained by differences in main prey species, as Taeniids and *Sarcocystis* sp. were found in 4/4 and 3/4 packs respectively. Similarly, relative importance of livestock and wild ungulates in the diet (Grottoli 2011) does not correlate with parasite richness. In the Villavalelonga pack, availability of parasites in the environment can be the major factor explaining elevated parasite richness detected in the pack. On the other hand, important parasite richness detected in the Orsara pack reflects more parasite diversity, and is related to the presence of uncommon parasites in the park, that were only found in this pack: *Alaria* sp., *T. canis* and *T. leonina*. Furthermore, *Physaloptera* sp. was only detected in Orsara and Villavalelonga packs.

In YNP, prevalence of *T. leonina* was best explained at the pack level (Table 5). The parasite was not found in the Blacktail Deer Plateau pack, but detected in 17.1% and 30.0% of samples from the Druid Peak and the Slough Creek pack respectively. These data can reflect an uneven distribution of the parasite in the environment, or differences in parasite availability between years.

Specific biological factors affecting parasite prevalence and richness

Individual

Given the behavioral biology of wolves, no differences are expected in exposure to parasite between the members of a pack. Genetic differences between individuals may however influence host susceptibility to infection by parasites (Wilson et al. 2002). However, our results show that, among the samples attributable to specific individuals in PNM and YNP, the prevalence of the most common parasites was not explained by individual wolf identity (Table 6 and Table 7).

Gender

Consistent with previous studies, we found no significant difference between samples from males and females in total parasite prevalence, parasite richness, or prevalence of specific parasite taxa (Bagrade 2009; Segovia et al. 2001; Guberti 1993). Even though gender is the second best explanatory factor for the presence of Taeniidea in a subset of samples in PNM and in YNP, these models did not receive sufficient support (Table 6 and Table 7).

Age

In a subset of samples from YNP, we report an age-related prevalence of *T. leonina*, with higher values in fecal samples collected from pups (<1 year old) than in older individuals. Such a trend was already reported in wolves in Canada (Holmes and Podesta 1968). An important infection rate of pups at the den site was suggested as the main reason for such age-related infection (Holmes and Podesta 1968). Acquired immunity might also be the reason for a lower prevalence of *T. leonina* in older

individuals (Bryan et al. 2012; Wilson et al. 2002; Custer and Pence 1981b). This hypothesis is further supported by our results, when considering all samples from YNP. In fact, almost no fecal sample contained eggs of *T. leonina* in the Blacktail Deer Plateau pack, the only studied pack of YNP in which no pup was present at the time of sample collection. In addition, prevalence of *T. leonina* nicely reflects the proportion of pups in the Druid Peak and the Slough Creek packs (Druid Peak: prevalence of 17.1% and proportion of pups = 37.5%; Slough Creek: prevalence of 30% and proportion of pups = 56.3%).

Alternatively, or in addition, such an age-related prevalence of *T. leonina* suggests a higher propensity of pups to search for and eat small rodents, possible paratenic host of the parasite (Bowman 2009; Foreyt 2001). Such behavior of pups was observed in YNP.

Monthly variations

Seasonality in parasite aggregation can be explained by host exposure to infective stage of a parasite (Wilson et al. 2002). Our study examined fecal samples from winter months only, so that seasonality could not be assessed. However, in a subset of samples from YNP, month of production best explained the prevalence of Taeniidae in fecal samples of wolves (Table 7). Further investigations on all samples collected from the park revealed that this result was attributable to the situation in the first winter of study in YNP (2007-2008). Heavy snowfalls started early winter in the park that winter and elk, an intermediate host of Taeniidae, is the main prey species of wolves in YNP. For the ungulate, important snow cover makes access to food more difficult and moving requires more energy expenditure. These conditions may have favored predation on elk most weakened by parasitic infection. The prepatent period (the time interval between infection and first detection of a diagnostic stage) for most Taeniids being of more or less two months (Bowman 2009), a selective predation on heavily infected elk in early winter could explain the peak of detected eggs in February.

Much thinner snow cover characterized the next winter (2008-2009) in YNP. In Europe, winter conditions were especially mild at the time of sample collection (2006-2007) and snow cover was limited (Réseau Loup/Lynx 2007; http://www.ncdc.noaa.gov/sotc/global/2007). In PNALM and PNM, as well as during the second winter of sample collection in YNP, prevalence of detected Taeniidae eggs clearly didn't follow a pattern similar to that of the winter 2007-2008 in YNP (Fig. 3).

Interference between most commonly detected parasite species

We did not detect clear mutual exclusion between any of the most frequently identified parasite taxa (>10% prevalence in a samples from a park) in the investigated areas. Similarly, the presence of a parasite taxa was generally not predicted by the presence of another taxa (Table 5). The only exception was *C. aerophila*, which was found solely in samples also containing *C. boehmi*. However, given the very high prevalence of *C. boehmi*, this result may not indicate an interaction between these two parasite species.

Unidentified nematode larvae

The nematode larvae that we detected in fecal samples from wolves can have different origins. A thorough morphometric and morphological examination is necessary to accurately identify first stage larvae of (1) hookworm (in our samples: *U. stenocephala*) that hatched from eggs eliminated in feces and exposed to adequate environmental conditions, (2) free-living or plant nematodes that migrated into the scat from the ground (Traversa et al. 2010), (3) larvae from infested prey eaten by the wolf, or (4) parasites of wolves eliminated as larvae. To a large extend, collection of samples in winter prevented the development of *U. stenocephala* larvae from eliminated eggs, as well as invasion of feces by free nematode larvae. As most larvae were found in samples collected in PNM (Appendix 3), and given the especially mild conditions that prevailed in Europe in the winter of sample collection, we cannot exclude these hypotheses. However, we then should have found similar results in samples from PNALM. Furthermore, we also found nematode larvae in samples from YNP. Thus, the detected larvae are more likely either parasites of wolves or parasites of prey species, which were fed on by the carnivore.

Different parasites of dog are eliminated at a larval stage in feces, including *Oslerus osleri, Strongyloides stercoralis, Angiostrongylus vasorum* or *Crenosoma vulpis* (Traversa et al. 2010) and most of these parasites have been reported in wolves (Bagrade et al. 2009; Popiołek et al. 2007; Segovia et al. 2001; Shimalov and Shimalov 2000; Erickson 1944). If the detected larvae belong to parasites infecting wolves, prevalence of helminths would double in PNM, while not changing much in PNALM and YNP (Appendix 3). If true wolf parasites, the high prevalence of larvae found in samples from PNM suggests the presence in the environment of at least one locally widespread nematode, possibly also infecting red foxes. Alternatively, elevated prevalence of unidentified larvae in the analyzed fecal samples from PNM could reflect important infection of wolf prey species in that area.

Parasites in prey species

Our study provides direct and indirect information on parasite communities present in prey species of wolves in the three investigated areas. Direct evidence is provided by the recovery of pseudoparasite helminth eggs (helminths infecting prey species) in fecal samples from the carnivore. The diversity of these pseudoparasites apparently reflects the diversity of prey species used by the wolf in the three national parks (Table 2; Appendix 2). The presence of *Trichuris suis* and *Metastrongylus* sp. in samples from PNALM reflects the important proportion of wild boar in the diet of wolves in the Italian national park during winter (Table 2). *Dicrocoelium dendriticum* and *Nematodirus* sp. are helminth parasites infecting mammals from the Bovidae, the Ovidae and Capridae family, while Capillariidae parasites also infect birds. The presence of these parasites can reflect use of either wild or domestic animals, through predation or scavenging of

abandoned carcasses (Grottoli pers. com.). *Toxocara cati* is a parasite of cats, and the finding of this parasite in wolf fecal sample results from coprophagy, scavenging or predation.

Pseudoparasite richness in packs (Appendix 2) does not reflect main prey species used by the different packs in PNALM (Grottoli 2011) and may be explained by prevalence of the detected parasite in more sedentary local prey species.

Our investigations also indirectly show the presence of *Alaria* sp., *Sarcocystis* sp., Taeniids, and the protozoan *Isospora* sp. in prey species of wolves. Particularly, our data show that Taeniids and *Sarcocystis* sp. are present in wolves prey species in all three investigated areas. Our data suggest that *Sarcocystis* sp. and Taeniidae are common in the elk population of YNP, while these parasites are less frequent or infect only some of the prey species of wolves in PNM and PNALM.

Conclusion

The characteristic helminth fauna of wolves is reported to be highly similar throughout the distribution range of the carnivore at a large geographical scale (Brand et al. 1995; Guberti et al. 1993; Custer and Pence 1981b; Holmes and Podesta 1968), so that the species lists reported in our study likely represent the helminth community of wolves at a large geographical scale in Southern Europe and in western North America.

Our study provides comparative data on wolf parasites in three different geographical areas, which can help our understanding of the spread of parasites in newly established populations. We also report information on the infection by helminths and protozoans that should be included in modeling population dynamics of free-ranging wolves, and considered in the management of recovering populations (Brand et al. 1995). Our data provide an important update of the parasite fauna of wolves in Italy, showing the presence of yet not reported parasite taxa, some of which may impact population dynamics through reduced dispersal capabilities. The two *Capillaria* species reported in our investigations are only rarely described in free-ranging canids in Italy, and the wolf population of PNALM might serve as a reservoir for *C. boehmi*. This parasite can however also be present in various canid populations and be mistaken for *C. aerophila*. Further research is definitely needed on *C. boehmi*, in order to better understand the transmission route of the parasite, the presence of this parasite in canid species, and the impact of this nematode on infected populations.

Consistent with previous studies, our results show that large geographical scales best predict parasite community in wolves, and that age or sex of individuals are not major determinants of the prevalence of most detected parasites. This result is in accordance with the behavioral biology of wolves, as group living enhances transmission of parasites between all pack members, most often independently of gender or age of individuals.

Our data suggest that the density of susceptible hosts and the availability of parasites in the environment are the main factors shaping the parasite community and determining parasite prevalence and richness in the studied wolf packs. For parasite species with an indirect life cycle, diet habits of wolves and prevalence of infection in prey species are additive factors that influence the presence of these parasites in wolf populations. Availability of infected prey species is partly determined by seasonality, in relationship with the behavioral biology of the prey (hibernation) or with extreme weather conditions, which can influence infection of wolves.

Life history of wolf populations further seems to shape the parasite communities infecting the species in different geographical areas. Directly transmitted parasite taxa are most common in the long established population of PNALM, where the density of wolves and other susceptible hosts is high. Thus, diet of the carnivore does not appear as the main factor shaping prevalence of parasites in that population. In areas of recent recolonization by wolves, the most prevalent parasite species (*Sarcocystis* sp. and Taeniids) are transmitted through a predator-prey cycle, and some of the species from these parasite taxa were probably maintained in the environment in the absence of wolves by sylvatic cycles established with the red fox or the coyote and different mammal preys. Our results suggest that the presence of alternative canid host species (i.e. coyotes) triggered the infection of reintroduced dewormed wolves in YNP. On the other hand, in PNM, density of these alternative hosts may have been too low to maintain some parasite species in the environment, which would explain the absence of some parasite species in that naturally recolonizing population. It can be expected that parasite diversity will increase in both these newly settled populations, as dispersing wolves keep arriving from neighboring areas.

Parasite diversity and richness are probably underestimated in our study due to (1) low sensitivity of coprology for different parasite taxa, (2) because different species of parasites are probably classified under the Taeniidae family and as (3) different species can also be represented by the parasites determined to the genera level (as *Sarcocystis* sp.) and among the unidentified nematode larvae.

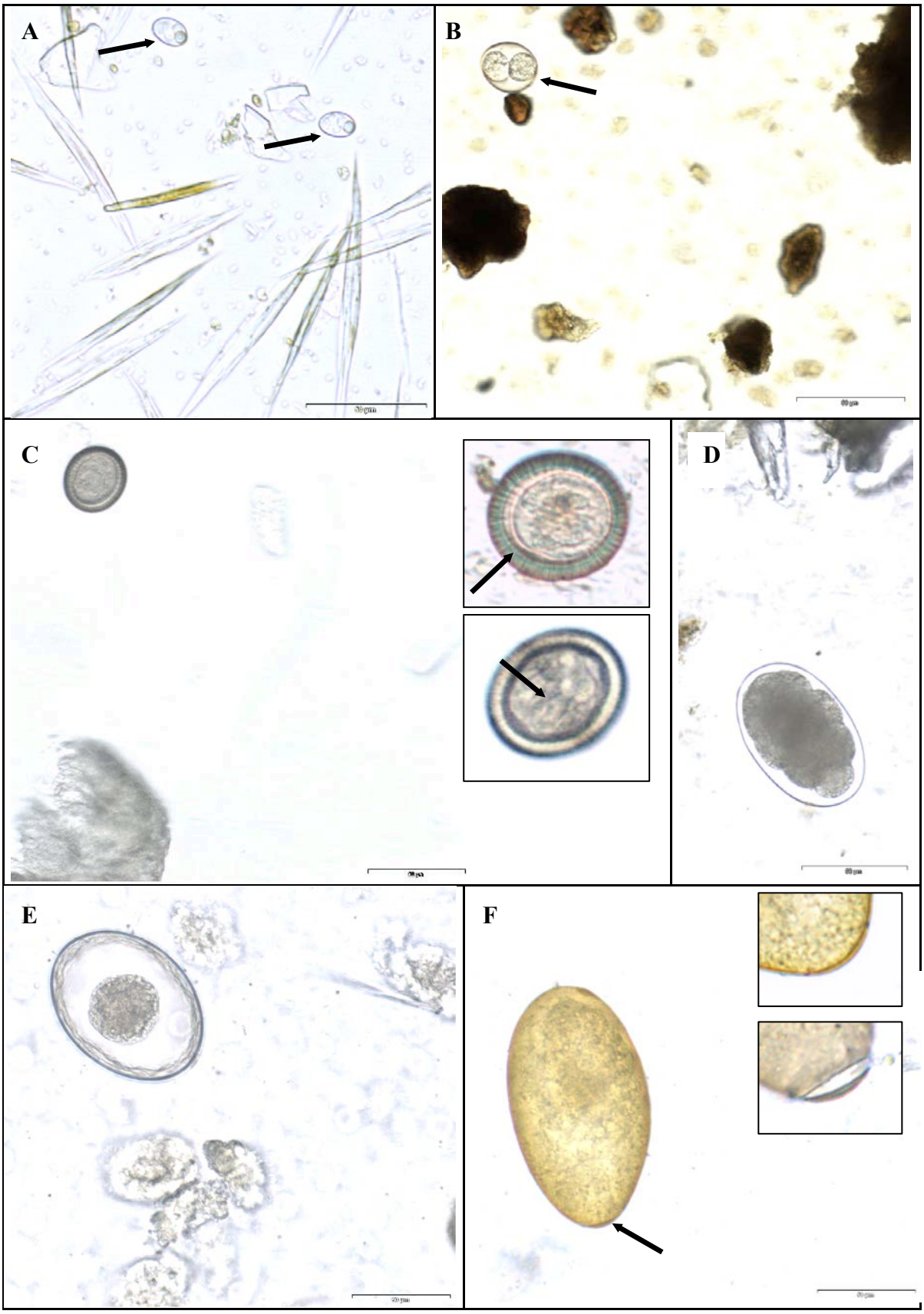
Little is known about the effect of most pathogens on carnivore population dynamics, particularly when effects seem sublethal or non-epidemic, or when symptoms are aggravated through interactions with other factors or diseases (Murray et al. 1999). However, significant impact of infection by helminths on the energy budget of hosts has been reported (Roberts et al. 2002). Thus, chronic infection frequently leads to increased metabolic rate and reduced body mass, which may impact host fitness in the long term (Roberts et al. 2002). In Europe, emerging helminth parasites have recently been reported in dogs (Traversa et al. 2009, 2010) that can potentially have an additional impact on wolf populations, thus underlying the importance of continuous monitoring of the helminth fauna of free-ranging wolves, dogs and other sympatric canids.

Parasite load is an important factor in determining severity of infection and subsequent symptoms (Holt and Dobson 2007), but cannot be precisely evaluated through coprological analyses. Considering that parasite diversity can also correlate with severity of symptoms, the wolf population in PNALM appears under elevated selective pressure and more threaten by parasitic infection than wolf populations in PNM and YNP. However, specific parasites can, by themselves, have an important impact on population dynamics. In particular, *T. canis* and *C. aerophila* are known to potentially cause death of infected individuals (Bowman 2009), further suggesting that the wolf population in PNALM is exposed to higher risks than those of YNP and PNM. As for *T. canis*, it is transmitted from an infected mother to the foetus *in utero* and, to a lesser extend, through lactation of newborns (Bowman 2009; Foreyt 2001), and can impact population dynamics by decreased pup recruitment.

Consequences of infection by helminths are aggravated when combined with viral or bacterial disease, or other weakening factors such as malnutrition (Kreeger 2003). Some parasitic infections can thus be predisposing factors to infection by canine parvovirus (CPV-2) (Kreeger 2003), and this may be true for some other viral infections. The two *Capillaria* species detected in our study infect the respiratory system and can by themselves severely impair infected hosts (Bowman 2000a, 2009). These parasite species possibly have an additive weakening effect on individuals in case of canine distemper virus (CDV) outbreaks, as this virus also infects the respiratory system (Deem et al. 2000). Such outbreaks have been repeatedly reported in wolves and coyotes in YNP (Almberg et al. 2009). All other parasite taxa detected in the present study are localized in the small intestine of their host, except for *T. vulpis* and *Physaloptera* sp., which were rarely found in the analyzed samples. Simple attachment to the intestinal wall (cestodes) or feeding on the host in that portion of the gut (various nematodes) can damage host tissues and facilitate bacterial and viral infections. Thus, virulence of viruses affecting the small intestine, such as CPV-2 and canine coronaviruses (CCoV) (Pratelli 2006; Evermann et al. 2005; Steinel et al. 2001), might be enhanced by the infection of helminths localized in the same anatomical portion of hosts. Both CPV-2 and CCoV have been detected in fecal samples of wolves in PNALM and PNM (cf. chapter 1), and exposure to CPV-2 has recently been reported in all sampled individuals in YNP (Almberg et al. 2009). In conclusion, the weakening effect of infection by helminths and protozoans might enhance the virulence of viruses infecting wolves in all three study areas. Parasites and viruses may act in synergy on a host's health and severely affect wolf population dynamics in the investigated national parks.

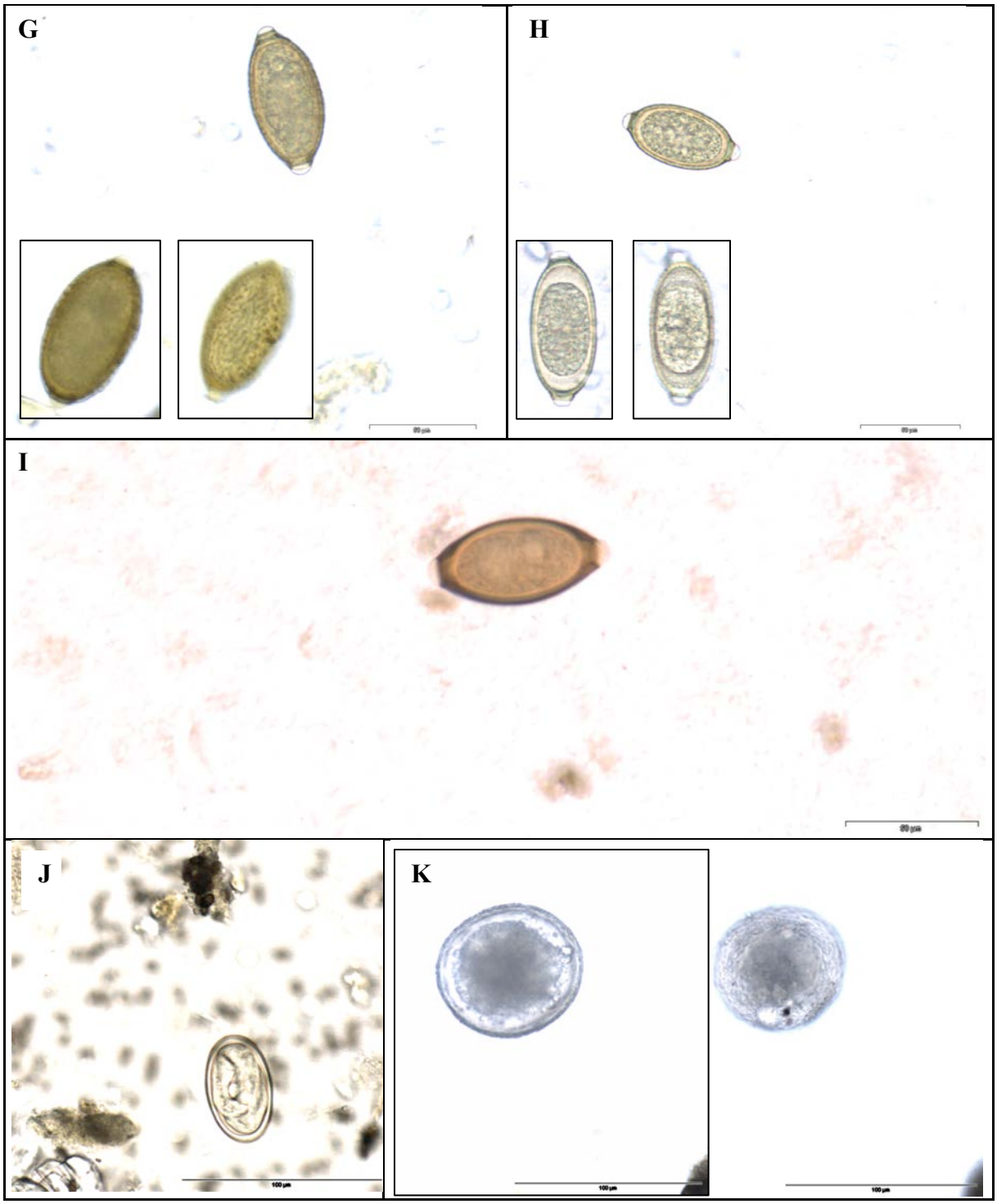
Appendix 1: Pictures of identified wolf parasite taxa.

A. *Sarcocystis* sp.; **B.** *Isospora* sp.; **C.** Taeniidae (*Taenids/Echinococcus*); inserts show the characteristic shell and hooks; **D.** *Uncinaria stenocephala*; **E.** *Toxascaris leonina*; **F.** *Alaria* sp., arrow points at operculum; inserts show closed and open operculum. Bar = 50µm.



Appendix 1 (continued): Pictures of identified wolf parasite taxa.

G. *Capillaria aerophila*, inserts show network of ridge on shell surface; **H.** *Capillaria boehmi*, inserts show pitted shell surface; **I.** *Trichuris vulpis*; **J.** *Physaloptera* sp.; **K.** *Toxocara canis*, insert shows dimpled pattern of shell surface; **G**, **H** and **I**: bar = 50µm; **J** and **K**: bar = 100µm.



Appendix 2: Pseudoparasite helminths identified in fecal samples of wolves.
Total number of tested samples (n), number of samples that tested positive (N), prevalence (P) and confidence intervals (CI) are figured. P and CI are expressed as percentages (%). (PNM: Mercantour National Park; PNALM: Abruzzo, Lazio e Molise National Park; YNP: Yellowstone National Park).

Pseudoparasites taxa	**PNALM (n=88)**			**PNM (n=68)**			**YNP (n=186)**			**TOTAL (n=342)**		
	N	**P**	**CI**	**N**	**P**	**CI**	**N**	**P**	**CI**	**N**	**P**	**CI**
Trematoda												
Dicrocoelium dendriticum	14	15.91	9.3-25.6	11	16.18	8.7-27.5	0	0	-	25	7.31	4.9-10.7
Nematoda												
*Capillariidae**	1	1.1	0.1-7.1	1	1.5	0.1-9.0	3	1.6	0.4-5.0	5	1.5	0.5-3.6
Metastrongilus sp.	4	4.5	1.5-11.9	0	0	-	0	0	-	4	1.2	0.4-3.2
Nematodirus sp.	0	0	-	1	1.5	0.1-9.0	0	0	-	1	0.3	0.0-1.9
Toxocara cati	1	1.1	0.1-7.1	0	0	-	0	0	-	1	0.3	0.0-1.9
Trichuris suis	3	3.4	0.9-10.3	0	0	-	0	0	-	3	0.9	0.2-2.8
Number of identified pseudoparasite taxa	5			3			1			6		

* Taxa identifiable to Family only

In each pack, 1 or 2 different pseudoparasites (i.e. parasites of prey species) were found, except for the Mainarde pack in PNALM (n=5) and the Slough Creek pack in YNP (n=0). These pseudoparasites are not taken into account in precedent analyses of data.

Appendix 3. Total parasite prevalence in fecal samples of wolves from the three national parks considering different parasitic structures.

Prevalence (P) considering helminhs only, helminths and protozoans, and including unidentified nematode larvaes are shown. Total number of tested samples (n), number of samples that tested positive (N) and confidence intervals (CI) are figured. P and CI are expressed as percentages (%). PNALM = Abruzzo, Lazio e Molise National Park, PNM = Mercantour National Park, YNP = Yellowstone National Park.

	PNALM (n=88)			PNM (n=68)			YNP (n=186)			TOTAL (n=342)		
Prevalence	**N**	**P**	**CI**	**N**	**P**	**CI**	**N**	**P**	**CI**	**N**	**P**	**CI**
Helminths only	77	87.5	78.3-93.3	19	27.9	18.1-40.3	136	73.1	66.0-79.2	232	67.8	62.6-72.7
Helminths and protozoans	78	88.6	79.7-94.1	20	29.4	19.3-41.9	152	81.7	75.2-86.8	250	73.1	68.0-77.7
Helminths, protozoans and unidentified larvae	80	90.9	82.4-95.7	38	55.9	43.4-67.7	152	81.7	75.2-86.8	270	78.9	74.2-83.1

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Environmental and intrinsic correlates of persistent stress in free-ranging wolf packs

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Introduction

"Stress" is a state in which homeostasis of the organism is lost, as a consequence of a real or perceived threat to the physical or psychological integrity of an individual. The cause of such loss in equilibrium is named a "stressor", and may be physical (environmental or physiological) or psychological (Reeder and Kramer 2005; Touma and Palme 2005).

When confronted with a stressor, an individual responds with numerous physiological and behavioral adaptations to cope with the situation and reestablish homeostasis (Reeder and Kramer 2005; Touma and Palme 2005; Romero 2004; Herman and Cullinan 1997). The collection of these adaptations allows the organism to give an adequate response to a stressor, and is named "stress-response" (Sapolsky 2004). Energy reserves are allocated to specific pathways that help the organism to cope with the stressor. Processes unnecessary in the short term, such as digestion, reproductive functions, growth or tissue repair are reduced or suppressed (Sapolsky 2004).

The stress-response can be subdivided into an initial alarm phase, a resistance or adaptation phase and an exhaustion phase (Sapolsky 2004). The resistance phase helps the organism to adapt and recover from the stressor. The exhaustion stage only takes place when the organism is exposed to a sustained stressor.

Glucocorticoids (GCs) are the steroid hormones involved in the resistance and in the exhaustion phases. In carnivores, cortisol is the main secreted glucocorticoid (Touma and Palme 2005; Millspaugh and Washburn 2004).

Adaptive in the short term, GCs can have deleterious effects on the organism when levels remain elevated, as in the case of exposure to a sustained stressor (Touma and Palme 2005; Sapolsky 1992, 2004; Selye 1973). Sustained stress can decrease the efficiency or even trigger the collapsus of the immune system, thereby favoring pathogenic infections (Reeder and Kramer 2005; Sapolsky 2004; Selye 1973).

The stress response has been studied in a variety of mammals but is still an emerging field of research in free-ranging individuals (Reeder and Kramer 2005). Recently developed technics allowing the measurement of glucocorticoid metabolites (GCMs) in feces (Möstl et al. 2005; Palme et al. 2005; Touma and Palme 2005) markedly enhanced the array of possible investigations of stress in free-ranging animals (Touma and Palme 2005). To date, these non-invasive techniques remain the only possible way

to collect data in numerous species (Reeder and Kramer 2005), and allow large scale and repeated investigations that do not affect the behavior or physiology of the studied individuals.

The wolf is a highly social and territorial species. Individuals typically live in family-based packs, consisting of a mated pair and their offspring of usually one or two generations; composition of unstable groups might however differ (Mech and Boitani 2003; Packard 2003). Such a pack composition is referred to as a "nuclear family" (Packard 2003). In this study, we consider two or more individuals travelling together as a pack. Wolves are essentially monogamous and mates usually pair for years, or even for a lifetime (Vonholdt et al. 2008; Packard 2003). Breeding season takes place from late January through April, depending on the latitude (Mech 1970). Females have a single annual estrous cycle (Seal et al. 1987) and sperm production is cyclic in males (testosterone is, with a peak from December to March), reaching a maximum during the breeding season (Kreeger 2003). In a population, estrous might last at least a month (Kreeger 2003); it mainly takes place in February in YNP (pers. obs.) and possibly late February and early March in PNALM (Ciucci pers. com.). In a stable pack, dominance-subordinate relationships are typically age-graded, and the parents are thus naturally at the top of the hierarchy (Packard 2003). The breeding dominant individuals in a wolf pack are also referred to as the "alpha" animals, as various authors reported two dominance hierarchies in wolf packs, one for each sex (Mech 1999; Zimen 1975; Schenkel 1947). The dominance hierarchy is often considered linear, although more complex relationships between pack members have been reported (Packard 2003; Zimen 1975). The highest ranking female and male are the only breeding individuals in a stable pack. Multiple litters within a pack may however occur, particularly when one or both original breeders are lost, or in newly founded packs (Smith et al. 2008, 2009, 2010; Packard 2003).

Environmental stressors include anthropogenic disturbances (Van Meter et al. 2009; Pereira et al. 2006; Creel et al. 2002), extreme temperatures (Reeder and Kramer 2005; Touma and Palme 2005) density of individuals (Mech 1970), and infection by pathogens (Sapolsky 2004).

In social species, agonistic or affiliative interactions with group members can respectively induce increase or decrease of stress in individuals (DeVries et al. 2003; Sachser et al. 1998). Sudden changes in the social environment, such as the loss of an attachment figure (mother, or mate in pair-bonding species) (Mason and Mendoza 1998; Hennessy 1997) or disruption of a relationship with a socially close member of the group (Aureli and Smucny 2000) are also reported social stressors. Low stress levels seem to be correlated with the stability of the social environment in various species (Sachser et al. 1998; Sapolsky 1992) and probably also apply to wolves (McLeod et al. 1996).

A correlation between social status and GC levels has been reported in various species of social mammals, and report higher GC levels in dominants, in subordinates, or

no difference related to social status (Sands and Creel 2004; Creel 2001; McLeod et al. 1996; Sapolsky 1992). In some species, an association between GC levels and sex, age, reproductive status or body condition has been shown (Reeder and Kramer 2005; Millspaugh and Washburn 2004; Weingrill et al. 2004). Further individual differences in stress response have been reported, possibly attributable to the temperament or personality of the animals (Anestis 2005; Reeder and Kramer 2005; McEwen 2002; Virgin and Sapolsky 1997; Sapolsky 1993, 2004).

In wolves, no seasonal or circadian variation in cortisol level was reported in captive individuals (McLeod et al. 1996; Seal et al. 1987). Wolves are well adapted to snow conditions and they are believed to suffer less from an important snow cover than do their ungulate prey species (Smith and Ferguson 2005). Anthropogenic disturbance was reported as possibly increasing stress in free-ranging wolves (Creel et al. 2002). Encounters with neighboring packs or dispersing conspecifics, or notification of their presence (scent marking, howling) can be important stressors for wolves, and stress level in a pack might therefore be dependent on wolf density on the regional scale (Mech 1970). In wolves, elevated stress level can thus be a consequence of interactions with neighboring packs or of conflicts with pack mates (Harrington and Asa 2003; Packard 2003; McLeod et al. 1996; Derix et al. 1993; Zimen 1975; Mech 1970).

The link between social status and stress level remains unclear in wolves, as different studies lead to contradictory results (Creel 2005; Sands and Creel 2004; McLeod et al. 1996). Higher levels of cortisol were reported in dominant than in subordinate individuals in a study of free-ranging animals (Sands and Creel 2004), while no such correlation was observed in a captive pack (McLeod et al. 1996). Gender differences in basal levels of fecal CMs were demonstrated in dogs (*Canis lupus familiaris*) (Schatz and Palme 2001) but not in wolves (Sands and Creel 2004; McLeod et al. 1996).

In the present study, we evaluate the relationship of specific environmental factors in stress levels measured in eleven packs of free-ranging wolves. In order to limit bias induced by persecution by humans, we selected wolf packs belonging to protected wolf populations. None of the investigated packs was subject to legal persecution or harvesting at the time of sample collection. We compare stress levels at the park scale and consider park size (linked with human exploitations in buffer zones), wolf density and the presence of free-ranging dogs as putative factors affecting stress levels in free-ranging wolves. We predict higher stress level in high-density wolf populations living in sympatry with free-ranging dogs. We further investigate the possible relationship of viral or parasitic infections to stress level, and expect a positive correlation between these variables. We finally evaluated monthly variations of the stress level in a pack in two consecutive winters.

We also expect differences in stress levels between packs, and investigate possible correlation between stress and pack stability. Attribution of part of the collected samples to specific individuals allowed us to consider age, gender and social status of

individuals as factors that may affect stress level in wolves. We expect an increase in stress levels during the annual breeding season. No difference between males and females or between alpha and subordinate individuals is predicted.

Material and methods

Study areas

The investigated packs are settled in three different national parks: Abruzzo, Lazio e Molise National Park (PNALM), in Italy, Mercantour National Park (PNM), in France, and Yellowstone National Park (YNP), in the United-States, all of them being government managed protected areas. The three study sites share a mountainous backcountry, constituted by partly forested and partly open areas, and are located at similar latitudes. Table 1 summarizes some characteristics of the three National Parks. In YNP, investigations took place on the northern winter range (Smith et al. 2003), the only area of the park accessible year round by car.

Table 1. Characteristics of the three national parks

Study area	**Creation year** [a]	**Location and coordinates** [a]	**Mountain range**	**Size** (km^2) [a]	**Wolf presence / return** [b]	**Wolf density** (ind./1000km2)
PNALM (included buffer zone)	1923	Central Italy (41°76' N; 13°84' E)	Apennines	1'297 (800)	Always present	57 [c]
PNM (included buffer zone)	1979	Southeastern France (44°18' N; 7°05' E)	Alps	2'835 (2'150)	Since 1992	11 [c]
YNP (included northern range)	1872	Northwestern USA (44°60' N; 110°55' O)	Rocky Mountains	8'890 (1'530)	Since 1995	50 [d]

[a] Source : World database on protected areas, http://www.wdpa.org/
[b] Berger and Smith 2005; Boitani 2003; Smith et al. 2003; Ciucci and Boitani 1998b; Bangs and Fritts 1996; Houard and Lequette 1993.
[c] Wolf density was calculated as the mean number of wolves per pack, divided by the mean size of packs' territory (PNALM: packs' territory: 100-120 km^2; Grottoli 2011; PNM: packs' territory: 260-350km^2, www.oncfs.gouv.fr; C. Duchamp pers. com.)
[d] Information for the northern range of YNP (Berger and Smith 2005; Smith et al. 2003).

At the time of sample collection, the species was considered as having reached carrying capacity in all three investigated areas. Different wild ungulates species are present in all three study sites, and in PNM and PNALM livestock is also present year-round (Table 2). Main prey species of wolves vary given the abundance and accessibility of the ungulates species present in their home range.

Table 2. Different ecological characteristics related to the investigated packs (source: Grottoli 2011; Ciucci and Boitani 2009; Smith et al. 2008; Espuno et al. 2004; Smith et al. 2003; Poulle et al. 1998; Houard and Lequette 1993).

Study area	Other canids[a]	Wild ungulates	Livestock	Main prey species of wolves in wintertime[c]
PNALM	Foxes, dogs	Chamois[b], roe deer, red deer and wild boar	Sheep, horses, cattle, few goats	Mainly wild boar; roe deer, red deer, domestic ungulates
PNM	Foxes, dogs	Chamois, European mouflon, roe deer, red deer, wild boar, ibex	Sheep, few goats and cattle	Mainly chamois and roe deer; some red deer, ibex, European mouflon, wild boar and few domestic ungulates (sheep and goats)
YNP	Foxes, coyotes, dogs	Red deer, bison, mule deer, white-tailed deer, moose, pronghorn antelope, big horn sheep, mountain goat	None	Mainly red deer; few bison, deer and moose

[a] Red fox (*Vulpes vulpes*), dog (*Canis lupus familiaris*), coyotes (*Canis latrans*). In PNM, dogs present on the studied packs' territory are working dogs (Shepard dogs and livestock-guarding dogs). In PNALM, dogs belonging to local owners, working dogs as well as feral dogs are all present. Dogs travelling with tourists are theoretically prohibited in PNALM, allowed in the buffer zone, but excluded from the core area of PNM (Duchamp pers. com.), and restricted to a range of 100 yards off roads and parking lots in YNP.

[b] Chamois (*Rupicapra rupicapra*), roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), wild boar (*Sus scrofa*), european mouflon (Ovis orientalis), alpine ibex (*Capra ibex*), bison (*Bison bison*), mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), moose (*Alces alces*), pronghorn antelope (*Antilocapra americana*), big horn sheep (*Ovis canadensis*), and mountain goat (*Oreamnos americanus*).

[c] Fecal samples collected in PNALM and PNM were submitted to dietary analyses, performed by Ciucci Paolo and collaborators in Italy (Ciucci and Boitani 2009) and Duchamp Christophe and collaborators in France (Réseau Loup/Lynx 2004, 2006c; Plan d'action national sur le loup 2008-2012). In YNP, main prey species are assessed through close monitoring of wolves (Smith et al. 2008, 2009, 2010).

Tourism is important in all three national parks. In YNP, cars and people are mainly concentrated on park roads, and much fewer visitors venture on trails. In PNM and PNALM, pastoralism is important and persists year round, while it is inexistent in YNP. Mainly sheep occur in PNM (Duchamp pers. com.), while cows and horses are also numerous in PNALM (Grottoli pers. com.). No changes in human activities happened in the three national parks during the winters concerned by sample collection.

Investigated packs

In PNM and PNALM, snow-tracking sessions in wintertime, as well as wolf-howling sessions performed in summertime (Grottoli 2011; Ciucci and Boitani 2009; Réseau Loup/Lynx 2005, 2006a, 2006b, 2007) provided information on the number of animals present in each pack for each winter, and on the success of reproduction in these packs. In this study, at least two individuals travelling together are considered as forming a pack. When fieldwork was conducted, in both PNM and PNALM, 7 packs were known to have part or the entire of their home range within the boundaries of the parks' buffer zone (Réseau Loup/Lynx 2005, 2006a, 2006b, 2007; Grottoli pers. com.). In each of these National Parks, 4 packs have been retained for the present study, based on the quality and quantity of collected fecal samples (Table 3). In YNP, 8 wolf packs' home range was entirely or partly included in YNP's northern range (Smith et al. 2008, 2009)

at the time of sample collection. Samples from 3 different packs have been collected, based on pack's visibility and accessibility of sample collection sites (Table 3).

Table 3. Characteristics of investigated packs.

Park and winter of data collection	Packs and dispersing individuals	Number of individuals/pack [a]
PNM		
2005-2006	Moyenne Tinée	2-3
	Haute Tinée	3-4
	Vésubie-Tinée	3-5
	Vésubie-Roya	3-4
	Dispersed/died [c]	5
	Total	**16-21**
2006-2007	Moyenne Tinée	2
	Haute Tinée	2-3
	Vésubie-Tinée	3-5
	Vésubie-Roya	4
	Dispersed/died [c]	6
	Total	**17-20**
PNALM		
2006-2007	Iorio	6
	Orsara	3
	Villavalelonga	7
	Mainarde	9
	Total	**25**
2007-2008	Iorio	4
	Orsara	6
	Villavalelonga	6
	Mainarde	5
	Total	**21**
YNP		
2007-2008	Slough Creek	14
	Druid Peak	16
	Blacktail Deer Plateau	(not constituted yet)
	Total	**30**
2008-2009	Slough Creek	(disappeared)
	Druid Peak	16
	Blacktail Deer Plateau	6-9
	Total	**22-25**

NI: no information available.

[a] In Europe, pack-size estimates are based on: in PNALM, snow-tracking sessions (Ciucci and Boitani 2009; Grottoli 2011), in PNM, snow-tracking sessions and genetic analyses performed on fecal samples (Réseau Loup/Lynx 2006a, 2007). In YNP pack size is known from direct observations; variations are due to dispersal and death.

[b] Presence of pups during the summer preceding sample collection. PNALM (Grottoli 2011; Ciucci and Boitani 2009) and PNM (Réseau Loup/Lynx 2005, 2006b). In YNP, the presence of pups is known from direct observations.

[c] One sample only per individual, as revealed by genetic analyses (Duchamp pers. com.); each winter, a sample from an unidentified individual that was present in the area but could not be assigned to a pack is also included in this category.

Collection of fecal samples

Fecal samples from wolves collected in two consecutive winters in each investigated national park were considered in our study (Table 3). In PNM and in PNALM, analyzed samples were collected by scientists and local co-workers and rangers (Grottoli 2011;

Ciucci and Boitani 2009; Duchamp and Marboutin 2007; Marboutin and Duchamp 2005) from the 1st of October to the 31st of March. In YNP, sample collection took place from the 1st of December to the 31st of March.

In PNALM and PNM, snow-tracking session allowing sample collection mainly took place as soon as possible after snowfalls. In YNP, the collected samples remained at or below freezing point from defecation to collection time, as attested by daily direct observation. In all three national parks, only well preserved samples were retained for analyses. Typically, scats partly consumed by birds, dried out, exposed to rain or temperatures obviously above freezing point were not retained for analysis. Samples composed mostly by hair (estimated as > 90% of the scat volume) did not contain sufficient fecal material allowing further analysis and were excluded. As well, scats over-marked with urine or less than 50 cm away from another scat were excluded from analysis.

Aiming for the detection of fecal cortisol metabolites (CMs) rapid freezing and storage at -20°C of the feces is advised (Palme 2005; Khan et al. 2002). In all three national parks, collection in wintertime was chosen to best match these recommendations, accessing samples that remained at the lowest possible temperature, in order to preserve hormonal metabolites of interest from degradation. On the day of collection, samples were transported at freezing temperature (0°C) in thermic bags, and stored at -20°C in labeled plastic bags. From then on, the cold chain was maintained at any time, during transportation or shipment of the samples, until analysis was performed.

Characterization of fecal samples

Genetic analyses performed on the samples from PNM were used to discriminate wolf scats from scats of other species (Miquel et al. 2006; Valière et al. 2003). Furthermore, these analyses allowed for the determination of the sex, and sometimes the identity of the contributing animals (Duchamp and Marboutin 2007). In PNALM, in wintertime, most samples were collected while snow-tracking the studied packs and thus directly identifying the contributing pack (Ciucci 1994). In lack of snow, samples were collected at known scent posts and at exploited carcasses (Grottoli pers. com.; Harrington and Asa 2003; Vilà et al. 1994). Additional criteria were used to conservatively discriminate wolf scats from those of other species (Ciucci and Boitani 1998b): a diameter ≥ 2,5-3,0 cm and an estimated volume ≥100 cm^3.

In YNP, fecal samples were collected following direct observation and filming of contributing individuals, and allowed the attribution of part of the collected samples to specific individuals. When an individual was observed defecating, the animal and the surrounding landscape were filmed meticulously (Canon XL-H1 camcorder, Canon EF Adapter XL, Canon EF 100-400mm f/4.5-5.6L IS USM photo lens, Canon Extender EF 2x II). Later that day, the recorded sequence was visualized on a computer and relevant pictures of the screen were taken using a digital still camera. The next day, or as soon as possible, fecal samples were collected using the following procedure. One person set a

spotting scope at the exact place from where the camera filmed the defecating individual. Using walkie-talkies, and with the help of the pictures taken with the still camera, that person guided a collaborator in the field to the sample. During these field trips, previously localized scats, as well as any scats found on wolf tracks were collected, if no tracks of other canids were found less than 1 meter away from the fecal sample. This collection procedure provided information on the sex, age and identity of part of the contributing individuals. Sample collection was abandoned when wolves not belonging to the studied pack were known to have used the area between production and collection time.

Measurement of fecal cortisol metabolites (CMs)

Adherent snow was cautiously removed from all fecal samples before proceeding. As soon as thawed enough, each sample was vigorously hand-mixed for 1 min through its plastic bag container, in order to avoid bias induced by the uneven distribution of CMs (Palme 2005; Touma and Palme 2005). A portion (0.50 ± 0.01 g) of each homogenized sample was suspended in 5 ml of methanol (80%) in order to extract cortisol metabolites (Palme 2005; Touma and Palme 2005; Schatz and Palme 2001). After thorough shacking for 30 minutes, followed by centrifugation at 2'500 g for 15 minutes, the supernatant was removed and stored at -20°C until further analysis. An aliquot was then processed by a cortisol enzyme immunoassay (EIA). Details of the EIA including cross-reactivity of the antibody are given elsewhere (Palme and Möstl 1997). Each sample was run in duplicate and results expressed as ng per g wet feces. When difference between the two measures exceeded 10%, the samples were reanalyzed.

The wolf and the dog are subspecies of *Canis lupus* (Wilson and Reeder 2005) and few genetic differences exist between them (Vilà et al. 1997). The cortisol EIA was successfully validated for dogs (Schatz and Palme 2001; Palme et al. 2001). However, in order to further guarantee the suitability of the EIA, a biological validation was performed on 4 captive wolves (3 females and 1 male) housed at the "Popoli Wolf Sanctuary", Italy. As these animals were not habituated to be handled by humans, capture (on day 0) for veterinarian control purposes was used as a biological stressor (Palme 2005; Touma and Palme 2005). From day -3 through day +5, fecal samples of the 4 individuals were collected within minutes after defecation and immediately stored in labeled plastic bags at -20°C until analysis. The EIA detected an increase in CMs concentrations in connection with the capture event followed by a return to normal, and thus confirmed that the cortisol EIA was also suited for the analysis of wolf scats.

Parasitological and virological data

A subset of the analyzed samples in which CMs levels were measured was also submitted to parasitological and virological analyses (see chapters 1 and 2 of this manuscript). Results of these investigations are used to examine the possible relationship between CMs levels and parasitic or viral infection.

Definition of pack stability

In YNP, detailed knowledge of pack's behavioral dynamics allowed us to investigate pack stability as a factor influencing stress in free-ranging wolves. In 2007-2008, the Druid Peak pack (n=71) was considered "stable", as constituted like a nuclear family (Packard 2003) and roaming on its long established territory. In contrast with this, the Druid Peak pack multiplied extensive extra-territorial movements in 2008-2009, and was thus considered unstable. To avoid confounding pack factor, data from the Druid Peak pack in the second winter were not included in analyses associated with pack stability. The Slough Creek pack was also labeled "unstable", as it experienced the death of two successive alpha males in late 2006 and in fall 2007, just before the winter of data collection (Smith et al. 2008). A new yearling male replaced the killed alpha male and recently joined the pack at the onset of our study. A third pack, the Blacktail Deer Plateau pack, was founded just prior to data collection, in November 2008, by six male dispersers from the Druid Peak pack and 4 female dispersers from a neighboring pack (the Agate Creek pack) (Smith et al. 2009). It subsequently lost 3 of its members during the winter: two juvenile males dispersed to other packs while a young adult female was probably killed in a confrontation with the Druid Peak pack. A fourth individual briefly left the pack from February to April (Smith et al. 2010). Considering this, the Slough Creek and the Blacktail Deer Plateau packs were considered as unstable at the time of data collection.

Statistical analyses

Considered as a whole, data from the three national parks did not follow a normal distribution (Kolmogorov-Smirnov test: $p<0.000$). Transformation of data did not allow normalization, except for data from PNM considered separately (Kolmogorov-Smirnov tests). In order to apply homogenous statistical analyses to all data sets, we used non-parametric statistics (Kruskal-Wallis (K-W), Mann-Whitney U (M-W) and Spearman's correlation), which allowed the inclusion of all available data in the statistical analyses. Furthermore, these statistical tests have the advantage of smoothing extreme values and allow testing of low sample sizes as well. Statistical analysis was performed with SPSS 20.0 (IBM® SPSS® Statistics). To adequately match our choice in statistical tools, median of CMs values and boxplots are used in the illustrations of our results. To facilitate the readability of figures, extreme outliers are not figured.

Results

In total, 461 samples from PNALM (n=165), PNM (n=132) and YNP (n=164) were analyzed. Measured cortisol metabolite (CMs) levels ranged from 1.1 to 397.72 ng/g of feces. These values were in the range of levels from individuals tested in our biological validation experiment (7.1-448.8 ng/g of feces).

In 95% of the samples collected in the three national parks measured CMs levels were below 52ng/g. This value fits maximum levels measured before capture in individuals used in our validation procedure. Thus, in this study, CMs measures above 52ng/g are labeled "extreme" values from here on.

Extreme levels of CMs (n=23) were particularly frequent in PNALM (n=19), while such values were measured in only two samples from PNM and two samples from YNP. Extreme values have been found in all packs and all years of the study in PNALM.

Environmental correlates of stress

Considering geographical scales: parks and packs

Environmental factors affecting stress level in free ranging wolf populations may act at various geographical scales. Our data show important differences in CMs levels between the three national parks (Fig. 1; K-W, n=461, p<0.000; M-W, p<0.005).

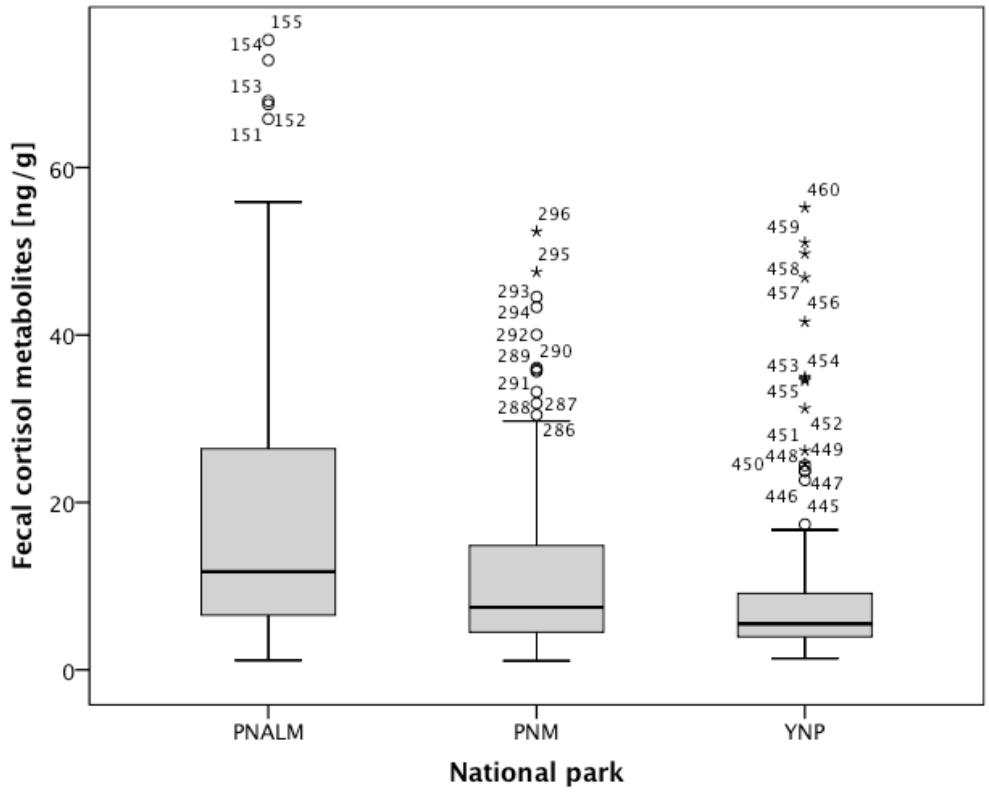

Figure 1. Fecal cortisol metabolite levels measured from wolf fecal samples collected in the three national parks. Block: lower and upper quartiles (25-75% of the data); box whiskers: 9th and 91st percentiles; solid horizontal line in block: median; circle: suspected outlier; star: outlier. (PNALM: Abruzzo, Lazio e Molise National Park; PNM: Mercantour National Park; YNP: Yellowstone National Park).

Comparison of CMs levels in the eleven packs studied confirms clustering of elevated values in PNALM, while differences between PNM and YNP appear more pack-dependent (Fig. 2). Statistical analyses of data (M-W; appendix 2) confirmed these tendencies.

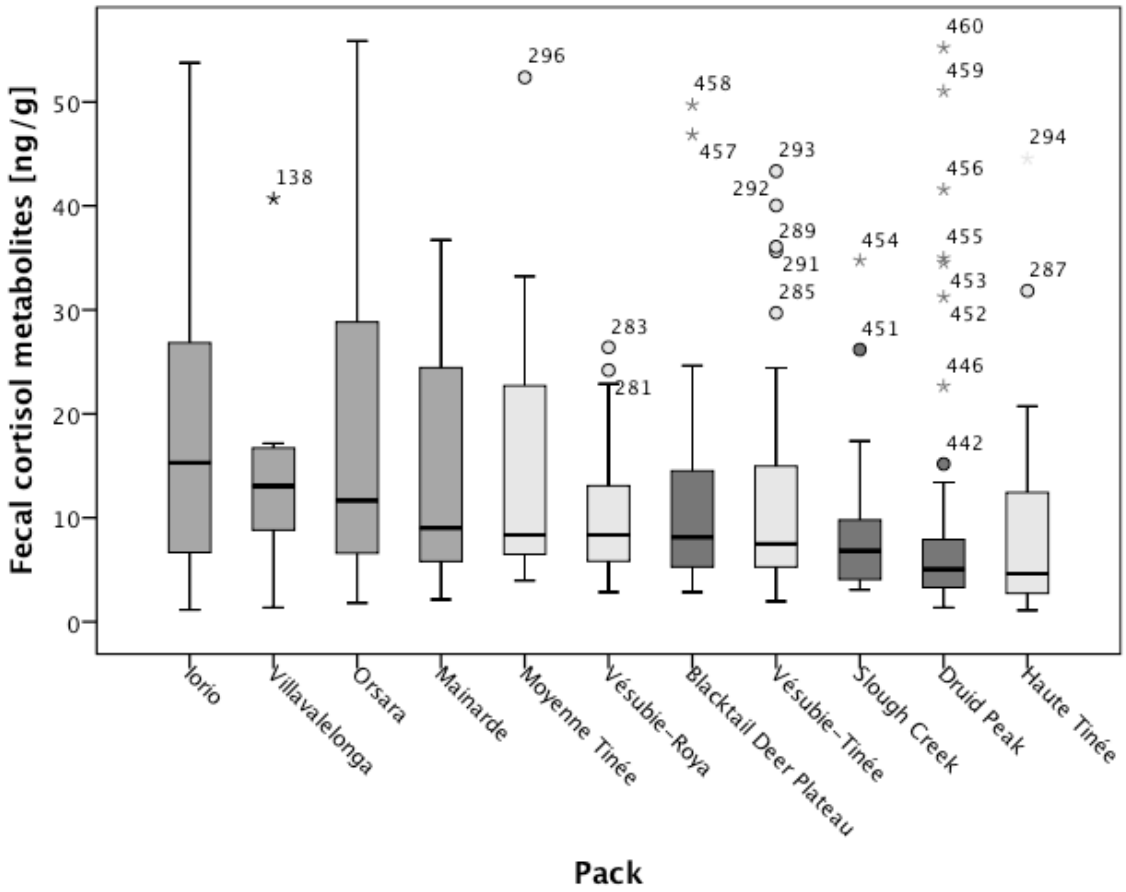

Figure 2. Fecal cortisol metabolite levels measured in the eleven investigated packs. Block: lower and upper quartiles (25-75% of the data); box whiskers: 9th and 91st percentiles; solid horizontal line in block: median; circle: suspected outlier; star: outlier. Parks: light grey: Mercantour National Park; medium grey: Abruzzo, Lazio e Molise National Park; dark grey: Yellowstone National Park.

Parasitic infection

For the subset of samples for which parasitological data were available (PNALM: n=79; PNM: n=66; YNP: n=164), we investigated the relationship of CMs levels to the number of parasite taxa detected in the same fecal samples (cf. Chapter 2 of this manuscript). In PNALM, CMs levels were positively correlated to the number of parasite taxa (rho Spearman: 0.266, n=79, p=0.018; Fig. 3). Similar results and an even stronger correlation were obtained when extreme values were discarded (rho Spearman: 0.314, n=67, p=0.010). No association was found between parasitic infection and CMs levels in PNM and YNP.

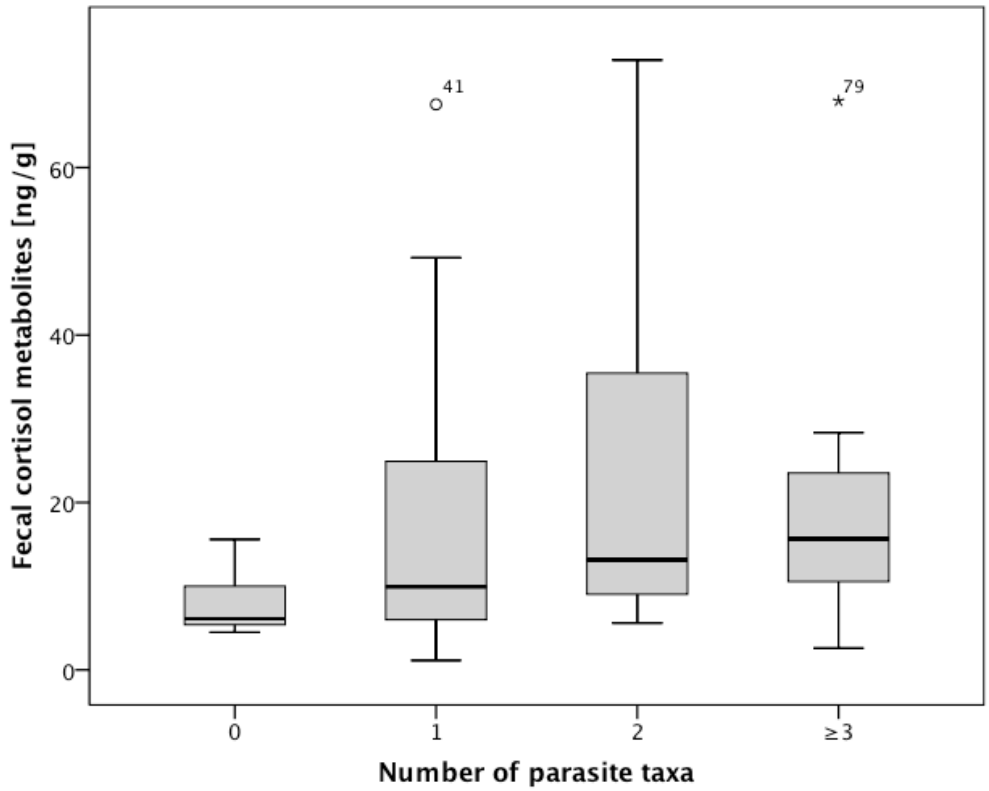

Figure 3. Fecal cortisol metabolite in samples containing 0, 1, 2 or ≥3 parasite taxa. Block: lower and upper quartiles (25-75% of the data); box whiskers: 9th and 91st percentiles; solid horizontal line in block: median; circle: suspected outlier; star: outlier.

Viral infection

Analysis of a subset of data from PNALM (n=79) and PNM (n=66) for which virological data were available (cf. chapter 1 of this manuscript) revealed no relationship between viral infection and CMs levels (M-W and Spearman correlation, $p>0.05$).

Yearly variations

We found no significant difference between CMs levels in two consecutive winters in PNALM (M-W, n=165, p=0.965) and in PNM (M-W, n=132, p=0.642). In YNP, important differences between the two winters considered (M-W, n=164, p=0.005) are best interpreted at the pack level, as different packs have been considered in the two winters of sample collection (Table 3).

To further investigate differences in CMs levels among packs in each national park, we compared data from two consecutive winters. We included data from dispersers considered as a group in PNM. We did not find differences in CMs levels between the groups in any of the two consecutive winters (K-W, $p>0.05$). Within the second winter, the Moyenne Tinée pack was discarded from the analysis, given the too small sample size (n=3).

In PNALM, no statistical difference in CMs level was detected between packs (K-W,

n=165, p=0.648). However, considering each year separately, CMs levels in the Orsara pack were statistically higher than in other packs in 2006-2007 (M-W, p<0.05) while no difference was found between other packs (M-W, p>0.05) (Fig. 4). This result correlates with the 6 extreme CMs values recorded in this pack in 2006-2007. Only 1-3 extreme values were measured each winter in other packs, and in the Orsara pack in 2007-2008.

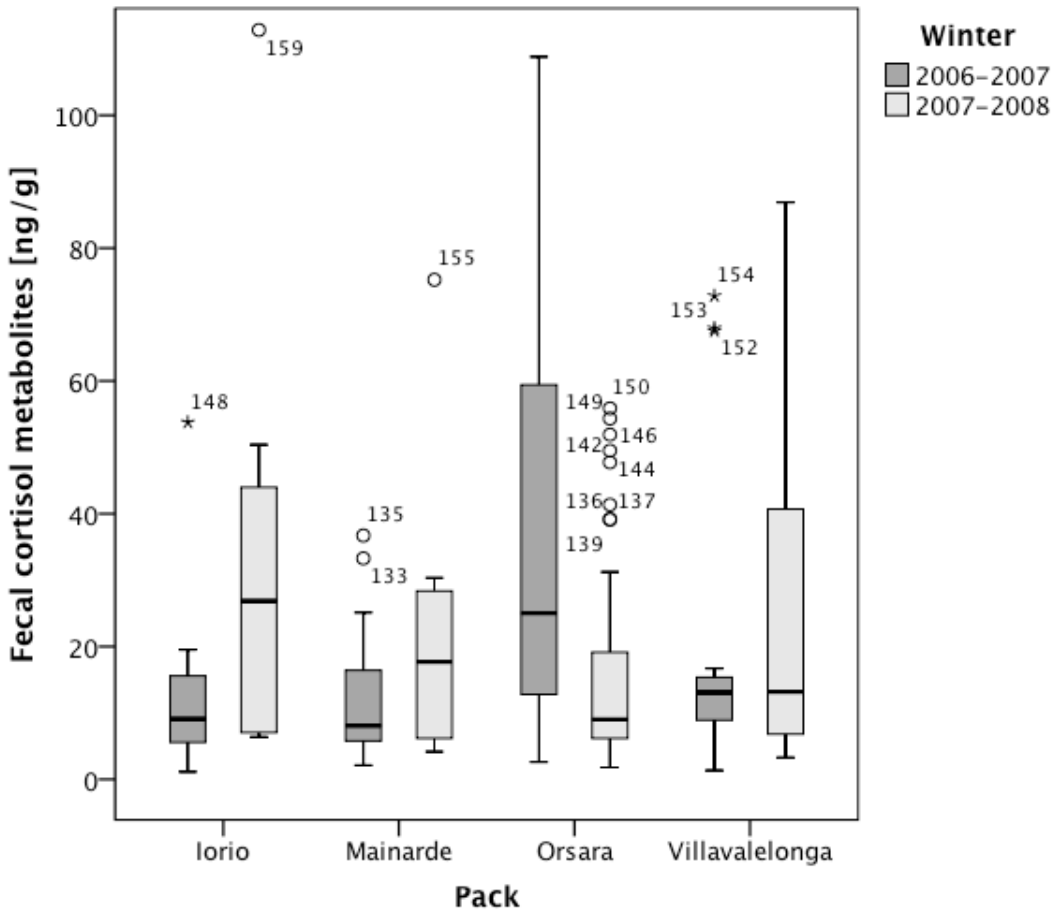

Figure 4. Fecal cortisol metabolites measured in packs from Abruzzo, Lazio e Molise National Park in winter 2006-2007 and 2007-2008. Block: lower and upper quartiles (25-75% of the data); box whiskers: 9th and 91st percentiles; solid horizontal line in block: median; circle: suspected outlier; star: outlier.

In the second winter of study in PNALM, no statistical difference was found among packs (K-W, n=86, p=0.222).

Intrinsic correlates of stress

Monthly variation within a pack

We tested for monthly variations in CMs levels as measured in wolf fecal samples.

In the Druid Peak pack, sufficient data were available to allow the comparison of monthly variations in CMs levels during two consecutive winters, with however no data from December 2008-2009. We found no significant differences in CMs values between

months in 2007-2008 (K-W, n=71, p=0.383). The next winter, however, data analyses revealed important differences between January, February and March (K-W, n=34; p<0.000), and all months differed from each other (Fig. 5; M-W, p<0.05).

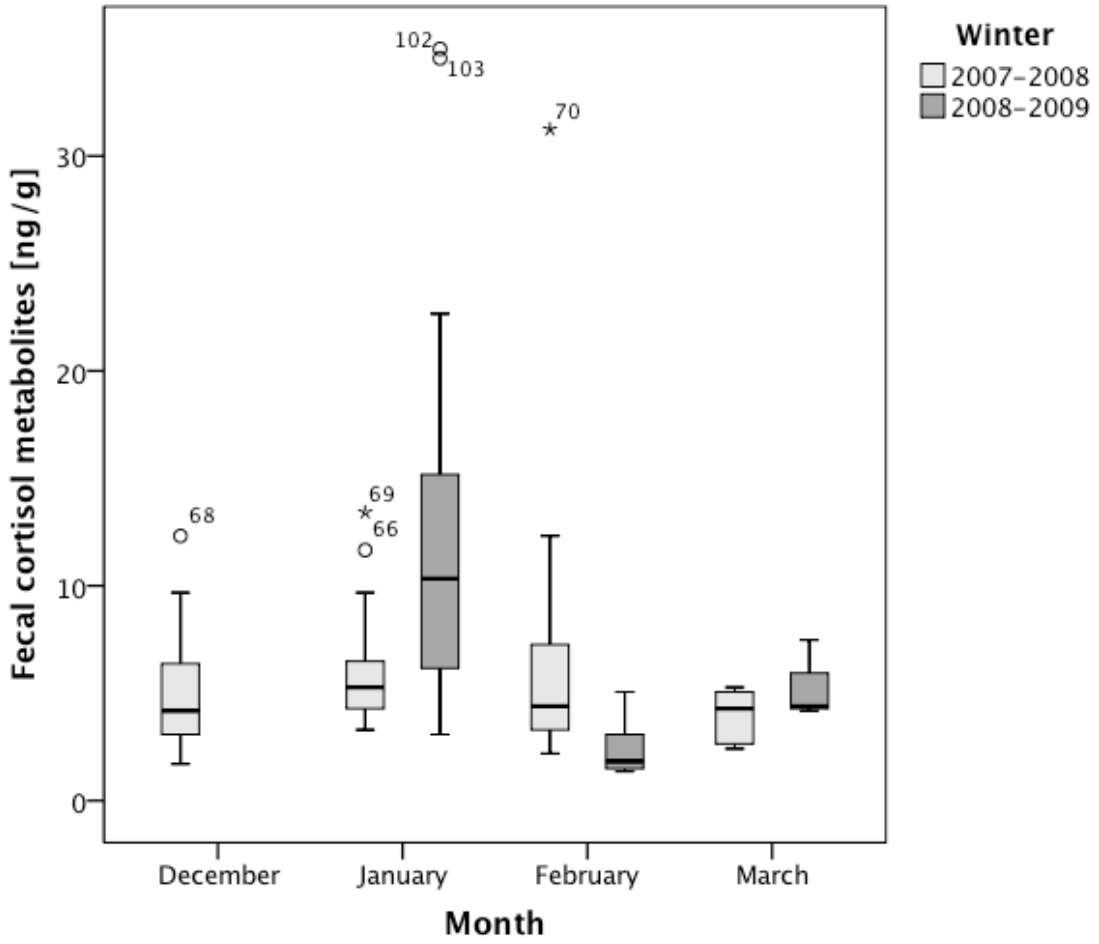

Figure 5. Fecal cortisol metabolites measured each month in the Druid Peal pack during two winters in Yellowstone National Park. Block: lower and upper quartiles (25-75% of the data); box whiskers: 9th and 91st percentiles; solid horizontal line in block: median; circle: suspected outlier; star: outlier.

Very high CMs levels are found in January, while comparatively low values characterize samples collected in February. Levels of March 2009 are comparable to monthly levels measured in the winter 2007-2008. In the winter 2008-2009, all values of CMs above 8 ng/g in the Druid Peak pack were measured in samples from January (n=15). These samples were from individuals of all three age classes (pup, yearling and adult) and from alpha as well as subordinate individuals.

In Europe, too many missing values prevented us to compare monthly CMs values in individual packs. CMs levels from PNALM did not show statistically significant difference between various months (K-W, n=165, p=0.599). In PNM, CMs values were low in December. The other months did not significantly differ in CMs levels (K-W, n=121,

p=0.411). In contrast, in YNP, CMs values importantly differ between months (K-W, n=164, p<0.000).

Pack stability

In YNP, a close year round monitoring of packs by local co-workers allowed us to consider pack stability as a factor in our investigations about stress in free-ranging wolves. Analyzed data are from the Druid Peak (n=71) and the Slough Creek (n=28) packs in the first winter (2007-2008) and from the Blacktail Deer Plateau pack (n=31) in the second winter (2008-2009).

Our results reveal significant differences in CMs levels between the three packs (K-W, n=130, p<0.000). Samples from the Druid Peak pack showed lower CMs levels than those from the Slough Creek pack (M-W, n=99, p=0.011) or the Blacktail Deer Plateau pack (M-W, n=102, p<0.000), while CMs levels measured in the Slough Creek and Blacktail Deer Plateau packs did not differ from each other (M-W, n=59, p=0.199).

When investigating monthly CMs level variations in each of the three packs (Fig. 6.), significant differences were only observed in the Blacktail Deer Plateau pack (K-W, n=31, p=0.003), in which CMs levels were lower in February than in December or January (M-W, p<0.05). No sample was available for this pack in March.

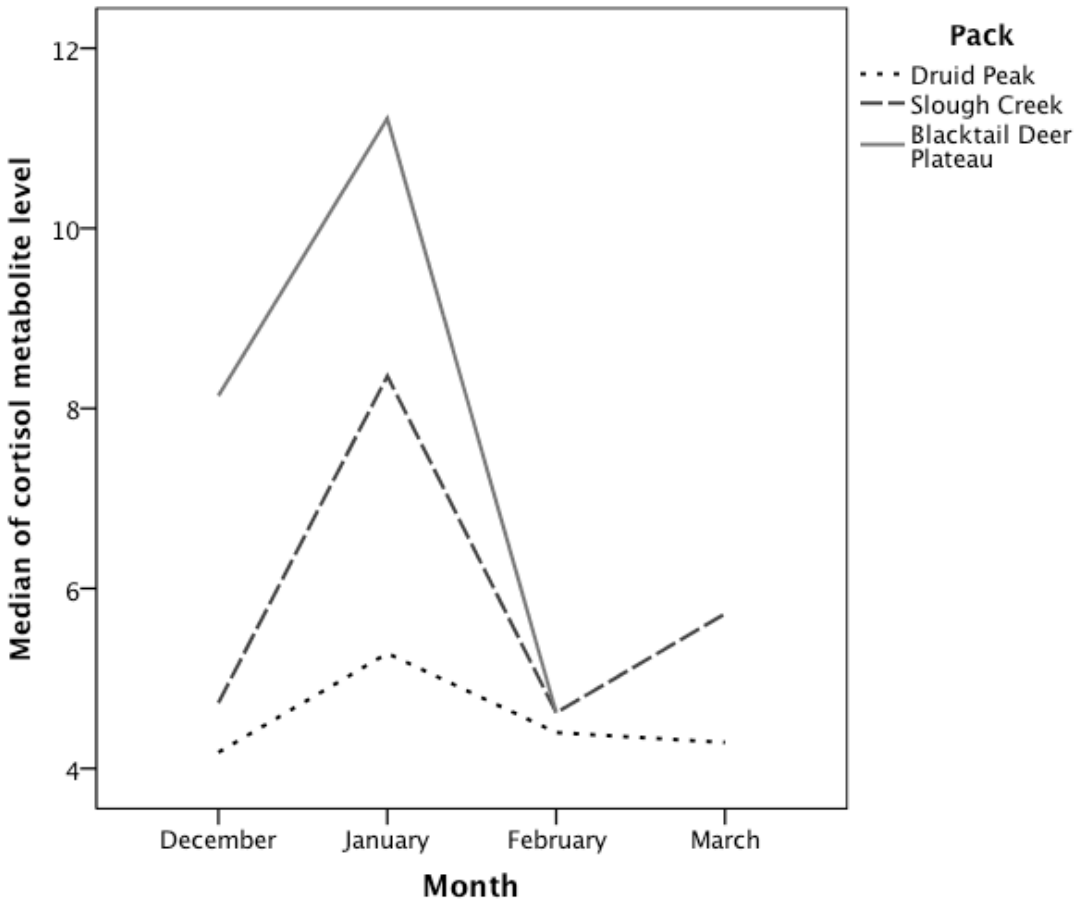

Figure 6. Monthly variations of fecal cortisol metabolites in a stable pack (Druid Peak 2007-2008) and two unstable packs (Slough Creek 2007-2008 and Blacktail Deer Plateau 2008-2009).

The three compared packs did not differ in CMs levels in February (K-W, n=130, p=0.950) and March (M-W, n=9, p=0.262). In January, CMs levels were significantly lower in the Druid Peak pack compared to the two other packs (M-W, $p<0.05$), which did not differ between each other (M-W, n=59, p=0.452). In December, CMs levels only differed between the Druid Peak and the Blacktail Deer Plateau packs (M-W, n=102, p=0.004).

A member of the Blacktail Deer Plateau pack was probably killed late in November 2008 in an encounter with the Druid Peak pack. Such an encounter could well explain very high CMs values measured in the Blacktail Deer Plateau pack in December 2008.

Gender

With respect to genders, we found no difference in CMs levels in subset of samples from PNM (n=99) and YNP (n=58) for which this information was available (M-W, $p>0.05$). We obtained the same results when considering separately each winter in PNM and YNP (M-W, $p>0.05$). Similarly, we found no differences between males and females in CMs levels when considering each pack separately (M-W, $p>0.05$).

Age

In a subset of samples from YNP (from the Druid Peak and the Blacktail Deer Plateau packs), for which the age of contributing individuals was known, we compared CMs levels between fecal samples from pups (n=18), yearling (n=19) and adults (n=20) and found no difference between these age groups (K-W, n=57, p=0.288).

Social status

We did not find a difference in CMs levels between samples from alpha individuals (n=11) and samples from subordinates (n=46) (Fig. 7; M-W, n=57, p=0.649). The same results were obtained separately in each of the two packs, in which such data was available (Druid Peak and Blacktail Deer Plateau packs).

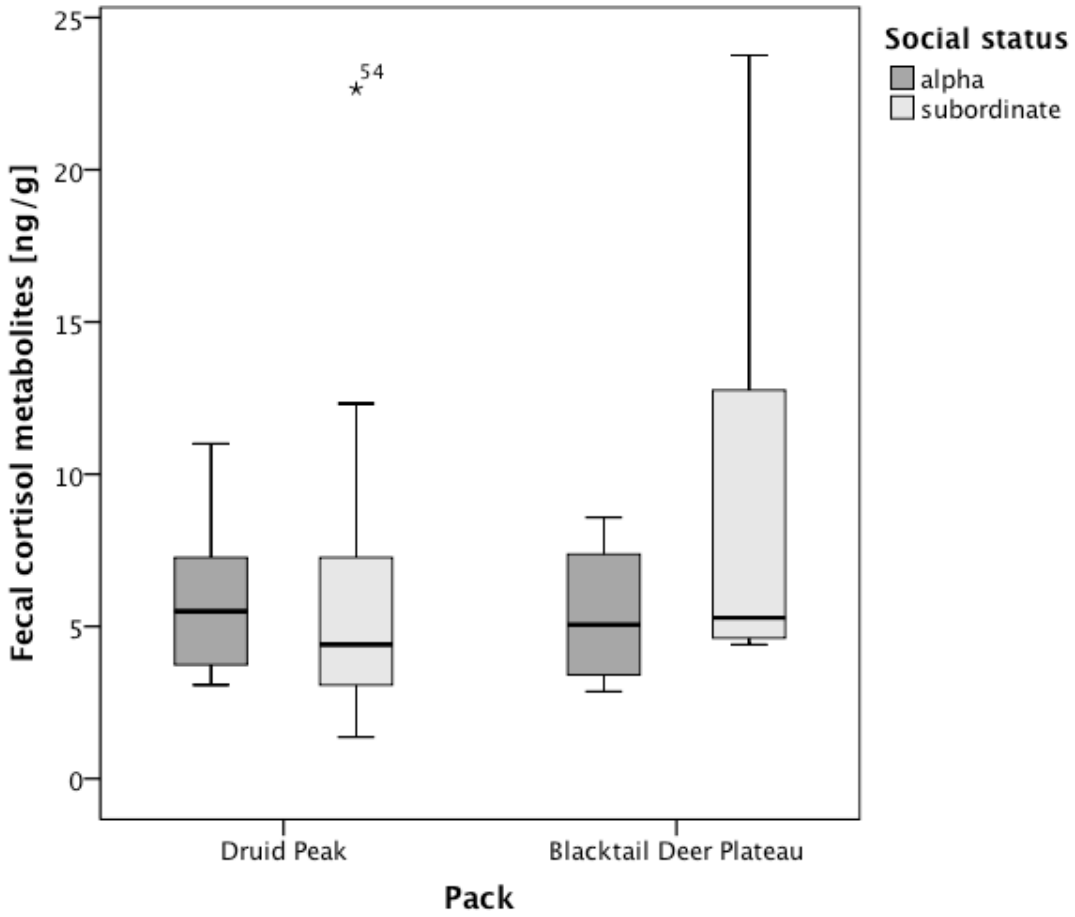

Figure 7. Fecal cortisol metabolite in alpha and subordinate individuals in two packs from Yellowstone National Park. Block: lower and upper quartiles (25-75% of the data); box whiskers: 9th and 91st percentiles; solid horizontal line in block: median; circle: suspected outlier; star: outlier.

Four samples were collected from an individual that left his second rank position in the Druid Peak pack (n=2, samples from December and February) to become the alpha male of the Blacktail Deer Plateau pack (n=2, samples from February). In this individual, mean CMs levels were lower when he was in an alpha position (5.06 ± 1.1) than as a second ranking male (10.67 ± 1.65).

Low sample sizes, partly due to the emigration of some individuals, prevented comparison of CMs levels in samples from the lowest ranking individuals with hormonal levels of other pack members.

Discussion

Environmental correlates of stress

Different geographical scales: parks and packs

We selected the three wolf populations of PNALM, PNM and YNP with the aim of minimizing anthropogenic and climatic differences that could biase data analyses. These study areas offer similar habitat for wolves in a mountainous backcountry. All three areas are exposed to comparable seasonal climatic variations and located at similar latitudes. We limited our study to colder months and standardized sample processing and analyses. Further multivariate modeling of the data are planned that may enhance presentation of our results.

We measured differences in stress level at different geographical scales: between the three national parks and, in each area, between packs. Important differences reported between the three national parks mainly highlight elevated CMs levels in PNALM (Fig. 1), and suggest that the wolf population of the Italian park is under sustained stress. Globally elevated levels of CMs measured in PNALM suggest that a stressor is acting on wolves in this national park at a large geographical scale (Fig. 2). The findings of extreme values of CMs in all packs in both winters studied in PNALM support this hypothesis. Elevated density can be an important stressor for free-ranging wolves (Mech 1970) and thus be a factor causing elevated CMs level at the regional scale in the Italian national park. However, wolf density being similar in PNALM and in YNP (Table 1), additional stressor(s) must be acting on the wolf population of the Italian national park. We suggest that the free-ranging dog population of PNALM (Table 2) represent a supplementary stressor for the wolf packs established in this area. As free-ranging dogs might prey on the same species as wolves in Central Italy (Ciucci and Boitani 1998a), dogs can be direct competitive carnivores of wolves in PNALM, and have a stressful impact related to the defense of packs' territory and associated resources.

The size of the national parks can be an additional determinant of stress levels in the studied wolf packs. As compared to YNP, the two European national parks are small, and part of the territory of most packs lies outside the core portion of the parks (Grottoli 2011; Duchamp pers. com.). These packs are therefore in contact with supplementary human exploitation in these areas (i.e. logging) that may enhance stress in individuals. Small park size and important wolf density in PNALM, as compared to PNM, can directly affect dispersal possibilities for individuals, which might in turn increase social stress within established packs.

Human activities also markedly differ among the three investigated areas. Tourism is important in all three national parks, and over two times more intense in PNALM (1 million visitors/year) than in PNM (800'000 visitors/year) and in YNP (3.3 million visitors in 2009), when reported to the dimensions of the parks (Table 1). This

important anthropogenic disturbances linked to touristic activities can be an additional stressor for the wolf population of PNALM. However, in all three national parks, touristic activities are fare more important in summer, thus having little influence on our measures of CMs levels in fecal samples collected in winter. Besides tourism, pastoralist activities are important and persist year round in both European national parks, while they are inexistent in YNP. Pastoralism and increasing human density, but not tourism, has been reported as a stressor in wild spotted hyenas (*Crocuta crocuta*; Van Meter et al. 2009). In our study, the statistically significant differences between CMs levels measured in PNALM and PNM suggest that pastoral activities have little impact on stress levels in the investigated wolf populations.

Parasitic and viral infection

Our results show that the wolf population of PNALM experienced sustained stress at the time of sample collection, unlike populations of PNM and YNP, and that elevated CMs levels measured in packs in the Italian national park are associated with important parasite richness and wide distribution of viral infection.

Immunosuppressive effects of cortisol can trigger a higher susceptibility of the organism to infections (Sapolsky 2004). Our results obtained with respect to PNALM indicate a positive correlation between parasite[1] richness (the number of parasite taxa identified in each fecal sample) and CMs levels (Fig. 3). In PNM and YNP, we did not find a relationship between parasitic infection and CMs levels. Lower parasite richness in these two national parks as compared to PNALM (see chapter 2 of this manuscript) can be the reason for this result. Alternatively, such a correlation might be a consequence of elevated CMs levels measured in the wolf population in the Italian national park, as infection by pathogens is a physical stressors for the organism (Reeder and Kramer 2005; Sapolsky 2004).

Taken together, our results suggest that infection by additional parasite species can be expected in individuals showing high CMs levels. Parasitic infection may cause increased stress level, and sustained stress decreases the ability of an organism to fight infection, thus favoring infection by additional parasites otherwise eliminated by the immune system.

Predisposing factors to infection by canine parvovirus type 2 (CPV-2), other than the age of the animal, include different parasitic infections and stress (Kreeger 2003). Stress is also reported as a risk factor increasing the virulence of canine coronaviruses (CCoV) infection (Pratelli 2008). Despite of this, we did not find a correlation between stress level and detected CPV-2 and CCoV infections (cf. chapter 1 of this manuscript). Individuals infected by either CPV-2 or CCoV commonly only shed viral particles for a few days in feces, which can be the reason why we did not find a correlation between viral infection and CMs level. However, we report infection by both these viruses in wolves in PNALM and PNM (cf. chapter 1 of this manuscript), and CPV-2 was recently detected in all sampled individuals from YNP (Almberg et al. 2009). Our results also

[1] For the purpose of this discussion, parasite refers to helminths and protozoans.

show that wolves are infected by various parasites in all three national parks (cf. chapter 2 of this manuscript). Stress is a risk factor, which can facilitate infection (Murray et al. 1999) and may also increase virulence of viral infections otherwise optimally handled by the immune system (Pence and Custer 1981). Stress-induced reduction of resistance to diseases might therefore have a broad impact on population dynamics (Millspaugh and Washburn 2004).

Yearly variations

Data collection during two consecutive winters in each national park allowed the assessment of yearly variations in CMs levels. In PNM, we did not observe statistically significant differences in stress levels between packs in any of the two winters considered. In PNALM, however, significantly elevated CMs levels were detected in the Orsara pack in 2006-2007, while no other difference was found between packs (Fig. 4). The small size of the Orsara pack that winter (Table 3) as compared to neighboring packs can be a factor explaining these results. The territory of the Orsara pack is located between the Canneto-Fondillo, the Iorio and the Terraegna packs, numbering respectively 3, 6 and 5 individuals in the winter 2006-2007 (Grottoli 2011). The next winter, the number of individuals in these neighboring packs was of 4, 4 and 6 respectively, thus much more balanced as compared to the 6 wolves composing the Orsara pack that winter (Grottoli 2011). Some territory overlaps likely exist between each of these packs and the Orsara pack. As these territories are relatively small (100-120 km^2, Grottoli 2011), stressful encounters may happen regularly, which usually are at the net disadvantage of a small pack.

Comparable dissimilarities in pack size exist in PNM, but packs' territories are much larger (260-350 km2; Duchamp pers. com.). Furthermore, the territory of the studied packs more or less fit the elongated geographical shape of the protected core area of the park (Duchamp pers. com.), and only few overlaps exist between them. As compared to the situation of the Orsara pack in 2006-2007, the territories of the two smaller packs investigated in PNM (Table 3) are mostly or entirely separated from that of other wolf packs (Duchamp pers. com.).

Pack size is an important factor determining the capacity of a pack to defend its territory and related resources against close neighboring groups. Thus, at a regional scale, we suggest that CMs level in each wolf pack is partly correlated with the number of individuals in neighboring groups, and the risk of between-pack encounters.

Intrinsic correlates of stress

Considering subsets of all available data, we investigated the link between CMs level and factors intrinsic to the studied packs and individuals. Attribution of part of the collected samples to specific individuals allowed us to consider gender, age, and social status as potential factors acting on stress levels. Further information on the stability of some packs was used to compare CMs levels in the light of this factor.

Monthly variations within a pack

In the Druid Peak pack sufficient number of data were available for almost each month during two consecutive winters to allow the comparison of monthly variations in CMs levels in two winters. Our results show striking differences between 2007-2008 and 2008-2009 (Fig. 5). While we did not observe monthly differences in CMs levels in the first winter, we found important variations in the second. In 2008-2009, all CMs values above 8 ng/g were measured in January (n=15), from pups, yearling and adults, and alpha as well as subordinate individuals. These results suggest that the entire pack was submitted to an acute momentary stressor. In the first winter (2007-2008), the Druid Peak pack was relatively sedentary, and spent most of its time in the core of its territory. In 2008, important changes in wolf packs' composition and territories occurred on the northern range of YNP (Smith et al. 2009). In November, 5 yearling and an adult male dispersed from the Druid Peak pack. In the winter 2008-2009, the Druid Peak pack undertook extensive extra-territorial movements, and was sometimes away from the core of its usual territory for extended periods of time. Extra-territorial movements were reported as stressful in a previous study on a social carnivore, the meerkat (*Suricata suricatta*) (Young and Monfort 2009). Stressful travels in an unknown countryside can furthermore be increased by aggressive encounters with neighboring groups. Considering late December 2008 and the month of January 2009, the Druid Peak pack experienced such encounter at least once, but possibly more often. This could explain the very high CMs levels measured in samples from that pack in January. Other possible stressors include anthropic causes, as the pack also travelled beyond the national park's boundaries.

Pack stability

We compared the Druid Peak pack in 2007-2008, labeled "stable", with the Slough Creek and the Blacktail Deer Plateau packs, both labeled "unstable". Our results show globally low CMs levels in the stable pack as compared to the unstable groups. Previous studies report a correlation between low stress levels and the stability of the social environment (Sachser et al. 1998; Sapolsky 1992). Stability of a pack greatly depends on the stability of the alpha pair (Brainerd et al. 2008). Loss of one or both of the alpha individuals in a pack creates important perturbations in the group, as highlighted by the elevated rate of dissolution of such packs (Brainerd et al. 2008). In agreement with these studies, we propose that the loss of an alpha individual in the Slough Creek pack was an important stressor for the members of the pack. Social instability also applies to the newly founded Blacktail Deer Plateau pack, and our results suggest that establishment of relationships with new pack members may be as stressful as exciting.

When comparing CMs levels in each month in the three considered packs, our results show two main trends: monthly variations within packs, and differences

between stable and unstable packs. Yet, we found only few statistically significant differences.

Regarding monthly variation within each pack, we report low CMs levels in February, as compared to December or January, in one of the unstable packs (the Blacktail Deer Plateau pack). No other significant monthly variation was found. However, graphic representation of our results indicates a peak in CMs levels in January followed by a downward trend in February in all three packs (Fig. 6). Elevated stress in January can be linked with proestrus in females, which lasts 15-60 days, and is characterized by increased attractiveness to males but refusal to allow mounting (Kreeger 2003). Conflict upon available future mating partners may arise during this period, especially in packs in which the alpha pair recently formed, as in both unstable packs. In accordance with this, we measured much lower stress levels in January in the stable pack than in the unstable packs (Fig. 6). In YNP, proestrus approximately ends early in February, and is characterized by a peak of the hormone estradiol in females. Thereafter, during estrus, which lasts 1-2 weeks, estradiol declines but progesterone levels rise in females. In males, testosterone levels are at their highest levels in winter, and correlate with sperm production, which peaks during the breeding season (Kreeger 2003). Estradiol, progesterone and testosterone interact in various ways with the hypothalamic-pituitary-adrenal (HPA) axis responsible for the release of glucocorticoids, and these interactions can at least partly explain the decrease in CMs levels in February (Viau and Meaney 1996; Kudielka and Kirschbaum 2005).

Taken together, our results suggest that proestrus in females triggers stress in packs in which the alpha pair is new, conceivably because different possible mating partners are available to new alpha individuals. An even higher stressful impact can therefore be expected when both leaders are new, as shown by our results for the Blacktail Deer Plateau pack founded just before the onset of sample collection. In the Slough Creek pack, in which we measured intermediate CMs levels, only one of the leading individuals recently joined the pack when we started sample collection. In a pack structured like a nuclear family (Packard 2003), as is the case of the Druid Peak pack, breeding status within the pack likely does not elicit much questioning, in accordance with incest avoidance commonly recognized in wolves (Packard 2003; Smith et al. 1997).

In Europe, our results showing no global increase in CMs levels in the breeding season may thus correlate with the stability of the breeding pair in the studied packs. In PNM, this is in accordance with genetic analyses proving that a couple of identified male and female, which are likely to be the alpha pair, remained permanently in each pack during the two consecutive winters considered (Duchamp, pers. com.).

Gender and age

In agreement with previous studies (Sands and Creel 2004; McLeod et al. 1996), we did not find an association between gender and CMs levels in wolves. Similarly, we did not observe an effect of age on the measured CMs levels. Our results show that individuals from the three defined age categories, namely pups, yearlings and adults, are

all subject to similar stress levels. It is however reasonable to consider that the nature of some stressors is different in these different age categories.

Social status

Our results show that no difference in stress level exists between alpha and subordinate individuals (Fig. 7). The only wolf that changed status from subordinate to alpha showed even a decrease in CMs level in his new leading position, even though this assessment is based on a too low sample size to allow statistical testing. Our results contradict previous work on free-ranging wolves that showed an elevated stress level in identified (Sands and Creel 2004) and probable (Barja et al. 2008) alpha individuals. These diverging results can reflect the importance of diverse additional factors acting on stress levels in dominant and subordinate individuals in the different wolf packs investigated so fare. We suggest that rather than being solely determined by social status, individual variations in stress levels depend on the temperament of individuals, as well as on the strength and quality of the relationships shared with other pack members.

Methodological issues

Samples lost to birds

Pulsatile secretion of glucocorticoids in response to a stressor results in uneven distribution of the corresponding metabolites (GCMs) within a fecal sample (Palme 2005; Touma and Palme 2005; Millspaugh and Washburn 2004), and each sample needs to be homogenized prior to analyses (Palme 2005; Wasser et al. 1996). Thus, partially consumed fecal samples cannot be included in such analyses. In YNP, despite undertaking collection of samples in best possible delays following the departure of wolves from an area, an important proportion of the samples were lost to coprophagy by birds. We report a loss of over 2/3rd of the recovered fecal samples due to their partial or total consumption by corvids. This represents an important cost in sampling efforts, especially regarding the valuable samples attributed to identified individuals.

Sampling in wintertime – the only option

Glucocorticoid metabolites are widely used as a measure of stress in mammals (Touma and Palme 2005). As environmental factors can significantly modify the concentrations of fecal GCMs levels, rapid freezing of collected fecal samples is advised (Palme 2005). Thus, sample collection in wintertime was chosen as the best possible strategy to avoid bias in the measurement of CMs levels, in our study of free-ranging wolves. We carefully excluded all samples known or suspected to have been exposed to rain or excessive temperatures (Millspaugh and Washburn 2004). An enzyme immunoassay (EIA) was used, which previously showed the best results for CMs

detection in dogs (Schatz and Palme 2001). Furthermore, a conclusive validation of the assays was also performed. This approach assured the reliability of the measured CMs levels, which therefore adequately reflect stress levels in the sampled individuals and wolf packs.

Conclusion

As opposed to social interactions between the members of a pack, most of the potential stressors identified in the present study have received little if any attention to date in wolves.

In our study, we compared free-ranging packs established in three different national parks. No circadian or seasonal variation in the production of cortisol has been reported in wolves (McLeod et al. 1996; Seal et al. 1987). Careful sampling strategy and validation of the hormonal assay thus allowed the comparative analysis of CMs levels in wolf packs from PNALM, PNM and YNP.

Our study shows that CMs levels in free-ranging wolves are associated with global as well as local factors, and can vary within a pack depending on specific environmental or intrinsic factors. The elevated CMs levels measured in samples from PNALM suggest the exposure of individuals to sustained stress. We propose that wolf density and the presence of a free-ranging dog population on packs' territory can be important determinants of the stress level at a global scale for wolves in the Italian national park.

Although the stress response is adaptive in the short term and helps the organism to cope with noxious stressors, a long-term activation of the HPA axis has deleterious consequences (Sapolsky 2004). Besides the immunosuppressive effect favoring pathogenic infections, elevated cortisol level can cause neuronal cell death, muscle and bone atrophy, poor wound healing, and growth inhibition (Reeder and Kramer 2005; Sapolsky 2004; Romero and Butler 2007). Therefore, the consequences of persistent stress cannot only severely impair health and survival of individuals (Touma and Palme 2005; Sapolsky 2004), but may impact population dynamics (Millspaugh and Washburn 2004).

Our results show that CMs levels are correlated with the number of parasite species infecting wolves from PNALM. In PNM and YNP, no such correlation was found. These results suggest that elevated CMs levels are associated with parasitic infection when the number of parasite taxa is superior to two. Various parasitic and viral infections are widespread in free-ranging animals and should be further investigated as possible correlates of stress.

Our study shows that exceptional monthly variations in stress levels reflect particular behavioral or social events. Extra-territorial movements expose individuals to unusual stressful challenges. Among these, encounter with neighboring packs can be an important stressor for wolves from protected free-ranging populations. Our results suggest that stability of a group is another important factor affecting stress level in free-

ranging wolf packs, while gender, age and alpha status of individuals did not influence stress levels in the studied packs.

Relatively low anthropogenic pressure in winter importantly rises in all three national parks in summer, and may cause an increase of stress in wolf populations. To better evaluate the impact of this factor, and given that a suitable technic is used avoiding bias of measured metabolites, CMs levels should be investigated year round. Cumulative effects of sustained elevated stress levels, and parasitic and viral infections need to be monitored in free-ranging mammals as they may cause significant populations declines.

Appendix 1

P-values of Mann-Whitney U tests comparing cortisol metabolite levels between all investigated packs. (Packs: 1: Iorio; 2: Mainarde; 3: Orsara; 4: Villavalelonga; 5: Haute Tinée; 6: Moyenne Tinée; 7: Vésubie-Roya; 8: Vésubie-Tinée; 9: Druid Peak; 10: Slough Creek; 11: Blacktail Deer Plateau).

	1	**4**	**3**	**2**	**6**	**7**	**11**	**8**	**10**	**9**	**5**
1	-	0.976	0.892	0.410	0.654	0.127	0.124	0.058	0.020	>0.000	0.001
4	0.976	-	0.894	0.380	0.409	0.068	0.062	0.046	0.007	>0.000	0.001
3	0.892	0.894	-	0.216	0.509	0.042	0.027	0.009	0.001	>0.000	>0.000
2	0.410	0.380	0.216	-	0.923	0.423	0.350	0.250	0.047	>0.000	0.001
6	0.654	0.409	0.509	0.923	-	0.464	0.475	0.311	0.097	0.001	0.013
7	0.127	0.068	0.042	0.423	0.464	-	0.993	0.888	0.222	0.001	0.033
11	0.124	0.062	0.027	0.350	0.475	0.993	-	0.809	0.199	>0.000	0.011
8	0.058	0.046	0.009	0.250	0.311	0.888	0.809	-	0.284	>0.000	0.013
10	0.020	0.007	0.001	0.047	0.097	0.222	0.199	0.284	-	0.045	0.150
9	>0.000	>0.000	>0.000	>0.000	>0.001	>0.001	>0.000	>0.000	>0.045	-	0.979
5	0.001	0.001	>0.000	0.001	0.013	0.033	0.011	0.013	0.150	0.979	-

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Conflict management in free-ranging wolves (*Canis lupus*)

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Introduction

Benefits of group living are almost invariably accompanied by costs related to conflicts between group members. Conflict management strategies are expected when these costs exceed the benefits of group living, such as protection from predators, enhanced foraging efficiency, territory defense or cooperative brood care. Conflicts often concern the access to food or to mating partners, and consequent costs include exclusion from the group, injuries, enhanced stress levels and alteration of relationships (Kutsukake and Clutton-Brock 2008; Aureli et al. 2002; Aureli and Smucny 2000; de Waal 2000). Conflict management strategies help prevent escalation of aggression and repair social damages caused by conflicts (Aureli et al. 2002). Such strategies are therefore of critical importance in the social life of numerous species living in stable social units (Aureli and de Waal 2000). Conflict management have been widely studied in the past decades, especially in non-human primates including macaques (Thierry 2000; Matsumura 1996; Aureli et al. 1993; Cords 1988; de Waal and Ren 1988), chimpanzees (Kutsukake and Castles 2004; Arnold and Whiten 2001), baboons (Leone and Palagi 2010; Colmenares and Silveira 2008) and prosimians (Norscia and Palagi 2011; Palagi et al. 2008). More recently, conflict management has been investigated in some other taxa, such as hyaenids (Wahaj et al. 2001), equids (Cozzi et al. 2010), canids (Palagi and Cordoni 2009; Cools et al. 2008; Cordoni and Palagi 2008) and corvids (Fraser and Bugnyar 2010; Seed et al. 2007).

Different forms of friendly post-conflict interactions have been described. These include "reconciliation", which refers to a friendly contact between former opponents shortly after a conflict (de Waal and Vanroosmalen 1979). Conflict management may also involve third parties that did not take part in the initial agonistic encounter (Watts et al. 2000). "Consolation" is defined as an increase in unsolicited affiliative interactions initiated by a third party towards the victim of a conflict (de Waal and Vanroosmalen 1979). A "solicited consolation" refers to an affiliative contact initiated by the victim towards a third-party (Watts et al. 2000). These conflict management strategies have been shown to reduce the likelihood of renewed post conflict aggression (Norscia and Palagi 2011; Fraser and Bugnyar 2010; Leone and Palagi 2010; Palagi and Cordoni 2009; Palagi et al. 2006; Aureli and van Schaik 1991) and restore tolerance between former opponents (Aureli et al. 2002; Cords and Aureli 2000; de Waal 2000). Conflict resolution reduces the social tension between individuals (Cheney et al. 1995; Cords 1992), as

measured by a decrease of stress level in former opponents (Fraser and Bugnyar 2010; Fraser et al. 2010; Cooper et al. 2007; Aureli and van Schaik 1991).

The valuable relationship hypothesis

While several hypotheses have been proposed to explain the occurrence of post-conflict affiliative behaviours, most findings are best interpreted according to the "Valuable Relationship Hypothesis" (de Waal and Aureli 1997), which proposes that the function of post-conflict friendly reunions is to restore relationships that are particularly important to individuals. Valuable relationships are necessary to maintain (a) group cohesiveness when individuals profit from cooperative interactions within a group, and (b) distinct social relationships such as alliances, partnerships or friendships, which have a high value to individuals.

The negative consequences of disturbed social relationships are accordingly distinct. In case of severe deterioration of the relationships to group members (a), the victim can leave the group. If a dyadic relationship is disturbed (b), future cooperative interactions between the two individuals can be impaired. For instance, investments in brood care (among pair partners), in cooperative breeding tasks (among helpers and parents) or in cooperative hunting (among involved group members) could be less efficient. Furthermore, group cohesion is of importance in species defending a territory against neighboring groups, and conciliatory mechanisms are therefore expected (Schino 2000). However, as relationships rarely are symmetric in terms of importance to social partners (van Schaik and Aureli 2000), differences are expected in the motivation of each former opponent to resolve a conflict (Aureli and Smucny 2000).

Social organization in free-ranging wolf packs

Wolves typically live in family-based groups, consisting of a mated pair and their offspring of usually one or two, but up to four, generations; composition of unstable groups may however be different (Mech and Boitani 2003; Packard 2003). While pups and yearlings typically remain in their natal pack, sexually mature individuals commonly disperse. Group membership is central in this highly social species. Pack members engage in cooperative breeding, cooperative hunting and cooperative territory defense against neighboring groups (Mech and Boitani 2003; Packard 2003). Wolves are essentially monogamous and mates usually pair for years, or even for a lifetime (Vonholdt et al. 2008; Packard 2003). In a stable pack, dominance-subordinate relationships are typically age-graded, and the parents are thus naturally at the top of the hierarchy (Packard 2003). Agonistic interactions are mainly observed between individuals of the same sex, and various authors report two dominance hierarchies in wolf packs, one for each sex (Mech 1999; Zimen 1975; Schenkel 1947). The dominance hierarchy is often considered linear, although more complex organizations have been reported (Packard 2003; Zimen 1975). Mostly, the highest ranking female and male are the only breeding individuals. However, multiple litters may occur within a pack, particularly when one or both original breeders are lost, or in newly founded packs

(Smith et al. 2008, 2009, 2010; Packard 2003). Sexual maturity and limited food availability probably trigger dispersal events (Mech and Boitani 2003; Packard 2003). Helped by established dominance relationships between pack members, overt aggressive conflicts and severe aggressions are rare.

Conflict management in wolves

Conflict management is expected in species living in stable social units, sharing long lasting individualized relationships, and experiencing within-group aggression and post-conflict hostility (Aureli et al. 2002; Cords and Aureli 2000). Conciliatory mechanisms are also expected in group-living species defending a territory and related food sources, as between-group competition can be important (Schino 2000). These characteristics all apply to the ecology and social life of free-ranging wolves (Mech and Boitani 2003; Packard 2003) and conflict management strategies in wolf packs are thus foreseeable.

The importance of pack membership to wolves, as well as the typically relaxed dominance relationships between group members (Zimen 1981; Mech 1977), also favor conflict management mechanisms (Thierry 2000). Additionally, a conflict between 2 individuals may disturb social cohesiveness by inducing elevated anxiety and stress level also in other members of the pack. Post-conflict affiliative behaviors between former opponents and possible third parties likely reduce the level of general social tension, and thus benefit each member of the group (Watts et al. 2000). Friendly post-conflict interactions have recently been reported in captive wolves (Palagi and Cordoni 2009; Cordoni and Palagi 2008) and domestic dogs (*Canis lupus familiaris*; Cools et al. 2008).

Material and methods

Study site and individuals

The fieldwork took place in Yellowstone National Park, United States (44°60' N; 110°55' O) from the 1st of November 2008 to the 31st of March 2009, in agreement with the Park policy (permits YELL-2008-SCI 5716 and YELL-2009-SCI 5716).

We studied two free-ranging packs of gray wolves *(Canis lupus),* the Druid Peak pack and the Blacktail Deer Plateau pack, whose home range was located on the northern range of the park (Smith et al. 2009, 2010). The Druid Peak pack was established in 1996 (Smith and Ferguson 2005) and consisted of sixteen wolves, twelve females and four males. It was structured like a nuclear family (Packard 2003) with all members born into the pack except for the alpha male. By the end of 2008, the pack consisted of 6 pups, 2 yearlings and 8 adults. The Blacktail Deer Plateau pack was founded in November 2008 and consisted of dispersing males from the Druid Peak pack and dispersing females from the Agate Creek pack. In November 2008, the pack consisted of 10 individuals, 7 yearlings (5 males) and 3 adults (1 male). One of these individuals (a

yearling female) probably died and two other (yearling males) dispersed during the winter (Smith et al. 2009, 2010; pers. obs.).

Fieldwork

We observed and filmed individuals daily, from dawn to dusk, given suitable weather conditions and distance to the animals. Wolves were commonly active all morning, and from late afternoon into the night. Radio-collared individuals, as well as tracks and howls, helped locating the animals.

We filmed the packs using an adapted camcorder (Canon XL-H1 camcorder, Canon EF adapter XL, Canon EF 100-400 mm f/4.5-5.6L IS USM photo lens, Canon extender EF 2x II). We recorded all possible social interactions among group members. Recording was interrupted when individuals went out of sight for more than 1 minute, or when wolves were resting. In total, 106 hours of observations were collected, 49 hours for the Blacktail Deer Plateau pack and 57 hours for the Druid Peak pack. Individuals were recognized based on distinctive external feature (presence/absence of a collar, fur colour and age-related characteristics, specific physiognomies, etc.) Distance to the studied animals ranged from 100 to 1500 meters approximately. In order to optimise recording of all possible social interactions, two observers guided the cameraman using spotting scopes. Wolves in YNP were accustomed to the daily presence of distant observers and our filming did not appear to affect behavior.

Video analyses

Videotapes were loaded on a computer and analyzed using the Noldus software, the Observer 5.0. We coded every single agonistic interaction between individuals using an all-occurrences sampling method (Altmann 1974). We focused on the victim, defined as the recipient of the aggression. For each aggressive encounter, we defined a post conflict (PC) period of ten minutes (Palagi and Cordoni 2009) starting 1 second before the aggressive interactions. Within PC periods, we coded all social behaviours involving the focal individual. When the focal individual went out of sight for more than 30 seconds, the PC period was considered over.

For each PC period, a corresponding matched control (MC) period was selected. In MC periods, the focal individual (i.e. the victim in corresponding PC period) was observed for up to 10 minutes in order to obtain data about baseline behaviour (i.e. without recent conflict). MC periods were selected according to the following criteria: the focal individual was not involved in any conflict during the 10 minutes preceding the onset of an MC period (de Waal and Yoshihara 1983); the opponents of the corresponding PC period were within 10 meters of each other and thus had the opportunity to interact (Palagi et al. 2008); inactive (e.g. sleeping, resting) episodes were not used as MC periods. Each MC period was selected on the first possible day following the corresponding PC period, with a maximum interval of a week between PC and MC periods. A MC period was considered as ended if the focal individual went out of sight for more than 30 seconds.

For each interaction in PC and MC periods, we identified the initiator and the recipient of the interaction. We recorded all behaviours listed in Table 1, the starting time and the duration of each interaction.

Aggressive interactions followed by submissive behaviors were excluded from further investigations. Instead, as in other studies of conflict management, we focused on aggressive encounters followed by friendly interactions.

Table 1. Ethogram of recorded wolf behaviors in YNP from November 2008 to March 2009.

Aggressive behaviors	
growl	growl at a conspecific with bared teeth, vocalization may be heard
approach dominant	move towards another individual, stiff forelegs and raised tail, piloerection possible
approach fast	move fast towards an individual, trotting or running, may stop or jump on the conspecific
chase	run after a conspecific, piloerection, ears flattened
snap	shut jaws in the air
T-position dominant	stand as dominant in the T formation, as the horizontal cross of the T, facing the chest of a conspecific
push	push hard a conspecific with part of body to make him/her move
ride up	mount or jump on a conspecific with forelegs, in an aggressive posture (raised tail, may growl, piloerection), without pushing him down
stand over	stand over a conspecific that is lying down, with piloerection, stiff tail
nip	brief inhibited bite, barely touching the conspecific, insufficient pressure to cause injury
knockdown	hit or push a conspecific down
bite	bite a conspecific, no inhibition, enough pressure to cause potential injuries
wrestle	fight with a conspecific, violent encounter
Affiliative behaviors	
inspect*	lick and/or sniff urogenital area of a conspecific
body contact	body parts in contact for several seconds, excluding tails
play	exaggerated, brief and/or inhibited motor patterns such as jump on, bite, chase… Play-bow.
lick	lick part of a conspecific's body, excluding urogenital area
sniff	sniff part of a conspecific's body, excluding urogenital area
greet	tail wagging, face oriented towards the face of a conspecific, sometimes licking and/or sniffing and/or prolonged nose touch, ears oriented forward
greeting ceremony	rally, more than 4 wolves, howling, greeting, tail wagging
nose touch	brief nose touch from a wolf to another, may be directed to the face or body. No tail wag, ears may be flattened
Submissive behaviors	
passive sub	lay on back or side, present stomach, throat and urogenital area, tail tucked between legs
light sub	tail tucked between legs, sometimes wagging, crouched, ears back, may lick or paw the dominant's muzzle
T-position submissive	stand as subordinate in the T formation, as the vertical cross of the T, facing the shoulder of the dominant
Other behaviors	
immobile	lay down, sit or stand, look around or sleep
travel	walk, trot or run
howl	howl (head back, muzzle upward, vocalizing)
Non social behaviors	
out of sight	out of sight
other	behaviors different from those described here above

This ethogram is based on personal observations, completed by published ethograms on domestic dogs (Cools et al. 2008) and captive wolves (Cordoni and Palagi 2008; Möslinger et al. 2008).

* Even though we consider "inspect" as a sexual and not a friendly interaction, we decided to include it in data collection in order to make our results comparable with previous studies on wolves (Palagi and Cordoni 2009; Cordoni and Palagi 2008) and domestic dogs (Cools et al. 2008).

Predictions and statistics

Statistical analyses were performed with SPSS statistical software (IBM® SPSS® Statistics version 18.0.0). As the data did not follow a normal distribution, we used nonparametric tests (chi square, Wilcoxon signed-rank and Spearman rank correlation. Due to small sample size, we pooled data from both studied packs. We treated each observation (i.e. each PC and MC periods) as an independent data point, even if the focal individual was the same in some of these observations. We consider this to be a valid assumption because the interval between observations was generally at least 30 minutes (with 5 exceptions, in which only once was the focal individual the same in two consecutive PC periods).

To discriminate between two consecutive aggressive interactions amongst the same aggressor and the same victim within a given PC period, we used a bout criterion interval method (Martin and Bateson 1993). We calculated the minimal time interval between two distinct conflicts by performing a log survivorship plot. This allowed us to determine that two consecutive events can be considered as independent when separated by more than two minutes. Thus, we considered a renewed aggression as occurring at least two minutes after the previous aggressive interaction in the same PC period. This is consistent with the definition of renewed aggression used in the study on captive wolves (Palagi and Cordoni 2009).

In order to determine whether individuals preferentially avoid contact or seek friendly interactions after a conflict, we compared the number of conflicts followed by affiliative contacts to the number of conflicts followed by no contact, focusing on the victim. We considered the first agonistic encounter of each PC period (n=34), and repeated the analysis taking also renewed aggressions (n=7) into account.

If conflict management takes place, affiliative interactions should occur earlier in PC periods than in corresponding MC periods. We recorded the latency between the end of the first agonistic interaction and the onset of the first affiliative behavior in PC periods. We compared this latency with the time interval between the beginning of MC periods and the onset of the first affiliative behavior involving the focal individual. We grouped the data for each minute following the end of the agonistic interaction in PC periods, and for each minute of MC periods. According to the time-ruled method (Aureli et al. 1989), we compared the number of first affiliative contacts occurring each minute in PC and MC periods.

Conciliatory tendency (CCT) gives a quantitative indication of the occurrence of reconciliation and is therefore used to compare groups or species (Veenema 1994). We calculated the conciliatory tendency in the investigated packs including all pairs of opponents. For each pair of PC and MC periods, we assessed the latency (in minutes) to the first affiliative interaction between former opponents, as described above. If the first friendly contact occurred earlier or only in the PC period, we labelled the pair "attracted", whereas if the affiliative contact occurred earlier or only in the MC period, we labelled the pair "dispersed" (Veenema 1994). Pairs in which affiliative behaviours took place after the same amount of time in both periods, or in which no such interaction occurred, were labelled "neutral" (Veenema 1994). The conciliatory

tendency is calculated as the number of attracted minus dispersed pairs, divided by the total number of pairs (Veenema 1994) and describes the tendency of individuals to reconcile after a conflict. Values close to 50 % correspond to a high conciliatory tendency whereas values close to 0 % are considered as low (Thierry 2000).

Conflict may induce elevated stress level and jeopardize further collaboration as well as group cohesiveness. To assess whether investment in reconciliation is correlated with the intensity of a conflict (estimated as the number of aggressive behaviors displayed in an interaction), we tested whether the total number of aggressive and affiliative behaviors within each PC period were correlated. We expect a positive correlation between the number of friendly behaviors initiated after conflict and the level of conflict escalation.

In order to find out whether a specific affiliative behavior was preferentially used in reconciliatory interactions, we recorded the occurrence of each specific affiliative behavior first performed in PC and MC periods. We compared the number of occurrence of all types of affiliative behavior involving the victim, considering PC periods and MC periods separately.

To investigate the pattern of conflict management in the studied packs we examined which individual, of the former opponents or a third party, initiated the first affiliative behavior in PC periods. We analyzed separately interactions between the former opponents and interactions involving the victim and a third party. We tested the observed values against predicted values based on a 50:50 ratio, corresponding to a random situation in which interactions are initiated equally often by both partners. We predict that mainly victims invest in post conflict affiliation and that friendly behaviors are primarily directed towards the former opponent, with whom the relationship is damaged.

Results

From 106 hours of filming, we collected a total of 68 observations (i.e. 34 PC and 34 MC periods). Renewed aggression occurred in 20.5% of PC periods (n=7) and never happened twice in the same period. Redirection of aggression by the victim towards a third-party only took place in two occasions, which were not considered as new aggressive events.

Considering the first friendly interaction following all conflicts (34 initial and 7 renewed conflicts) in PC periods, reconciliation, consolation and solicited consolation occurred following 61.0% (n=25), 4.9% (n=2) and 19.5% (n=8) of the conflicts respectively. The victim was not involved in any affiliative interaction following 6 of the total number of conflicts (n=41).

Do free-ranging wolves engage in post-conflict affiliation?

Following a conflict, former opponents more often engaged in affiliative interactions (n=29) than they avoided each other (n=5) ($\chi2$=16.941, n=34, df=1, p<0.000), even when also considering the 7 renewed aggressions (contact: n=34; no contact: n=7) ($\chi2$=17.780, n=41, df=1, p<0.000). The first affiliative contact occurred significantly earlier after a conflict (PC) than in the matched control period (MC) (Wilcoxon 2-sided, n=34, T=-2.9, ties=1, p=0.004; Fig. 1).

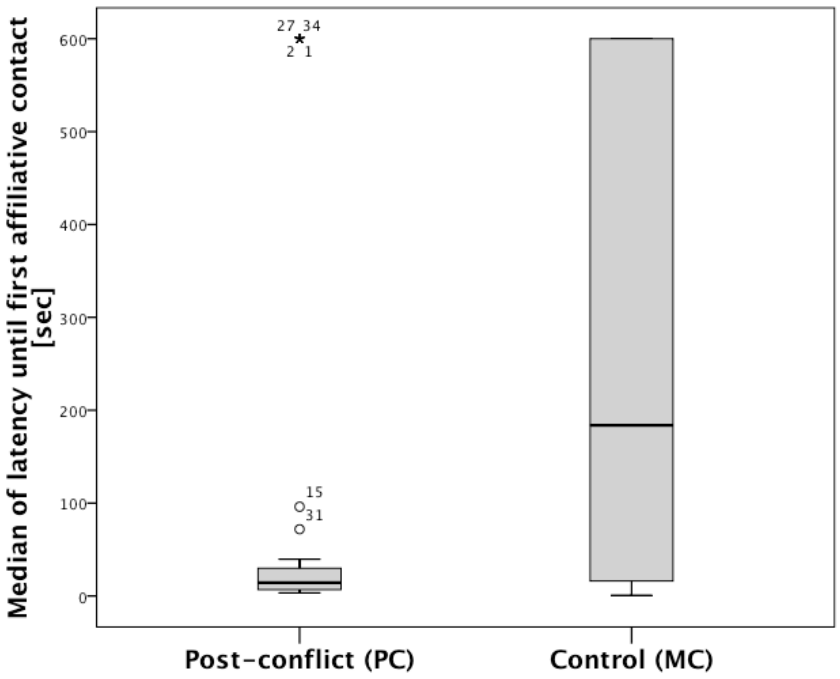

Figure 1. Latency until first affiliative contact after a conflict (PC) and in control periods (MC).

The number of first affiliative contacts performed by or towards the focal individual (victim) during the first minute was significantly higher after a conflict (PC) compared to a random situation (MC) ($\chi2$, n=42, df=1, $\chi2$=6.095 p=0.014). After that, there was no significant difference between both periods (Fig. 2).

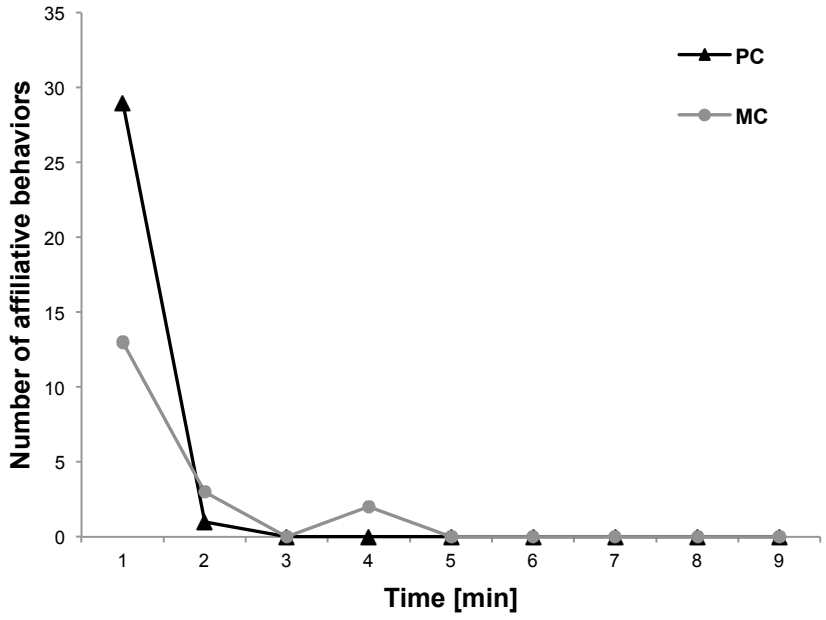

Figure 2. Temporal distribution of the first affiliative contact involving the victim after a conflict (PC) and in matched control periods (MC).

Furthermore, the total number of affiliative behaviors initiated by or towards the victim, was significantly higher in PC than in corresponding MC periods during each of the first four minutes ($\chi 2$, n=34, df=1, $p<0.05$). After that period of time, no significant difference was found between both periods.

The calculated conciliatory tendency, which describes the likelihood of a friendly interaction between former opponents after an aggressive encounter, was of 44.1%. We found a positive correlation between the number of affiliative and aggressive behaviors displayed during PC periods (Spearman's rank correlation, n=34, rs=0.379, p=0.027), indicating that more affiliative behaviours were displayed in response to repeated aggressive behaviours.

What specific affiliative behaviors are first displayed after a conflict?

We investigated the type of affiliative behavior first displayed in PC and in MC periods, between the former opponents or between the victim and a third party. The 34 initial and the 7 renewed aggressions were considered in PC periods. We found a significant difference in the occurrence of three specific behaviors: "Nose touch" occurred more often in PC periods ($\chi 2=4.995$, n=51, df=1, p=0.025), while "play" ($\chi 2=5.844$, n=51, df=1, p=0.016) and "greeting" ($\chi=17.395$, n=51, df=1, $p<0.000$) were displayed more often in MC periods (Fig. 3). The behavior "inspecting" occurred only once as first non-aggressive interaction in PC periods.

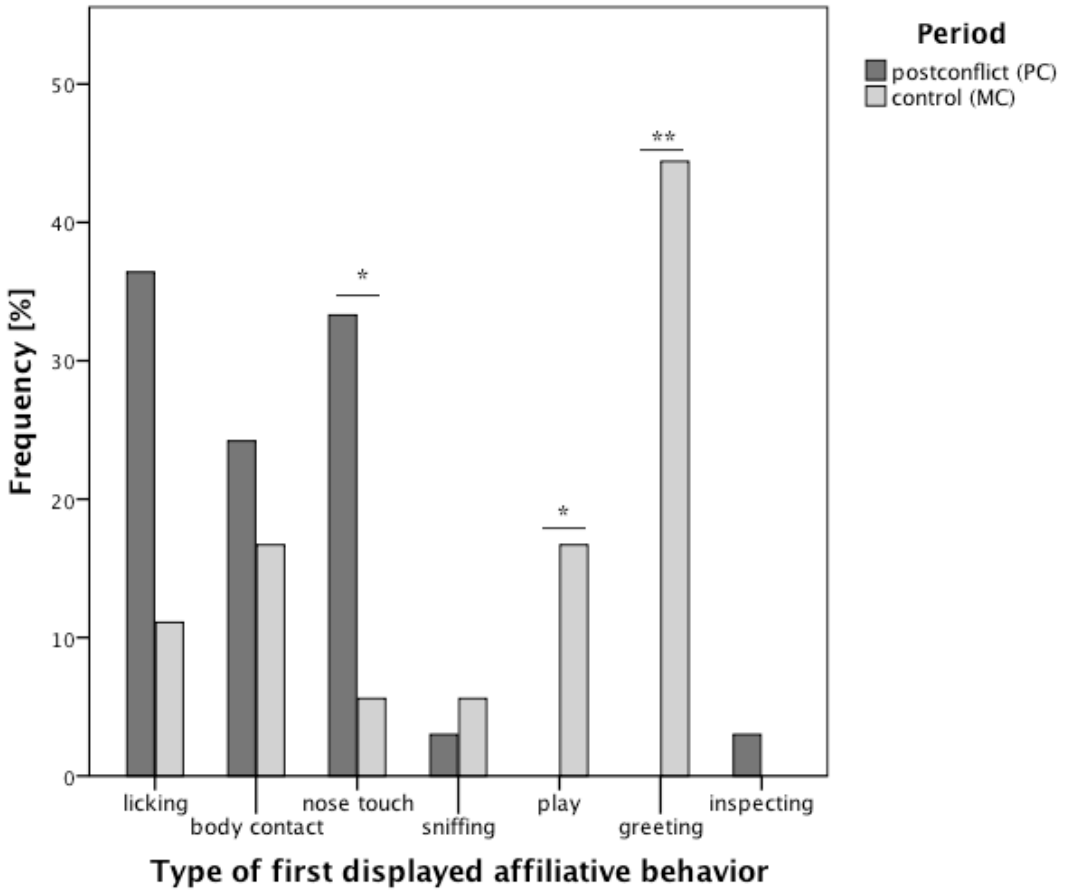

Figure 3. Occurrence of each type of affiliative behavior performed in first affiliative contact, after a conflict (PC) and in control periods (MC). Within PC and MC periods respectively, the frequency (%) of each first performed behavior is figured.

Who initiates post-conflict affiliation?

Focusing on the first affiliative interaction following a conflict, we examined who was the initiator of these interactions. We considered the 34 initial and the 7 renewed aggressions that occurred in PC periods, and treated interactions with former opponent and those with a third party separately. Regarding former opponents, victims were more likely to initiate post-conflict friendly interactions than aggressors ($\chi 2=13.542$, N=51, df=1, $p<0.000$). However, we found no statistical difference in the propensity of the victim or a third party in initiating such affiliative contact ($\chi 2=1.978$, N=20, df=1, $p=0.16$; Fig. 4).

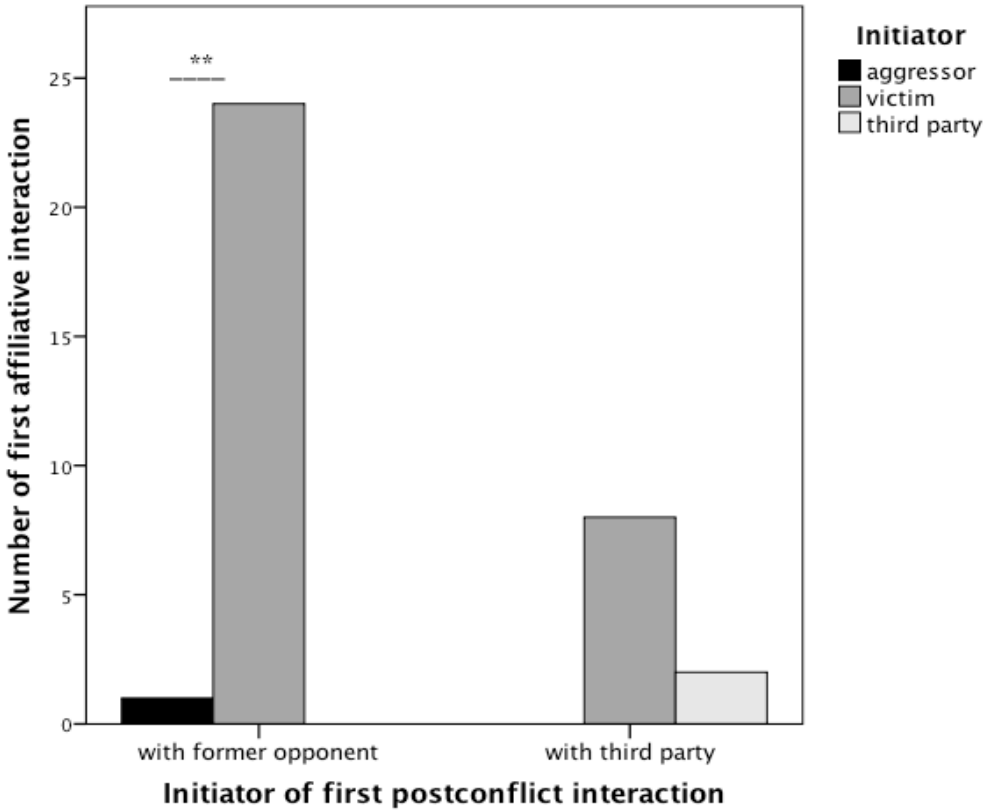

Figure 4. Initiators of first post-conflict affiliative behaviors in PC periods. Details for the interactions between former opponents and interactions between the victim and a third party.

Discussion

Our results provide the first reported evidence for post-conflict affiliation serving as conflict management in free-ranging wolves. Affiliative behaviors occurred earlier and more often after a conflict as compared to control periods. These post-conflict friendly behaviours were mainly initiated by the victim and primarily directed towards the aggressor. Our data show that reconciliation and both solicited and unsolicited consolation took place between pack members. We found a positive correlation between the number of aggressive behaviours and the number of affiliative behaviours in PC periods, and our results show that a specific behavior was mainly used in post-conflict periods. Overall, our results suggest that post-conflict affiliative behaviors are an essential component of the social life of free-ranging wolves, and highlight the importance of relationships between pack members.

What types of conflict management occur in wild wolves?

Instead of avoiding each other, former opponents engaged in post-conflict affiliative reunions, which are typical of conflict management (Aureli et al. 2002). Among these friendly interactions, we mainly observed reconciliation events. Hence, affiliative behaviours appear selectively directed towards those individuals with whom conflict

resolution is necessary to reduce social tension (de Waal and Yoshira 1983). These results are consistent with recent studies of captive wolves (Cordoni and Palagi 2008) and domestic dogs (Cools et al. 2008).

In 24.4% (n=10) of first friendly interaction following a conflict, we found evidence for third-party affiliation, in which the first friendly interaction of victims was with individuals different from the former opponent. Victims mostly initiated these interactions (n=8) and actual consolation, initiated by a third-party towards the victim (Kutsukake and Castles 2004), was rare (n=2; Fig. 5). However, we found no statistical difference regarding the propensity of the victim or a third party in initiating these interactions. Former findings in captive wolves (Palagi and Cordoni 2009) and domestic dogs (Cools et al. 2008) also reported the occurrence of solicited and unsolicited third-party affiliation between group members. Considering all consolation interactions, unsolicited consolation accounts for 17.3% of contacts. This result shows that mainly victims seek social support from other group members in free-ranging wolves.

Does conflict management relate to the social system of wolves?

Wolf sociality is characterized by an elevated degree of cooperation (Mech and Boitani 2003; Packard 2003) resulting in high value of group membership. Wolves typically live in stable social units and share long lasting individualized relationships (Vonholdt et al. 2008). Members of wolf packs establish dyadic dominance relationships, which help prevent escalated aggression as signals mostly replace aggressive encounters in potentially competitive situations (Preuschoft and van Schaik 2000). Aggressive encounters however happen, and post-conflict hostility is evidenced by the occurrence of renewed aggression. These aspects have been suggested to be important ingredients for the occurrence of conflict management (Aureli et al. 2002; Cords and Aureli 2000).

Two related hypothesis might explain post-conflict affiliation in free-ranging wolves. Conflicts often damage established relationships and may jeopardize further cooperation and associated benefits (Kutsukake and Clutton-Brock 2008; Aureli et al. 2002). Accordingly, individuals should invest in maintaining good relationships with group members to promote group cohesion (Schino 2000). The valuable relationship hypothesis (de Waal and Aureli 1997) predicts that conflict resolution mechanisms should prevail between individuals sharing relationships particularly important to each other. In cooperative breeding species, parents and older siblings are typically valuable partners to offspring. Siblings are also particularly prized play partners. Similarly, mating partners are likely to be important to each other (Aureli et al. 2002; Schaffner and Caine 2000) and probably even more so in monogamous species like wolves. A stable group as the Druid Peak pack is family-based, and strong bonds likely connect parents and offspring of different ages. In the newly founded Blacktail Deer Plateau pack, all new relationships were established with potential mating partners, and thus of high value. In social and territorial carnivores like wolves, relationships to all group members may be important when individuals need to cooperate against external threats (Aureli et al. 2003). As competition between neighboring groups may be high, group

membership is thus of importance in the defense of a territory and related resources (Schino 2000), as well as in insuring a more secure environment for individuals. Hence, the elevated conciliatory tendency revealed by our study is in accordance with the high importance of group membership to free-ranging wolves.
Alternatively, the type and degree of post conflict affiliation may be related to the dominance style prevailing in a species, ranging from despotic to tolerant (Thierry 2000). Conflict management is expected in species with a clear hierarchy but tolerant dominance relationships (Thierry 2000). Consistent with our observations in both studied packs, the dominance style in wolves has been described as a "qualified democracy" (Zimen 1981), in which pack members may protest their leader's actions (Mech 1977), so that dominance style can be considered "tolerant". Species with such a tolerant dominance style usually show less intensive aggression between group members and an elevated conciliatory tendency, consistent with important social cohesiveness (Thierry 2000). Accordingly, we found a high conciliatory tendency between individuals (CCT = 44.1%), comparable to data from a captive wolf pack (CCT = 53.3%; Cordoni and Palagi 2008) and from macaque species showing a relaxed dominance hierarchy (CCT > 40%; Thierry 2000). In contrast, in species sharing despotic dominance relationships, conciliatory tendencies are much lower (rhesus macaques (*Macaca mulatta*): 9.0% (de Waal and Ren 1988), chimpanzees (*Pan troglodytes*): 15.5% (Kutsukake and Castles 2004), brown lemur (*Eulemur fulvus*): 26.6% (Norscia and Palagi 2011).

Reconciliation is the most commonly used mechanism to restore social cohesiveness (Leone and Palagi 2010) and thus relieve the negative consequences of a conflict by inducing reassurance and appeasement in previous opponents (Aureli et al. 2002; Aureli and Smucny 2000). However, a conflict between 2 individuals can be stressful for other members of the group (Aureli et al. 2002), so that any post-conflict affiliative behaviors between former opponents and possible third parties likely reduce general social tension of group members, and consequently benefit each member of the group (Watts et al. 2000). Thus, the importance of maintaining group membership and cohesiveness may explain the occurrence of solicited and unsolicited consolation in free-ranging wolves, and the correlation we found between the level of conflict escalation (i.e. the number of aggressive behaviors) and the number of affiliative behaviors initiated in PC periods. Victims were not discouraged by repeated agonistic interactions and kept investing in reconciliatory behaviors.

Who initiated post-conflict affiliation?

Consistent with our prediction, reconciliation was mainly initiated by the victims, mostly the younger and lower-ranking individuals, towards the older and higher-ranking aggressors. Dyadic relationships are seldom symmetric in terms of importance (i. e. value) to social partners (van Schaik and Aureli 2000), and differences are thus expected in the motivation of each former opponent to resolve a conflict (Aureli and Smucny 2000). It has been proposed that variation in initiating post-conflict reunions

may also be related to the dominance style (de Waal and Luttrell 1989). As suggested by Sterck et al. (1997), in species in which tolerant dominance relationships prevail, victims should be more inclined to initiate reconciliation than aggressors, as the risk of renewed aggression is low (Sterck et al. 1997). Correspondingly, victims are the main initiator of post-conflict affiliative behaviours in species with a high level of tolerance (Palagi et al. 2008), while post-conflict reunions were mostly initiated by aggressors in species characterised by despotic dominance relationships (de Waal and Yoshihara 1983). Wolves show tolerant dominance relationships, which are based on an age-graded hierarchy (Packard 2003). Consistent with this, our results show that victims are the main initiators of post-conflict friendly behavior, and that renewed aggression is rare (in 20.5% of PCs), as reported in highly tolerant species.

In contrast to our results, Cordoni and Palagi (2008) found no difference in the propensity of former opponents to initiate post-conflict affiliation in a captive wolf pack, and report a high rate of renewed aggression. These results highlight differences in post-conflict management behavior in the same species when studied in captive and in free-ranging conditions.

A specific behavior is used for conflict management

One specific behavior ("nose touch") was particularly important for conflict management, as it occurred significantly more often after a conflict compared to the control situations. We suggest that "nose touch" could be interpreted as an "excuse". The use of a special behavior pattern in post-conflict situation is characteristic of species with frequent post-conflict interactions (Aureli et al. 2002), in agreement with the elevated conciliatory tendency that we measured in the studied packs. Similarly, specific affiliative behaviors have been described to frequently occur after conflicts, but rarely in other situations, in chimpanzees and in macaques (Thierry 2000; Aureli et al. 1993; de Waal and Ren 1988). Specific affiliative behaviors are also displayed in post-conflict interspecific interactions by cleaner fish towards client reef fishes (Bshary and M. Würth 2001).

In contrast, "playing" and "greeting" virtually never occurred in PC periods. Hence, particular behaviors seem to be suppressed shortly after conflicts.

Methodological issues

Due to our small sample size, we could not analyse the reconciliatory tendency at the individual or pack level. However, given that the interval between successive interactions among individuals was mostly larger than 30 minutes, we consider that interactions can be treated as independent data points.

Interestingly, we observed an elevated number of affiliative behaviours in the first minute of the MC situations (see Fig.2 and Fig.3). This can likely be explained by the way we selected matched controls. MC periods were recorded when the former opponents were active and within close proximity to each other. Under these circumstances, the probability was high that these individuals would interact with each other.

Conclusion

This is the first study of post conflict affiliative interactions in free-ranging wolves. As compared to other species, we report a very high conciliatory tendency and the occurrence of solicited and unsolicited consolation, only rarely reported in non-primate species. Our results suggest that a specific behavior, “nose touch”, is most often used as the first post-conflict interaction between pack members. We suggest that the important occurrence of friendly post-conflict behaviors reported in our study of free-ranging wolves serves to maintain group cohesiveness, a central component to this highly social species.

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General discussion

A multidisciplinary study

We investigated four interconnected research topics in 3 different countries on 2 continents: conflict management, stress, parasitology[2], and virology. This is the first report of comparative research combining viral, parasitological and hormonal data in free-ranging wolves of different geographical areas. Conflict management strategies have not been investigated before in free-ranging wolf packs.

The topics of interest of this research are to date still poorly investigated in free-ranging wolves of Europe and northwestern America. The results of this multi-disciplinary study provide information of importance on the presence of various pathogens in the studied wolf packs. These are central data to consider for the conservation of the species in the investigated areas, but also elsewhere on the planet. Information regarding infection by various pathogens of wolves in the three different national parks is likely representative of the situation at a much larger geographical scale.

Numerous pathogens have been reported as infecting wolves. These include protozoans, viruses, bacteria, fungi, helminths and various ectoparasites (Archer et al. 1986; Brand et al 1995; Ratti et al. 1999; Kreeger 2003; Kloch et al. 2005). The present study focused on the detection of helminths and selected viruses, and further reports infection by some protozoans.

Infection by helminths usually is not fatal to wolves, but can have indirect effects on the fitness and survival of individuals (Brand et al. 1995; Kreeger 2003). However, helminthiasis associated with viral or bacterial disease can be lethal (Kreeger 2003; Brand et al. 1995). Predisposing factors to infection by canine parvovirus type 2 (CPV-2), other than the age of the animal, include concurrent diseases, different parasitic infections and stress (Kreeger 2003). Stress is further reported as a factor favoring the development of clinical disease in case of infection by canine coronaviruses (CCoV) (Pratelli 2008). Such aggravated outcome of infection is probably common to other co-infections by viruses and parasites. Additionally, sustained stress is known to reduce efficiency of the immune system, therefore decreasing the ability of an organism to fight infections. Such stress-induced reduced resistance to diseases in individuals may have a broader impact on population dynamics (Millspaugh and Washburn 2004).

Even in the absence of infection, sustained stress can have harmful physiological consequences on mammals. Prolonged exposure to elevated levels of cortisol

[2] For the purpose of this discussion, parasite refers to helminths and protozoans.

metabolites (CMs) is believed to enhance reproductive failure, decreased resistance to diseases, reduced wound healing as well as developmental and growth dysfunctions. Such consequences can directly affect health and survival of individuals, and therefore further modulate population dynamics. Besides social stress, identification of variables modulating stress level at different geographical scales (i.e. at the pack and the park scale, in our study) is therefore of conservation interest.

We investigated parasitic and viral infections in protected wolf populations in which such data were absent or reported only a long time ago. We also measured stress level in the same wolf packs. We conducted detailed comparative analyses in an attempt to determine what environmental and biological factors play a role in influencing these variables. Collected information regarding exposure to pathogens (parasites and viruses) and CMs levels in the studied wolf packs can help understanding population dynamics in free ranging wolf populations, locally and more globally. We suggest that modeling of population dynamics of free-ranging wolves should be adapted taking these variables into account. Awareness of these weakening factors, acting in the study areas but also elsewhere on the planet, should also encourage the adoption of measures aiming to a better conservation of protected populations, such as vaccination of free-ranging dogs in Abruzzo, Lazio e Molise National Park (PNALM).

This work also aimed to enhance our understanding of wolf's social behavior by exploring an important component of the social life of the species, namely management of conflicts. We provide the first study of conflict management in free-ranging wolves, based on detailed information on the behavioral mechanisms used to settle conflicts between pack members.

All investigated research topics are closely related to wolf sociality. As a highly social species, wolves live in groups and share close relationships with pack members. Elevated sociality favors transmission of diseases between group members. Frequent physical contacts during social interactions, shared food and resting places, are all occasion of pathogen transmission. The fidelity of wolves to their den sites and rendezvous areas further helps maintenance and transmission of parasites.

Social stress has previously been studied in wolf packs. We compared CMs levels in alpha (dominant) and subordinate individuals. We further investigated this variable at a larger scale, comparing the influence of pack stability on measured CMs levels, and also examined differences between the three national parks.

Conflict management is obviously directly linked with sociality. Group living is invariably associated with arousal of conflict between pack members, and such disturbance of established bonds needs to be managed in order to maintain benefits of group memberships.

Choice of study areas

In this study, we considered eleven wolf packs belonging to three populations. We chose protected wolf populations of temperate areas, at similar latitude, and settled in similar habitat in order to minimize influence of climatic factors (e.g. seasonality) and human disturbance that can affect variables of interest. When fieldwork was conducted, the animals were protected in the three parks and none of the investigated packs was exposed to legal harvesting by humans.

Furthermore, owing to climatic conditions (cold winter temperature) and accessibility of sample collection sites, all three national parks were also well suited for the present study. Of major importance, the permanent collection of wolf fecal samples by local teams in both European national parks allowed us to access a maximum number of samples, from which those suitable for our study were selected.

The study sites, all government managed protected areas, were further selected because of the different history of wolf settlement in these areas. While wolves never disappeared from central Italy (Boitani 2003), they were exterminated in France in the 1930s and probably disappeared from the southeastern corner of the country in the first decade of the 20th century (Houard and Lequette 1993). Wolves from Italy are dispersing towards France and Switzerland, and first signs of their return in France were confirmed in 1992, in Mercantour National Park (PNM) (Valière et al. 2003; Fabbri et al. 2007; Houard and Lequette 1993). In Yellowstone National Park (YNP), wolves were reintroduced in 1995 and 1996, through release of individuals captured in Alberta and British Columbia, Canada (Bangs and Fritts 1996; Philips and Smith 1996). Thus, first individuals settled back in YNP and PNM at about the same time. In the American national park, investigations took place on the northern winter range (Smith et al. 2003), the only area of the park accessible year round by car. The northern range is located at a lower altitude and receives less snow than the rest of the park, and is thus home of numerous migrating ungulates and sedentary predators. Wolf density was elevated in PNALM and on the northern range of YNP, while about five times lower in PNM. Such characteristics of the three wolf populations allowed us to investigate the impact of these different factors on variables of interest at a large geographical scale, comparing wolf populations of the three national parks.

Results of genetic analyses performed on samples from PNM provided by the National Game and Wildlife Service in France (ONCFS) gave access to detailed information regarding individual wolves. In YNP, direct observation of defecating individuals allowed for identifying specific contributing individuals, giving access to precious specific information regarding part of the collected samples. Thus, direct identification (in YNP) and genetic analyses (in MNP) of part of the samples provided information allowing to test for important individual variables, such as gender or age, in the statistical analysis of data.

Yellowstone National Park was among the very few possible choices regarding the behavior part of our study, as it is probably the most accessible place where relevant behavioral data can be collected on free-ranging wolf packs. It was perfectly suited for data collection and allowed us to collect detailed information on interactions between individuals.

In Europe, only one winter was considered for viral and parasitological investigations, as the same packs were studied in two consecutive winters and little change in exposure to these pathogens was expected from one year to the next. Regarding parasitological data, this was corroborated by results in the Druid Peak pack showing no important variation in detected species and prevalence between the two consecutive winters of sample collection.

Choice of sampling in winter

Fecal sample collection during winter was the most appropriate for the present study, as low temperatures preserve hormonal metabolites of interest (Palme 2005) as well as most helminth eggs and larvae (Marquard-Petersen 1997). Cold temperatures also prevent the development of eliminated hookworm eggs into larval stages, as well as invasion of feces by free-living or plant nematode larvae. For the purpose of fecal CMs measurement, fast freezing of the collected fecal samples is advised (Khan et al. 2002; Palme 2005), and rapid storage at -20°C without any previous chemical treatment has been shown to be the most reliable storage method, across species (Hunt and Wasser 2003; Millspaugh and Washburn 2004). In winter, rapid freezing was assured by natural weather conditions, so that samples were maintained at cold temperature until collection. Additionally, sample collection strategies used in each national park allowed for fast storage of samples at -20°C. Thus, winter was the time of the year best adapted to our hormonal and parasitological investigations.

Non-invasive and efficient tool for multidisciplinary analyses: fecal samples

Fecal sample analyses allowed us to access hormonal, parasitological and virological information about the investigated free-ranging wolf populations. This very complete set of data regarding specific samples allowed for testing of detailed hypothesis. In addition to this information, the same fecal samples were analyzed in both PNALM and PNM to assess diet of wolves (Grottoli 2011; Réseau Loup/Lynx 2004, 2006), and in PNM for genetic identification of individuals. To our knowledge, no such wide range of information was previously collected from the same fecal samples. This additional information was considered in data analyses or interpretation of our results. All these

analyses performed on each sample represent a great advantage in terms of spared fieldwork time and sample use optimization.

Furthermore, fecal sample analysis allowed for continuous large-scale investigation of free-ranging wolf packs in all three national parks, over four to six months for up to two consecutive winters in each park. This non-invasive tool has the additional advantage of having no impact on the behavior and physiology of individuals, thus ensuring collection of reliable behavioral and hormonal data. Fecal sample analyses provide data considered acceptable regarding the parasite community in canids (Torres et al. 2001). As regards total parasite prevalence, results obtained by examination of feces are usually lower than from necropsy due to a relatively low sensitivity of coprology, as shown in comparative studies in canids (Magi et al. 2009; Torres et al. 2001). Similarly, prevalence of helminths in wolves determined by coprology (44.0-63.5%; Kloch et al 2005; Popiołek et al. 2007; Marquard-Petersen 1997) is usually much lower than found by necropsy (80-100%, Moks et al. 2006; Bagrade et al. 2009; Guberti et al. 1993; Segovia et al. 2001; Shimalov and Shimalov 2000; Rausch and Williamson 1959). Considering this, the overall prevalence of parasites in PNALM and in YNP reported in our study is high, but in accordance with results for wolves from few studies that used similar methods (88.4%, Szafrańska et al. 2010; 95.8% Archer et al. 1986). Thus, our results are an acceptable estimate of the parasite community infecting the investigated packs, even though parasite richness reported in our study represents minimal values only.

Environmental factors and life history of studied populations

In our study, large geographical scale appears to be the main factor structuring parasite community in free-ranging wolves, and major differences are noted among the three national parks. The park scale is further an important determinant of total parasite prevalence and CMs level in the studied wolf populations.

All parasite taxa found in PNM were also identified in PNALM. Detected parasite diversity is different in all three investigated areas, supporting the idea that different geographical and biological factors structured parasite community of wolves in the three national parks. In areas of recent return of wolves, namely PNM and YNP, the other canid species present probably maintained in the environment several parasite taxa that they share with wolves. In such recently established wolf populations, parasites spread through a predator-prey interaction are the most common, while directly transmitted parasites are the most prevalent in the long-established population of PNALM. Some differences in parasite diversity between Europe and North America were expected as previously reported (Craig and Craig 2005). Our results show large dissimilarities in parasite diversity and prevalence between the two connected wolf populations in Europe, which can be a consequence of the loss of uncommon parasite taxa in the course of a slow recolonization process. Additionally, major differences in susceptible canid populations living in sympatry with wolves in the two national parks

can determine parasite availability in the environment, and thus be an important factor explaining the presence of *Capillaria aerophila* and *Capillaria boehmi* in PNALM and their absence from the wolf population of PNM. Consequently, we suggest that a slow natural recolonization process and low density of susceptible hosts in newly occupied areas triggered loss of parasite taxa in the wolf population of PNM.

Elevated density of wolves implies an increased number of interactions with ungulates, intermediate hosts of different wolf parasites, and with other canids, definitive hosts of parasites infecting wolves (Choquette et al. 1973; Messier et al. 1989; Guberti et al. 1993; Ratti et al. 1999). A high density of susceptible hosts further induces an elevated contamination of the environment (eg. eggs in feces) (Marquard-Petersen 1997; Messier et al. 1989). Different canids live in sympatry with wolves in all three national parks. Coyotes (YNP) and foxes (YNP, PNM and PNALM) share some of the parasites infecting wolves. The wolf and the dog belong to the same species (Wilson and Reeder 2005) and are thus expected to host the same pathogens. Therefore, in addition to the high wolf density in PNALM, the free-ranging dog population present in the park can be a major factor favoring maintenance of parasites and viruses in the environment. This dog population living in sympatry with wolves may also have facilitated the introduction of some of the pathogens reported in our study into the wolf population (Brand et al. 1995; Guberti et al. 1993).

Elevated host density also enhances maintenance of viral infections in the ecosystem and chances of transmission. We detected the presence of canine parvovirus type 2 (CPV-2) and canine coronaviruses (CCoV) in both European populations.

As free-ranging dogs might prey on the same species as wolves in central Italy (Ciucci and Boitani 1998), the dog population of PNALM can moreover represent competitive carnivores for wolves in the Italian national park. Such competition would directly affect defense of wolf packs' territory and associated resources, and thus be an important factor causing the elevated CMs levels measured in the wolf population of PNALM.

In addition to the density of alternative susceptible hosts, availability of infected prey species is an important factor determining the infection of wolves by parasites with an indirect life-cycle. Thus, this study gave indirect information on some of the parasites infecting prey species of wolves in the three investigated national parks, the helminth community in the carnivore being partly consistent with its feeding habits (Marquard-Petersen 1997). As the elk is the main prey species of wolves in YNP (Smith et al. 2008, 2009, 2010), our study indicates that Taeniids and *Sarcocystis* sp. apparently infect a large proportion of the elk population of the American national park. On the contrary, our results suggest that these parasites are less prevalent in prey species of wolves in PNALM and PNM, or infecting only part of the ungulate species preyed on by wolves in these areas. Diversity of the diet can therefore impact on the prevalence of parasites with an indirect life cycle that infect free-ranging wolves. Besides these parasites using ungulates as intermediate hosts, we also report the presence of other parasites of prey

species that do not infect wolves, based on the presence of eggs in feces of wolves that fed on infected preys.

Conservation issues

In our comparative study of wolves from three different national parks, the population of PNALM clearly stands out. We report the highest parasite diversity and richness and most elevated CMs levels in the Italian park, as opposed to PNM and YNP. Although total parasite prevalence and wolf density are similar in PNALM and YNP, the presence of free-ranging dogs in the Italian national park can contribute to observed differences. History of wolf settlement can be another important factors influencing dissimilarities between study areas. Parasitic infection might have a stronger impact on population dynamics in areas where a long established relationship evolved between the carnivore and its parasite fauna (Holt and Dobson 2007). Poor diversity of parasites infecting wolves in PNM and deworming procedure applied to individuals released in YNP possibly favored the successful and rapid expansion of wolves at the onset of their return (Holt and Dobson 2007).

The value of disease screening is acknowledged in a growing number of wild canid conservation projects (Macdonald and Sillero-Zubiri 2004). Wolves from Italy, dispersing towards France and Switzerland, belong to a specific subspecies (*Canis lupus italicus*), only present in that area. Besides reporting parasitic infection in wolves in the two European national parks, our results indicate infection of individuals from various packs by two highly contagious viruses in the connected wolf populations. Presence of CPV-2 and CCoV in wolves is here first investigated at a large scale in PNALM and PNM, and we report infection in various wolf packs in both national parks. The impact of these viruses on pup recruitment is well known, and co-infection can increase deleterious impact of the viruses. In YNP, repeatedly occurring population declines were attributed to viral infections (Almberg et al. 2009). The potential weakening effect of parasitism on populations experiencing viral outbreaks is recognized (Holt and Dobson 2007) and co-infection with other pathogens is a considered explanation for periodic high level of mortality in YNP (Almberg et al. 2009). The assessment of the helminth parasite taxa harbored by wolves in the park is therefore important to further precise the impact of infection by pathogens on population dynamics in the area.

This study first reports the infection of wolves by a specific helminth, *C. boehmi*. Infection by other parasites and viruses are here shown for the first time in geographical areas in which they were not previously described in the species. The impact of infection by these parasites is yet unclear, and is most likely underestimated due to the secretive behavior of wolves and their large home range.

Emergence of new viral strains and different helminths is reported in Italian canids (Traversa et al. 2009, 2010; Origgi et al. 2012; Le Poder 2011; Monne et al. 2011; Decaro and Buonavoglia 2008; Evermann et al. 2005; Martella et al. 2005; Buonavoglia et al.

2001, 2006) that can have an additional negative impact on wolf populations, if not yet present in the investigated areas. Existing and emerging pathogens may drive rapid changes in the numerical abundance and genetic composition of free ranging host populations (Altizer et al. 2003). Parasitic and viral infection can impact population dynamics by affecting pup recruitment and dispersal of individuals (Brand et al. 1995). Furthermore, sustained stress has a weakening effect on the organism and decreases immune defense of individuals. Cortisol metabolite levels measured in PNALM are clearly higher than those from the other two national parks and also higher than in individuals used in the biological validation of our hormonal assays. Such elevated values can affect health of individuals, concurrently or not with infection by pathogens, and might thus impact population dynamics.

This is the first study investigating post conflict affiliative interactions in free-ranging wolves. We found a very high conciliatory tendency in wolves, in accordance with values reported in primate societies in which individuals share relaxed dominance relationships. We also report the occurrence of solicited and unsolicited consolation, highlighting benefits of conflict resolution for different group members. The frequent occurrence of post-conflict friendly behaviors is in accordance with the importance of group cohesion in this highly social and cooperative species. We also report the use of a specific behavior in post-conflict interactions, which is further characteristic of species frequently engaging in affiliative behaviors after a conflict (Aureli et al. 2002). Cooperation among group members is central to breeding of pups and defense of a territory and related resources against neighboring groups, and common occurrence of conflict management strategies was expected.

Small geographical scale: the packs and individual characteristics

We report only little differences in parasite richness or total prevalence between neighboring free-ranging wolf packs.

In accordance with our expectations, our results suggest that group stability is an important determinant of CMs levels in free-ranging wolf packs. Extraterritorial travels are correlated with elevated CMs levels, which can be caused by encounters with neighboring packs or signs of their presence. Additionally, variations between packs can be due to social stress, in connection with the composition and stability of packs as well as dispersal possibilities, and is likely influenced by individual differences.

Consistent with previous reports, our data show that males and females do not differ in parasitic infection variables. Similarly, we found no gender difference in measured CMs levels. With the exception of a specific parasite species, we found no effect of age on parasitism in the studied packs, and measured CMs levels did not differ statistically between pups, yearlings or adults. Furthermore, our results show that the alpha social status does not correlate with an elevated stress level in the studied packs.

Conclusion

The results of this research increase our understanding of social behavior in free-ranging wolves, and also provide information on hormonal, parasitological and virological factors that can have an important impact on the species' health, ecology, as well as the population dynamics.

Our results show that life history of a population and density of susceptible hosts might be important factors shaping parasite community and infection by viruses. The long-established wolf population of PNALM is especially exposed to viral and parasitological infection, as well as elevated CMs levels, factors that can importantly affect population dynamics, although difficult to monitor in free-ranging wolves. The important population of free-ranging dogs inhabiting wolf packs' territory in PNALM probably influences infection of wolves by parasites and viruses, as well as CMs levels measured in the different Italian packs. This dog population can have facilitated the introduction and/or maintenance of some of the parasite taxa reported in our study in that area (Brand et al. 1995; Guberti et al. 1993). Parasite community of coyotes was also at least partly transmitted to dewormed wolves reintroduced into YNP. Thus, monitoring the presence of pathogens in canids living in sympatry with wolf populations is of importance to better understand potential risks of pathogen transmission and consequent concerns for the conservation of protected populations.

Regarding infections by pathogens shown by our study in the investigated populations, our results are of local as well as more global interest for the survey of the species, and we advise future monitoring of these variables. As Murray et al. (1999) stated, "it would be naïve to assume that such diseases fail to affect carnivore demography simply because they do not cause outbreaks". As infection by helminths and viruses may act in synergy, they should both be considered in future disease screenings of animals, and particularly of protected populations.

Additional pathogens (helminths, protozoans, fungi, viruses, bacteria and/or ectoparasites) likely have a supplementary harmful impact on the studied populations and should also be investigated. As an example, heartworm disease was only recently reported in an Italian wolf (Pascucci et al. 2007). This supports the importance of continuous monitoring of pathogens infecting free-ranging wolves, dogs and other sympatric canids.

Sustained stress in individuals can impact population dynamics, notably by facilitating infection by pathogens. Our results suggest that some factors determine CMs levels at a large geographical scale, while other influence specific packs and are dependent of intrinsic factor such as pack stability. When possible, factors inducing elevated CMs levels in a population at a large geographical scale should be minimized, as cumulative effects of sustained high stress levels, and parasitic and viral infections may cause significant populations declines.

We are the first to report detailed behavioral analysis of conflict management in free-ranging wolf packs. Results of our investigations suggest that conflict resolution mechanisms are an important component of social interactions in free-ranging wolves. This study is one of the rare reports on conflict management in free-ranging vertebrates and of consolation behavior in a non-primate species. The common occurrence of friendly post-conflict behaviors reported in our study is in agreement with the importance of group cohesion in this highly social and cooperative species, and thus in accordance with our expectations.

For the purpose of the present study, the use of non-invasive methods proved very efficient and satisfactory, and allowed for the collection of representative behavioral, hormonal, virological and parasitological data.

Our study reports data from winter months only, as environmental factors best fitted investigation tools used in the analyses of fecal samples. However, if adapted collection and analyses techniques can be used, further investigations should be conducted during warmer months, in order to study the impact of additional factors on infections by pathogens and CMs levels in free-ranging wolves.

Our work provides baseline information of importance that should be considered prior to human interventions on free-ranging wolf populations.

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Appendix A

Table 1: Simple and multiple infections of protozoans and helminths in samples from the three investigated national parks. Only samples in which parasites were found are shown (n=250). Total number of tested samples (n), number of samples that tested positive (N) and prevalence (P) are figured. P is expressed as percentages (%). (PNM: Mercantour National Park; PNALM: Abruzzo, Lazio e Molise National Park; YNP: Yellowstone National Park; Pro: Protozoans; Tre: Trematodes; Ces: Cestodes; Nem: Nematodes).

Parasite taxa	**PNALM (n=78)**		**MNP (n=20)**		**YNP (n=152)**		**TOTAL (250)**	
	N	**P**	**N**	**P**	**Nb**	**P**	**Nb**	**P**
Simple infection								
Pro	1*	1.3	1*	5.0	15 (93.3% *)	9.9	17	6.8
Tre	0	-	0	-	0	-	0	-
Ces	1**	1.3	17**	85.0	69**	45.7	87	34.9
Nem	38 (89.5% *C. boehmi*)	48.7	0		5 (100% *T. leonina*)	3.3	43	17.3
Total	**40**	**51.3**	**18**	**90.0**	**89**	**58.9**	**147**	**59.0**
Multiple infection								
Pro/Tre	0	-	0	-	0	-	0	-
Pro/Tre/Ces	0	-	0	-	0	-	0	-
Pro/Tre/Ces/Nem	0	-	0	-	1	0.7	1	0.4
Pro/Ces	0	-	1	5.0	36	23.8	37	14.9
Pro/Nem	7	9.0	1	5.0	6	4.0	14	5.6
Pro/Tre/Nem	0	-	0	-	0	-	0	-
Pro/Ces/Nem	2	2.6	0	-	6	4.0	8	3.2
Tre/Ces	0	-	0	-	1	0.7	1	0.4
Tre/Ces/Nem	0	-	0	-	1	0.7	1	0.4
Tre/Nem	2	2.6	0	-	0	-	2	0.8
Ces/Nem	11	14.1	0	-	11	7.3	22	8.8
Nem	16	20.5	0	-	0	-	16	6.4
Total	**38**	**48.7**	**2**	**10.0**	**62**	**41.7**	**102**	**41.0**

(* = *Sarcocystis* sp.; ** = Taeniidae)

35.8% of all multiple infections are due to co-infection by protozoan and cestodes (Taeniidae), while a co-infection by cestodes and nematodes occurred in 21.2% of the samples. 58.9% of the single infections are due to cestodes. The high prevalence of cestodes in these results is not surprising as, taken together, Taeniidae are present in 45.9% of the samples.

Taeniidae probably correspond to a quiet large group of different species, including the genera *Taenia* and *Echinococcus*. Multiple infections caused by nematodes only were observed in 6.8% of the samples.