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Endoparasitic infections in farmed ring-necked pheasant (*Phasianus colchicus*) and red-legged partridge (*Alectoris rufa*) (Galliformes: Phasianidae) in the Czech Republic

Ph.D. Thesis

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Annotation

Even though parasitic diseases of phasianid birds have been extensively studied in the past, some of them still represent a health and economic problem in intensive artificial breeding programmes in the Czech Republic. To increase our current knowledge on the endoparasitic infections of reared birds, during 2012–2015, 836 pooled faecal samples of the ring-necked pheasant (*Phasianus colchicus*) and 900 of the red-legged partridge (*Alectoris rufa*) were collected and examined. Additionally, some post-mortem examinations were carried out on 514 pheasants and 99 partridges. This study details the course of spontaneous infections of protozoan and helminth infections (single or coinfection) and describes the proportion of positive samples, prevalence, and age dynamics from hatching until birds are released for hunting purposes into the open area. We identified main infections of both hosts with protozoans *Eimeria* spp. and *Cryptosporidium* spp., as well as nematodes, such as Capillariidae gen. sp., *Heterakis gallinarum* and *Syngamus trachea*. Moreover, this study elucidates the critical periods of parasite occurrence under intensive artificial breeding programme conditions, thus forming the basis for preventing possible outbreaks. Cryptosporidial infections in these facilities have not been adequately studied and our aim about the species spectrum and modes of transmission during the entire artificial rearing period was accomplished. Results showed differences in susceptibility to parasite infections depending on species and age of both game birds, and also a graphical representation of infection pathway during a complete rearing season, including the translocation of birds were described in detail for cryptosporidial infections. Finally, molecular characterization of oocysts isolates of *Cryptosporidium* spp. from both hosts was performed. Identification of *C.*

meleagridis highlights the potential transmission of cryptosporidiosis from game birds to humans. The situation as described here may serve as a model for the spread of cryptosporidial infections of other commercial domestic and exotic birds.

Keywords: coinfection; coprology; game birds; helminth; infection pathway; molecular analyses; percentage positivity; post-mortem examination; prevalence; protozoan

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Anotace

Parazitární onemocnění volně žijících ptáků z čeledi bažantovitých byla v minulosti rozsáhle studována, přesto některá z nich představují zejména v intenzivních umělých chovech v České republice i v současné době zdravotní i ekonomické problémy. K získání nových poznatků o endoparazitárních infekcích bylo v období let 2012–2015 odebráno a koprologicky vyšetřeno celkem 836 směsných vzorků trusu od bažantů obecných (*Phasianus colchicus*) a 900 od orebic rudých (*Alectoris rufa*). Postmortálně bylo vyšetřeno 514 bažantů a 99 orebic. Studie u této pernaté zvěře podrobně popisuje průběh spontánních protozoárních a helmintózních infekcí (monoinfekce nebo koinfekce), počet pozitivních vzorků, prevalenci v závislosti na věkové dynamice od vylíhnutí ptáků až po jejich vypuštění do otevřeného prostoru pro účely lovu. Identifikovali jsme hlavní původce nákaz u obou hostitelů, a sice prvoky *Eimeria* spp. a *Cryptosporidium* spp., a hlístice, Capillariidae gen. sp., *Heterakis gallinarum* a *Syngamus trachea*. Práce objasňuje kritická období výskytu parazitů v intenzivních umělých chovech a tvoří podklad k zabránění vzniku možného ohniska nákaz. Problematika kryptosporidiových infekcí v těchto zařízeních nebyla doposud dostatečně studována a splněním cíle byly získány nové poznatky o jejich druhovém spektru a způsobu přenosu během celého období umělého chovu. Výsledky ukázaly rozdíly ve vnímavosti k parazitárním infekcím v závislosti na druhu i věku obou druhů ptáků, graficky byly znázorněny cesty šíření kryptosporidiových infekcí v daném chovu v souvislosti s přesuny zvěře. Nálezy oocyst *Cryptosporidium* spp. u obou hostitelů a jejich izoláty dovolily molekulárními analýzami provézt druhovou typizaci. Identifikace *C. meleagridis* upozorňují na možnost přenosu kryptosporidiózy z pernaté zvěře na člověka. Situace, jak je zde popsána může sloužit jako model šíření kryptosporidiových infekcí i ostatní užitkové domácí drůbeže a exotického ptactva.

Klíčová slova: helminti; koinfekce; koprologie; molekulární analýzy; pernatá zvěř; post-mortální vyšetření; prevalence; procento pozitivních; prvoci; zdroje a způsoby šíření infekce

Declarations

I hereby declare that this thesis entitled ,,Endoparasitic infections in farmed ring-necked pheasant (*Phasianus colchicus*) and red-legged partridge (*Alectoris rufa*) (Galliformes: Phasianidae) in the Czech Republic“ is based on my own work and all other sources of information have been acknowledged.

Date..... Signature.....…

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List of author papers

The Ph.D. thesis is based on the following papers (listed chronologically):

I. Máca O, Pavlásek I. 2015. First finding of spontaneous infections with *Cryptosporidium baileyi* and *C. meleagridis* in the red-legged partridge *Alectoris rufa* from an aviary in the Czech Republic. Veterinary Parasitology 209:164–168 (IF = 2.60).

II. Máca O, Pavlásek I. 2016. *Cryptosporidium* infections of ring-necked pheasants (*Phasianus colchicus*) from an intensive artificial breeding programme in the Czech Republic. Parasitology Research 114:2933–2939 (IF = 2.37).

III. Máca O, Pavlásek I. 2018. Protozoan and helminthic infections of farm-reared *Alectoris rufa* (Galliformes: Phasianidae) before releasing for hunting in the Czech Republic: infection pathway and potential risks. Journal of Parasitology (submitted, major revision).

Co-author statement related to the Ph.D. thesis

Máca Ondřej made a major contribution to the work in the research phase and in the writing phase.

Date..... Signature.....…

Table of Contents

1. Introduction

During the last century, wild populations of game birds, especially those of the grey partridge (*Perdix perdix*) and ring-necked pheasant (*Phasianus colchicus*), have been high in the number of individuals. In that time, extensive farming of poultry (e.g., free-range, poultry exported in the form of nomadism on the harvested fields of cereals, etc.) was very common. By using such systems there always exists the possibility of mutual transmission of various infectious and parasitological diseases among game birds and domestic animals. Since hunting continues in our country, the study of the parasite fauna in these wild and commercial birds has been achieved by several parasitologists. The main objective of those studies was to identify the species spectrum of endoparasites in both groups of birds (*P. perdix* and *P. colchicus*) and propose measures to reduce the real possibility of mutual transmission of parasitic infections. In parallel with the intensification of the Czech agriculture and reduced levels of cropping, a significant reduction in wild pheasant populations occurred. Individual hunting associations started to develop artificial breeding programmes for pheasants, with subsequent release in private lands of pheasant and some other game birds, such as red-legged partridges (*Alectoris rufa*). In the Czech Republic, there currently are several artificial breeding facilities with tens of thousands pheasants and thousands of partridges, in which a high concentration of birds in a relatively small area creates favourable conditions for the rapid spread of infections, including bacteria and parasites. In many cases, co-infections occur and some galliform farms have a high prevalence of helminth and protozoan parasites, potentially resulting in disease outbreaks and increased morbidity and mortality (Bolognesi et al. 2006; Pavlásek 2006; Pagès-Manté et al. 2007; Pavlásek & Šverma 2009).

To increase the production of artificially bred *P. colchicus* and *A. rufa* (Galliformes: Phasianidae), parasitological examinations were carried out. We focussed on protozoan and helminth endoparasites, with the high occurrence of *Eimeria* Schneider, 1875, *Cryptosporidium* Tyzzer, 1907; as well as lower percentage of positivity of *Histomonas meleagridis* Tyzzer, 1924. Three nematodes (Capillariidae gen. sp., *Heterakis gallinarum* Schrank, 1788, *Syngamus trachea* Montagu, 1811) and two cestodes (*Choanotaenia* Railliet, 1896, *Raillietina* Fuhrmann, 1920) are common in similar breeds. Particularly, cryptosporidial infections in artificial breeding programmes

of pheasants and partridges in the Czech Republic and other countries need more attention due to its importance.

This study details the course of spontaneous infections of protozoan and helminths and describes the proportion of positive samples, prevalence, age dynamics and a graphical representation of infection pathway during a complete rearing season, including the translocation of birds. Moreover, it elucidates the critical periods of parasite occurrence under intensive artificial breeding programmes conditions in order of preventing possible outbreaks.

2. Aims of the research

1) To determine the parasite fauna of the ring-necked pheasant (*P. colchicus*) and red-legged partridges (*A. rufa*).

2) To assess infection pathway and onset of parasitic infections throughout the breeding process, from hatching up to release of birds to open area.

3) To evaluate the species diversity, occurrence and prevalence of spontaneous cryptosporidial infections with identification by molecular analyses.

3. Literature overview

Although parasitic diseases of game-farmed phasianid birds have been extensively studied worldwide (e.g., Anderson & Shapiro 1955; Bickford & Gaafar 1966; Boyd & Fry 1971; Arora & Chandra 1983; Dowell et al. 1983), including the Czech Republic (e.g., Páv & Zajíček 1968; Bejšovec 1970, 1972, 1973; Pavlásek 1999), some pathogens still represent a problem in breeding conditions (Goldová et al. 2006; Pagès-Manté et al. 2007; Villanúa et al. 2007, 2008; Forejtek & Chroust 2010a, b; Andreopoulou et al. 2011; Santilli & Bagliacca 2012). Even though most of the important parasites of game birds have been described, research on the parasite fauna and, in some cases, experimental studies for possible transmission pathways and impact of the wide spectrum of pathogens between wildlife and domestic birds, are needed.

There are other parasitoses, especially those of *Cryptosporidium* spp., whose contribution to complex diseases and economic losses are almost completely unknown. For this reason, attention has been paid to cryptosporidial infections in artificial breeding programmes of game birds in the Czech Republic and other countries (e.g., Pavlásek & Šverma 2009; Wang et al. 2012). Coming studies should include more details on the course of spontaneous infections, description of positive samples, prevalence, age dynamics and graphical representations of the pathway of *Cryptosporidium* infections during a complete rearing season. Moreover, they should also consider the translocation of birds and where diseases could become a major health problem with impact on health and economics. Because of their very frequent occurrence, cryptosporidian infections, especially those of *C. meleagridis*, are an important issue of possible zoonotic potential, as described recently by many authors (e.g., Xiao & Feng 2008; Silverlås et al. 2012; Baroudi et al. 2013; Wesołowska et al. 2016).

In comparison to the parasite fauna of pheasants, situation is very different in the case of *A. rufa*. To our best knowledge, no data have been published about the parasite fauna of partridges in the Czech Republic.

In farms for hunting purposes, animals harbour high level of parasites, which could result in economic and biological losses during the production process (Wójcik et al. 1999). Studies have shown that birds represent a health risk in the pathogen

transmission after bird releasing (Calvete et al. 2003; Millán et al. 2004; Tampieri et al. 2005; Buenestado et al. 2009; Santilli & Bagliacca 2012). According to Villanúa et al. (2007), the anthelmintic treatment used and recommend for partridge farms in Spain is not effective and can seriously compromise wild and released bird populations.

Prevention, treatment and control of endoparasites are very difficult. A selected and approved anti-parasitic treatment is used, but there is a clear lack of data for appropriate medication, based on the study of the dynamics of spontaneous parasite occurrence. Moreover, it is important to carry out routine and periodical faecal examinations of whole breed to protect animals. For those reasons, it is relevant to expand the knowledge about gallinaceous game birds, as well as their veterinary and epidemiological significance.

Taxonomy and general biology have been described in many review articles and books, and there is no need to repeat this information here. In the following text, we included the most common and important parasites of the examined bird species in our study, from the epidemiological point of view.

3.1 Protozoan infections

The most common protozoan infections causing mortality or pathogenicity in birds are *Cryptosporidium* spp., *Eimeria* spp., and *Histomonas meleagridis*. Most hosts are domestic birds or birds under intensive artificial breeding conditions (e.g., *A. rufa*, *Colinus virginianus*, *Coturnix coturnix japonica*, *Gallus gallus*, *Meleagris gallopavo*, *P. colchicus*) (see Atkinson et al. 2008). Some species can be highly pathogenic and cause outbreaks with high losses to breeders. Host specificity varies from being species-specific to infect a wide variety of birds. In pheasants and red-legged partridge breeding, protozoans like *Eimeria* and *Cryptosporidium* are the most widespread disease in the Czech Republic.

3.2 Cryptosporidial infections in birds

With the increasing number of breeding farms for gallinaceous birds, special attention is paid to prevention and control of significant emerging pathogens, such as apicomplexan protozoans of the genus *Cryptosporidium*. Infections in birds are mainly

caused by three species: *C. baileyi* Current, Upton et Haynes, 1986, *C. galli* Pavlásek, 1999, and *C. meleagridis* Slavin, 1955 (see Slavin 1955; Current et al. 1986; Pavlásek 1999; Ryan et al. 2003). Recently, a new species was accepted, *C. avium* Holubová, Sak, Horčičková, Hlásková, Květoňová, Menchaca, McEvoy et Kváč, 2016, along with several avian *Cryptosporidium* genotypes (11) (e.g., Ng et al. 2006; Ryan 2010; Nakamura & Meireles 2015; Holubová et al. 2016; Zahedi et al. 2016). Some of these species have been found and described in birds in the Czech Republic. Pavlásek (1985, 1993, 1994a) reported for the first time the occurrence of *C. baileyi* in domestic chickens (*G. gallus*) and wild birds (black-headed gull, *Larus ridibundus*), as well as *C. meleagridis* in turkey (*M. gallopavo*) and salmon-crested cockatoo (*Cacatua moluccensis*), and *C. galli* in domestic chickens (*G. gallus*) and red-cowled cardinal (*Paroaria dominicana*). Later, numerous isolates were biological, morphological and molecularly identified and characterized from various avian host species from the Czech Republic (see Pavlásek 1999; Ryan et al. 2003; Ng et al. 2006). Problematic of cryptosporidial infections in breeding facilities were studied in the past. Sun et al. (2005) reported that *C. baileyi* caused 75% mortality in the Japanese quail (*C. coturnix japonica*), while Pavlásek (2006) observed only 15–20% mortality in these birds. Wang et al. (2012) described *C. baileyi* as a widespread parasitic species in birds.

Until recently, the only species of *Cryptosporidium* parasitizing the respiratory and intestinal tracts of the red-legged partridge *A. rufa* was *C. meleagridis*, genetically characterized after an outbreak in Spain (Pagès-Manté et al. 2007). According to the authors, avian cryptosporidiosis was accompanied by up to 50% mortality and 60–70% morbidity between May and July, and even up to 89% mortality during July in later hatches. In the course of a long-term study in the Czech Republic, *C. meleagridis* from red-legged partridge was first detected and diagnosed during 2006–2007 in an aviary in the Mladá Boleslav region (Pavlásek, unpublished data).

On the other hand, *Cryptosporidium* spp. parasitizing *P. colchicus* were previously found in the respiratory tract (Whittington & Wilson 1985), trachea and bursa of Fabricius (Randall 1986; Pavlásek & Kozakiewicz 1989; Sironi et al. 1991). Musaev et al. (1998) and Ashraf et al. (2015) detected oocysts in pheasant faeces in Azerbaijan, whereas Pavlásek (2004) reported the occurrence of *C. baileyi* and *C. galli* in ring-necked pheasants from aviaries of the Czech Republic. Some experimental

infections have also been carried out (e.g., O'Donoghue et al. 1987; Pavlásek & Kozakiewicz 1989; Lindsay & Blagburn 1990; Pavlásek 1999). Recently, Holubová (2016) carried out experimental infections on age categories (1-day-old, 21-day-old and adult) of pheasant, and birds were not susceptible to *C. avium* infection. A high percentage of *C. baileyi* infections were also described by Pavlásek and Kozakiewicz (1989), who reported its occurrence in all four pheasant aviaries examined on Polish farms. Musaev et al. (1998) reported *Cryptosporidium* sp. infections in 7.9% (16/203) pheasants. In the study by Barvíř (2012) presented only negative results for the presence of *Cryptosporidium* during the examination of two breeds from the Czech Republic.

Several factors, such as stress, rearing densities, infection seasonality, contamination of the environment, oocyst excretion, persistence and infectivity may generate immune suppression and thus affect the seasonal occurrence and prevalence of cryptosporidial infections in bird farms (Blanco et al. 2001; Moller et al. 2003; Villanúa et al. 2006).

Molecular data were presented only by Pagès-Manté et al. (2007), who used PCR-RFLP techniques and sequencing of oocyst wall protein (COWP) gene fragment from faecal samples of *C. meleagridis* from red-legged partridge. To our best knowledge, characterizations by molecular analyses have never been done from isolates from *P. colchicus*. Because of the current lack of more detailed information on these parasitic species, and the common breeding of their bird hosts in many countries, further research on both bird species is required for a better understanding of cryptosporidian epidemiology, and to establish a sub-genotype analysis at the 60-kDa glycoprotein (GP60) locus.

3.3 *Eimeria* infections in game birds

The protozoan parasites of the genus *Eimeria* (Apicomplexa: Eimeriorina: Eimeriidae) represent a large group of species (>2000 described forms, >79 species from Galliformes). Most species have been described based on oocysts morphology and host species. Species of *Eimeria* are often considered (majority) to be strictly host-specific, but some are not restricted to a single host species (Vrba & Pakandl 2015). Representatives of this genus cause coccidiosis, a worldwide economically important

disease because of the ability of parasite to development resistance against drugs. According to Duszynski et al. (2000) and Fernández-Álvarez et al. (2016), six *Eimeria* species have been identified from the intestine of *A. rufa*, namely: *E. caucasica* Yakimoff et Buewitsch, 1932; *E. gonzalezcastroi* Lizcano-Herrera et Romero-Rodríguez, 1975; *E. kofoidi* Yakimoff et Matikaschwill, 1936; *E. legionensis* Cordero de Campillo et Pla Hernández, 1966; *E. padulensis* Romero-Rodríguez et Lizcano-Herrera, 1974; and *E. procera* Haase, 1939. An outbreak of coccidiosis (*E. kofoidi* and *E. legionensis*) in red-legged partridges was reported by Bolognesi et al. (2006) in a game farm in Italy, which caused high mortality, 52.1% and 46.5%, respectively. Another outbreak of coccidiosis caused by three species (*E. caucasica*, *E. kofoidi* and *E. legionensis*) was described by Naciri et al. (2011) in France, who described the pathogenicity by experimental infections and provided oocyst characteristics. Naciri et al. (2014) experimentally infected *A. rufa* with three coccidia species and concluded that depressed growth is higher in dual and triplet infections in comparison with single ones, and found that only *E. legionensis* caused mortality (4.3%).

The occurrence of coccidia in the breeding of pheasants is also described, due to their spreading and common use for hunting in many countries. Much more authors (see below) have dealt with the problematic of *Eimeria* from pheasants. According to Duszynski et al. (2000), there are 7 *Eimeria* species identified in *P. colchicus*: *E. colchici* Norton, 1967; *E. duodenalis* Norton 1967; *E. langeroni* Yakimoff et Matschousky, 1937; *E. megalostoma* Ormsbee, 1939; *E. pacifica* Ormsbee, 1939; *E. phasiani* Tyzzer, 1929; and *E. tetartooimia* Wacha, 1973. The most significant pathogens from pheasants are *E. colchici*, *E. duodenalis*, and *E. phasiani*.

There have been several review articles and theses focused on the pathogenicity, chemotherapy, prevalence, species spectrum, endogenous development of coccidia, and immunological responses of host to these parasites (Goldová et al. 1998, 2000, 2001; Liou 2001; Loószová et al. 2001; Šavlík et al. 2005, 2007; Fuller et al. 2008; Pavlović et al. 2012a; Griffith et al. 2014). Prevalence of four species (*E. duodenalis*, *E. pacifica*, *E. phasiani*, and *E. tetartooimia*) in game-farmed pheasants in Iowa was studied by Fisher (1973). In that study, the author mentioned the first study on coccidia from pheasants by M'Fadyean (1894) from England on a group of birds, which exhibited a high mortality rate (see Fisher 1973).

Eimeria species were studied from pheasants by Páv and Zajíček (1968), Bejšovec (1970, 1972, 1973), and Chroust (1990) in the Czech Republic. In those times, it was assumed that their *Eimeria* species spectrum was identical with that of *G. gallus* and thus leading to mutual exchange. According to the above mentioned authors, traditional intensive rearing of *G. gallu*s in fields of harvested grains and presence of high numbers of pheasants in environment, led to transmissions. Pavlásek (1971) compared the antigenicity of the most pathogenic species, *E. tenella* (Railliet et Lucet, 1891) Fantham, 1909, from hens (*G. gallu*s) and pheasants and found no binding of the labelled antibodies in pheasant sera to antigen from chicken species. He warned that the mutual exchange between domestic chicken and pheasant is not possible and both species have their specific coccidia. Similarly, Doran (1978) informed about the ability of *E. dispersa* Tyzzer, 1929 to complete its life cycle in gallinaceous birds (chicken, partridge, pheasant) from Turkey, but were not confirmed by Vrba and Pakandl (2015), who realized cross-transmission experiments with coccidia from *M. gallopavo* and *G. gallus* to *A. rufa* and *P. colchicus* with no ability to complete the life cycle.

Host specificity of *Eimeria* species is still in focus of researchers. According to new data by recent studies, the main problem with the specific identification is due to incomplete/mistaken taxonomy (Vrba & Pakandl 2015; Fernández-Álvarez et al. 2016). Many researchers have re-described most pathogenic species by using experimental infections, molecular diagnostic, morphological features and life cycles (Bolognesi et al. 2006; Yang et al. 2014, 2016; Vrba & Pakandl 2015; Fernández-Álvarez et al. 2016).

Problematic of pheasant coccidiosis is still alive also from the view of high morbidity and mortality after many years of research. Goldová et al. (2006) after examination of faecal samples reported prevalence of coccidiosis 64% (up to 2-weeks-old) and 73% (2–8-week-old) of pheasant chicks in confined systems in Slovakia. Gassal and Schmăschke (2006) detected three species of coccidia (*E. duodenalis*, *E. phasiani* and *E. tetartooimia*) in 41% of all examined pheasants in two pheasantries from Germany. Higher prevalences of *E. phasiani* (49.7%) and *E. colchici* (19.0%) resulted from faecal examinations from pheasanteries, which were different than those from natural conditions (*E. phasiani* 17.4% and *E. colchici* 3.3%) (Konvičková 2008). From 2011 to 2012, the prevalences of *Eimeria* spp. were 30.4% and 22.5% in two breeds from the Czech Republic (Barvíř 2012).

3.4 Helminthic infections

Parasitologists have long studied helminth infections in game birds. According to our knowledge and experiences from diagnostic of helminth infections during routine post-mortem examinations and coprology in the laboratory of parasitology, State Veterinary Institute Prague, nematodes (*Capillaria* s.l., *Heterakis gallinarum*, *Syngamus trachea*) and cestodes (*Choanotaenia* spp., *Raillietina* spp.) are the most common helminth parasite of pheasant. Examinations of partridges are rare in comparison with other game bird species, but have similar nematode fauna than pheasants. There are checklists of the helminth parasites of wild and captive birds of the genus *Alectoris* (including *A. rufa*) from Spain, Italy, and Portugal (Villanúa et al. 2007; Millán 2009). Helminth infections are usually accompanied by protozoans (Pavlásek & Šverma 2009), and vice versa; therefore, it is often difficult to assess the impact on the host in terms of individual groups or parasite species and more studies on the parasite fauna must be done, like those of Gassal and Schmăschke (2006), Goldová et al. (2006) and Konvičková (2008). Individual groups of helminths were found and described by many authors around the world in game bird species, where the incidence or prevalence in intensive farming conditions is often very high. Pavlović et al. (2003) found helminth infections in 42% of up to 14 weeks old pheasants and in 33% of adult birds. Often, helminths are considered as the main reason for increased mortality (which also applies to protozoan infections), morbidity or bird production, thus resulting in huge economic losses for breeder.

3.5 Capillariid infections in game birds

Capillariid nematodes (Capillariidae Railliet, 1915) have been reported in domestic and wild birds from many countries (Anderson 2000). According to Moravec (2001), capillariids of *P. colchicus* are: *Aonchotheca burzata* (Freitas et Almeida, 1934); *A. caudinflata* (Molin, 1858); and *Baruscapillaria obsignata* (Madsen, 1945) in the small intestine of birds; *Capillaria phasianina* Kotlán, 1940 in caeca; *Eucoleus annulatus* (Molin, 1858) in oesophagus, beak and buccal cavity; *E. contortus* (Creplin, 1839) in the mucosa or submucosa of oesophagus, beak, buccal cavity and stomach. The taxonomic history of this family is described in Moravec (2001), although most

medical and veterinary literature ignores this classification and considers all capillariids as *Capillaria* spp.

There are several studies dealing with the prevalence and species spectrum of capillariids in pheasants and partridges. For example, Pavlović et al. (2003) investigated 12 pheasanteries in Serbia and found *C. gallina*e (syn. *C. caudinflata*) (Kowalewski, 1859), *C. columbae* (syn. *C. obsignata*) (Rudolphi, 1819) and *C. phasianis* (Kotlan, 1940) with low prevalences (< 10%) in *P. colchicus*. Gürler et al. (2012), on a farm from Turkey, found faecal samples positive to *Capillaria* spp. eggs (28.6%), and five species during necropsy: *C. annulata* (17.6%), *C. bursata* (35.3%), *C. caudinflata* (23.5%), *C. contorta* (64.7%), *C. obsignata* (5.9%). A similar situation occurred in Slovakia (prevalence 38.4%), and Germany (prevalence 67.5%, five species: *C. annulata*, *C. bursata*, *C. contorta*, *C. perforans*, *C. phasianina*) (Goldová et al. 2006; Gassal & Schmäschke 2006). Lower prevalence of *Capillaria* spp. (24.1% and 27.5%) was reported by Barvíř (2012) from two aviaries in the Czech Republic.

Studies on the helminth fauna of *A. rufa* are common, especially due to the releasing of farmed birds into the wild, which may introduce parasites in some regions. Millán et al. (2004) carried out a study on red-legged partridges and found a prevalence of 10.7% for *E. contortus* and 13.9% for *A. caudinflata* in farmed birds, while wild birds were negative to both parasite species. Similarly, Villanúa et al. (2008) studied the helminth community of *A. rufa* from Central Spain, with findings of *A. caudinflata* (prevalence 6.5%) and *E. contortus* (prevalence 2.2%) in managed areas, and no findings in wild. Occurrence of capillariid species from *A. rufa* has not been yet documented from the Czech Republic.

3.6 Ascaridida infections in birds

Nematodes of the genera *Heterakis* Dujardin, 1985 and *Ascaridia* Dujardin, 1845 are the most commonly recognized parasites affecting a wide variety of wild and domestic bird species. *Heterakis* is associated with the transmission of *Histomonas meleagridis* via eggs and causes the so called blackhead disease (histomoniasis), larval and adult nematodes occur in caecum. *Heterakis dispar* (Schrank, 1790), *H. gallinarum* (Schrank, 1788), *H. isolonche* (Schrank, 1798), and *H. tenuicaudata* (Linstow, 1863) are the most common species diagnosed in wild populations and in higher occurrence

under intensive artificial breeding systems of ring-necked pheasants and red-legged partridges in Europe (Calvete et al. 2003, 2004; Goldová et al. 2006; Villanua et al. 2007, 2008; Pavlović et al. 2012a). An exhaustive list of phasianid avian hosts infected with *Heterakis* spp. is lacking, although their geographic location was summarized by Atkinson et al. (2008) in the book focuses on parasitic protozoans, helminths, leeches, and ectoparasitic arthropod of wild birds. In areas where *Heterakis* spp. are common in *P. colchicus*, prevalence can reach high percentage 31.7%–100% (Draycott et al. 2000; Goldová et al. 2006; Gürler et al. 2012). Gürler et al. (2012) reported 17.2% of faecal samples positive to *Heterakis* spp., and 58.8% of necropsied birds were infected by *H. gallinarum* in a Turkey farm. In Czechoslovakia, *H. gallinarum* has been reported in a number of studies with high prevalence up to 86.5%) (Páv & Zajíček 1968; Prokop et al. 1969). In the Czech Republic, Barvíř (2012) observed 3.8% and 7.5% of positivity at two aviaries, with age related dynamics positivity (0.6% of chicks and 3.0% of adults). For the first time, *H. gallinarum* and *H. phasianina* from *A. rufa* were found by Clapham (1935) and two management models were examinated. *Heterakis gallinarum* with prevalence 3.8% in wild populations and 17.4% in managed area (farm) were detected by Villanúa et al. (2008).

Phasianus colchicus and *A. rufa* are also parasitized by members of the genus *Ascaridia*, such as *A. galli* (Schrank, 1788), *A. columbae* (Gmelin, 1790), and *A. compar* (Schrank, 1790) (see Pavlović et al. 2003; Millán et al. 2004, partly summarized by Atkinson et al. 2008). In up to 14 week-old pheasants, prevalence of *A. galli* and *A. columbae* was 13 and 10.3%, respectively; higher prevalence in adult pheasants reached 23.1% for *A. galli* and 18.2% for *A. columbae* (Pavlović et al. 2003). Santilli and Bagliacca (2012) studied farm-reared birds as possible carriers of parasites and diseases to natural populations in Italy, and found eggs of *Heterakis* spp. and *Ascaridia* spp. (7.6% in restocking areas vs. 3.9% in wild areas) in both groups. Similarly, *A. galli* and *A. compar* were found in farm-reared *A. rufa* in Spain, with a prevalence of 1.6% and 25.4%, respectively (Millán et al. 2004). Baruš (1966) informed that gray partridges (*P. perdix*) were host of *A. compar* (prevalence 2.6%) in Czechoslovakia.

3.7 *Syngamus* infections in game birds

Syngamiasis is caused by parasitic tracheal nematodes of the genus *Syngamus* (Siebold, 1836) and has a worldwide distribution. The most common representative species is *S. trachea* (Montagu, 1811), which has been documented in domestic, exotic and wild birds (e.g., Bejšovec 1976; Nevárez et al. 2002; Pavlović et al. 2003; Pavlásek & Šverma 2009; Andrepoulou et al. 2012). In pheasants, *S. trachea* has been reported in many studies (see Wójcik et al. 1999; Pavlović et al. 2003; Gassal & Schmăschke 2006; Goldová et al. 2006), whereas in reared partridges were also mentioned as a potential host of this non-specific parasite, for example, Pavlásek and Šverma (2009) reported a positivity percentage from 20–100% in *P. perdix*; while Andrepoulou et al. (2012) found a case of syngamosis in *A. chukar* from a backyard farm, which causes mortality. *Alectoris rufa* can serve as host species for *S. trachea* (see Millán 2009).

3.8 General methodology of parasitic examinations during the study of spontaneous endoparasitic infections

For determination of parasites in animals under intensive artificial breeding conditions, examination by coprological methods is essential. The qualitative detection of parasitic developmental stages (cysts or oocyst of protozoa, eggs or larvae of helminths) can be carried out by various flotation solutions with specific gravity (1.2–1.3 are commonly used). The standard method used during a routine examination of animals is the flotation-centrifugation coprological technique, according to Breza (1957), although many other methods are also available (see Kváč et al. 2003). This standard method was described in detail and is still valid by Team of authors (1989). The intensity of occurrence of endogenous stages is evaluated in 1–3 g of excrement sample in most cases. Intensities are expressed by crosses (1–5), according to the number of parasite stages in the entire preparation or number at a certain microscope magnification in a single vision field. During quantitative examination, 1 g of faeces is used. Intensity of oocysts, cysts, eggs, or larvae are determined by various techniques, like the so-called chambers (e.g., McMaster or Bürker-Türk counting chambers) (see Vadlejch et al. 2013). Chambers are recommended especially when determining the effectiveness of chemotherapeutics or during experimental infection process. Evaluation

using this method during the dynamics of spontaneous infection is very problematic and often may not reflect the specific situation in the breed.

Incomplete helminthological autopsies are performed by using the native preparation or coprological methods. To detect protozoan, the most commonly used technique are the smears of mucosal epithelium of organs or tissues, which are processed by conventional staining methods (Giemsa) or histology. For detection of helminth species, the examination is based on the gradual washing and review of individual sections.

Nowadays, new diagnostic kits, sets, equipment for simplification and flotation, automatic counting machines and computer programs, are presented and utilized in some laboratories (e.g., Mini-FLOTAC, FLOTAC, FECPAK, FECRT). Recently, Slusarewicz et al. (2016) described the first smartphone-based parasite faecal egg counting technique. Comparisons of the results obtained by those new techniques are presented in many scientific papers (Rinaldi et al. 2014; Godber et al. 2015; Lima et al. 2015).

All above mentioned techniques are routinely use and cheap in comparison with molecular methods. Molecular detection, differentiation and genotyping are becoming more applied in both diagnostic and research settings; advantages of those methods are numerous (see Chalmers & Katzer 2013; Verweij & Stensvold 2014).

3.9 Treatment

Using different management practices means struggling with different bacterial and parasitic infections (Díaz-Sánchez et al. 2012; Santilli & Bagliacca 2012). Rearing under intensive breeding conditions causes problems with the frequency of parasitic infections in game birds, as in the case of *G. gallus* and *M. gallopavo*, where breeders and veterinary supervision deal with the occurrence of protozoan and helminth infections. Therefore, in numerous breeding facilities preventive measures or treatment of farmed birds are necessary, but could be very expensive and sometimes ineffective due to many reasons. Prevention is frequently mentioned in connection with the breeding of animals, since treating a disease is much more difficult, especially without early diagnosis and administration of an effective treatment against infections that causes high economic losses in bird rearing industry. Even though various treatments

have long been permitted, some chemotherapeutics are limited by the legislation for game bird species, according to Commission Regulation (EC) No. 2205/2001 (EC 2001).

Anthelmintic and anticoccidial resistant parasites are an increasingly serious problem. Especially, in the case *Eimeria*, one of the most economically important bird parasites, whose resistance pressures rise. Although animal health research has provided effective prevention strategies for the major parasite infections, billions of dollars are still payed to control coccidiosis in poultry industry and also in intensive artificial breeder systems of game birds. After more than 80 years of research on coccidiosis, scientists are still working on this problematic and maybe it will be for 40 more years. Coccidia will still require control by providing to farmers a larger armoury and control strategies (see Shirley & Lillohoj 2012).

Coccidiosis in pheasant was commonly treated by using amprolium. Patton et al. (1984) tested its efficacy against *E. colchici*, *E. duodenalis*, and *E. phasiani* and got successful survival in treated birds. After many years, this drug has lost its efficacy to control coccidiosis on pheasants. Nowadays, sulfachlorpyrazine (Esb3), sulfonamides and sulfonamide combinations (Sulfadimidin, Sulfakombin, Sulfacox T) or toltrazuril (Baycox) are being used for the treatment of pheasants and partridges in the Czech Republic (Forejtek & Chroust 2010a). According to the European Commission of Regulation (Regulation No. 2205/2001), amprolium was excluded from the list of approved anticoccidials (EC 2001).

Parasitic infections caused by nematode genera *Capillaria*, *Heterakis*, *Syngamus* are treatable with fenbendazole (Panacur), mebendazole, ivermectin, flubendazole (Flubenol) or levamisole (Chemisol) (Šavlík et al. 2005, 2007; Forejtek & Chroust 2010b; Pavlović et al. 2012b).

In recent studies, more researchers have focused on developing highly sensitive and specific molecular diagnostic tests for accurate diagnosis. The use of new technologies and new reagents, derived from analyses of the genome sequence, reveals more knowledge about anthelmintic resistance. Chapman and Jeffers (2014) conclude that alternation of drug during vaccination in the field could result in restoration of drug sensitivity, by introduction of drug-sensitive parasites into the houses.

4. Material and methods

This study was carried out during seasons 2012–2015 in an intensive artificial breeding programme in the Czech Republic, based on long tradition and experiences of ring-necked pheasants and red-legged partridges breeding.

4.1 Partridges

4.1.1 Area and breeding technology characteristics

During three rearing seasons (July-December 2012–2014), faecal pooled samples (n= 900) and post-mortem examinations (n= 99) of *A. rufa* from an aviary in the Central Bohemian Region (Czech Republic) were processed. The annual hatching at this site reaches many thousands of individual *A. rufa* and *P. colchicus*, where the arrangement of breeding facilities for both species prevents mutual contact. The reserved area is divided into separate uphill and downhill sections, each containing a system of eleven closed sheltering aviaries. Later, ~5week-old birds were released to five paddock groups, separated by wire fences with shelters in each section.

4.1.2 Sample collection

Pooled samples (fresh excrements with contents of caecum and other parts of the intestinal tract) were collected from the same area in the morning before noon. Pooled samples represent one aviary or later, after combining, one paddock group (approximately 20–30 individual excrements of the red-legged partridges). Screening was performed over 7 day intervals and daily after first detection of *Cryptosporidium* spp. oocysts. All samples were directly transported to the Department of Pathology and Parasitology, State Veterinary Institute in Prague and stored at 4°C.

4.1.3 Coprological examination

The flotation-centrifugation coprological method was used for examination of faeces and intestinal contents and the detection of parasitic stages (oocyst of protozoa,

eggs of helminths) according to Breza (1957). *Cryptosporidium*-positive faecal samples were processed in wet mounts using glycerin (see Pavlásek 1991). Oocysts of *Eimeria* spp. and eggs of Capillariidae gen. sp. were identified up to genus and family levels, respectively, whereas oocysts and eggs of *Cryptosporidium baileyi*, *C. meleagridis*, *Heterakis gallinarum* and *Syngamus trachea* were specifically identified.

Unsporulated coccidian oocysts (genus *Eimeria*) were put into Petri dishes with 2.5% potassium dichromate, left to sporulate at room temperature 23°C (± 2°C), then identified up to the species level. All samples positive for *Cryptosporidium* spp. were also stored in 2.5% potassium dichromate at 4°C for further processing by molecular analysis.

4.1.4 Post-mortem examination

Ninety-nine red-legged partridges were provided to the laboratory for incomplete parasitological evaluation using three methods (flotation-centrifugation, native preparations, and native preparation using glycerin). For the detection of parasitic stages, scrapings (unstained wet mounts) of the larynx, trachea, crop, livers, intestinal mucosa, bursa of Fabricius and cloaca were examined. Helminthic specimens were isolated and fixed in 4% formalin, 70% and 100% alcohol, and identified to the species level with the help of available identification keys and other specialized literature (e.g., Moravec 2001; Anderson et al. 2009). For detection of *Cryptosporidium* developmental stages, one positive specimen from the post-mortem group of birds (scrapings of intestinal mucosa, bursa fabricii and cloaca) was used for air-dried smears fixed with methyl alcohol, stained in Giemsa solution, and fixed in 10% formalin for histopathology. Examinations were carried out using a Leica DMLB optical microscope and a Leica MZ6 stereomicroscope.

4.1.5 Drug administration

Drug administration (active constituents: flubendazole, levamisole, sulfachlorpyrazine, sulphadimidine, toltrazuril used during study, according to breeder documentation) was without the intervention and influence of the author and was carried out under veterinary supervision.

4.1.6 Molecular analysis

Genomic DNA was extracted from concentrated oocysts isolated from faeces using Sheather sugar solution, followed by bead disruption homogenization and using the QIAamp®DNA Stool Mini Kit (Qiagen) with minor modifications to the manufacturer's instructions. Molecular characterization was conducted by nested polymerase chain reaction (PCR) analysis of molecular markers: ~400 bp HSP70, ~800 bp actin and ~900 bp fragment of GP60 genes. Gene amplification and sequencing were performed according to Morgan et al. (2001), Ng et al. (2006) and Stensvold et al. (2014). PCR products were visualized on an agarose gel, and successful amplification products were purified using the QIAquick®Gel Extraction Kit (Qiagen) in accordance with the manufacturer's instructions. Sequencing was carried out in both directions using secondary PCR primers at SVI Prague and the resulting contigs were compared with sequences published in GenBank using BLAST. Representative partial sequences of isolates were compared and deposited in the GenBank database under accession Nos. KM822866, KM822867, KP703166 - KP703170.

4.2 Pheasants

Game birds, *P. colchicus*, have historically been artificially bred annually, in lots of 10,000 individuals, in selected facilities in the Central Bohemian Region (Czech Republic).

4.2.1 Breeding facilities

In general, pheasant chicks were placed within brooder houses after hatching, for different periods of time (up to 17 weeks); some were then moved into flight pens (age class >7 weeks) and then released to open areas (from >8 weeks). Movements or relocations are described in Máca and Pavlásek (2016) (see **Appendix II;** Figs. 1 and 2). Breeding technologies were almost identical in all locations and facilities, and birds were monitored daily with dead individuals being collected. One of these facilities also harboured specimens of red-legged partridge (*A. rufa*), although without contact with other bird species.

4.2.1.1 Brooder house (BH)

All selected BHs (n= 7) had concrete floors (covered by a paper carpet during the first weeks), divided into several connected parts that are cleaned mechanically and chemically annually. From hatching until approximately 3–5 weeks, birds remained in BHs with or without outside runs. Later, depending on temperature and humidity, some birds went outside (up to an age of 17 weeks) to sandy, clay/muddy or grassy grounds. Well water and commercial food for pheasants were given daily in feeders and bowls. Some facilities were used annually for more hatchings (BHs 1, 2a, 2b, 7) or restocked from BH 7 (BHs 4, 5), but previous pheasants were all moved out before new birds were introduced. BHs 1 and 6 harboured many enclosures inside one building, with access to outdoor runs. BHs 2a, 3, 4, and 5 were small separated buildings connected with outdoor runs. BH 2b was an experimental wooden facility with an outdoor run designed for breeding 50–200 pheasants. BH 7 differed in respect of a lack of access to outdoor runs, and birds after age 3–5 weeks were moved to BHs 4 and 5.

4.2.1.2 Flight pen (FP)

Depending on weather, some pheasants were released and combined in different outdoor enclosures, such as flight pens or enclosed paddocks that had clay/muddy or grassy surfaces. These pens were also supplemented with water and food.

4.2.1.3 Open area (OA)

Older age classes of birds were released to open areas (release sites), and supplemental feeding continued, but with the possibility of free feeding, water supply and movement in a neighbouring agricultural landscape.

4.2.2 Sampling method

All pooled faecal samples (approximately 5–10 individual excrements) were collected from newly hatched up to released birds. The majority of fresh samples were

collected at 7-day intervals, placed in labelled plastic bags and transported within 12 h to the Department of Pathology and Parasitology, State Veterinary Institute Prague.

4.2.3 Coprological examination

The flotation-centrifugation coprological method was used for the detection of oocysts according to Breza (1957); positive findings were reviewed using glycerin according to Pavlásek (1991). Faecal samples and intestinal contents, where morphologically identical oocysts of *Cryptosporidium baileyi* and *C. meleagridis* were found, were mixed with 2.5% potassium dichromate, stored at 4°C and used as isolates for molecular analyses. In 2014, oocysts morphologically similar to *C. galli* were obtained from a single examination of four pooled faecal samples of pheasants (36-week-old group) from another artificial breeding site in the same region. Examination processes were similar to those of Chapter 4.1.3. A total of 85 pooled faecal samples were processed for examination during 2012 (July to November) in a preliminary study. Subsequently, 173 and 238 faecal samples were examined over the whole breeding seasons of 2013 and 2014 (May to December). In 2015, examination of 340 faecal samples was carried out during May to August.

4.2.4 Post-mortem examination

Post-mortem methods were chosen to confirm the findings of the coprological examinations. Dead birds were transported to the laboratory within 12–24 h, in most cases, in various stages of autolysis. Incomplete parasitological evaluations of birds were carried out in the same way like partridges (see Chapter 4.1), with minor changes. During 2012 (July to November), 36 post-mortem examinations of pheasants were processed. Subsequently, 219 and 168 were examined during the whole breeding 2013 and 2014 seasons, respectively. During 2015, 91 dead pheasants were examined only by flotation-centrifugation method.

4.2.5 Molecular analysis

Cryptosporidium baileyi (n= 8) and *C. meleagridis* (n= 3) isolates from different housing facilities in 2014 were used for molecular characterization. During April 2014, *C. galli* (n= 1) oocysts were obtained by coprological examination of pheasants in different breedings from the same region. DNA was extracted from isolated oocysts using the QIAamp®DNA Stool Mini Kit (Qiagen) according to the manufacturer's protocol. Molecular characterization was conducted by nested PCR analyses of the small subunit (SSU) of ribosomal RNA (rRNA), heat shock protein 70 (HSP70) and actin and glycoprotein 60 (GP60) loci. Gene amplification and sequencing were carried out using published methods of Morgan et al. (2001), Ng et al. (2006) and Stensvold et al. (2014). PCR products were visualized on an agarose gel, purified using the QIAquick® Gel Extraction Kit (Qiagen) or High Pure PCR product purification kit (Roche) and sequenced in both directions at SVI Prague, and the resulting contigs were compared with sequences published in GenBank using BLAST (Basic Local Alignment Search Tool). The representative sequence data from this study were deposited in the NCBI database (nos. KU221101 to KU221106).

Sequence alignments were performed using BioEdit v7.2.5. Submission of sequences to GenBank was done using Sequin Application v15.10.

4.2.6 Drug administration

Drug administration was similar to that of Chapter 4.1.5.

5. RESULTS

5.1 Determination of the parasite fauna of the ring-necked pheasant (*P. colchicus*) and red-legged partridges (*A. rufa*)

A total of 1736 pooled excrement samples (836 from pheasants and 900 from partridges) were examined for determination of the parasite fauna. Post-mortem examinations were also carried out on 613 dead birds (514 pheasants and 99 partridges), during 2012–2015 from an intensive artificial breeding programme/aviary in the Czech Republic.

The result of examinations clearly shows occurrence of protozoan and helminth parasite taxa.

5.1.1 Coprological and post-mortem examination results

Most pooled faecal samples were collected from newly hatched up to released birds, in order to describe in detail the spontaneous infections in both hosts, as well as the percentage of positive samples and age-associated dynamics. Main infections of *A. rufa* (rearing season 2012–2014) and *P. colchicus* (2013–2014) comprised representatives of the protozoan parasites *Eimeria* and *Cryptosporidium*; as well as the nematodes Capillariidae gen. sp., *Heterakis gallinarum* and *Syngamus trachea* **(Appendix III**, **V**, Table I, II).

To confirm the findings of the coprological examinations, post-mortem examinations were performed during four seasons (2012–2015) and partly summarized for pheasants **(Appendix V**, Table II); for partridges during three seasons (2012–2014) see **Appendix III**.

5.1.2 Endoparasite fauna summary

A total of four protozoans species were found in *A. rufa*: *C. baileyi* (sporadic occurrence), *C. meleagridis*, *E. kofoidi* and *E. legionensis*. Moreover, four helminths: *Capillaria phasianina*, *Eucoleus perforans*, *H. gallinarum* (sporadic findings), and *S. trachea* were found.

On the other hand, the parasite fauna in *P. colchicus* was also composed of four protozoan species: *C. baileyi*, *C. meleagridis* (sporadic occurrence), *E. duodenalis* and *E. phasianina*, and other *Eimeria* spp. (sporadic occurrence), and five helminths: *C. phasianina*, *E. perforans*, *H. gallinarum*, *S. trachea*, and *Choanotaenia* eggs. Both host species were also infected by *Histomonas meleagridis*-like lesions on liver (one partridge and seven pheasants were supposedly positive).

5.2 Infection pathway and onset of parasitic infections until hunting season

Infection pathway and onset were determined and the critical periods of parasite occurrence under intensive artificial breeding programmes conditions are described in **Appendix II, V** for pheasants and in **Appendix I, III** for partridges, from hatching up to release of birds to open area.

5.2.1 *Eimeria* and helminth spontaneous infections of partridges

Oocysts of *Eimeria* first occurred at age 2–3 weeks, with maximum intensities during weeks 7–8 (2012) and 6, 20–21 (2013) in *A. rufa*, percentages of positivity were 35% and 27% during whole rearing seasons. During 2014, oocysts first occurred at age 4 weeks (**Appendix III**).

Eggs of Capillariidae gen. sp. occurred in 47% and 57% samples obtained from *A. rufa* at age 11–22 weeks, with a highest positivity of 85% and 100% at age 15–18 and 19–21 weeks, respectively. Findings of *S. trachea* eggs in *A. rufa* were in 29% and 40% samples at age 8 and 10–22 weeks, with highest positivity (65%) at age 13–14 and 19–21 weeks during 2012–2013. During 2014, eggs of Capillariidae gen. sp. first occurred at age 5–7 and *S. trachea* in 8–12 weeks. Sporadic findings of *H. gallinarum* eggs were in older groups only during 2013 and 2014 (**Appendix III**).

During 2012, examination of 51 birds showed that *S. trachea* had the highest prevalence (20%) in 6–19 week-old birds. Capillariidae gen. sp. was found in 4 dead birds, in 2 of them *E. perforans* in the crop and *C. phasianina* in the cecum. *Cryptosporidium meleagridis* was diagnosed in 3 birds along with *E. perforans* or *S.*

trachea, and *E. perforans* with *C. phasianina* and *S. trachea*, respectively (**Appendix III**).

In the 2013 season, 38 dead birds were examined and the prevalence of *S. trachea* was again the highest (26%) in 7–18 week-old birds as single infections. There were also 3 mixed infections: *Eimeria* spp. and *S. trachea*, and two cases of *E. perforans* and *S. trachea*. *Eimeria* spp. single infections were also present (8% prevalence) (**Appendix III**).

For 2014, there were only 10 dead birds examined and results were similar to those of year 2013. The prevalence of *S. trachea* was 20% and occurred in 12–13 week-old birds. Only one case of *Eimeria* spp. (single infection) was diagnosed in a 6 week-old bird. A mixed infection (*Eimeria* spp. and *S. trachea*) occurred in 12 week-old birds (**Appendix III**).

5.2.2 *Eimeria* and helminth spontaneous infections of pheasants

Other protozoan and helminthic infections were also found in pheasants, as in the case of *A. rufa*. Oocysts of *Eimeria* were the most common finding (82% total percentage of positivity during 2013 and 2014), first occurred at age 1–2 weeks during both seasons, maximum intensities occurred during many weeks (see **Appendix V**, Table I), percentage of positivity were 52% to 100% during whole rearing seasons. Eggs of Capillariidae gen. sp. occurred in 18% and 42% samples at almost all age classes, with a highest positivity (57% and 88%) at ages 15–18 and 19–24 weeks, respectively. Findings of *S. trachea* eggs were in 25% and 40% of samples at same age classes as Capillariidae gen. sp., with highest positivity (50% and 72%) at ages 13–14 and 5–7 weeks during 2013 and 2014 (**Appendix V**).

For all four seasons, *Eimeria* spp. was the most prevalent (31–56%) infection with *E. phasiani* as the most common species. During 2012, examination of 36 birds showed that *Eimeria* spp. (56%) and *H. gallinarum* (53%) had the highest prevalences. *Syngamus trachea* had the highest prevalence (28%) in 2012. Capillariidae gen. sp. was found with 17% prevalence, with finding of *E. perforans* in the crop. For 2013, 219 dead birds were examined and the prevalence of *Eimeria* spp. was the highest (47%) first occurred at first week, but also common in older birds. Coccidial infections were

followed by Capillariidae gen. sp. (7%), *S. trachea* (7%), *H. gallinarum* (6%) and other. In 2014, *Eimeria* spp. was the highest (31%) with *H. gallinarum* (20%), *E. perforans* in the crop and *C. phasianina* in the cecum were also found. *Choanotaenia* eggs were found during this season. Season 2015 was nearly the same like in 2013, with findings of *Eimeria* spp. (51%) followed by Capillariidae gen. sp. (6%), *S. trachea* (6%) and *H. gallinarum* (6%) (**Appendix V**).

Mixed infections are common in older bird classes in 2012 in comparison to season 2015, where samples were collected from younger birds. Seasons 2013 and 2014 were very similar in the amount of single and mixed infections (**Appendix V**). The most common single infection of pheasants was *Eimeria* spp. with prevalences of 36% (13/36), 33% (72/219), 19% (32/168) and 45% (41/91) during seasons 2012–2015, respectively.

5.3 Cryptosporidial infections

5.3.1 Cryptosporidial spontaneous infections of partridges

Parasitological examination was carried out on two groups of partridges at age of 1 day until 19 or 22 weeks. Analysis of the results from 2012–2014, were made from a total of 900 pooled samples of excrement during three semesters (see **Appendix I, III**).

The first report of spontaneous infection with *C. baileyi* and *C. meleagridis* in the red-legged partridge was described. *Cryptosporidium meleagridi*s occurred in 42 samples (prevalence 17%) in 2012, 99 samples (24%) in 2013, 18 samples in 2014 (8%) mainly in 13, 15–18, 19–21, and 22 week-old age classes; whereas 4 samples (1%) of the 5–7 week-old age class in 2013 and 1 sample (0.4%) in 2014 harboured *C. baileyi* (**Appendix I, III**).

Oocysts were present in ileal and cecal (proximal and middle part) contents during 2012 in three individuals out of 51 examined (prevalence 6%) within the 18–20 week old age class. Developmental stages were found in the microvillous region of the ileum and in the epithelium of the proximal cecum, at low and high levels, respectively

(**Appendix I**, Fig. 1). Results of all other post-mortem examinations were *Cryptosporidium*-negative.

5.3.2 Cryptosporidial spontaneous infections of pheasants

In pheasants, from July to November 2012, preliminary coprological examinations were carried out to 85 pooled faecal samples of different aged ring-necked pheasants, where *Cryptosporidium* oocysts were detected in 12 samples (14.1%). This finding was supported by the presence of *C. baileyi* and *C. meleagridis* oocysts in intestinal, cloacal contents, and/or bursa of Fabricius in 9 from 36 examined dead pheasants (prevalence 25%) (**Appendix II**).

Main results are from complete rearing seasons 2013 and 2014. Most important were the description and study of cryptosporidial infections as mono or co-infections, which have never been studied before. *Cryptosporidium*-positive were 14.5% of pooled samples tested during 2013 (173 tested/25 positive) and 18.1% during 2014 (238/43). All tests were verified as being *Cryptosporidium* positive in 9 from 219 (prevalence 4.1%) and 4 from 168 (prevalence 2.4%) post-mortem examinations. Significantly, *C. baileyi* was found more frequently in faeces, with positivity ranging from 11.1 to 100% (4–>16-week-old pheasants). Oocysts of *C. meleagridis* were detected at ages 6–>15 weeks ranging from 7.1 to 100 % in faeces during the rearing seasons. The burdens of *C. baileyi* (7 of 14 and 10 of 16) and *C. meleagridis* (5 of 14 and 7 of 16) for each year, in monitored brooder houses, flight pens and spread across all open areas were finished (**Appendix II**). The study focused on showing pathways (graphically described in detail) of infection of *C. baileyi* and *C. meleagridis* during the whole rearing seasons of pheasants concluded by consideration that released birds from breeding to open area are potential source of parasitic infections (**Appendix II**, Figs. 1, 2).

5.3.3 Results of molecular analysis

To our best knowledge, characterizations by molecular analyses have never been done with isolates from *A. rufa* in the Czech Republic. Our findings extend the host

range for *C. baileyi*. The GP60 subtype was identified for the first time from *A. rufa*, as the IIIe family, named IIIeA16G2R1 variants b and c (see **Appendix I**).

Molecular characterization of oocysts of *C. baileyi* and *C. meleagridis* obtained from pheasant examinations, and *C. galli* (obtained in another aviary from 36-week-old pheasants) was conducted by nested PCR analyses of the small subunit (SSU) of ribosomal RNA (rRNA), HSP70, actin. Highly variable GP60 of *C. meleagridis* were also identified for the first time and was identical with IIIeA16G2R1c (**Appendix II**).

Because of their very frequent occurrence, cryptosporidian infections, especially of *C. meleagridis*, are an important issue of possible zoonotic potential. The data described here represent new knowledge on the occurrence and epizootiology.

5.4 Application of antiparasitic drugs

All results of excrement and dead bird examinations were completed by drug administration used during study. Drugs were served in order to suppress the occurrence of the most common and pathogenic endoparasites, according to breeder documentation without the intervention and influence of the author. It was carried out under veterinary supervision.

Anticoccidic treatment was used during the whole study of red-legged partridges, for details see **Appendix III**, Fig. I. The graphical representation of weekly age-related infection dynamics over three seasons, complemented by drug administration, clearly show that toltrazuril was the most effective during 2013. All seasons finished with lower *Eimeria* percentage of positivity, but oocysts were still present in faecal samples. Very different situation occurred in pheasants, where *Eimeria* was the most common finding (**Appendix V**). Effectivity of schedule, based on our results (**Appendix V**), is low and this problematic need re-evaluation. None of breeder schedule or combinations of drug active constituents fully prevented birds, only decreased coccidial infection. After administration, situation was even worst and usually required repeating of serve, e.g., 4–5 times treatment during first 5 weeks mostly by variation of 2–3 constituents.

For both host species, administration of anthelminthic drugs prevented of possible outbreak and losses, but situation deserves new procedures and reassessment in

suppression of infections in the future. Especially, from partridges, we demonstrated that long-term anthelminthic drug administration, as used in the aviary, was ineffective in stopping the occurrence of *S. trachea* and Capillariidae gen. sp. eggs in faecal samples. Interestingly, egg release after treatment (during 2012 and 2013) exceeded the control level, suggesting that antiparasitic drugs should also be implemented in older age classes. During season 2014, no anthelminthic drugs were given, thus resulting in the lowest occurrences (positivity percentages) of helminth eggs from three seasons (**Appendix III**).

Based on these results, effective schedule for the application of antiparasitic drugs must include co-infections, which makes it difficult. Further work to adjust the dosing regimen may be necessary to minimizing the survival of coccidian and nematodes.

Due to the very similar parasite fauna found in both host species, there is a real possibility of mutual exchange. For these reasons, mainly from the epidemiological point of view, I recommend to avoid the transport of animals from one area (brooder house, aviary, etc.) to another. Moreover, a sort of quarantine or isolation of animals between the original and newly accepted animals is required.

6. Discussion

In this study, the parasitic fauna of two bird species under intensive artificial breeding from a Czech aviary was examined. *Alectoris rufa* harboured four protozoan and four helminth species that represent a lower helminths richness when compared with that of wild (9–13 spp.) and farmed (6 spp.) specimens in Spain (Calvete et al. 2003; Millán et al. 2004). Moreover, the specific composition of helminths was also different (nematodes vs. nematodes, cestodes and trematodes) and protozoans were not included/detected. These differences may be due to sample size (99 vs. 296–587 birds), and the broader geographical area examined (1 vs. 16). Until now, this is the first study on such a large scale of both protozoan and helminth spontaneous infections from *A. rufa*.

In both hosts, *Histomonas meleagridis*-like lesions were observed in livers, in all cases, with the same morphology (circular yellow necrotic tissue surrounded with white rings). For definitive identification, according to our opinion, molecular identification is necessary, because the autolytic changes occur very quickly. Only 8 birds had lesions on livers, so the problematic of *Histomonas* was not studied in detail.

Pheasant coccidiosis is still alive, because high morbidity and mortality occur after many decades of research. *Eimeria* spp. appeared also in red-legged partridges throughout the whole sampling period, but no mortalities or outbreaks were produced by this parasite. Naciri et al. (2011, 2014) also found no mortalities in *A. rufa* when *E. kofoidi* was present, although low mortality was observed in experimental birds while infected by *E. legionensis*. Mixed infections (dual or triple) considerably increased the effects on growth. Bolognesi et al. (2006) described an outbreak of *Eimeria* spp. in a game-bird farm in Italy, with high mortalities (46.5–52.1%). It is clear that attention should be paid to the presence of mono and mixed infections with *Eimeria* in others avian species. Different situation was for *P. colchicus*, where *E. phasiani* and *E. duodenalis* have higher pathogenicity in comparison with those of *E. kofoidi* and *E. legionensis* in *A. rufa*. *Eimeria phasiani* was the most common finding in faeces and problematic for breeder, even when they use drugs very frequent (3–5 times during first 5 weeks of age), the amount of oocysts in faeces is high and losses still occur. The most pathogenic species is *E. colchici*, but it was not found during this study. This species is

common in other aviaries across the country (personal observation; e.g., Konvičková 2008). The birds that will be transported in future will spread this coccidian and other species across whole breeding facilities. Therefore, higher pathogenicity or resistance will arise.

Cryptosporidial infections especially that of *C. meleagridis*, are an important issue of possible zoonotic potential, and the data described herein represent new knowledge on the occurrence and epizootiology. *Cryptosporidium meleagridis* in farmed *A. rufa* was described for the first time in the Czech Republic. This bird also represents a new host for *C. baileyi*. Species determination was carried out by comparing the localization of endogenous developmental stages in infected birds and oocyst morphology with the original descriptions of Slavin (1955) in turkeys and Current et al. (1986) in chickens. The findings for these *Cryptosporidium* species were also identical with the data of Pavlásek (1994b), who described infection in turkeys, chickens and parrots. The identity of the species was also confirmed by sequence analysis and compared with previously described *C. meleagridis* subtype from a human host in Japan (GenBank accession No. AB539718) (Abe 2010). Our sequences differed by 8 to 10 bases within the region downstream of the trinucleotide repeat (98.8% and 98.6%, respectively), resulting in7 to 9 amino acid changes. Based on these significant differences, we suggest two distinct subtype variants. BLASTn analysis showed that IIIeA16G2R1b and IIIeA16G2R1c were also similar to subtypes IIIeA20G2R1 (GenBank accession No. AB539721) parasitizing a cockatiel in Japan (Abe & Makino 2010), IIIeA17G2R1 and IIIeA19G2R1 (GenBank accession Nos. KJ210608, KJ210620), representing infection of a human from India/Nepal and Uzbekistan, respectively (Stensvold et al. 2014). Our results might be compared with that of Pagés-Manté et al. (2007), who describe an outbreak of *C. meleagridis* in one farm with red-legged partridges in Spain. According to these authors, avian cryptosporidiosis was accompanied by up to 50% mortality and 60–70% morbidity between May and July, and even up to 89% mortality during July. Unlike the data from Spain, we detected the first *C. meleagridis* oocysts in the partridge excreta at the age of 13 and 17 weeks, over a 28-day period of infection, with no clinical symptoms of disease and no increased mortalities, during 2012–2013. Although various tests have been performed (Pagés-Manté et al. 2007) in partridge breeding, unfortunately these were without detection in

specific parts of the intestine, and endogenous developmental stages are not obvious from the descriptions or pictures of histological sections of *Cryptosporidium*. In contrast to these authors, we have studied in detail the localization of this protozoan. Detecting the source of *C. meleagridis* and *C. baileyi* infection in red-legged partridge is difficult, although it is possible that pheasants may be the source of infection, because they are primarily bred annually in aviaries. It also cannot be excluded that wild birds could contaminate the environment of an aviary with their excrements. Game bird diseases could become a major health problem with impact on health and economics. Because of their very frequent occurrence, cryptosporidian infections, especially of *C. meleagridis*, are an important issue of possible zoonotic potential, as described recently by many authors (e.g. Xiao & Feng 2008; Silverlås et al. 2012; Baroudi et al. 2013). Apparently, *A. rufa* does not act as a reservoir host for *C. baileyi*, since it is less parasitized than grey partridges, chickens, Pekin ducks, pheasants or quails (see Pavlásek & Kozakiewicz 1989; Sun et al. 2005; Pavlásek & Šverma 2009; Wang et al. 2010, 2012; Máca & Pavlásek 2016).

The presence of generalist parasites in *A. rufa* could be a potential risk for other hosts when birds are released into the open area and have contact with wild bird populations. Calvete et al. (2003), Millán et al. (2004) and Villanúa et al. (2008) suggested that released *A. rufa* could share similar parasitic fauna with wild bird populations. In the case of *C. meleagridis*, releasing positive partridges represents a risk for animals and humans, since this species has zoonotic potential (Silverlås et al. 2012; Wang et al. 2014).

Previous studies on quails (*Coturnix coturnix japonica*) from China showed a higher occurrence of *C. baileyi* (237 positive from 1818 examined samples) than *C. meleagridis* (2/1818) (Wang et al. 2012). Likewise, we also found higher positive samples with *C. baileyi* (19/173 and 36/238) than *C. meleagridis* (7/173 and 10/238). In contrast, *A. rufa* was more sensitive to *C. meleagridis* (42/242 and 99/421) than to *C. baileyi* (0/242 and 4/421) during 2012 and 2013 (Máca & Pavlásek 2015, **Appendix I**). The most widespread cryptosporidial infection in quails in China was *C. baileyi* (5/5) rather than *C. meleagridis* (1/5) (Wang et al. 2012). Similarly, in the present study of *P. colchicus*, more breeding facilities harboured *C. baileyi* (7/14 and 10/16) than *C. meleagridis* (5/14 and 7/16). A high percentage of *C. baileyi* infections were also

described by Pavlásek and Kozakiewicz (1989), who reported only the occurrence of *C. baileyi* in all four pheasant aviaries examined on Polish farms. Musaev et al. (1998) reported *Cryptosporidium* sp. infections in 7.9 % (16/203) of pheasants, although they did not determine this to the species level, but only presented oocyst measurements of 3.3–7.4 × 2.5–7.4 μm (mean length 4.4, mean width 3.9 μm). Unlike Musaev et al. (1998), we and many other authors (Slavin 1955; Current et al. 1986; Lindsay et al. 1989; Ng et al. 2006) have not seen such variability in the size of *Cryptosporidium* oocysts from different host species, and the mean data most likely correspond to *C. meleagridis*. No previous studies have characterized *Cryptosporidium* species using molecular analyses to support parasite identification, especially in the case of a highly variable locus, such as the GP60 of *C. meleagridis* from ring-necked pheasants. A higher prevalence in 2012 could be explained by the fact that several birds were examined only in preselected facilities during an assumed outbreak of *C. baileyi* and *C. meleagridis* infections, compared to 2013 and 2014 across each complete rearing season. Interestingly, during the present study, a co-infection of *C. baileyi* and *C. meleagridis* occurred in one bird during 2012, although only mono infections in pheasants were previously published. The situation in intensive artificial breeding may serve as a model for the spread of cryptosporidial infections during fattening of chickens and other commercial birds (e.g. ducklings, goslings) or game birds bred in brooder houses and later moved to an open area. Pheasants act as possible disseminators of *Cryptosporidium* for gull chicks after their release (Pavlásek 1993, 2004), as well as other potential host species. Different aviaries in the same region of the Czech Republic were chosen to obtain *C. galli* isolates from pheasants that were positive during routine examinations during April 2014 and May 2015. Pavlásek and Šverma (2009) examined birds at the same place, and 36-week-old pheasants were repeatedly positive to *C. galli* for many seasons. *Cryptosporidium galli* was diagnosed in many avian host species bred in backyards and aviaries or held as exotic pets (Pavlásek 2004; Ng et al. 2006). An isolate obtained from *P. colchicus* was identical to previously identified species (e.g. Jiang et al. 2005; Ryan et al. 2003). Several factors such as stress, rearing densities, infection seasonality, contamination of the environment, oocyst excretion, persistence and infectivity may generate immune suppression and thus affect the occurrence and prevalence of cryptosporidial infections during seasons in breeding or bird farms

(Blanco et al. 2001; Moller et al. 2003; Villanúa et al. 2006). In the future, it will be important to compare other isolates derived from different aviaries infected with *C. meleagridis* using GP60 genotyping for comparisons of intensive breeding of game birds across the Czech Republic, and also the possible occurrence of zoonotic transmission of *C. meleagridis* (Silverlås et al. 2012; Stensvold et al. 2014; Wang et al. 2014). We conclude that coprological and post-mortem examinations confirmed that *Cryptosporidium* spp. are important parasites in intensive artificial breeding programmes of pheasants. Moreover, special attention should be paid to the real possibility of infection spread, caused mainly by translocations of pheasants and the mixing of birds in different farming systems and the close contact between infected and uninfected birds. This fact increases the duration of cryptosporidial infections. Villanúa et al. (2006) showed an increase in the excretion of parasites at different developmental stages and parasite burdens after the release of captive pheasants. In this context, we consider the ring-necked pheasants released from artificial breeding programmes to open areas as a new potential source of cryptosporidial infections for avian and other potential hosts.

Further research is required in the Czech Republic for a better understanding of cryptosporidian epidemiology and to establish a subgenotype analysis at the GP60 locus. Because of the current lack of more detailed information on these parasite species, and the common breeding of its hosts in many European countries, the data described herein represent new knowledge on the occurrence and epizootiology of cryptosporidian infections.

Chosen aviary was nearly ideal for the study of all objectives, especially on *A. rufa*, because breeder left birds till hunting, but this was not the case of *P. colchicus*. We demonstrated that long-term anthelminthic drug administration, as used in the aviary in *A. rufa*, was ineffective in stopping the occurrence of *S. trachea* and capillariid eggs in faecal samples; therefore, drug efficacy should be investigated. Interestingly, egg release after treatment (during 2012 and 2013) exceeded the control level, suggesting that antiparasitic drugs should also be implemented in older age classes, according to suggestions by Andreopoulou et al. (2011). The release (expulsion) of oocysts/eggs into faecal samples highlights the presence of an ongoing infection and the possibility of spreading the infection throughout the whole population. On the other

hand, some studies have shown that anthelminthic treatments reduce nematode infections, but increase the prevalence of other gastrointestinal parasites (Pedersen & Antonovics 2013), such as *Eimeria* in wild mice (Knowles et al. 2013). *Syngamus trachea* caused mortalities of *A. rufa* throughout the study, especially in young birds during 2012 and 2013, similar to that reported in partridges (*A. chukar*) in Greece (Andreopoulou et al. 2011), although drug administration positively reduced it. Because body condition could be negatively affected by the helminthic infection (see Gethings et al. 2016), young birds must be treated when this parasite is present. Dynamics patterns and onset of parasitic infections could help to breeder, but resistance, proper dosing or targeted treatment deserve more attention, especially in the case of pathogenic *Eimeria* species as those of *E. phasiani* and *E. duodenalis* in *P. colchicus*.

Mixed infections are very common in populations of various host species (Petney & Andrews 1998; Graham et al. 2007), although most studies only included single-parasite infections. One of my aims was to study this problem in complex parasitic infections, where the parasites may interact (Graham 2008; Telfer et al. 2010; Ezenwa 2016; Naciri et al. 2014) synergistically or antagonistically (Cox 2001).

In conclusion, prevention, treatment and control of endoparasites are very difficult under conditions of intensive artificial breeding. Some parasitic species, such as *C. baileyi* and *C. meleagridis*, need more attention, especially in hosts with multi-specific infections where they could have important impacts on bird losses and animal production efficiency. Parasite infections and their prevalence varied from one breed to another, so it is important to pay particular attention to parasitic fauna during translocations of birds from different parts of aviary/facilities or when purchasing new birds, which are going to bird population in intensive artificial breeding programmes. Also, it is important to expand our knowledge of parasitic infections in gallinaceous game birds, as well as their veterinary and epidemiological significance, in order to avoid the introduction and transmission of new pathogens to wild populations.

7. Conclusions

The spontaneous parasite infections of reared ring-necked pheasants and red-legged partridges were studied for the first time on a large scale. Both bird species are exposed to be infected by a group of protozoans and helminths that could be spread into the wild when birds are released for hunting. Parasite fauna of both game birds was very similar and there is a real possibility of mutual exchange, but percentage positivity and prevalence were different. Data described here represent new knowledge on their occurrence and epizootiology.

Age dynamics and a graphical representation of the pathway of *C. baileyi* and *C. meleagridis* infections during a complete rearing season were followed. Results suggest differences in susceptibility to infection. The resulting data highlight the real risk of transmission of *Cryptosporidium* to susceptible wild birds and other potential hosts, especially due to zoonotic *C. meleagridis*.

Administration of anthelminthic drugs prevented possible outbreak and high mortality, but situation deserves new procedures and reassessment in suppression of infections in the future and considering nutritional and immune status of the host. Studies focused on anticoccidial drugs for pheasants and anti-*Cryptosporidium* drugs generally are needed, although effective treatments and vaccines are not yet available for cryptosporidosis.

Breeders should avoid transporting animals from one area to another and provide quarantine or isolation routines to all birds. Economic losses due to parasite infections could be extremely high; therefore, any preventive steps, e.g., rotation program or different breeding technology, could help to reduce occurrence of parasitic infections.

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Appendix I

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First finding of spontaneous infections with *Cryptosporidium baileyi* and *C. meleagridis* in the red-legged partridge *Alectoris rufa* from an aviary in the Czech Republic

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ABSTRACT

This paper represents the first report of spontaneous infection with *Cryptosporidium baileyi* and *Cryptosporidium meleagridis* in the red-legged partridge (*Alectoris rufa*), as well as the percentage of positive samples and age-associated dynamics of cryptosporidial infections in an aviary in the Czech Republic. The entire infection process was monitored over two semesters (July–December 2012 and 2013) until release of birds for hunting purposes. Coprological examination of 663 pooled fecal samples and 89 post-mortem examinations of red-legged partridges were carried out. Our results indicated that infections with *C. baileyi* only occurred in 5–7 week-old birds during 2013 (percentage of positivity, 1%) and those with *C. meleagridis* in 18–22 week (17%) and 17–21 week-old birds (24%) during 2012 and 2013, respectively. Molecular characterization of isolates of *C. baileyi* and *C. meleagridis* heat shock protein 70 and actin genes were analyzed in order to support our coprological results. DNA sequence analysis of the 60 kDa glycoprotein gene was used to subtype *C. meleagridis*. Our findings extend the host range for *C. baileyi*.

1. Introduction

With the increasing number of breeding farms for gallinaceous birds, special attention is paid to prevention and control of significant emerging pathogens, such as apicomplexan protozoans of the genus *Cryptosporidium* Tyzzer, 1907. Infections in birds are mainly caused by three species: *Cryptosporidium meleagridis* Slavin, 1955, *C. baileyi* Current, Upton and Haynes, 1986, and *C. galli* Pavlásek, 1999 (see Slavin, 1955; Current et al., 1986; Pavlásek, 1999; Ryan, 2010), along with several avian *Cryptosporidium* genotypes (e.g. Ryan et al., 2003; Ng et al., 2006; among others).

The three mentioned parasitic species have been found and described in birds in the Czech Republic. Pavlásek (1985, 1993, 1994a) reported for the first time the occurrence of *C. baileyi* in domestic chickens (*Gallus gallus*) and wild birds (black-headed gull, *Larus ridibundus*), as well as *C. meleagridis* in turkey (*Meleagris gallopavo*) and salmon-crested cockatoo (*Cacatua moluccensis*). Later, numerous isolates were biologically, morphologically and molecularly identified and characterized from various avian host species from the Czech Republic (see Pavlásek, 1999; Ryan et al., 2003; Ng et al., 2006).

To date, the only species of *Cryptosporidium* parasitizing the red-legged partridge (*Alectoris rufa* Linnaeus, 1758) is *C. meleagridis*, which was found in the

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respiratory and intestinal tracts, and genetically characterized (Pagés-Mante et al., 2007) after an outbreak in Spain. In the course of a long-term study, *C. meleagridis* from *A. rufa* was first detected and diagnosed during 2006–2007 in an aviary in the Mladá Boleslav region, Czech Republic (Pavlásek, unpublished data). This paper focuses on spontaneous infections with two *Cryptosporidium* spp. obtained by a targeted and systematic study of red-legged partridge breeding in the Czech Republic. Identification of oocyst isolates of *C. baileyi* and *C. meleagridis* were supported and characterized by nested PCR of heat shock protein 70 (HSP70), actin and 60 kDa glycoprotein (GP60) genes.

2. Materials and methods

2.1. Area and breeding technology characteristics

Examinations for the presence of coccidial oocysts in *A. rufa* were carried out during two semesters (July–December 2012 and 2013) in an aviary in the Central Bohemian Region (Czech Republic). The annual hatching at this site reaches many thousands of *A. rufa* and *Phasianus colchicus*, where the arrangement of breeding facilities for both species prevents mutual contact. Breeding technology is the same for both species. The reserved area is divided into separate uphill and downhill sections, each containing a system of eleven closed sheltering aviaries. Later, ~5 week-old birds were released to five paddock groups, separated by wire fences with shelters in each section.

2.2. Sample collection and examination

Pooled samples (fresh excrements with contents of cecum and other parts of the intestinal tract) were collected from the same area in the morning before noon. Pooled samples represent one aviary or later, after combining, one paddock group (approximately 20–30 individual excrements of the red-legged partridges). Screening was performed over 7 day intervals and daily after first detection of *Cryptosporidium* spp. oocysts. A total of 663 pooled fecal samples were collected on the first day after hatching until release of the birds (Table 1). All samples were directly transported to the Department of Pathology and Parasitology, State Veterinary Institute in Prague and stored at 4 °C. The flotation-centrifugation coprological method was used for the detection of oocysts according to Breza (1957), and those positive fecal samples and intestinal contents were processed as native preparations using glycerin (see Pavlásek, 1991). All samples positive for *Cryptosporidium* spp. were stored in 2.5% potassium dichromate at 4 °C for further processing by molecular analysis. Eighty nine whole red-legged partridges were provided to the laboratory for incomplete parasitological evaluation using three methods (flotation-centrifugation, native preparations, and native preparation using glycerin), during both years. For detection of developmental stages, one positive specimen from post-mortem exanimate birds (scrapings of intestinal mucosa, bursa fabricii and cloaca) was used for air-dried smears fixed with methyl alcohol, stained in Giemsa solution, and fixed in 10% formalin for histopathology. Examinations and figures were carried out with the aid of a Leica DMLB optical microscope equipped with a Leica DFC420 digital camera.

2.3. Molecular analysis

Genomic DNA was extracted from concentrated oocysts isolated from feces using Sheather sugar solution, followed by bead disruption homogenization and using the QIAamp® DNA Stool Mini Kit (Qiagen) with minor modifications to the manufacturer's instructions. Molecular characterization was conducted by nested polymerase chain reaction (PCR) analysis of molecular markers: ~400 bp HSP70, ~800 bp actin and ~900 bp fragment of GP60 genes. Gene amplification and sequencing were performed according to Morgan et al. (2001), Ng et al. (2006) and Stensvold et al. (2014). PCR products were visualized on an agarose gel, and successful amplification products were purified using the QIAquick® Gel Extraction Kit (Qiagen) in accordance with the manufacturer's instructions. Sequencing was carried out in both directions using secondary PCR primers at SVI Prague and the resulting contigs were compared with sequences published in GenBank using BLAST.

3. Results

3.1. Coprological examination

The total percentage positivity to *Cryptosporidium* spp. after the examination of 663 pooled fecal samples was 22%, with higher occurrence of *C. meleagridis* (21%) in comparison to *C. baileyi* (0.6%) (Table 1). During 2012, 242 samples were examined, of which 42 (17%) had *C. meleagridis*, mainly in 15–18, 19–21, and 22 week-old age classes. Positive fecal samples were found in only the uphill section of the aviary. On the other hand, examination of 421 samples in 2013 revealed that 99 (24%) samples of 15–18 and 19–21 week-old age classes were positive, whereas 4 samples (1%) of the 5–7 week-old age class harbored *C. baileyi*. Oocyst of *C. meleagridis* and *C. baileyi* were released over approx. 28 days in both sections of the aviary and in uphill sections for 15 days, respectively.

3.2. Post-mortem examination

Oocysts were present in ileal and cecal (proximal and middle part) contents during 2012 in three individuals out of 51 examined (prevalence 6%) within the 18–20 week age class. Developmental stages were found in the microvillous region of the ileum and in the epithelium of the proximal cecum, at low and high levels, respectively (Fig. 1). Results of all 38 post-mortem examinations in 2013 were negative. There were no clinical signs of disease, no evidence of losing weight, diarrhea, etc.

3.3. Molecular analysis

The HSP-70 and actin genes of 8 isolates were successfully amplified and two *Cryptosporidium* species were identified. Sequence analysis of isolates from both

Table 1
Results of parasite occurrence according to host age classes and semesters completed by percentage positivity.

Year	Age group (weeks)	No. of			
		Examined	Positive	*C. baileyi* positive (%)	*C. meleagridis* positive (%)
2012	1–4	39	0	0	0
	5–7	33	0	0	0
	8–12	55	0	0	0
	13–14	12	0	0	0
	15–18	23	2	0	2 (9)
	19–21	60	39	0	39 (65)
	22	20	1	0	1 (5)
Total		242	42	0	42 (17)
2013	1–4	40	0	0	0
	5–7	30	4	4 (13)	0
	8–12	40	0	0	0
	13–14	20	0	0	0
	15–18	110	18	0	18 (16)
	19–21	155	81	0	81 (52)
	22	26	0	0	0
Total		421	103	4 (1)	99 (24)
Grand total		663	145	4 (0.6)	141 (21)

years confirmed the occurrence of *C. meleagridis* and *C. baileyi*. Representative partial sequences of isolates were compared and deposited in the GenBank database under accession Nos. KM822866, KM822867, KP703166 to KP703168.

The results of all HSP70 gene sequences demonstrated 100% similarity with known isolates e.g. *C. meleagridis* from human and *C. baileyi* from quail already deposited in GenBank (accession Nos. AF402283 and AF221539, respectively) and with each other.

In the actin gene of *C. meleagridis*, there was a single nucleotide polymorphism (G-to-A) between isolates during 2012 (isolate D1) and 2013 (isolate D11), without amino acid changes. Nucleotide sequence comparison with other isolates e.g. from a cockatiel in Japan, available in GenBank (accession No. AB471662) revealed 100% and 99.9% identity (768/769) to the reported sequences. The actin gene sequence of *C. baileyi* was 100% identical with existing sequences e.g. from chicken isolates in GenBank (accession Nos. AF382346 and GQ227482).

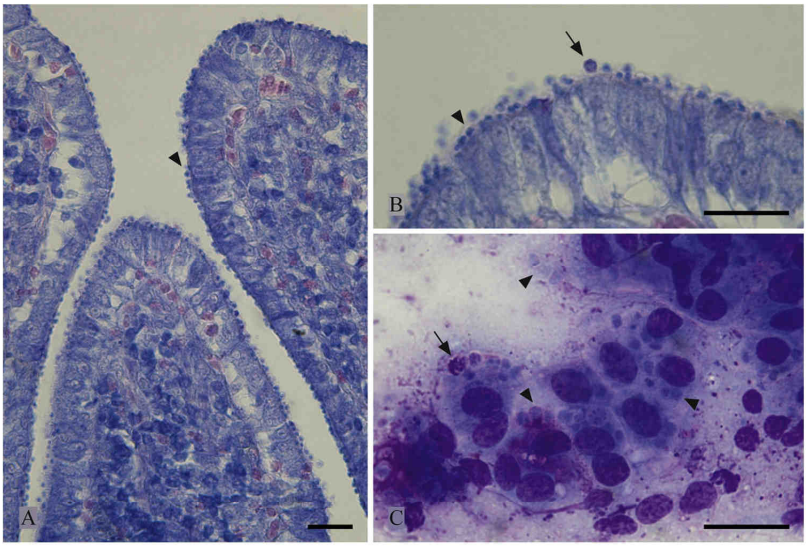

Fig. 1. *Cryptosporidium meleagridis* in microvillous regions of the proximal cecum. (A) Histological preparation stained with Giemsa (arrowhead indicates mucosa with various developmental stages). (B) Detail of mucosa with various developmental stages (arrowhead), meront (arrow). (C) Smear of intestinal mucosa (Giemsa) showing massive infection of *C. meleagridis* in the proximal cecum. Scale bars = 20 μm.

Fig. 2. Comparison of amino acid differences in the GP60 gene between sequences acquired in the study and those retrieved from GenBank of the *C. meleagridis* IIIe subtype family. The alignments are identified by names of subtype and GenBank accession numbers. Identical alignment positions are indicated by a dot, gaps by (–) and the variants are shown. The amino acid sequences were aligned using the program BioEdit (v7.2.5).

The gp60 subtype of 4 isolates was identified as the IIIe family, which had 16 serine-coding TCA repeats and 2 TCG repeats in the trinucleotide repeat region, followed by 1 repeat of ACATCA. In addition, there were two nucleotide differences in the non-repeated region in the GP60 gene between our isolate sequences obtained from years 2012 and 2013 (Fig. 2). These were therefore named IIIeA16G2R1 variants b and c (submitted to GenBank under accession Nos. KP703169, KP703170), based on the established gp60 nomenclature (Sulaiman et al., 2005; Stensvold et al., 2014).

4. Discussion

In this study, *C. meleagridis* in farmed *A. rufa* was detected for the first time in the Czech Republic. This bird also represents a new host for *C. baileyi*. Species determination was carried out by comparing the localization of endogenous developmental stages in infected birds and oocyst morphology with the original descriptions of Slavin (1955) in turkeys and Current et al. (1986) in chickens. The findings for these *Cryptosporidium* species were also identical with the data of Pavlásek (1994b), which described infection in turkeys, chickens and parrots. The identity of the species was also confirmed by sequence analysis with 100% agreement for HSP70, actin and GP60 genes. Compared to the previously described *C. meleagridis* subtype from a human host in Japan (GenBank accession No. AB539718) by Abe (2010) and identified as IIIeA16G2R1 variant a (see Stensvold et al., 2014), our sequences differed by 8 to 10 bases within the region downstream of the trinucleotide repeat (98.8% and 98.6%, respectively), resulting in 7 to 9 amino acid changes (Fig. 2). Based on these significant differences, we suggest two distinct subtype variants, with lower case letters b and c (Fig. 2). BLASTn analysis showed that IIIeA16G2R1b and IIIeA16G2R1c were also similar to subtypes IIIeA20G2R1 (GenBank accession No. AB539721) parasitizing a cockatiel in Japan (Abe and Makino, 2010), IIIeA17G2R1 and IIIeA19G2R1 (GenBank accession Nos. KJ210608, KJ210620), representing infection of a human originating from India/Nepal and Uzbekistan, respectively (Stensvold et al., 2014). Our results might be compared with that of Pagés-Mante et al. (2007), who describe an outbreak of *C. meleagridis* in one farm with red-legged partridges in Spain. These authors examined feces from four necropsied birds at ages from 4 to 25 days. According to the authors, avian cryptosporidiosis was accompanied by up to 50% mortality and 60–70% morbidity between May and July, and even up to 89% mortality during July with later hatches. In comparison with Pagés-Mante et al. (2007), the situation was monitored in one game farm over two years, always from July to December. Parasitological examination was carried out on two groups of partridges at age of 1 day until 22 weeks. Analysis of the results were made from a total of 663 pooled samples of excrement, where *C. meleagridis* oocysts were detected in 17–24% of samples. In this study, a total of 89 birds were dissected (prevalence 3%). Unlike the data from Spain, we detected the first *C. meleagridis* oocysts in the partridge excreta at the age of 17 weeks, over a 28-day period of infection, with no clinical symptoms of disease and no increased mortalities. Although various tests have been performed (Pagés-Mante et al., 2007) in partridge breeding, unfortunately these were without detection in specific parts of the intestine, and endogenous developmental stages are not obvious from the descriptions or pictures of histological sections of *Cryptosporidium*. In contrast to these authors, we have studied in detail the localization of this protozoan. Oocysts of *C. baileyi* appeared in excrements of partridges much earlier (5–7 week-old) in comparison to *C. meleagridis*. From the total number of examinations, only 1% of the samples were positive.

The methodology for collection of pooled samples of excrement, as commonly used when determining the

presence of endoparasites in domestic, commercial, wild and exotic animals, was used in our work. Samples were taken at specific intervals, and over approximately the same area and specific sites within the enclosure (aviaries, paddocks). Therefore, we believe that based on the examination of 663 samples, using techniques commonly practised in most veterinary diagnostic parasitology laboratories, objective results were obtained.

Conflicting published data shows that further studies should focus on *Cryptosporidium* species. Whereas Sun et al. (2005) reported that *C. baileyi* caused 75% mortality in the Japanese quail (*Coturnix coturnix japonica*), Pavlásek (2006) observed only 15–20% mortality in these birds. Wang et al. (2012) described *C. baileyi* as a widespread parasitic species in birds, although in our investigation the dominant species was *C. meleagridis*.

Detecting the source of *C. meleagridis* and *C. baileyi* infection in red-legged partridge is difficult. Because of the general low host specificity of *Cryptosporidium* spp. it is possible that pheasants, which are primarily bred annually in the thousands, in aviaries, may be the source of infection, although they were examined in the same area as our study, with negative results. It also cannot be excluded that wild birds could contaminate the environment of an aviary with their excrements.

Game bird diseases could become a major health problem with impact on health and economics. Because of their very frequent occurrence, cryptosporidian infections, especially of *C. meleagridis*, are an important issue of possible zoonotic potential, as described recently by many authors (e.g. Xiao and Feng, 2008; Silverlas et al., 2012; Baroudi et al., 2013). Further research is required in the Czech Republic for a better understanding of cryptosporidian epidemiology, and to establish a sub-genotype analysis at the GP60 locus. Because of the current lack of more detailed information on these parasitic species, and the common breeding of its hosts in many European countries, the data described here represent new knowledge on the occurrence and epizootology of cryptosporidian infections.

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Appendix II

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ORIGINAL PAPER

Cryptosporidium infections of ring-necked pheasants (*Phasianus colchicus*) from an intensive artificial breeding programme in the Czech Republic

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Abstract From July to November 2012, preliminary coprological examinations were carried out on 85 pooled faecal samples of different aged ring-necked pheasants (*Phasianus colchicus*) (hatches from May until July) from an intensive artificial breeding programme in the Czech Republic. *Cryptosporidium* oocysts were detected in 12 samples (14.1 %) of ages >12 weeks (August–September). These results were supported by findings of *Cryptosporidium baileyi* and *Cryptosporidium meleagridis* oocysts in intestinal, or cloacal contents, and/or the bursa of Fabricius in 9 from 36 examined dead pheasants (prevalence 25 %). We describe in detail the various age groups of pheasants after hatching and present graphically the overall results of coprological examinations, showing pathways of infection of *C. baileyi* and *C. meleagridis* during the full rearing seasons of 2013 and 2014. We found very similar mean proportions of *Cryptosporidium*-positive samples over the entire 2013 period in pheasantry (173 pooled samples tested, 25 positive, 14.5 %) and 2014 (238 samples tested, 43 positive, 18.1 %). All tests were verified as being *Cryptosporidium* positive in 9 from 219 (prevalence 4.1 %) and 4 from 168 (prevalence 2.4 %) post-mortem examinations. Significantly, *C. baileyi* was found more frequently in faeces, with positivities ranging from 11.1 to 100 % (4–>16-week-old pheasants). Oocysts of *C. meleagridis* were detected at ages 6–>15 weeks ranging from 7.1 to 100 % in faeces during the rearing seasons. The burdens of *C. baileyi* (7 of 14 and 10 of 16) and *C. meleagridis* (5 of 14 and 7 of 16) for each year, in monitored brooder houses, flight pens and spread across all open areas were recorded. Oocysts of *C. baileyi* and *C. meleagridis* obtained from this study, and *Cryptosporidium galli* (obtained in another aviary from 36-week-old pheasants), were sequenced, and we characterized the highly variable 60-kDa glycoprotein gene of *C. meleagridis*. These results highlight the real risk of transmission of *Cryptosporidium* to susceptible wild birds and other potential hosts after termination of rearing and release.

Keywords *Cryptosporidium meleagridis* · *C. baileyi* *C. galli* · GP60 · Prevalence · Dynamics and transmission

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Introduction

Although parasitic diseases of game-farm pheasants have been extensively studied worldwide (e.g. Anderson and Shapiro 1955; Bickford and Gaafar 1966; Dowell et al. 1983; Goldová et al. 2006; Villanúa et al. 2006; Santilli and Bagliacca 2012; Ashraf et al. 2015), some pathogens still need more attention. Common breeding of pheasants in many countries is frequently affected by high parasitic loads, causing economic losses to breeders.

Parasitic fauna of *Phasianus colchicus* Linnaeus, 1758 (Galliformes: Phasianidae) in the former Czechoslovakia reflects the developmental history of Czech parasitology. It was a period when pheasants were an important part of the agricultural landscape and authors dealt mainly with the helminthic fauna and coccidia (genus *Eimeria*) of this host species (Páv and Zajíček 1968; Bejšovec 1970, 1972, 1973; Chroust 1990). In parallel with the intensification of Czech agriculture

and reduced levels of cropping, a significant reduction in wild pheasant populations occurred. Individual hunting associations started to develop artificial breeding programmes for pheasants, with subsequent release to private lands. In the Czech Republic, there currently are several artificial breeding facilities with tens of thousands of pheasants, in which a high concentration of birds in a relatively small area creates favourable conditions for the rapid spread of infections, including bacteria and parasites. In many cases, co-infections occur and some galliform farms have a high prevalence of helminths and protozoan parasites, potentially resulting in outbreaks of disease and increased morbidity and mortality (Bolognesi et al. 2006; Pavlásek 2006; Pavlásek and Šverma 2009; Pagès-Manté et al. 2007).

To increase the production of artificially bred ring-necked pheasants, parasitological examinations were carried out. We focussed on protozoan parasites of the genus *Cryptosporidium* (Tyzzer, 1907), which were previously found in the respiratory tract (Whittington and Wilson, 1985), trachea and bursa of Fabricius (Randall 1986; Pavlásek and Kozakiewicz 1989; Sironi et al. 1991) of pheasants. Musaev et al. (1998) and Ashraf et al. (2015) detected oocysts in pheasant faeces, and Pavlásek (2004) reported the occurrence of *Cryptosporidium baileyi* and *Cryptosporidium galli* in ring-necked pheasants from aviaries of the Czech Republic. Some experimental infections have also been carried out (e.g. O'Donoghue et al. 1987; Lindsay and Blagburn 1990; Pavlásek and Kozakiewicz 1989; and Pavlásek 1999).

Because little attention has been paid to cryptosporidial infections in artificial breeding programmes of pheasants in the Czech Republic and other countries, this study details the course of spontaneous infections and describes the proportion of infected samples, prevalence, age dynamics and a graphical representation of the pathway of *C. baileyi* and *C. meleagridis* infections during a complete rearing season, including the translocation of pheasants. This work also describes the molecular characterization of three species, *C. meleagridis* Slavin, 1955, *C. baileyi* Current, Upton and Haynes, 1986, and *C. galli* Pavlásek, 1999, obtained from pheasants in the Czech Republic. The resulting data highlight the real risk of transmission of *Cryptosporidium* to susceptible wild birds and other potential hosts, especially due to zoonotic *C. meleagridis*.

Materials and methods

Game birds, *P. colchicus*, have historically been artificially bred annually, in lots of 10,000 individuals, in selected facilities in the Central Bohemian Region (Czech Republic).

Breeding facilities

In general, pheasant chicks were placed within brooder houses after hatching, for different periods of time (up to 17 weeks); some were then moved into flight pens (age class >7 weeks) and then released to open areas (from >8 weeks). Movements or relocations are described in Figs. 1 and 2. Breeding technologies were almost identical in all locations and facilities, and birds were monitored daily with dead individuals being collected. One of these facilities also harboured specimens of red-legged partridge (*Alectoris rufa*), although without contact with other bird species.

Brooder house (BH)

All selected BHs ($n = 7$) had concrete floors (covered by a paper carpet during the first weeks), divided into several connected parts that are cleaned mechanically and chemically annually. From hatching until approximately 3–5 weeks, birds remained in BHs with or without small outside runs. Later, depending on temperature and humidity, some birds went outside (up to an age of 17 weeks) to sandy, clay/muddy or grassy grounds. Well water and commercial food for pheasants were given daily in feeders and bowls. Some facilities were used annually for more hatchings (BHs 1, 2a, 2b, 7) or restocked from BH 7 (BHs 4, 5), but previous pheasants were all moved out before new birds were introduced. BHs 1 and 6 harboured many enclosures inside one building, with access to outdoor runs. BHs 2a, 3, 4, and 5 were small separated buildings connected with outdoor runs. BH 2b was an experimental wooden facility with an outdoor run designed for breeding 50–200 pheasants for more detailed data. BH 7 differed in respect of a lack of access to outdoor runs, and birds after age 3–5 weeks were moved to BHs 4 and 5.

Flight pen (FP)

According to weather, some pheasants were released and combined in different outdoor enclosures, such as flight pens or enclosed paddocks that had clay/muddy or grassy surfaces. These pens were also supplemented with water and food.

Open area (OA)

Older age classes of birds were released to open areas (release sites), and supplemental feeding continued, but with the possibility of free feeding, water supply and movement in a neighbouring agricultural landscape.

Sampling method

All pooled faecal samples (approximately 5–10 individual excrements) were collected from newly hatched up to released birds. The majority of fresh samples were collected at 7-day

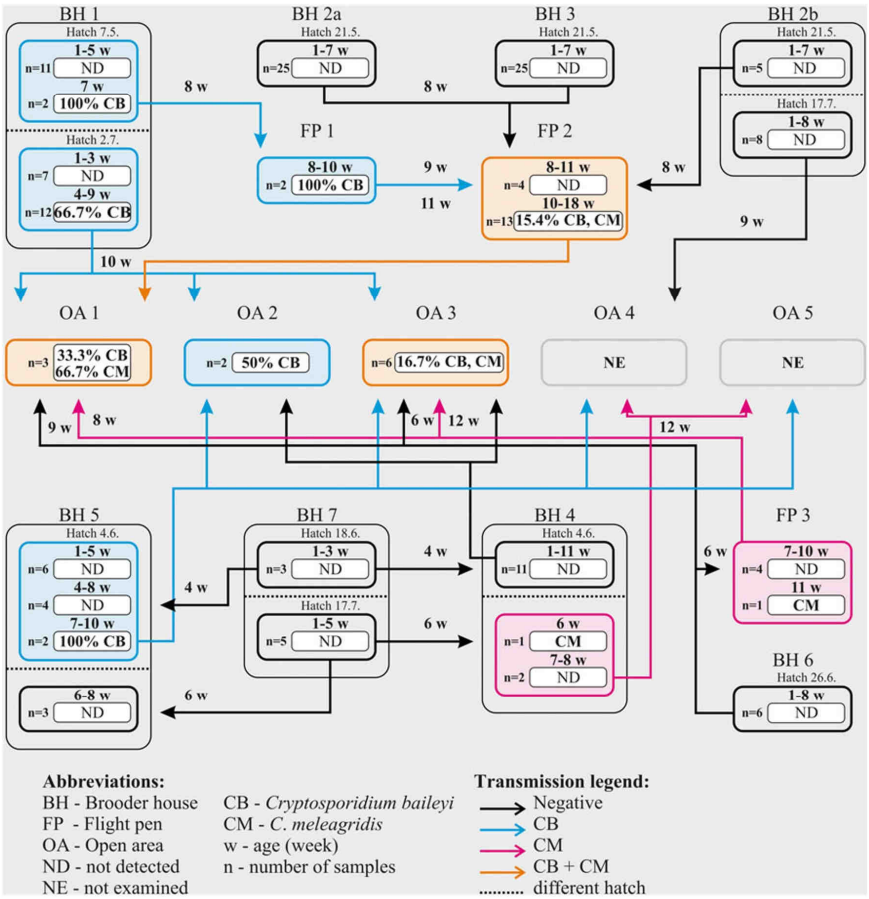

Fig. 1 Schematic presentation of the transmission of cryptosporidial infections during rearing in brooder houses (BHs), flight pens (FPs) and open areas (OAs) until bird release in 2013

intervals, placed in labelled plastic bags and transported within 12 h to the Department of Pathology and Parasitology, State Veterinary Institute Prague (see Máca and Pavlásek 2015).

Examination methodology

Coprological examination

The flotation-centrifugation coprological method was used for the detection of oocysts according to Breza (1957); positive findings were reviewed using glycerine according to Pavlásek (1991). Faecal samples and intestinal contents, where morphologically identical oocysts of *C. baileyi* and *C. meleagridis* were found, were mixed with 2.5 % potassium dichromate, stored at a temperature of 4 °C and used as isolates for molecular analyses. Oocysts morphologically similar to *C. galli* were obtained from a single examination of four pooled faecal samples of pheasants (36-week-old group) from another artificial breeding site in the same region, in 2014. Examination processes were similar to that of Máca and Pavlásek (2015).

A total of 85 pooled faecal samples were processed for examination during 2012 (July to November) in a preliminary study. Subsequently, 173 and 238 faecal samples were examined over the whole breeding seasons of 2013 and 2014 (May to December).

Post-mortem examinations

Post-mortem examination methods were chosen to confirm the findings of the coprological examinations. Dead birds

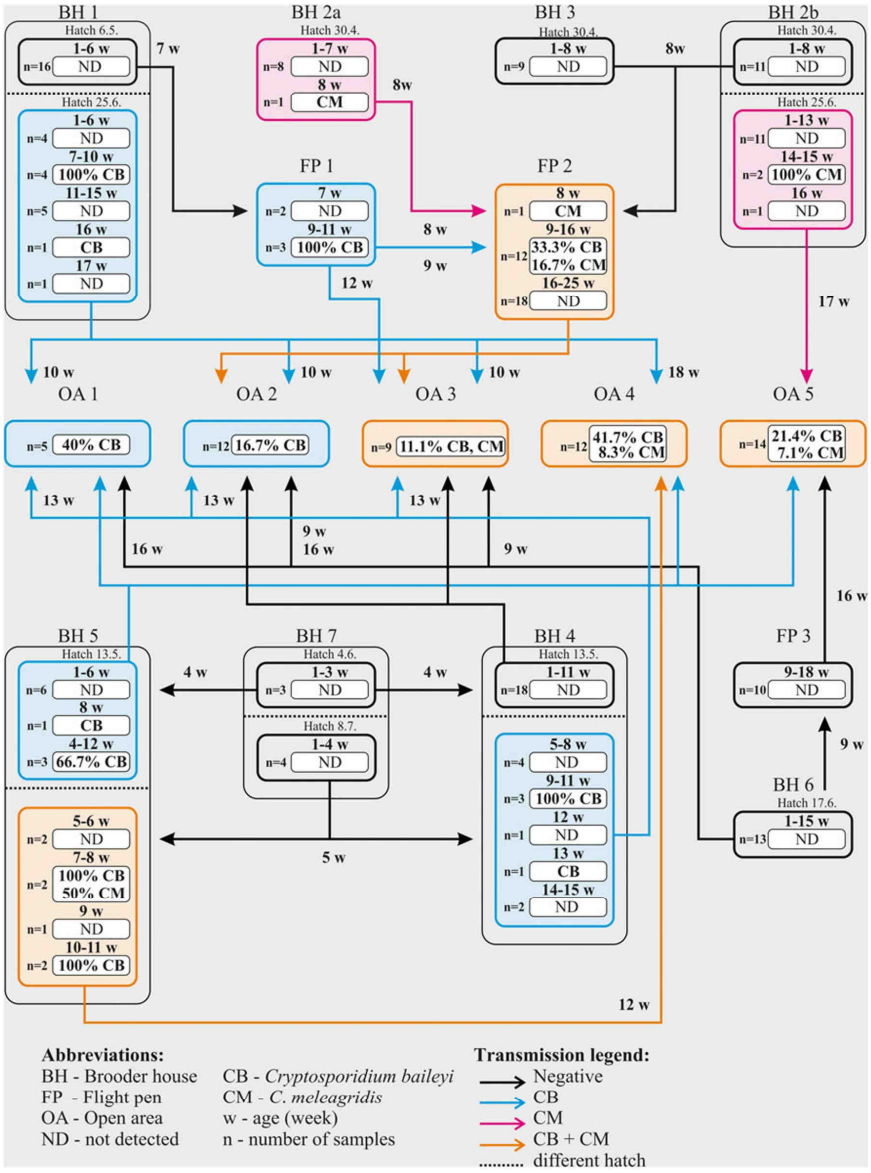

Fig. 2 Schematic presentation of the transmission of cryptosporidial infections during rearing in brooder houses (BHs), flight pens (FPs) and open areas (OAs) until bird release in 2014

were transported to the laboratory within 12–24 h, in most cases, in various stages of autolysis. Incomplete parasitological evaluations of birds were carried out using native preparations and coprological methods; air-dried smears were fixed with methyl alcohol, stained in Giemsa solution and fixed in 10 % formalin for histopathology. For the detection

of oocysts and developmental stages, scrapings of the larynx, trachea, intestinal mucosa, bursa of Fabricius and cloaca were examined, using a Leica DMLB optical microscope. During 2012 (July to November), 36 post-mortem examinations of pheasants were processed. Subsequently, 219 and 168 post-mortem examinations were examined during the whole breeding seasons of 2013 and 2014, respectively.

Molecular analysis

C. baileyi ($n = 8$) and *C. meleagridis* ($n = 3$) isolates from different housing facilities in 2014 were used for molecular characterization. During April 2014, *C. galli* ($n = 1$) oocysts were obtained by coprological examination of pheasants in different breedings from the same region and also analysed. DNA was extracted from isolated oocysts using the QIAamp® DNA Stool Mini Kit (Qiagen) according to the manufacturer's protocol. Molecular characterization was conducted by nested PCR analyses of the small subunit (SSU) of ribosomal RNA (rRNA), heat shock protein 70 (HSP70) and actin and glycoprotein 60 (GP60) loci. Gene amplification and sequencing were carried out using published methods of Morgan et al. (2001), Ng et al. (2006) and Stensvold et al. (2014). PCR products were visualized on an agarose gel, purified using the QIAquick® Gel Extraction Kit (Qiagen) or High Pure PCR product purification kit (Roche) and sequenced in both directions at SVI Prague, and the resulting contigs were compared with sequences published in GenBank using BLAST (Basic Local Alignment Search Tool). The representative sequence data from this study were deposited in the NCBI database (nos. KU221101 to KU221106).

Results

Coprological examination

From July to November 2012, 85 pooled excrement samples from 1–>20-week-old pheasants were examined, of which 12 (14.1 %) harboured oocysts of two *Cryptosporidium* species. According to their morphometric parameters, they were identified as *C. baileyi* and *C. meleagridis*. Results of the 2-year examinations of pheasants in breeding facilities are graphically presented (Figs. 1 and 2) and are complemented with data on infections (percent positive), age classes and translocations of birds during their breeding season.

In 2013, *C. baileyi* oocysts were found in 19 samples (11 %), the first findings being made in 4-week-old birds. Oocysts were found in BH 1 during the first (group of 7-week-old birds) and second (group of 4-week-old birds) hatches, and in BH 5. All birds within BHs 1 and 5 were moved to other places but were still positive for *C. baileyi* after translocation. All remaining BHs ($n = 5$) were negative. Infections with *C. baileyi* were later in FPs 1 and 2 and OAs 1–3. During the 2014 season, *C. baileyi* oocysts were identified in 36 samples (15.1 %), more than the previous year, and they were spread across the breeding facilities. Infections were monitored in up to 16-week-old birds in BH 1, but with a different occurrence of infection during the first (9-week-old) and second (7-week-old) hatches. Birds in BH 5 were again infected. For the first time, oocysts were also present in BH 4 and 5 facilities after restocking of birds from BH 7. Infections with *C. baileyi* were later again in FPs 1 and 2 and all OAs ($n = 5$).

C. meleagridis oocysts were detected in seven pooled faecal samples (4 %), from 6-week-old birds in BH 4, where birds from BH 7 during the 2013 season were also housed. Six BHs were negative for *C. meleagridis*, although FPs 2 and 3 and OAs 1 and 3 were positive. During the 2014 season, *C. meleagridis* was detected in 10 samples (4.2 %) from the 7-week-old group, in BHs 5, 2a and 2b. In other facilities (FP 2, OAs 3–5), *C. meleagridis* oocysts were also detected.

An overall analysis showed that the mean contamination with total *Cryptosporidium* spp. oocysts during 2013 and 2014 was 14.5 and 18.1 % of the samples, respectively. For *C. baileyi*, infections ranged from 11.1 to 100 % of the samples, and for *C. meleagridis*, 7.1–100 %, over both seasons. *C. baileyi* was more common in 7 out of 14 facilities in 2013, while *C. meleagridis* was only positive in 5. In 2014, *Cryptosporidium* spp. occurred in more places than in the previous year (*C. baileyi* in 11 and *C. meleagridis* in 7 out of 16 places examined).

Post-mortem examination

Post-mortem examinations were performed on 423 ring-necked pheasants to determine the prevalence of *Cryptosporidium* spp. The overall prevalence was 5.2 % (22 infected/423 examined) during 2012–2014 at the ages of 1 to >25 weeks (4.5 % *C. baileyi* and 1 % *C. meleagridis*).

Cryptosporidium oocysts mainly infected the intestinal contents, bursa of Fabricius and cloaca, but all samples were negative for the presence of developmental stages due to advanced autolytic processes of the mucosal epithelium. During 2012, *C. baileyi* oocysts were found in the bursa of Fabricius in nine cases (facilities BHs 1 and 5, FP 1, OAs 3 and 4). In one case, *C. baileyi* and *C. meleagridis* co-infected the bursa of Fabricius and caecum, respectively, in BH 1. In 2013, *C. baileyi* had already occurred in 3-week-old birds without any sighting of *C. meleagridis*. Nine positive birds supported our results of species found during coprological examinations, with the only difference being the occurrence of *C. baileyi* (7-week-old bird) in BH 4. During 2014, one case of *C. baileyi* and three cases of *C. meleagridis* were found, and all findings were confirmed by examination of faecal samples.

Oocysts of *Cryptosporidium* were only found in intestinal contents, the bursa of Fabricius and the cloaca.

Molecular analysis

Three *Cryptosporidium* species from *P. colchicus* in 2014 were examined using molecular tools. Nucleotide sequences of the HSP70 and actin genes of *C. baileyi* and *C. meleagridis* isolates were identical across different breeding places.

Partial sequences of the HSP70 gene of *C. baileyi* isolates from *P. colchicus* were identical to those of *A. rufa* (GenBank accession no. KM822866) from the Czech Republic and quail from Australia (GenBank accession no. AF221539). All *C. meleagridis* isolates were identical to human isolates (GenBank accession nos. JX024761, AB539715) and also with those of *A. rufa* from the Czech Republic (GenBank accession no. KM822867).

Sequences at the actin gene locus of *C. baileyi* and *C. meleagridis* isolates were identical to those (e.g. *C. baileyi* from *Gallus gallus* f. dom.) from Brazil (GenBank accession no. GQ227482), and *C. meleagridis* isolates were 99.9 % (730/731) similar to sequences of human isolates from Australia (GenBank accession no. JX471003). Both of our isolates from pheasants were identical to *A. rufa* sequences from the Czech Republic (GenBank accession nos. KP703168 and KP703167).

The *C. galli* isolate ($n = 1$) was identical to previously obtained sequences in the SSU rRNA gene locus from storm water samples in the USA (GenBank accession no. AY737590) and isolates of spontaneously infected hens (*Gallus gallus*) in the Czech Republic (GenBank accession no. AY168847). We did not attempt to amplify *C. galli* at the actin locus, similarly to Ng et al. (2006) in different avian hosts.

Sub-typing of *C. meleagridis* was used in the present study, and isolates were identical to IIIeA16G2R1c at the GP60 locus from the *A. rufa* reference sequence (GenBank accession no. KP703170) from the Czech Republic.

Discussion

Intensive rearing of pheasants in different breeding facilities can be problematic since it is associated with high numbers of birds in a relatively small area, thus increasing the possibility of bacterial, viral and parasitic diseases and their rapid spread, compared with wild birds. Cryptosporidial infections in these facilities have not been adequately studied, and it was our aim to gain new knowledge about the species spectrum, age sensitivity and modes of transmission during the entire artificial rearing period of pheasants. We used pooled samples (in numbers that represented a few percent of the total birds) as a basic method for investigating fresh faecal material. Tracking of individual birds was not possible due to translocations among different breeding facilities in high concentrations of each selected group.

Previous studies on quails (*Coturnix coturnix japonica*) from China (Wang et al. 2012) showed a higher occurrence of *C. baileyi* (237 positive from 1818 examined samples) than *C. meleagridis* (2/1818). Likewise, we also showed higher numbers of positive samples with *C. baileyi* (19/173 and 36/238) than *C. meleagridis* (7/173 and 10/238). Although a different host, the patterns of infection were similar. In contrast, *A. rufa*, which is bred in the same region and under the same conditions as pheasants, was more sensitive to *C. meleagridis* (42/242 and 99/421) than to *C. baileyi* (0/242 and 4/421) during 2012 and 2013 (Máca and Pavlásek 2015). The most widespread cryptosporidial infection in quails across study areas in China was from *C. baileyi* (5/5) rather than *C. meleagridis* (1/5) (Wang et al. 2012). Similarly, in the present study, more breeding facilities harboured *C. baileyi* (7/14 and 10/16) than *C. meleagridis* (5/14 and 7/16). A high percentage of *C. baileyi* infections were also described by Pavlásek and Kozakiewicz (1989), who reported the occurrence of *C. baileyi* in all four pheasant aviaries examined on Polish farms. During rearing, *C. baileyi* was diagnosed once in 3-week-old pheasants and more often in 7–10-week-old birds. In FP and OA, after merging of age classes, positive samples were lower but infection still circulated. The occurrence of *C. meleagridis* was not as common as *C. baileyi*, supported by our previous findings of cryptosporidial infections from many localities across the Czech Republic (unpublished data). Musaev et al. (1998) reported *Cryptosporidium* sp. infections in 7.9 % (16/203) of pheasants, although they did not determine this to the species level, but only presented oocyst measurements of 3.3–7.4 × 2.5–7.4 μm (mean length 4.4, mean width 3.9). Unlike Musaev et al. (1998), we and many other authors (Slavin 1955; Current et al. 1986; Lindsay et al. 1989; Ng et al. 2006) have not seen such variability in the size of *Cryptosporidium* oocysts from different host species, and the mean data most likely correspond to *C. meleagridis*. No previous studies have characterized *Cryptosporidium* species using molecular analyses to support parasite identification, especially in the case of a highly variable locus, such as the GP60 locus of *C. meleagridis* from ring-necked pheasants.

C. baileyi was the most prevalent species, infecting mostly the bursa of Fabricius, cloaca, trachea or larynx, while *C. meleagridis* occurred in the microvillus region of the ileum and epithelium of the proximal caecum of pheasants; it was also diagnosed during our previous routine examinations (unpublished data). A higher prevalence in 2012 could be explained by the fact that several birds were examined only in preselected facilities during an assumed outbreak of *C. baileyi* and *C. meleagridis* infections, compared to 2013 and 2014 across each complete rearing season.

Interestingly, during the present study, a co-infection of *C. baileyi* and *C. meleagridis* occurred in one bird during 2012, although only mono infections in pheasants were previously published.

The situation as described here for intensive artificial breeding may serve as a model for the spread of cryptosporidial infections during fattening of chickens and other commercial birds (e.g. ducklings, goslings) or game birds bred in brooder houses and later moved to an open area. Pheasants act as possible disseminators of *Cryptosporidium* after their release for, e.g., gull chicks, of which up to 100 % harbour *C. baileyi* (Pavlásek 1993, 2004), as well as other potential host species.

Máca and Pavlásek (2015) characterized isolates of *Cryptosporidium* spp. from an aviary of *A. rufa* that were bred in the same area/region. All isolates of *C. baileyi* and *C. meleagridis* from pheasants were identical to sequences from *A. rufa* at the HSP70, actin and GP60 loci. Based on these findings, we carried out a study and preliminary data that support a possible transmission of *C. baileyi* and *C. meleagridis*, by co-infection from *P. colchicus* to *A. rufa* (unpublished data).

Different aviaries in the same region of the Czech Republic were chosen to obtain *C. galli* isolates from pheasants that were positive during routine examinations during April 2014 and May 2015. Pavlásek and Ševerma (2009) examined birds at the same place, and 36-week-old pheasants were repeatedly positive to *C. galli* for many seasons. *C. galli* was diagnosed in many avian host species bred in backyards and aviaries or held as exotic pets (Pavlásek 2004; Ng et al. 2006). An isolate obtained from *P. colchicus* was identical to previously identified species (e.g. Jiang et al. 2005; Ryan et al. 2003).

Several factors such as stress, rearing densities, infection seasonality, contamination of the environment, oocyst excretion, persistence and infectivity may generate immune suppression and thus affect the occurrence and prevalence of cryptosporidial infections during seasons in breeding or bird farms (Blanco et al. 2001; Moller et al. 2003; Villanúa et al. 2006).

In the future, it will be important to compare other isolates derived from different aviaries infected with *C. meleagridis* using GP60 genotyping for comparisons of intensive breeding of game birds across the Czech Republic, and also the possible occurrence of zoonotic transmission of *C. meleagridis* (Silverlas et al. 2012; Stensvold et al. 2014; Wang et al. 2014).

We conclude that coprological and post-mortem examinations confirmed *Cryptosporidium* spp. as important parasites in intensive artificial breeding programmes of pheasants. Moreover, special attention should be paid to the real possibility of infection spread, caused mainly by translocations of pheasants and the mixing of birds in different farming systems and, thus, to close contact between infected and uninfected birds. This fact increases the duration of cryptosporidial infections. Villanúa et al. (2006) showed an increase in the excretion of parasites at different developmental stages and parasite burdens after the release of captive pheasants. Infections and their prevalence varied from one breed to another, so it is important to pay particular attention to parasitic fauna during translocations of birds from different parts of aviary/facilities or when purchasing new birds. Most likely, this also applies to *C. meleagridis*. In this context, in accordance with the data above, we consider the ring-necked pheasants released from artificial breeding programmes to open areas as a new potential source of cryptosporidial infections for avian and other potential hosts.

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Appendix III

Máca O, Pavlásek I. (2018) Protozoan and helminthic infections of farm-reared *Alectoris rufa* (Galliformes: Phasianidae) before releasing for hunting in the Czech Republic: infection pathway and potential risks. Journal of Parasitology (submitted, major revision)

PROTOZOAN AND HELMINTHIC INFECTIONS OF FARM-REARED *ALECTORIS RUFA* (GALLIFORMES: PHASIANIDAE) BEFORE RELEASING FOR HUNTING IN THE CZECH REPUBLIC: INFECTION PATHWAY AND POTENTIAL RISKS

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Abstract: Age-related dynamics of protozoan and helminthic infections in the red-legged partridge *Alectoris rufa* were studied in an intensive breeding aviary in the Czech Republic before releasing birds for hunting purposes. Pooled fecal samples (n= 900) were examined over three rearing seasons. A total of four protozoans species, *Cryptosporidium baileyi* (sporadic occurrence), *C. meleagridis*, *Eimeria kofoidi* and *E. legionensis*, and four helminths, *Capillaria phasianina*, *Eucoleus perforans*, *Heterakis gallinarum* (sporadic findings), and *Syngamus trachea* were found. The most common parasite was *S. trachea* (prevalence 20–26%) in dead birds (n= 99); therefore, it represents a high risk for breeders. Co-occurrence of protozoa and helminths indicated similarities in dynamics throughout the three breeding seasons. Mixed infections of *C. baileyi* and *C. meleagridis* with other parasitic species are discussed for the first time. Our findings provide new insight into breeding of *A. rufa* and may help improve the efficacy of disease control strategies and prevention, especially with the potential for spread of parasitic infections to wildlife through released birds. The presence of *C. meleagridis* also requires attention because of its zoonotic potential.

Breeding of galliform game birds represents an important commercial activity worldwide. Aviary breeding of red-legged partridge, *Alectoris rufa* (Galliformes:

Phasianidae) has become more popular in some parts of the Czech Republic and other European countries, due to the high economic value of the bird, although it is vulnerable to parasitic infections that cause losses during breeding. However, despite the existence of data on the spectrum of endoparasitic species from this bird (e. g., Calvete et al. 2003; Pagés-Manté et al. 2007; Villanúa et al. 2007; Millán, 2009; Naciri et al. 2011), no studies have focused on mixed infections with protozoans and helminths.

Breeding facilities harbor high concentrations of partridges in a relatively small area, thus creating favorable conditions for the rapid spread of infectious diseases of different origins, including parasites. In many cases, mono- or multiple infections are frequent and contribute significantly to the prevalence of protozoan or helminthic parasites that could initiate disease outbreaks, morbidity, mortalities (Bolognesi et al. 2006; Pavlásek, 2006; Pagès-Manté et al. 2007; Pavlásek and Šverma, 2009), or pathogen transmission between released birds and wildlife (Millán et al. 2004; Villanúa et al. 2008; Sánchez et al. 2012). Currently, insufficient data are available on cryptosporidial infections, especially focusing on protozoan and helminthic mixed infection age-related dynamics in game bird species such as *A. rufa* under conditions of intensive aviary breeding.

Aviary production may be increased by controlling parasites in artificially bred *A. rufa*. Fecal and post-mortem examinations were therefore carried out on birds from one aviary in the Czech Republic. This study details the course of spontaneous infections of protozoan and helminthic parasites and describes the proportion of positive samples, prevalence, and age dynamics over the period of three rearing seasons. We also describe critical periods of parasite occurrence under intensive farming conditions and discuss the schedule for proper application of anti-parasitic drugs.

MATERIALS AND METHODS

Sample collection

During three rearing seasons (July-December 2012–2014), fecal pooled samples (n= 900) and post-mortem examinations (n= 99) of *A. rufa* from an aviary in the Central Bohemian Region (Czech Republic) were processed. The rearing area was divided into

uphill and downhill sections, each section containing a system of 11 closed brooder houses (BHs) with the same stocking densities (approximately 440 birds from hatching to 4 week-old per BH), which were later combined into five paddocks. Samples were collected from newly hatched up to released birds for hunting. Pooled samples of BHs were represented by approximately 20–30 individual excrements. BHs had concrete floors (covered by a paper carpet during the first weeks) without outside runs. Later, depending on temperature and humidity, birds were placed outside on sandy, clay/muddy or grassy grounds to paddocks and were sampled individually as with BHs. Well-water and commercial food for partridges were provided daily in feeders and bowls.

The majority of fresh samples were collected in the morning at 7-day intervals (or daily, during semester 2013, after the first detection of *Cryptosporidium* spp. oocysts), placed in labelled plastic bags, transported within 12 h to the Department of Pathology and Parasitology, State Veterinary Institute Prague and stored there at 4°C. Dead birds (in several stages of autolysis) were transported (cooled) to the laboratory.

Drug administration (active constituents: flubendazole, levamisole, sulfachlorpyrazine, sulphadimidine, toltrazuril used during study, according to breeder documentation) was without the intervention and influence of the author and was carried out under veterinary supervision.

Coprological examination

The flotation-centrifugation coprological method was used for examination of feces and intestinal contents and the detection of parasitic stages (oocyst of protozoa, eggs of helminths) according to Breza (1957). *Cryptosporidium*-positive fecal samples were processed in wet mounts using glycerin (see Pavlásek 1991). Oocysts of *Eimeria* spp. and eggs of Capillariidae gen. sp. were identified up to genus and family levels, respectively, whereas oocysts and eggs of *Cryptosporidium baileyi*, *C. meleagridis*, *Heterakis gallinarum* and *Syngamus trachea* were identified to species levels.

Unsporulated coccidian oocysts (genus *Eimeria*) were put into Petri dishes with 2.5% potassium dichromate, left to sporulate at room temperature 23°C (± 2°C), then identified up to the species level. *Cryptosporidium* spp. oocysts were previously

molecularly characterized as a part of this study by Máca and Pavlásek (2015), and those data are used here.

Post-mortem examination

For the detection of parasitic stages, scrapings (unstained wet mounts) of the larynx, trachea, crop, livers, intestinal mucosa, bursa of Fabricius and cloaca were examined. Helminthic specimens were isolated and fixed in 4% formalin, 70% and 100% alcohol, and identified to the species level with the help of available identification keys and other specialized literature (e. g., Moravec, 2001; Anderson et al. 2009). Examinations were carried out using a Leica DMLB optical microscope and a Leica MZ6 stereomicroscope.

RESULTS

Coprological examination

A total of four protozoans species were found in *A. rufa*, *C. baileyi* (sporadic occurrence), *C. meleagridis*, *E. kofoidi* and *E. legionensis*. Moreover, four helminths, *Capillaria phasianina*, *Eucoleus perforans*, *H. gallinarum* (sporadic findings), and *S. trachea* were found. The occurrence of parasites showed similar dynamics over three breeding seasons, each starting with the presence of *Eimeria* spp. followed by other protozoan and helminthic infections (see Fig. 1).

Eimeria spp. oocysts occurred in fecal samples over the whole monitoring period (2012–2014) and in all age classes. The percentage positivity ranged from 27–44% (positive samples: 85–114) in the three years, with a maximum of 88%. *Cryptosporidium meleagridis* was positive in 18–99 samples (8–24%) with the highest positivity in the 19 week-old age class during 2012 (88%) and 2013 (72%). On the other hand, *C. baileyi* was only present in 4 samples (1%) of the 5–7 week-old age class in 2013 and 1 (0.4%) of the 17 week-old class in 2014 (Table I). The most commonly found helminths during this study were eggs of Capillariidae gen. sp. (mostly *E. perforans*) in 63–240 samples (27–57%), with a maximum of 100% in 19 and 20–21

week-old age classes (Table I, Fig. 1). *Syngamus trachea* eggs were present in 56–168 samples (24–40%) in 8 week-old birds and older, with a maximum of 68%. There were rare findings of *H. gallinarum* eggs in 2 samples (0.5%) in 2013, and 2 more (1%) in 2014 (Table I). Neither cestodes nor trematodes were found during this study.

The most common findings were single infections (34%), especially those caused by *Eimeria* spp. Mixed infections with 2 taxa (26%) were mainly in combinations of Capillariidae gen. sp. and *S. trachea*; and those with 3 taxa (12%) were with *C. meleagridis*, Capillariidae gen. sp. and *S. trachea*. Only 2% had mixed infections with 4 taxa (Table II).

Post-mortem examination

During 2012, examination of 51 birds showed that *S. trachea* had the highest prevalence (20%) in 6–19 week-old birds. Flotation examination of dead birds was positive for *S. trachea* eggs in 14 cases. In a 13 week-old *A. rufa*, *H. meleagridis*-like lesions were found in the liver. Capillariidae gen. sp. were found in 4 dead birds, in 2 of them *E. perforans* in the crop and *C. phasianina* in the cecum. *Cryptosporidium meleagridis* was diagnosed in 3 birds along with *E. perforans* or *S. trachea*, and *E. perforans* with *C. phasianina* and *S. trachea*, respectively (see Table III).

In the 2013 season, 38 dead birds were examined and the prevalence of *S. trachea* was again the highest (26%) in 7–18 week-old birds as single infections (Table III). There were also 3 mixed infections: *Eimeria* spp. and *S. trachea*, and two cases of *E. perforans* and *S. trachea*. *Eimeria* spp. single infections were also present (8% prevalence).

For 2014, there were only 10 dead birds examined and results were similar to those of year 2013. The prevalence of *S. trachea* was 20% (Table III) and occurred in 12–13 week-old birds. Only one case of *Eimeria* spp. (single infection) was diagnosed in a 6 week-old bird. A mixed infection (*Eimeria* spp. and *S. trachea*) occurred in 12 week-old birds.

DISCUSSION

In this study, we examined the parasitic fauna of *A. rufa* under intensive artificial breeding from a Czech aviary, and demonstrated populations comprising four protozoan and four helminthic species that represent a lower helminth richness (4 spp.) when compared with that of wild (9–13 spp.) and farmed (6 spp.) *A. rufa* in Spain (Calvete et al. 2003; Millán et al. 2004), and of a different composition (nematodes vs. nematodes, cestodes and trematodes). These differences may be due to sample size (99 vs. 296–587 birds), and the broader geographical area (1 vs. 16).

Eimeria spp. occurred throughout the whole sampling period, but no mortalities or outbreaks were produced by this parasite. Naciri et al. (2011, 2014) also found no mortalities in *A. rufa* when a mono-infection with *E. kofoidi* occurred, but a low level of mortality from *E. legionensis* was observed in experimental birds. Mixed infections (dual or triple) considerably increased the effects on growth. Bolognesi et al. (2006) described an outbreak of both *Eimeria* spp. in a game-bird farm in Italy, with high mortalities (46.5% and 52.1%). It is clear that attention should be paid to the presence of mono- and mixed infections with *Eimeria*, as with others avian host species.

Recently, attention has been paid to the issue of cryptosporidial infections of game birds, similarly to other host species. Máca and Pavlásek (2015) studied spontaneous infections of *A. rufa* with *C. baileyi* and *C. meleagridis*, where 5–7 week-old (2013); 18–22 (2012) and 17–21 (2013) week-old birds, respectively were infected, with rapid dispersal throughout the enclosures. This study however, did not include other parasitic species. We have therefore complemented those studies with the finding of *C. baileyi* in 17 week-old birds without spreading, thus suggesting that birds at this age are less sensitive to infection. Moreover, we reported six other parasitic species. Apparently, *A. rufa* does not act as a reservoir host for *C. baileyi*, since it is less parasitized than grey partridges, chickens, Pekin ducks, pheasants or quails (see Pavlásek and Kozakiewicz, 1989; Sun et al. 2005; Pavlásek and Šverma, 2009; Wang et al. 2010, 2012; Máca and Pavlásek, 2016).

The presence of generalist parasites in *A. rufa* could be a potential risk for other hosts when birds are released into the open area and have contact with wild bird populations. Calvete et al. (2003), Millán et al. (2004) and Villanúa et al. (2008)

suggested that released *A. rufa* could share similar parasitic fauna with wild bird populations. In the case of *C. meleagridis*, releasing positive birds represents a risk for animals and humans, since this species has zoonotic potential (Silverlås et al. 2012; Wang et al. 2014).

We demonstrated that long-term anthelminthic drug administration, as used in the aviary, was ineffective in stopping the occurrence of *S. trachea* and Capillariidae gen. sp. eggs in fecal samples; therefore, drug efficacy should be investigated. Interestingly, egg release after treatment (during 2012 and 2013) exceeded the control level, suggesting that antiparasitic drugs should also be implemented in older age classes, according to suggestions by Andreopoulou et al. (2011). The release (expulsion) of oocysts/eggs into fecal samples highlights the presence of an ongoing infection and the possibility of spreading the infection throughout the whole population. On the other hand, some studies have shown that anthelminthic treatments reduce nematode infections, but increase the prevalence of other gastrointestinal parasites (Pedersen and Antonovics, 2013), such as *Eimeria* in wild mice (Knowles et al. 2013). *Syngamus trachea* caused bird mortalities throughout the study, especially in young birds during 2012 and 2013, similar to that reported in partridges (*A. chukar*) in Greece (Andreopoulou et al. 2011), although drug administration positively reduced it. Because body condition could be negatively affected by the helminthic infection (see Gethings et al. 2016), young birds must be treated when this parasite is present.

Mixed infections are very common in populations of various host species (Petney and Andrews, 1998; Graham et al. 2007), although most studies only included single-parasite infections. Our aim was to study this problem in complex parasitic infections, where the parasites may interact (Graham, 2008; Telfer et al. 2010; Ezenwa, 2016; and Naciri et al. 2014) synergistically or antagonistically (Cox, 2001).

In conclusion, prevention, treatment and control of endoparasites are very difficult under conditions of intensive artificial breeding. Some parasitic species such as *C. baileyi* and *C. meleagridis* need more attention, especially in hosts with multi-specific infections where they could have important impacts on bird losses and animal production efficiency. Therefore, it is important to expand our knowledge of parasitic infections in gallinaceous game birds, as well as their veterinary and epidemiological

significance, in order to avoid the introduction and transmission of new pathogens to wild populations.

ACKNOWLEDGEMENTS

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Table captions

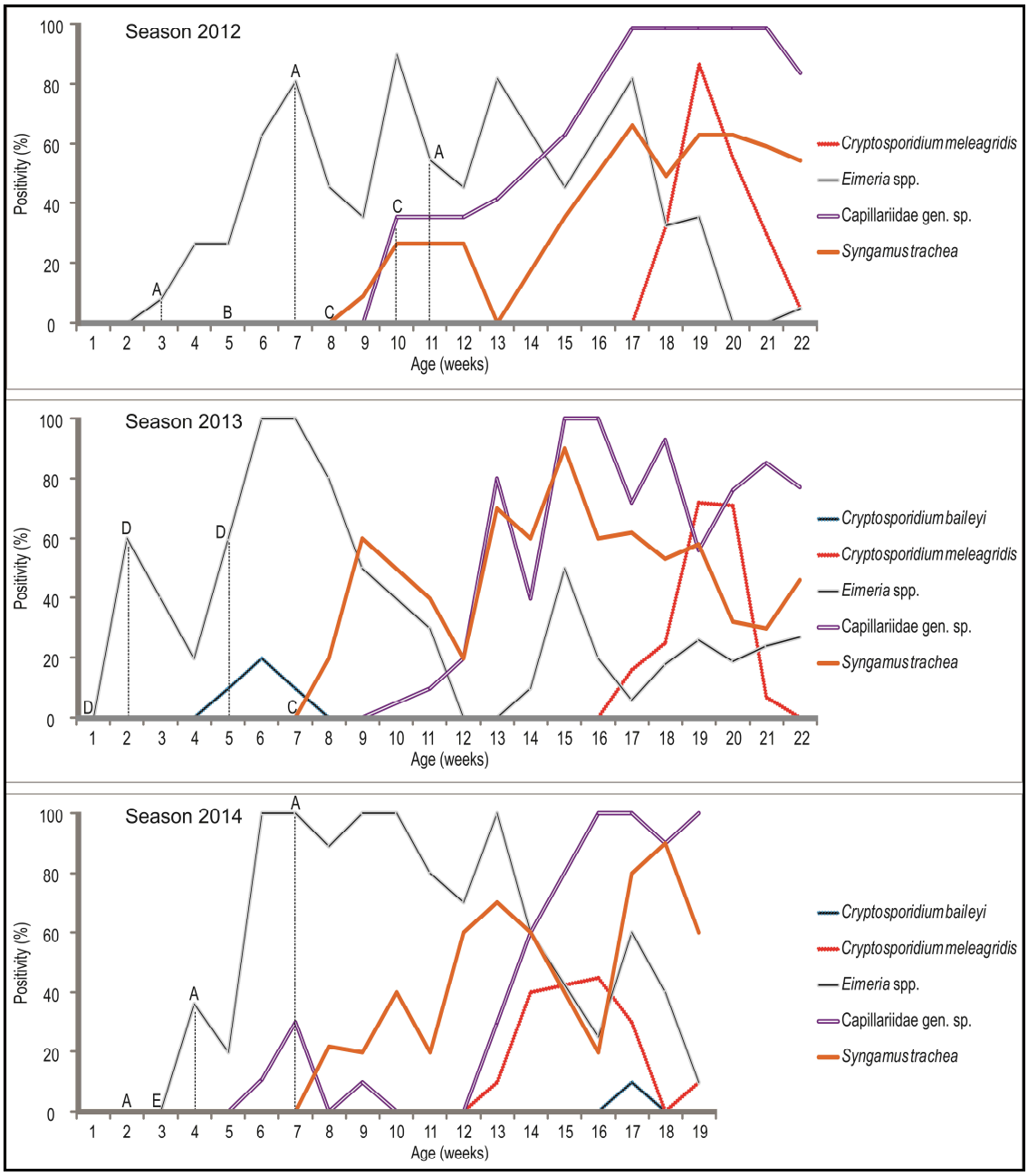
Table I. Parasite occurrence in host age classes and seasons, expressed as percentage of positivity.

Table II. Single and mixed infections obtained from coprological examinations of pooled fecal samples (n= 900), expressed as percentage of positivity.

Table III. Prevalence of protozoan and helminthic infections from post-mortem examinations of *A. rufa* (n= 99).

Figure caption

Figure 1. Graphical representation of weekly age-related infection dynamics over three seasons, complemented by drug administration. Abbreviations: A – sulfachlorpyrazine, B – flubendazole, C – levamisole, D – toltrazuril, E – sulphadimidine.


Season 2012
Positivity (%)
Age (weeks)
A
B
C
Cryptosporidium meleagridis
Eimeria spp.
Capillariidae gen. sp.
Syngamus trachea
Season 2013
D
C
Cryptosporidium baileyi
Season 2014
E

Year	Age group (weeks)	Examined	*C. baileyi* positive (%)	*C. meleagridis* positive (%)	*Eimeria* spp. positive (%)	Capillariidae gen. sp. positive (%)	*Syngamus trachea* positive (%)	*Heterakis gallinarum* positive (%)
2012	1-4	39	-	-	4 (10)	-	-	-
	5-7	33	-	-	19 (58)	-	-	-
	8-12	55	-	-	30 (55)	12 (22)	9 (16)	-
	13-14	12	-	-	10 (83)	5 (42)	-	-
	15-18	23	-	2 (9)	12 (52)	19 (83)	11 (48)	-
	19	25	-	22 (88)	9 (36)	25 (100)	17 (68)	-
	20-21	35	-	17 (49)	-	35 (100)	22 (63)	-
	22	20	-	1 (5)	1 (5)	17 (85)	11 (55)	-
Subtotal		**242**	-	**42 (17)**	**85 (35)**	**113 (47)**	**70 (29)**	-
2013	1-4	40	-	-	12 (30)	-	-	-
	5-7	30	4 (13)	-	26 (87)	-	-	-
	8-12	40	-	-	16 (40)	3 (8)	14 (35)	-
	13-14	20	-	-	1 (5)	12 (60)	13 (65)	-
	15-18	110	-	18 (16)	17 (16)	93 (85)	67 (61)	1(1)
	19	50	-	36 (72)	13 (26)	28 (56)	29 (58)	-
	20-21	105	-	45 (43)	22 (21)	84 (80)	33 (31)	1(1)
	22	26	-	-	7 (27)	20 (77)	12 (46)	-
Subtotal		**421**	**4 (1)**	**99 (24)**	**114 (27)**	**240 (57)**	**168 (40)**	**2 (0.5)**
2014	1-4	89	-	-	8 (9)	-	-	-
	5-7	29	-	-	21 (72)	4 (14)	-	-
	8-12	49	-	-	43 (88)	1 (2)	16 (33)	-
	13-14	20	-	5 (25)	16 (80)	9 (45)	13 (65)	-
	15-18	40	1 (3)	12 (30)	15 (38)	39 (98)	21 (53)	2 (5)
	19	10	-	1 (10)	1 (10)	10 (100)	6 (60)	-
Subtotal		**237**	**1 (0.4)**	**18 (8)**	**104 (44)**	**63 (27)**	**56 (24)**	**2 (1)**
Total		**900**	**5 (0.6)**	**159 (18)**	**303 (34)**	**416 (46)**	**294 (33)**	**4 (0.5)**

Infections (overall positivity)	Positivity percentage (%)		
	2012 (n= 242)	2013 (n= 421)	2014 (n= 237)
Single (1 taxa) (34 %)	**30**	**39**	**29**
Cryptosporidium meleagirdis	-	4	-
Eimeria spp.	21	12	24
Capillariidae gen. sp.	8	17	5
Syngamus trachea	2	6	0.4
Mixed (2 taxa) (26%)	**24**	**31**	**20**
Cryptosporidium baileyi + *Eimeria* spp.	-	1	-
C. baileyi + Capillariidae gen. sp.	-	-	0.4
C. meleagridis + *Eimeria* spp.	-	0.2	-
C. meleagridis + Capillariidae gen. sp.	6	7	0.4
C. meleagridis + *S. trachea*	-	3	-
Eimeria spp. + Capillariidae gen. sp.	6	2	4
Eimeria spp. + *S. trachea*	1	2	8
Capillariidae gen. sp. + *S. trachea*	12	16	6
Mixed (3 taxa) (12%)	**14**	**14**	**8**
C. meleagridis + Capillariidae gen. sp. + *S. trachea*	8	6	2
C. meleagridis + *Eimeria* spp. + *S. trachea*	-	1	1
C. meleagridis + *Eimeria* spp. + Capillariidae gen. sp.	1	2	1
Eimeria spp. + Capillariidae gen. sp. + *H. gallinarum*	-	0.2	-
Eimeria spp. + Capillariidae gen. sp. + *S. trachea*	4	6	4
Mixed (4 genera) (2%)	**2**	**1**	**1**
C. meleagridis + *Eimeria* spp. + Capillariidae gen. sp. + *S. trachea*	2	1	0.4
C. meleagridis + Capillariidae gen. sp. + *H. gallinarum* + *S. trachea*	-	0.2	0.4
Eimeria spp. + Capillariidae gen. sp. + *H. gallinarum* + *S. trachea*	-	-	0.4

Infection (overall prevalence)	Prevalence (%)		
	2012 (n= 51)	2013 (n= 38)	2014 (n= 10)
Single (26%)	**20**	**34**	**30**
Eimeria spp.	-	8	10
Syngamus trachea	20	26	20
Mixed (2 taxa) (7%)	**6**	**8**	**10**
Cryptosporidium meleagridis + *Eucoleus perforans*	2	-	-
C. meleagridis + *S. trachea*	2	-	-
Eimeria spp. + *S. trachea*	2	3	10
E. perforans + *S. trachea*	-	5	-
Mixed (3 taxa) (1%)	**2**	**-**	**-**
Eimeria spp. + *E. perforans* + *S. trachea*	2	-	-
Mixed (4 taxa) (2%)	**4**	**-**	**-**
C. meleagridis + *Capillaria phasianina* + *E. perforans* + *S. trachea*	2	-	-
C. phasianina + *E. perforans* + *Heterakis gallinarum* + *S. trachea*	2	-	-

Appendix IV

Máca O, Pavlásek I. (2015) Endoparasitic infections of *Alectoris rufa* in an aviary in the Czech Republic; 25th WAAVP conference, GB

Endoparasitic infections of *Alectoris rufa* in an aviary in the Czech Republic

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Introduction:

Red-legged partridges *Alectoris rufa* (Galliformes: Phasianidae) are an important game bird species in many countries, but are vulnerable to parasitic infections that cause losses to rearers during breeding. Data on the spectrum of endo-parasitic species from these birds are, however, sporadic (e.g. Calvete et al. 2004; Pagés-Manté et al. 2007; Villanúa et al. 2007; Millán 2009; Naciri et al. 2011). We have studied the age dynamics of endoparasitic infections in farm-reared birds from the Czech Republic over the period 2012 and 2013.The main objective was to identify critical periods during breeding, and our preliminary experiments validated antiparasitics drugs against coccidia of the genus *Eimeria*, and nematodes of *Capillaria* and *Syngamus*. After thorough analysis of data, in close cooperation with the breeder, a preventive antiparasitic scheme will now be prepared. Special emphasis was placed on coccidia of the genus *Cryptosporidium* (Máca and Pavlásek, 2015), since this genus has been extensively studied from different host species in the laboratory of parasitology at the State Veterinary Institute in Prague.

Materials and methods

Characteristics of area and breeding technology

- Two semesters (July-December 2012 and 2013)
- 22 closed sheltering aviaries, later, (~5 week-old birds) 10 paddocks

Sample collection and examination

- 663 pooled samples (fresh excrements with contents of caecum and other parts of the intestinal tract, app. 20-30 individual excrements)
- 7 day intervals, later, daily
- Flotation-centrifugation coprological method (Breza, 1957)
- Native preparations using glycerin (Pavlásek, 1991)
- Parasitological evaluation of 89 whole red-legged partridges
- Developmental stages (scrapings of intestinal mucosa, bursa Fabricii and cloaca)
- Histopathology

Molecular analysis of *Cryptosporidium* spp.

- Sheather sugar solution, followed by bead disruption homogenisation and using the QIAamp® DNA Stool Mini Kit
- Nested PCR analysis of molecular markers: ~400 bp HSP70, ~800 bp actin and ~900 bp fragment of GP60 genes
- Products were purified using the QIAquick® Gel Extraction Kit
- Contigs were compared with sequences published in GenBank using BLAST

Results:

Occurrence of parasites showed variability between semesters, for detailed information on the results see Tables 1 and 2.

1. Coprological examination

Protozoans:

- *Cryptosporidium meleagridis* Slavin 1955 occurred during both years 2012 and 2013 (Fig. 1)
- *C. baileyi* Current, Upton and Haynes, 1986 only occurred in 2013
- *Eimeria* spp. was presented during the entire monitoring

Helminths:

- *Capillaria* spp.
- *Syngamus trachea* (Montagu 1811)

2. Post-mortem examination

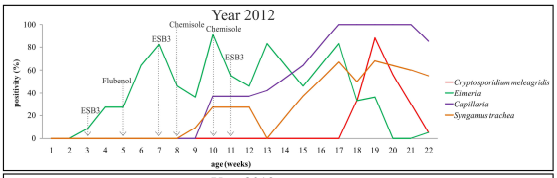
- Most losses were caused by *S. trachea*, prevalence 29% (2012) and 34% (2013)

3. Molecular analysis

- Sequence analysis of isolates from both years confirmed the occurrence of *C. meleagridis* and *C. baileyi*. The GP60 subtype was identified as the IIIe family, named as IIIeA16G2R1 variants b and c (Fig. 2)

Year	Age group (weeks)	Examined	No. of *C. baileyi* positive (%)	No. of *C. meleagridis* positive (%)	No. of *Eimeria* spp. positive (%)	No. of *Capillaria* spp. positive (%)	No. of *Syngamus trachea* positive (%)
2012	1-4	39	0	0	4 (10)	0	0
	5-7	33	0	0	19 (58)	0	0
	8-12	55	0	0	30 (55)	12 (22)	9 (16)
	13-14	12	0	0	10 (83)	5 (42)	0
	15-18	23	0	2 (9)	12 (52)	19 (83)	11 (48)
	19-21	60	0	39 (65)	9 (15)	60 (100)	39 (63)
	22	20	0	1 (5)	1 (5)	17 (85)	11 (55)
Total		**242**	**0**	**42 (17)**	**85 (35)**	**113 (47)**	**70 (29)**
2013	1-4	40	0	0	12 (30)	0	0
	5-7	30	4 (13)	0	26 (87)	0	0
	8-12	40	0	0	16 (40)	3 (8)	14 (35)
	13-14	20	0	0	1 (5)	12 (60)	13 (65)
	15-18	110	0	18 (16)	17 (16)	93 (85)	67 (61)
	19-21	155	0	81 (52)	35 (23)	112 (72)	62 (40)
	22	26	0	0	7 (27)	20 (77)	12 (46)
Total		**421**	**4 (1)**	**99 (24)**	**114 (27)**	**240 (57)**	**168 (40)**
Grand total		**663**	**4 (0.6)**	**141 (21)**	**199 (30)**	**353 (53)**	**238 (36)**

Table 1: Results of parasite occurrence according to host age classes and semesters completed, expressed as percentage positivity.



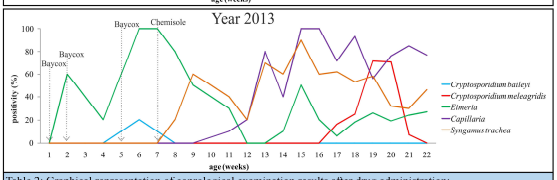


Table 2: Graphical representation of coprological examination results after drug administration; Baycox (Toltrazurilum), Chemisole (Levamisolum), ESB3 (Sulfaclozinum), Flubenol (Flubendazolum).

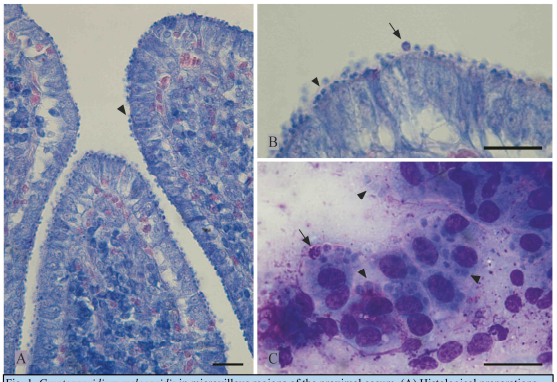


Fig. 1. *Cryptosporidium meleagridis* in microvillous regions of the proximal cecum. (A) Histological preparations (arrowhead indicates mucosa at various developmental stages). (B) Detail of mucosa, developmental stages (arrowhead), meront (arrow). (C) Smear of intestinal mucosa; Scale bars = 20 µm (reproduced from Máca and Pavlásek, 2015).

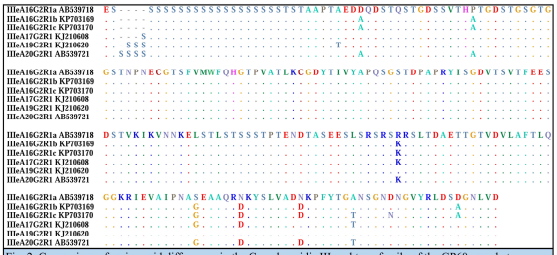


Fig. 2. Comparison of amino acid differences in the *C. meleagridis* IIIe subtype family of the GP60 gene between sequences acquired in the study and those retrieved from GenBank (reproduced from Máca and Pavlásek, 2015).

Conclusions:

The main infections of *A. rufa* comprised representatives of the coccidia genera *Eimeria* and *Cryptosporidium* and of the nematode genera *Capillaria* and *Syngamus*. Further research will be focused on the potential to design control measures for these parasites using appropriate antiparasitic drugs.

Acknowledgements:

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Appendix V

Endoparasitic infections of *Phasianus colchicus* from an intensive artificial breeding programme in the Czech Republic (unpublished data).

Table I. Parasite occurrence in host (*P. colchicus*) age classes and seasons, expressed as percentage of positivity.

Year	Age group (weeks)	Examined	*C. baileyi* positive (%)	*C. meleagridis* positive (%)	*Eimeria* spp. positive (%)	Capillariidae gen. sp. positive (%)	*Syngamus trachea* positive (%)	*Heterakis gallinarum* positive (%)
2013	1-4	58	1 (2)	-	30 (52)	-	-	-
	5-7	66	8 (12)	1 (2)	66 (100)	8 (12)	24 (36)	-
	8-12	22	7 (32)	3 (14)	20 (91)	10 (46)	10 (46)	5 (23)
	13-14	4	-	1 (25)	4 (100)	2 (50)	2 (50)	4 (100)
	15-18	7	1 (14)	1 (14)	7 (100)	4 (57)	2 (29)	7 (100)
	Older*	16	2 (13)	1 (6)	14 (88)	7 (44)	5 (31)	3 (19)
Subtotal		**173**	**19 (11)**	**7 (4)**	**141 (82)**	**31 (18)**	**43 (25)**	**19 (11)**
2014	1-4	53	2 (4)	-	40 (76)	2 (4)	2 (4)	-
	5-7	39	2 (5)	1 (3)	39 (100)	5 (13)	19 (72)	2 (5)
	8-12	47	18 (38)	5 (11)	46 (98)	25 (53)	28 (60)	7 (15)
	13-14	14	2 (14)	1 (7)	14 (100)	8 (57)	7 (50)	8 (57)
	15-18	22	3 (14)	1 (5)	22 (100)	17 (77)	10 (46)	14 (64)
	19-24	16	-	-	16 (100)	14 (88)	9 (56)	11 (69)
	Older*	47	9 (19)	2 (4)	47 (100)	30 (64)	19 (40)	19 (40)
Subtotal		**238**	**36 (15)**	**10 (4)**	**224 (94)**	**101 (42)**	**94 (40)**	**61 (26)**
Total		**411**	**55 (13)**	**17 (4)**	**365 (89)**	**132 (32)**	**137 (33)**	**80 (20)**

*Older = various age groups

Table II. Prevalence of protozoan and helminthic infections from post-mortem examinations of *P. colchicus* (n= 514).

Infection (overall prevalence)	Prevalence (%)			
	2012 (n= 36)	2013 (n= 219)	2014 (n= 168)	2015 (n= 91) *
Capillariidae gen. sp.	17	7	11	6
Cryptosporidium baileyi	25	4	1	-
Cryptosporidium meleagridis	3	-	2	-
Eimeria spp.	56	47	31	51
Heterakis gallinarum	53	6	20	6
Histomonas meleagridis-like	3	1	2	-
Choanotaenia	-	-	1	-
Syngamus trachea	28	7	15	6
Single (1 taxa)	**28**	**35**	**28**	**46**
Mixed (more taxa)	**64**	**17**	**21**	**7**

* During 2015 only flotation examinations were carried out

Appendix VI

Curriculum Vitae

CURRICULUM VITAE

Name:	Ing. **Ondřej Máca**
Date of birth:	3.6.1985
Telephone number:	+420737436528
E-mail:	biola.biola@seznam.cz

Education:

2012 – now PhD studies at the **Faculty of Tropical AgriSciences** CULS Prague

2010 – 2012 master's degree at **Institute of Tropics and Subtropics** CULS Prague; Wildlife Management in Tropic and Subtropics (Defended Diploma thesis: The parasite fauna of some domestic and wild ruminant species in Bandia and Fathala Reserve in Senegal)

2005 – 2009 bachelor's degree at **Faculty of Science** University of South Bohemia in České Budějovice (Defended Bachelor thesis: Parasitic crustaceans from Neotropical freshwater fish)

2001 – 2005 **Grammer school**, Pierra de Coubertina in Tábor

Experiences:

2009 – now **State Veterinary Institute Prague, Pathology and Parasitology Department**, Prague, Czech Republic

2014 **El Colegio de la Frontera Sur**, Unidad Chetumal, Mexiko, laboratory work (coprology and post-mortem examinations) and field work (birds breeding programs, sampling), 1 month

2011 **reserve Bandia and Fathala** (an expedition with the members of the *ex situ* conservation programme for protecting the western Derby eland (*Taurotragus derbianus derbianus*) in Senegal, 1 month

2006 – 2009 **Biology Centre, ASCR, v.v.i., Institute of Parasitology**, Czech Republic

Received Awards:

2016 **Prize of Josef Hlávka**

Received Grants:

2014 – 2016 **Internal Grant Agency of the Czech University of Life Sciences in Prague** (CIGA)

- Project: The dynamics of endoparasitic infections in farmed birds ring-necked pheasant (*Phasianus colchicus*) and red-legged partridge (*Alectoris rufa*) (Galliformes: Phasianidae)

Conferences:

2015 poster (Title: Endoparasitic infections of *Alectoris rufa* in an aviary in the Czech Republic) **25th WAAVP conference**, GB

2008 poster (Title: Amazon fish crustacean parasites) **Helmintologické dny conference**, CR

Certificate:

2015 Qualifications and competence in the field of experimental animals according to § 15 paragraph 2, letter d) of Act no. 246/1992 Coll., On protection of animals against cruelty, as amended

Publications:

Máca O. 2018. Molecular identification of *Sarcocystis lutrae* in the European otter (*Lutra lutra*) and the European badger (*Meles meles*) from the Czech Republic. Parasitology Research 117:943–945.

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Máca O. 2012. Description of a new species of *Eimeria* Schneider, 1875 (Apicomplexa: Eimeriidae) from the western Derby eland *Taurotragus derbianus derbianus* Gray (Artiodactyla: Bovidae) in Senegal. Systematic Parasitology 82:121–3.