Conquering Switzerland: emergence of *Angiostrongylus vasorum* over three decades and rapid regional increase in the fox population contrasts with the stable prevalence of lungworms

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Abstract

Angiostrongylus vasorum, *Crenosoma vulpis* and *Capillaria aerophila* are the most common lungworms of domestic and wild canids. We investigated the short- and long-term lungworm prevalence changes in the Swiss fox population with a focus on *A. vasorum*. Between 2012 and 2017, lungs and hearts of 533 foxes from north-eastern Switzerland were necropsied and blood samples tested for circulating *A. vasorum* antigen. *Angiostrongylus vasorum* prevalence increased steadily from 21.5% in 2012 to 81.8% in 2017. In contrast, *C. aerophila* and *C. vulpis* prevalences fluctuated between 41.8% and 74.7%, and 3.6% and 14.9%, respectively. Based on 3,955 blood samples collected between 1986 and 2017 from three geographic areas, antigen seropositivity increased from 2.4%, to 62.0%. In north-eastern Switzerland seropositivity was initially low (1.9%, and 1.7%, respectively) but increased in the following two decades to 22.2% and 62.0%, respectively. Our findings depict the spectacular expansion of *A. vasorum* in the past three decades. Regionally the prevalence in foxes increased fourfold in six years in some regions. This underpins the important role of foxes as reservoir hosts, likely explaining the increasing number of cases of canine angiostrongylosis in Switzerland. Our findings may help anticipating future developments in areas where *A. vasorum* is present but (still) infrequent.

Key words: fox, *Angiostrongylus vasorum*, *Crenosoma vulpis, Capillaria aerophila,* nematode, serology, necropsy, prevalence, worm burden, Switzerland

Introduction

Angiostrongylus vasorum, *Crenosoma vulpis* and *Capillaria aerophila* (syn. *Eucoleus aerophilus*) are the most common lungworms of domestic and wild canids in Europe (Saeed *et al.* 2006; Taylor *et al.* 2015; Tolnai *et al.* 2015; Hermosilla *et al.* 2017; Maksimov *et al.* 2017; Martinez-Rondan *et al.* 2019; Traversa *et al.* 2019). The red fox (*Vulpes vulpes*) represents the best known wildlife reservoir host for transmission of parasitic diseases to domestic dogs (Otranto *et al.* 2015). The cardiopulmonary nematode *A. vasorum* resides in the right heart and pulmonary arteries of infected definitive hosts; slugs and snails act as intermediate hosts (Guilhon and Cens 1973). The parasite is endemic in Europe, South America and Newfoundland, Canada (Jefferies *et al.* 2010) and has also been reported from Uganda (Bwangamoi 1972). Importantly, canine angiostrongylosis can manifest with respiratory signs, bleeding disorders and other clinical signs; they can be severe, and fatal in 10-15% of cases (Chapman *et al.* 2004; Koch and Willesen 2009). Especially in Europe, where the parasite was first discovered (Serres 1854; Cuillé and Darraspen 1930), the occurrence of *A. vasorum* has been increasingly reported in the past decades, in known and in newly recognised endemic areas (Helm *et al.* 2015; Jolly *et al.* 2015; Lurati *et al.* 2015; Maksimov *et al.* 2017). For instance, in British and Danish fox populations, increased *A. vasorum* prevalences have been observed between 2005-2006 and 2013-2014 (Morgan *et al.* 2008; Taylor *et al.* 2015) and between 1997-2002 and 2006-2008 (Saeed *et al.* 2006; Al-Sabi *et al.* 2014), respectively. However, infected foxes were also reported from countries without previous wildlife hosts reports (Demiaszkiewicz *et al.* 2014; Kistler *et al.* 2014).

Adult *C. vulpis* are found in the bronchi of canids. Like *A. vasorum,* this parasite requires gastropods as intermediate hosts (Wetzel 1940). *Capillaria aerophila* infects canids, felids, mustelids and hedgehogs; adults reside in the trachea and bronchi of their definitive hosts and can be transmitted directly or via earthworms (Chandler 1922; Christenson 1938; Holmes and Kelly 1973; Deplazes *et al.* 2016). *Capillaria aerophila* may cause zoonotic infections in rare occasions (Otranto and Deplazes 2019). *Crenosoma vulpis* is endemic in Europe and North America and *C. aerophila* occurs worldwide (Conboy 2009). These two parasites have been increasingly reported, but evidence for an actual spread to new areas is lacking, despite the partial overlapping spectrum of definitive and intermediate hosts with *A. vasorum* (Sreter *et al.* 2003; Saeed *et al.* 2006; Morgan *et al.* 2008; Al-Sabi *et al.* 2014; Taylor *et al.* 2015; Tolnai *et al.* 2015; Maksimov *et al.* 2017).

In foxes, infections with lungworms are mostly detected by necropsy and, less frequently, by examination of faecal samples processed by the Baermann technique or faecal flotation, or by DNA

detection in bronchoalveolar fluid (Willingham *et al.* 1996; Houpin *et al.* 2016; Koller *et al.* 2019). ELISAs for detection of circulating *A. vasorum* antigen and specific antibodies, developed for dog samples (Schnyder *et al.* 2011; Schucan *et al.* 2012), were evaluated for their use in foxes (Gillis-Germitsch *et al.* 2017). The antibody response in foxes was highly variable, hence, the assay showed low sensitivity. Antigen detection, instead, revealed high sensitivity and specificity, and a positive correlation between worm burdens and optical density values. Since fox necropsies are time-consuming, while blood collection from dead animals is easy and fast, antigen detection was proposed as an efficient method for mass-screening, also in fox populations (Gillis-Germitsch *et al.* 2017).

The aim of this study was to investigate the short- and long-term lungworm prevalence changes in the Swiss fox population with a focus on the emergence of *A. vasorum*. To do so, lungs and hearts of foxes from north-eastern Switzerland were necropsied over six years and their blood samples analysed for the presence of circulating *A. vasorum* antigen. Additionally, countrywide *A. vasorum* prevalence changes over a 30-year period of time were investigated in a retrospective analysis of Swiss fox blood samples sampled between 1986 and 2017.

Material and Methods

Fox lung and heart necropsy

Between 2012 and 2017, 533 red foxes (*Vulpes vulpes*) were killed during hunting seasons in the frame of control and management measures of fox populations by game wardens and local hunting associations and brought to the Institute of Parasitology in Zurich, Switzerland. All animals originated from the north-east of Switzerland, which, based on defined biogeographic characteristics, is part of the Swiss Plateau (Gonseth *et al.* 2001). As of 2013, foxes were shot exclusively in the canton of Zurich. Five hundred foxes were killed in rural areas and 33 in the city of Zurich. The animals were necropsied in the first two months of the year: 79 in 2012 (of these, 39 foxes were necropsied in October), 87 in 2013, 42 in 2014, 88 in 2015, 83 in 2016 and 154 in 2017. Sex and age were determined (either 'young' (<12 months of age) or 'adult' (>20 months of age), according to tooth wear). Lungs and hearts were removed in one piece and frozen at -20 °C until further examination. Blood samples, collected directly during necropsy and/or recovered from hearts and lungs before dissection, were stored at -20 °C for later mass-screening. As of 2014, the lungs were photographed and scored macroscopically from 0-3 according to macroscopic pathological changes. The classification key, adapted from Poli *et al.* (1991), was as follows: 0 = healthy lung without pathological

changes (no discoloration, regular tissue consistency, no adhesions); 1 = mild changes (discoloration, regular tissue consistency, no adhesions); 2 = moderate pathologies (discoloration, nodules and/or increased tissue consistency in lung lobes, no adhesions), and 3 = severe pathologies (discoloration, nodules and/or increased tissue consistency in all lung lobes, adhesions amongst lung lobes, with pericardium and/or mediastinum). After thawing at 4° C, lungs and hearts were transferred to a conical glass, which was filled with tap water. Ventricles, atria and pulmonary arteries were opened with surgical scissors. Then, bronchi, bronchioles and all visible blood vessels and airways were cut open, until the lung only consisted of a thin layer of tissue. During the dissection process the organs were repeatedly 'washed' in the water recipient. Lungs and hearts were then removed and parasites were allowed to settle down in the glass for at least 30 minutes. The supernatant was discarded, the sediment was transferred to a large petri dish and examined under a stereomicroscope. Parasites, if present, were counted; species and sex were determined based on Guilhon and Cens (1973), Wetzel (1940) and Christenson (1935).

Analysis of fox blood samples

Since 1986 foxes had been investigated in the frame of different projects and studies performed at the Institute of Parasitology in Zurich (Ewald 1993; Hofer *et al.* 2000; Fischer *et al.* 2005; Tanner *et al.* 2006; Hegglin *et al.* 2007; Guerra *et al.* 2014). A total of 3,955 blood samples were available from Swiss foxes collected between 1986 and 2017, including samples from the above mentioned 533 foxes necropsied between 2012 and 2017. The samples were therefore kept frozen at -20 °C for several years until examination for this study. All samples were tested by ELISA for detection of circulating *A. vasorum* antigen as described in Schnyder *et al.* (2011) and cut off values from fox studies performed by Gillis-Germitsch *et al.* (2017) were applied. To confirm that antigen can still be detected in old samples we retested 63 8 to 13-year-old stored blood samples from *A. vasorum* experimentally infected dogs and compared the resulting OD values.

In order to investigate prevalence changes over time samples were assigned to four different periods of time: 1986 to 1992 (period 1, n=1729), 1993 to 2002 (period 2, n=1524), 2003 to 2012 (period 3, n=249) and 2013 to 2017 (period 4, n=453). The coordinates of hunting locations from 3,704 foxes were used to create maps with the program Quantum GIS Version 3.6 Noosa (shown in Fig. 1); coordinates were missing for 251 samples. In addition, Fig. 1 displays the three regions from which samples were collected: the north-eastern part of Switzerland (encompassing the canton of Zurich)

consisting of the Swiss Plateau and Prealps, the south-eastern part that comprises alpine areas and the southern part of the Alps, and the third area in the western part of Switzerland including different biogeographic regions (Swiss Plateau, Alps and Jura) (Gonseth *et al.* 2001).

Statistical analysis

Statistical analysis was performed in R (v. 3.5.1). For prevalence calculation, an animal was considered infected with *A. vasorum* based on antigen detection above cut off (Gillis-Germitsch *et al.* 2017) and/or presence of worms at necropsy. Prevalences for *C. vulpis* and *C. aerophila* were calculated based on the necropsy results only. The exact binominal 95% confidence intervals (CI) were calculated according to Clopper and Pearson (1934). Relationships between *A. vasorum* infection status ('infected' or 'uninfected') and age ('young' or 'adult'), sex, as well as the hunting years were analysed using a generalised linear (multivariate) model. The data were further stratified to produce odds ratios on significant model predictors. Foxes for which data on sex or age were missing (n = 30/533) were excluded from the analysis.

The relationships between lung scores reflecting pathological changes and *A. vasorum*, *C. aerophila*, and *C. vulpis* worm burdens as well as hunting years, age and sex were analysed by ordinal logistic regression. Lung scoring data were missing for foxes hunted in 2012 and 2013. Foxes for which data on sex or age were missing (n = 11/367) were excluded from the analysis. Average worm burden and lung score differences between years were analysed using one-way ANOVA. Mean worm burdens stratified by age groups were tested in a non-parametric Wilcoxon rank test.

Results

Necropsy results

In total, 244 females, 275 males and 14 foxes for which the sex was not recorded, were necropsied. Out of the hunted 533 foxes 312 were adults and 191 were juveniles; the age of 30 foxes was not determined. Yearly lungworm prevalences for *A. vasorum*, *C. vulpis* and *C. aerophila* are displayed in Tab.1. Of the 533 foxes 72 did not have any lungworms, 253 were infected with one lungworm species, 184 with two species and 24 with all three (see Fig. 2). Overall, 3,453 intact adult lungworms were recovered: 1,572 *A. vasorum* (1,002 females, 570 males), 270 *C. vulpis* (157 females, 113 males) and 1,611 *C. aerophila* (854 females, 757 males). Exact hunting locations of foxes with the three lungworm species are displayed in Fig. 3a-c.

Between 2012 and 2017, based on necropsy the prevalence of *A. vasorum* infection in the fox population within the study area increased steadily and significantly ($p < 0.001$) from 10.1 % (8 out of 79; 95% CI = 4.5-19.0) to 71.4 % (110 out of 154; 95% CI = 63.6-78.4) (Tab. 1). Differences in particular were significant between 2012 and 2014 (and the following years), between 2013 and 2015 (and the following years), and between 2014 and 2016 and 2017. The yearly arithmetic mean *A. vasorum* worm burden per infected animal increased by trend, especially between 2014 and 2017 (Fig. 4): on average, foxes harboured 5.0 adult *A. vasorum* specimens in 2014 (n=19), 5.9 in 2015 (n=52), 8.5 in 2016 (n=59), and 10.3 in 2017 (n=110) ($p = 0.06$). During the period under examination, neither the infection status (odds ratio (OR) = 1.19, $p = 0.39$) nor the average worm burden ($p = 0.77$) differed across age groups (young versus adult). Males were more often infected than females, however below the statistical significance level (OR = 1.32, $p = 0.14$). No worm burden differences were observed between sexes.

Prevalences of *C. vulpis* and *C. aerophila* fluctuated each year. *Capillaria aerophila* prevalence ranged between 41.8 % and 74.7 % but the year-to-year variation did not show any trend (Tab. 1). Similarly, the mean worm burden oscillated between a minimum of 2.0 (2012) and maximum 10.7 (2014), and ranged from 1 to 99 specimens (Fig. 4). Male foxes tended to be more often infected with *C. aerophila* than females (OR = 2.5, $p < 0.001$) and with a higher worm burden ($p < 0.01$); there was no correlation between the infection status and age. Among *C. aerophila* infected animals, the mean worm burden was not significantly different between both age groups ($p = 0.27$). *Crenosoma vulpis* was less commonly found, infecting between 3.6 % (2016) and 14.9 % (2013) of the fox population. Mean worm burdens fluctuated between 1.3 (2016) and 10.4 (2015), and ranged from 1 to 49 adult specimens (Fig. 4). Male foxes tended to be more often infected than females (OR = 4.4, $p < 0.001$), as did young animals (OR = 1.8, $p = 0.06$). Worm burdens were not different between sexes or age groups.

Macroscopic lung scores

A total of 366 lungs were scored according to their macroscopic appearance and were distributed as follows: score 0, n=7; score 1, n=39; score 2, n=153; score 3, n=167, indicating different degrees of pathological alterations. For illustrative images of lungs scoring 0 to 3 see Supplementary Figs. S1a-d. Among *A. vasorum* infected foxes (n=269), 3 scored 0 (1.1%), 17 scored 1 (6.3%), 85 lunges scored 2 (31.6%) and 164 lungs scored 3 (61.0%). Mean lung scores increased from 2.21 (± 0.7) to 2.77 (± 0.46) between 2014 and 2017 ($p = 0.06$). *Angiostrongylus vasorum* infection was significantly

associated with high macroscopic lung scoring ($p < 0.001$). For every increase in *A. vasorum* worm burden, the odds of being more likely to show a higher lung score is multiplied by 1.21 (95 % CI = 1.15-1.28). For the other lungworms there was no significant association.

Serological results

In line with necropsy data, prevalence rates determined by the antigen ELISA for foxes shot between 2012 and 2017 in the north-east of Switzerland increased year by year, from overall 20.3% to 76.6%. Differences were significant between 2012 and 2015 (and following years), between 2013 and 2015 (and following years) and between 2014 and 2017. The comparison between necropsy and serological results showed that both procedures were in agreement (Tab. 1). Combined necropsy and serology results lead to an increasing prevalence from 21.5% (17 out of 79; 95% CI = 13.1-32.2) in 2012 to 81.8% (126 out of 154; 95% CI = 74.8-87.6) in 2017.

Out of overall 3,955 fox blood samples, originating from the whole country, 463 (11.7%) were positive for circulating *A. vasorum* antigen (Fig. 1). Antigen seropositivity increased over time periods 1 to 4 from 2.4%, to 3.9%, to 32.9%, and to 62.0%, respectively (Fig. 5a-d). North-eastern Switzerland is the only area represented in all four time intervals: a drastic increase can be seen when comparing time periods 1 to 4. Samples from south-eastern Switzerland were available from 3 time periods (1, 2 and 3), and those from western Switzerland were collected during periods 1 and 3 only. An increase in number of positive samples in the southern part of Switzerland from the second to the third time span was observed.

The retested 8 to 13-year-old blood samples from *A. vasorum* experimentally infected dogs showed slight to moderate deviations in their OD values in both directions (de- or increase) when compared to initial values. The greatest variations were observed in samples with high initial OD values. A decrease in highly positive samples did not lead to values below the cut-off (Supplementary Results S2).

Discussion

Our necropsy data show that *A. vasorum* prevalence increased fourfold in the fox population in the canton of Zurich in only 6 years. In addition, the parasite burdens increased as well, with higher mean worm loads per infected fox each year. This suggests that infected foxes may get continuously reinfected, accumulating worms in their cardiopulmonary tissues. In line with this, experimental trials

showed that foxes do not develop protective immunity and that *A. vasorum* worm burdens increase in animals upon reinfection (Woolsey *et al.* 2017), which also resulted in continuous and increasing numbers of excreted first stage larvae (L1). Our results support the hypothesis that infected foxes are a continuous source for environmental contamination with larval stages that allow the infection of gastropod intermediate hosts (Woolsey *et al.* 2017). This, on the one hand, explains the successful spread and establishment of *A. vasorum* in novel areas, and on the other hand, confirms the important role of foxes as wildlife reservoir hosts, likely contributing to dog infection dynamics in the same areas.

In contrast, the prevalences of other common lungworms of foxes, i.e. *C. vulpis* and *C. aerophila,* were not increasing, but rather fluctuated from year to year. Also, contrasting with *A. vasorum,* the *C. vulpis* and *C. aerophila* worm burdens did not increase over time, despite the fact that *A. vasorum* and *C. vulpis* share the same intermediate hosts (Lange *et al.* 2018). Hence, one may have expected parallel increasing prevalences for both parasites. In agreement with our results, in Hungary, *A. vasorum* prevalence in foxes increased from 5% to 18% between 2002 and 2013/2014, while prevalences of *C. vulpis* (24% in both studies) and *C. aerophila* (66% versus 62%) were constant (Sreter *et al.* 2003; Tolnai *et al.* 2015). Similarly, in Copenhagen, Denmark, a long known endemic region for *A. vasorum*, the parasite's prevalence was of 49% in 1997-2002 and of 80% in 2006-2008, while the prevalences of other lungworms remained approximately constant (*C. vulpis*: 17 versus 23%; *C. aerophila*: 74.5 versus 87%) (Saeed *et al.* 2006; Al-Sabi *et al.* 2014). In Great Britain, *A. vasorum* prevalence increased from 7 to 18% in 8 years, while *C. vulpis* increased from 2 to 11% and *C. aerophila* remained at high levels of 39 and 32%, respectively (Morgan *et al.* 2008; Taylor *et al.* 2015). Overall, *C. aerophila* was the most abundant lungworm in these three countries and prevalences were comparable with the present Swiss prevalence. In Switzerland *C. vulpis* was the least common lungworm recovered (10.1%), closely paralleling the prevalence in Great Britain (11%), but below the observed proportions in Danish and Hungarian fox populations (17-23% and 24%, respectively).

Prior data on *A. vasorum* and other lungworms in Swiss foxes are scant. In a single Swiss-wide investigation from 2010-2012 performed on fox faeces an overall *A. vasorum* prevalence of 8.8% was established (Koller *et al.* 2019). This low prevalence obtained before 2012 is in line with the hypothesis of increasing numbers of infected animals over a relatively short time but also supports the superiority of necropsy data over faecal examinations performed on frozen samples. Surprisingly, *Capillaria* spp. were less prevalent (8.3%) and *C. vulpis* more prevalent (21.4%) (Koller *et al.* 2019), compared to the present observations (62.9 and 10.1%, respectively). This is likely due to the ability of *C. vulpis* L1 to

resist deep frost, even at -80 °C (Saeed *et al.* 2006), unlike *A. vasorum* L1 and *Capillaria* eggs. Both *A. vasorum* and *C. vulpis* were detected frequently in the Swiss Plateau, while both were less commonly detected in the southern (warmer) part of Switzerland, also suggesting that these parasites may be more adapted to survive in colder climates (Koller *et al.* 2019).

Clustered distributions of *A. vasorum* and *C. vulpis* were observed in several countries, with either overlapping or diverging occurrences. This was attributed to distinct temperature and precipitation optima favouring the life cycle of *A. vasorum* (Jeffery *et al.* 2004; Taylor *et al.* 2015; Tolnai *et al.* 2015; Cabanova *et al.* 2018). However, findings about the influence of climate on *A. vasorum* and *C. vulpis* occurrence in fox populations are conflicting. This is likely due occurrence of *A. vasorum* and *C. vulpis* in local endemic spots, which may also be defined by local gastropod populations (Aziz *et al.* 2016; Lange *et al.* 2018).

In 2009, Morgan *et al*. used a model that relied on long-standing known endemic foci of *A. vasorum* and on climatic data to predict potential areas in which *A. vasorum* could establish in the future, even without climate change. Switzerland was entirely included, with differing levels of predicted suitability (Morgan *et al.* 2009). The spread of *A. vasorum* predicted by the model has also been recently described from previously parasite-free areas (or areas with unknown presence) such as from Romania (Deak *et al.* 2017), Belgium (Jolly *et al.* 2015), the Czech Republic (Hajnalová *et al.* 2017), Slovakia (Hurnikova *et al.* 2013) and even from mainland North America (Priest *et al.* 2018). Here we show that *A. vasorum* has successfully established in the Swiss fox population, reaching regional prevalences of more than 80%. Particularly interesting is the marked emergence of *A. vasorum* in the Swiss fox population at around the start of the new millennium (from study time span 2 to 3). The timing of this marked increase correlates with first accumulations of cases of canine angiostrongylosis occurring between 1999 and 2004 in southern and northern Switzerland (Staebler *et al.* 2005). The very first cases of *A. vasorum* in the country were nonetheless already reported in the 1960's from a dog breeding facility in the canton of Zurich (Wolff *et al.* 1969). Although after these first cases there were no further reports for approximately 40 years, we actually illustrate that since 2013 the whole canton of Zurich represents a hot spot. More recently, there is accumulating evidence in support of a spread in the dog population (Glaus *et al.* 2010; Lurati *et al.* 2015; Sigrist *et al.* 2017).

In Switzerland, a drastic increase in the number of foxes in rural and urban areas has been observed in the 1980s and 1990s (Breitenmoser *et al.* 2000). In the city of Zurich, fox density is estimated at more than 10 adult foxes per square kilometre (Gloor 2002). This increased number of foxes could

result in more contact between foxes and humans and pets (Gloor 2002; Deplazes *et al.* 2004), increasing the risk of parasite transmission (Saeed *et al.* 2006; Otranto *et al.* 2015). It can be hypothesised that transmission of *A. vasorum* among foxes started to increase at the end of the 20st century due to higher density of foxes, increasing contamination of the environment and therefore infecting intermediate hosts, as well as dogs. Fox populations were suggested to reliably reflect the parasite occurrence because unbiased by factors such as increased disease awareness or anthelmintic treatments (Taylor *et al.* 2015). The same authors advanced that fox data on parasite distribution and infection intensity over time will promote our understanding of the epidemiology and anticipate future trends (Taylor *et al.* 2015).

In general, the lung necropsy findings observed in foxes are similar to the ones observed in experimentally (Schnyder *et al.* 2010) and naturally (Bourque *et al.* 2008) infected dogs. *Angiostrongylus vasorum* infected foxes necropsied in our study displayed severe lung pathology, such as partial lung fibrosis, lung lobe consolidation and adhesions. Increasing worm burdens were associated with higher lung scores. Despite the fact that we necropsied hunted animals, and that post mortem changes such as haemorrhages, discoloration and trauma may macroscopically affect some lungs, a more severe degree of lung pathology was nevertheless associated with higher worm burdens. In Italy, Poli *et al.* (1991) described similar findings in necropsied foxes and confirmed slight changes in 6.5% of *A. vasorum* positive foxes, mild alterations in 92.5% of infected foxes, and severe pathology in 1% of the animals. Eleni *et al.* (2014) recently scored 27 lungs of *A. vasorum* infected Italian foxes and identified no lesions in 18.5%, light changes in 33.3%, mild changes in 22.2% and severe pathology in 25.9 % of the lungs, respectively. Unlike in dogs (Stockdale and Hulland 1970), foxes infected with *C. vulpis* showed little pathological lesion in the lungs (Jeffery *et al.* 2004), explaining the lack of correlation between *C. vulpis* infection and higher lung scoring. Generally, reports from naturally infected foxes with apparent clinical illness are rare (Simpson 1996; Philbey and Delgado 2013), and experimentally infected foxes did not show any clinical signs (Webster *et al.* 2017). However, affected fitness in foxes with high worm burdens and severe lung pathology cannot be excluded.

There was no significant correlation between age and any lungworm infection, indicating that foxes get infected at a young age. Males were significantly more frequently infected with *C. vulpis* and *C. aerophila* and were by trend more frequently infected with *A. vasorum* than females. Males of several mammalian species were described to be more often infected with nematodes than females (Poulin

1996). This is, among others, attributed to testosterone induced immunosuppression in male individuals (Folstad and Karter 1992) and/or different behaviour between the sexes (Klein 2004): males tend to have larger home ranges and therefore are more likely to forage in endemic areas, explaining more frequent infections. Worm burden, however, did not seem to have a sex bias (Poulin 1996), while we observed a sex bias in *C. aerophila* worm burdens only (males harboured more worms).

In the present study, calculated prevalences determined by necropsy and antigen detection are similar. The sensitivity for necropsy stated by Houpin *et al.* (2016) was slightly lower (84.1%) than what we could achieve by detecting circulating *A. vasorum* antigen by ELISA in fox blood (91.2%) (Gillis-Germitsch *et al.* 2017). In foxes with low worm burden *A. vasorum* specimens may be missed upon necropsy, whilst these animals may be antigen positive. On the other hand, if foxes are necropsied during the prepatent period (5 to 10 weeks post inoculation), one may already find subadult specimens in the heart and pulmonary arteries whilst the animal will still show seronegative. This may explain the slightly diverging prevalences by necropsy and serology. Therefore, without necropsy expertise, *A. vasorum* serology has higher sensitivity and can fully substitute fox necropsy. Although for both procedures foxes are usually killed, serology is more efficient, as it is less time-consuming; it is compatible with whole blood samples and even bloody fluids of hunted foxes (Gillis-Germitsch *et al.* 2017; Houpin *et al.* 2016). The age of the stored samples may represent a limitation. However, we showed that antigen can still be reliably detected even after several years of storage. Given that a clear decrease in OD values of highly positive samples did not lead to values below the cut-off, we assume that the age of samples should not influence the overall findings of this study.

The fact that a considerable number of samples originated from the city and surroundings of Zurich and that some areas of Switzerland were not sampled may represent a further limitation of the study. Relying on samples collected previously in the frame of independent projects, the 30-year retrospective analysis was not based on equal numbers of samples for all four time periods and on all areas of Switzerland. However, due to the large number of examined fox blood samples (3,955) originating mainly from the Swiss Plateau (which is also the most densely populated area) but also from alpine areas, from the south of the Alps and the Jura, we hypothesise that our findings are representative for the whole country.

In conclusion, serological antigen detection by ELISA applied on fox blood samples from 1986 to 2017 and across the country represented a unique opportunity to analyse the spread of *A. vasorum* in

Switzerland retrospectively. Our findings for the Swiss fox population stand for a prime example of a drastic *A. vasorum* emergence from 2.4% to 62.0% in the past three decades. Locally the prevalence increased fourfold in only 6 years. This underpins the important role of foxes as reservoir hosts and explains the concomitantly increased number of cases of canine angiostrongylosis in Switzerland at the turn of the millennium. It also helps to understand the increasing number of dog cases along with significant prevalences in the fox populations in other European countries in the last decade. The reasons behind such prevalence developments and why these are not observed for other lungworms of foxes like *C. vulpis* and *C. aerophila* are still under debate. Our findings may anticipate future developments and support disease awareness in areas where *A. vasorum* is indeed present but (still) in low prevalence.

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Conflicts of Interest

None.

Ethical Standards

Ethical standards were fulfilled.

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Table 1: Prevalence of lungworms, worm burden and seropositivity for *Angiostrongylus vasorum* antigen detection in 533 necropsied foxes from Switzerland over six consecutive years (CI: confidence intervals).

		Angiostrongylus vasorum						*Crenosoma vulpis*			*Capillaria aerophila*		
year	n	Necropsy and sero-positive (n)	Combined prevalence % (95% CI)	Necropsy positive (n)	Necropsy prevalence % (95% CI)	Sero-positive (n)	Seropositivity % (95% CI)	Mean worm burden (range)	Necropsy positive (n)	Prevalence % (95% CI)	Mean worm burden (range)	Necropsy positive (n)	Prevalence % (95% CI)	Mean worm burden (range)
2012	79	17	21.5 (13.1-32.2)	8	10.1 (4.5-19.0)	16	20.3% (12.0-30.8)	7.1 (1-30)	6	7.6 (2.8-15.8)	2.7 (1-5)	33	41.8 (30.8-53.4)	2.0 (1-6)
2013	87	36	41.4 (30.7-52.9)	31	35.6 (25.6-46.6)	32	36.8 (26.7-47.8)	5.0 (1-18)	13	14.9 (8.2-24.2)	6.6 (1-33)	65	74.7 (64.3-83.4)	5.7 (1-51)
2014	42	21	50.0 (34.2-65.8)	19	45.2 (29.8-61.3)	18	42.9 (27.7-59.0)	5.0 (1-17)	5	11.9 (4.0-25.6)	3.3 (1-9)	24	57.1 (41.0-72.3)	10.7 (1-99)
2015	88	59	67.0 (56.2-76.7)	52	59.1 (48.1-69.5)	56	64.4* (53.4-74.4)	5.9 (1-42)	7	8.0 (3.3-15.7)	10.4 (1-48)	65	73.9 (63.4-82.7)	9.2 (1-39)
2016	83	64	77.1 (66.6-85.6)	59	71.1 (63.6-78.4)	57	68.7 (57.6-78.4)	8.5 (1-44)	3	3.6 (0.8-10.2)	1.3 (1-2)	54	65.1 (53.8-75.2)	4.8 (1-30)
2017	154	126	81.8 (74.8-87.6)	110	71.4 (63.6-78.4)	118	76.6 (69.1-83.1)	10.3 (1-126)	20	13.0 (8.1-19.3)	6.7 (1-49)	94	61.0 (52.9-68.8)	4.5 (1-48)
Total	533	323	60.6 (56.3-64.8)	279	52.3 (48.0-56.7)	297	55.8 (51.5-60.1)	8.4 (1-126)	54	10.1 (7.7-13.0)	6.3 (1-49)	335	62.9 (58.6-67.0)	6.2 (1-99)

* Of one fox no blood samples were collected (and therefore no ELISA result).

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Figure legends

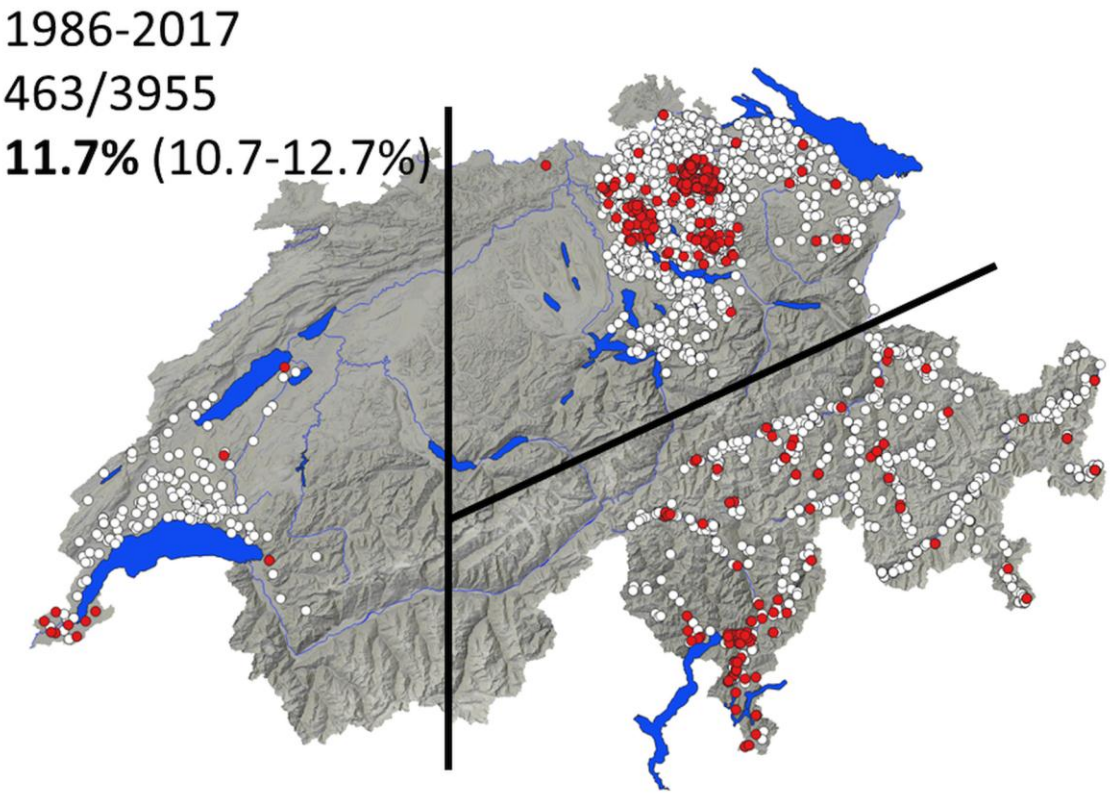

Figure 1: Map of Switzerland showing three different areas and origin of foxes hunted between 1986 and 2017. Blood samples were tested for circulating *Angiostrongylus vasorum* antigen. The coordinates of 3,704 out of 3,955 fox blood samples were available: red dots (n=446) indicate seropositive samples, white dots (n=3,258) negative samples.

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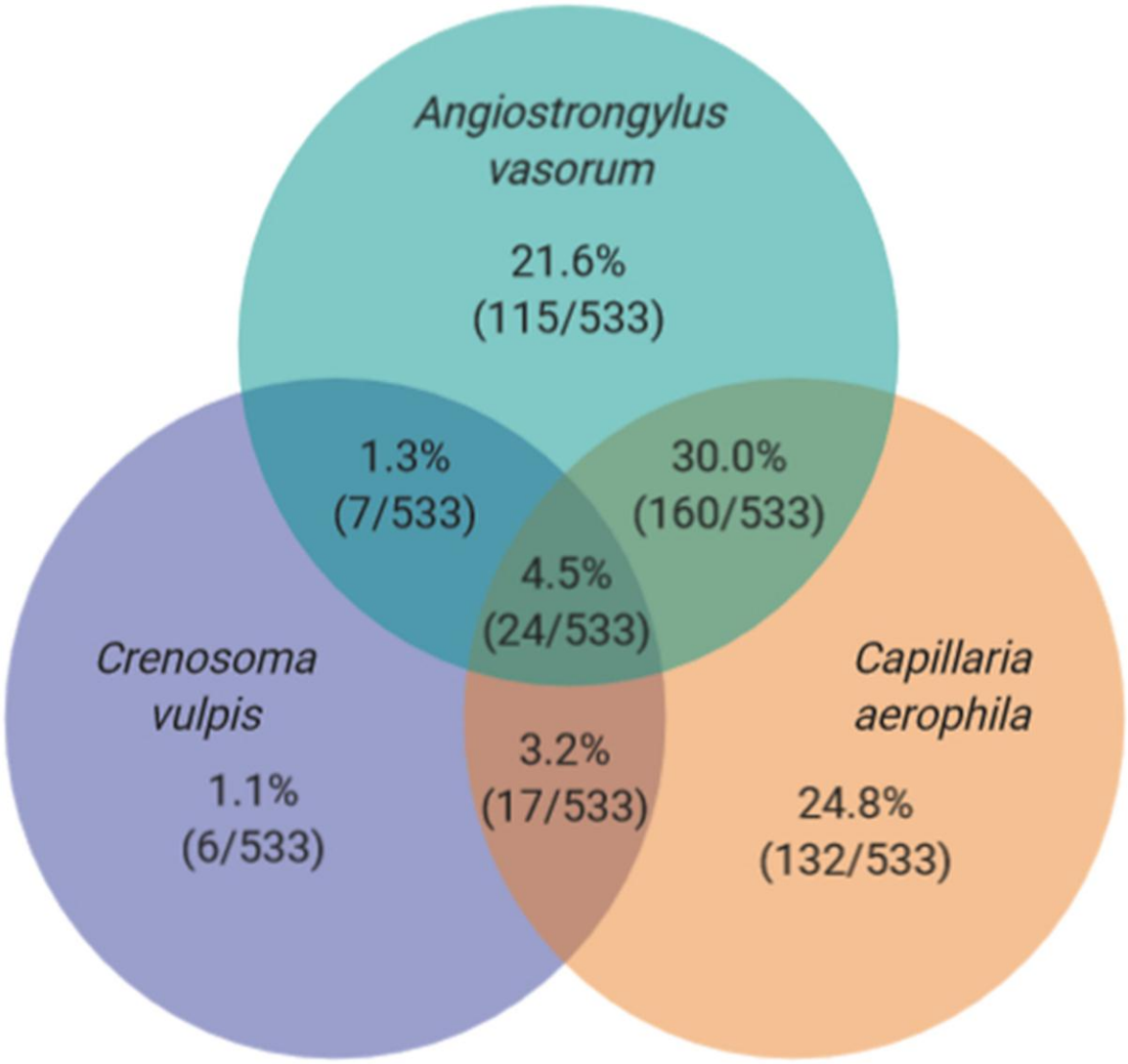
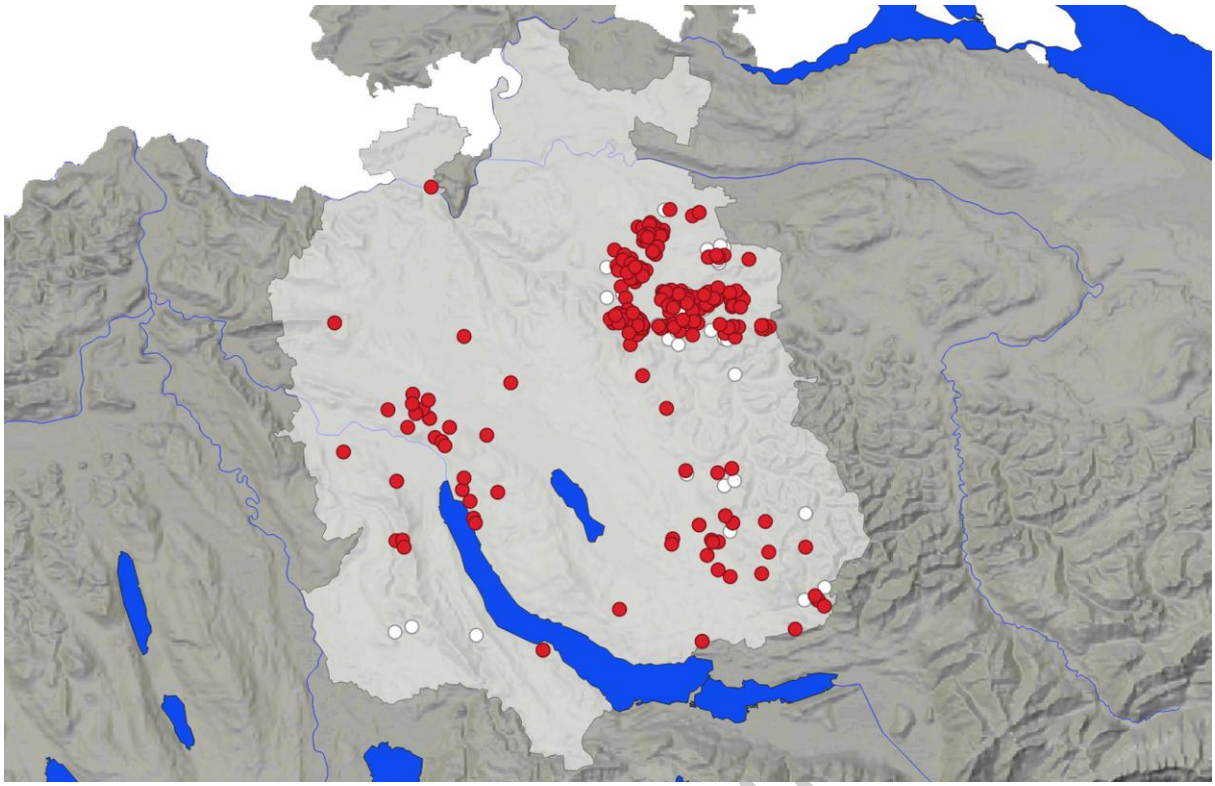
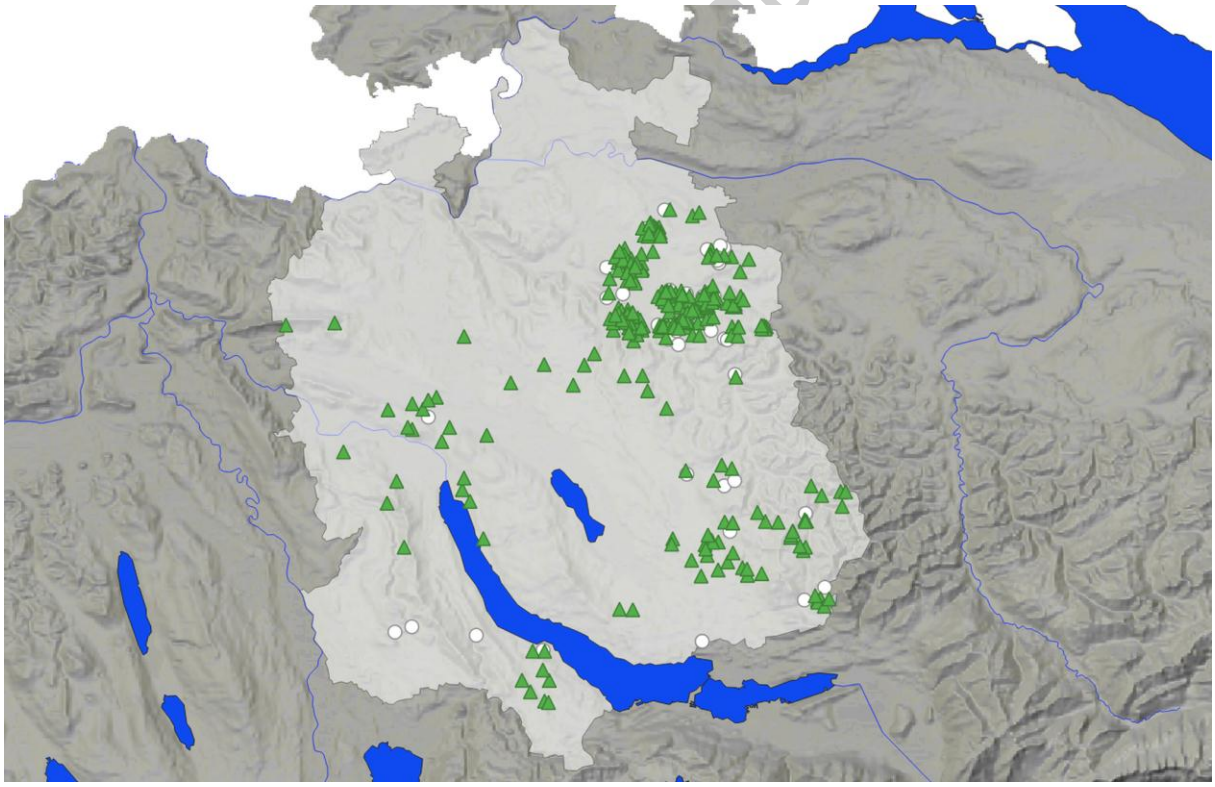


Figure 2: Single and multiple infections with *Angiostrongylus vasorum*, *Crenosoma vulpis* and *Capillaria aerophila* of 533 necropsied Swiss foxes examined for lungworms. Seventy-two foxes (13.5%) were negative for lungworms.





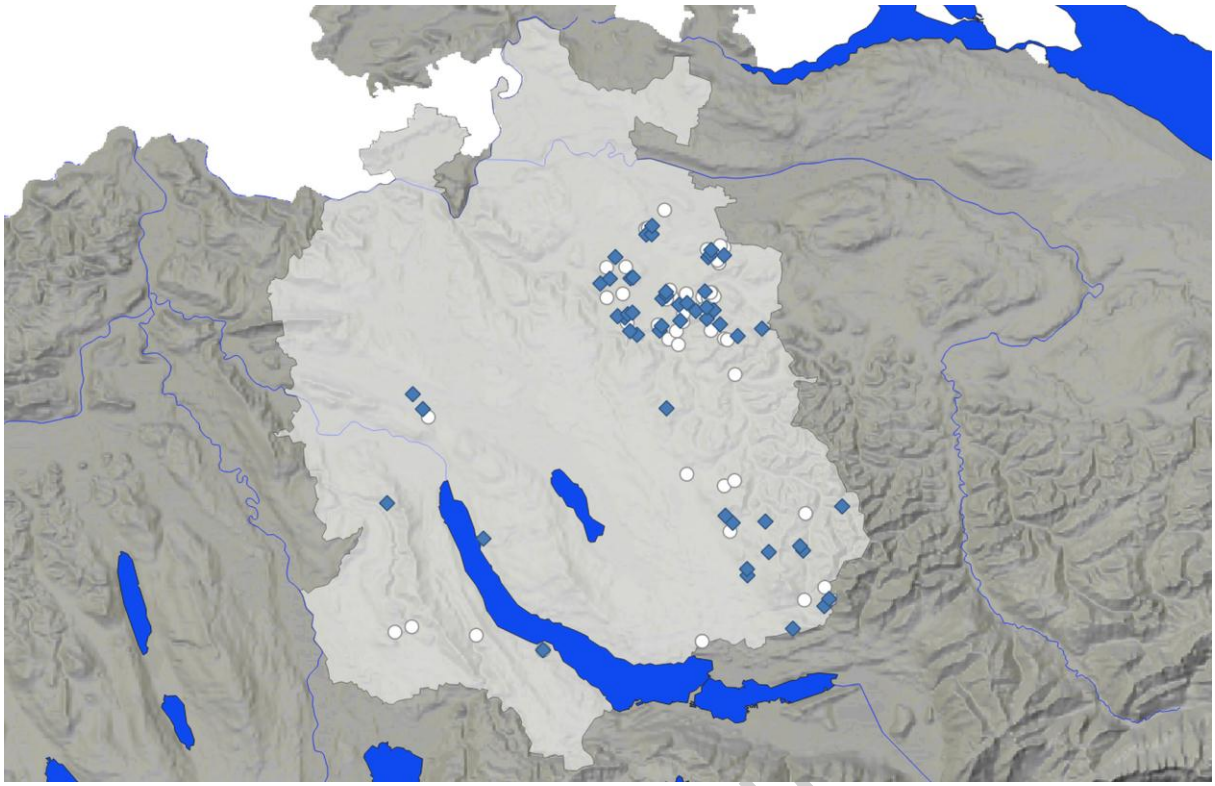


Figure 3a-c: Map of the canton of Zurich (light grey) showing the location of 475 foxes hunted and necropsied between 2012 and 2017 and examined for lungworms (of 58 foxes locations were not available). (a) red dots: *Angiostrongylus vasorum* positive foxes (n=269); (b) green triangles: *Capillaria aerophila* positive foxes (n=313); (c) blue diamonds: *Crenosoma vulpis* positive foxes (53). White dots: lungworm-free foxes (n=53).

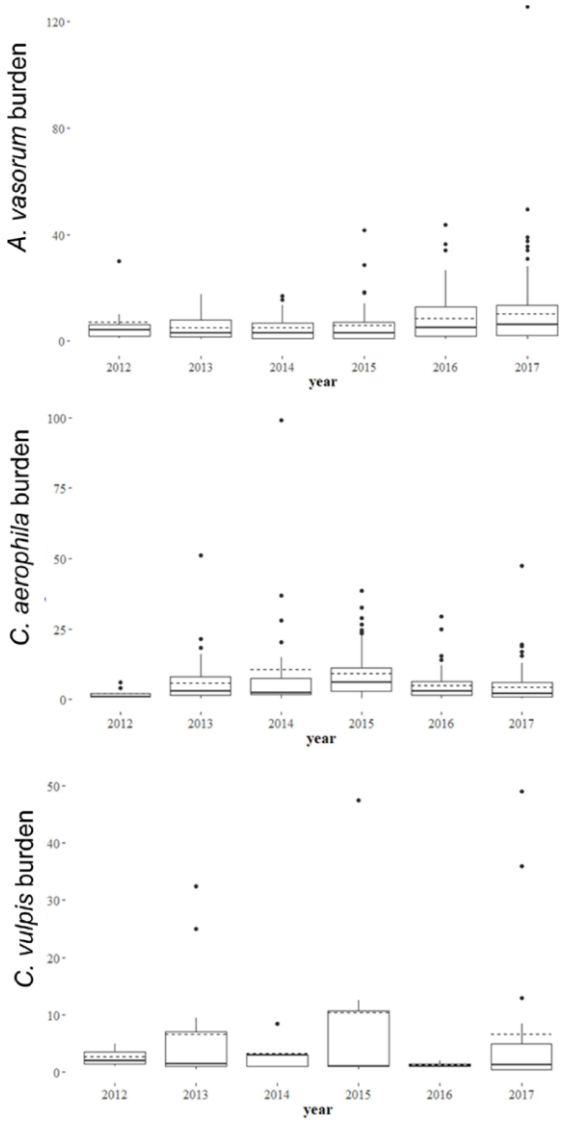
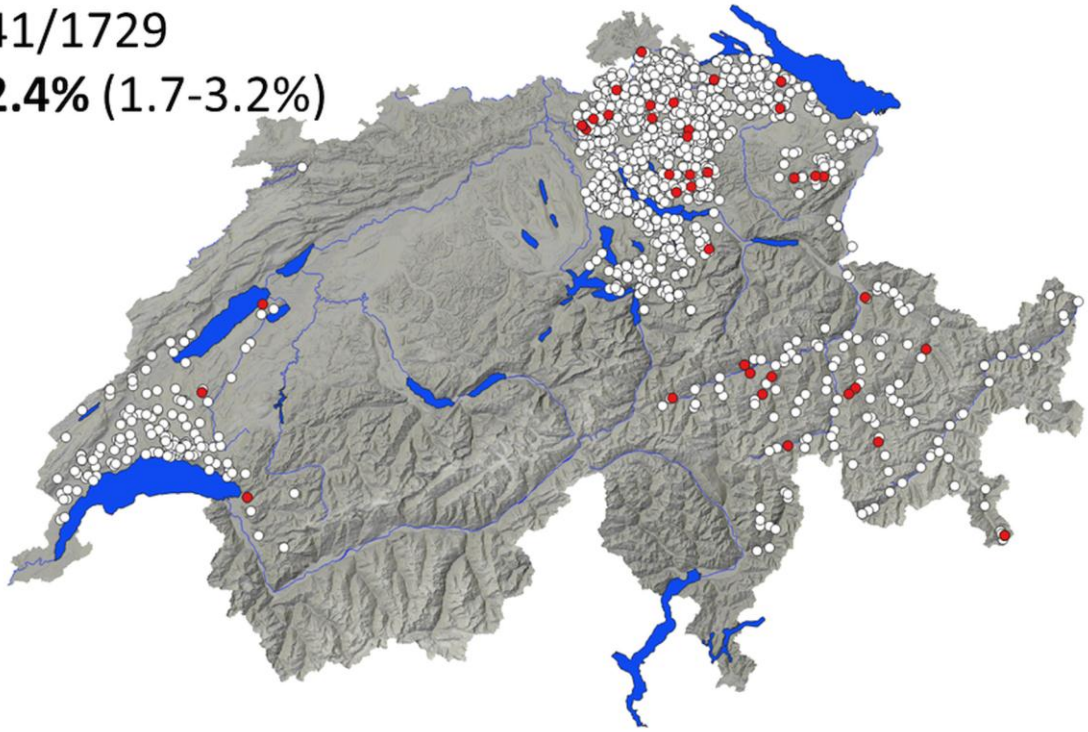
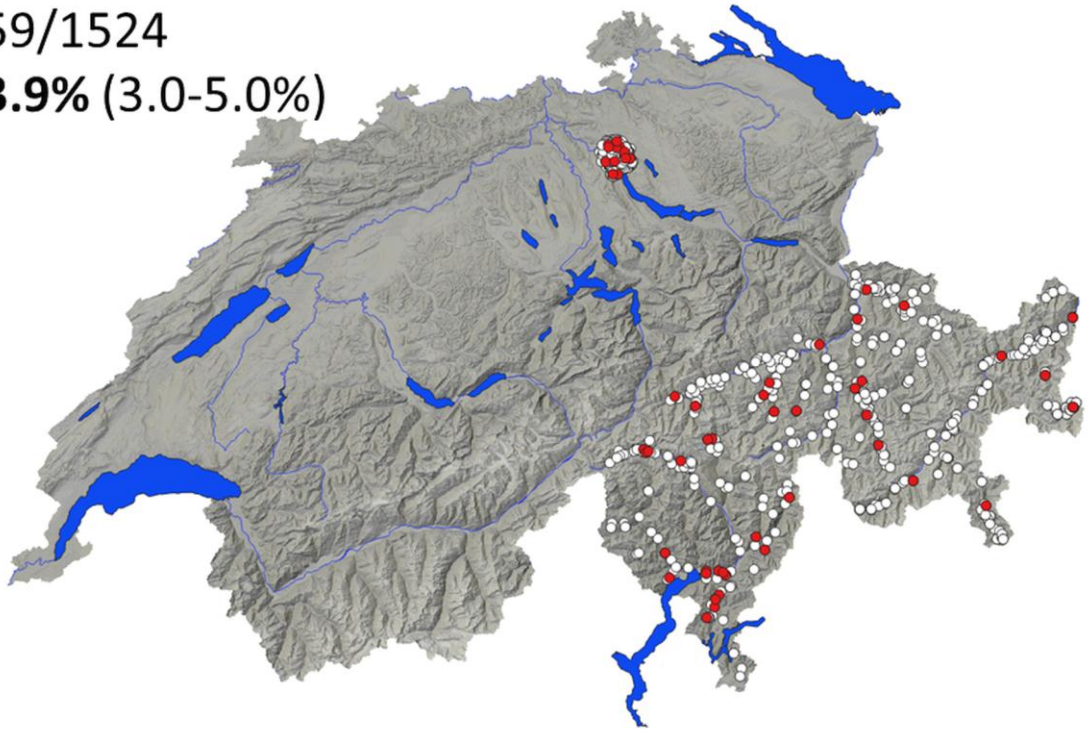


Figure 4: *Angiostrongylus vasorum*, *Crenosoma vulpis* and *Capillaria aerophila* worm burdens in 533 red foxes dissected between 2012 and 2017. Solid line: median worm burden; dashed line: mean worm burden.

1986-1992
41/1729
2.4% (1.7-3.2%)



1993-2002
59/1524
3.9% (3.0-5.0%)



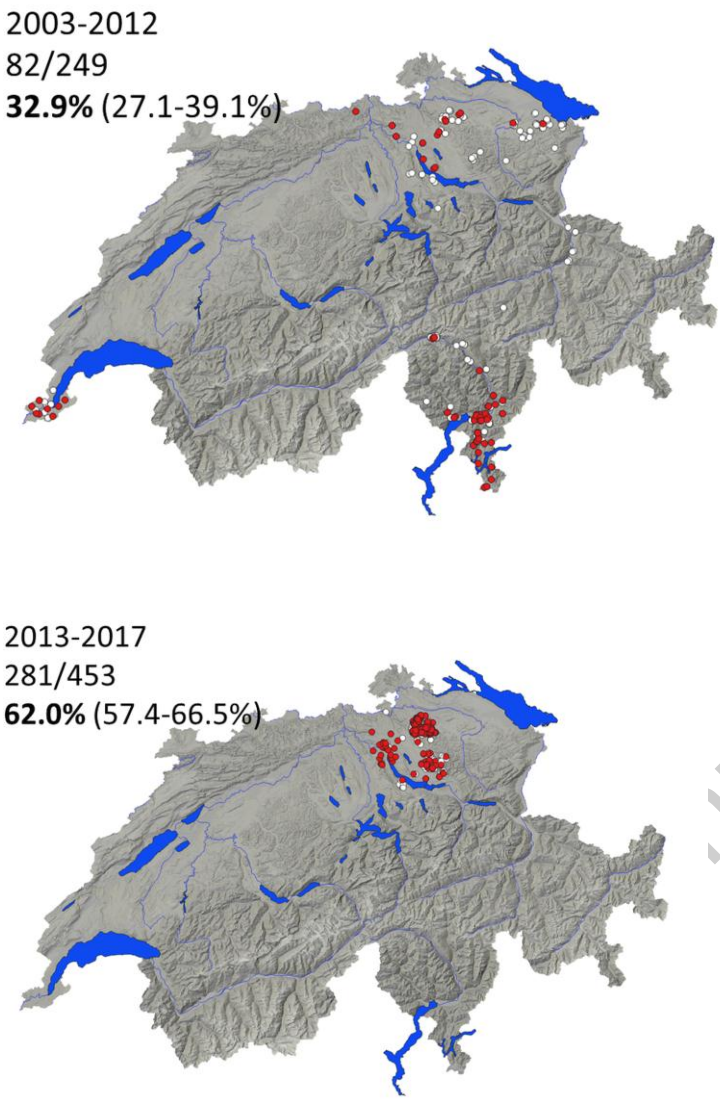


Figure 5a-d: Maps of Switzerland indicating the time period of collection, the location of the examined fox blood samples of which coordinates were available, the number of positive vs. the number of totally analysed samples, seropositivity and 95% confidence intervals. Samples were collected (a) between 1986 and 1992 (of 103 foxes no coordinates were available); (b) between 1993 and 2002 (of 120 foxes no coordinates were available); (c) between 2003 and 2012 (of 15 foxes no coordinates were available); (d) between 2013 and 2017 (of 13 foxes no coordinates were available). Red dots: samples tested seropositive, white dots: tested negative for *Angiostrongylus vasorum* antigen.