Seasonal variation of spirurian nematode *Mastophorus muris* in water voles (*Arvicola amphibius)* in southern Sweden

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Declaration

I, Bijaya Neupane, declare that this thesis is a result of my research investigations and findings. Sources of information other than my own have been acknowledged and a reference list has been appended. This work has not been previously submitted to any other university for award of any type of academic degree.

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Abstract

I studied the spirurian nematode (*Mastophorus muris*) in water voles (*Arvicola amphibius*) in southern Sweden. Limited information is known about this nematode particularly in water voles. Trapping was done using topcats in the spring and fall of 2013 in three regions of Sweden: Uddevalla, Katrineholm and Gnesta/Nyköping. Rodents were frozen and stomach content and feces were examined after thawing for presence of *M. muris*. Prevalence, mean abundance and mean intensity of infection of *M. muris* were calculated. A GLM model was used to examine the effect of sex, functional group, season, and region on the numbers of *M. muris* and presence or absence of *M. muris* in each vole. Forty-seven of 181 (26%) voles were infected with *M. muris*. Infected voles had up to 74 worms. The overall mean intensity [worms per infected vole, 95% CI] was 15, 10-21 and mean abundance [average numbers of worms in all voles, 95% CI] was 4, 2-6. Mean abundance was also calculated for sex [females 5, 2-7; males 3, 1-6], functional group [adults 5, 2-9; subadults/juveniles 4, 1-6; unknown 3, -1-8], season [spring 7, 3-12; fall 3, 1-5], and region [Gnesta/Nyköping 6, 1-11; Uddevalla 4, 1-6; Katrineholm 4, 1-6]. Model output indicated a significant effect of season ($p<0.05$) and tendency effect of region ($p=0.053$). Altogether, 10 different categories of parasite eggs were found in fecal samples. Typical *M. muris* eggs were present in only 7 (4%) out of 178 total samples, whereas *Trichuris* like eggs were the most abundant egg type and present in 66 (37%) samples. This is the first report of *M. muris* in water voles in Sweden. My results indicate a significant seasonal effect and a tendency for a regional effect of *M. muris* infection, which was independent of sex and functional group of the investigated voles.

Keywords: nematode, Spirurida, *Mastophorus muris*, *Arvicola amphibius*, functional group and *Trichuris*

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1. Introduction

Helminth parasites of wild rodents are common and have been studied over many years throughout Europe. Most of these studies have focused on factors (both intrinsic and extrinsic) such as host species, age, sex, season, site of study and year (Abu-Madi et al., 2000; Behnke et al., 1999; Burlet et al., 2011; Kataranovski et al., 2011; Lõhmus & Albihn, 2013). Although *M. muris* has been previously studied in different rodents (Grzybek et al., 2014; Lafferty et al., 2010; Smith & Kinsella, 2011; Vukićević et al., 2007), there are, to my knowledge, no studies about this nematode in water voles.

M. muris is a stomach nematode (order Spirurida, family Spirocercidae) in rodents. Male nematodes are 17 to 56 mm long whereas females are 23 to 87 mm long; males have copulatory bursae with spicules and the size of unembryonated eggs in female is 0.051 to 0.055 mm long and 0.031 to 0.032 mm wide (Wertheim, 1962). The life cycle is indirect where insects (beetles, locusts, earwigs, cockroaches, etc.) are intermediate hosts (Quentin, 1970). The first stage larva (L1) of this nematode hatches after the eggs are consumed by insects and develop into third stage larva (L3) that are infective to final or definite hosts after around 2 weeks (Quentin, 1970). After being ingested, it takes in rats (*Rattus rattus*) about 28 days for the L3 stage to develop into breeding adults in the stomach.

Among several studies on the effects of season on abundance of helminths in different wild rodents (Abu-Madi et al., 2000; Charleston & Innes, 1980; Langley & Fairley, 1982), only a few have reported seasonal variation in prevalence of *M. muris* in rodents. For example, a study conducted in New Zealand on seasonal influences on *M. muris* in rats (*R. rattus*) showed higher prevalence of infection from March to June (around 40%) and lower infection in other months (less than 15%) (Charleston & Innes, 1980). However, no studies have reported seasonal influences on number of *M. muris* in water voles.

Regarding the influences of habitat or regions on the prevalence of helminths, Roberts et al. (1992) concluded that habitat utilization was the most important factor shaping the prevalence of helminth parasites in rat populations. Local variations in endoparasite prevalence and intensity were observed in bank voles (*Clethrionomys glareolus*) within ecologically similar sites (Barnard et al., 2002). Also, a study performed on *M. muris* in rats (*R. rattus*) in the central Pacific Line Islands in the Pacific Ocean has attributed the importance of habitat consisting of locally dominant plant species *Cocos nucifera* to the

abundance of this nematode (Lafferty et al., 2010). The sites dominated by this plant species had more abundance of *M. muris* in rats. Nevertheless, the knowledge about the differences in the prevalence of *M. muris* within similar and other habitats is very limited.

One indirect method of diagnosing nematode infections in animals is by the examination of fecal samples for nematode fecal egg count (FEC). It is widely used in parasitological studies for estimating a wide range of parasites. This method was used to study intestinal helminths of spiny mice in Sinai, Egypt (Behnke et al., 2000). Not only in rodent species, FEC was performed in studying gastrointestinal parasite infecting grey squirrels (*Sciurus carolinensis*) in northern Italy (Romeo et al., 2014), diagnosing parasitic infection of feral Soay sheep population (*Ovis aries*) in Hirta, St. Kilda (Craig et al., 2007) and studying two nematode species of Svalbard reindeer (*Rangifer tarandus platyrhynchus*) in northernmost part of Norway (Irvine et al., 2001). However, realiability in egg counts may depend mainly on fluctuation in concentration of eggs in fecal materials and type of method used to count eggs (Sinniah, 1982). Some studies have compared various egg counting methods for estimating worm intensities (Seivwright et al., 2004), while I only used the McMaster egg counting method in this study.

Although one study has shown a direct relationship between number of eggs in feces and parasite abundance in hosts (Seivwright et al., 2004), FEC may not always be a reliable index of worm intensity because of biological factors like density dependence constraits on fecundity of worms (Anderson & Schad, 1985; Romeo et al., 2014) and variation of worm egg production in different seasons (Romeo et al., 2014). Additionally, freezing fecal samples at very low temperature of -40°C and storing fecal samples at optimum temperature of 5°C for more than 3 weeks decreases concentration of parasite eggs in fecal samples (Seivwright et al., 2004).

In this thesis, I present data on the stomach nematode *M. muris* in the water vole (*A. amphibius*) along with information on prevalence of different nematode eggs found in feces. I particularly focused on factors that may affect infection rates of *M. muris* in the stomach of water voles. My aim was to assess the various levels of infection (prevalence, abundance and intensity) in relation to intrinsic factors (host sex and functional group) and extrinsic factors (season and region) that could explain variation in worm burdens in water voles. Additionally, I used FEC in an attempt to correlate number of *M. muris* worms from the stomach with nematode eggs found in feces of water voles.

2. Materials and methods

2.1 Study area

Water vole (*A. amphibius)* trapping was done as part of a larger project studying *Echinococcus multilocularis* (EM) in rodents (EMIRO, www.emiro.org). The survey was conducted in three different regions of Southern Sweden: Uddevalla, Katrineholm and Gnesta/Nyköping (Fig. 1). The study sites of Uddevalla (20 x 20 km) and Katrineholm (20 x 20 km) were chosen because they were the sites of the original EM findings (Wahlström et al., 2012). The region of Gnesta/Nyköping (~25x25km) is part of a national environmental/wildlife-monitoring program (FoMA, www.slu.se/en/environment). Because EM had not yet been identified here, this region was chosen for comparison. Although a fourth region, near Växjö, had also been included in the EMIRO/FoMA activities, it was excluded from this study as no water voles were caught there in 2013. Each region consists of forests, fields, pastures and areas with human settlement.

Fig. 1. Water vole trapping sites in southern Sweden. The red stars inside the map (with names) represent the regions where the study was conducted.

2.2 Ecology of water vole

The water vole *(A. amphibius)* is a small mammal belonging to rodent family, which is characterized by a rounded body, blunt nose, short rounded ears, chestnut brown fur and a long hairy tail (Forder, 2006) http://www.nottinghamshirewildlife.org/animal-facts/water-vole). The adult weighs 200-300 g and prefers riparian habitats (Melis et al., 2013; Stoddart, 1970), such as vegetated bank of rivers, streams, ditches, canals, ponds and marshes with still water or little flow. It can swim and dive well in water. Water voles are generally herbivore that eats lush stems, grasses, roots, sedges, reeds and leaves of plant found around their habitats but sometimes supplement their diet with insects. The species is considered a farm pest because they prefer to eat roots of farm crops such as potatoes and to chew the base of trees in orchards (Jansson, Albertsson, & Svensson, 2010). It is territorial only during the breeding season and produces up to 5 litters annually (Isakova, Nazarova, & Evsikov, 2012). Reproduction occurs between end of March and September (Stoddart, 1970) with a short gestation period of 20-22 days. It is less active with a high mortality (up to 70%) during the winter (Forder, 2006) http://www.nottinghamshirewildlife.org/animal-facts/water-vole). Individual water voles have a life span of up to 2 years (Isakova et al., 2012). They live in small, discrete colonies composed of a few individuals (Aars et al., 2006).

There are two main species of water vole separated by their habitat use: *A. amphibius* and *A. scherman* (Taberlet et al., 1998). *A. amphibius* is a larger vole having shaggy pelage and ortodont incisors and lives in aquatic environment, whereas *A. scherman* is smaller having softer pelage and forward projecting upper incisors and lives in fossorial habits (Baillie, Hilton-Taylor, & Stuart, 2004). As these taxa have different life styles and morphological features, they are distributed in different climatic and geographical regions of Europe. *A. amphibius* is mainly present in northern and central Europe excluding Spain whereas *A. scherman* is restricted to the Alps in central Europe and other mountainous areas of northern Spain, Portugal and Romania (Piras et al., 2012). However, *A. scherman* is absent on the Scandinavian Peninsula including Sweden.

2.3 Trapping procedures

Topcat traps (AndermattBiocontrol AG) were used for trapping (Fig.2) water voles in this study. This is a stainless steel snap trap that is highly sensitive for mechanical release (Source: http://www.topcat.ch/Description-1_1.html). The topcat traps are placed in water vole tunnels and catch the voles as they move through their tunnel systems. The voles investigated herein were collected during 4-6 weeks in the spring (April/May) and fall (September/October) of 2013. Trapping sites were selected after identifying typical water vole signs (tunnels and mounds) in the fields in each of the three regions. Traps were set for minimum of 2 hours with frequent checks. Collaborating landowners, who had been loaned topcats, donated 19 voles in spring 2013. All of the specimens were immediately frozen at -20°C until dissection. In total, 181 *A. amphibius* were trapped: Uddevalla 76 (31 spring, 45 fall), Katrineholm 74 (12 spring, 62 fall) and Gnesta/Nyköping 31 (1 spring, 30 fall).

Fig.2. Topcat used for trapping water voles (http://ww.export.biocontrol.ch/sites/products/rodent-control/topcat.html)

2.4 Laboratory procedures

2.4.1 Dissection and collection of worms

All water voles collected in 2013 were dissected in the Parasitology lab, at the Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden. Morphologic characteristics, sex, and weight were recorded upon dissection. They were also categorised into three functional groups based on their reproductive status: adult (breeding), subadult/juvenile (non-breeding) and unknown (likely non-breeding). The "adult" voles were those voles with signs of active breeding. In females this was based mostly on presence of embryos or scars in the uterus, evidence of lactation, and an open vagina, while in males it was based mostly on size of testicles (>15mm). "Subadults" were those voles that were matured but not breeding and "juveniles" were sexually immature. The focus of the dissections was to examine the livers for metacestodes of the tapeworm *E. multilocularis*. However, other

organs including the gastrointestinal (GI) tract were saved from each water vole and stored at -20°C for further analysis in other studies.

For my study, the GI-tract was first weighed. Then fecal pellets formed in the latter part of the small intestine were taken out from the large intestine with help of scissors and tweezers and collected in 100 ml plastic tube. The feces were weighed and put in the refrigerator for later enumeration of parasite eggs. The stomach was separated from the intestines and put in a petri dish with water. The stomach was then cut open and contents washed with water.

All *M. muris* found were counted and collected before putting back the washed stomach with the intestines. Stomach content was then sieved through a tea strainer (mesh size ~510-610 µm) to eliminate coarse food material and washed into a counting tray. The counting tray with stomach content and water was examined with a dissecting microscope. All parasites found were collected. Immature and mature *M. muris* were collected separately from all other parasites for each vole. All other parasites were collected and stored in individual small glass tubes with 70% alcohol for use in other studies. The remainder of the intestines and stomach were put in the original bag and stored in the freezer at -20°C.

2.4.2 McMaster techniques

The McMaster method is a commonly used technique for counting nematode eggs in fecal samples. The McMaster chamber consists of two compartments, each with grids on the upper and lower surface of the covering glass (Fig. 3). The main principle of this technique is that the chamber is filled with a diluted fecal suspension where the eggs float to the underside of the glass with the grids where they can be counted. This technique is based on a predetermined weight of feces and from that, the number of egg per gram of feces (EPG) is estimated.

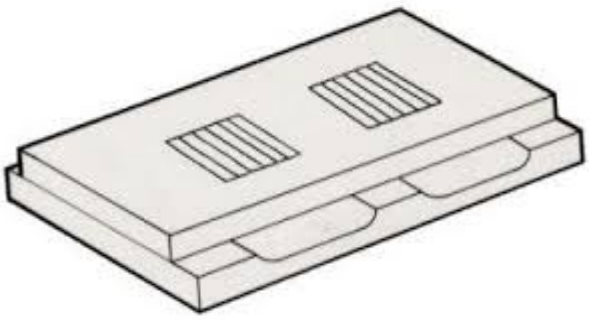


Fig.3. Picture of McMaster slide ***(Source**: http://www.vetslides.com/two-chamber-mcmaster-counting-slides)*

Since there were several fecal weights less than 3 g, the normal McMaster technique was modified and scaled down to lower volumes to account for the lower fecal weights of rodents. Therefore, an adjusted amount of water was put in the tube according to the weight

of the feces to get a dilution factor of 15 to provide a diagnostic sensitivity of 50 eggs per gram of feces (Appendix 1). The tube was hand-shaken until the feces were dissolved and contents sieved through 150-µm meshes into another container. Samples with less than 1.2 g feces were put into a small 15 ml plastic tube whereas those with ≥ 1.2 g feces were sieved into a larger 60 ml plastic tube. The level of sample solution in tubes was marked, and the samples centrifuged at 1500 rpm for 3 minutes (G-force of 425 G). After centrifugation, water was poured out and the tube was slightly vortexed before adding saturated zinc chloride ($ZnCl_2$) up to the previously marked level. After testing McMaster with different salt solutions and methods, this $ZnCl_2$ salt solution was used because it allowed many different types of eggs to flotate and reduced the number of bubbles in the McMaster chamber. The $ZnCl_2$ solution has a density of 1.45 g/ml, which means that most nematode eggs can be floated. The mixed $ZnCl_2$ salt solution was pipetted into both sides of the McMaster chamber and allowed to stand for at least 5 minutes to flotate the eggs. The number of different egg types within the grids (ignoring those outside the squares) of each McMaster chamber were counted at a magnification of 20 X. Eggs were grouped into categories based on size and shape (Appendix 2). The number of eggs of each category was multiplied by 50 to calculate the number of each egg per gram of feces (EPG).

2.5 Statistical analysis

Two data sets were produced from the laboratory work: parasitic worm data and fecal egg count data. Although data were obtained for both *M. muris* and other parasitic worms, I focused on *M. muris* for statistical analysis. Similarly, 10 different categories of parasite egg data were produced but only two categories of eggs: *M. muris* eggs and the predominant *Trichuris* like eggs were analysed and interpreted.

A database was constructed and data were analyzed using "R x 64 3.0.1 (http://cran.r-project.org/)". Prevalence, mean intensity and mean abundance of infection of *M. muris* were calculated by using following formulas as defined by Margolis et al. (1982).

Prevalence (proportion of infected animals) = Number of infected water voles / total number of investigated water voles

Mean intensity of infection= Number of worms (M. muris) / number of infected water voles

Mean abundance of infection= Number of worms (M.muris)/ total number of both infected and non-infected water voles

For the analysis of worms in individual voles, modelling was done using R version 3.0.1 (R Core Development Team) with the number of *M. muris* as the dependent response variable, and sex, functional group, season and region as factors or explanatory variables. The assumptions of normality, linearity, homogeneity and independent observations were checked and it was found that most of these assumptions were not fulfilled. So, GLM (generalised linear model) was used for the analysis of worm intensity and abundance with "Poisson distribution and log-link function" because the response variable was a count and numeric. Overdispersion was checked by observing "summary table" (if the residual deviance of summary table is more than 2 times the degree of freedom, then the data is over dispersed). In all models, the data was found to be overdispersed, so "quasipoisson" was used instead of "Poisson distribution". All the variables were entered into our initial models. Then, backward selection method (step-wise removal of non-significant variables or factors) was implemented and developed the final model with significant predict variables for which the likelihood ratio of χ^2 was significant (i.e., $p \leq 0.05$). Similarly, nominal logistic regression was used for analyzing prevalence of infection. Full models were incorporated with sex, functional group, season and region as independent variables and infection as binary factor (presence or absence of worms) in my logistic regression models. Overdispersion was checked and it was found that the data was not overdispersed. Backward selection procedure was then implemented to derive minimum sufficient models, as above. The significance was set at $P \leq 0.05$. Confidence intervals (95% CI, lower limit-upper limit) were calculated using library package "lsmeans" in R x 64 3.0.1.

3. Results

3.1 *M. muris* in water voles

3.1.1 Overall infection of *M. muris* in water voles

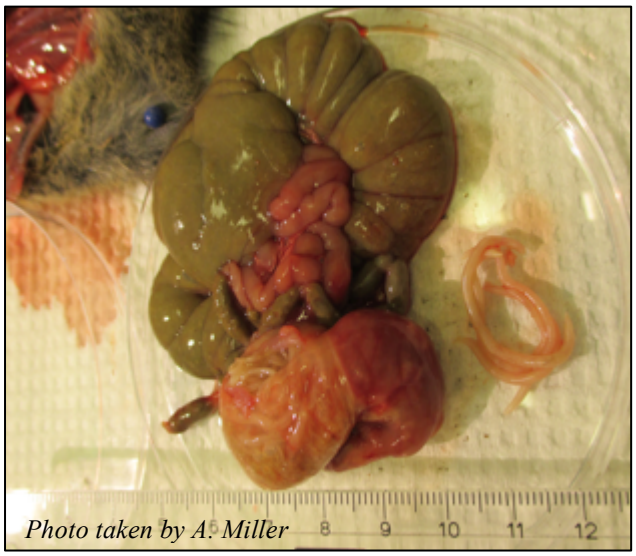


Fig. 4. *M. muris* observed in the stomach of water vole sample

Overall, 47 out of 181 voles (26%) were infected with adult *M. muris* (Fig. 4) with a slightly higher prevalence among female voles compared with males (Table 1). Prevalence of infection (%) varied among three functional groups, between two seasons and among three regions. However these differences were marginal with overlapping confidence intervals (CI) (Table 1). The total mean intensity and mean abundance of infection (95% CI) were 15 (10-21) and 4 (2-6) respectively (Table 1). The majority of voles had less than 10 worms with only a few voles having more worms (Fig. 5).

Table 1. Prevalence (in percentage), mean intensity and mean abundance of infection as well as maximum number of worms categorized according to sex, functional group, season and region

Category		Prevalence (95% CI)	Mean intensity (95% CI)	Mean abundance (95% CI)	Max
Total (n=181)		**26 (19-33)**	**15 (10-21)**	**4 (2-6)**	
Sex	Male (n= 81)	25 (15-40)	14 (6-22)	3 (1-6)	74
	Female (n=100)	27 (18-36)	17 (8-25)	5 (2-7)	65
Functional group	Adult (n= 48)	21 (9-33)	15 (7-24)	5 (2-9)	63
	Subadult/Juvenile (n= 109)	31 (22-40)	17 (8-26)	4 (1-6)	74
	Unknown (n= 24)	13 (-1-26)	11 (-4-26)	3(-1-8)	56
Season	Spring (n= 44)	41 (26-56)	18 (9-27)	7 (3-12)	63
	Fall (n= 137)	21 (14-28)	14 (6-22)	3 (1-5)	74
Region	Uddevalla (n= 76)	22 (13-32)	16 (6-26)	4 (1-6)	63
	Katrineholm (n= 74)	27 (17-37)	13 (4-23)	4 (1-6)	74
	Gnesta/Nyköping (n= 31)	32 (15-49)	18 (6-30)	6 (1-11)	54

n= number of water voles *(A. amphibius)*; Max, maximum number of worms ; CI= confidence interval

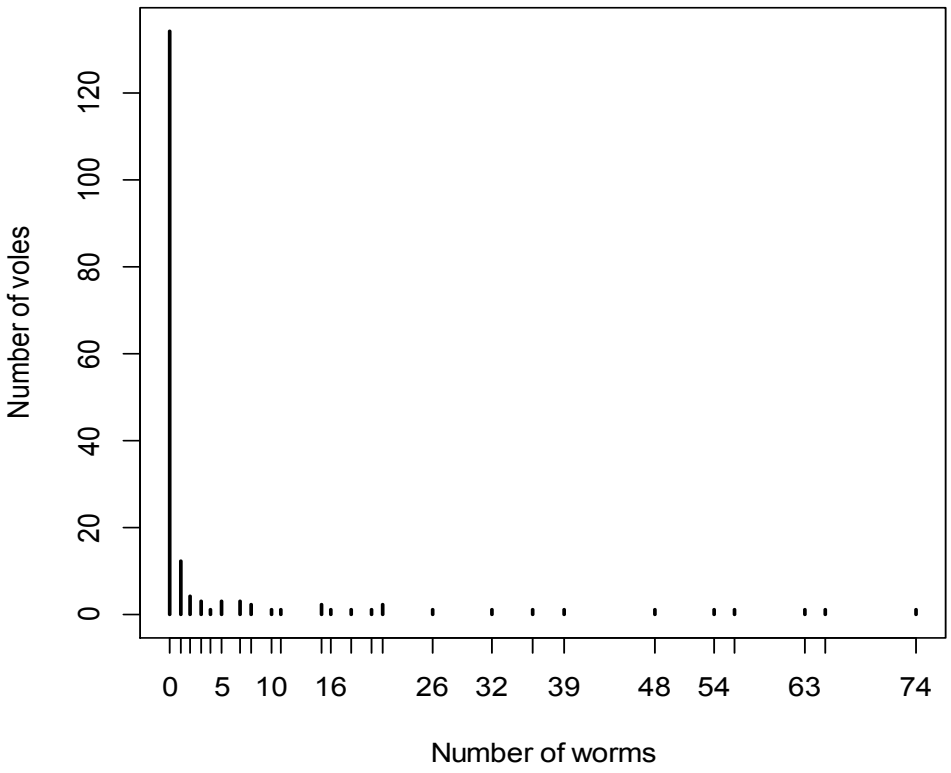


Fig. 5. Number of water voles and their corresponding number of *M. muris* worms.

3.1.2 Infection of *M. muris* in relation to sex

Although prevalence of infection of females was slightly higher than males, this difference was not significant ($\chi^2_{1,179} = 0.036$; $p = 0.850$) from logistic regression model. The highest number of worms was detected in a male (Table 1; Fig. 7.A). Both the mean intensity and mean abundance of infection were higher in females than in male voles but not statistically significant (Mean intensity: $\chi^2_{1,45} = 932.98$; $p = 0.50$ and mean abundance: $\chi^2_{1,179} = 2671.3$; $p = 0.764$) from quasipoisson models.

3.1.3 Infection of *M. muris* in relation to functional group

The prevalence of infection was highest in subadult/juvenile voles (Table 1). Total number of *M. muris* per body length category was also found higher (n= 238) in medium body length voles (150-160 mm) (Fig. 6). From the original data, only 7 out of 48 adult voles were in the medium body length category. Most of the subadult/juvenile voles ranged from 140 to 160 mm in body length. This category had higher numbers of worms as shown in Fig. 6. So, this confirms that subadults contain the most worms, which is also supported in Fig. 7.B. However, there was no significant difference between the three functional groups regarding the proportion of infected animals ($\chi^2_{2,177} = 0.4132$; $p = 0.813$) from logistic regression model. The mean intensity of infection was highest in subadult/juvenile voles but not statistically significant ($\chi^2_{2,44} = 982.01$; $p = 0.378$), whereas mean abundance of infection was highest in adult voles but also not statistically significant ($\chi^2_{2,178} = 2723.9$; $p = 0.469$) from quasipoisson models (Table 1).

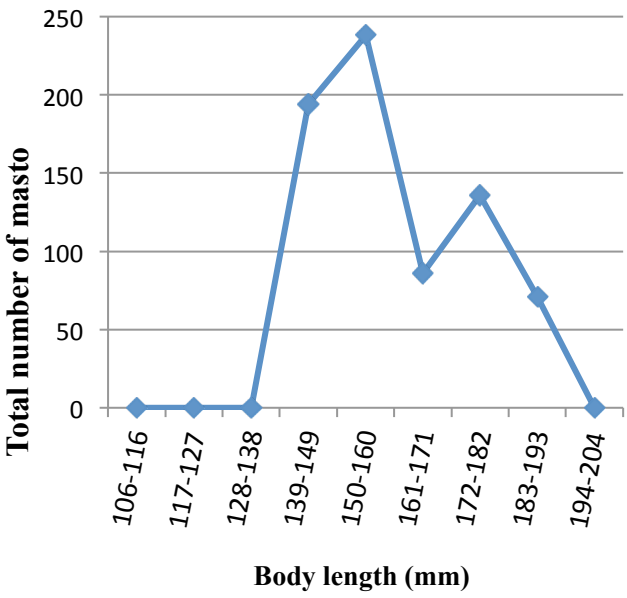


Fig. 6. Number of *M. muris* plotted against different body length classes of water voles. Total data of body lengths were classified into 9 different classes of 10 mm length intervals. The body lengths of two samples were missing

3.1.4 Infection of *M. muris* in relation to season

There was a significant seasonal effect on prevalence of infection of *M. muris (slope ± SE* from the logistic regression model *=0.9471 ±0.3712;* $\chi^2_{1,179} = 6.3554; p=0.012)$, with a higher prevalence of infection in spring than in fall (Table 1). There was also a higher mean intensity and mean abundance of infection in spring than in the fall. The difference was statistically significant in terms of mean abundance ($\chi^2_{1,179} = 2918.6; p= 0.022$) but not with mean intensity ($\chi^2_{1,45} = 990.90; p= 0.564$). The maximum number of worms was higher in the fall than in spring (Table 1; Fig. 7.C).

3.1.5 Infection of *M. muris* in relation to region

There was a tendency of regional effect on mean abundance of infection (*slope ± SE* from the quasipoisson model *=0.72 ± 0.60;* $\chi^2_{2,177} = 2723.9; p=0.053)$ with highest prevalence of infection in Gnesta/Nyköping among the 3 regions (Table 1). Similarly, mean intensity of infection was highest in Gnesta/Nyköping. Katrineholm had higher prevalence of infection but lower mean intensity of infection than Uddevalla. These two regions had similar mean abundance of infection. There was no significant difference in prevalence and mean intensity of infection among three regions (prevalence: $\chi^2_{2,177} = 4.8066; p= 0.090$ and mean intensity*:* $\chi^2_{2,40} = 921.57; p= 0.810)$. The maximum numbers of adult worms were observed in Katrineholm (Table 1; Fig. 7.D).

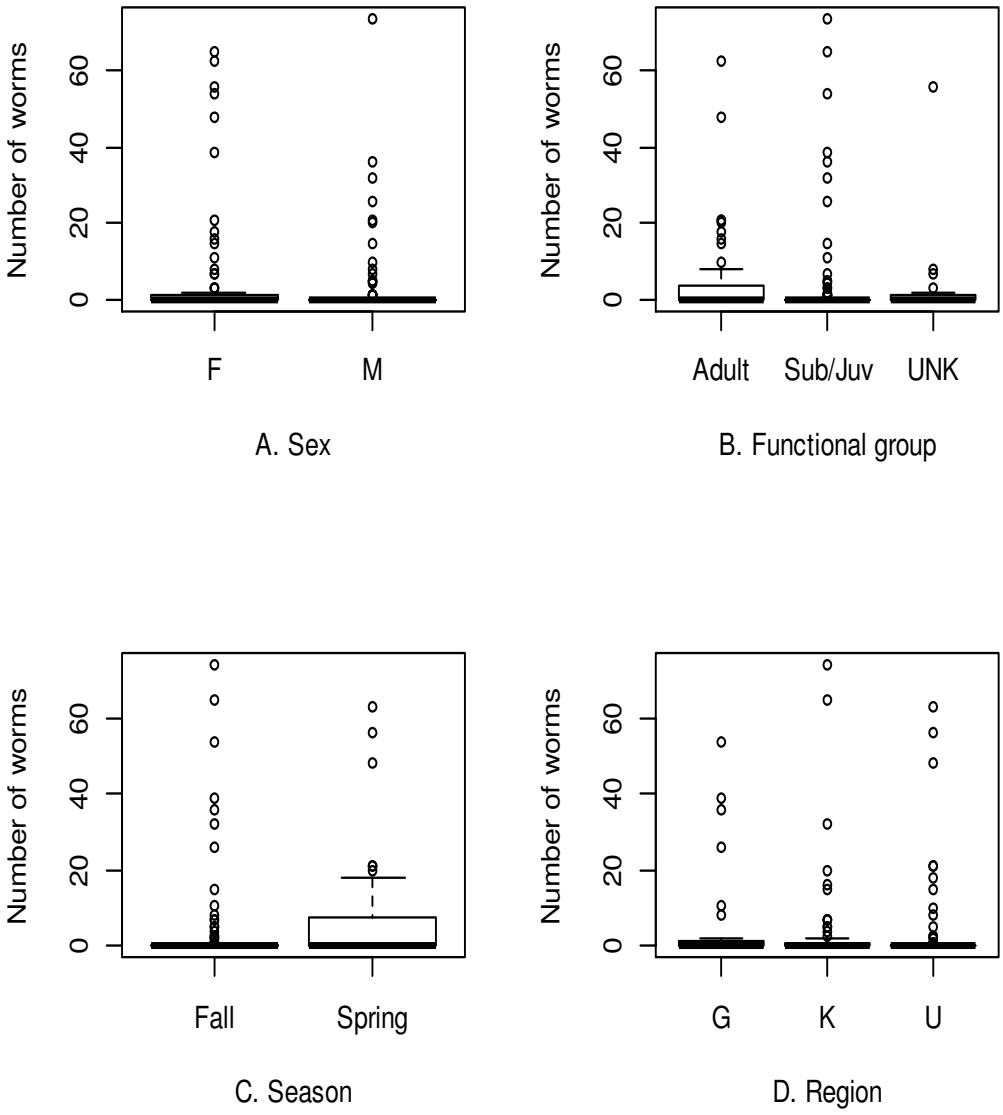


Fig.7. Number of *M. muris in A. amphibius* according to sex, functional group, season and region. "F" and "M" refers to female and male (Fig. A), "Sub/Juv" and "UNK" refers to Subadult/Juvenile and Unknown (Fig. B) and "K", "G" and "U" refers to Katrineholm, Gnesta/Nyköping and Uddevalla respectively (Fig.D). The black bold lines in each figure indicate that many observed values are near to zero.

3.2 Fecal egg counts

3.2.1 Summary of fecal egg results

Fig. 8. *Trichuris* like egg (left) and *Mastophorus muris* egg (right) observed in fecal samples of water vole *(Photo taken during lab work, SLU, Uppsala, Sweden; 29/05/2014)*

Feces could not be collected in 3 out of 181 vole samples because two contained no feces and one had a damaged large intestine. Out of the 178 fecal samples, 109 (61%) were egg positive. Ten different categories of helminth eggs were observed but only seven of these were identified. Among these categories, two were dominant: *Trichuris* like eggs (Fig. 8) and a 30 x 30 micrometer round unidentified eggs. Only 7 samples (4%) were observed with the typical *M. muris* eggs (Fig. 8). *Trichuris* like eggs were present in 66 samples (37%). For further analysis and description, the overall egg categories were summarised into 4 main categories: feces with eggs, feces with *Masto* eggs, feces with *Trichuris* like eggs and feces with other eggs (Table 2).

3.2.2 Prevalence of different types of fecal eggs

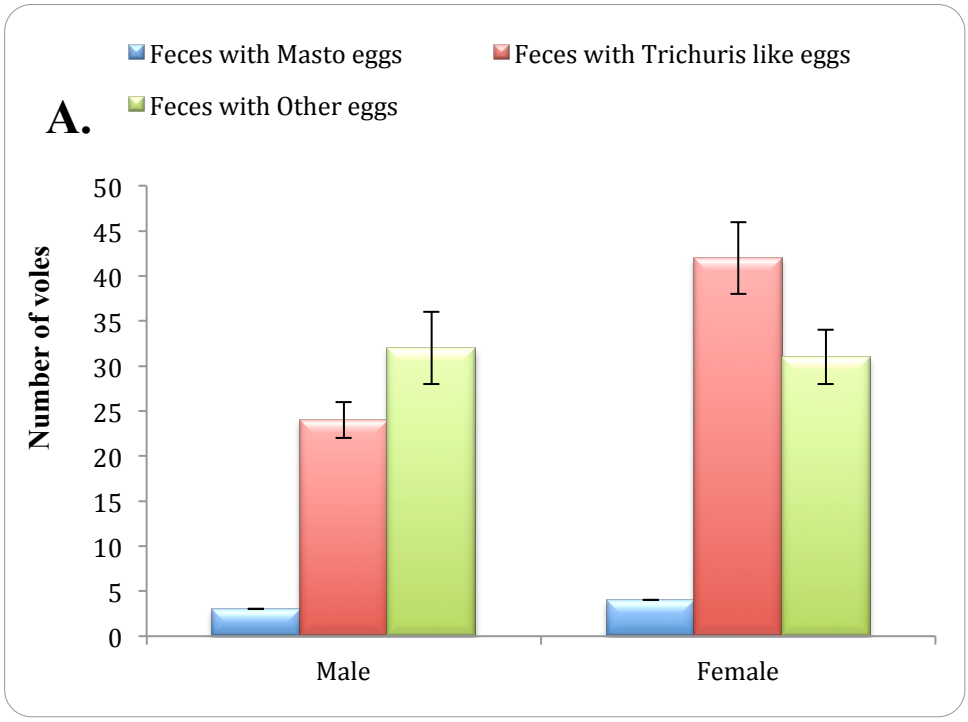
As shown in Table 2, nematode eggs were higher in fecal samples from females than from males, from samples from adults than from the other functional groups, and in samples from spring than from fall. With regards to region, prevalence of eggs was highest in Katrineholm. Very low prevalence (95% CI) of *M. muris* eggs was observed, 0.04 (0.01-0.07) and with no *M. muris* eggs in spring samples from Uddevalla (Table 2). The overall prevalence of *Trichuris* like eggs (95% CI) was 37% (30-44%) with some variation in sex, functional group, season and region (Table 2). Differences in prevalence of different egg categories in different groups were not statistically compared.

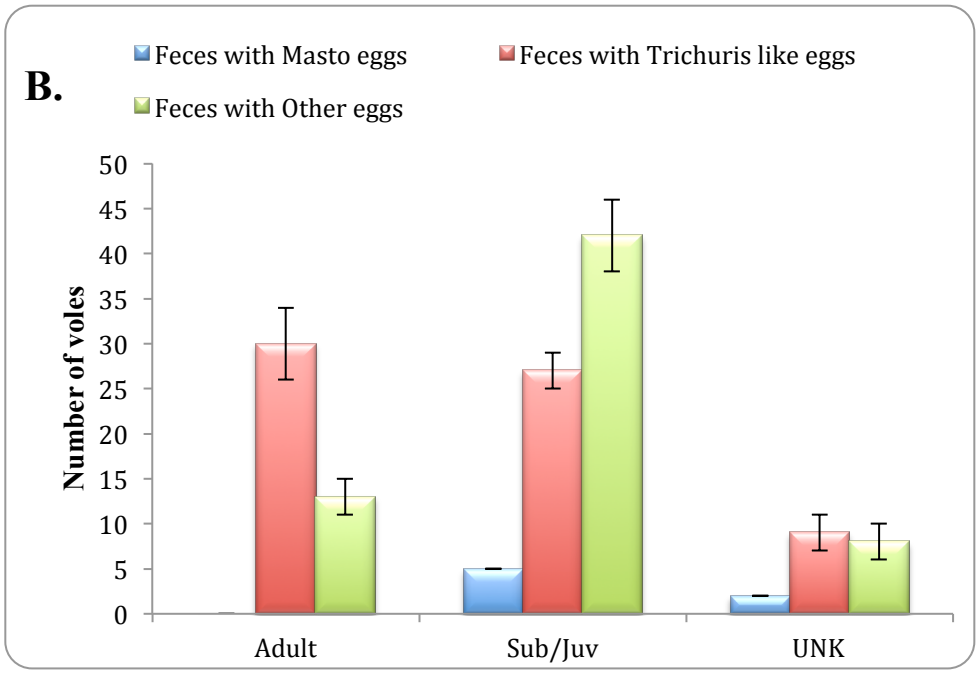
Table 2: Prevalence (in percentage and 95% CI) of different categories of eggs found in fecal samples grouped according to sex, functional group, season and region. Some fecal samples have more than one egg type present.

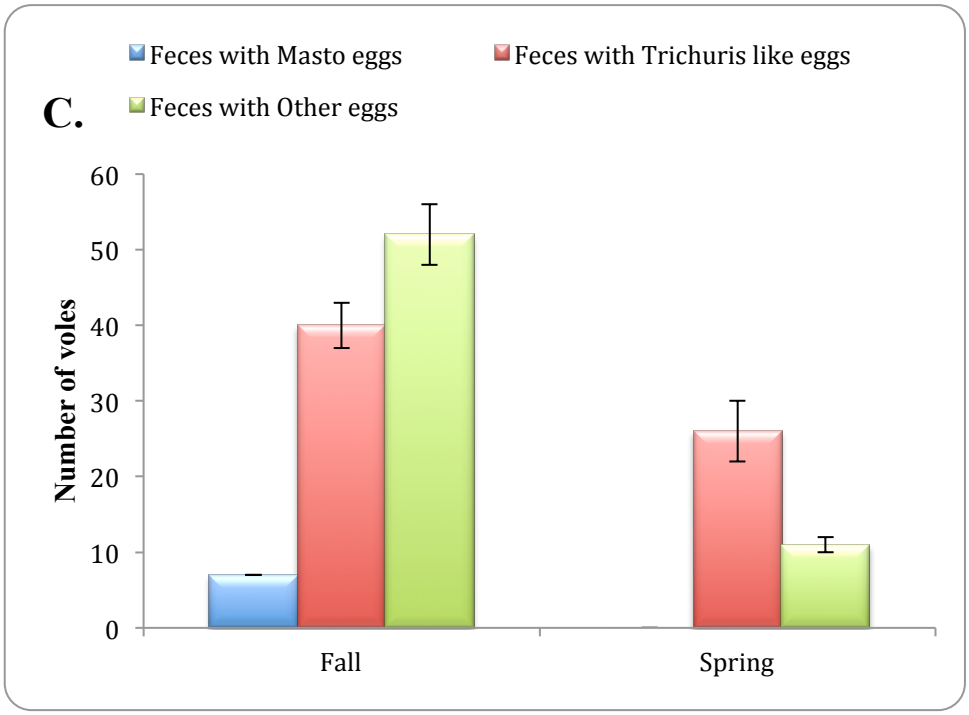
Category		Feces with eggs	Feces with *Masto* eggs	Feces with *Trichuris* like eggs	Feces with other eggs
Total		**61 (54-69)**	**4 (1-7)**	**37 (30-44)**	**35 (28-43)**
Sex	Male	56 (45-67)	4 (0-8)	30 (20-40)	40 (29-51)
	Female	65 (56-75)	4 (0-8)	43 (33-53)	32 (22-41)
Functional group	Adult	73 (60-86)	0	63 (48-77)	27 (14-40)
	Subadult/ Juvenile	56 (46-65)	5 (1-9)	25 (17-34)	40 (30-49)
	Unknown	63 (42-83)	8 (-3-20)	38 (17-58)	33 (14-53)
Season	Spring	68 (54-82)	0	59 (44-74)	25 (12-38)
	Fall	59 (50-67)	5 (1-9)	30 (22-38)	39 (30-47)
Region	Uddevalla	57 (45-68)	0	29 (18-39)	38 (27-49)
	Katrineholm	74 (64-84)	8 (2-15)	48 (36-60)	40 (28-51)
	Gnesta/ Nyköping	41 (23-60)	3 (0-10)	31 (14-49)	17 (3-32)

3.2.3 Fecal eggs in voles in relation to sex, functional group, season and region

Trichuris like eggs were present more often in females (42/178) than in males (24/178). However, more males contained other parasite eggs than females (Fig. 9.A). *Trichuris* like eggs were present more often in adult voles, whereas other helminth eggs were more common in subadult/juvenile voles (Fig. 9.B). Both *Trichuris* like eggs and eggs of other nematodes were found more frequently in voles collected from fall than in spring (Fig. 9.C). Similarly, the number of voles carrying both *Trichuris* like eggs and other helminth eggs was higher in Katrineholm than the other two regions (Fig. 9.D). Though different categories of eggs in different variables (sex, functional group, season and region) seems to be higher or lower in each variables but their overlapping confidence intervals provides additional information that there may not be so much differences.


A.
Feces with Masto eggs
Feces with Trichuris like eggs
Feces with Other eggs
Number of voles
50
45
40
35
30
25
20
15
10
5
0
Male
Female


B.
Feces with Masto eggs
Feces with Trichuris like eggs
Feces with Other eggs
Number of voles
50
45
40
35
30
25
20
15
10
5
0
Adult
Sub/Juv
UNK



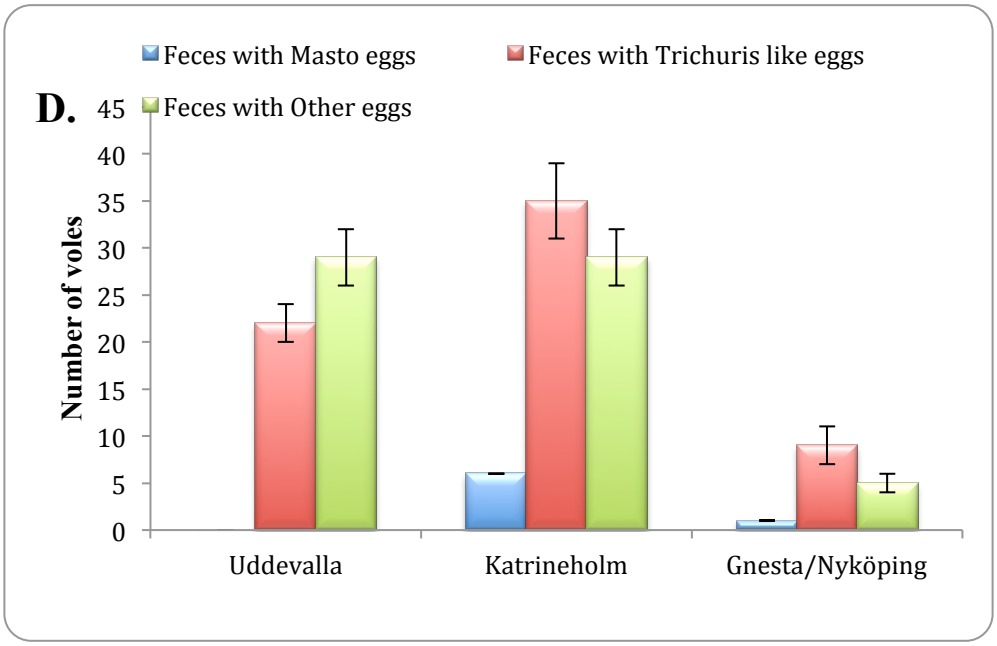


Fig. 9. Variation in number of voles with 95% CI with different categories of eggs relative to sex (Fig A), functional group (Fig B), season (Fig C) and region (Fig D).

3.3 Comparison of *M. muris* worm burden with *M. muris* eggs found in feces

The result showed that *M. muris* worm data was not correlated with data of fecal eggs of *M. muris*. The comparison between *M. muris* worm data and fecal eggs of *M. muris* was done by selecting three regions (Fig. 10.). The figure showed that number of voles infected with adult female worms were much higher than number of voles shedding eggs. Besides, no *M. muris* eggs were observed from the voles captures in region Uddevalla.

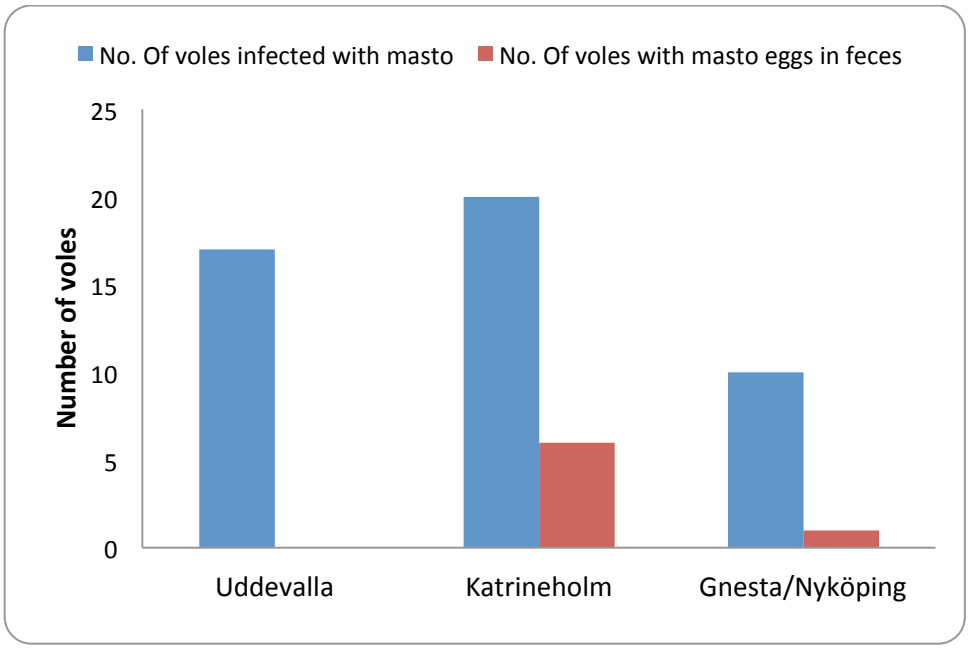


Fig. 10. Comparison between number of voles infected with Masto worms and number of voles with masto eggs with respect to three different regions

4. Discussion

M. muris infection in water voles was investigated in terms of host sex, functional group, season and different regions within southern Sweden. Alhough the nematode parasites of wild rodents from different countries of Europe have been studied previously, most documented reports are dealing with intestinal parasites and those located in other parts of the body rather than in the stomach (Burlet et al., 2011; Kataranovski et al., 2011; Lõhmus & Albihn, 2013; Milazzo et al., 2003; Pétavy, Tenora, & Deblock, 2003). Nonetheless, there are studies available on the prevalence of the gastric nematode *M. muris* in wild rodents, according to our knowledge no previous study has assessed the situation in voles of the genus *Arvicola* (Grzybek et al., 2014; Vukićević-Radić et al., 2007). Furthermore, few previous studies have focused on nematodes other than *M. muris* in water voles (Gerlinskaya et al., 2013). The quantitative analyses of actual parasite burdens and fecal egg counts measuring prevalence, intensity and abundance of *M. muris*, therefore make novel contributions to understanding the effects of intrinsic factors (sex and functional group) and extrinsic factors (season and region) on the number of *M. muris* in water voles in 3 different sampling sites in Sweden.

As stated above, *M. muris* has been previously reported from several rodents in different regions worldwide. For example, it was recorded in house mouse (*Mus musculus*) from the suburban area of Belgrade, Serbia (Vukićević-Radić et al., 2007), rats (*R. rattus*) in coconut habitat at Palmyra Atoll of central Pacific Line Islands (Lafferty et al., 2010) and bank voles (*Myodes glareolus*) of north-eastern region of Poland (Grzybek et al., 2014). It has also been described from different species including striped possums (*Dactylopsila trivirgata*), which is a small arboreal marsupial in Australia (Smith & Kinsella, 2011). Thus, it seems like this parasite can infect a wide range of hosts, in different ecological and geographical ranges. My study, like previous studies, is based on morphological identification. Further investigation using genetic markers may show that worms from different hosts represent different genotypes or species.

An earlier study presented an overall prevalence of *M. muris* as 59% in a sample of 165 rats from areas of central Pacific Line Islands (Lafferty et al., 2010), which is greater than in the present study (26%) on water voles of 181 samples. They found that the mean intensity and mean abundance of infection with 95% CI as 12 (10-14) and 7 (5-9). Thus, my study showed

greater mean intensity but lesser mean abundance (Lafferty et al., 2010). In contrast, other studies have mentioned low prevalence of *M. muris* in other rodents. The prevalence of *M. muris* in *R. rattus* was measured as 20% out of total 191 samples from New Zealand (Charleston & Innes, 1980). Recently, Grzybek et al. (2014) reported even lower prevalence of infection of *M. muris* (14% out of total 922 samples) in the study conducted in north-eastern region of Poland in wild bank voles (*M. glareolus*). Another study reported prevalence of *M. muris* as 11% out of 61 samples from spiny mice (*Acomys cahirinus dimidiatus*) of Sinai, Egypt (Behnke et al., 2000). However, it is still unknown whether *M. muris* prefer certain rodents than other or if it is represented by species a complex that may be adapted to different hosts. Further studies with different potential host species of *M. muris* are therefore required.

The level of infection with nematode parasites in general varies seasonally according to availability of food resources and fluctuation in the availability of insects (intermediate host) (Tauber & Tauber, 1981). It is known that *M. muris* must pass through an arthropod intermediate host such as cockroaches, beetles, locusts, earwings and fleas to complete its life cycle and requires 28 days to reach infectivity in its intermediate host (Quentin, 1970). Although individual worms sizes were not measured in my study, different sizes of *M. muris* worms were observed in most of the infected voles, which was also supported by finding of numerous *M. muris* of varying sizes in the stomach of the striped possum (*D. trivirgata*) in Australia (Smith & Kinsella, 2011). The most interesting finding in this study was that there was a seasonal difference in the infection levels of *M. muris*. Although, the sampling period was limited to 4 to 6 weeks in fall and spring, it is evident from wide ranges of worms of different sizes that exposure to the infection had been appearing, may be for some months prior to both sampling periods.

Few studies have been published regarding seasonal changes in infection of *M. muris* in rodents. For example, Abu-Madi et al. (2000) has established significant seasonal variation in prevalence and abundance of three intestinal helminths of the wood mouse (*Apodemus sylvaticus*) from south-east England. They mentioned that the infection was higher in winter and spring whereas lower in summer and autumn. Similar to my study, a study performed in rats (*R. rattus*) in New Zealand also showed higher prevalence of *M. muris* from March to June but lower prevalence from July to October (Charleston & Innes, 1980). Similarly, a study performed on bank voles (*C. glareolus*) in north boreal zone of Finland also showed higher prevalence of *M. muris* in early summer which peaked in July and then declined in

late summer (Haukisalmi, Henttonen, & Tenora, 1988). The seasonal differences in levels of infections in voles may be due to seasonal changes in food availability, immune system function and age structure. Higher infection of *M. muris* was found in spring in my study. This may be due to low resistance of voles with low food availability and high mortality of adult voles in spring. At the same time, the voles are breeding during spring that could add stress to them owing to higher infection.

There was no difference in infection levels between male and female voles in my study. Similar results were reported previously from male and female rats (*R. rattus*) in New Zealand (Charleston & Innes, 1980). Most studies on parasites in rodents have shown higher infections in male than in female hosts. For example, a study performed on the cestode *Hymenolepsis diminuta* in adult brown rats (*R. norvegicus*) in Doha, Qatar found that male rats were more heavily infected than females (Abu-Madi et al., 2005). The few studies which are available on *M. muris* have so far shown a female host sex bias (Grzybek et al., 2014; Zain, Behnke, & Lewis, 2012). Grzybek et al. (2014) further discussed that higher infection of *M. muris* in female bank voles (*M. glareolus*) in Poland is possibly due to pregnancy and the lactating period of females during which a higher protein diet is required. During this situation, female voles in general consume more insects, which increase their chances of being exposed to infection. Thus, the lack of differences in infection of male and female voles in my study may be due to higher number of captured subadults/juvenile voles. The results in a previous study with juvenile voles show there was no detectable difference in infection rates (Abu-Madi et al., 2005). In adult rodents, sex hormones may result in males having different behaviours and exposing them to higher infection risk. This idea is supported by a study from southwest Nigeria, which found higher infection rates of *M. muris* in adult male brown rats (*R. rattus*) than in females and attributed this to the larger home ranges of males, hypothesizing that this gave them more exposure to infections (Mafiana, Osho, & Sam-Wobo, 1997) .

The term “functional group” has been used commonly in previous rodent studies describing various reproductive stages of the population. Although significant differences in levels of infection among functional groups was not supported by my data, a previous study found that levels of helminths infections differed significantly with regard to different functional groups of bank voles (*C. glareolus*) from northern Finland (Haukisalmi et al., 1988). A higher level of *M. muris* infection was observed in overwintered adult females than in non-breeding voles studied by Haukisalmi et al. (1988). In contrast, another study on helminth

communities in *Microtus oeconomus* and *M. miurus* performed in north slope of Alaska showed highest infection levels in overwintered adult males (Haukisalmi, Henttonen, & Batzli, 1995). There is no general explanation for differences observed in infection levels among functional groups within different host populations. However, it can be predicted that higher exposure to the environment increases chances of infections in voles that have larger home ranges. For example, Lambin, Krebs, and Scott (1992) mentioned that sexually mature males have larger home ranges than females in the study of a population of tundra voles (*Microtus oeconomus*) in Canada. Furthermore, Barger (1993) showed that males are more often infected than females because of reduced resistance to helminth infections due to the male sex hormone (testosterone) whereas female sex hormones (oestrogen) increased resistance. Since fewer adults or sexually mature voles were captured compared to subadults/juveniles in my study, this could explain why there were no significant differences between the functional groups. However, note that I did not differentiate between sexes within the functional groups in my study.

With respect to regional differences in infection levels, it was found that the prevalence, mean intensity and mean abundance of infection with *M. muris* were highest in Gnesta/Nyköping. The difference in abundance of infection among regions was near to significant ($p= 0.0532$). Although very few water voles were captured in Gnesta/ Nyköping compared to Uddevalla and Katrineholm, more *M. muris* were found in Gnesta/Nyköping. This may reflect the relative abundance of intermediate hosts and possibly also intrinsic factors of host adaptation to different local environments. A study conducted in water vole from Switzerland showed significant spatial variations in prevalence of infection for two cestode parasites *E. multilocularis* and *Taenia taeniaeformis* (Burlet et al., 2011). Similarly, Roberts et al. (1992) reported a strong effect of habitat influencing the prevalence of helminth parasites including *Brachylaima apoplania*, *Capillaria hepatica*, *M. muris*, etc. of Polynesian rats (*R. exulans*) from 3 habitats (forest, grassland and lighthouse-farm) of New Zealand. It was found that both prevalence and abundance of infection of *M. muris* was higher in forest rats than grassland and lighthouse-farm rats. They discussed that the possible reason may be due to various groups of tree-dwelling orthopteran (intermediate host insect of *M. muris*) that were more dominantly found from the stomach content analysis of forest rats. Similarly, a study conducted in 3 localities of Poland on bank voles (*C. glareolus*) also showed variations in different helminth infections including *M. muris* with respect to sites (Behnke et al., 2001). Another study performed in *M. muris* in rats *R. rattus* in coconut (*C.*

nucifera) habitat at central Pacific Line Islands discussed that both rats and those insects which serves as intermediate host for *M. muris*, feed on same coconut that increases more chances of *M. muris* infection (Lafferty et al., 2010).

In addition to analysis of *M. muris*, fecal egg counts (FEC) (McMaster method) was used to assess different types of nematode eggs in feces. However, analysis and explanations were focused on *M. muris* eggs and predominant *Trichuris* like eggs because other types of eggs were rarely found and some egg types were never identified. *Trichuris muris* is an intestinal nematode of rodents belonging to family Muridae. Unlike *M. muris*, it has a direct life cycle. However, like *M. muris* the host range is wide and it also widely distributed in different geographical regions (Callejón et al., 2010). The eggs of *T. muris* are barrel shaped with a bipolar plug at both ends (Sharma et al., 2013).

Behnke et al. (2000) concluded that FEC is a non-invasive method of quantifying parasites but which required refinement and modification. Based on the fecal examination, *Trichuris* like eggs were found in 37% of samples, whereas *M. muris* eggs were only present in 4%. Similar results were produced from a study of fecal samples of water voles where prevalence for of the protozoan parasite (*Giardia sp.*) was 30% and several other pathogens were positive by less than 5% (Gelling et al., 2012). Similarly, examination of fecal samples from spiny mice of Sinai, Egypt identified *D. kuntzi* eggs in 7% of the samples and those of *A. africana* by 2% (Behnke et al., 2000). In my study, it was found that *M. muris* worm burden was not correlated with eggs of *M. muris* found in feces. So, this would be further evidence that the worm burden is not always correlated to egg burden.

However, there seems to be shortcomings in analysis protocol and method of FEC used in the present study. Some authors have recommended precautions in the use of FEC to estimate infection intensity since the number of eggs may not always reflect numbers of adult parasites (Gillespie, 2006). For example, Seivwright et al. (2004) mentioned that freezing the samples with feces at very low temperature of –40°C and even storing fecal samples at optimum temperature of 5°C for longer period of time (more than 3 weeks after collecting samples) decreases the concentration of parasite eggs in fecal samples. Additionally, there are multiple factors that influence the rate of eggs produced such as seasonal variations in fecundity (Romeo et al., 2014). Thus, lower prevalence of *M. muris* eggs in my study may be due to the long period of storage of my samples in the freezer (more than 8 months). Although $ZnCl_2$ appeared suitable for flotation (flotated many

different types of eggs) and was efficient in reducing number of bubbles in McMaster chambers, its use was not validated for *M. muris*. The use of $ZnCl_2$ could be validated by a study comparing the flotation of *M. muris* from frozen and fresh samples using the McMaster technique.

This is the first paper that has reported quantitative worm burden data of *M. muris* infecting *A. amphibius* captured from different locations in Sweden. There were seasonal and regional variations in levels of infections of *M. muris*. Although *Trichuris* like eggs were predominantly found, only a few fecal samples harboured *M. muris* eggs. How these parasites affect host fitness and life history remains unknown. Therefore, more research and comprehensive studies are essential to understand the variation of *M. muris* in relation to different intrinsic and extrinsic aspects and the ecological consequences of the infection of *M. muris* in water voles.

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Permits

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Appendix 1. Dilution factor for EPG estimate

Kammare	Volume (ml)	Feces (gm)	Total	Density	EPG
0.3	**42**	**3**	45	15	50
0.3	**40.6**	**2.9**	43.5	15	50
0.3	**39.2**	**2.8**	42	15	50
0.3	**37.8**	**2.7**	40.5	15	50
0.3	**36.4**	**2.6**	39	15	50
0.3	**35**	**2.5**	37.5	15	50
0.3	**33.6**	**2.4**	36	15	50
0.3	**32.2**	**2.3**	34.5	15	50
0.3	**30.8**	**2.2**	33	15	50
0.3	**29.4**	**2.1**	31.5	15	50
0.3	**28**	**2**	30	15	50
0.3	**26.6**	**1.9**	28.5	15	50
0.3	**25.2**	**1.8**	27	15	50
0.3	**23.8**	**1.7**	25.5	15	50
0.3	**22.4**	**1.6**	24	15	50
0.3	**21**	**1.5**	22.5	15	50
0.3	**19.6**	**1.4**	21	15	50
0.3	**18.2**	**1.3**	19.5	15	50
0.3	**16.8**	**1.2**	18	15	50
0.3	**15.4**	**1.1**	16.5	15	50
0.3	**14**	**1**	15	15	50
0.3	**12.6**	**0.9**	13.5	15	50
0.3	**11.2**	**0.8**	12	15	50
0.3	**9.8**	**0.7**	10.5	15	50
0.3	**8.4**	**0.6**	9	15	50
0.3	**7**	**0.5**	7.5	15	50
0.3	**5.6**	**0.4**	6	15	50
0.3	**4.2**	**0.3**	4.5	15	50
0.3	**2.8**	**0.2**	3	15	50
0.3	**1.4**	**0.1**	1.5	15	50

Appendix 2. Different types of nematode eggs

(Source: Veterinary Parasitology, 3rd Edition: M.A. Taylor, R.L., Coop, R.L. Wall)

(Note: G1, G2…G7 in the diagram below are just the codes given to different types of eggs based on their shapes and sizes)

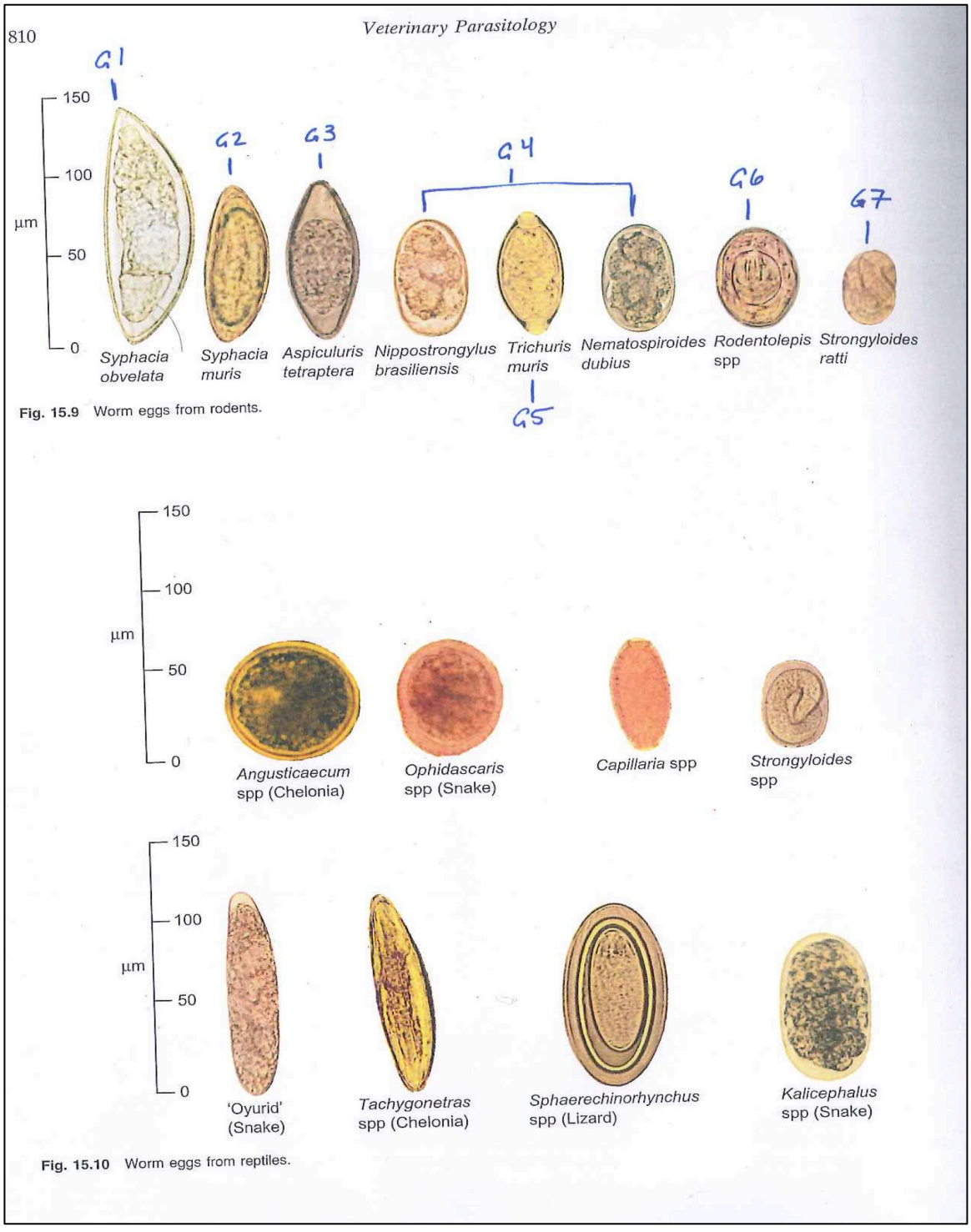

Fig. 15.9 Worm eggs from rodents.

Fig. 15.10 Worm eggs from reptiles.