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Gregarina typographi (Eugregarinorida: Gregarinidae) in the Bark Beetle *Ips typographus* (Coleoptera: Curculionidae): Changes in Infection Level in the Breeding System

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Summary. Changes in *Gregarina typographi* Fuchs, 1915 (Eugregarinorida: Gregarinidae) infection levels were studied in a population of the bark beetle *Ips typographus* (L., 1758) (Coleoptera: Curculionidae), which lives in association with Norway spruce. Beetles were repeatedly collected from three logs of trap trees during 2009 and 2010 at one study site in the Czech Republic with a high level of *G. typographi* infection (seasonal mean of 15.7% in 2009 and 19.8% in 2010). Infection levels did not differ statistically between sexes, logs, and trap trees but did differ among sampling dates. During the beetle reproductive period, the infection levels nearly doubled in 2009 (from 10.7 to 19.8%) and more than tripled in 2010 (from 9.3 to 31.3%). We infer that the continuous increase in the *G. typographi* infection level within each of the two years resulted from transmission among beetles in nuptial chambers during the May–June reproductive and egg-laying period.

Key words: Beskids, Gregarinidae, horizontal transmission, protozoan disease, spruce bark beetle.

INTRODUCTION

Several authors (Wegensteiner 2004, Takov *et al.* 2010, Wegensteiner *et al.* 2010) have studied pathogens in *Ips typographus* (L., 1758). The spruce bark beetle *I. typographus* is the most damaging insect attacking spruce forests (*Picea abies* (L., 1753) Karsten, 1881) in Eurasia (Christiansen and Bakke 1988). Adult beetles of *I. typographus* emerge from the forest litter and tree bark on warm spring days and fly to stressed host trees. These bark beetles use an aggregation pheromone to attract more individuals of the same species to the tree for the purpose of weakening the tree and mating. The pheromone attracts both sexes. The attracted males join the attack and secure an area for mating and oviposition. This area consists of a hole and a chamber beneath the bark known as a "nuptial chamber". The females construct a tunnel ("maternal gallery") beneath the nuptial chamber in which to lay eggs. In all species of the *Ips* genus, several females (usually two or three) join each male in his nuptial chamber (Wermelinger 2004). At lower elevations, overwintering *I. ty-*

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pographus beetles emerge in April/May and produce one or two generations of offspring per year with the main peaks of bark beetle emergence in July and August/September. At elevations above 1,000 m a.s.l., this species produces only one generation of offspring per year (Wermelinger 2004). The emergence and migration of beetles of monovoltine generations depend on the geographical latitude: northern populations emerge later and fewer beetles move before overwintering than in southern populations (Forsse 1991).

Gregarina typographi Fuchs, 1915 has been found in the midgut lumen in a number of members of the Scolytinae subfamily (Wegensteiner 2004, Kereselidze *et al.* 2010, Takov *et al.* 2010). Insects usually are infected when they ingest oocysts contained in host faeces or in dead or living (in the case of cannibalism) body parts of the host. Most of authors (Wegensteiner 2004, Takov *et al.* 2010) monitored levels of infection in the populations; only Wegensteiner *et al.* (2010) tried to determine the impact of *G. typographi* on the demographic structure of *I. typographus*. Because gregarines develop rapidly, horizontal transmission of infection among parental beetles inhabiting a single nuptial chamber could be significant (MacDougall 1942, Smith *et al.* 2007). Each male beetle possesses a harem that usually consists of 2–3 females, and the male and females inhabit a gallery system (nuptial chamber) for several weeks (Wermelinger 2004). In this study, we observed changes in the infection level of *G. typographi* in *I. typographus* in the nuptial chamber during two seasons from the time when beetles begin burrowing into the trees until the end of the egg-laying period.

MATERIALS AND METHODS

Study site

Gregarina typographi and *I. typographus* were studied at the Smrk Nature Reserve in the Beskids (Czech Republic). The Beskids bioregion has a terrain characterized by mountains with typical elevations of 500–1,200 m a.s.l. Forest cover exceeds 70%, and more than 70% of that cover is represented by spruce (Culek 1996). The old-growth forest at the Smrk Nature Reserve consists of beech and spruce trees. The study site was situated in the center of the reserve (coordinates: 49°30′N, 18°22′E) within an elevation range of 1,180 to 1,200 m a.s.l. In 2009, the average temperature varied 4–5°C, and the total precipitation was about 1,400 mm (www.chmu.cz).

Spruce bark temperature was calculated from average monthly air temperature and global radiation (obtained from www.chmu.cz) during the study period in each year according to Baier *et al.* (2007). The mean bark temperature in 2009 was around 7.7°C in May and 10.7°C in June and was slightly higher the following year at around 11.8°C in June and 15.3°C in July.

The Reserve is a protected area with only minimal forestry management, represented by limited sanitation felling, trap-tree importation and removal, and beetle trapping in pheromone traps.

Trapping of beetles

The study involved 11 trap trees (*Picea abies*) in 2009 and 5 trap trees (*P. abies*) in 2010. Trap trees were imported from neighboring managed forests. Each trap tree (about 30 cm in diameter, 30 m in height) was cut into three logs of the same length (log I–III), which were situated in parallel with 1–2 m spacing in the center of the nature reserve. The logs were located in a clearing perpendicular to a trail through the forest, and the logs from each tree were ca. 50 m distant from the logs of the other trees. Trap trees were placed at the site on 12 May 2009 and 13 May 2010. Mature beetles were collected under a strip of bark from each log of trap tree during 2009 (26 May, 18 June) and 2010 (at 10-day intervals in June and July). Each sample area was 1 m long and about 0.5 m wide (half the tree circumference). The samples collected on different dates were separated by 1 m.

All mature beetles in nuptial chambers and maternal galleries beneath each strip of bark were collected. Analyzed beetles from each sample date and bark strip were always chosen at the same stage of development of offspring in the breeding system (from nuptial chambers to callow beetles). Thus only beetles from the same period of infestation, i.e. at the end of May (for 2009) and in early June (in 2010), were chosen. The entry holes were counted, and the individual beetles were placed into 2-cm^3 Eppendorf micro test tubes. A piece of wet gauze was added to the test tube to maintain 100% relative humidity. The beetles were immediately frozen and stored at –4°C. The tissues of beetles thus prepared remained intact. The entire body cavity (including intestines, Malpighian tubules, adipose tissue and gonads) were dissected in a water drop within 4 months of collection and checked for pathogens.

During the last analysis of beetles (20 July 2010), we collected a samples of frass and faeces from maternal galleries (length 60–80 mm) and entry holes. Frass was removed with a brush and crushed into drops of water. The final suspension was examined under light microscope and checked for presence of gregarine oocysts.

Determination of infection and statistical analysis

In 2009 and 2010, each dissected beetle was examined with a light microscope at magnifications of 40–400 × to determine its sex and whether it was infected with *G. typographi* or any other pathogen. For beetles collected in 2010, life stages of *G. typographi* were measured and photographed with an Arsenal LPE 5013i-T microscope (Arsenal s.r.o., Prague, Czech Republic). When trophozoites, gamonts and gametocysts occurred together, we recorded all these stages and the data were included (individually) in the analysis of individual life stages. To compare the total prevalence of *G. typographi*, we used only the presence/absence of a pathogen (all stages combined). The following structures of trophozoites and gamonts were measured: length of deutomerite (LD), length of protomerite (LP), total length (TL), width of deutomerite (WD), width of protomerite (WP), ratio of the length of protomerite to total length (LP : TL), ratio of the width of protomerite to the width of

deutomerite (WP : WD), and ratio of the width of protomerite to the length of protomerite (WP : LP).

Infections were compared by year (Kruskal-Wallis test), collection date (one-way ANOVA), sex of the beetle (Wilcoxon rank-sum test), and trap tree (one-way ANOVA), such that infections of all beetles collected from a trap tree (> 100 beetles) were calculated. For comparison of infections among the individual trap trees (one-way ANOVA), data from beetles collected from a sample were used (> 20 beetles). The R 2.10.1 software program and STATISTICA 9 were used for statistical analyses.

RESULTS

Gregarina typographi morphometrics and life stages

G. typographi gametocysts were spherical with a diameter of 93.6 ± 20.2 μm (n = 30) and were observed only in the proctodeum. The dimensions of gregarine trophozoites (Fig. 1) and gamonts are reported in Table 1. Both stages were always found from the stomodeum to midgut.

In 2010, the presence of *G. typographi* life cycle stages in different samples were recorded (Fig. 2). The percentage of beetles with gamonts (Fig. 2B), and gametocysts (Fig. 2C) did not differ among the sampling times. The percentage of beetles with trophozoites, however, tended to increase over time and was 3.45 times greater at the last sampling date than at the first sampling date (Kruskal-Wallis test; chi-square = 9.50, $p < 0.05$) (Fig. 2A).

Infection rates

The mean infection rate of *G. typographi* in *I. typographus* was 15.7% in 2009 and 19.8% in 2010. *G. typographi* infection levels did not significantly differ between the individual years (Kruskal-Wallis test; chi-square = 1.00, $p > 0.05$).

For 2009, a total of 1,624 *I. typographus* beetles were dissected. Data for infection levels for individual trap trees in 2009 were normally distributed (Shapiro-Wilk test for normality; $W = 0.97$, $p > 0.05$). Infection levels differed significantly among the sampling dates in 2009 (ANOVA; $F = 5.69$, $p < 0.05$) (Fig. 3) but did not differ among trap trees or logs. *G. typographi* infection averaged 10.7% in May 2009 (n = 792 beetles) and 19.8% in June 2009 (n = 454 beetles). The only other pathogen detected in 2009 was a single microsporidium of *Chytridiopsis typographi* (Weiser 1954) Weiser 1970. *G. typographi* infection levels for females (16.1%) and males (15.9%) did not differ in 2009 (Wilcoxon rank-sum test; $W = 2638.5$, $p > 0.05$). In 2009, there were 0.6 entry holes of *I. typographus* per dm^2 of trap tree in June.

In 2010, a total of 822 *I. typographus* beetles were dissected. Data for infection levels for individual trap trees in 2010 were normally distributed (Shapiro-Wilk test for normality; $W = 0.94$, $p > 0.05$), and therefore one-way ANOVA ($F = 3.57$, $p < 0.05$) was used. Mean values of *G. typographi* infection did not differ among trap trees (ANOVA; $F = 3.41$, $p > 0.05$). According to multivariate analysis, the infection rate was significantly lower on 10 June 2010 than on 1 July and 10 July 2010 (ANOVA; $F = 5.33$, $p < 0.05$) (Fig. 4). More males (28.3%) than females (16.8%) were infected, but this difference was not statistically significant (Wilcoxon rank-sum test; $W = 147.5$, $p > 0.05$). No other pathogen was found in any organ or haemolymph in

Table 1. Morphometrics of trophozoites (n = 30) and gamonts (n = 30) of *Gregarina typographi* in *Ips typographus.* Measurements are in μm.

Stage		TL	LP	LD	WP	WD	LP : TL	WP : WD	WP : LP
Trophozoites	Min	103.5	20.1	55.0	7.3	37.2	0.2	0.1	0.2
	Max	277.2	52.1	186.3	29.7	148.4	0.3	0.4	1.0
	Mean	163.0	35.1	109.1	16.6	79.7	0.2	0.2	0.5
	SD	53.5	9.7	45.9	6.7	28.1	0.1	0.1	0.2
Gamonts	Min	105.0	16.1	82.1	31.4	49.2	0.2	0.4	0.8
	Max	241.5	99.7	182.6	82.8	143.5	0.4	0.7	2.2
	Mean	186.1	48.4	130.7	60.2	107.3	0.3	0.6	1.4
	SD	40.8	22.4	27.6	14.5	27.6	0.1	0.1	0.5

TL – total length; LP – length of protomerite; LD – length of deutomerite; WP – width of protomerite; WD – width of deutomerite; LP : TL – the length of protomerite to total length; WP : WD – the width of protomerite to the width of deutomerite; WP : LP – the width of protomerite to the length of protomerite; Min – minimum value; Max – maximum value; SD – standard deviation.

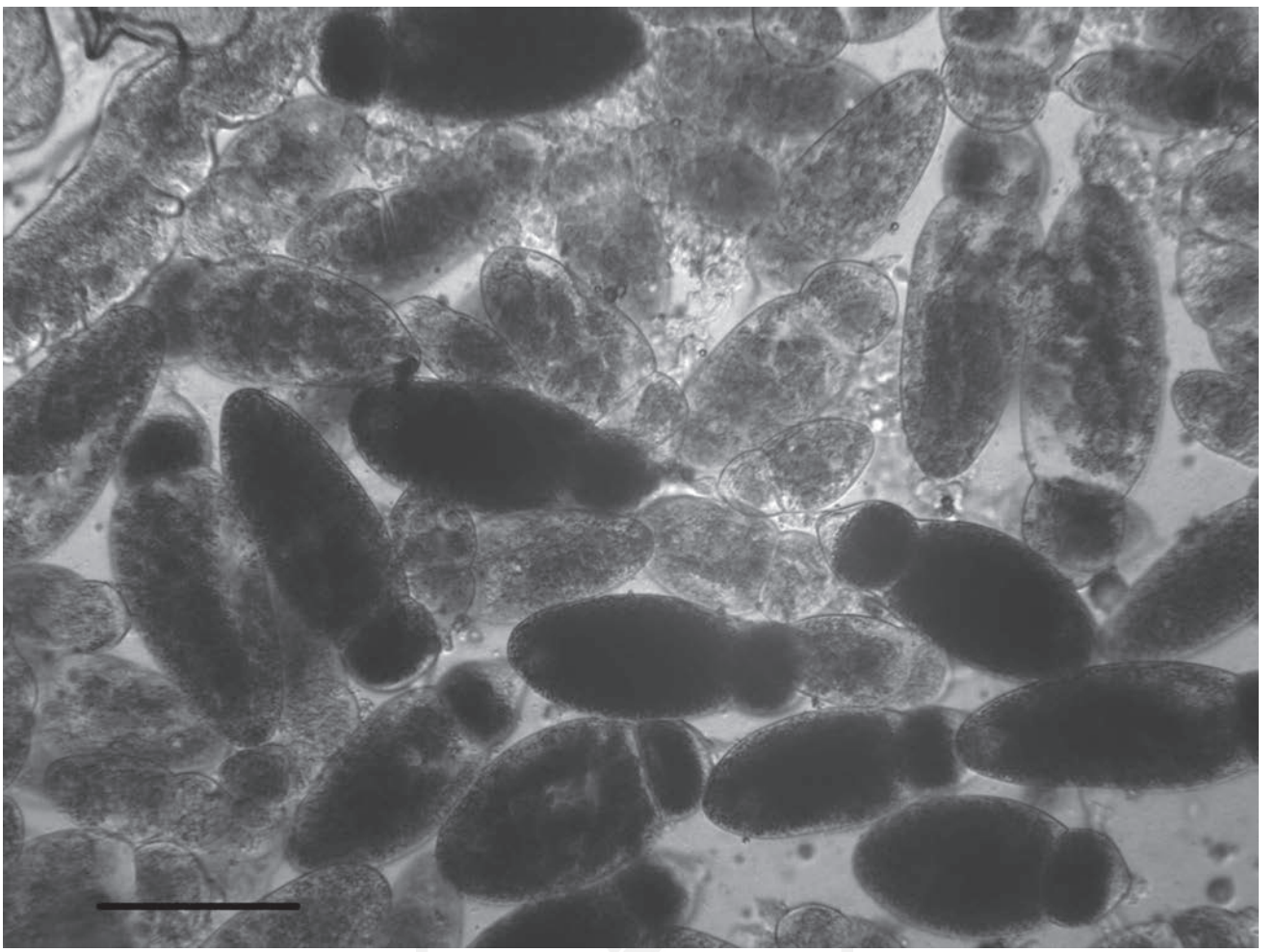


Fig. 1. *Gregarina typographi* trophozoites outside the body cavity of *Ips typographus* obtained from trap trees in 2010. Bar: 100 μm.

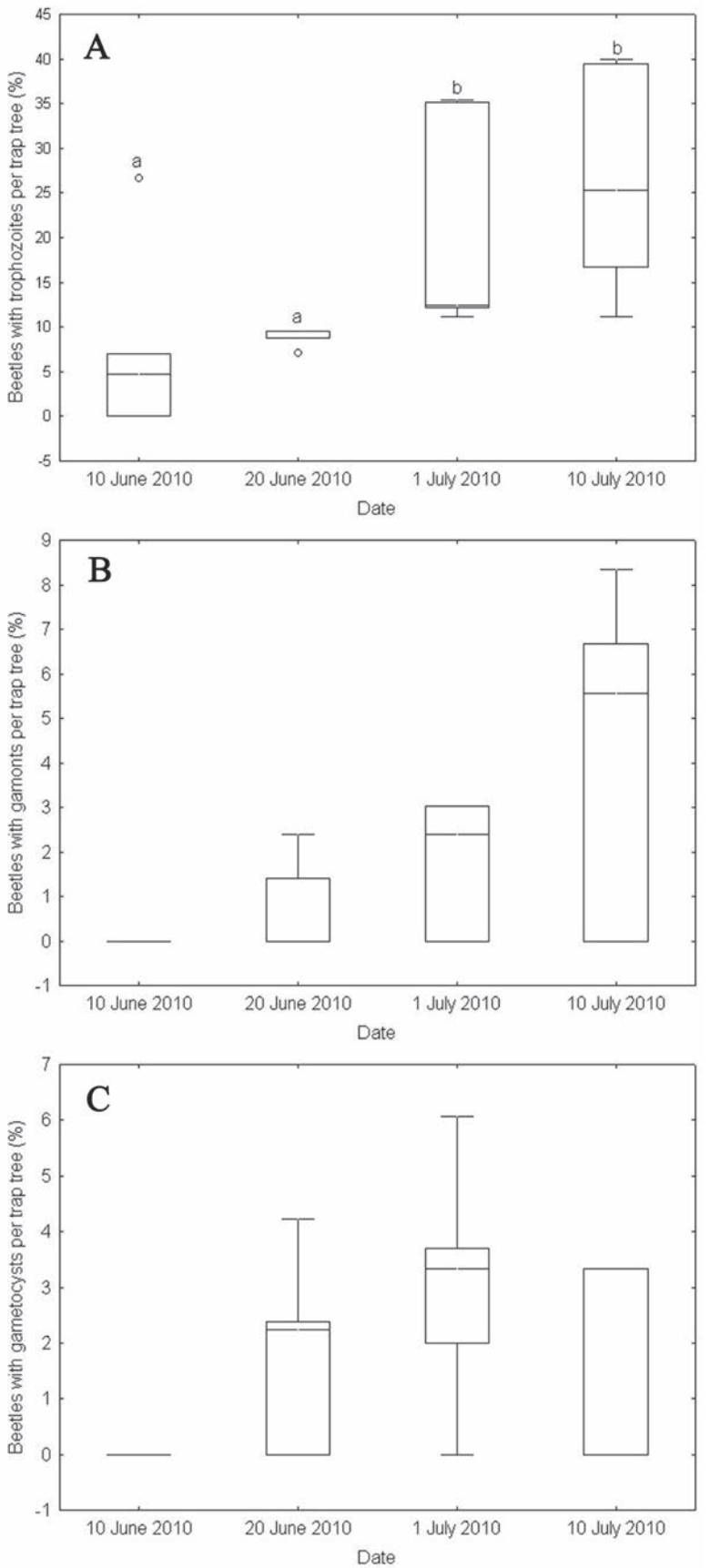
Fig. 2. Percentage of *Ips typographus* infected by *Gregarina typographi* in 2010 by stage: **(A)** trophozoites, **(B)** gamonts, and **(C)** gametocysts. Boxplots show median plus upper and lower quartiles. Minimum and maximum values are shown by the upper and lower whiskers (1.5 × interquartile range), and outlying values are depicted as circles. In the trophozoites graph (A), different letters above each bar indicate a significant difference between mean values (Kruskal-Wallis test, $p < 0.05$).

2010. The average population density on one trap tree was 0.4 entry holes of *I. typographus* per dm^2.

No gregarine oocysts were found in host frass and faeces from maternal galleries (n = 50) and entry holes (n = 20).

DISCUSSION

Gregarina typographi is the only representative of eugregarines found in the genus *Ips* (Wegensteiner 2004). From *Ips acuminatus* (Gyllenhal 1827) is far closer blank recorded species of the genus *Gregarina* sp. (Takov *et al.* 2007). Based on the morphology and morphometrics reported by other authors (Yaman 2007, Takov *et al.* 2007) for the individual stages of *Gregarina*, it is certain that the species observed in this study was also *G. typographi*. Precise detection of this parasite is problematic, because intracellular stages (sporozoites) of the eugregarine life cycle are difficult to identify with a light microscope (Bjørnson 2008, Yaman 2008). This could result in a slight underestimation



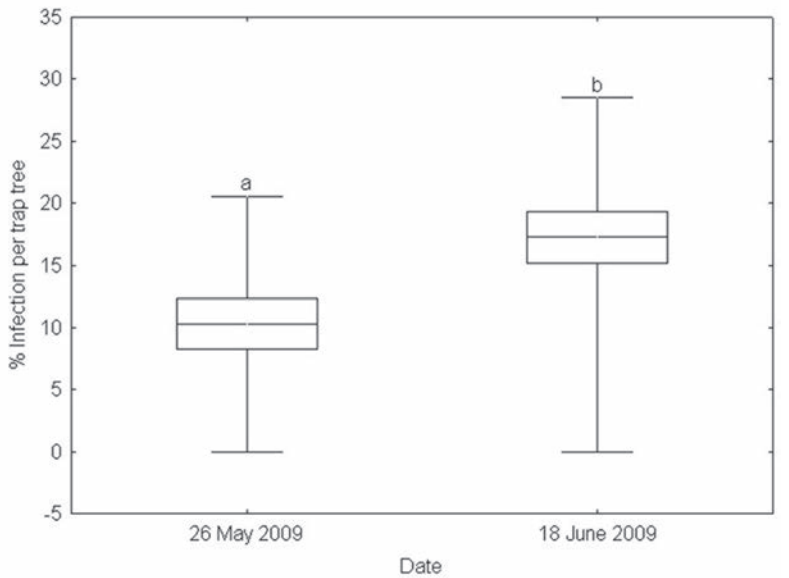


Fig. 3. Percentage of *Ips typographus* infected by *Gregarina typographi* per trap tree in 2009. Boxplots show mean ± standard deviation. Minimum and maximum values are shown by the upper and lower whiskers. Different letters above each bar indicate a significant difference between mean values (ANOVA, $p < 0.05$).

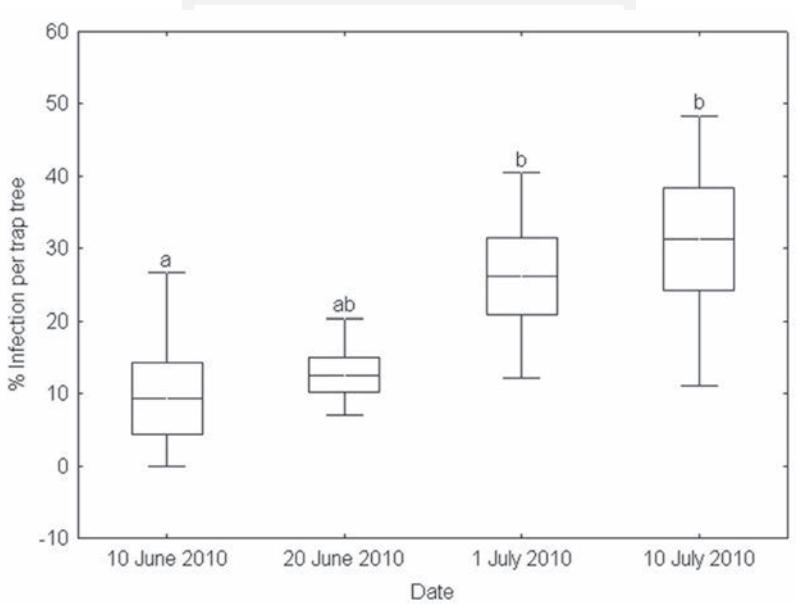


Fig. 4. Percentage of *Ips typographus* infected by *Gregarina typographi* per trap tree in 2010. Boxplots show mean ± standard deviation. Minimum and maximum values are shown by the upper and lower whiskers. Different letters above each bar indicate a significant difference between mean values (ANOVA, $p < 0.05$).

of infection. Oocysts are easily overlooked, especially in the early stages of infection (after ingestion) when they may occur only in small numbers. Most studies of bark beetle pathogens record only the trophozoite, gamont, and gametocyst stages or do not differentiate these stages at all (Händel *et al.* 2003, Wegensteiner and Weiser 2004, Yaman 2007).

Gregarina typographi is a quite common and non-specific pathogen of bark beetles. About 53% of bark beetle species are hosts to *G. typographi* (Takov *et al.*

2010), and in some cases the prevalence in a population can be high (up to 44%) (Wegensteiner *et al.* 1996, Wegensteiner 2004). The similar level of *G. typographi* infection in the current study area was relatively high. The greater was the density of gallery systems at a given location, the greater was the probability of the pathogen transmission between beetles. Thus, even the recently matured beetles can become infected when they cut through another gallery while feeding (Wegensteiner and Weiser 1996). When their population densities are low, bark beetles only rarely encounter other beetles outside their own galleries, and the spores cannot therefore be ingested with frass or the remnants of dead infected beetles (in the case of infections attacking the fat body) (Wegensteiner and Weiser 1996).

The ratio of infected males and females was similar in the two years, which corresponds to the report by Wegensteiner *et al.* (1996). Both sexes come in contact with contaminated faeces at the same time during breeding, egg-laying and removal of frass from entry holes. These findings show that the risk of infection is similar for both sexes and that the infection spreads more or less evenly among males and females.

Increasing infection level of *G. typographi* is probably the result of mutual transmission of gregarines over a very short time. In both years there was a statistically significant increase in infection levels between the first and last samples. Due to unfavourable weather (low temperatures, snow and rainfall) in 2010 (10 June) a time of the emergence of beetles was later compared to 2009 (26 May). But the time period from the moment of landing of beetles on the logs and excavation of nuptial chambers to their leaving the trap trees (the pupal stage and callow beetles of F1 generation) was similar (about one month) in both years. The duration of the ontogenetic development of gregarines is relatively short, even though the development rate depends on temperature and humidity (MacDougall 1942, Smith *et al.* 2007) or the efficiency of the host's immune system (Corbel 1968). The infection cycle of *Gregarina rigida* (Hall, 1907) Ellis, 1913 requires about 10–11 d from the sporozoites stage to reproductive stage (Allegre 1948). *G. cubensis* Peregrine, 1970 requires more time to complete its development at 27°C (216 h) than at 15°C (144 h) (Smith *et al.* 2007). The temperature of spruce phloem depends on air temperature and solar radiation (Baier *et al.* 2007). During flight activity of bark beetles, the average daily temperature of phloem in shaded areas of the tree stem is balanced around 15°C, while the sunlit parts vary between 15 and 30°C (Netherer *et al.* 2003). We measured a slightly lower bark temperature (7–16°C) due to the high altitude and low temperature of the study site. This means that gregarines' development cycle may be carried out several times during the continued presence of beetles in the breeding. We infer that some infected beetles that were establishing galleries were only in the initial stage of infection and could not transmit the infection to other beetles co-inhabiting the nuptial chamber until sufficient time had passed for *G. typographi* to complete its life cycle. These data explain why the infection level did not increase in the current study unless there were at least 20 d between samplings (see Fig. 4).

The life cycle of species in the genus *Gregarina* Dufour, 1828 is direct, i.e. there is no intermediate host or vector (Clopton and Gold 1996). Gametocysts are shed with the host's frass (Zuk 1987). Upon maturation, gametocysts dehisce to release hundreds of infective oocysts into the environment (Zuk 1987). Faeces along with frass are pushed out from the breeding system by males (Christiansen 2008). During this process, individual oocysts adhere to the walls of the breeding system and are then eaten accidentally when beetles excavate galleries and feed on phloem. Unfortunately, no oocysts were detected in any smear of samples from 50 maternal galleries and displaced frass from 20 entry holes from the sampling date 10 July 2010. Considering the failure to find oocysts in the frass, we assume that the probability of oocysts ingestion was very low in the study population of *I. typographus* and the rise of gregarine infection levels took a longer time than we expected. After ingestion, excystation of gregarines is activated in the gut lumen and the sporozoites leave behind the oocysts (Clopton and Gold 1995). The sporozoite grows into a large trophozoite, the feeding stage of the life cycle. Trophozoites undergo a period of extracellular growth during which they remain attached to the intestinal epithelium by means of an epimerite (Omoto *et al.* 2004, Smith *et al.* 2007, Valigurová *et al.* 2009). Trophozoites as well as gamonts were the most frequently observed stages in the stomodeum and midgut, while gametocysts occurred most frequently in the proctodeum, where they become part of the faeces.

In the Smrk Nature Reserve, where forestry management is limited, beetles are abundant and pathogen transmission is therefore favored. As beetle numbers increase, the level of *G. typographi* infection in *I. typographus* gradually increases (see Holuša *et al.* 2009, Wegensteiner *et al.* 2010). Given what is known about the biology of the pathogen and the beetle, the doubling

of the *G. typographi* infection prevalence in 2009 and its more than tripling in 2010 could be a result of disease transmission among beetles in nuptial chambers, i.e. by horizontal transmission during reproduction and egg-laying (May–June).

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