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Seasonality of *Harpella melusinae* Léger and Duboscq (Harpellales) in black fly larvae in Northern Thailand

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Abstract – The influence of season and water temperature on the occurence of *Harpella melusinae* in black fly (Diptera: Simuliidae) larvae was investigated in three streams in northern Thailand. A total of 1260 black fly larvae were examined and 86.3% (CI = 82-88%) were colonized by *H. melusinae*. Both prevalence and level of colonization by *H. melusinae* varied depending on season ($P < 0.01$). The highest prevalence was during the cool season (November, 96-100%), compared to the wet season (July to October, 80-92%) and dry season (April to June, 72-89%). The levels of colonization (or numbers of thalli per midgut) by *H. melusinae* also showed a similar pattern. Regression analysis was significant for season and prevalence at all sites, whereas the level of colonization varied across sites. Prevalence and level of colonization varied with water temperature ($P < 0.05$), and both were higher in colder waters. This study helps to redress the paucity of ecological information concerning this group of fungi but further studies with different taxa, hosts and habitats will undoubtedly increase our limited knowledge about the ecology of this group of obligately endosymbiontic microorganisms.

Aquatic insects / ecology / gut fungi / Kickxellomycotina / Simuliidae / Zygomycota

INTRODUCTION

We are carrying out studies on the ecology and biodiversity of fungi from various habitats in northern Thailand (e.g. insects: Aung *et al*., 2008; leaf litter: Duong *et al*., 2008; wood: Kodsueb *et al*., 2008a, b; monocotyledons; Pinruan *et al*., 2007; Thongkantha *et al*., 2008) which have previously been poorly studied. In the present study we investigated the ecology of *Harpella melusinae* (Trichomycetes) occuring in black fly larvae in streams in Northern Thailand.

Trichomycetes is derived from Greek word “tricho” meaning hair – due to the hair-like appearance of this fungi within the gut – and “mycetes” meaning fungi. The name has recently been redefined and used to describe an ecological group of microorganisms inhabiting the digestive tracts of arthropods (“gut fungi”) but was previously a Class of fungi within the Phylum Zygomycota (Lichtwardt *et al*., 2001). The traditional class Trichomycetes, however, has been shown to be polyphyletic with two orders in the true fungi (Harpellales and

Asellariales) and two others that are protists (Amoebidiales and Eccrinales) (Benny and O'Donnell, 2000; Cafaro, 2005; White *et al*., 2006a). It is now recommended that "trichomycetes" be used to describe an ecological group comprising insect gut fungi and protists following the re-evaluation of the classification (Hibbett *et al*., 2007).

Trichomycetes have been reported throughout many parts of the world wherever suitable hosts occur. More than 200 described species of trichomycetes are known from 15 orders of arthropods (mostly Insecta, but also Crustacea and Diplopoda) from various habitats – freshwater, marine and terrestrial (Lichtwardt *et al*., 2001). Nearly all hosts from freshwater habitats are immature stages of aquatic insects – black flies, mosquitoes, midges, and soldier fly larvae or stonefly and mayfly nymphs – with one exception, Isopoda (White, 1999; Lichtwardt *et al*., 2003; Strongman and White, 2008).

Black fly (Diptera: Simuliidae) larvae are host to eleven genera of trichomycetes. Two genera belong to order Amoebidiales – *Amoebidium* and *Paramoebidium*, and eight are Harpellales – *Barbatospora*, *Genistellospora*, *Graminelloides*, *Harpella*, *Pennella*, *Simuliomyces*, *Smittium*, *Stachylina* and *Stipella*. *Harpella* and more rarely *Stachylina* (Harpellaceae) inhabit the midgut peritrophic matrix (sometimes referred to as the peritrophic membrane) and are characterized by unbranched thalli with more or less determinate growth, whereas the remaining are all Legeriomycetaceae – which inhabit the hindgut and are characterized by branched thalli and a more indeterminate growth (Misra and Lichtwardt, 2000; Lichtwardt *et al*., 2001). The digestive tract of black fly hosts can have multiple species of gut fungi but growth is not such that the lumen is blocked by the endosymbionts. Two harpellids, *H. melusinae* and *Pennella simulii*—and perhaps *Genistellospora homothallica* – have been reported infecting the ovaries of female black flies. Their occurence as ovarian cysts (or chlamydospores) have been confirmed as a stage in the life cycle of gut fungi (Labeyrie *et al*., 1996; White *et al*., 2006b).

Although trichomycetes have been collected and identified from many parts of the world, surveys of tropical gut fungi have been limited (Misra, 1998; White *et al*., 2000; Valle *et al*., 2008; Valle and Cafaro, 2008). Most trichomycete studies have dealt with taxonomy, while ecology of trichomycetes have received less attention (Beard and Adler, 2002). To redress the paucity of ecological information (especially in the tropics), we examined the interaction between season and water temperature on the prevalence and level of colonization of *H. melusinae* in black fly larvae in three streams in Northern Thailand.

MATERIALS AND METHODS

Collections of 30 black fly larvae were made at three sites: Huai Khang Pla Waterfall; New Waterfall and Pong Dueat Hotspring stream, every two weeks, from April to November 2008. Larvae were collected randomly from the substrates using forceps and placed on moist filter paper in Petri dishes and transported on ice to the laboratory. Description of the collecting sites are listed in Table 1.

Seasons were defined as follows: hot-dry season, March-June; hot-wet season, July-October; and cool-dry season, November-February. In the hot-dry season humidity was very low, air temperature was quite high, and streams usually became shallow. In hot-wet season, heavy rains occurred on most days, air

Table 1. Collecting sites for black fly larvae in Northern Thailand, April to November 2008.

Site	*Location*	*Elevation (m)*	*Description*
(1) Huai Khang Pla Waterfall, Chiang Rai Province	N 20°05' E 099°46'	512	Permanent, cascading stream with boulders, mostly bamboo trees along the riverbank
(2) New Waterfall, Chiang Mai Province	N 19°52' E 098°41'	781	Permanent, cascading stream, muddy, heavily shaded
(3) Pong Dueat Hotspring Stream, Chiang Mai Province	N 16°06' E 099°43'	711	Permanent, clear cascading stream, boulders, and mature trees along the riverbank

temperature was high, and streams had increased silt. In the cool-dry season, air temperature decreased sharply and there was no precipitation.

We followed the dissection methods provided by Lichtwardt *et al.* (2001). Black fly larvae were dissected on a drop of water under the dissecting microscope. Larvae were cut anteriorly, near the head, and optionally the last segment, near the anus, was severed with a razorblade or scalpel. The peritrophic matrix was removed from the anterior end of the midgut and cleared of gut contents by lifting it on a drop of water for several times, or by gently pressing the food bolus out with a mounted needle or fine forceps. The cleared peritrophic matrix was mounted on a microscope slide and the gut fungi examined using a compound microscope. The living fungi were photographed live (Nikon coolpix 4500) or drawn after infiltration with lactophenol-cotton blue (using a Lucida microscope). Fungal identifications were made with the Lucid keys available at the University of Kansas Trichomycetes Website (www.nhm.ku.edu/fungi/) (Lichtwardt, 2004). All microscope slide vouchers, made by first infiltrating lactophenol-cotton blue and than sealing the edge of the coverslip with clear fingernail polish, are deposited at Mae Fah Luang University Herbarium.

The ratio of black fly larvae colonized by *H. melusinae* compared to the total number collected was calculated as prevalence. The actual number of thalli present in the peritrophic matrix was calculated as the level of colonization. We follow the methods provided by Beard and Adler (2002), and use a scale due to the difficulties of counting the large number of thalli, the branching thalli (for the Legeriomycetaceae) and the three dimensional nature of the gut, with some adjustments: 0, indicating no thalli; 1, indicating fewer than ten thalli; 2, indicating fewer than 20 thalli; and 3 indicating more than 20 thalli.

To determine whether the prevalence and level of colonization by *H. melusinae* varied by season or water temperature, analysis of variance (ANOVA) was performed. Whenever variation was detected, data was then analyzed by regression analysis to determine whether season or water temperature gave any significant effect on the prevalence and level of colonization of *H. melusinae*. All data were transformed to arcsin prior to analysis.

RESULTS AND DISCUSSION

Over the eight months of the study period, 86% (CI = 82-88%) of the 1260 black fly larvae examined contained *H. melusinae* with Site 3 having the highest mean prevalence and level of colonization. This was not surprising, perhaps, as this site always had clear water, even following heavy rains. Clear water is assumed to be an important condition that determines the density of aquatic insect populations as the abrasive action of suspended silt results in high insect mortality (Elzinga, 2000). Host density itself is another factor that regulates infestations of the gut fungi, in addition to temperature and suspended solids (Taylor *et al*., 1996).

Prevalence of *H. melusinae*. The prevalence of *H. melusinae* varied seasonally at all sites except Site 3 ($P > 0.05$). The prevalence was highest during the cool-dry season, February (> 95%) and reached 100% in Site 3. During the hot-dry season, April to June (72-89%) and hot-wet season, July to October (80-92%), the prevalence was lower (Table 2). Prevalence was positively related to season (S) at all sites (Site 1, $Y = 0.770+0.156S$, $F = 4.73$, $R^2 = 0.11$, $P = 0.036$; Site 2, $Y = 0.581+0.273S$, $F = 14.85$, $R^2 = 0.27$, $P = 0.000$; Site 3, $Y = 0.936+0.209S$, $F = 8.95$, $R^2 = 0.18$, $P = 0.005$). Prevalence of *H. melusinae* in black fly larvae collected from South Carolina was also reported to be seasonal at one of three sites sampled, being lowest during the winter (67%) versus the other seasons (96-100%) (Beard and Adler, 2002). However, regression analysis of prevalence and season was not included in their analysis. We also found that the prevalence of *H. melusinae* in black fly larvae varied with water temperature ($P < 0.05$) (Table 3). There was a tendency for colder waters to have both higher prevalence and higher levels of colonization. Prevalence of *H. melusinae* was inversely related to water temperature (T) with values of tightness of this relationship as well: Site 1, $Y = 2.317-0.059T$, $F = 6.21$, $R^2 = 0.13$, $P = 0.017$; Site 2, $Y = 3.469-0.111T$, $F = 19.15$, $R^2 = 0.32$, $P = 0.000$; Site 3, $Y = 2.263-0.043T$, $F = 2.86$, $R^2 = 0.07$, $P = 0.099$.

Level of colonization by *H. melusinae*.

Level of colonization by *H. melusinae* varied seasonally ($P < 0.01$) (Table 2). Regression analysis using the variable of season (S) produced significant regression at all sites except Site 3 ($P > 0.1$) (Site 1, $Y = 0.713+0.230S$, $F = 6.72$, $R^2 = 0.04$, $P = 0.000$; Site 2, $Y = 1.347+0.147S$, $F = 3.43$, $R^2 = 0.01$,

Table 2. Seasonal prevalence and level of colonization (LC) (mean of proportional sample prevalences ± SE) of *Harpella melusinae* at three study sites in Northern Thailand, April to November 2008.

Site	*Season*	*Prevalence*	*LC*
Site 1	Hot-dry	0.81 ± 0.11 [a]	1.02 ± 0.05 [a]
	Hot-wet	0.80 ± 0.04 [a]	1.10 ± 0.04 [a]
	Cool-dry	0.96 ± 0.09 [b]	1.50 ± 0.09 [b]
Site 2	Hot-dry	0.72 ± 0.06 [a]	0.98 ± 0.06 [a]
	Hot-wet	0.85 ± 0.06 [b]	1.73 ± 0.04 [b]
	Cool-dry	0.98 ± 0.07 [c]	2.55 ± 0.09 [c]
Site 3	Hot-dry	0.89 ± 0.09 [a]	1.16 ± 0.06 [a]
	Hot-wet	0.92 ± 0.06 [ab]	1.86 ± 0.05 [b]
	Cool-dry	1.00 ± 0.00 [b]	2.76 ± 0.07 [c]

Notes. Means within sites followed by a different letter are significantly different ($P < 0.01$).

Table 3. Prevalence and level of colonization (LC) (mean of proportional sample prevalences ± SE) of *H. melusinae* in each recorded water temperature at three study sites in Northern Thailand, April to November 2008.

Site	*T (°C)*	*Prevalence of H. melusinae*	*LC of H. melusinae*
Site 1	19	0.90 ± 0.10 [b]	1.28 ± 0.06 [a]
	21	0.82 ± 0.05 [ab]	1.19 ± 0.06 [a]
	22	0.70 ± 0.08 [a]	0.70 ± 0.08 [b]
	24	0.80 ± 0.07 [ab]	1.03 ± 0.05 [a]
Site 2	19	0.93 ± 0.10 [b]	2.08 ± 0.11 [b]
	20	1.00 ± 0.00 [a]	2.60 ± 0.08 [a]
	21	0.83 ± 0.11 [cd]	1.13 ± 0.14 [d]
	22	0.73 ± 0.03 [d]	1.22 ± 0.08 [d]
	23	0.70 ± 0.06 [d]	1.07 ± 0.11 [d]
	24	0.87 ± 0.10 [bc]	1.67 ± 0.12 [c]
Site 3	19	0.91 ± 0.13 [ab]	1.78 ± 0.13 [b]
	20	1.00 ± 0.00 [a]	2.22 ± 0.11 [a]
	21	0.99 ± 0.05 [a]	1.91 ± 0.09 [ab]
	22	0.83 ± 0.12 [b]	1.36 ± 0.11 [bc]
	24	0.91 ± 0.11 [ab]	1.75 ± 0.09 [bc]
	25	0.90 ± 0.00 [b]	1.80 ± 0.13 [c]

Notes. Means within sites followed by a different letter are significantly different ($P < 0.05$ for prevalence and $P < 0.01$ for level of colonization).

$P = 0.065$). A significant difference was not detected at Site 3 ($P > 0.05$) although it had the highest recorded level of colonization by *H. melusinae* in the cool-dry season (2.76). In contrast with our results, Beard and Adler (2002) did not find that the level of colonization of *H. melusinae* varied by season, although their highest level of colonization was in autumn (September to November). They indicated, however, that a relationship might have been detected if larger sample sizes had been available (Beard and Alder, 2002). Their work was also conducted in a temperate region (about N 34^{o} and E 082^{o}), whereas our result represents the first ecological study of the gut fungi from the tropics. We also found that the level of colonization by *H. melusinae* varied with water temperature ($P < 0.01$) (Table 3). Level of colonization by *H. melusinae* was related to water temperature (T) with values for the tightness of this relationship as follows: Site 1, $Y = 2.295\text{-}0.054T$, $F = 8.77$, $R^2 = 0.02$, $P = 0.003$; Site 2, $Y = 5.644\text{-}0.188T$, $F = 44.78$, $R^2 = 0.10$, $P = 0.000$; Site 3, $Y = 3.330\text{-}0.071T$, $F = 8.16$, $R^2 = 0.02$, $P = 0.000$.

Both prevalence and level of colonization were highest in cool-dry season, and there was a tendency for both parameters to be highest in cooler water temperatures. The lowest recorded average water temperature (19^{o}C) was the cool-dry season as compared with the other two seasons. Hynes (1970) proposed that the constancy of the photoperiod plays a role in governing seasonality of univoltine aquatic insects. This might result in trichomycetes being directly or indirectly affected by the photoperiod. In our study, the highest prevalence and level of colonization by *H. melusinae* were at Site 3. This site, however, was neither the most open area (Site 1) nor the most shaded area (Site 2). It is unclear whether the infection rate of trichomycetes correlates with habitat or canopy cover and concomitant presence of shade. We note that Site 3, which had average canopy cover, it was easier to locate 30 black fly larvae, but it was difficult to locate this number of insect larvae at both the opened and closed sites. Thus, factors impacting host population density at collection sites may well account for some of the difference observed and are worth incidence of black fly larvae.

The water temperatures during our study were relatively warm, ranging from 19-25°C. Some species of trichomycetes are known to be restricted to certain ranges of water temperature. *Harpella tica*, for example, was found in streams in Misiones Province (Argentina) where the stream temperature was higher (15-24°C), whereas in Tierra del Fuego Province where the water temperature was lower (9-15°C) *H. tica* was not found (Lopez Lastra *et al.*, 2005). *Harpella meridianalis*, dissected from black fly larvae collected in Argentina, in contrast, was restricted to colder streams (13.5-20°C) and *H. tica* was found in warmer streams (17-24°C) (Lichtwardt *et al.*, 2000). Lopez Lastra *et al.* (2005) also found that the diversity of trichomycetes was higher in colder streams (seven harpellid taxa) as compared to warmer streams (five harpellid taxa). *H. melusinae* is one of the most widespread trichomycetes in the northern hemisphere (Lichtwardt *et al.*, 2004) has not been reported to be restricted to certain water temperatures, although its prevalence has been reported to be seasonal (Beard and Adler, 2002) and has been reported from Thailand (Takaoka and Adler, 1997).

We have shown that season and water temperature influence the infestation of trichomycetes in black fly larvae. As noted by Beard *et al.* (2003), the relationship of trichomycetes can be viewed as a triangular relationship involving the fungus, host (black fly) and environment (streams). This study, however, only represents one taxon of trichomycetes known from Thailand, and only measured two independent variables; season and water temperature. Other stream conditions such as canopy cover, conductivity, depth, discharge, dissolved oxygen, dominant stream-bed particle size, pH, riparian vegetation, seston, velocity and width have been reported to be a useful predictors of the occurence of trichomycetes (McCreadie and Adler, 1998). Further ecological studies, including other taxa, hosts, habitats and other parameters are warranted in Thailand and globally to understand how environmental factors influence fungus-host relationships and this interesting symbiosis.

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