

Co-Occurrence of *Haematoloechus complexus* and *Rhabdias joaquinensis* in the Plains Leopard Frog from Nebraska

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ABSTRACT: Species of *Haematoloechus* and *Rhabdias* both are ubiquitous lung parasites of frogs, yet surprisingly little research has been conducted on the interactions between these worms. In a small Nebraska stream, 256 *Rhabdias joaquinensis* and 225 *Haematoloechus complexus* were found in the lungs of *Lithobates* (=*Rana*) *blairi*. Thirty-six of the 44 (82%) frogs were co-infected with *H. complexus* and *R. joaquinensis* whereas 5 (11%) frogs were infected with only 1 species and 3 (7%) frogs were uninfected. Tests for association between *H. complexus* and *R. joaquinensis* found a significant positive relationship between the 2 parasites. Significant positive relationships in intensity of infection were found when both lungs were combined but not when lungs were considered individually. Tests for association between *H. complexus* and *R. joaquinensis* found no relationship between the worms in the anterior and posterior portions of the lungs. These data suggest that *H. complexus* and *R. joaquinensis* do not competitively exclude each other from the lungs of *L. blairi* in southeastern Nebraska.

Since Holmes' discovery (1961, 1962a, 1962b) that *Hymenolepis diminuta* and *Moniliformis dubius* (=*moniliformis*) displayed apparent competitive interactions in rats but not in hamsters, parasitologists have debated whether parasite communities are interactive or isolationist (reviewed by Janovy, 2002). This debate continues today primarily because (1) appropriate experimental systems using multiple parasite species are rare (Holland, 1987; Janovy, 2002; Poulin, 2007), (2) a multiplicity of factors structure natural parasite communities (Janovy, 2002), and (3) predictions of co-occurrence in nature rely on imperfect presence-absence matrices and models to infer interactions (see Gotelli and McCabe, 2002; Poulin and Luque, 2003). Throughout this debate, in general, positive interactions are often attributed to ecological conditions such as favorable transmission conditions for 2 or more species in space and time (Janovy, 2002), whereas negative interactions are commonly reported as interspecific competition, especially where 2 or more parasite species could co-occur in the same niche (Poulin, 2007).

Most examples of interspecific interactions have been found in invertebrate intermediate hosts (Sousa, 1992; Kuris and Lafferty, 1994; Janovy et al., 1997; Poulin and Rohde, 1997; Carney and Dick, 2000; Barger and Esch, 2001; Paul et al., 2002; Leung and Poulin, 2011), whereas unambiguous examples of interspecific interactions are rare within vertebrate final hosts (but see Holmes, 1961; Holland, 1984, 1987; Patrick, 1991). Poulin (2007) proposed that strong asymmetrical interactions between parasites in vertebrate hosts are rare and likely only occur under severe resource limitations, such as reported by Jackson et al. (1998), who found interspecific competition in a space-limited habitat within the monogenean genus *Protopolyystoma*. However, Poulin's (2007) resource limitation hypothesis is poorly tested, especially for parasites that reside outside of the gastrointestinal tract, due primarily to a lack of good vertebrate systems that have co-occurring parasites amenable to experimental manipulation and with life histories of both hosts and parasites elucidated.

Dare and Forbes (2009) reported apparent competitive exclusion between *Haematoloechus* spp. and *Rhabdias* spp. within the lungs of northern leopard frogs (*Lithobates* [=*Rana*] *pipiens*) and wood frogs (*Lithobates* [=*Rana*] *sylvaticus*). While that study was the first to quantitatively and specifically address competitive interactions between these lung parasites, it was not the first to note the potential for competition between species of *Haematoloechus* and *Rhabdias*. Mazurmovich (1957) observed that *Haematoloechus* spp. and *Rhabdias bufo* rarely occurred together in the same frog. Holmes (1973) thought that these 2 lung parasites must competitively exclude each other because of this low co-occurrence in nature. Kennedy (1975) pointed out that the low co-occurrence of the 2 worms only suggests, but does not prove, competition. The current study contributes to this on-going discussion; specifically, we sampled *Haematoloechus complexus* and *Rhabdias joaquinensis* from plains leopard frogs (*Lithobates* [=*Rana*] *blairi*) to determine if these parasites co-occur in nature. In addition, we discuss the feasibility of using the frog–*Haematoloechus*–*Rhabdias* system as a new vertebrate model for conducting experimental infections to test parasite interaction dynamics.

In October 2008 a total of 44 plains leopard frogs were collected from Elk Creek (40°53.145'N, 96°50.048'W), Lancaster County, Nebraska. Elk Creek is a small stream that meanders through open fields (active and fallow agriculture) and small woodlands. Live frogs were collected from an area with a stream width of 2–3 m and steep, muddy banks of 3–5 m. The creek had an overstory canopy through much of the collection site, and numerous larval and adult odonates were noted at this location. Non-breeding adult frogs were collected immediately prior to winter hibernation by partially submerging 6 minnow traps (42-cm, Frabill Deluxe Black Minnow Traps, Memphis Net, Memphis, Tennessee) in the stream and also by using opportunistic hand captures. Snout-to-vent length was measured in centimeters for each frog. Host sex was determined by gonad examination.

During necropsy, the lungs were immediately removed and teased apart with forceps to locate lung parasites. The location of worms, anterior (near trachea) or posterior portion of each lung, was simultaneously noted for the last 17 frogs collected in an attempt to quantify whether worms segregate in the lungs (see Dare and Forbes, 2009). It is noteworthy to acknowledge that Dare and Forbes (2009) collected road-kill frogs, which may have biased their results because both lungworms and lung flukes wander following host death (M. G. Bolek and G. J. Langford, pers. obs.). The initial necropsy was followed by a complete exploration of all organs and body cavities for parasites. All nematodes were removed and fixed in 70% ethanol, cleared in glycerol, and identified on temporary mounts according to Kuzmin et al. (2003). All flukes were placed in aged tap-water for 24 hr and allowed to release eggs, then stained with acetocarmine, dehydrated in a graded ethanol series, cleared in xylene, and mounted in Canada balsam. Trematodes were identified according to Bolek and Janovy (2007a, 2007b) and references within.

Prevalence, mean intensity (MI), and mean abundance of lungworms (*R. joaquinensis*) and lung flukes (*H. complexus*) are reported in accordance with Bush et al. (1997). The chi-square test for independence was calculated to compare differences in prevalence among worms whereas a Mann–Whitney *U*-test was used to compare MI between worms. A Kolmogorov–Smirnoff 1-sample test showed that a Fisher exact

test was required to test for association, i.e., presence or absence, between lungworms and lung flukes. Lastly, to test for relationships between intensity of infection, Spearman's rank correlation was performed to compare the rates of infection of *H. complexus* and *R. joquinensis*. All tests were performed separately for left lung ($n = 44$), right lung ($n = 44$), both lungs ($n = 44$), anterior portion of the lungs ($n = 17$), and posterior portion of the lungs ($n = 17$). Voucher specimens of *H. complexus* (HWML 49759) and *R. joquinensis* (HWML 67158) from *L. blairi* were deposited in the H. W. Manter Parasitology Collection, University of Nebraska, Lincoln, Nebraska.

In total, 256 *R. joquinensis* and 225 *H. complexus* adults were found in the lungs of *L. blairi*. An additional 26 juvenile *Rhabdias* spp. were collected from the body cavity of the frogs. Thirty-six of the 44 (82%) frogs sampled were co-infected with *H. complexus* and *R. joquinensis* whereas 5 (11%) frogs were infected with only 1 species and 3 (7%) frogs were uninfected. The prevalence of *H. complexus* (91%) was higher than that of *R. joquinensis* (84%), but the $MI \pm SD$ of *R. joquinensis* (6.9 ± 4.1) was higher than that of *H. complexus* (5.5 ± 3.3); neither difference was significant ($P > 0.05$). Tests for association between *H. complexus* and *R. joquinensis* found a significant positive relationship between the parasites when both lungs were considered together ($P = 0.04$) but not when lungs were considered individually (left lung $P = 0.13$, right lung $P = 0.091$). Significant positive relationships in intensity of infection were found between *H. complexus* and *R. joquinensis* in the left lung ($r = 0.255$, $P = 0.048$, $df = 42$) and when both lungs were combined ($r = 0.269$, $P = 0.028$, $df = 42$), whereas worms from the right lung did not show a positive relationship ($r = 0.234$, $P = 0.059$, $df = 42$). Tests for association between *H. complexus* and *R. joquinensis* found no relationship between the worms in the anterior ($P = 0.69$) or posterior ($P = 0.23$) portions of the lungs. A significant positive relationship in intensity of infection was found between *H. complexus* and *R. joquinensis* in the posterior portion of the lungs ($r = 0.435$, $P = 0.04$, $df = 15$), whereas a significant negative relationship was found in the anterior portion of the lungs ($r = -0.144$, $P = 0.28$, $df = 15$).

Our study found *R. joquinensis* and *H. complexus* occurring together in significantly more frogs than either species occurred alone, which suggests that these worms are either isolationist or positively interacting but not competitively excluding each other from the lungs of plains leopard frogs. We also found that co-occurring worms did not segregate into different portions of the lungs. Our findings are contrary to the suggestions of Dare and Forbes (2009) that *Haematoloechus* spp. and *Rhabdias* spp. undergo competitive exclusion and, in the rare chance when they co-occur, that the worms practice site segregation. However, when taken together, our studies concur with Holmes (1961, 1962a, 1962b), who found that each host-parasite combination may result in different competitive outcomes for co-occurring parasites.

Dare and Forbes (2009) suggested that lung damage caused by *Haematoloechus* spp. inhibits *Rhabdias* spp. feeding, which results in spatial partitioning within the lung and, thus, the low number of concurrent infections found in their study. Our findings do not reject this hypothesis because we found no fibrous cysts and only mild disturbance to the pleural lining associated with *H. complexus* infections. However, because Dare and Forbes (2009) did not identify any of the lung flukes in their study to species, it is difficult to speculate if these observed differences are due to specific host-parasite interactions or differences in pathology caused by different frog lung fluke species. The formation of fibrous cysts by lung flukes have been found in 2 studies (Shields, 1987; Dare and Forbes, 2009) whereas other studies have failed to find cysts or substantial pathology, even during high-intensity infections (Krull, 1931; Hsu et al., 2004; this study). Consequently, it would be interesting to determine if the formation of fibrous cysts by different frog lung fluke species exists and, if so, are the fibrous cysts always associated with a reduced incidence of dual infection.

Alternatively, an ecological explanation for the contrary results found in our study and in Dare and Forbes (2009) seems reasonable; specifically, their collection times in both the spring and breeding season were not

conducive to collecting frogs with co-occurring infections (discussed by Dare and Forbes, 2009). Collecting frogs in the spring provides a census of the flukes and nematodes that survived overwintering in the host before new worms are recruited. While Woodhams et al. (2000) found that *Rhabdias ranae* in *Lithobates sylvaticus* (a freeze-tolerant frog) had low overwintering mortality, nothing is known about overwintering in lung flukes; thus, we suggest that early spring is not an ideal time to test for associations between these worms. Likewise, breeding anurans are not ideal to test for competitive interactions because frogs lose lung fluke infections during the spring (Bolek and Coggins, 2000) and lungworms begin infecting new hosts during the breeding season (Baker, 1979).

We collected frogs for our study in October, based on parasite seasonal dynamics and host physiology, because the fall allows for the best opportunity to observe co-occurrence in *Haematoloechus* spp. and *Rhabdias* spp. In most species of lung flukes and lungworms annual patterns of recruitment show development of new worm cohorts in mid-summer, with some additional recruitment through the rest of summer, which culminates in an accumulation of primarily adult worms in the lungs during the fall (Baker, 1979; Bolek and Coggins, 2000, 2001). Given the high prevalence and intensity of worms in anurans during the fall season, it seems likely this period of potential high association should shape the competitive interactions, if any, between *Haematoloechus* spp. and *Rhabdias* spp.

We propose that our study found frequently co-occurring *H. complexus* and *R. joquinensis* for 2 equally important ecological reasons. First, as discussed above, our sampling period coincided with the greatest opportunity for lung parasites to overlap based on seasonal transmission dynamics. Second, our collection site was conducive to transmission of both parasites. For co-occurrence to be likely, a sampling location must provide suitable and equal conditions for transmission of both species (Janovy, 2002). Locating an ideal habitat that favors equal transmission for lung flukes and lungworms is no trivial task because each life cycle is dependent on a multitude of ecological conditions and evolutionary constraints. Lung flukes require a snail first intermediate host, followed by an invertebrate second intermediate host (typically an odonate), and a final anuran host (Bolek and Janovy, 2007a, 2007b) whereas lungworms must survive, mate, and produce infective offspring in a specific soil microhabitat before encountering an anuran host (Langford and Janovy, 2009, 2013). In nature, these 2 worms often are found infecting the same populations of frogs in North America (Goldberg et al., 1998; Bolek and Coggins, 2000, 2001; Dare and Forbes, 2009; Cabrera-Guzman et al., 2010), but this does not mean that transmission dynamics are equal for each species of lung parasite over space and time (Janovy, 2002). Thus, when ecological conditions provide transmission advantages for 1 species over another, it may falsely suggest competition (Poulin, 2007).

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