IDENTIFICATION AND MORPHOLOGICAL VARIATION OF AN INVASIVE PARASITE IN INTRODUCED AND NATIVE LIZARDS

A Thesis

by

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ABSTRACT

Exotic species can threaten biodiversity by introducing parasites to native hosts. Thus, it is critical to identify if the same parasite species is infecting both native and exotic hosts. Developmental or environmentally induced variation in morphology, however, may complicate identification. Geckos are one of the most successful invasive families of vertebrates and are known to host lung parasites, pentastomids of the genus *Raillietiella*. Raillietiellids have a cosmopolitan distribution, which in part, may have been facilitated by the introductions of their hosts. Indeed, *Raillietiella frenatus*, a Southeast Asian parasite, has been reported in Texas (TX) from the exotic Mediterranean gecko, *Hemidactylus turcicus*. Here we report on the recent introduction (between 1998 and 2008) of a *Raillietiella sp*. into an established population of *H. turcicus* in Louisiana (LA). More critically, we found infections in native green anoles, a new host record for pentastomes. Upon sequencing 604 bp of the pentastome's cytochrome *c* oxidase gene, we observed identical sequences from parasites of anoles and geckos. In fact, there was no sequence variation between published sequences of *R. frenatus* from geckos and cane toads in Australia. Interestingly, we found that traditional taxonomic analyses based on hook dimensions would have led to the false conclusion of two pentastome species within *H. turcicus*. But, as in Kelehear et al. (2011), when pentastome body size is accounted for the distinction between the two groups disappears. These results along with prior moulting studies on *R. frenatus* suggest hook size varies ontogenetically. Nonetheless, even after accounting for pentastome body size, hook

dimensions differ significantly between host species. This result suggests these traits may be plastic as a result of host environment, but quantitative genetic experiments will be needed to disentangle phenotypic plasticity from genetic variation.

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NOMENCLATURE

ANCA	*Anolis carolinensis*
CO1	Cytochrome c oxidase subunit 1
HEFR	*Hemidactylus frenatus*
HETU	*Hemidactylus turcicus*
PCR	Polymerase chain reaction
RHMA	*Rhinella marina*
SVL	Snout-vent-length

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INTRODUCTION

In the continental United States, approximately 42% of native species listed as endangered or threatened are as a result of direct or indirect consequence of invasive species (Pimentel 2011). Indeed, invasive species cost the United States, the British Isles, Australia, Europe, South Africa, India, and Brazil, combined, as much as $300 billion per year in damages and control (Pimentel 2011). The effects of invasive species are often studied for their direct ecosystem impacts on nutrient cycling or habitat structure (Simberloff 2011). However, there could also be indirect impacts where the effects may be more subtle, but not necessarily inconsequential in driving changes in the native community (Simberloff 2011). One possible indirect effect can arise from co-invasive parasites, which are cases where an exotic host brings an exotic parasite and the parasite subsequently infects native hosts (Lymbery et al. 2014). This invasion dynamic is referred to as spillover (Kelly et al. 2009). The conservation concern in spillover is that an invasive parasite represents a novel infection that could reduce the fitness of native hosts and hence, negatively impact the native community.

A critical initial step in studying the potential impacts of parasites in species invasions is to determine if there is a shared parasite between native and alien hosts and if so, determine if the parasite is native or alien. This initial step in itself requires correct identification of the parasite. Unfortunately, determining whether a parasite is native or introduced can be problematic and thus, lead to cryptogenic status, i.e., alien or native status cannot be ascertained (Carlton 1996). Anthropogenic transport of species prior to

taxonomic surveys was recognized early as one factor leading to cryptogenic status (Carlton 1996). With many parasite species, an additional issue leading to cryptogenic status is taxonomic ambiguity (Lymbery et al. 2014), which may result from a scarcity of morphological traits, investigator induced phenotypic variation during parasite fixation, extensive underlying genetic variation of phenotypes, or environmental-induced (especially host-induced) phenotypic variation (Criscione and Font 2001; Perkins et al. 2011). Clarifying cryptogenic species as alien or native is important for understanding several aspects of biological invasions such as knowledge of invasion corridors, susceptibilities of communities to invasions, and frequencies of introductions and successful invasions (Carlton 1996). Moreover, establishing if a parasite is native or exotic is important for differentiating between spillover and spillback effects. The latter refers to when an exotic host acquires a native parasite and subsequently amplifies the parasite population in native hosts (Kelly et al. 2009). Both spillover and spillback may manifest as an apparent competition dynamic between a native and alien host, but the underlying cause is different. Spillover results from the introduction of a novel enemy whereas spillback is the amplification of a preexisting enemy in the native host.

The subject of our study is a pentastome parasite that infects the lungs of the invasive Mediterranean gecko, *Hemidactylus turcicus*, in the southern U.S.A. Prior to our study there were reports of two species of pentastomes infecting *H. turcicus* in the continental U.S.A.: *Raillietiella frenatus* [sic] in Hidalgo, Texas (Pence and Selcer 1988) and *Raillietiella teagueselfi* newly described in Houston, Texas (Riley et al. 1988). Recently, Kelehear et al. (2011) drew attention to ambiguities in interpreting key

taxonomic traits of raillietiellids. Specifically, Kelehear et al. (2011) demonstrated how anterior and posterior hook measurements were correlated with pentastome body size; an indication that hook size covaries with development. They also found that hook measures varied significantly with pentastome sex and host species effects after controlling for body size. With confirmation from DNA sequence data, Kelehear et al. (2011) concluded that the same pentastome species infected two exotic host species, *Hemidactylus frenatus* (Asian house gecko) and *Rhinella marina* (cane toad), and the native tree frog *Litoria caerulea* in Australia (Kelehear et al. 2011). They concluded the raillietiellid species infecting these three hosts was *R. frenatus* [sic] (correct spelling should be *R. frenata*, discussed in Poore (2012)). However, Poore (2012) lists *R. frenata* as a junior synonym to *R. indica*. Therefore, we refer to the pentastome as *R. indica* henceforth.

Here, we report that *R. indica* (confirmed with sequence data) infects both the invasive Mediterranean gecko and the native green anole, *Anolis carolinensis*, in the southern U.S.A. As our study system was distinct (both in terms of location and host species) from that of Kelehear et al. (2011), we took this as an opportunity to provide an independent assessment, though differing in 2 key aspects of the analysis, as to whether key pentatstome taxonomic traits differed according to various host and parasite characteristics. First, we tested if the key morphometric hook measurements were themselves highly correlated and thus, would essentially represent a single trait. Second, using traditional raillietiellid morphometric analyses along with prior life cycle work (Ali and Riley 1983), we *a priori* designated individual pentastomes to distinct instar

stages. Taking these aspects (instar stage and correlated hook variables) into account, we were able to directly test the role of, and subsequently control for development, as proposed by the results of Kelehear et al. (2011), on a composite hook measurement. In addition, a large sample size of pentastomes from *H. turcicus* enabled us to test if new factors such as host body size, host sex, and parasite density-dependence influenced hook morphology. Lastly, we combined data from our study and Kelehear et al. (2011) to test for broader host species effects on pentastome morphology.

METHODS

Sampling

Geckos were captured by hand from locations in Metairie, LA; Ingleside, TX; and Port Aransas, TX at various times from 2011 through 2013. Anoles were captured from a location in Metairie, LA in 2012. Details on the sampling locations are given in Caballero et al. (2015) and Criscione and Font (2001). Data recorded from lizard hosts included weight, total length, snout-vent-length (SVL), and sex. The research protocols i.e., capture, handling, and sacrifice (decapitation followed by pithing) prior to dissection in this study were approved by the Institutional Animal Care and Use Committee at Texas A&M University (AUP: 2009-23; 2012–023). Live pentastomes were recovered from the lungs and placed in 0.7% saline solution and then placed at 4 C for a few minutes to relax them. Next, 90 C water was poured on the pentastomes to heat-kill and fix. Pentastomes were then stored in 70% ethanol at 4 C.

DNA Extraction and Amplification

We sequenced 24 individuals: 16 from *H. turcicus* (8 from Metairie, LA; 4 from Port Aransas, TX; and 4 from Ingleside, TX) and 8 from *A. carolinensis* from Metairie, LA. For DNA extractions, a 1 mm^3 piece of tissue from an individual worm was placed into 200 µL of 5% chelex containing 0.2 mg/mL of proteinase K. Samples were incubated at 56 C for 2 hours then boiled at 100 C for 8 minutes. As in Kelehear et al. (2011), we amplified the cytochrome *c* oxidase subunit 1 (CO1) of the mitochondria

with the primer pair LCO1490 (5'-ggtcaacaaatcataaagatattgg-3')/HCO2189 (5'-taaacttcagggtgaccaaaaaatca-3') (Folmer et al. 1994). PCR amplification was performed with a hot start of 95 C for 3 minutes, followed by 36 cycles of 94 C for 45 s, 55 C for 30 s, and 72 C for 45 s, followed by a final extension of 72 C for 7 minutes. PCR products were purified with the Ultra Clean PCR clean-up Kit (MO BIO Laboratories, Inc., Solana Beach, CA) and then sent to the DNA Analysis Facility on Science Hill at Yale University (New Haven, CT) for sequencing.

Morphological Measurements

Traditional pentastome identification relies on measurements of the two pairs of anterior and posterior hooks surrounding the buccal cavity on the anterior end of the worm (e.g., see Figure 4 of Riley (1986)). Anterior and posterior hook measures, body length, shape and size of the male copulatory spicules, and number of annuli have been used as the primary morphological traits for species identification among raillietiellid pentastomes. However, Riley (1986) states "annulus counts are sometimes too close and overlapping to be of diagnostic value" especially among raillietiellid pentastomes (see Kelehear et al. (2011) for similar difficulties in using annuli counts). Therefore, we did not consider annuli counts in our study.

All measurements were based on microscopy photographs of whole specimens or morphological traits. The pictures were analyzed with the segmented-line and straight line tools in ImageJ software (Schneider et al. 2012) where photos of a micrometer scale taken at corresponding magnifications were used to calibrate pixels/mm per each

magnification setting. Full body length measurements were done by taking pictures of the worms under a dissection microscope set to the highest magnification that allowed the entire worm to be visible. To take close-up photos, the anterior end of pentastomes was removed and soaked in a lactophenol solution for 10 minutes to clear the tissue before the sample was mounted on a slide with glycerol under a cover slip. Body length measurements were taken with the segmented-line tool by using enough points to keep the line in the middle of the worm. Anterior and posterior hook measurements followed that of Ali et al. (1981) (see their figure 5). Briefly, hook length measurements are split into two separate, straight-line measurements: blade length (AB) and shank length (BC). AB measurements are taken by dragging the straight line tool from the tip of the hook's barb (point A) to the small projection formed where the hollow back closes (point B). BC measurements were taken by dragging the straight line tool from point B to the bottom of the hook's flared base (point C). Kelehear et al. (2011) introduced the new measure of hook bluntness, which is measured by taking the area at the tip of the hook. The area is estimated by outlining the edges of the hook tip from point A up to 20µm along the hook shaft. In males, copulatory spicule length measures were taken by placing line points in the middle of the spicule from the base to the tip of the hook and width was taken at the widest part of the base. As the traits above are all paired (e.g., left and right anterior hook), we used the average measurement for all pairs of anterior hooks, posterior hooks, and spicules.

We make special note that in these pentastomes the anterior hooks are smaller and sharper than the posterior hooks and as such we found these were much more

difficult to measure accurately, especially from a single focal plane photograph. Hence, we *a priori* expect anterior measures to contain more error. Accordingly, we had more missing data for the anterior hook measures due to difficulty in orienting the smaller hooks on the slides. These issues are compounded in males because males are smaller, and hence have smaller structures, than females.

Analyses

Data in Kelehear et al. (2011) from *Rhinella marina* (cane toad) and *Hemidactylus frenatus* (Asian house gecko) host species were incorporated where possible in order to look for more global patterns and draw more robust and generalized conclusions. For simplicity, we abbreviate the host names in the presentations of the statistical analyses: HETU, *H. turcicus*; ANCA, *A. carolinensis*; RHMA, *R. marina*; and HEFR, *H. frenatus*. Also, in the tests below, we analyzed male and female morphometrics separately, as sexual dimorphism in these organisms has been pointed out by previous studies (Ali and Riley 1983; Kelehear et al. 2011).

Trait relationships: Prior studies have treated the different hook measures as independent traits, so our first objective was to test for possible relationships among the 6 hook measurements used in our study (AB and BC of anterior and posterior hooks and the anterior and posterior hook bluntness areas). With both the female and male data sets, we conducted a Principle Components Analysis (PCA) to examine for the latent relationships among the hook measures. PCA was conducted with the psych package v1.7.2 in R v3.3.3 and (Revelle 2017; R Core Team 2017) using Varimax rotation.

Factor loadings of >|0.5| were considered significant considering our sample sizes (Hair et al. 1998). The female PCA data set consisted of *n* = 196 total pentastomes (*n* = 152 from 34 HETU, *n* = 23 from 4 ANCA, *n* = 15 from 5 RHMA, and *n* = 6 from 4 HEFR). The male PCA data set consisted of *n* = 109 total pentastomes (*n* = 91 from 28 HETU, *n* = 3 from 2 ANCA, *n* = 7 from 5 RHMA, and *n* = 8 from 4 HEFR). A simple linear model was used to test for a correlation between spicule length and width in male worms. The data set for the latter was the same used in the PCA of hook measurements of male pentastomes.

Based on the results of the PCA, downstream analyses on hook measurements used a summated score of the AB and BC measures of the posterior hooks (justification given in Results). For simplicity, we refer to this summated score as 'hook size'.

2D plot of posterior hook AB and BC: Historically, a 2D plot of female posterior BC by AB hook measurements were used to view clusters, which in turn were used to delimit species. However, Kelehear et al. (2011) showed that in these 2D plots, discrete clusters disappeared after accounting for body size. They concluded that the clusters were likely driven by pentastome development. The latter was an important finding because body size would need to be accounted for in downstream analyses of hook measurements. To examine the generality of Kelehear et al. (2011) results we repeated their analysis on our ANCA and HETU samples. A 2D plot was constructed with raw values and then repeated with residuals of body size regressions.

At this point, we note that females of *R. indica* can be gravid in distinct instar developmental stages inside their final host (Ali and Riley 1983). Superimposing onto

our data and that of Kelehear et al. (2011) the instar specific hook measurements from Ali and Riley (1983) shows that the clusters in the 2D-plot of AB by BC posterior hook measurements correspond to the 7th, 8th, and 9th instar stages of *R. indica* (*R. frenatus* [sic]) (see Results). We mention this here because accounting for instar stage would provide a more explicit and discrete means of accounting for development (e.g., tests for stage-specific patterns) while also allowing us to account for body size as a separate variable. Thus, downstream analyses will categorize female individuals into instar stages based on cluster cutoffs (see Results). Males are mature only in a single instar stage (Ali and Riley 1983) and thus, are not expected to form clusters based on the 2D plot when examining worms from a single host species. Indeed, this is what we observed (data not shown; see also Fig. 5 in Kelehear et al. 2011); hence, males were not subdivided into instar stages.

Testing factors that could influence morphology: We tested whether two host factors (SVL and host sex) and a context specific factor (i.e., parasite density-dependence) were associated with morphological traits in order to ascertain the potential for environmental induced morphological variation. These tests were conducted separately for the 8th and 9th instar females, and males. Tests were conducted in the R packages lme4 v1.1.12 and lmerTest v2.0.36 (Bates et al. 2015; Kuznetsova et al. 2017) using linear mixed effect models. We first used pentastome body length as the dependent variable. Host sex, total intensity, and SVL of the host were the main effects. The random effects were sampling location and individual host nested within location. All 2-way interactions of main effects were tested; if non-significant, they were pooled.

Next, we used hook size as the dependent variable and again conducted tests separately for 8th and 9th instar females and males. Main and random effects were as given above, but we also included pentastome body length as a covariable main effect. The inclusion of body length in these models was to simply control for any additional growth differences that may occur independently of instar stage; hence, we did not test for interactions with pentastome body length. In males, we also repeated the same analyses above, but used either spicule length or width as dependent variables.

The female 8th instar data set consisted of n = 59 total pentastomes from 24 HETU, while the 9th instar dataset consisted of n = 110 total pentastomes from 22 HETU. The male data set consisted of n = 105 total pentastomes from 30 HETU.

Testing host species as a factor: For the analyses testing for a host species effect on female traits, only 9th instar female worms were used (n = 110 from 22 HETU, n = 21 from 4 ANCA, n = 4 from 3 HEFR, and n = 8 from 4 RHMA) as there were too few samples from some host species at the 8th instar category. We first tested for a host species effect on pentastome body size, where host species was the main effect and individual host ID was the random effect. To test for an effect on hook size, host species and pentastome body size were used as the main effects with host ID as the random effect. The same models as above were use in male worms, but with an additional test for an effect on spicule length (n = 105 from 30 HETU, n = 5 from 3 ANCA, n = 8 from 4 HEFR, and n = 10 from 5 RHMA). We note that we could not incorporate the variables of host sex, SVL or total intensity in the above analyses because sample sizes were too small from some host species or the information itself was not available for

HEFR and RHMA from the study Kelehear et al. (2011). As we did not find any consistent effects for host sex, SVL or total intensity within HETU samples alone (see Results), we do not believe the analyses of host species effects are unduly affected by the exclusion of these variables.

RESULTS

DNA and Sampling Data

Distributional data of pentastome infections at Ingleside and Port Aransas (combined) are given in Caballero et al. (2015). In short, in 2012 collections 36 out of 48 geckos were infected with a mean intensity of 10.14, and in 2013 collections 40 out of 70 geckos were infected with mean intensity of 9.45. Of the 88 geckos sampled from Metairie, LA in 2012, 52 were infected with a mean intensity of 7.21, ranging from 1 to 43 worms per host. Five of the 22 anoles sampled from Metairie, LA in 2012 were infected with a mean intensity of 6.6, ranging from 1 to 15 worms per host. We note that five of these anoles, one of which was infected, were actually collected from a house in River Ridge, LA, approximately 5.4 miles from the Metairie location.

The CO1 Genbank sequence (JF975594.1) of Kelehear et al. (2011) is 617 bases long. We note the last 13 bases on the 3' end match the reverse primer and thus, should not be considered. All 24 samples, which included 8 from anoles from LA, 16 from geckos (8 from TX and 8 from LA) matched 100% across the 604 bases with JF975594.1. Along with the genetic data, the club-shaped base of the male spicules (an important taxonomic trait; Riley 1986) matched between worms from green anoles and Mediterranean gecko hosts (Fig. 1; n = 105 pentastomes from geckos, n = 5 pentastomes from anoles examined) and importantly, to previous reports of *R. indica* (see Plate 2C in Ali and Riley 1983; Fig. 4 in Kelehear et al. 2011; Fig. 4, Barton and Riley 2004). Based on the above evidence, we identified the pentastomes in our study as *R. indica*.

Analyses

Trait relationships: The PCA results from both the female and male data sets show that 5 of the hook measurements load very highly and significantly onto a single factor and that area of the anterior hook tip loads by itself on a second factor (Table 1: A and B). Validation of the factors is evidenced by the congruence in the results of the two independent data sets (Hair et al. 1998). With the exception of anterior hook bluntness, these results indicate that the traditional hook measurements highly covary and as such, treating each as independent runs the risk of pseudoreplicating a single underlying trait. For this reason and the following reasons, we have chosen to focus our subsequent analyses on a summated score (i.e., average) of the AB and BC measures of the posterior hooks (i.e., hook size). First, the AB and BC posterior hook measures have traditionally been used in 2D plots to delimit species (Ali and Riley 1983; Ali et al. 1981; Riley 1986) and instar developmental stages (Ali and Riley 1983). Second, summated scales are an appropriate way to summarize correlated variables of the same trait, i.e., an aspect of hook size in these pentastomes, while also helping to reduce measurement error (Hair et al. 1998). Moreover, a summated score is comparable across studies whereas standardized factor scores are only comparable within data sets. Third, although the anterior hook bluntness could represent an independent trait, we also had the most difficulty in measuring this trait. Hence, its loading on a separate factor could also be an indicator of more measurement error in this variable. Indeed, as we noted in the methods, we had more missing data for anterior hooks in general. Fourth, the missing data is why we excluded the anterior hook AB and BC as we could include more

samples into our analyses. Lastly, although the posterior hook bluntness also loads highly onto the first factor (Table 1), we did not include it in the summated score as it is an area as opposed to linear measure. Simply put, a summated score of the posterior hook AB and BC measures should reflect overall hook size while reducing error.

Spicule length and width of males were correlated (F = 13.45, p = 0.00038, and r^2 = 0.1034). However, because of the low r^2, we analyzed spicule length and width separately.

2D plot of posterior hook AB and BC: Figure 2A shows the 2D plot of the female posterior AB and BC measures wherein 3 clusters are readily observed when looking at the collective samples from HETU and ANCA. However, when allometrically correcting for body length, as in Kelehear et al. (2011), distinction among the clusters disappears (Fig. 2B). This independent analysis is concordant with the result observed in Kelehear et al. (2011) and suggested that hook size is affected by development. Indeed, after superimposing data from the experimental infections of Ali and Riley (1983) onto our data and that of Kelehear et al. (2011), it was clearly evident that the clusters represented three female instar stages: 7, 8, and 9 (Fig. 3). These clusters and the data of Ali and Riley (1983) enabled us to delimit female samples into distinct instar stages, which in turn provided a more explicit control variable for development in subsequent analyses. We demarcated instar stages using the posterior AB measurements as follows: 7th instar, less than 138µm; 8th instar, inclusive measurements 138 µm through 233µm; 9th instar, measurements greater than 233µm (Fig. 1). We recognize these cutoffs are somewhat subjective when data from all host species are combined, but are relatively unambiguous

when looking at worms from a single host species. When looking at samples from all host species, ambiguous cutoffs would be expected if indeed there were host species effects on these measures (we address host effects below).

Factors that could influence morphology: No interactions or main effects were found to be significant on body length of 8th instar (though host sex was marginally non-significant, with pentastomes in females being larger than those in males; $F_{1, 20.70} = 4.29$, $p = 0.051$; Table 2A). No interactions were significant on body length of 9th instar female worms. Total pentastome intensity was found to be negatively related with body length of 9th instar worms ($F_{1, 14.06} = 7.51$, $p = 0.02$; Table 2B), a pattern consistent with density-dependence. For male body length, no interactions were significant, but there was a negative relationship ($F_{1, 17.34} = 7.13$, $p = 0.02$; Table 2C) with host SVL.

There were no interactions for hook size in the 8th or 9th instar stage of females. Also, hook size was not associated with the potential environmental variables of host sex, SVL, or parasite intensities in the 8th or 9th instar stage of females, though body size was positively associated with hook size at the 9th instar. ($F_{1, 102.68} = 13.21$, $p < 0.001$; Table 3B). For male worms, there was a significant interaction between total intensity and host sex ($F_{1, 13.58} = 5.18$, $p = 0.04$; Table 3C); where total intensity was found to be positively correlated with hook size in female hosts, but negatively correlated in male hosts (Fig. 4). For spicule length, the final model showed that total intensity had a negative relationship even when controlling for body length, which itself was positively correlated to spicule length ($F_{1, 4.79} = 11.10$, $p = 0.02$; $F_{1, 96.52} = 4.85$, $p = 0.03$, respectively; Table 4A). Spicule width's negative correlation with intensity was

marginally non-significant ($F_{1, 8.19}$ = 4.64, p = 0.06*;* Table 4B).

Testing host species as a factor: Female body length was significantly different among host species ($F_{3, 30.29}$= 11.035, p < 0.001; Table 5A). A *post hoc* pairwise comparison analysis (difflsmeans function from the lmerTest v2.0.36 R package; Brockhoff and Christensen 2017) showed pentastome body size was significantly different and decreasing in size from ANCA, HETU, and RHMA (Table 5B, Fig. 5). Pentastomes in HEFR (n = 4) overlapped with worms in ANCA and HETU. In males, host species also had a significant effect on body length ($F_{3, 51.11}$ = 12.209, p = < 0.001; Table 5C) where a *post hoc* pairwise comparison analysis showed male worms from ANCA and HETU were larger than those from HEFR and RHMA (Table 5D, Fig. 6). Controlling for body size (itself with a positive relationship $F_{1, 135.91}$ = 16.528, p < 0.001; Table 6A), hook size in females was significantly different among host species ($F_{3, 39.28}$ = 18.562, p < 0.001; Table 6A; Fig. 7). *Post hoc* pairwise comparison analysis (Table 6B) showed hooks are the largest in HETU (Fig. 7). Similarly, hook size was significantly different among host species in male worms ($F_{3, 54.72}$ = 21.4475, p < 0.001; Table 6C) where again HETU tended to have the larger hooks (*post hoc* analysis; Table 6D, Fig. 8). Spicule length was not related to host species while controlling for body size (positive relationship; $F_{1, 122.81}$ = 7.4315, p = 0.007347; Table 7A). Spicule width's association was marginally non-significant ($F_{3, 51.10}$ = 2.74, p = 0.053; Table 7 B).

DISCUSSION AND CONCLUSIONS

The key findings of our study indicate that there are three critical considerations regarding Raillietiellid (possibly pentastomes in general) taxonomy and hence resolution of cryptogenic status. First, our results indicate that the majority of hook traits are likely not independent and thus, should not be treated as separate variables in different analyses. Second, our results explicitly indicate that key traits vary according to instar (developmental) stage. Third, host species is associated with significant differences in morphological variation of important taxonomic traits in pentastomes, even when accounting for body length and instar stage. Below we discuss these issues in detail and argue that the pentastome, *R. indica*, is an invasive parasite which lacks host specificity and has spilled-over into a native host.

Variation in Taxonomic Traits

As noted by Riley (1986), taxonomic studies of pentastomids are hindered by environmental and developmental factors which affect morphological variation, and a lack of external structures suitable for fixation and thus identification. Metrics such as body shape, hook morphology, annulus number, and the position of the female gonopore are suitable for broad generic identification but can exhibit too much intraspecific variation for reliable species identification (Riley 1986). Another problem is the generally few specimens upon which species are described, as such, the full range of morphological variation is poorly understood. For these reasons, taxonomic analyses

have primarily focused on the rigid structures, e.g. hooks and copulatory spicules, as they are less susceptible to artifacts due to processing and handling.

Traditionally, cluster patterns observed in plots of hook shank length (BC) by hook blade length (AB) of female worms were used for species identification, but, as noted by Ali et al. (1981) and Riley (1986), this assumes all measurements are from fully adult females because hook size increases with each subsequent moult, i.e. instar stage, in the definitive host. Thus, as noted by Riley (1986) and Kelehear et al. (2011), it is necessary to establish developmental, i.e. instar, stage to meaningfully compare morphology of species, but there is no clear way of doing this outside of controlled infections. Ali and Riley (1983) reported the percentage of fully developed eggs in the uterus was correlated with instar stage, but, as noted by Kelehear et al. (2011), this is undoubtedly a tedious and time consuming metric to obtain. The issue of morphological taxonomy is further confounded by the variation in pentastome morphology induced by host species, as reported by Kelehear et al. (2011), and possibly other environmental factors, e.g. intensity of infection, which we discuss further.

Our findings are a significant contribution to help resolve some of the ambiguity in the taxonomic issues mentioned above. First, our PCA revealed strong evidence that the hook measurements highly covary in both male and female pentastomes, and as such, treating each as independent runs the risk of pseudoreplicating a single underlying trait. Based on the PCA results, our downstream analyses used a summated score of the posterior hook AB and BC measurements, which is comparable across studies.

Second, we were able to replicate the results of Kelehear et al. (2011) in that when we allometrically corrected for body length the distinction among the clusters disappeared from the 2D-plot of AB by BC posterior hook measures (Fig. 2). Based on the latter result, Kelehear et al. (2011) hypothesized that hook size was affected by development. Here, we explicitly make the connection between the 2D-plot clusters and discrete instar developmental stages by superimposing data from the experimental infections of Ali and Riley (1983) onto our data and that of Kelehear et al. (2011). By using hook morphology data from Ali and Riley's (1983) experimental infections to delimit instar stage, we were more explicitly able to control for development in our analyses.

Third, we found that pentastome body length and hook size varied among different host species. Our results showed body length of 9th instar female worms and male worms differed significantly among host species where worms in ANCA, HETU, and RHMA tend to be largest to smallest, respectively. The body size of worms in HEFR vary between the male and female worms, but this is also the host with the smallest sample sizes. Hook sizes of 9th instar female worms and male worms for a given body size tended to be generally larger in HETU and HEFR than those from ANCA and RHMA. Curiously, pentastomes from ANCA are largest in terms of body length, but smallest in terms of hook size. The above patterns suggest that for a given trait, host species effects male and female worms similarly, but that patterns may differ among the traits themselves.

In contrast to the above, we did not observe any effect of host species on male

spicules. There was a marginally non-significant affect of host species on spicule width, but in general, spicule measures were robust to host species of origin; a result that reinforces the use of spicules as a species diagnostic trait.

In general, the host-induced variation we and Kelehear et al (2011) observed may explain, in part, much of the taxonomic confusion as presented by Poore (2012). We suspect the difficulty in assessing the maturity of female worms is what lead to the initial description of *R. frenatus* [sic] as a different species from *R. indica*. As discussed in Kelehear et al. (2011), it is plausible that the species description of *R. frenatus* [sic] was based on a later instar stage of *R. indica*. As stated by Kelehear et al. (2011), traditional morphological analyses of hook measurements alone would have lead them to identify the pentastome infections of the introduced cane toad as two species, *R. indica* and *R. frenatus* [sic]. They ultimately concluded these were the same pentastome species based on molecular data and allometrically correcting hook size for body length, effectively correcting for development. They identified their pentastome species as *R. frenatus* [sic], but, according to a taxonomy review by Poore (2012), *R. frenatus* [sic] is a junior synonym of *R. indica*.

Lastly, taking advantage of the large sample collections from HETU, we tested for other factors that may induce environmental variation. In 9th instar female worms, body length was found to be negatively correlated with intensity. This pattern is consistent with what would be expected of a density dependent limitation on worm body length; as intensity increases, body length of the terminal moult decreases. However, we did not find a density dependent effect on 8th instar female body length, suggesting

density-dependence is manifested in an instar specific manner. In males, there was a negative relationship found between worm body length and snout-vent-length of the host, indicating male worms are smaller in larger geckos. When looking at hook size we did not see any significant main effects in 8 or 9th instar females.

But in male worms there was a significant interaction between total intensity and host sex; where total intensity was found to be positively correlated with hook size in female hosts, but negatively correlated in male hosts. This host-sex specific relationship indicates there is some sex specific behavioral or physiological factor impacting the development of male hooks. However, we did not see consistent patterns across these tests of environmentally induced variation on pentastome morphology, so interpretation of results should be regarded with caution. Especially if one regards we are doing multiple tests. It may be that some of the patterns are real, but additional studies and under more controlled conditions would be needed for confirmation.

In contrast to the above we did find a consistent density-dependent effect with regards to male spicules. Spicule length was negatively associated with intensity while width was marginally so. So while robust to host species environments, this trait could be influenced by intraspecific crowding effects.

Sequence data

The CO1 sequence from our samples translates according to the invertebrate mtDNA code 5; however, the lack of variation from all our samples and that in Australia is a strange result given the generally high mutation rate of mtDNA (Ballard and

Whitlock 2004). The lack of variation may suggest this amplified locus is a nuclear pseudogene, but more flanking sequence would be needed to test this hypothesis. Nevertheless, a pseudogene would not negate its use as a marker to determine if it is the same parasite species in each host; the main caveat is that the lack of variation precludes inferences on colonization history. As such, for future studies, it may be necessary to develop more markers to aid in taxonomy or within-species population history. Nevertheless, based on the 100% similarity in the CO1 sequences between our pentastomes and those from Australia, as well as the similarity between spicule shape (Fig. 1), we are confident that our pentastomes are the same species as those from Australia and are the same between Mediterranean gecko and green anole hosts within the southern U.S.A.

Range, Host Expansion, and Resolving the Cryptogenic Status

To our knowledge, the first report of a species of *Raillietiella* sampled from within continental U.S.A. came from Pence and Selcer (1988) wherein *R. indica* (*R. frenatus* [sic]) was reported from *H. turcicus* in the far south of Texas (Edinburg) in 1981. Since then, *Raillietiella teagueselfi* was described from *H. turcicus* in Houston, Texas by Riley et al. (1988). Here, we report a range expansion of *R. indica* into Port Aransas, Texas and surrounding areas (in 2012; Caballero et al. 2015) as well as Metairie, Louisiana (part of the metropolitan area of New Orleans). It is noteworthy to highlight that survey collections from 1997-1999 at the Metairie location sampled herein did not reveal any pentastome infections in *H. turcicus* (*n* = 42) or *A. carolinenesis* (*n* =

11) (Criscione 2000, Criscione and Font, 2001). Moreover, an additional 184 geckos sampled from 5 additional locations in southeastern Louisiana in 1998 also did not have pentastome infections (Criscione and Font, 2001). In 2008, a single Mediterranean gecko from the Metairie site was found infected with *R. indica* (Criscione, unpublished). Indeed, this finding prompted the additional 2012 surveys in Metairie from both the exotic Mediterranean gecko and the native green anoles on which our current study is based. Importantly, we here document a host expansion into the native green anole.

We speculate on three possible routes of pentastome colonization in Louisiana. First, the Port of New Orleans is part of one of the largest port systems in the world, offering a path for infected geckos or possible intermediate hosts (e.g., roaches) to be transported into the city from around the globe. However, *H. turcicus* has been reported in the New Orleans area since 1949 (Etheridge 1952) and the 1997-1999 surveys did not show any pentasome infections. Second, although anecdotal, we note that Hurricane Katrina occurred in 2005. Recovery and cleanup soon thereafter involved transient workers from southern Texas. So, it is plausible that infected gecko or roach intermediate hosts were transported from southern Texas. Third, the Cuban brown anole (*Anolis sagrei*) has recently invaded and rapidly spread in New Orleans (Lever 2003). Interestingly, *R. indica* (*R. frenatus* [sic]) has been reported from brown anoles in Hawaii (Barton and Riley 2004). So, another plausible scenario is that invading populations of brown anoles also harbored pentastome infections.

Given the collective data in our study and Kelehear et al. (2011), we are of the opinion that most reports of *R. frenatus* [sic] are likely all *R. indica*. Poore (2012)

provides an excellent discussion of taxonomy in the genus *Raillietiella* (including details *R. hebitihamata*, *R. frenata* and *R. indica*) and concludes that the name *R. indica* has seniority. Although we are able to resolve the morphological issues and identify the species as *R. indica*, there is still another problem that remains in order to resolve the cryptogenic status.

As noted in our introduction, anthropogenic transport of host species prior to taxonomic surveys complicates identification of exotic parasite origins (Carlton 1996). This would be especially problematic in geckos as they represent one of the most successful establishing families of alien reptiles or amphibians known (Detwiler and Criscione 2014). Indeed this issue has been raised before for other parasites found in the Mediterranean gecko in the southern USA. In particular, Criscione and Font (2001) discuss how tapeworm species of the genus *Oochoristica* may have colonized new areas before many of them were ever described. In the case of *R. indica*, which appears to have a lack of host specificity, the original description was from an anuran host at the Indian Museum in Calcutta, but no specific type locality is given (Gedoelst 1923). Most subsequent reports of *R. indica* or *R. frenatus* [sic] have occurred throughout southeast Asia (Poore 2012), including Taiwan (Ali et al. 1982) and Malaysia (Ali et al. 1981, Ali et al. 1985). More recent reports of *R. indica* include Australia (Kelehear et al. 2011), Hawaii (Barton and Riley 2004). With the exceptions of Pence and Selcer (1988) and Riley et al. (1988), we are not aware of reports of *Raillietiella* spp. in the continental USA.

The preponderance and historical dates of reports suggests that *R. indica* is

indeed an invasive parasite that now has spilled-over into a native host (green anoles) in the southern USA. The spillover of *R. indica* into green anoles is important because of the potential for adverse fitness effects pentastomes may impose on their hosts. For example, Pence and Selcer (1988) found pentastome infections in geckos were associated with a reduction in the number of oviductal eggs. In addition, Caballero et al. (2015) found the recovery time of recently active geckos increased with the number of pentastomes, demonstrating a potential mechanism of fitness reduction in the gecko itself. These effects, along with the mounting pressure green anoles already face from the introduction of brown anoles Campbell (2000), gives cause for concern. Given the apparent lack of host specificity demonstrated by *R. indica* there could very well be other native reptile and amphibian species infected.

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APPENDIX

Figure 1. Copulatory spicules of *R. indica* from *A. carolinensis* (left) and *H. turcicus* (right).

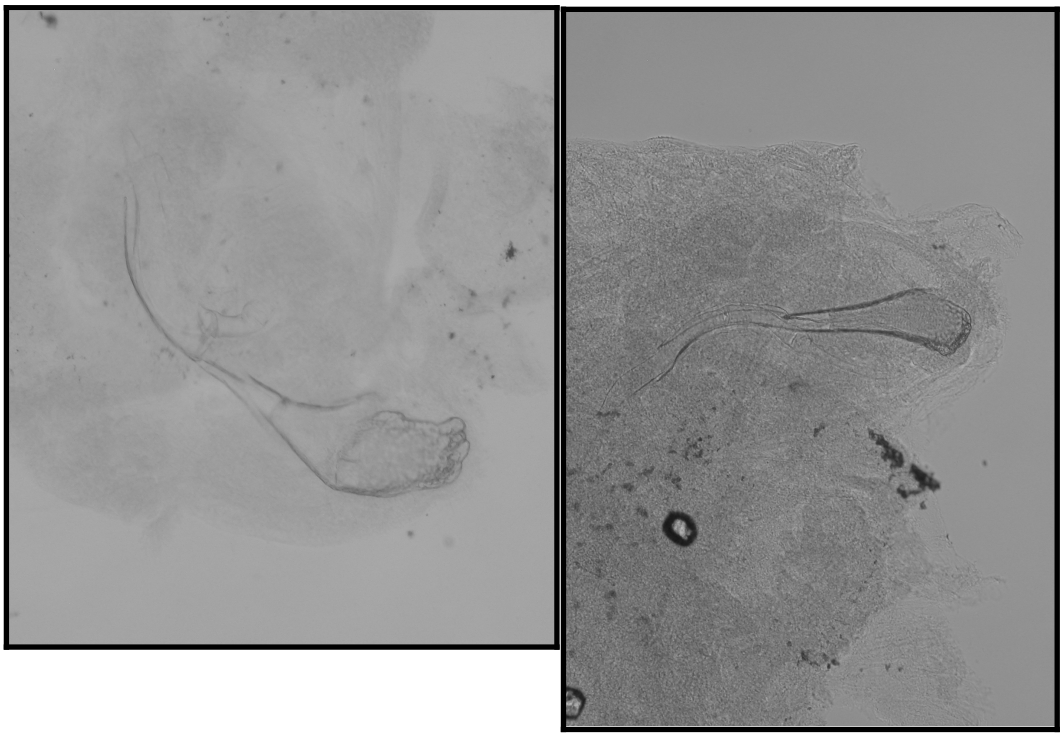

Figure 2. (A) Plot of posterior hook BC measurements by posterior hook AB measurements of female *R. indica* from *H. turcicus* and *A. carolinensis* shows 3 distinct groups. (B) Plot of BC residuals against AB residuals (removing the effect of body length). The distinction of the three groups is no longer apparent.

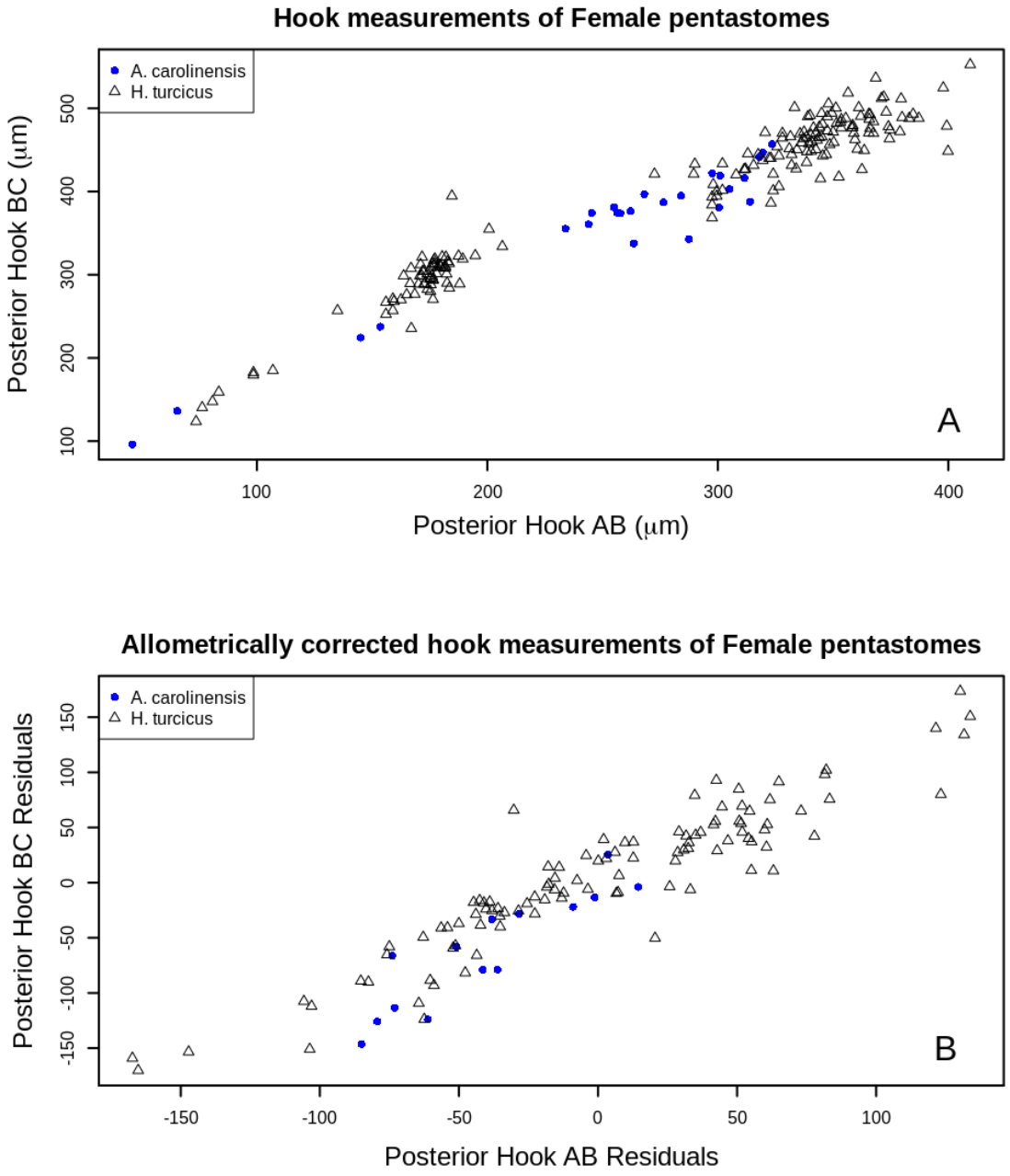

Figure 3. Posterior hook BC by posterior hook AB of *R. indica* from *A. carolinensis* and *H. turcicus*, and *H. frenatus* and *R. marina*, adapted from Kelehear et al. (2011). The red crosses represent the min, max, and means (the crux) of female *R. frenatus* [sic] in the 7th, 8th, and 9th instars adapted from Ali et al. (1981). A general trend appears of host associated variation in hook measurements where pentastomes from *A. carolinensis* and *R. marina* tend to have smaller hook sizes relative to pentastomes in the gecko hosts.

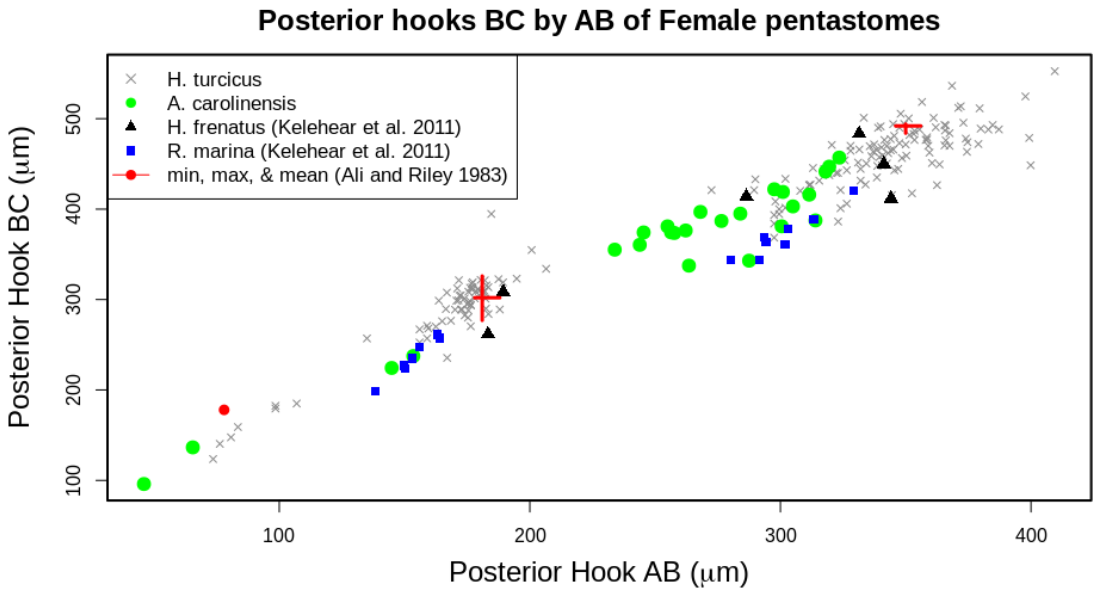

Table 1. PCA results on hook measurments: variable factor loadings, factor eigenvalues, and proportion of total variance explained by each factor from the Varimax rotated correlation matrix of all sampled worms (see main text for sample sizes).

A) Female Hook Measurements	Varimax rotated loading matrix	
	Factor 1	Factor 2
Posterior Hook AB (µm)	0.97	0.08
Posterior Hook BC (µm)	0.97	0.01
Anterior Hook AB (µm)	0.89	0.24
Anterior Hook BC (µm)	0.93	0.16
Mean Area of the Posterior Hook (µm^2)	0.94	0.04
Mean Area of the Anterior Hook (µm^2)	0.09	0.99
Rotated eigenvalues	4.43	1.07
Percent total variance explained	0.74	0.18

B) Male Hook Measurements	Varimax rotated loading matrix	
	Factor 1	Factor 2
Posterior Hook AB (µm)	0.78	-0.32
Posterior Hook BC (µm)	0.89	-0.20
Anterior Hook AB (µm)	0.67	0.16
Anterior Hook BC (µm)	0.81	0.22
Mean Area of the Posterior Hook (µm^2)	0.79	-0.25
Mean Area of the Anterior Hook (µm^2)	-0.04	0.92
Rotated eigenvalues	3.13	1.12
Percent total variance explained	0.52	0.19

Table 2. Tests for factors associated with pentastome body length. *p*-values were calculated using the Satterthwaite approximation (fixed effects) using the lmerTest package.

A) Body Length of 8th instar Female Worms				
lmer(Body Length~Host Sex+Intensity+Snout Vent Length+(1|Location/Host ID))				
Fixed Effect	***F*-value**	**df num.**	**df den.**	***p-value***
Host Sex	4.29	1	20.70	0.051
Intensity	0.05	1	19.17	0.833
Snout Vent Length	0.02	1	18.22	0.90
Random Effects		**Variance**		
Host ID:Location		1.29		
Location		0		
Residual		0.69		
Total		1.98		

B) Body Length of 9th instar Female Worms				
lmer(Body Length~Host Sex+Intensity+Snout Vent Length+(1|Location/Host ID))				
Fixed Effect	***F*-value**	**df num.**	**df den.**	***p-value***
Host Sex	2.29	1	14.06	0.15
Intensity	7.51	1	9.54	**0.02**
Snout Vent Length	1.66	1	14.61	0.22
Random Effects		**Variance**		
Host ID:Location		1.56		
Location		0		
Residual		4.04		
Total		5.6		

C) Body Length of Male Worms				
lmer(Body Length~Host Sex+Intensity+Snout Vent Length+(1|Location/Host ID))				
Fixed Effect	***F*-value**	**df num.**	**df den.**	***p-value***
Host Sex	0.74	1	8.25	0.41
Intensity	0.29	1	18.24	0.59
Snout Vent Length	7.13	1	17.35	**0.02**
Random Effects		**Variance**		
Host ID:Location		0.07		
Location		0		
Residual		0.38		
Total		0.45		

Table 3. Tests for factors associated with pentastome hook size**.** *p*-values were calculated using the Satterthwaite approximation (fixed effects) using the lmerTest package.

A) Hook Size of 8th instar Female Worms				
lmer(Hook Size~Host Sex+Intensity+Snout Vent Length+Body Length+(1|Location/Host ID))				
Fixed Effect	***F*-value**	**df num.**	**df den.**	***p-value***
Host Sex	1.12	1	17.10	0.30
Intensity	1.47	1	14.58	0.24
Snout Vent Length	1.40	1	9.48	0.27
Body Length	0.82	1	51.46	0.37
Random Effects		**Variance**		
Host ID:Location		553.7		
Location		116.1		
Residual		506.3		
Total		1176.1		

B) Hook Size of 9th instar Female Worms				
lmer(Hook Size~Host Sex+Intensity+Snout Vent Length+Body Length+(1|Location/Host ID))				
Fixed Effect	***F*-value**	**df num.**	**df den.**	***p-value***
Host Sex	0.24	1	16.12	0.21
Intensity	1.71	1	12.25	0.63
Snout Vent Length	1.85	1	17.15	0.19
Body Length	13.21	1	102.68	< **0.001**
Random Effects		**Variance**		
Host ID:Location		667.4		
Location		627.4		
Residual		154.2		
Total		1449		

C) Hook Size of Male Worms				
lmer(Hook Size~Host Sex+Intensity+Snout Vent Length+Body Length+Intensity:Host Sex+ (1|Location/Host ID))				
Fixed Effect	***F*-value**	**df num.**	**df den.**	***p-value***
Host Sex	5.16	1	17.72	**0.036**
Intensity	0.008	1	13.22	0.93
Snout Vent Length	0.16	1	15.77	0.69
Body Length	0.43	1	97.53	0.51
Intensity:Host Sex	5.18	1	13.58	**0.04**
Random Effects		**Variance**		
Host ID:Location		88.63		
Location		0		
Residual		238.69		
Total		327.32		

Figure 4. Plot of fitted male hook sizes from lmer model by intensity from male and female *H. turcicus* hosts. Red and blue lines (offset on the x-axis for clarity) indicate the lower and upper bound confidence intervals of female and male hosts, respectively.

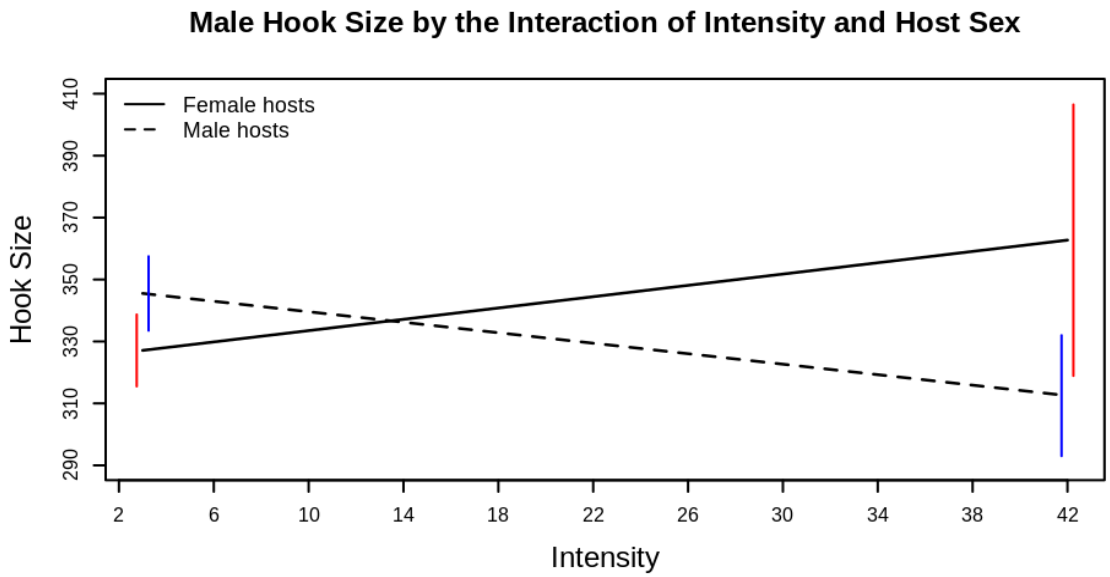

Table 4. Tests for factors associated with male pentastome spicule length. *p*-values were calculated using the Satterthwaite approximation (fixed effects) using the lmerTest package.

A) Spicule Length of Male Worms				
lmer(Spicule Length~Host Sex+Intensity+Snout Vent Length+Body Length+(1|Location/Host ID))				
Fixed Effect	***F*-value**	**df num.**	**df den.**	***p-value***
Host Sex	0.43	1	15.63	0.52
Intensity	11.10	1	4.79	**0.02**
Snout Vent Length	0.05	1	15.79	0.82
Body Length	4.85	1	96.52	**0.03**
Random Effects		**Variance**		
Host ID:Location		107.78		
Location		62.22		
Residual		1601.91		
Total		1771.91		

B) Spicule Width of Male Worms				
lmer(Spicule Width~Host Sex+Intensity+Snout Vent Length+Body Length+(1|Location/Host ID)				
Fixed Effect	***F*-value**	**df num.**	**df den.**	***p-value***
Host Sex	0.67	1	16.55	0.42
Intensity	4.64	1	8.19	0.06
Snout Vent Length	0.42	1	18.08	0.52
Body Length	0.04	1	99.84	0.83
Random Effects		**Variance**		
Host ID:Location		10.09		
Location		0		
Residual		45.02		
Total		55.11		

Table 5. Tests for a host species association with pentastome body length**.** *p*-values were calculated using the Satterthwaite approximation (fixed effects) using the lmerTest package.

A) Body Length of 9th Instar Females

lmer(Body Length~Host Species+(1|Host ID))

Fixed Effect	***F*-value**	**df num.**	**df den.**	***p-value***
Host Species	11.04	3	30.29	**< 0.001**
Random Effects		**Variance**		
Host ID		1.62		
Residual		4.07		
Total		5.69		

B) Pairwise Comparison of Female Body Lengths

					95% Confidence Interval for Difference	
(I) Host	**(J) Host**	**Mean Difference (I-J)**	**Std. Error**	***p-value***	**Lower**	**Upper**
ANCA	HEFR	1.4	1.49	0.365	-1.65	4.39
	HETU	2.7	0.88	**0.006**	0.88	4.55
	RHMA	7.2	1.28	**< 0.001**	4.60	9.83
HEFR	HETU	1.3	1.32	0.31	-1.29	4.00
	RHMA	5.8	1.61	**< 0.001**	2.61	9.10
HETU	RHMA	4.5	1.06	**< 0.001**	2.34	6.66

C) Body Length of Males

lmer(Body Length~Host Species+(1|Host ID))

Fixed Effect	***F*-value**	**df num.**	**df den.**	***p-value***
Host Species	12.21	3	51.11	**< 0.001**
Random Effects		**Variance**		
Host ID		0.11		
Residual		0.37		
Total		0.48		

Table 5. (continued)

D) Pairwise Comparison of Male Body Lengths

(I) Host	(J) Host	Mean Difference (I-J)	Std. Error	*p-value*	95% Confidence Interval for Difference	
					Lower	**Upper**
ANCA	HEFR	1.3	0.45	**0.005**	0.43	2.23
	HETU	0.0	0.35	0.97	-0.72	0.70
	RHMA	1.2	0.42	**0.007**	0.34	2.04
HEFR	HETU	-1.3	0.31	< **0.001**	-1.96	-0.73
	RHMA	-0.1	0.38	0.72	-0.91	0.63
HETU	RHMA	1.2	0.27	< **0.001**	0.67	1.74

Figure 5. *Post hoc* pairwise comparisons of female pentastome body length between host species (*n* = number of worms). Red bars indicate 95% lower and upper bound confidence intervals. Letters denote similarities or significant differences in the pairwise tests.

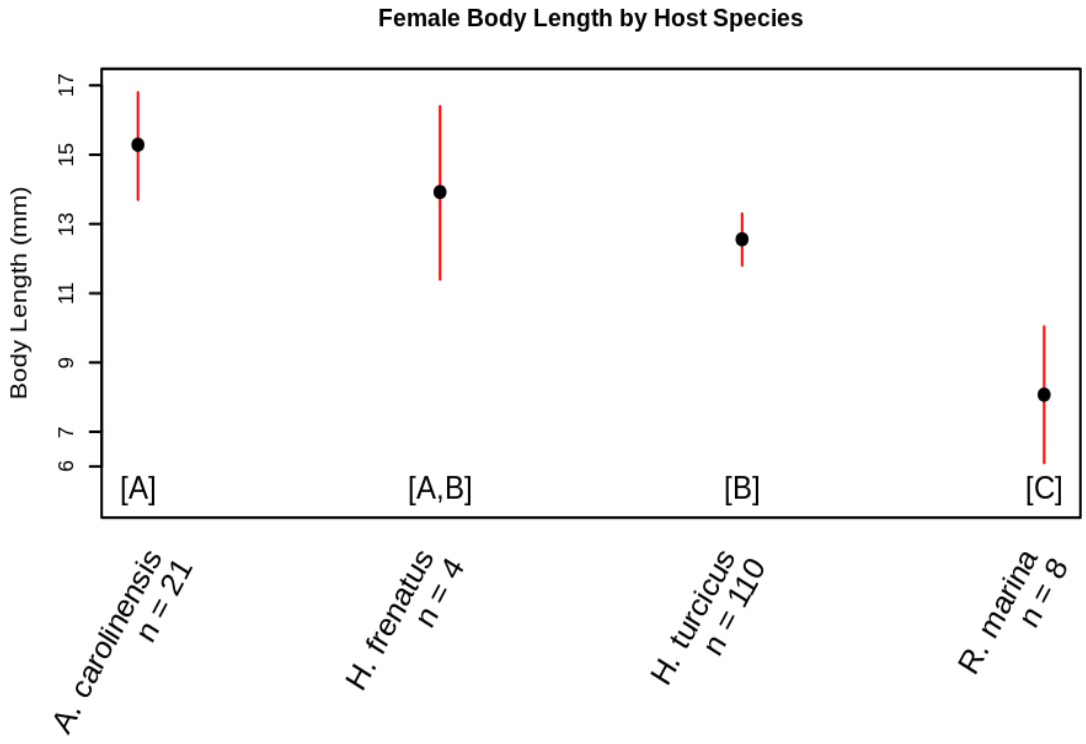

Figure 6. *Post hoc* pairwise comparisons of male pentastome body length between host species (*n* = number of worms). Red bars indicate 95% lower and upper bound confidence intervals. Letters denote similarities or significant differences in the pairwise tests.

Table 6. Tests for a host species association with pentastome hook size. *p*-values for were calculated using the Satterthwaite approximation (fixed effects) using the lmerTest package.

A) Hook Size of 9th Instar Females

lmer(Hook Size~Host Species+Body Length+(1|Host ID))

Fixed Effect	***F*-value**	**df num.**	**df den.**	***p-value***
Host Species	18.56	3	39.28	< **0.001**
Body Length	16.53	1	135.91	< **0.001**
Random Effects		**Variance**		
Host ID		1194		
Residual		1452		
Total		2646		

B) Pairwise Comparison of Female Hook Size

					95% Confidence Interval for Difference	
(I) Host	**(J) Host**	**Mean Difference (I-J)**	**Std. Error**	***p-value***	**Lower**	**Upper**
ANCA	HEFR	-76.4	34.2	**0.03**	-145.5	-7.25
	HETU	-138.7	21.9	< **0.001**	-183.7	-93.82
	RHMA	-28.2	32.3	0.39	-93.5	37.02
HEFR	HETU	-62.4	29.4	0.04	-121.4	-3.33
	RHMA	48.1	37.4	0.20	-26.80	123.00
HETU	RHMA	110.5	25.6	< **0.001**	59.00	162.05

C) Hook Size of Male Worms

lmer(Hook Size~Host Species+Body Length+(1|Host ID))

Fixed Effect	***F*-value**	**df num.**	**df den.**	***p-value***
Host Species	21.45	3	54.72	< **0.001**
Body Length	0.85	1	121.60	0.36
Random Effects		**Variance**		
Host ID		113.60		
Residual		240.70		
Total		354.3		

Table 6. (continued)

D) Pairwise Comparison of Male Hook Size

(I) Host	(J) Host	Mean Difference (I-J)	Std. Error	*p-value*	95% Confidence Interval for Difference	
					Lower	Upper
ANCA	HEFR	-25.2	13.04	0.06	-51.35	0.86
	HETU	-27.8	9.98	**0.007**	-47.84	-7.81
	RHMA	33.6	12.27	**0.008**	9.05	58.17
HEFR	HETU	-2.60	9.23	0.78	-21.06	15.90
	RHMA	58.9	10.88	< **0.001**	36.99	80.72
HETU	RHMA	61.40	8.10	< **0.001**	45.20	77.66

Figure 7. *Post hoc* pairwise comparison of female pentastome hook size between host species (*n* = number of worms). Red bars indicate 95% lower and upper bound confidence intervals. Letters denote similarities or significant differences in the pairwise tests.

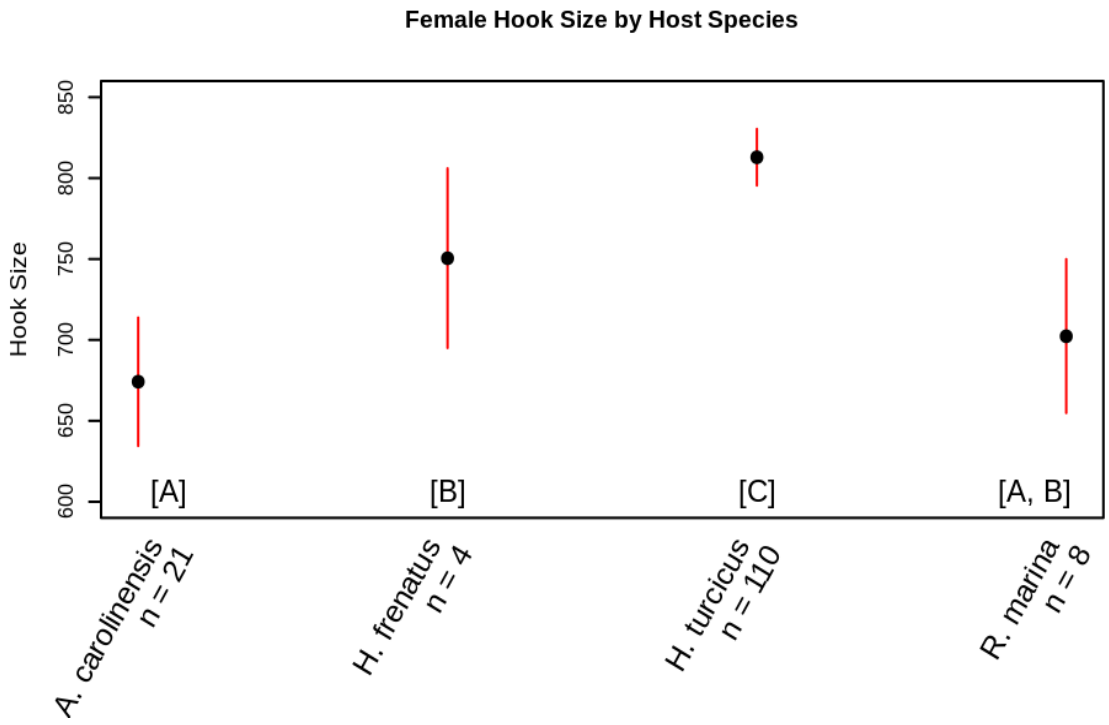

Figure 8. *Post hoc* pairwise comparison of male pentastome hook size between host species (*n* = number of worms). Red bars indicate 95% lower and upper bound confidence intervals. Letters denote similarities or significant differences in the pairwise tests.

Table 7. Tests for a host species association with male pentastome spicule length. *p*-values were calculated using the Satterthwaite approximation (fixed effects) using the lmerTest package.

A) Spicule Length of Male Worms				
lmer(Spicule Length~Host Species+Body Length+(1|Host ID))				
Fixed Effect	***F*-value**	**df num.**	**df den.**	***p-value***
Host Species	0.28	3	61.19	0.84
Body Length	7.43	1	122.81	**0.007**
Random Effects		**Variance**		
Host ID		353.60		
Residual		1480.90		
Total		1834.5		

B) Spicule Width of Male Worms				
lmer(Spicule Width~Host Species+Body Length+(1|Host ID)				
Fixed Effect	***F*-value**	**df num.**	**df den.**	***p-value***
Host Species	2.74	3	51.10	0.053
Body Length	0.03	1	120.97	0.86
Random Effects		**Variance**		
Host ID		22.17		
Residual		43.77		
Total		65.94		