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EFFECT OF AGE OF THE INTERMEDIATE HOST *TRIBOLIUM CONFUSUM* (COLEOPTERA) ON INFECTION BY *HYMENOLEPIS DIMINUTA* (CESTODA)

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ABSTRACT: A cross-sectional study of 27 cohorts of *Tribolium confusum* aged 2–78 wk was done to examine effects of host age on exposure to eggs of *Hymenolepis diminuta* under standardized conditions. Pre-exposure, fasting, and postexposure mortality were low, sex ratio was equal, and fecundity of hosts was high during the first 30 wk, followed by increasing mortality and male bias of the sex ratio, and declining fecundity, in older beetles. These changes in the host were not associated with pronounced changes in infection results. Prevalence of infection was higher in females than males, but was unaffected by age in both sexes. Intensity of infection was similar between sexes in beetles up to 30 wk old, and thereafter declined in females, but not in males. Age-related changes in hosts were gradual, but unexpected levels of short-term variation in infection results suggest that some undetermined proximate factors may override general host age effects on the infection process.

Host age affects infections of insect hosts where age correlates with body size, such as in larval insects infected with parasitoids (Colinet et al., 2005) or where defensive capabilities of invertebrate hosts change with age (Blaser and Schmid-Hempel, 2005). Beetles (Tenebrionidae) are commonly used as experimental hosts for protozoan and helminth parasites, particularly the cestode *Hymenolepis diminuta*. Control of host age is common in these experimental studies, but only 3 studies have specifically examined the effect of varied host age on parasite establishment following a controlled exposure of flour beetles *Tribolium confusum* to eggs of the cestode *H. diminuta*. Kelly et al. (1967) infected virgin beetles 4–51 wk old at the time of infection. Dunkley and Mettrick (1971) infected beetles that were 4–12 days old. Keymer (1982) infected beetles over the range 2–14 wk posteclosion. All studies reported age effects. Other studies (Soltice et al., 1971; Mankau, 1977; Yan and Norman, 1995; Robb and Reid, 1996) have used a single age class of beetle, virgin or mated, but only up to 6 wk old. Collectively, these studies fall short of encompassing the normal life span of *T. confusum*, which can live 77 wk or more (Pearl et al., 1941), particularly for mated hosts, which are more likely to reflect the status of older individuals. Moreover, comparisons among previous studies are hampered by variations in methods used to infect beetles.

This article reports a cross-sectional study on cohorts of mixed-sex, adult *T. confusum*, aged 1–78 wk at the time of exposure to eggs of *H. diminuta*. Patterns of host survival and fecundity were used to identify different phases of adult life, and prevalence and intensity of infection and parasite growth were compared among these phases.

MATERIALS AND METHODS

Animals

History and general maintenance conditions of hosts and parasites are described elsewhere (Shostak et al., 2006). Twenty-seven cohorts of known-age beetles were established between August 2004 and August 2006 by removing up to 200 pupae of *T. confusum* at a time from stock cultures, and storing them at 26 C in the dark in a 10-cm-diameter glass dish with 15 g of flour/brewer's yeast medium. The time of pupa collection was termed age 0. The actual time of emergence of adults was not recorded, but typically would occur 1–7 days after collection of pupae. Each 4 wk or less the contents of each dish were passed through a sieve, adults (live and dead) were censused to determine pre-exposure survival, and live adults were transferred to 15 g of fresh medium; any eggs, larvae, and pupae were discarded. To minimize injury of beetles during the censuses, beetles were poured onto an 18.5-cm-diameter filter paper, which they generally gripped well. Inversion of the paper allowed eggs and debris to fall off, and the adults were counted as they were brushed gently off the paper. Any live adults that fell off the paper were transferred by scooping them gently onto a small piece of filter paper. Eggs of *H. diminuta* for this specific study were obtained from the same 2 rats, each infected with 10 cysticercoids 8 mo prior to the first exposure. Fresh fecal pellets were collected on each day of exposure, washed through sieves, cleaned with the use of sucrose gradient centrifugation, and diluted to the required concentration in distilled water.

Experimental design

Five of the oldest cohorts died out before any infections were attempted, but these provided data on host mortality rates. Experimental infections were initiated after heavy mortality in the oldest of the remaining 22 cohorts (February 2005) indicated that those beetles were also nearing the end of their life span. Two of 22 cohorts contained a large number of surviving beetles and were subdivided and exposed on separate occasions, so 24 exposures in all were done. Exposures were done over a period of 3 mo for logistic reasons. The order in which cohorts were exposed was selected to minimize gaps in host age in the final data set.

The medium in a dish selected for infection was passed through a sieve. Up to 35 beetles were removed for infection and placed in a clean dish without food (26 C, dark) for 4 days. If ≥35 live beetles remained, they were placed on fresh medium to age further and be infected at a later date. Following the 4-day fast, beetles were censused to determine fasting survival and up to 32 were selected at random for infection. Beetles were placed individually in infection arenas comprising a 44-mm inside diameter acrylic ring placed on a 55-mm-diameter Whatman no. 1 filter paper, on which was placed 400 μl distilled water estimated to contain 1,500 eggs from a suspension mixed with the use of a magnetic stirrer. For logistic reasons, a quick initial estimate of egg density was required and was done with the use of counts on 8 20-μl samples from the stirred suspension, followed by concentration or dilution of the egg suspension to the required density. Some beetles landed on their back when added to the arena. The time required for the beetle to right itself was noted and the midpoint of the range for all beetles in an exposure was recorded as a representative righting time. The arena was covered with a glass dish to prevent beetle escape and to retard drying of eggs and left for 6 hr (room temperature and lighting), then beetles were removed and stored individually in 25-mm glass vials containing 1 g fresh medium for 2 wk (26 C, dark). After beetles had been added to arenas, a more time-consuming total count of eggs in 3 separate, 400-μl samples of the egg suspension was done to confirm the actual exposure dose. At 2 wk, hosts were again censused to determine "post-exposure survival". The remaining hosts were killed, sexed, and dissected to count and photograph cysticercoids for size determination according to published procedures (Shostak et al., 2006). Beetle egg counts were done after passing the medium from each vial through a 250-μm sieve.

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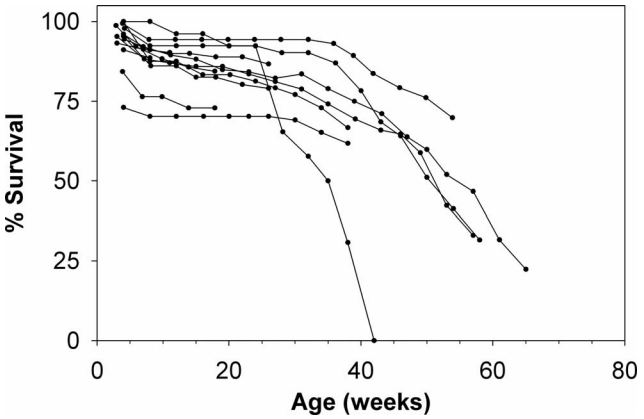

FIGURE 1. Percentage survival over time for different cohorts of uninfected *Tribolium confusum*. Each line represents a different cohort and each point represents a census day.

Analysis

Pre-exposure survival was assessed by 2 methods. Census records for some cohorts were complete from the time of adult emergence and these enabled expression of survival over time relative to the original number of adults. Census records for these as well as the remaining cohorts also provided initial (N_0) and final (N_t) numbers of live hosts for each cohort and census interval of t wk from which a weekly mortality rate, $\lambda = \log_e(N_0/N_t)/t$ (Sheil et al., 1995) was calculated. Statistical analysis was done with the use of SAS 9.1 (SAS Institute Inc., Cary, North Carolina) PROC CORR, PROC GLM, and PROC FREQ, with the exception of comparisons of prevalence (percent of hosts with 1 or more parasites) and intensity of infection (number of parasites per infected host), which were done with the use of Quantitative Parasitology 3.0 (Rozsa et al., 2000). Data are reported as mean ± SD unless indicated otherwise. Statistical significance was determined with the use of $\alpha = 0.05$.

RESULTS

The maintenance procedure appeared to maintain each cohort of beetles free of contamination by younger beetles developing from eggs to adults within the 4-wk cleaning cycle. Development even to the pupa stage was rare, occurring at a rate of 1 pupa per 100 beetles per 10 cycles. Census results also did not reveal any increases in numbers of beetles per dish.

This study used 2,250 adult beetles, of which 553 were exposed and survived to the time of necropsy. It was observed casually during censuses that older beetles moved more slowly and were less able to hold onto the filter paper used to transfer them among containers. Young beetles that landed on their back when added to the arena righted themselves almost immediately, but older beetles took up to 20 sec to right. In cohorts for which righting time was measured, there was a strong correlation with age (Spearman $R_{16} = 0.69$, $P = 0.003$).

All cohorts of hosts exhibited a similar pattern of mortality (Fig. 1). There was initial heavy mortality of newly emerged adults during the first census period, then a period of relative stasis until 25–40 wk old, followed by a second period of high mortality. There were differences among cohorts in the magnitude of initial mortality, with 2 cohorts exhibiting low survival to the first census time, and differences in the time of onset of the second period of mortality, with 1 cohort exhibiting a sharp decrease in survival as early as 25 wk.

The 5 aspects of host biology that were monitored varied in a complex manner over time (Fig. 2A–E). Although infections of each cohort started with 6–32 hosts (mean = 26), mortality during pre-exposure fasting and following exposure reduced the number of hosts that were necropsied to 1–32 per cohort (mean = 22), with only 1–22 infected hosts per cohort (mean = 7). Therefore, data were pooled into 6 age classes to increase sample sizes for statistical analysis of infections and to smooth age-related patterns. The boundaries of each class were chosen to achieve a compromise between a consistent age range of each class and number of cohorts included, and the possession of a unique set of properties by each class (Fig. 2F–J). Class 1 (0–5 wk) were hosts with low pre-exposure mortality rates (λ), high fasting and postexposure survival, an even sex ratio, and moderate fecundity. Class 2 (5–15 wk) were hosts with low pre-exposure mortality rates, high fasting and postexposure survival, an even sex ratio and higher fecundity. Class 3 (15–30 wk) were hosts with the lowest pre-exposure mortality rates, high fasting, and postexposure survival, a slightly male-biased sex ratio but the highest fecundity. Class 4 (30–45 wk) were hosts with moderate pre-exposure mortality, reduced fasting, and postexposure survival, a slightly male-biased sex ratio and low fecundity. Class 5 (45–60 wk) were hosts with higher pre-exposure mortality rates, reduced fasting, and postexposure survival, a strongly male-biased sex ratio, and low fecundity. Class 6 (>60 wk) were hosts with the highest (although variable) pre-exposure mortality rates, the lowest fasting and postexposure survival, and with females absent.

Age-related trends in host biology (Fig. 2F–J) were not always unidirectional with age. Weekly mortality rates were initially low, dropped to negligible levels, then increased markedly (Fig. 2F). The proportion of beetles that survived the pre-exposure fast (Fig. 2G) increased initially, then decreased ($\chi^2_5 = 113.3$, $P < 0.001$). The proportion of beetles that survived the 2-wk period from exposure through to necropsy (Fig. 2H) was initially high and then declined ($\chi^2_5 = 148.0$, $P < 0.001$). The sex ratio (Fig. 2I) was initially even, but became gradually more male biased until the oldest beetles were male only ($\chi^2_5 = 62.2$, $P < 0.001$). Egg production by females was low initially, increased for a period, then declined sharply; the oldest females produced few eggs (Fig. 2J).

Prevalence and intensity of infection in individual cohorts (Fig. 3A, B) were pooled across sexes because of low sample sizes. There was irregular variation that did not appear to relate monotonically to host age. Some of the largest fluctuations occurred in younger beetles (Fig. 3A, B), where the sex ratio was uniformly near 50% (Fig. 2D). Spearman correlations were used to assess whether other experimental variables contributed to this pattern. The order in which the exposures were done was not correlated with prevalence ($R_{22} = 0.05$, $P = 0.83$) or intensity ($R_{22} = -0.17$, $P = 0.42$). Similarly, the date on which each cohort was removed from the source culture was not correlated with prevalence ($R_{22} = -0.01$, $P = 0.99$) or intensity ($R_{22} = -0.04$, $P = 0.85$). The number of eggs per arena within an exposure was fairly uniform, with the coefficient of variation (CV = mean/SD) averaging 5% in the 24 exposures. There was variation in mean number of eggs/arena used in different exposures (CV = 8.7%) with the actual number being 1,440 ± 126 in the 24 exposures. However, the actual number of eggs per exposure of each cohort was not correlated with resulting prevalence ($R_{22} = 0.35$, $P = 0.09$) or intensity ($R_{22} = 0.13$, $P = 0.55$).

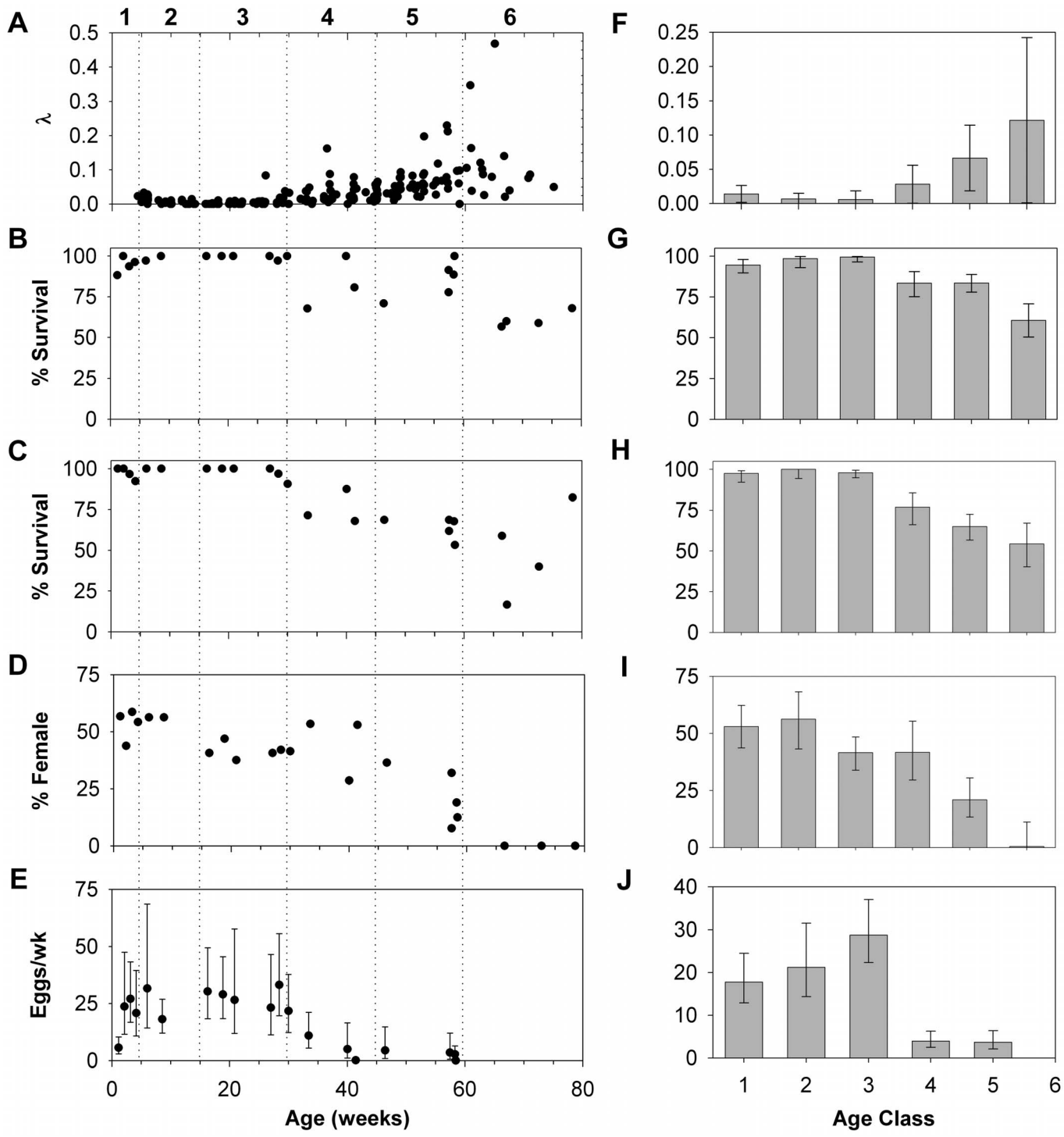

FIGURE 2. Changes in host properties over time determined for individual cohorts of *Tribolium confusum* (**A–E**) or following grouping of cohorts into age classes (**F–J**). (**A, F**) Weekly pre-exposure mortality rate (λ). (**B, G**) Percentage survival during a 4-day pre-exposure fast. (**C, H**) Percentage survival from the day of exposure to 2 wk postexposure. (**D, I**) Percentage of female beetles alive at 2 wk postexposure. (**E, J**) Mean weekly egg production by female hosts that did not acquire an infection. All error bars are 95% CL. Vertical dotted lines and numbers above (**A–E**) indicate the boundaries of the 6 age classes used in (**F–J**).

Hosts were pooled into 6 age classes as before to determine if prevalence and intensity were affected by the different combinations of host properties represented by that classification. Prevalence (Fig. 3C) did not differ among age classes in female hosts ($\chi^2_4 = 2.38$, $P = 0.665$), but it did differ among male hosts ($\chi^2_5 = 17.02$, $P = 0.004$). Examination of confidence limits (Fig. 3C) suggests that low prevalence in age class 3 contributed to the difference in males. Prevalence in males was consistently

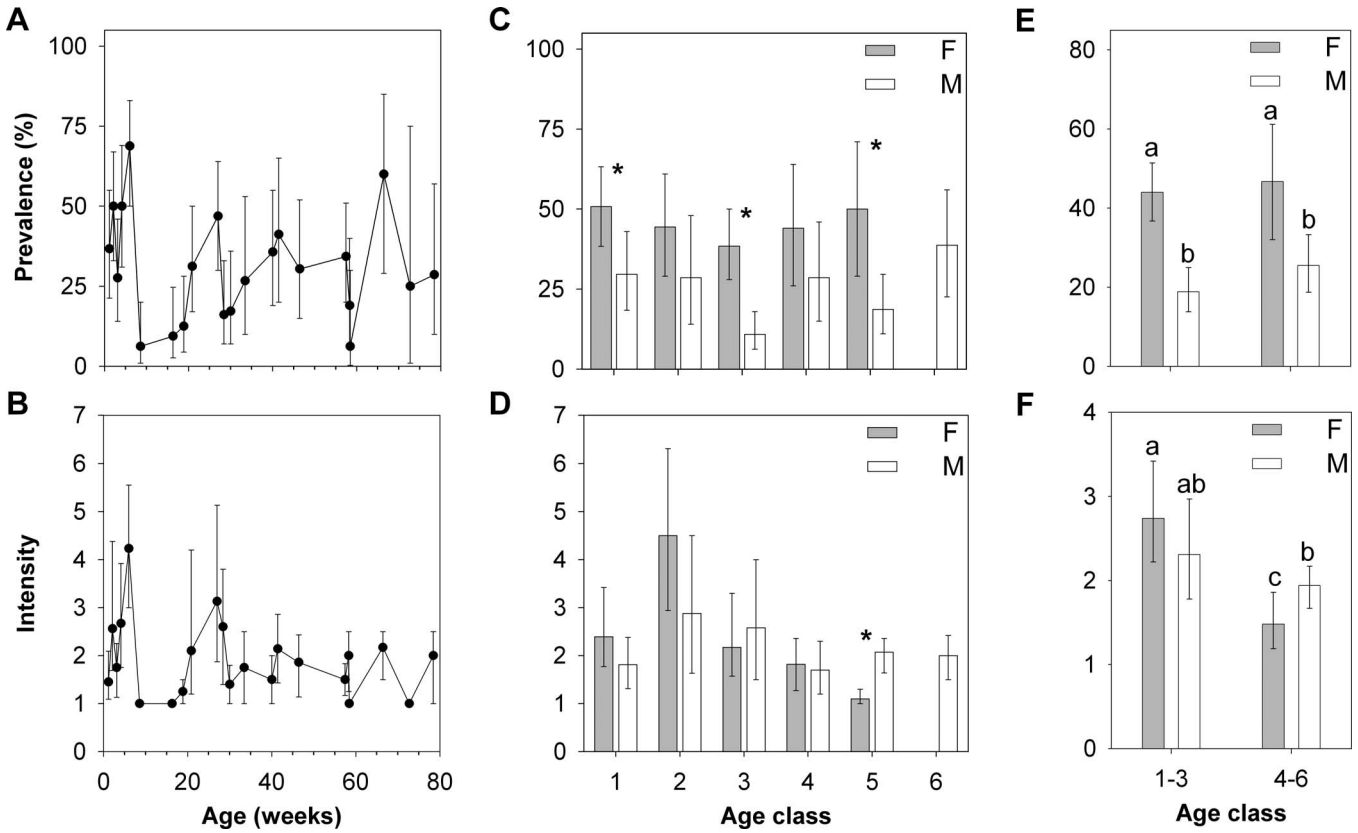

FIGURE 3. Changes in infection over time resulting from exposure to eggs of *Hymenolepis diminuta* for *Tribolium confusum* in individual cohorts (**A–B**), grouped into 6 narrow age classes (**C–D**) or grouped into 2 broad age classes (**E–F**). (**A, C, E**) Prevalence (percentage of beetles with 1 or more cysticercoids). (**B, D, F**) Intensity (mean number of cysticercoids per infected beetle). All error bars are 95% CL. F = female beetles. M = male beetles. In (**C–D**), * = significant difference ($P < 0.05$) in comparisons between sexes within an age class. In (**E–F**), samples with the same letter above the bar do not differ ($P > 0.05$).

lower in males than females (Fig. 3C), but statistical testing (Fisher exact test) supported this only for age classes 1 ($P = 0.024$), 3 ($P < 0.001$), and 5 ($P = 0.008$). Overall prevalences, ignoring host age, were 44.5% in 220 females and 21.6% in 333 males (Fisher's exact test, $P < 0.001$). Differences of intensity among age classes (Fig. 3D) were marginally significant in female hosts (Mood's median test, $P = 0.080$), tending to decline with age, but were not different in male hosts ($P = 0.956$). Intensity was similar between sexes except in age class 5, where intensity in males was significantly higher (Mood's median test, $P = 0.003$). Overall mean intensities, ignoring host age, were 2.47 in females and 2.12 in males (bootstrap *t*-test, $P = 0.254$).

The analysis of prevalence and intensity based on 6 age classes of hosts produced few significant results with respect to age. This may be due to a lack of effect, or that the magnitude of effects was too low to be detected given the distribution of sample sizes. Hosts were further pooled into just 2 classes: young and old. Young hosts (age classes 1–3, age 0–30 wk) had generally high survival and fecundity and old hosts (age classes 4–6, age 30 wk or older) had generally poor survival and low fecundity (Fig. 2). Prevalence (Fig. 3E) did not vary between young and old female beetles or young and old male beetles, but male beetles in both age classes had a lower prevalence than in females. Intensity in young male and female beetles (Fig. 3F) was similar. Intensity did not decline in old males, but old females had lower intensity than young females and also than old males (Fig. 3F).

Mean volume of cysticercoids from single-parasite infections was 0.0195 ± 0.0034 mm^3 (n = 73). Effects of host age and sex were tested by factorial ANOVA following $\log_{10}$ transformation of volumes. There was no effect of host sex ($F_{1,72} = 1.56$, $P = 0.216$) or age ($F_{4,72} = 1.08$, $P = 0.137$) and there was no host sex/age interaction ($F_{4,72} = 1.18$, $P = 0.329$).

DISCUSSION

This is the first study to evaluate infections of *H. diminuta* over the entire life span of *T. confusum*. With dramatic changes noted in host mortality and fecundity as the hosts aged, it was surprising that their parasite infections seemed relatively insensitive to these changes.

The basic survivorship curves for *T. confusum* in this study were similar in shape and duration to those reported previously (Pearl et al., 1941), with periods of initial and late high mortality. The only major difference noted was that the intervening period of relatively low mortality in this study was longer than observed by Pearl et al. (1941). Similarly, temporal changes in fecundity were similar to patterns reported previously (Maema, 1986), with fecundity increasing for several weeks and then declining. The results in the present study would appear to en-

compass and reflect the normal lifetime of a typical population of *T. confusum* under culture conditions.

Previous studies exposed *T. confusum* of different ages to eggs of *H. diminuta*. Dunkley and Mettrick (1971) reported that the number of cysticercoids recovered per beetle increased with beetle age over the narrow range from 5–6 to 15–16 days (corresponding to class 1), which is corroborated by the continuous increase in prevalence and intensity during the first 5 wk of the present study. Keymer (1982) reported between 2 and 14 wk posteclosion (corresponding to classes 1 and 2) an exponential decline from about 16 to 2 mean parasites per host. Kelly et al. (1967) examined young, middle-aged, and old (corresponding to classes 1, 3, and 5) virgin *T. confusum* and reported that both prevalence and intensity were high in young and middle-aged females and declined in the old females, but were highest in middle-aged, compared to young or old, males. These 2 studies (Kelly et al., 1967; Keymer, 1982) contrast with each other and with results of the present study, which indicated that prevalence, although lower in males than females, did not vary with age in either sex, and that intensity declined with age in females, but not males. The methods used in the other studies were not reported with sufficient detail to assess whether a methodological explanation for the differences exists. Strains of *T. confusum* vary in susceptibility to *H. diminuta* (Yan and Norman, 1995) and perhaps the populations of *T. confusum* used by Kelly et al. (1967), Keymer (1982), and in the present study also vary in their age-related response to infection by *H. diminuta*.

This is the first study to examine the size of cysticercoids resulting from infection of such an age range of hosts, and no age-related variation was found. This corroborates results from separate experiments (Shostak et al., 2006) in which the shape of cysticercoids varied minimally, or not at all, in hosts 2–52 wk old at the time of exposure.

The considerable variation among cohorts of similar age was surprising because it was expected that age-related changes in infections would be gradual and smooth, as were the changes in host properties. The source of variation may be host-age–dependent variation in the infection process, host-age–independent variation among cohorts in some property that affects the infection process, or variation resulting from the methodology. Many aspects of *T. confusum* change markedly with age, often over relatively short time spans. Response to pheromones increases dramatically during the first 3 wk of life, then declines markedly in females but not males (Obeng-Ofori and Coaker, 1990). Egg production increases during the first 7 wk, then declines until at least 12 wk of age (Maema, 1986), although in *Tribolium castaneum* a general decline in egg production from about 1 to 17 wk is punctuated by periods of sharp increases (Mertz, 1969). The overlap of different physiological and behavioral processes as the host ages could result in irregular patterns of susceptibility to infection such as those observed. Populations of *Tribolium* spp. in culture undergo fluctuations in numbers and demographics for a variety of reasons (Mertz, 1969). Some cohorts in the present study exhibited unusual survivorship curves. It may be that pupae removed from the stock cultures at different times vary in properties that affect infection probability when adult. The nature of these properties is unknown, although laboratory populations of *T. confusum* subject to selection have yielded strains with large variation in susceptibility to *H. diminuta* (Yan and Norman, 1995). The stock colony of *T. confusum*, from which pupae were chosen to populate each cohort, has been allowed to breed freely without intentional selection, but presumably harbors a similar range in genetically based susceptibility. Subtle shifts in genetic makeup as the colony goes through population cycles could result in age-independent variation among cohorts. These explanations must be regarded at present as hypotheses requiring specific testing, such as through longitudinal studies in which the same cohort is tested at different times.

Methodological explanations for variation in infection among cohorts can be addressed more directly. Beetles were exposed individually, not in groups, reducing the chance that some event during the exposure would affect the infection results for the whole group. There was small variability in number of eggs placed in different arenas, and in average number of eggs used among treatments. Large differences in egg density could affect resulting infections (Keymer, 1981), but parasite egg density was not a significant factor in this study. The logistic requirement of conducting the infections over a period of about 3 mo raises the possibility of a systematic change in infectivity of eggs of *H. diminuta* that beetles were exposed to, or in ambient conditions under which exposures were conducted. This would be evident as an effect of the order in which infections were done, but again this effect was not significant. Without doubt, some of the variation among cohorts relates simply to the sample size of hosts available. Prevalence and intensity are difficult to estimate with high confidence due to the aggregated distribution typical of helminth infections (Rozsa et al., 2000). Although the present study used about 30 hosts in each exposure, which is typical (Kelly et al., 1967; Dunkley and Mettrick, 1971), sample-size problems become more acute in attempts to evaluate infections in beetles nearing the end of their life where, by definition, few have survived.

The only major observed effect for host age on infection was a decline of infection intensity in the older (class 4–6) females. This corresponded with a decline in the proportion of females, indicative of differential mortality at some point, which can occur in the absence of infection (Pearl et al., 1941). Host mortality severely confounds the interpretation of intensity determined at the time of necropsy. Intensity-dependent mortality is reported for *T. confusum* infected with *H. diminuta* (Keymer, 1980) and would remove heavily infected individuals, resulting in lower intensity at necropsy than immediately after infection. The present study did not generate the high intensities that Keymer (1980) did using multiple exposures, and a more recent study (Hurd et al., 2001) suggests that infection with *H. diminuta* may actually increase life span in female *Tenebrio molitor*. In the present study, host sex was determined only at the time of necropsy, as insufficient beetles were available to sample sex ratios at earlier times. Although there may have been mortality of heavily-infected females, it should also be noted that there was increased mortality in older beetles even during the preexposure fast, suggesting that mortality in the older beetles was more likely due to a general weakening of the beetles and not by the parasites per se. Therefore, although the low-intensity infections indicate clearly that the old females would contribute relatively few cysticercoids to the transmission process, it cannot be resolved whether they failed to acquire the infection initially, or whether they acquired similar or even higher num-

bers of parasites than younger beetles, followed by increased parasite-induced mortality.

There are a number of behavioral and physiological barriers in the infection process that must be overcome to produce a successful infection of *H. diminuta* in *T. confusum*, even under simplified laboratory conditions. The initial barriers are behavioral, where the beetle must first encounter a viable egg and then ingest it (although these behaviors may be influenced by underlying physiological processes). Subsequent barriers are largely physiological. The oncosphere must be released from the egg membranes and enter the hemocoel, the parasite must survive within the hemocoel long enough to develop to the cysticercoid stage, and the host must survive the infection. It is this very complexity of the infection process that may explain the relatively uniform results of infection over the life span of *T. confusum* when such dramatic changes are taking place in the host. Some barriers may become less effective as the host ages and others become more effective. Previous studies (Kelly et al., 1967; Keymer, 1982) attribute age-related differences in infections of *T. confusum* to changes in host susceptibility or resistance, although other explanations such as differences in host food intake associated with growth and maturation of the host (Dunkley and Mettrick, 1971) have been offered. The present study observed a general decline in beetle activity with age, which may reduce encounters and feeding on parasite eggs and hence reduce levels of infection in older hosts. The increased fasting and postexposure mortality of the hosts suggests a weakened physiological state of the host that might promote establishment of parasites, and hence increase the proportion of ingested parasites that successfully establish in older hosts. However, the similarity of parasite sizes in hosts of all ages suggests that the older hosts are still acquiring sufficient nutrients to support normal parasite growth. Intriguing as these observations may be, critical experiments are necessary to discriminate among potential barriers and these are lacking at even a single age, let alone across the entire life of *T. confusum*.

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