The development of spicules in *Heligmosomoides bakeri* (Nematoda, Heligmosomidae)

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Abstract

The spicules of male parasitic nematodes are key morphological features, which vary between species in shape and length and are used often for species identification. However, little is known about spicules and particularly if/how their length varies during growth. We first assessed the degree of variation in spicule length of male *Heligmosomoides bakeri* 21 days post infection (PI), and then in two follow-up experiments measured spicule lengths at half daily/daily intervals between days 6 and 14 PI. Mean spicule length in 21-day worms was 0.518 mm with a range of 94 µm, and variation between the two spicules of individual worms from 2 to 32 µm. Spicules were first detectable on day 6–6.5, after which their lengths increased until day 7 PI (mean = 0.61 and 0.59). This was followed by significant contraction, initially relatively quickly over the following 48 h and then more slowly over a longer period, stabilizing by days 10–14, with only minor further reduction in length. We conclude that the length of spicules varies significantly over the first few days after they have formed, and, consequently, the age of worms is an important factor for consideration when spicule lengths are measured for experimental/diagnostic or taxonomical purposes.

Introduction

Spicules are cuticular structures present in the reproductive system of most (e.g. *Trichostrongylus* spp., *Caenorhabditis elegans*), although not all (e.g. *Aspiculuris tetraptera*), species of nematodes. They are usually paired, and members of a pair may be of equal size or unequal (Crofton, 1966), may differ in shape and length (Baylis, 1928) and can be twisted or untwisted as, for example, in *Heligmosomoides bakeri* (fig. 1). Spicules are important in nematode taxonomy because they vary in length and shape between species, even between closely related ones (Croll & Matthews, 1977). Hence, the characteristics of spicules are exploited as useful morphological features for distinguishing between nematode species (e.g. in the genera *Trichostrongylus* and *Haemonchus*; MAFF, 1986; Lichtenfels *et al.*, 1994; Kaufmann, 1996; Taylor *et al.*, 2015).

Although in some cases spicules may function as true intromittent organs, by forming a duct down which spermatozoa pass into the vagina of female worms, they are generally considered not to be true intromittent organs (Crofton, 1966). Nevertheless, they play an essential role in facilitating the entry of sperm cells into the reproductive system of female worms (Crofton, 1966; Barr & Garcia, 2006). Guided by the gubernaculum, spicules are inserted into the female's vulva and help to attach the male to the female during copulation, as well as dilating the vulva to allow sperm transfer against the high internal hydrostatic pressure present in the pseudocoelomic cavity, within which the uterus and ovary of the female worms reside (Barr & Garcia, 2006).

Little is known about the development of spicules in parasitic nematodes (although see Emmons (2005) and Barr & Garcia (2006) for *C. elegans*). In *C. elegans*, they arise from spicule cells that are first evident in third-stage larvae (L3), and are followed by initiation of spicule formation in the early fourth-stage larvae (L4). The spicule elongates in the late L4 stage following the anterior migration of two B-lineage proctodeal cells, which attach to the base of each spicule. These form a ridge, which constitutes a mould for the final shape of the spicule and around which spicule cells spread. As the spicules elongate, a hardened sclerotic cuticle forms around them (Sulston *et al.*, 1980; Emmons, 2005). However, their rate of growth and the extent to which spicules vary in length over the life span of nematodes have not been studied, but it is likely that age accounts for some of the variation in recorded spicule length within species, particularly during early stages of infection during spicule formation and when worms are still growing. Therefore, to shed some light on the development of spicules in *Heligmosomoides* spp., experiments were conducted using the laboratory mouse passaged *H. bakeri*, a species that is very closely related to *Heligmosomoides polygyrus*, and often considered to be a subspecies (Durette-Desset *et al.*, 1972). The aim of these experiments was
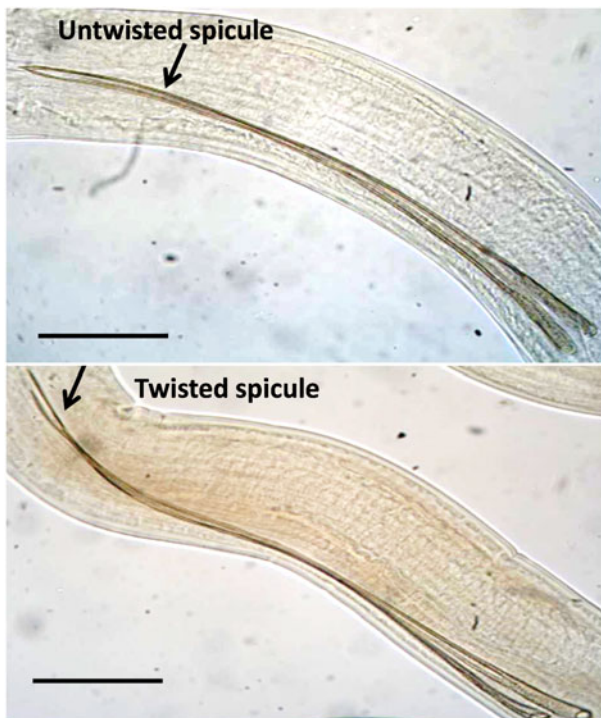
Fig. 1. Spicules of *Heligmosomoides bakeri*. Worms were collected from BKW mice at day 14 PI, and preserved in 80% ethanol until the spicules could be photomicrographed. Black arrows point to the untwisted and twisted spicule pair. Scale bars: 0.01 mm.

first to assess the degree of variation in the length of spicules in mature male worms, and then to determine when spicules first appear during the development of worms, and the degree to which spicule length changes as the worms mature.

Materials and methods

Mice and parasites

In this paper we refer to *H. bakeri* (previously known as *Nematospiroides dubius*, *H. polygyrus* and *H. p. bakeri*; Cable *et al.*, 2006; Behnke & Harris, 2010), as the parasite maintained in laboratory mice and *H. polygyrus* as that in wild wood mice (*Apodemus sylvaticus*) from Europe. Laboratory mice (BKW strain, purchased from Bantin and Kingman Universal Ltd, Grimston, Aldbrough, Hull, North Humberside, HU11 4QE , UK) were housed under standard conditions in individually ventilated cages in which water and standard mouse chow were provided *ad libitum*. Two replicate experiments were conducted, differing only in dose of infection administered to the mice, and some variation in days when mice were culled for provision of worms (details in the Results). Male *H. bakeri* were obtained from mice that had been infected orally with 300 (Experiment 1) or 250 (Experiment 2) infective (L3) larvae. Mice were killed humanely using the approved Schedule 1 method of exposure to an increasing concentration of carbon dioxide (CO_2) gas in an enclosed chamber. They were culled at times corresponding to exact 12 or 24 h intervals from day 6 post infection (PI) and their small intestines were placed into a Petri dish containing Hanks' phosphate-buffered solution on an incubator maintained at 37°C. After one hour, worms were seen to have migrated into the solution. The parasites were carefully collected with a pipette into a storage tube containing 80% ethanol, and were stored at room temperature for about one month prior to microscopic examination and photomicrography.

Measurement of worms and their spicules

Preserved individual worms were carefully picked out and placed onto glass slides using fine watch-makers forceps. They were then covered with one or two drops of lactic acid to clear them and to enable visualization of the spicules. Slides were examined under an Olympus light microscope and the spicules were photomicrographed using a high-resolution optic camera with a digital eye lens at ×20 magnification. Digital images were then visualized on a computer screen and each spicule of a pair, on each randomly selected worm, was measured four times with an Image J application (Lewis *et al.*, 2006; Schneider *et al.*, 2012). The length of the male worm was also recorded in each case.

Statistics

Heligmosomoides bakeri has two spicules of almost equal length. The average length of each of the two individual spicules from each selected worm was then calculated, converting Image J units to millimetres based on a photomicrograph at ×20 of a calibrated slide, on which each division represented 0.01 mm. The average of four measurements of the calibration slide was used as a standard conversion scale for all measurements. For each worm measured, spicule length of each of the two individual spicules was first expressed as the mean value of four measurements and these means were used to calculate group mean lengths. Spicule lengths are expressed as means ± standard error of the mean (SEM). The length of the worms was also measured by an identical procedure to that for spicules, and spicule length was then expressed as a percentage of the length of male worms. All measurements are given in millimetres, micrometres or as percentages. Statistical analyses were carried out in IBM SPSS version 24 (1 New Orchard Road, Armonk, New York 10504-1722, USA). Spearman's test of correlation was used to assess the significance of changes in length of spicules and worms over time, and the Mann–Whitney *U* test for comparisons between values on two different days within experiments or on the same day between experiments. $P \leq 0.05$ was considered to be significant.

Results

Spicule length in mature worms

First, we measured spicule length in worms from mice that had been infected for 21 days and harboured mature worms. Female worms from these mice would have been laying eggs for about 12 days. The average length of 50 spicules was 0.52 ± 0.003 mm, with a range of 0.094 mm from the shortest at 0.467 to the longest at 0.561 mm. This represented a mean of $8.35 \pm 0.123\%$ of the body length of male worms ($n = 50$,) with a range from 7.01% to 11.08%. The frequency distribution of spicule lengths is shown in fig. 2, where it can be seen that 58% of the spicules fell within the 0.50–0.52 mm limits.

For the 25 worms used in this analysis, we compared the length of the individual spicules in each pair, and the difference
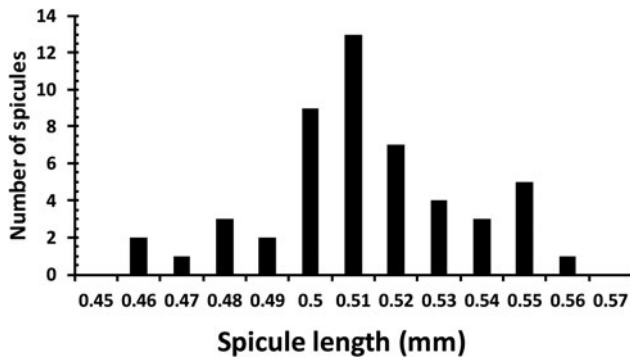
Fig. 2. Frequency distribution of the spicules of 21-day-old worms. Fifty spicules from twenty five, 21-day-old worms were measured.

in lengths varied from 2 μm to 32 μm (data not illustrated). In ten of the worms the difference in length of the two spicules was ≤10 μm, and in 11 worms ≥15 μm (but up to a maximum of 32 μm). These differences represent a range of 0.36–6.86% of the length of spicules, and since variation between the spicules was small, in further analyses the lengths of each of the two spicules of each worm were treated as two independent estimates.

Growth of worms from the L4 to adult stage

Two experiments were carried out in which mice were infected with either 250 or 300 infective larvae and culled at a range of time points between days 6 and 14 PI. The growth of worms during this period, as reflected in changes in the length of the worms, is illustrated in fig. 3. The results of both experiments were similar, although growth was slower initially in Experiment 1, in which the mice had been infected with a higher dose of larvae. The length of worms increased significantly from day 6 until day 14 in both experiments (fig. 3; Experiment 1, $r_s = 0.794$, $n = 57$, $P < 0.001$; Experiment 2, $r_s = 0.863$, $n = 154$, $P < 0.001$), but the slight increase between day 10 and day 14 in Experiment 2 was not significant ($U_{20,20} = 268.0$, $P = 0.068$), indicating that growth had ceased by day 10 PI. By day 14 the mean length of worms did not differ significantly between the two experiments (7.29 ± 0.118 and 7.07 ± 0.070 mm, for Experiments 1 and 2, respectively; $U_{10,20} = 59.0$, $P = 0.074$).

Spicule length in worms during growth from the L4 to adult stage

In Experiment 1, no discrete spicules were evident on day 6 PI, although in some worms there appeared to be signs of spicule formation (not illustrated). In Experiment 2 spicules were already detectable in some worms six days PI, and in those worms that had some signs of spicule formation, spicules were seen to be very thin, and some were very short (fig. 4), with length ranging from 0.30 to 0.64 mm (fig. 5) and with a mean of 0.46 ± 0.023 mm (fig. 6a). At this time, worms were observed to be moulting from the L4 to the pre-adult stage, as reported by Bryant (1974), so the variation in length on day 6 reflected initiation of spicule formation.

In Experiment 1, spicules were first evident in some worms on day 6.5 PI, with a mean length of 0.33 ± 0.025 mm, whereas by this time in Experiment 2 spicules appeared more elongated along the length of the body (fig. 4), with a mean length of
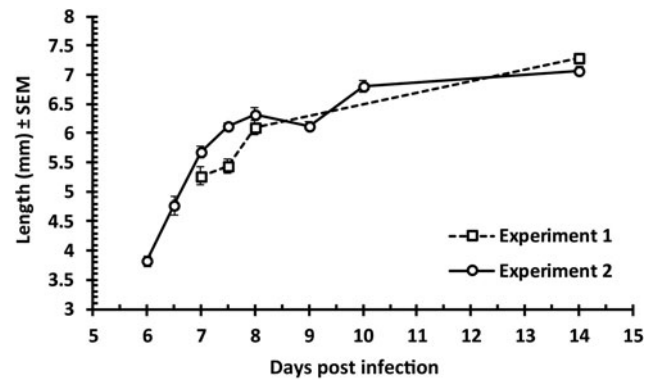

Fig. 3. Growth of male *Heligmosomoides bakeri* between days 6 and 14 PI in Experiments 1 and 2. In Experiment 1, the range of values (mm) and sample size (*n*) for days 7 to 14 were as follows: 4.43–6.70 (17), 4.24–5.91 (10), 5.77–6.86 (10) and 6.55–7.79 (10), respectively. In Experiment 2, the range of values for days 6 to 14 were as follows: 3.21–4.42 (14), 3.54–6.00 (20), 4.71–6.24 (20), 5.76–6.66 (20), 5.49–7.26 (20), 5.51–6.56 (20), 6.07–7.44 (20) and 6.54–7.88 (20), respectively.

0.59 ± 0.008 mm (fig. 6a). In both experiments spicule length peaked on day 7 PI, with mean values from both experiments very similar (0.61 ± 0.011 mm in Experiment 1 and 0.59 ± 0.006 mm in Experiment 2), differing only by 19 μm, and in each case with a smaller range of individual lengths than earlier (Fig. 5 for Experiment 2). The elongation of spicules by this time PI can be seen in fig. 4 (Experiment 2).

After day 7 PI, spicules in both experiments contracted in length over the following 24 h (fig. 6a at a similar rate, with lengths still very similar on day 8 PI (Experiment 1 = 0.57 ± 0.005 and Experiment 2 = 0.56 ± 0.004) and thickened in appearance (fig. 4). From day 9 PI spicule length began to stabilize, with a length of 0.56 ± 0.004 mm in Experiment 2 (figs 4 and 6a), but somewhat shorter in Experiment 1 (0.53 ± 0.003 mm). In both experiments there was then little further change in spicule length, although mean values in Experiment 1 were marginally shorter than those recorded in Experiment 2, until day 14 PI (Experiment 1, 0.51 ± 0.005; Experiment 2, 0.53 ± 0.004).

In Experiment 1, the reduction in spicule length from the peak mean length on day 7 until day 14 was highly significant ($r_s = -0.762$, $n = 96$, $P < 0.001$) as was that in Experiment 2, between day 7 and day 10 PI ($r_s = -0.518$, $n = 240$, $P < 0.001$). In both experiments there was a highly significant difference between spicule lengths recorded on days 7 and 14 (Experiment 1, $U_{20,20} = 89$, $P = 0.002$; Experiment 2 $U_{40,40} = 114$, $P < 0.001$). There was also a significant difference in the length of spicules between days 10 and 14 PI in Experiment 2 ($U_{40,40} = 580$, $P = 0.034$), indicating further shrinkage, although the reduction in length was smaller than recorded earlier during infection (fig. 6a).

Temporal changes in spicule length as a percentage of worm length

In Experiment 1, the mean length of spicules, expressed as a percentage of body length of male worms, shortened consistently from day 7 PI (12.86 ± 0.289%) until day 14 PI (fig. 6b), when the mean value was 7.1 ± 0.12%. The change in values over time was highly significant ($r_s = -0.902$, $n = 74$, $P < 0.001$). In Experiment 2 (fig. 6b), percentage changes followed the same pattern, with peak percentage of body length slightly earlier on day 6.5 (12.55 ± 0.287%), after which values fell consistently until
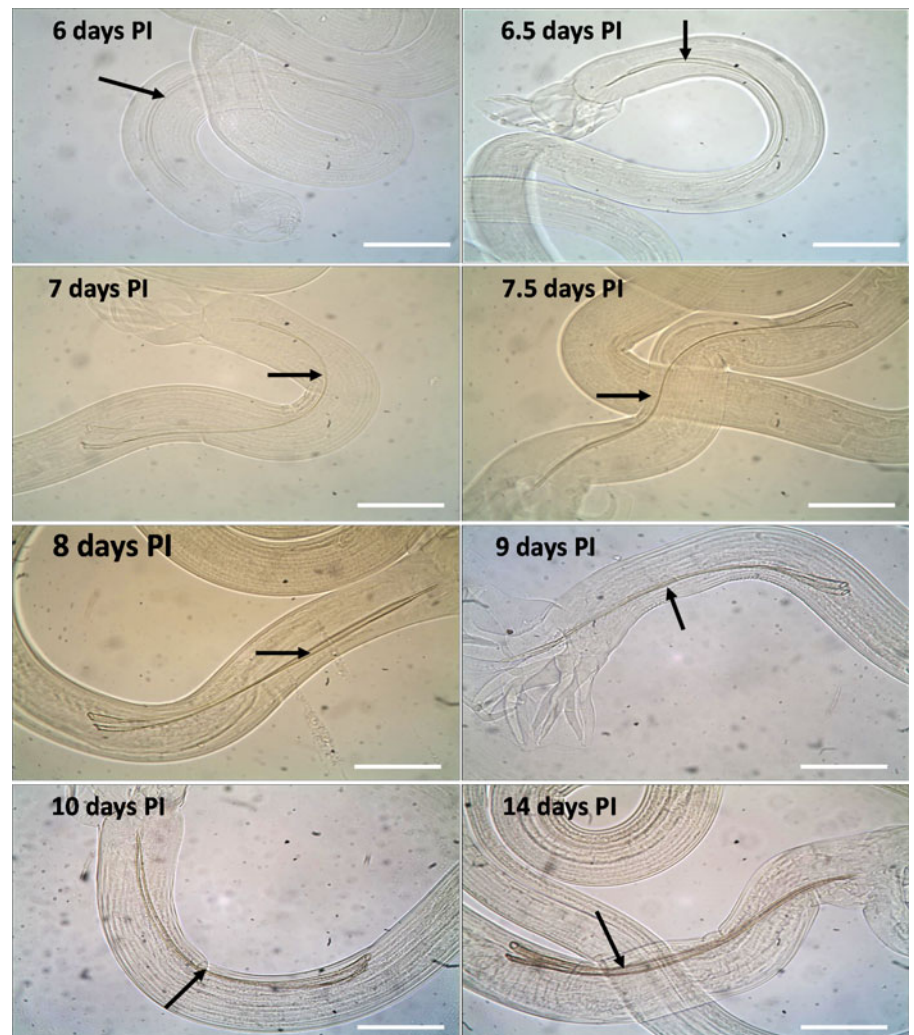
Fig. 4. Spicule development at half-daily and daily intervals (Experiment 2). Black arrow points to the spicules' developmental progression, initially showing a thin elongation along the length of the body before shortening and thickening as the worm matures. For further details, see text. Scale bars: 0.01 mm.

day 14 (7.54 ± 0.067%). This fall in values from day 6 to day 14 was highly significant ($r_s = -0.838$, $n = 280$, $P < 0.001$).

Discussion

In this paper we have shown that the age of male *H. bakeri* worms is a crucial factor affecting spicule length. Spicules can be detected microscopically first only six/six and a half days after infection, after which they lengthen, peaking on day 7 and then shrinking in length over the following week, even marginally between days 10 and 14 PI. The lengths recorded on days 10, 14 and 21 PI are largely in agreement with those recorded by Roe (1929) and Durette-Desset *et al.* (1972), both of whom measured worms from house mice, rather than wood mice.

The life cycle of *H. bakeri* in laboratory mice has been investigated by several authors (Spurlock, 1943; Ehrenford, 1954; Fahmy, 1956; Bryant, 1973). However, Spurlock (1943), Ehrenford (1954) and Fahmy (1956) did not give a complete account of the life cycle of the parasite. Bryant (1973) provided the most comprehensive account of the life cycle of the parasite from free-living to parasitic stages, reporting that the second parasitic moult takes place between six and seven days (144–166 h) after infection. At this stage, the L4 synthesize a new cuticle

from the hypodermis and then ex-sheath from the old cuticle as pre-adults, and this agrees closely with our own observations (6–7 days PI). In the current work, growth of worms was fastest between days 6 and 7 PI, after which it slowed, but did not cease entirely after the L4-juvenile moult, continuing for a few more days as reported also by Bryant (1974), before stabilizing by days10–14 PI.

In their descriptions of *H. polygyrus* and *H. bakeri*, different authors have given varying values for the length of the spicules of these parasites, ranging from 0.420 to 0.620 mm (Baylis, 1926; Roe, 1929; Dujardin, 1845; Durette-Desset *et al.*, 1972; Genov & Jančev, 1981), but none seem to have taken account of the age of the parasite on which the measurement was taken, nor considered that worms recovered from *Mus* spp. and *Apodemus* spp. may, in fact, be different species (Durette-Desset *et al.*, 1972; Tenora & Barus, 2001; Tenora *et al.*, 2003; Cable *et al.*, 2006). Most workers have relied on worms obtained from culled wild rodents (*A. sylvaticus*, *Apodemus flavicollis*, *Mus musculus*) and, therefore, could not account for the age of the parasite when the spicules were measured. In each case, a mixture of worms of different ages was most likely examined, since wild mice acquire worms throughout their lives and worm burdens are heavier in older age classes (Elton *et al.*, 1931; Gregory *et al.*, 1992; Abu-Madi *et al.*, 1998; Behnke *et al.*, 1999, 2001).
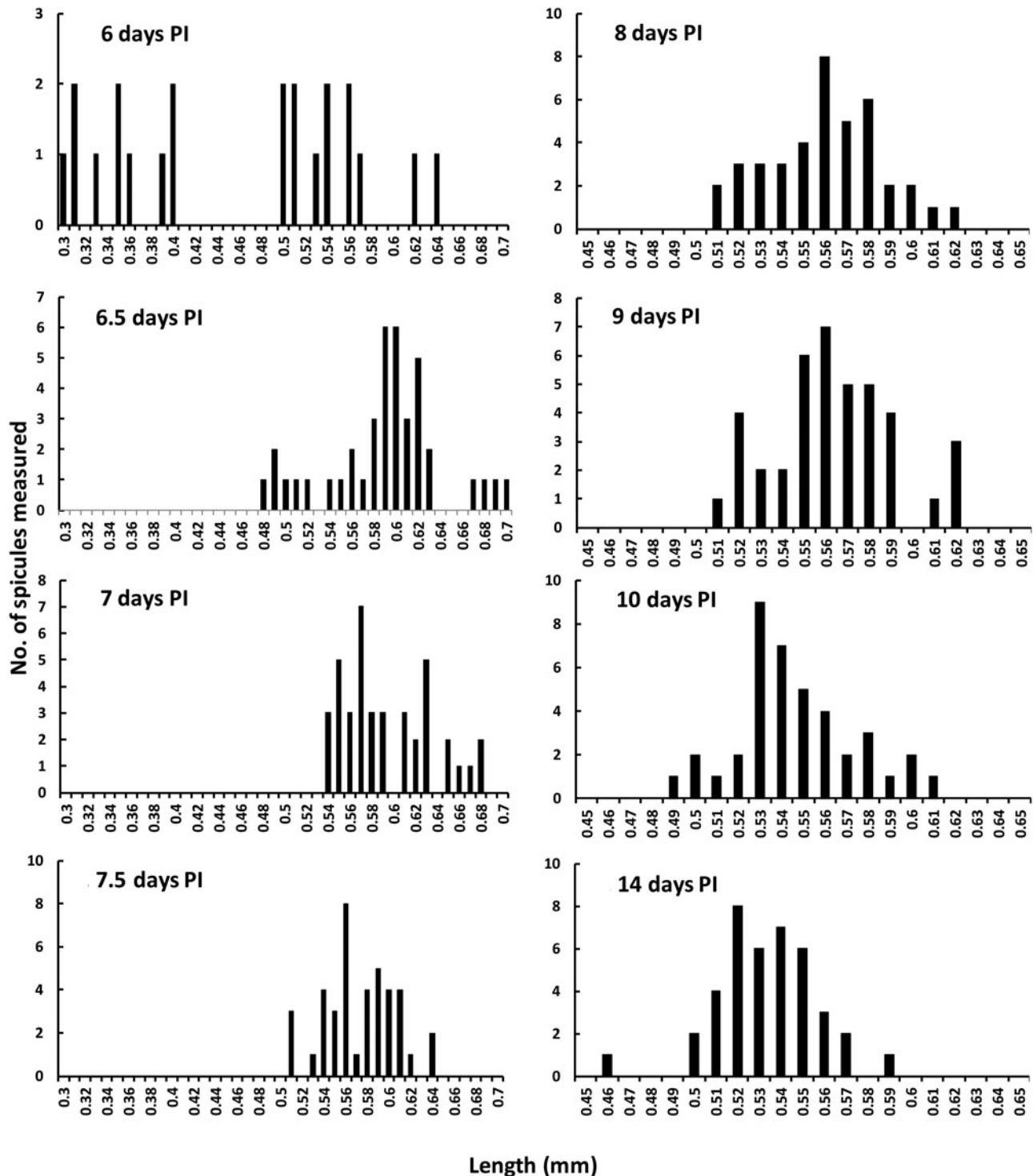
Fig. 5. Frequency distribution of spicule lengths of *H. bakeri* in Experiment 2 between days 6 and 14 PI, inclusive. Worms were photomicrographed and spicule lengths were measured as described in the text. Each pair of spicules, on each randomly selected worm, was measured separately four times and values were averaged for individual spicules. Spicule lengths are means in mm, and sample sizes were 22 for day 6 PI, and 40 for all other time points illustrated in the figure. Note that the increments for length classes differ between the set of panels on the left and right, to emphasize in each case sequential changes in frequency distribution over time.

Although it may have been possible to infect anthelmintic-treated wild-caught wood mice with *H. polygyrus* for the current study, the possibility that the animals may have been previously infected, and growth of worms may have been affected by acquired immunity, could not be eliminated (Bartlett & Ball, 1974). Hence, the focus of our work was on the laboratory-maintained *H. bakeri* during primary infections in naive laboratory mice.

The marked increase in spicule length observed in the present study between day 6 and 7 PI (fig. 6a) coincided with and followed the L4-juvenile moult (Bird & Bird, 1991). At this stage,
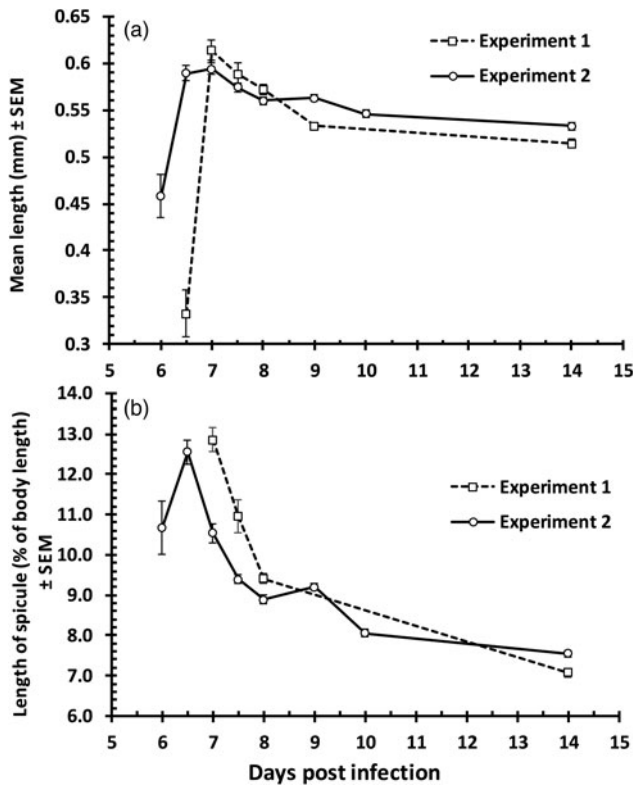
Fig. 6. Changes in the length of spicules during growth. (a) Temporal changes in spicule length. The range of values and sample sizes (*n*) in Experiment 1 for days 6.5 to 14 were as follows: 0.239–0.400 (6), 0.534–0.664 (16), 0.510–0.678 (20), 0.529–0.608 (20), 0.501–0.550 (20) and 0.471–0.541 (20), respectively. The range of values and sample size (*n*) in Experiment 2 for days 6 to 14 were as follows: 0.303–0.637 (22), 0.479–0.700 (40), 0.538–0.684 (40), 0.506–0.644 (40), 0.514–0.617 (40), 0.510–0.621 (40), 0.491–0.606 (40) and 0.462–0.589 (40), respectively. (b) Temporal changes in spicule length as a percentage of worm body length (% of body length). In Experiment 1, the range of values and sample size (*n*) for days 7 to 14 were as follows: 11.26–14.30 (14), 9.28–16.00 (20), 8.50–10.54 (20), 6.05–7.88 (20) and 7.01–11.08 (20), respectively. In Experiment 2, the range of values and sample size (*n*) for days 6 to 14 were as follows: 7.60–14.91 (16), 9.56–17.09 (40), 8.72–13.85 (40), 8.16–11.18 (40), 7.83–11.13 (40), 8.17–10.09 (40), 6.75–9.25 (40) and 6.73–8.45 (40), respectively.

it is most likely that proteins comprising the spicule are first laid down by the secreting cells. Cuticle protein secretion of some nematodes – for example, *C. elegans* – has been found to be higher during moulting (Bird & Bird, 1991). The increase in length of the spicule is paralleled by the rapid growth of the whole worm at this stage. After the L4-juvenile moult *H. bakeri* emerge from their histotrophic sites of development below the serosa in the *muscularis externa* (Liu, 1965) where they have been developing as L3 and L4 larvae, and then become entirely lumen-dwellers. The L4-juvenile moult facilitates greater flexibility of feeding (Bird & Bird, 1991), enabling the mouthparts of pre-adult worms to be adapted to a new environment and mode of feeding. Adult, lumen-dwelling stages feed on enterocytes from the villi (Bansemir & Sukhdeo, 1994), rather than on the tissues and fluids that surrounded them during their histotrophic phase in the intestinal walls.

Contraction in the length of the spicules from day 7 to 10 PI was reflected in both real terms (mm) and as a percentage of the body length of male worms. The latter was attributable initially to the continuing growth of worms in the period days 8–10 PI and, in both cases, also to the spicule proteins consolidating into the final structure (Bird & Bird, 1991; see Emmons, 2005 for

C. elegans). Thus, the relative stability in the length of the spicule observed after day 10 PI in the present work is because the worms at this point have fully matured with all of their reproductive structures now complete. Bryant (1973) reported that by 191 h PI, worms had passed from the mucosa into the lumen where they assume adult position coiling around intestinal villi in the duodenum (Bansemir & Sukhdeo, 1996). She first detected eggs in host faeces 240 h (ten days) after infection, slightly later than Ehrenford (1954). The latter author reported that eggs were first seen in faeces nine days PI and this agrees with our own observations that occasionally a few eggs can be detected on day 9 PI, followed by increasing numbers until day 14 PI when egg production tends to stabilize (Behnke & Parish, 1979). Therefore, by day 9 PI the spicules must be fully functional in some worms, although a degree of further shrinkage in length was apparent in our experiments over the following week. The reduction in mean percentage length (% of adult worm length) in this period is consistent with our observation that on average the worms were still showing some, albeit small, continuing increase in length after day 8 before achieving their final stable length 10–14 days PI.

The key conclusion from this study is that spicule length does change during worm growth and development, initially increasing from a barely detectable thin shape that first appears as the constituent proteins are being laid down on day 6 PI, and then expands for a day or so before contracting slightly as the structure is consolidated. The downward drift in the mean length of spicules, and the mean percentage of male worm length they represent, from days 8 to 14 PI is relatively smaller and indicates further contraction as the worms achieve their final mature length. Consequently, the age of worms is an important factor for consideration when spicule lengths are measured for experimental/diagnostic or taxonomical purposes: both the lowest and highest recorded values in a study may be attributable to relatively young worms in which the spicules have not yet consolidated to their eventual full adult size.

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