Chapter 5. The effects of amount and distribution of simulated rainfall on free-living development of *Haemonchus contortus* under controlled conditions

5.1 Introduction

Haemonchus contortus is the major species of sheep gastro-intestinal nematode in tropical and subtropical climates, and in regions were rainfall is summer-dominant, such as in the Northern Tablelands of NSW, Australia. In these regions, outbreaks of haemonchosis can result in high mortality of sheep. The ability to predict the timing and magnitude of *H. contortus* infective larval populations, and plan grazing and worm control strategies accordingly, is a key means of preventing these losses.

Little is known about the effects of rainfall- and moisture-related variables on egg hatch and transition to infective larvae (L3), although it is widely acknowledged (Gordon, 1948; Levine & Todd, 1975; Rossanigo & Gruner, 1995) that moisture availability is a key determinant of magnitude of pasture infectivity. However, there have been few attempts to quantify the relationship between rainfall and *H. contortus* development under conditions where moisture is under experimental control.

Plot experiments (reported in Chapter 4) to test the effects of timing, distribution and amount of simulated rainfall on *H. contortus* development showed the importance of the timing of rainfall relative to egg deposition. Recovery of pre-infective larvae from faeces was high (20-25%) following a single simulated rainfall event in the early stages of the experiment (1 or 4 days post-deposition), whereas a comparatively small proportion of eggs (15-16%) hatched in faeces which remained un-watered until 8 or 15 d post-deposition. Recovery of pre-infective larvae also increased with amount of rainfall (12-32 mm), and was higher at 4 d post-deposition when rainfall was administered in a single event over 1-5 h, compared with 3 split events over 36 h. There was negligible development to infective larvae (L3) in these

experiments, possibly due to the high evaporation rates experienced (typically 4-5 mm/day) leading to a rapid drying of faeces after simulated rainfall.

To test this hypothesis the present experiment re-examined the effects of rainfall amount and distribution under controlled conditions with lower evaporation rates. In addition, loss of free-living stages due to predation or disturbance of faeces by insects was eliminated (Waller & Faedo, 1996) by working with sterile soil samples.

5.2 Materials and methods

5.2.1 Conditions and experimental design

The experiment was conducted in two programmable incubators (MIR 253, Sanyo Electric Biomedical Co., Ltd.) in which temperature was regulated to mimic diurnal summer ground temperature recorded during the summer 2005 experiment reported in Chapter 4. Over the 14 d experiment, the mean daily mean temperature was 22°C, with a mean daily maximum and minimum of 39°C and 11°C, respectively.

Each incubator contained four shelves, on each of which 25 experiment units were placed. Experimental units consisted of a polycarbonate cylinder (100 mm height, 60 mm diameter) containing a layer of steam sterilised gravel (approximately 30 mm depth) underneath steam sterilised soil (screened loam; approximately 55 mm depth). All cylinders had a small drainage hole soldered into the side wall, at approximately 25 mm above the base, and a plastic ring at the top to prevent migration of larvae over the lip (see Figure 3.3).

Faeces used to contaminate experimental units (see Chapter 3, section 3.2.2) was mixed thoroughly by hand before worm egg count was determined on 3 x 9 g subsamples of the pooled faeces by a modified McMaster method (1 egg = 60 epg). A 9 g subsample of faeces (1708 epg; s.d. 267 epg) was placed on the soil surface of each cylinder on d 0. The experimental design was a 3 x 2 x 2 x 3 factorial, with 3 amounts (12, 24 or 32 mm) of simulated rainfall applied as either of two distributions, namely as a single application on the

day after faecal deposition (d 1; 16 h post-deposition) or in three smaller and equal split applications (ie. each application was one third of the total amount) on days 1, 3 and 6. Each combination of rainfall amount and distribution was applied in either the first week only, or repeated in the second week such that by d 14 the repeated application had received twice the total volume (ie. 24, 48 or 64 mm). In the second week, simulated rainfall was applied on d 8 (single application) or days 8, 10 and 13 (split application). The multi-factorial design of the experiment is shown in Table 5-1. The method used to simulate rainfall is described in Chapter 3 (section 3.3).

Table 5-1: Simulated rainfall event treatments by day, according to amount, distribution and repetition of rain. Blanks indicate no simulated rain applied.

Treatment factors			Amount of simulated rainfall applied (mm)						
			Day						
Amount (mm)	Distribution	Repetition	1	3	6	8	10	13	Total rain (mm)
12	Single	1st week	12						12
		1st & 2nd week	12			12			24
	Split	1st week	4	4	4				12
		1st & 2nd week	4	4	4	4	4	4	24
24	Single	1st week	24						24
		1st & 2nd week	24			24			48
	Split	1st week	8	8	8				24
		1st & 2nd week	8	8	8	8	8	8	48
32	Single	1st week	32						32
		1st & 2nd week	32			32			64
	Split	1st week	10.7	10.7	10.7				32
		1st & 2nd week	10.7	10.7	10.7	10.7	10.7	10.7	64

Harvesting of faeces and soil occurred at either of d 4, 7 or 14. There were 3 replicates per treatment combination, with each treatment combination allocated uniformly to incubator and shelf. In addition, 3 unwatered control cylinders were harvested at each sampling event to determine *H. contortus* development in the absence of added moisture.

5.2.2 Sampling & measurements

Temperature was recorded hourly on each shelf using Tinytag® climate data loggers (Gemini Data Loggers, Chichester, UK). Evaporative losses (mm/day) were determined from the disappearance of water contained in two polycarbonate cylinders (100 mm height, 60 mm diameter) located on each shelf.

All samplings were destructive. At each, faeces were collected, separated into two subsamples of approximately equal size and weighed. One subsample was dried to determine faecal moisture content (FMC; see Chapter 3, section 3.6) and the other subsample was used for intra-pellet enumeration of free-living stages (see Chapter 3, section 3.5). For enumeration of larval stages which had migrated from the faeces into the soil fraction (extra-pellet), the top 25 mm of soil from the experimental units was collected and processed according to the methodology described in Chapter 3 (section 3.8).

5.2.3 Statistical analysis

Recovery of each *H. contortus* free-living stage was expressed as a percentage of the total number of eggs deposited per experimental unit. Response variables investigated were recovery of: embryonated eggs; degenerate eggs; intra-pellet L1 and L2; intra-pellet L3, extra-pellet L3 and total L3. General linear models (GLM) were constructed to test the effects of experimental factors on each response variable. The main effects in the model were amount, distribution and repetition of simulated rainfall, day post-faecal deposition, incubator, shelf and all two-way interactions. In addition the continuous variable of FMC at sampling was included in the analysis. Treatment effects on FMC were also analysed with a GLM. Where the distribution of response variables in the GLM departed significantly from normality, a cube-root transformation was used and the suitability of the transformation confirmed using the Shapiro-Wilks test. All non-significant effects ($p>0.05$) were removed from the analyses and are not discussed. Least squares means and 95% confidence intervals (back-transformed where appropriate) are presented in the results. In some cases, raw means and standard errors

are also presented. All analyses were carried out using JMP-IN 5.1 software (SAS Institute Inc., NC, USA).

Cumulative daily evaporation (mm/day) was averaged within shelf (across incubators) and combined with cumulative daily precipitation rate (mm/day) to establish a Precipitation/Evaporation ratio (P/E) for each day, within each amount x distribution x repetition treatment. For example, if 24 mm was applied on d 1, and mean evaporation was 1.4 mm/day, the cumulative P/E at d 4 was $(24+0+0+0) \div (1.4+1.4+1.4+1.4) = 4.3$. Forward stepwise regression models were constructed to determine the day on which P/E best described mean extra-pellet L3 recovery (averaged over day of sampling within each treatment combination).

5.3 Results

5.3.1 Temperature and evaporation

Daily mean, minimum and maximum temperatures and mean daily evaporation rates are shown in Figure 5-1. The values are averaged across each incubator and shelf, and there was negligible difference in temperature between locations. For 36 h between d 6 and d 8, an error in the programming of the incubators resulted in the temperature in both chambers remaining at a constant 21° C. There were small differences in evaporation rate between incubators, however these were not significant ($p=0.247$). Shelf had a significant effect on evaporation ($p=0.003$), with the daily rate on the uppermost shelf (shelf 1; 2.9 mm/day) higher ($p<0.05$) than on the two lower shelves (shelves 3 and 4; 1.6 and 1.1 mm/day, respectively) but not significantly higher than shelf 2 (1.9 mm/day).

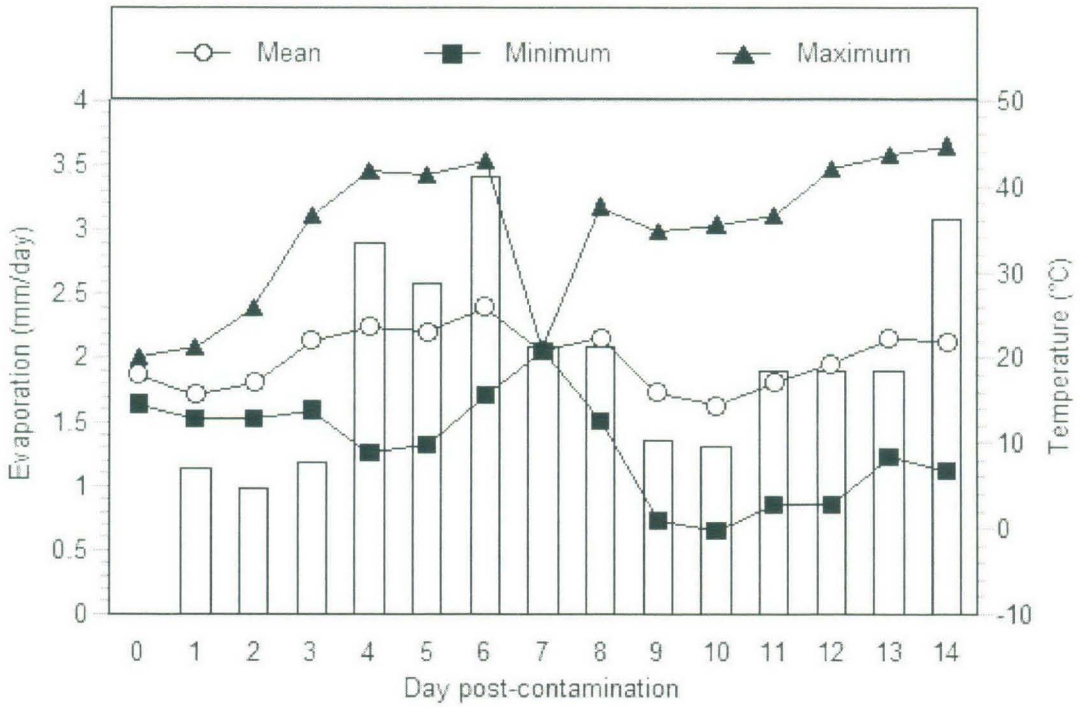

Figure 5-1: Mean daily evaporation rate and minimum, maximum and mean daily temperatures (averaged across incubators and shelves).

5.3.2 Recovery of infective larvae

Extra-pellet infective larvae were the dominant source of L3 throughout the experiment (81% of total L3 recovery), while the contribution of intra-pellet L3 to the total was small (19%) and decreased over time. Mean FMC was marginally higher in faecal samples containing L3 than those with no intra-pellet L3 ($p=0.033$; 66% compared to 59%) and the total amount of water applied was lower ($p=0.010$).

Recovery of extra-pellet L3 averaged across rainfall treatments increased over time from zero at d 4 to 6% at d 7 and 10% at d 14 ($p<0.001$). Both amount ($p<0.001$) and distribution ($p<0.001$) of simulated rainfall had a significant effect on L3 recovery. Recovery of L3 increased with each increment of simulated rainfall (Figure 5-2), while there were fewer L3 under the split distribution (Figure 5-3). The effect of repetition of rainfall was not significant ($p=0.21$). Recovery differed between shelves ($p<0.001$), with fewer L3 on the uppermost shelf (2.0%) than on the two lower shelves (shelf 3: 3.7%; shelf 4: 4.5%). Recovery from shelf 2 (2.3%) was also significantly lower than from shelf 4.

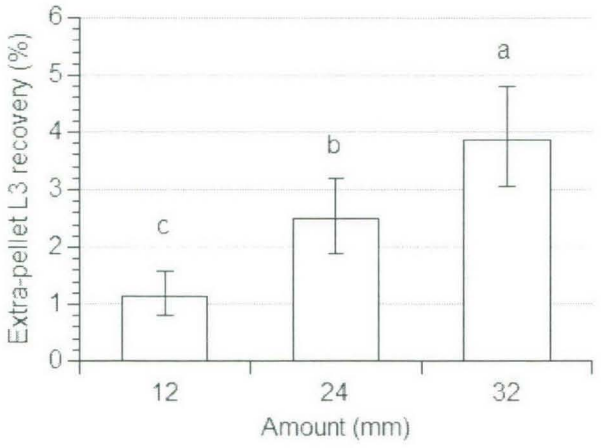

Figure 5-2: Back-transformed least squares means (±95% C.I.) of extra-pellet L3 recovery by amount of simulated rainfall. Means not sharing a common letter differ significantly ($p<0.05$).

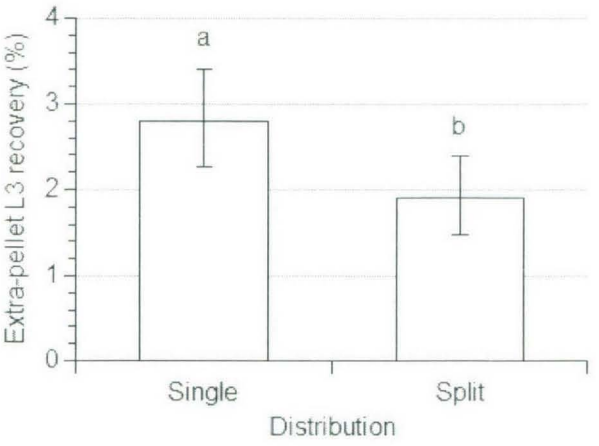

Figure 5-3: Back-transformed least squares means (±95% C.I.) of extra-pellet L3 recovery by distribution of simulated rainfall. Means not sharing a common letter differ significantly ($p<0.05$).

When FMC at sampling was fitted as a covariate, it fully accounted for the effect of rainfall amount on extra-pellet L3 recovery and was positively correlated with recovery at d 7 and 14 (R^2 = 0.41 & 0.22, respectively; Figure 5-4). When average FMC at d 4 for each simulated rainfall treatment combination was fitted against extra-pellet L3 recovery at d 7 and d 14 (Figure 5-5), the correlation was stronger for recovery at d 14 (R^2 = 0.42), though similar for d 7 recovery (R^2 = 0.35).

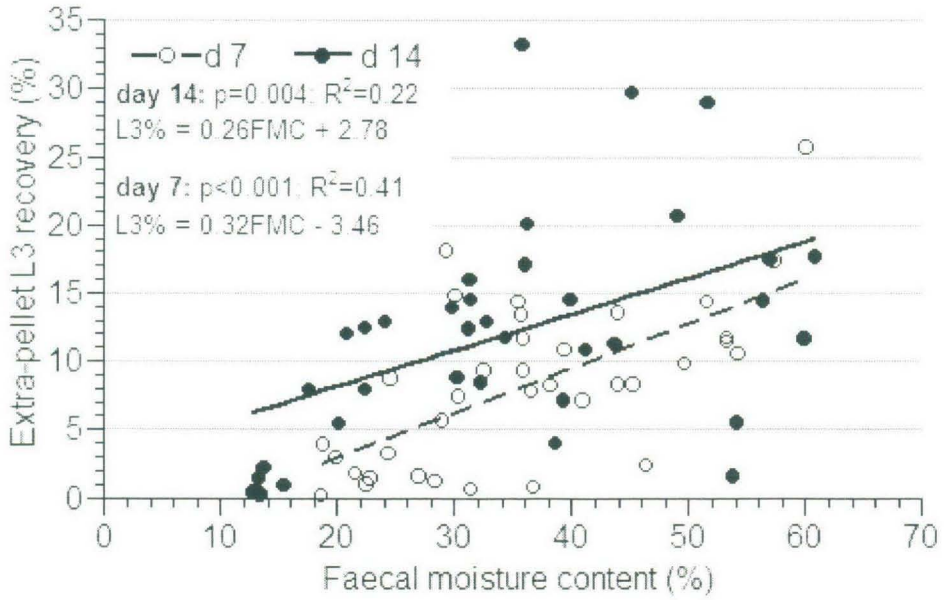

Figure 5-4: Extra-pellet L3 recovery regressed on faecal FMC, within day of sampling (raw data & linear fits).

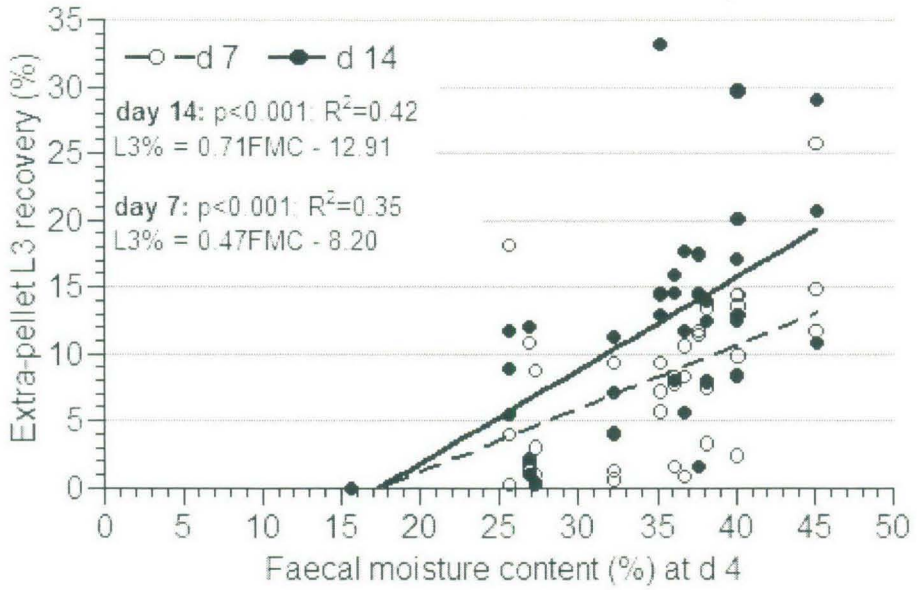

Figure 5-5: Extra-pellet L3 recovery at d 7 and 14 (raw data) by mean FMC at d 4 (raw means and linear fits).

Step-wise linear regression of extra-pellet L3 recovery (averaged over time) with the respective cumulative P/E values, for treatments watered in the first week only, indicated that the P/E value at d 5 was most closely associated with recovery ($p=0.001$; $R^2=0.40$;). The cumulative P/E value at d 8 was the best predictor of extra-pellet L3 recovery for treatments that were watered in both weeks ($p<0.001$; $R^2=0.49$). The regression plots are shown in

Figure 5-6 and Figure 5-7. For both repetition treatments, extra-pellet recovery increased with P/E and there was no evidence that recovery had reached a plateau within the range of P/E values reported.

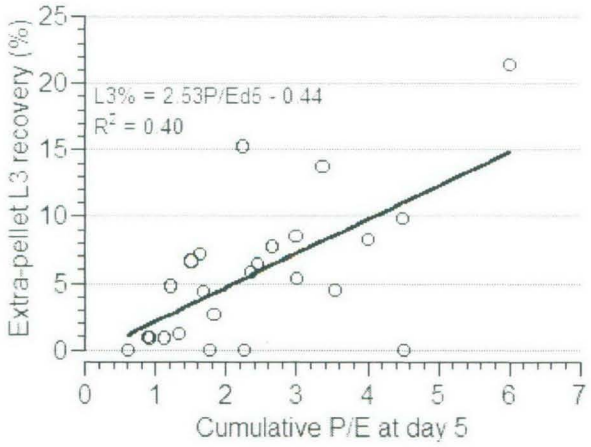

Figure 5-6: Regression plot of extra-pellet L3 recovery (averaged over time) with cumulative P/E at d 5 in treatments not subjected to repeat rainfall in week 2. Each point is the mean of a treatment combination within a shelf in the incubators.

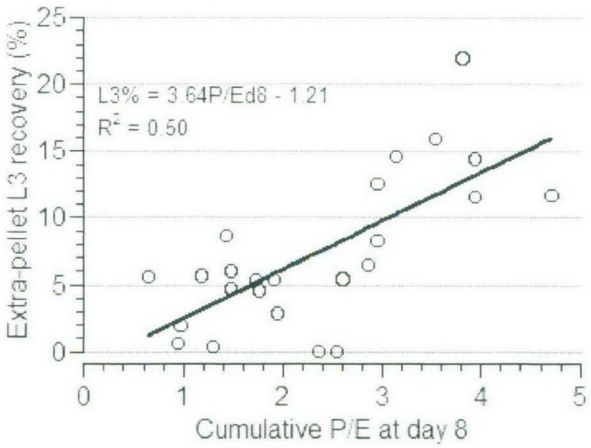

Figure 5-7: Regression plot of extra-pellet L3 recovery (averaged over time) with cumulative P/E at d 8 in treatments subjected to repeat rainfall in week 2. Each point is the mean of a treatment combination within a shelf in the incubators.

Total L3 (extra- and intra-pellet L3) recovery increased over time and with rainfall amount ($p<0.001$), with significantly fewer L3 recovered from the 12 mm treatment than greater amounts (1.7% at 12 mm compared with 3.2 and 4.9% at 24 and 32 mm, respectively). The interaction between the effects of amount and day post-faecal deposition was because total recovery did not differ among rainfall amounts at d 4, was significantly higher in the 32 mm treatment compared with the 12 mm treatment at d 7, and by d 14 was higher in both the higher rainfall treatments than the 12 mm treatment. Neither distribution nor repetition had statistically significant effects on total recovery of L3 ($p=0.507$ and $p=0.654$, respectively). There were no L3 (extra- or intra-pellet) recovered from unwatered controls at any stage.

5.3.3 Recovery of pre-infective larvae

Recovery of extra-pellet L1 and L2 was negligible, at less than 0.3% throughout the experiment, and hence was not included in the analysis. Recovery of pre-infective larvae from the faeces was highest on d 4 (29%) and declined to 2% by d 14 ($p<0.001$). There was an effect of distribution ($p=0.007$), with recovery from the split distribution (14%) significantly higher than from the single rainfall event (8%), however the interaction between distribution and sample day ($p=0.029$) indicated that the difference was largely due to d 7 samples, with no significant difference between distribution treatments at d 4 or 14. There was no effect of amount or repetition of rainfall on recovery of pre-infective larvae. FMC at the time of sampling significantly influenced recovery of pre-infective larvae ($p=0.021$), however the relationship was only significant at d 14 ($p=0.005$; $R^2=0.21$; L1L2% = -0.232FMC + 15.34). Recovery of intra-pellet L1 and L2 over time from the unwatered controls was significantly lower than all other amounts of rainfall ($p=0.001$). At d 4, 1.7% of eggs had hatched, declining to 0.1% at d 7 before increasing to 2.9% at d 14.

The LS means of pre-infective (intra-pellet) and infective (extra-pellet) larvae recovery over time for each amount are shown in Figure 5-8. The decline in recovery of L1 and L2 over time occurred regardless of rainfall amount or distribution, and under all treatments a concurrent increase in total L3 occurred, although the increase did not fully and quantitatively account for the loss of L1 and L2.

5.3.4 Egg development & recovery

Un-embryonated eggs were recovered in significant quantities at d 4 only, with 11% of eggs showing no signs of development. There were no treatment effects on embryonated egg recovery, although recovery decreased with FMC ($p<0.001$). At d 4, 14.5% of eggs had embryonated, increasing to 24.4% by d 7 before declining, as eggs hatched, to 14% by d 14. Recovery of degenerate eggs also decreased with FMC on d 14 ($p=0.006$) and again, there were no treatment effects on recovery. Overall, eggs were the dominant stage recovered from

experimental units, making up 60-65% of all free-living stages present at each sampling event.

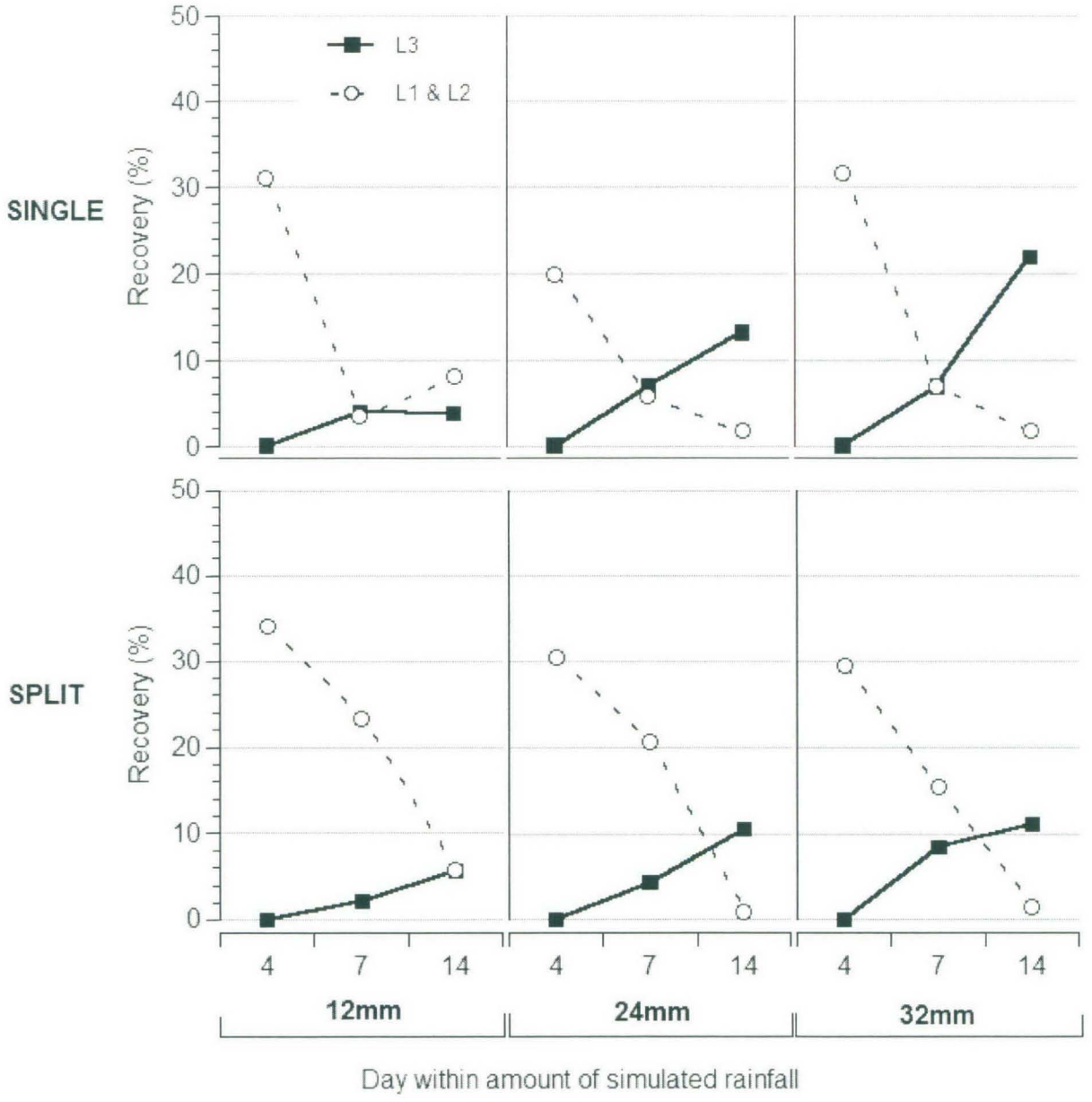

Figure 5-8: Back-transformed least squares means of L1 & L2 (intra-pellet) and L3 (extra-pellet) recovery by day post-faecal deposition within amount of simulated rainfall for both levels of distribution.

5.3.5 Recovery of free-living stages – overall

Overall recovery of free-living stages from both faeces and soil was highest on d 4, at 78%, declining to 70% at d 7 and 58% at d 14 (p=0.004). The contribution of extra-pellet larvae (L1, L2 or L3) to overall recovery was small, peaking at 12% on d 14 compared to 46% recovery of intra-pellet free-living stages at the same stage (Figure 5-9). Recovery of live

free-living stages declined over time ($p<0.001$), from 58% of eggs deposited at d 4 to 35% at d 14 (Figure 5-10). Over the same period there was no significant ($p=0.428$) increase in recovery of degenerate eggs (13-18%), indicating that some free-living stages were unrecoverable, most likely due to decomposition.

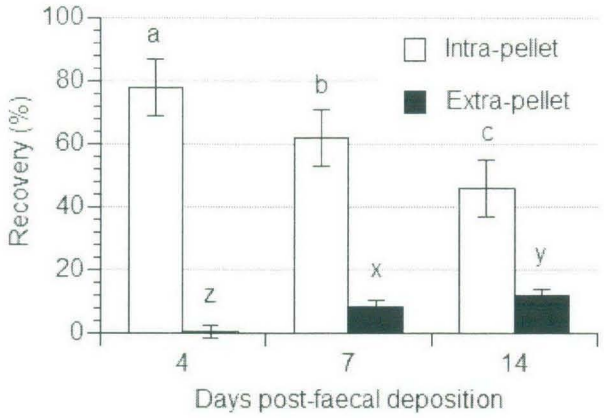

Figure 5-9: Least squares means (±95% C.I.) of recovery of total extra- and intra-pellet free-living stages by day post-faecal deposition. Means not sharing a common letter within each column type differ significantly ($p<0.05$).

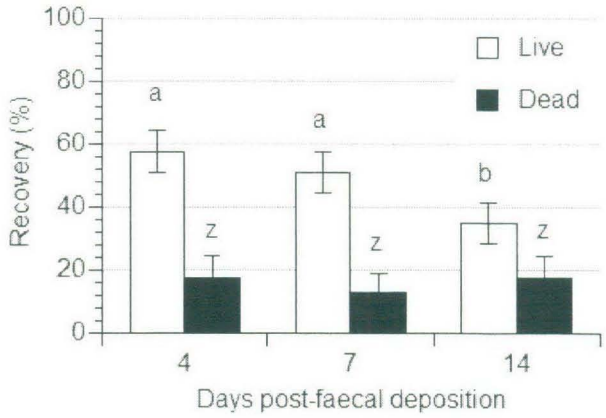

Figure 5-10: Least squares means (±95% C.I.) of recovery of degenerate eggs ("Dead") and live free-living stages by day post-faecal deposition. Means not sharing a common letter within each column type differ significantly ($p<0.05$).

5.3.6 Effects on faecal moisture content

The mean FMC of fresh faeces at the time of deposition onto experimental units was 53%. Amount and repetition of simulated rainfall were significant influences on FMC as measured at each sampling event, and there were significant interactions between day post-faecal deposition and repetition. There was no significant difference between distribution treatments ($p=0.054$).

When averaged over the three sampling times, FMC increased with amount ($p<0.001$), from 28% at 12 mm to 36% at 24 mm and 43% at 32 mm. There was no difference between repetition treatments until d 14, since the repeat watering event was yet to occur at earlier sampling events. At d 14, the first week only treatment had a mean FMC of 25% compared to 44% under the repeat watered treatment ($p<0.001$). The raw means of FMC over time for each

simulated rainfall treatment combination are shown in Figure 5-11. FMC in unwatered control treatments declined to 35% by d 4, before reaching a plateau at 26% on d 7 and 25% on d 14.

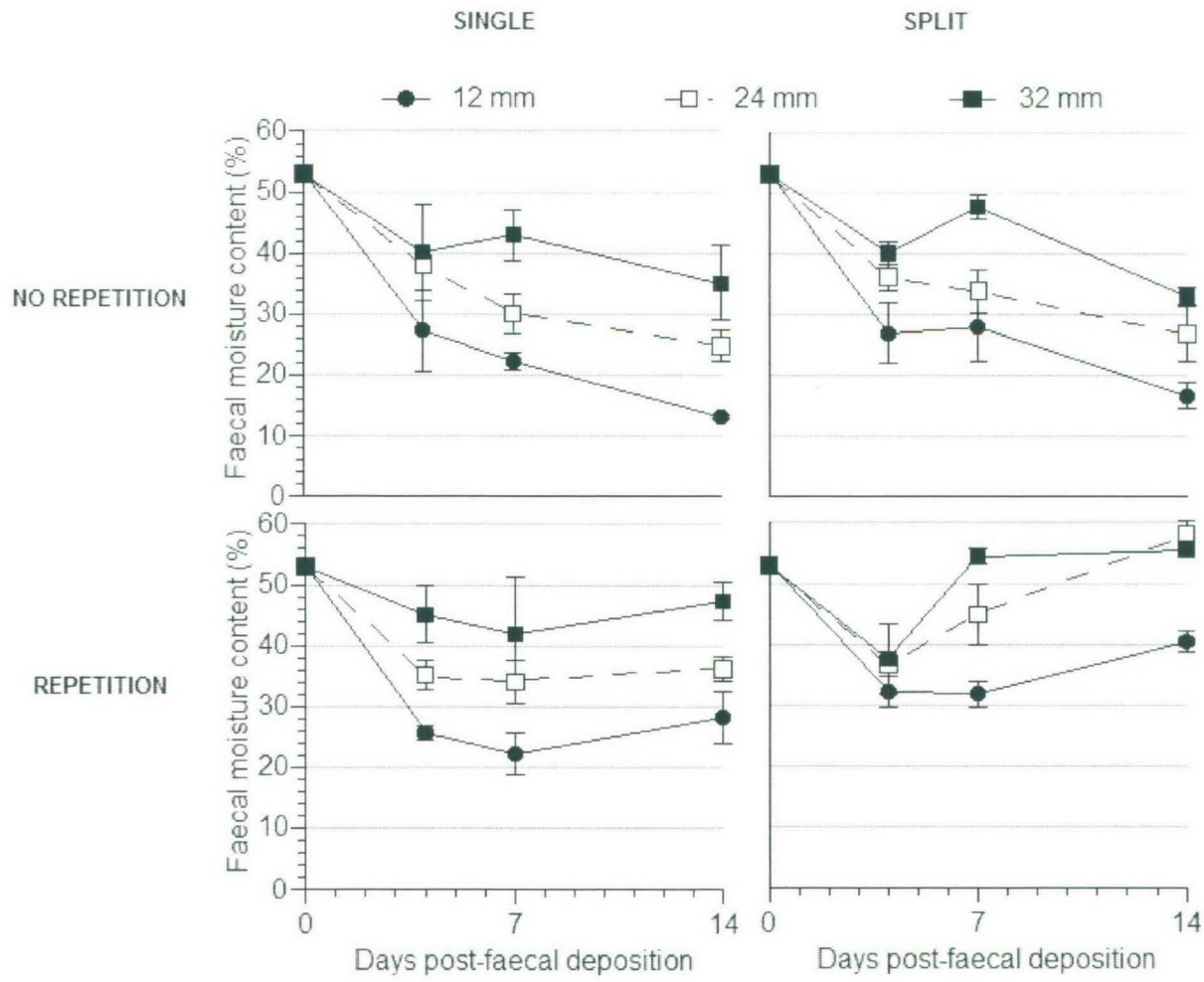

Figure 5-11: FMC (raw means and s.e.) by day post-faecal deposition for each combination of rainfall amount, distribution and repetition.

5.4 Discussion

The provision of larger amounts of simulated rainfall to infected faeces, within the first week after deposition, stimulated greater development success of the free-living stages, ultimately resulting in higher recovery of extra-pellet L3 under a temperature regime typical of the temperate Northern Tablelands summer. Similarly, application of each rainfall amount in a single event provided a development advantage, with more L3 recovered relative to the split distribution. The effect of repetition of simulated rainfall on recovery of L3 was minimal even

though these treatments received twice the amount of simulated rainfall than those watered in the first week only.

The evaporation rates prevailing in the incubators in the present experiment were considerably lower than those recorded on the plots in Chapter 4. Evaporation in the incubators was a consequence of the low rate of air flow in the incubators, not a deliberate design feature, and unfortunately could not be increased or manipulated within this system. This limitation will be rectified in subsequent experiments.

Overall recovery of L3 was higher (raw means of up to 20% on d 14) than that reported elsewhere in Mediterranean or temperate climate field studies – for example, up to 3% by Besier and Dunsmore (1993b), less than 0.3% by Levine *et al.* (1974) and up to 6% by Rose (1963) – a result attributable in part to both the controlled environment (low evaporation, no direct sunlight or wind, and sterile soil) and high moisture availability, and also because sampling loss of free-living stages was limited by the closed system. Despite the experimental design, a proportion of the free-living stages was unrecoverable with overall recovery ranging from 78% at d 4 to 58% at d 14. It is likely that death and subsequent decomposition of the pre-infective stages accounted for the majority of the losses, but movement below the top 25 mm of soil in experimental units could not be excluded. Similarly, upward movement of larvae into the watering device, although unlikely given the downward movement of water, was not measured and therefore may have been a source of loss.

Reports of the effect of rainfall amount on *H. contortus* free-living development are rare, with a consensus amongst many studies that other, more descriptive rainfall variables are better correlated with development (Besier, 1992; Besier & Dunsmore, 1993b; Levine & Todd, 1975). However, several attempts at modelling free-living populations of both *H. contortus* (Barger *et al.*, 1972) and *T. colubriformis* (Barnes *et al.*, 1988) have been made, and in each case the moisture requirements for development to L3 were expressed as the ratio of, or

difference between, cumulative precipitation and evaporation. In plot studies conducted at temperatures that were simulated in this study, there was no detected effect of simulated rainfall amount on L3 recovery (Chapter 4). However evaporation rates were substantially lower in the incubators (overall mean of 1.9 mm/day) than in the equivalent plot study (overall mean of 4.1 mm/day). As a result, moisture applied as simulated rainfall between d 0 and d 6 was still present in the microclimate and influencing development when L2 moulted to L3, after d 4. In the plot study, the cumulative P/E ratio was less than 1 by d 4 at the highest simulated rainfall amount (24 mm), indicating that most moisture applied on d 1 was rapidly lost to evaporation and thus had negligible influence on development. Under the same treatment in the laboratory study, P/E was still above 1 at d 14.

In contrast to that reported for infective larvae, there was no significant influence of simulated rainfall amount on pre-infective larval recovery, suggesting that the low evaporation rates led to non-limiting moisture conditions in the first few days post-deposition. The minimum amount of rain required for emergence of L3 was not established, as L3 were recovered from all treatments in which simulated rainfall was applied. The lack of development beyond the pre-infective larval stage in unwatered controls suggested that some addition of rain is required for development to L3 even at the low evaporation rates prevailing in the incubators.

Even though evaporation in the incubators was substantially lower than typical field evaporation, the difference between the top and the bottom shelves of approximately 1.8 mm/day resulted in a significant difference in recovery of L3 between the upper and lower shelves, regardless of rainfall treatment applied. The difference highlights the ecological significance of evaporation in regulating moisture availability for *H. contortus* free-living stages. That differences were apparent even at very low drying rates indicates that future ecological studies should account for both precipitation and evaporation when quantifying the effects of moisture on *H. contortus* development. The influence of cumulative P/E ratio on

development indicated that this variable was correlated with moisture conditions at the microclimate level under the conditions tested, and may have potential for the prediction of L3 development in the field, based on prevailing evaporative and rainfall conditions.

Interestingly, the regression of L3 recovery with P/E values revealed an effect of the repetition treatment which was not apparent in the initial statistical analysis. The LS means models suggested that moisture applied beyond 7 d after deposition had no significant additive effects on L3 recovery when moisture was also available prior to d 7. However in treatments which received the repeated rainfall, recovery was most closely correlated with the P/E ratio describing moisture conditions at d 8 – the day on which both the repeated single distribution event and the first of the repeated split distribution events occurred. In comparison, recovery from treatments which did not receive the repeat rainfall events was best described by P/E at d 5. This suggests that although the majority of development to L3 occurred in response to simulated rainfall events in the first 7 days, there was a small subsequent increase in development to L3 as a result of rainfall events that occurred post-d 7.

The influence of rainfall distribution on *H. contortus* development has been poorly quantified, most likely a consequence on the infinite combinations of intermittent rainfall events post-egg deposition. However Besier and Dunsmore (1993b) reported anecdotally that higher recovery of L3 was observed when 8 mm of rainfall fell across 4 consecutive days compared to a 26 mm event on 1 day. The distribution treatment tested in this study compared a single rainfall event with the same amount spread over 3 events in 6 days. That the single distribution was more beneficial for L3 development is in accordance with the findings from earlier plot studies (Chapter 4) in which a single simulated rainfall event applied within 1 or 4 d of egg deposition led to significantly higher rates of pre-infective development than events applied at 7 d or more post-deposition. These effects illustrate the importance of moisture availability soon after deposition for complete development – the split distribution provided only one-

third of the moisture to faeces that was available under the single distribution within 48 h of egg deposition, hence development may have been constrained by insufficient early moisture. The minimal influence of the repeat simulated rainfall events between d 7 and 14 on extra-pellet L3 recovery substantiates the conclusion that moisture availability within a few days of deposition is a key determinant of development success.

It is possible that the complexity of moisture effects on *H. contortus* free-living development can be simplified by defining the effect of FMC on development, along with the relationship between macroclimatic moisture conditions and FMC (O'Connor *et al.*, 2006). Rossanigo and Gruner (1995) measured *H. contortus* free-living development under a range of FMC, finding that the optimum level of moisture for development of L3 was 70% (23°C), at which approximately 50% of eggs reached L3 stage. At higher FMC, development success was lower, while the minimum FMC at which L3 appeared (1 L3 per 100 eggs) was 39%. However, these workers maintained FMC for the duration of the experiment. In the present experiment, FMC started at 53% and was only recorded above this value (up to 60%) when 24 or 32 mm of rainfall was applied in the split treatment over both weeks. The intermittent nature of sampling events meant that short term changes in FMC, such as those in the 24 h after rainfall events, were not detected. However substantially lower recovery of L3, compared to that of Rossanigo and Gruner (1995) suggests that moisture was not unlimiting under any treatments.

Intuitively FMC would seem to be the most accurate measure of moisture availability to pre-infective stages in the faeces, and results of this study indicate that different levels of the simulated rainfall treatments are detectable in FMC measurements. What was clear, however, was that FMC soon after deposition (in this case d 4) was at least as strongly correlated with development potential as FMC at 7 d post-deposition or later. This correlation of development with FMC soon after faecal deposition is likely to be especially apparent under field

conditions where faecal drying is rapid. A shortcoming in the experimental design was that there was no measure of FMC from d 1 to d 3. In hindsight, such a measure may have provided a sound explanation for the difference in L3 recovery between the single and split distribution, as the means for each distribution at d 4, 7 and 14 were largely an artefact of the timing of split applications in relation to sampling events. Such measurements may also have provided suitable data for quantification of FMC influences on L3 development. The FMC data collected at d 7 and 14 sampling events was of limited use except to illustrate trends, as it appears that moisture availability straight after egg deposition was the key determinant of development success.

The present study indicates that under conditions of low evaporation, the magnitude of *H. contortus* development will be significantly influenced by rainfall amount and distribution under typical Northern Tablelands summer temperatures. Both cumulative P/E and FMC may provide useful predictors of development success by integrating the range of variables affecting moisture availability to the free-living stages into a single function. However, whether the relationships observed in this study hold under field conditions, where macroclimatic conditions are substantially more limiting to free-living development, is yet to be determined. An investigation of the effects of a range of rainfall-related variables under conditions more similar to those prevailing in the field would allow more accurate quantification of the timing and magnitude of infective *H. contortus* pasture populations.

Chapter 6. The effects of amount and distribution of simulated rainfall on free-living development of *Haemonchus contortus* at high and low evaporation rates

6.1 Introduction

In regions where *Haemonchus contortus* is a major threat to sheep production, it is typically the combination of warm temperatures and rainfall that promotes the favourable conditions required in faecal material for development of the free-living stages. Previous studies of the effects of rainfall on *H. contortus* development are limited to observational experiments, in which development in plots or in the field has been measured in response to naturally-occurring rainfall events. The bioclimatographs developed by Gordon (1948) and Levine (1963) were based on the occurrence of the first outbreak of haemonchosis each year and mean monthly rainfall. Both specified 50 mm as the threshold amount of monthly rainfall required for significant development of L3 on pasture. Besier and Dunsmore (1993b) observed that rainfall distribution and green herbage were better correlated with *H. contortus* development in pasture plots than amount of rainfall or evaporation, but did not quantify the effect of either rainfall distribution or amount on L3 development. Barger *et al.* (1972) found good agreement between predicted and observed results using a model of the free-living ecology of *H. contortus*. The percentage of pre-infective larvae that developed to infective larvae was based on the assumption that cumulative precipitation must exceed cumulative evaporation within three weeks of egg hatch, suggesting that these two variables may be appropriate for describing moisture availability to the free-living stages. The effects of faecal moisture content (FMC) on *H. contortus* development have also been investigated, with Rossanigo and Gruner (1995) reporting that 50% of *H. contortus* eggs developed to L3 in faecal culture at 23°C when FMC was maintained at 70%, but development success was reduced at higher or lower FMC. Studies of the effects of irrigation on *H. contortus* (Bullick & Andersen, 1978; Gruner *et al.*, 1989) have shown greater development and survival rates

when water is freely available in the pasture microclimate due to flood or spray irrigation, but quantitative relationships between amount or timing of watering were not established.

An understanding of the quantitative effects of rainfall and moisture on the free-living stages of *H. contortus* will facilitate prediction of the developmental consequences of a given rainfall event on recently deposited eggs. There is a need to determine the relationships between development and variables such as rainfall amount and distribution, faecal moisture content and evaporation rate, and also to be able to apply knowledge of these relationships to grazing systems using available meteorological data. Meteorological data such as rainfall and evaporation are the most likely candidates for inclusion in models for prediction of larval development, given their ease of measurement and wide availability as indicators of moisture in the macroclimate. FMC, in contrast, measures moisture availability to free-living stages at the microclimate level, and is likely to be the mechanism by which both macroclimate and microclimate variables interact to regulate larval development in individual faecal deposits. Therefore, although impractical to measure and incorporate into field scale models, FMC offers considerable scope for quantifying moisture effects on *H. contortus* at a fundamental level. Current control strategies aimed at *H. contortus* would benefit significantly if prediction of the timing and magnitude of paddock infectivity based on recent weather conditions following grazing events could be improved.

The plot experiments reported in Chapter 4 investigated the effect of different amounts of simulated rainfall, occurring at various times post-egg deposition, on the development of the free-living stages of *H. contortus*. Egg hatch increased with amount of simulated rainfall, and when rain fell within 4 d of egg deposition compared to 8 or 15 days. More pre-infective larvae were also recovered when a given amount of rain was applied over 36 h, compared with 1-4 h. However there was negligible development to L3, despite the non-limiting temperatures, and no effect of simulated rainfall timing, amount or distribution on

development, presumably due to the rapid loss of moisture from the system through evaporation and run off. In the previous laboratory experiment (Chapter 5), in which rainfall simulations more closely mimicked the natural rate of rainfall and predation on or disturbance of free-living stages was eliminated, development to L3 under simulated summer temperatures was successful and rapid under the same amounts of rainfall applied in the plot study. Recovery of L3 increased with amount of rainfall, and also when water was applied in a single event compared to three smaller, equal-sized events split over six days. However the difference in evaporation rate between the field and laboratory experiments was significant, with daily evaporation rates between 4 and 5 mm/day in the field compared with 1-3 mm/day in the laboratory. These findings suggest that when evaporation was low, as under laboratory conditions, the effect of simulated rainfall amount and distribution on L3 development was large because the slow drying rate meant that treatments effects were still present when pre-infective larvae moulted to infective larvae. In comparison, significantly higher daily evaporation rates in the field meant that by the time eggs hatched to pre-infective larvae and were ready to develop to L3, most of the simulated rainfall applied initially had been lost to evaporation and hence there was little remaining influence of treatments.

Clearly evaporation and its interaction with rainfall plays a key role in determining moisture availability to the free-living stages. The present experiment therefore measured the effect of variable amounts and distributions of simulated rainfall under high and low evaporation conditions in a controlled environment simulating summer temperatures in order to a) confirm the effects of rainfall amount and distribution observed in earlier studies, b) establish and quantify the effect of interaction between evaporation rate and amount and distribution of rainfall on larval development and FMC, c) determine if there are threshold combinations of rainfall and evaporation conditions below which *H. contortus* development is prohibited, and d) determine the moisture-related variables that best predict development of *H. contortus* eggs to infective larvae.

6.2 Materials and methods

6.2.1 Conditions and experimental design

The experiment was conducted in two programmable incubators (MIR 253, Sanyo Electric Biomedical Co., Ltd.) in which temperature was regulated to mimic diurnal summer ground temperature, as recorded hourly during the summer 2005 experiment reported in Chapter 4, and simulated in the previous laboratory study in Chapter 5. Over the 14 d experiment, the mean daily mean temperature was 22°C, with a mean daily maximum and minimum of 39°C and 11°C, respectively. One incubator was set up to operate at low evaporation rate and the other at high evaporation rate, by varying the degree of air exchange.

The experimental design was a 3 x 4 x 2 x 3 factorial, with three replicates of each treatment combination blocked over time such that the complete set of experimental treatments and measurements was run three times. Three amounts of simulated rainfall (12, 24 or 32 mm) were applied in one of four distributions, namely as a single event on d 1 (08:00 h) or in 2, 3 or 4 smaller and equal split events (beginning at 08:00 h on the respective day) spread over 2, 3 or 4 d post-deposition (Table 6-1). Simulated rainfall events were conducted using the method described in Chapter 3 (section 3.3). Each combination of simulated rainfall amount and distribution was tested at two evaporation rates (Low and High), with each level tested in a separate incubator. Each treatment combination was designated to one of three sampling events (day 4, 7 or 14), and each treatment x sampling event replicate allocated to a different shelf (shelf 1, 2 or 3; upper to lower), so that all combinations were tested on every shelf during the experiment. External to the factorial design were unwatered control treatments, which were placed on the lowest shelf of each incubator (shelf 4). Also external to the full factorial design was an extra experimental unit for each treatment combination, which was used for partial sampling of faeces on d 1, 2 and 3 (approximately 16, 40 and 64 h post-deposition, respectively) for determination of faecal moisture content.

Table 6-1: Daily simulated rainfall events by amount and distribution.

Simulated rainfall treatment		Amount (mm) applied by day			
		Day (post-deposition)			
Amount	Distribution	1	2	3	4
12 mm	1	12.0			
	2	6.0	6.0		
	3	4.0	4.0	4.0	
	4	3.0	3.0	3.0	3.0
24 mm	1	24.0			
	2	12.0	12.0		
	3	8.0	8.0	8.0	
	4	6.0	6.0	6.0	6.0
32 mm	1	32.0			
	2	16.0	16.0		
	3	10.7	10.7	10.7	
	4	8.0	8.0	8.0	8.0

Each experimental unit consisted of a polycarbonate cylinder (100 mm height, 60 mm diameter) containing steam sterilised soil (screened loam; approximately 80 mm depth). All cylinders had a small drainage hole soldered into the side wall, at approximately 25 mm above the base. A 10 g subsample of intact faeces was placed on the soil surface of each experimental unit at 15:00 h on d 0. Cylinders were topped with a plastic ring to prevent migration of larvae over the lip of the experimental unit (see Figure 3.3).

Faeces were obtained from worm-free sheep artificially infected with *Haemonchus contortus* larvae (see Chapter 3, section 3.2.2). They were mixed thoroughly by hand and a worm egg count determined on 3 x 10 g subsamples of faeces using a modified McMaster method with a lower limit detection (1 egg counted) of 60 eggs per gram. Contamination rates for each replicate are shown in Table 6-2.

Table 6-2: Contamination rates and standard deviations of worm egg counts for the three replicates

	Replicate 1	Replicate 2	Replicate 3
Date	9 – 22 May, 2006	29 May – 12 June, 2006	21 August – 4 September, 2006
Duration	142 days	14 days	14days
Mean contamination rate (eggs per gram)	22356	21372	15387
Standard deviation (eggs per gram) [n]*	1566 [3]	762 [3]	2060 [4]
Mean contamination rate (eggs per plot)	223560	213720	153870
Weight of faeces per plot (g)	10	10	10

* Number of replicates from which standard deviation is derived.

6.2.2 Sampling & measurements

Temperature was recorded hourly on each shelf using Tinytag® (Gemini Data Loggers, Chichester, UK) climate data loggers. Evaporation rate (mm/day) was determined from the disappearance of water contained in two polycarbonate cylinders (100 mm height, 60 mm diameter) located on each shelf of each incubator.

From the experimental units designated to partial sampling of faeces on d 1, 2 and 3 for determination of FMC (see Chapter 3, section 3.6), a subsample of faeces was harvested at each time point. The remaining three experimental units from each treatment combination in the full factorial design were destructively sampled the designated sampling event (d 4, 7 or 14), with both faeces and soil collected. A subsample of the faeces was dried for determination of FMC. Evaluation of intra-faecal pellet parasitic stages (intra-pellet eggs and larvae) was conducted using the intra-pellet enumeration process described in Chapter 3 (section 3.5). Faeces and soil were also harvested from two replicates of the unwatered controls at d 4, 7 and 14.

For enumeration of larval stages in the soil fraction (extra-pellet L1 and L2, and L3), the soil profile of each experimental unit was separated into three strata (top 25 mm, next 25 mm and

remaining soil) and processed according to the method of soil larval enumeration described in Chapter 3 (section 3.8).

6.2.3 Statistical analysis

Recovery of each free-living *H. contortus* stage was expressed as a percentage of the total number of eggs deposited per experimental unit. Stages enumerated from faecal samples (intra-pellet) and analysed as response variables were: embryonated eggs (E2), degenerate eggs (d.eggs), pre-infective larvae (L1 and L2) and infective larvae (L3). Extra-pellet stages (recovered from soil) enumerated and analysed as response variables were: L1 and L2, and L3. Recovery of extra-pellet L3 at d 14 was divided by recovery of total L1 and L2 at d 4 (averaged across shelf for each treatment combination) and expressed as a percentage to determine the success of development from pre-infective larvae to infective larvae. Response variables were transformed with a cube-root transformation where necessary to better meet the assumptions of analysis of variance. General linear models were constructed to test the effects of experimental factors including amount and distribution of simulated rainfall, evaporation rate and day of sampling. Also included were the experimental design effects of shelf (1=top, 2=middle, 3=bottom) and block (replicates 1-3). FMC at sampling was fitted as a covariate in the models, along with all two-way interactions. Treatment effects on FMC at sampling were also analysed in a separate GLM. All non-significant effects ($p>0.05$) were removed from the analyses and are not discussed. Significant differences between means were determined using Tukey's HSD test or specific linear contrasts within the model. Back-transformed least squares means (LS means) and 95% confidence intervals are presented in the results for the relevant analyses. In some cases, raw means and standard errors are also presented. All analyses were carried out using JMP-IN 5.1 software (SAS Institute Inc., NC, USA).

Cumulative precipitation and cumulative evaporation ratios (P/E; see Chapter 5, section 5.2.3) were established for each day during the experiment based on the cumulative amounts of

rainfall and evaporation to that point for each treatment x shelf combination. Stepwise regression was used to select the P/E variables which best described the recovery of extra-pellet L3 and total L1 and L2 at d 7 and d 14, along with success of development from L1 and L2 to L3 (as described above). Stepwise regression was also used to determine which day during the experiment FMC was best correlated with d 7 and 14 extra-pellet L3 recovery, and d 4 L1 and L2 and degenerate egg recovery.

6.3 Results

6.3.1 Temperature and evaporation

Daily mean, maximum and minimum temperatures recorded in the incubators during the experiment closely mimicked those observed during the original summer plot experiment (Chapter 4) and are shown in Figure 6-1. Values are averaged across incubator, shelf and block.

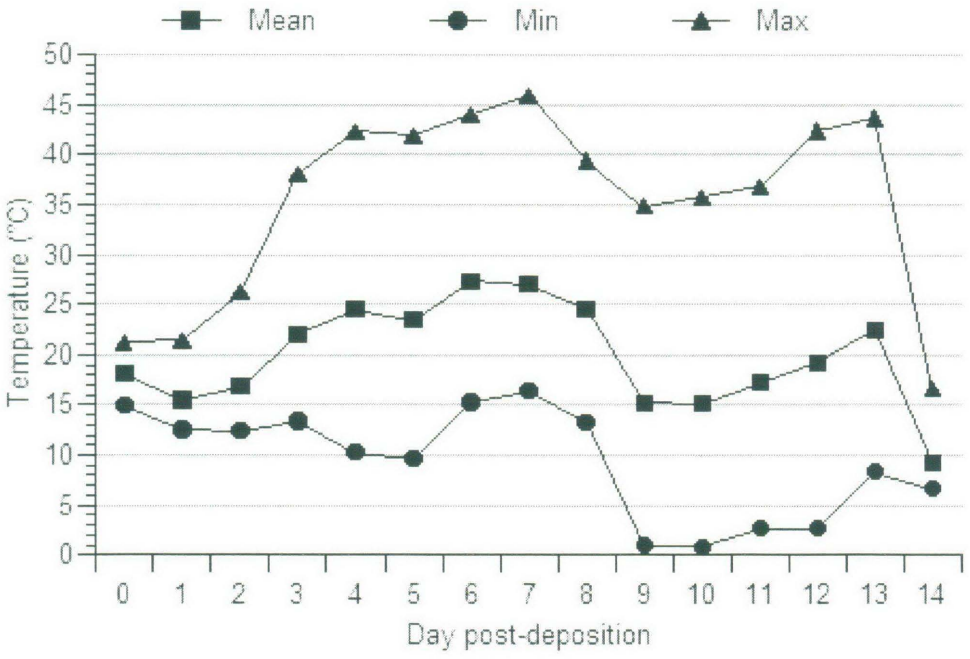

Figure 6-1: Daily minimum, maximum and mean temperature during the experiment. Temperatures are averaged across incubator, shelf and block. Standard error bars are not visible beyond the symbols.

Mean daily evaporation rates are shown in Figure 6-2 for the High and Low evaporation treatments. Values are averaged across block and shelf for each evaporation treatment. Within

each evaporation treatment, the evaporation rate varied among shelves (Table 6-3). Evaporation tended to decrease towards the base of each incubator, so that evaporation was highest on the upper shelf and lowest on the bottom shelf.

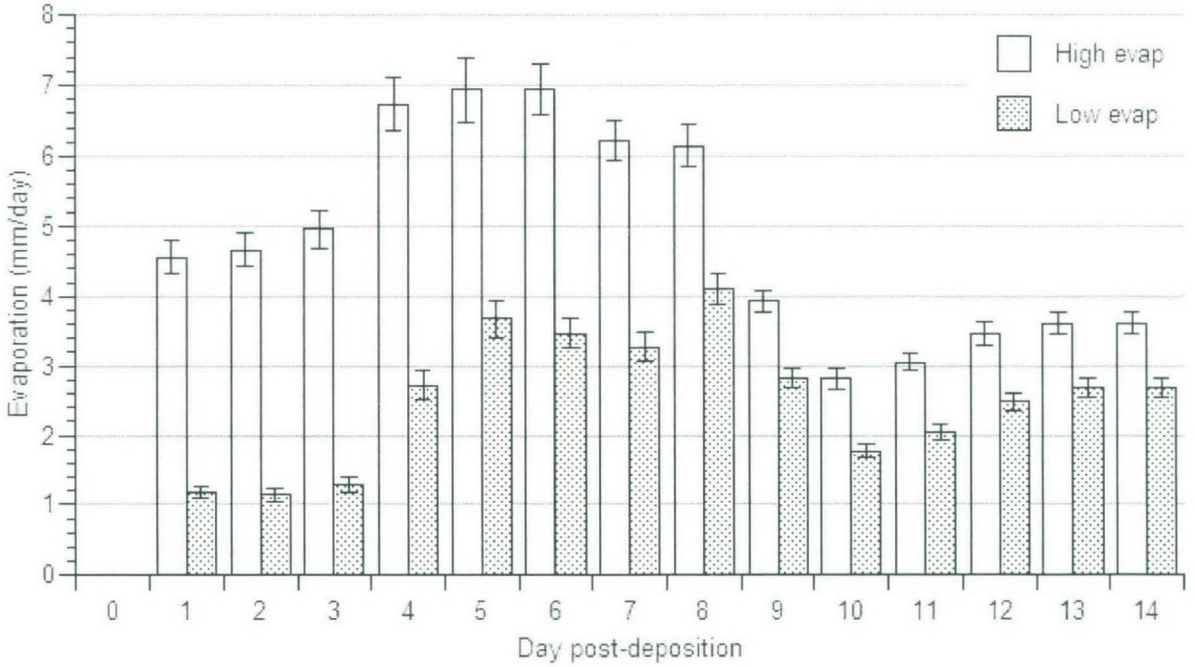

Figure 6-2: Mean daily evaporation rate (± s.e.) for High and Low evaporation treatments. Evaporation rates are averaged across block and shelf.

Table 6-3: Least squares means of daily evaporation rates, averaged over time and block for each shelf within evaporation treatment (High or Low). Means with no superscript in common differ significantly ($p<0.05$)

Evaporation treatment	High				Low			
Shelf	1	2	3	4	1	2	3	4
Evaporation (mm/day)	6.1a	4.6b	4.8ab	3.8bc	3.4bcd	2.4cd	2.1d	2.1d

6.3.2 Controls

No extra-pellet L3 were recovered from control units under either evaporation treatment. Raw recovery of intra-pellet L3 peaked on d 7 at 0.55% however there was no significant effect of evaporation rate (p=0.945) or day of sampling (p=0.500; Figure 6-3a). Raw recovery of intra-pellet L1 and L2 was highest on d 14 at 15.4%, increasing from 1.9% at d 7, while recovery of

extra-pellet L1 and L2 was low, peaking at 0.1% on d 7. The effect of evaporation rate was significant ($p<0.001$) with more L1 and L2 recovered under Low evaporation than High evaporation (Figure 6-3b).

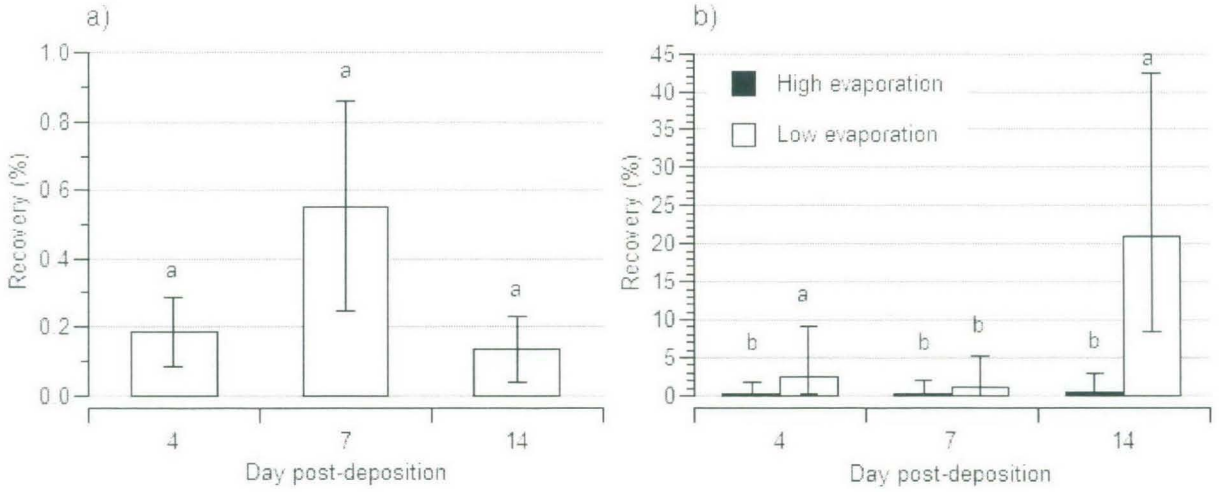

Figure 6-3: Back-transformed least squares means (± 95% C.I.) of larval recovery from unwatered controls over time; a) Recovery of intra-pellet L3; b) Recovery of intra- and extra-pellet L1 and L2. Means not connected by a common letter within each plot effect differ significantly ($p<0.05$)

6.3.3 Recovery of infective larvae (L3)

The raw means of L3 recovery indicated that initially the bulk of the total L3 pool consisted of intra-pellet L3, with only 3% of total L3 recovered from outside the faeces at d 4. By d 7 this had increased to 59%, and to 89% of the total L3 pool by d 14 (Figure 6-4).

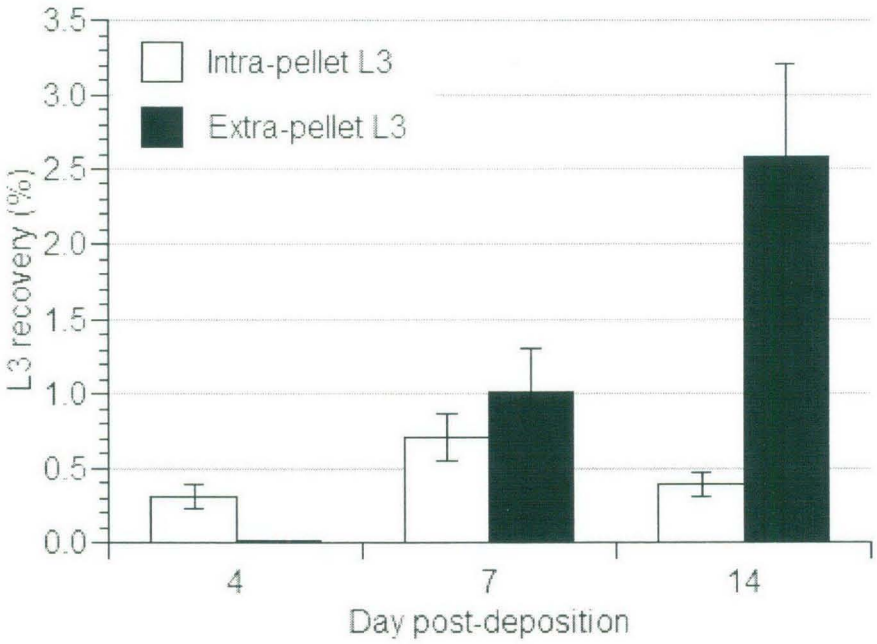

Figure 6-4: Raw means (± s.e.) of intra- and extra-pellet L3 recovery over time.

Treatment effects on total L3 are shown in Table 6-4, but all subsequent L3 recovery results and discussion are restricted to extra-pellet L3, as we are interested in treatments effects on pasture infectivity. Averaged across all treatments and days, 88% of total extra-pellet L3 were recovered from the upper stratum of soil (top 25 mm), 11% from the second stratum (second 25 mm), and 1% from the lower stratum (soil below 50 mm depth). The proportion of L3 in the two lower strata, relative to total extra-pellet L3, increased over time, and was higher under the Low evaporation treatment (Table 6-5). The proportion of total extra-pellet L3 recovered in the lower two strata also increased as amount of simulated rainfall increased (Table 6-6).

Table 6-4: P-values and back-transformed least squares means of total L3 (intra- and extra-pellet) recovery for sampling event, simulated rainfall treatment, and evaporation and shelf level. Means not connected by a common letter within model effect differ significantly ($p<0.05$).

Effect in model	Level	Total L3 recovery (%; intra- and extra-pellet)
		P<0.001
Day of sampling (post-deposition)	4	0.19[b]
	7	0.73[b]
	14	1.07[a]
		p=0.028
Amount of simulated rainfall	12 mm	0.07[c]
	24 mm	0.54[b]
	32 mm	1.54[a]
		p=0.142
Distribution of simulated rainfall	1	0.35[a]
	2	0.43[a]
	3	0.62[a]
	4	0.66[a]
		p<0.001
Evaporation	High	0.17[b]
	Low	1.12[a]
		p<0.001
Shelf	1 (top)	0.26[b]
	2 (middle)	0.53[ab]
	3 (bottom)	0.94[a]

Table 6-5: Proportion of mean extra-pellet L3 recovered from each soil stratum (within evaporation treatment; raw means).

		Proportion (%) of extra-pellet L3 by soil stratum		
Evaporation	**Extra-pellet L3 recovery (%)**	**1 (0-25 mm)**	**2 (26-50 mm)**	**3 (> 50 mm)**
High	0.09	97.6	2.0	0.4
Low	1.46	87.5	11.2	1.3

Table 6-6: Proportion of mean extra-pellet L3 recovered from each soil stratum (within amount of simulated rainfall; raw means).

		Proportion (%) of extra-pellet L3 by soil stratum		
Amount (mm)	**Extra-pellet L3 recovery (%)**	**1 (0-25 mm)**	**2 (26-50 mm)**	**3 (> 50 mm)**
12	0.05	99.0	0.99	0.01
24	0.80	91.8	7.4	0.8
32	1.82	86.5	12.0	1.5

Least squares means recovery of extra-pellet L3 recovery increased over time from zero at d 4 to 1.1% at d 14 ($p<0.001$). There were significant effects of amount of simulated rainfall (12 mm: 0.02%, 24 mm: 0.21%, 32 mm: 0.57%; $p<0.001$) and evaporation rate (Low: 0.06%, High: 0.44%; $p<0.001$) but not simulated rainfall distribution ($p=0.126$). The interaction of rainfall amount with evaporation ($p=0.008$) was because there was no difference between the 12 and 24 mm treatments under the High evaporation rate but significant differences between all rainfall amounts under Low evaporation (Figure 6-5). There was also an interaction between the effects of sampling day and rainfall amount ($p<0.001$). At d 4 there was no difference between amounts, by d 7 the 12 mm treatment had significantly lower values than the larger two amounts, and by d 14 all amounts were significantly different from each other (Figure 6-5).

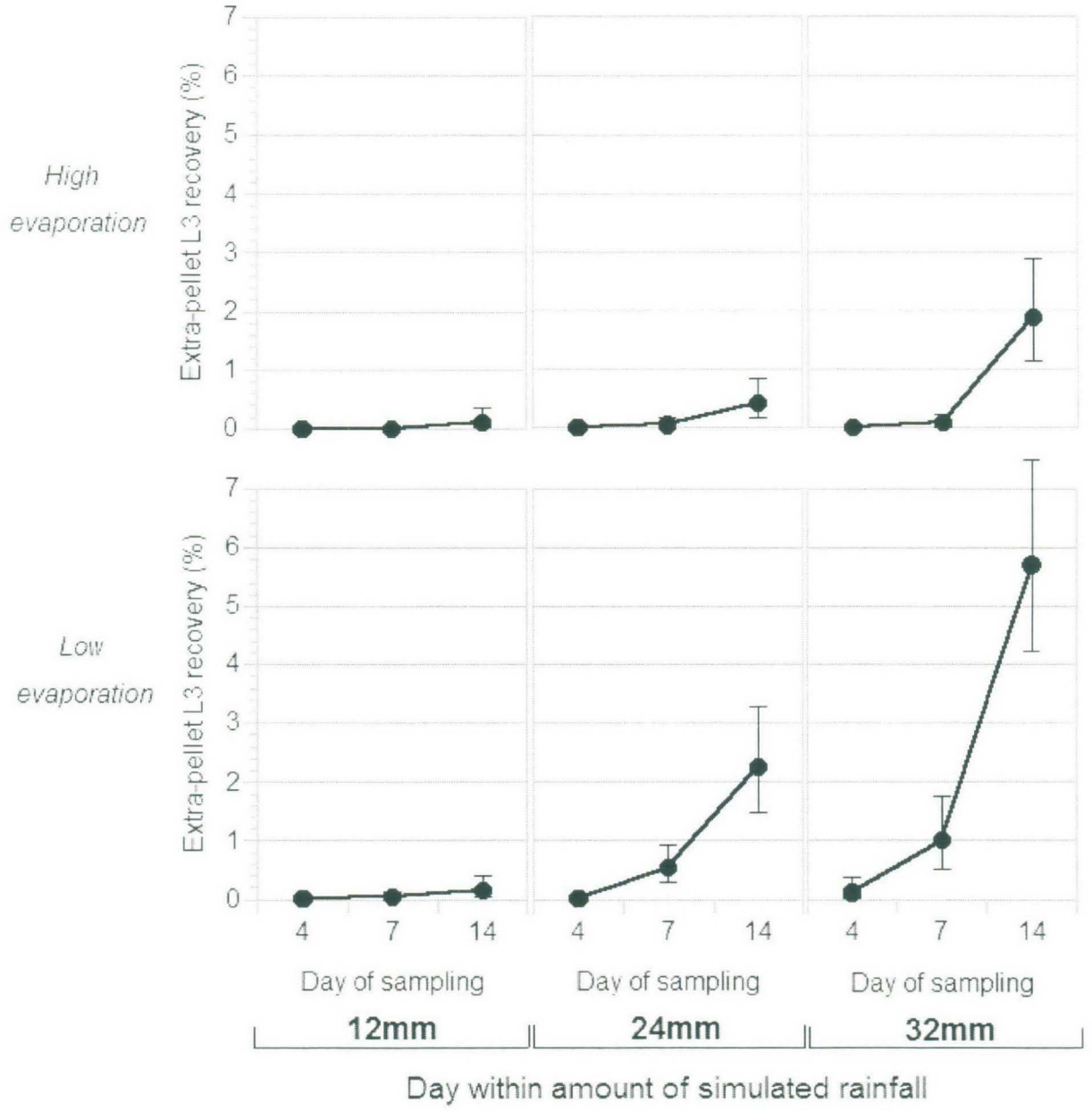

Figure 6-5: Back-transformed least squares means (± 95% C.I.) of extra-pellet L3 recovery within amount of simulated rainfall and evaporation rate, over time.

Recovery from the 1st block was lower than from later blocks (0.03% compared with 0.3%; $p<0.001$), and recovery from the lower two shelves (shelves 2 and 3; 0.21 and 0.31%, respectively) was higher than from the uppermost shelf (0.08%; $p<0.001$). FMC, fitted as a covariate, was significantly and positively associated with extra-pellet L3 recovery at d 7 and 14 ($p<0.001$; Figure 6-6).

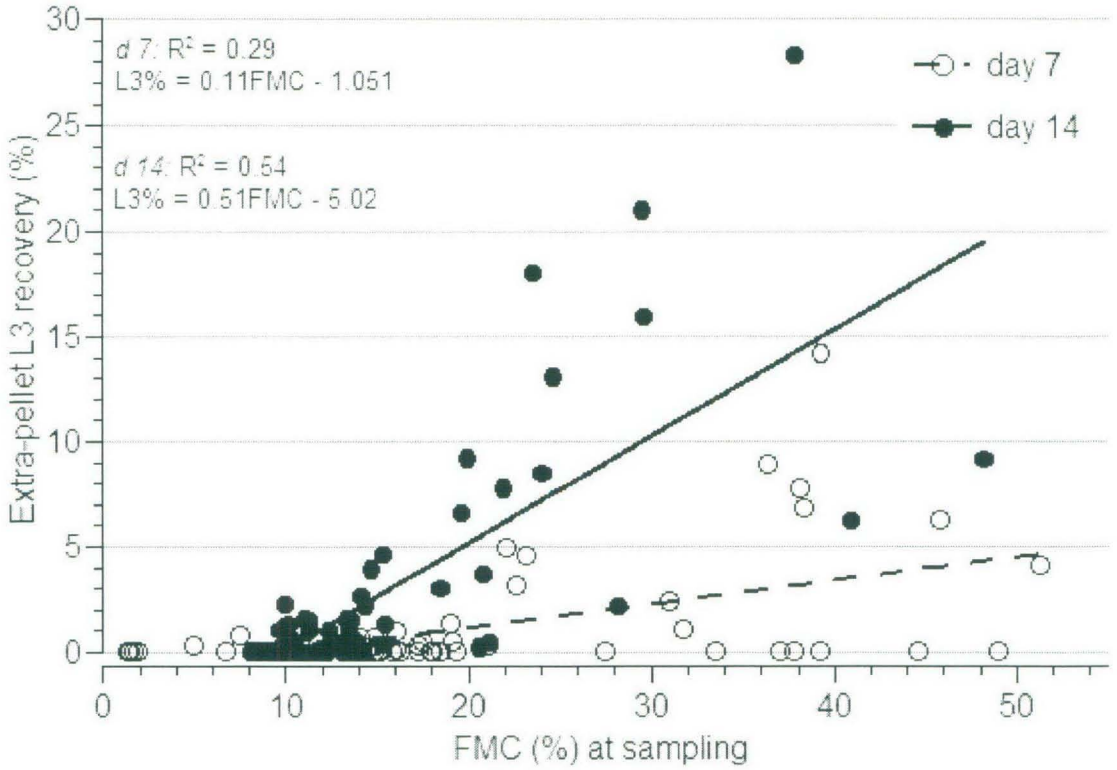

Figure 6-6: Regression of extra-pellet L3 recovery at d 7 and 14 with FMC at sampling (raw means and linear fits).

6.3.4 Recovery of pre-infective larvae

The raw means of total L1 and L2 recovery indicated that the bulk of the population was recovered from faecal material, with intra-pellet L1 and L2 contributing to 97-99% of the total L1 and L2 population between d 4 and 14. Raw mean recovery was considerably higher than recovery in the LS means model using transformed data, peaking at 16.8% on d 4, and declining to 10.4% at d 7 and 7.6% at d 14.

Least squares means recovery of total L1 and L2 decreased over time from 9.6% at d 4 to 4.4% at d 14 ($p<0.001$). Evaporation rate was a significant treatment effect ($p<0.001$), with greater total recovery under the Low (17.5%) than the High evaporation rate (1.3%). Amount of simulated rainfall was significant within Evaporation rate ($p<0.001$), with no significant difference between amounts at Low evaporation (average recovery 17.6%) but at High evaporation recovery from the 12 mm treatment (0.4%) was significantly lower than from larger amounts (average recovery 2.0%). There was no significant effect of distribution

(p=0.678) of simulated rainfall on L1 and L2 recovery, but FMC at sampling was significantly and positively associated with recovery (p=0.024; R^2=0.42). Recovery from shelf 2 (8.7%) was higher than from both other shelves (4.9% for shelf 1 and 5.7% for shelf 3; p<0.001).

Within each treatment combination, recovery of L1 and L2 invariably declined over time (data not shown) however the loss in L1 and L2 was not fully and quantitatively accounted for by the concurrent increase in extra-pellet L3 recovered over the same period. Success of development from pre-infective larvae to L3 was positively influenced by rainfall amount (p<0.001; 12 mm: 0.01%, 24 mm: 5.93%, 32 mm: 38.58%). Neither evaporation rate (p=0.558) nor rainfall distribution (p=0.191) were significant effects on success, and there was no effect of the d 4 or 14 FMC when fitted as a covariate.

6.3.5 Prediction of free-living development

Stepwise regression of extra-pellet L3 recovery (averaged over block) with P/E ratios indicated that recovery at d 7 was best described by P/E values at d 4 (R^2 = 0.48; Figure 6-7a), though d 3, 5, 6 and 7 P/E also provided good linear fits to recovery (R^2 = 0.45-0.46). However a 2nd order polynomial regression appeared to be a better fit (R^2 = 0.60) than the linear models (Figure 6-7a). Recovery of extra-pellet L3 at d 14 was best described by P/E at d 3 (R^2 = 0.45; Figure 6-7b), with P/E ratios for each day between d 4 and d 12 also good predictors (R^2 = 0.43-0.44).

Stepwise regression of success of development from pre-infective larvae to infective larvae with P/E ratios indicated that correlation was linear and positive but not very strong, with P/E at d 14 the best fit (p<0.001; Success = 32.47*d14P/E* -3.72; R^2 = 0.18).

The P/E ratios over time for each amount of simulated rainfall and evaporation rate are plotted in Figure 6-8 for two of the distribution treatments (simulated rainfall applied in 1 or 3 events). The raw means of extra-pellet L3 recovery over time for each treatment combination

is also shown. Under the High evaporation rate, P/E was less than 1 by d 3 , d 5 and d 6 following 12, 24 and 32 mm rainfall events, respectively (Figure 6-8a), and in each case recovery of L3 was less than 1%. In comparison, under the Low evaporation treatment P/E remained above 1 for 6, 8 and 13 d for the 12, 24 and 32 mm treatments, respectively (Figure 6-8b), and recovery of L3 was up to 8% under the 32 mm treatment. When rainfall was spread over 3 days, the decline in P/E was similarly rapid to the single event at the High evaporation rate, and recovery of extra-pellet L3 was never greater than 2.4% (Figure 6-8c). For the same distribution under Low evaporation, P/E remained above 1 until d 6, 10 and 13 for the 12, 24 and 32 mm treatments, respectively, and recovery of extra-pellet L3 was up to 16.6% under 32 mm of simulated rainfall (Figure 6-8d).

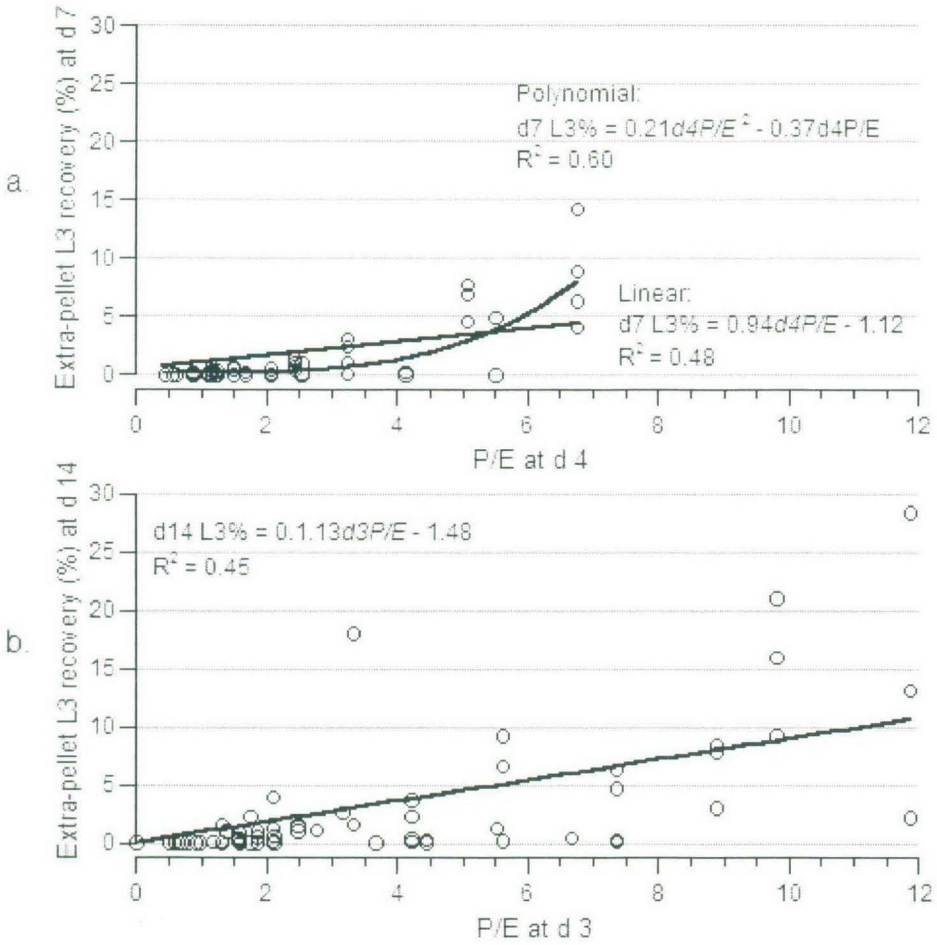

Figure 6-7: Regression of recovery of extra-pellet L3 with the most strongly correlated measure of cumulative P/E. a) Extra-pellet L3 recovery at d 7 with P/E at d 4. Raw means, and linear and polynomial fits; b) Extra-pellet L3 recovery at d 14 with P/E at d 3. Raw means and linear fit.

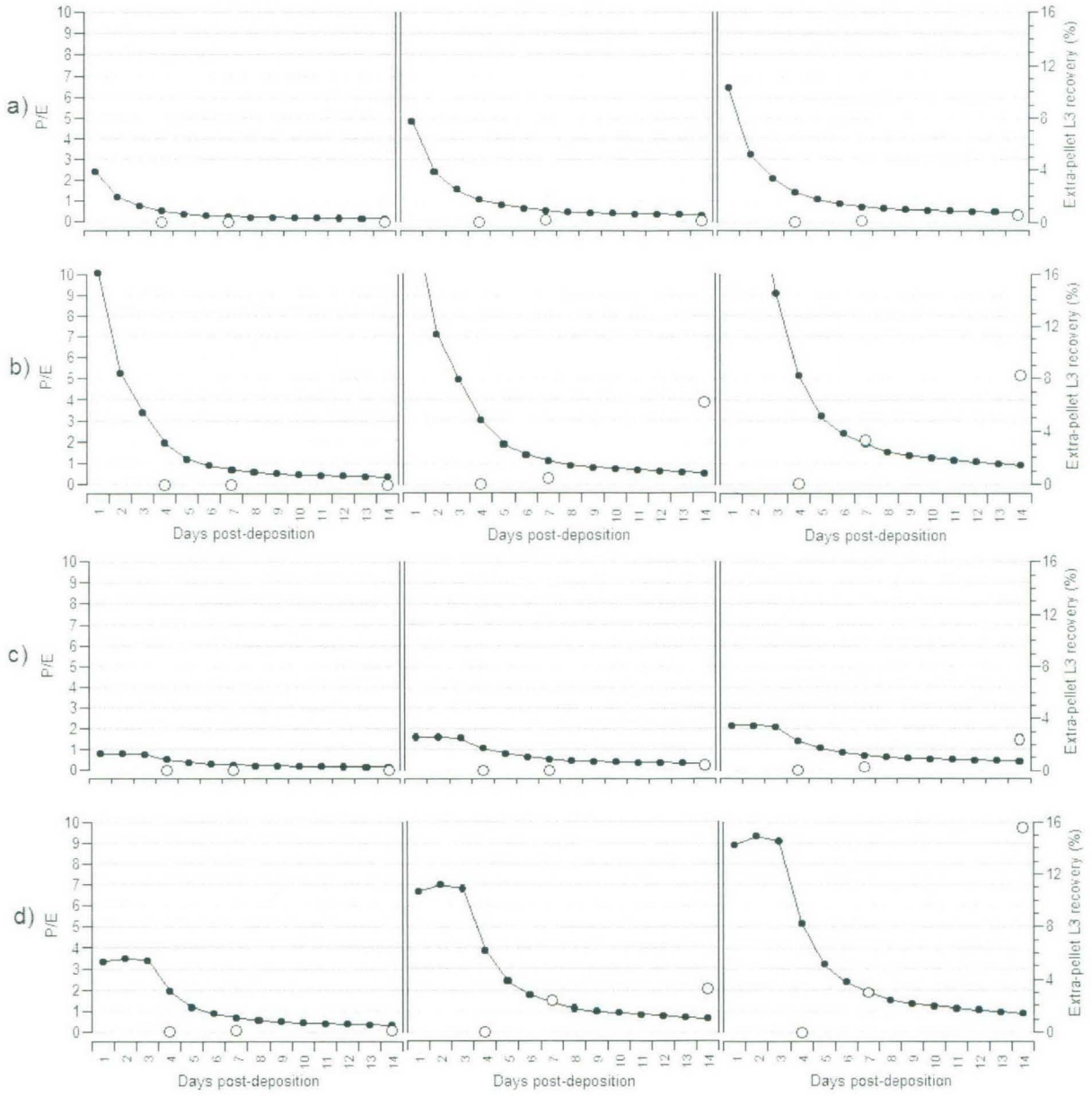

Figure 6-8: Cumulative P/E ratio (filled circle) and extra-pellet L3 recovery (hollow circle; raw means) over time within amount of simulated rainfall (12, 24 & 32 mm, left to right). Red dashed line indicates the point at which P/E < 1. a) Single simulated rainfall event at High evaporation rate; b) Single simulated rainfall event at Low evaporation rate. P/E values above 10 not shown. At 24 mm, P/E at d 1 was 12.9. At 32 mm, P/E at d 1 was 26.8; c) Simulated rainfall applied in 3 events at High evaporation rate; d) Simulated rainfall applied in 3 events at Low evaporation rate.

Figure 6-9 plots the mean recovery of extra-pellet L3 (averaged over time) against the number of days until the respective P/E value was less than 1. There was a strong positive association, with recovery increasing as the time between deposition and P/E < 1 increased. When P/E

was less than 1 within 4 days of deposition, no L3 were recovered in 11 out of 16 cases, and when L3 were present, recovery was never greater than 0.1%, and generally less than 0.03%.

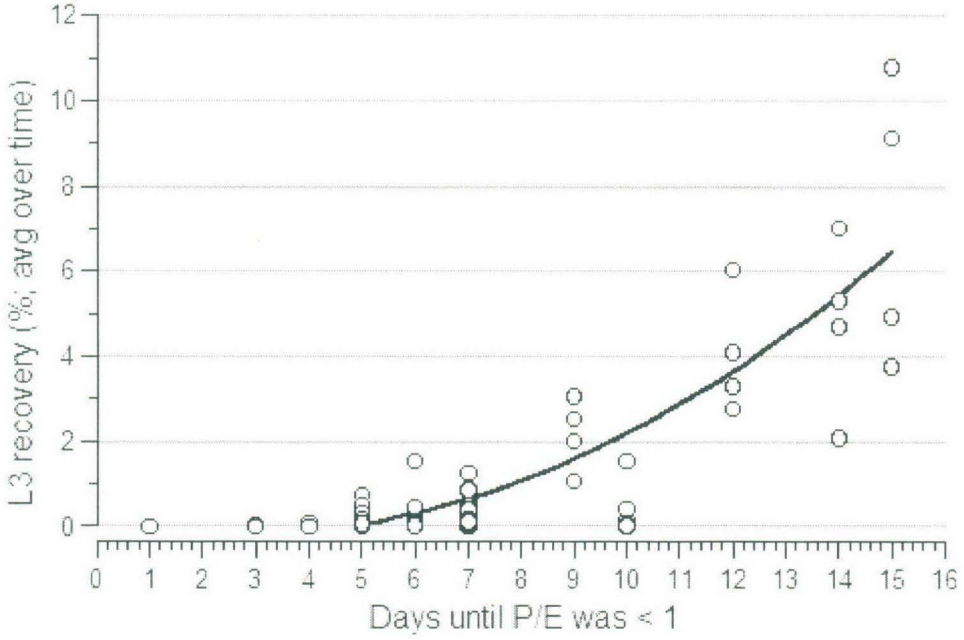

Figure 6-9: Regression of recovery of extra-pellet L3 (averaged over time; raw means) with days until cumulative P/E was less than 1. Polynomial fit to data indicated by solid line ($L3\% = 0.042P/E^2 - 0.21P/E$).

Forward stepwise regression of extra-pellet L3 recovery at d 7 with FMC at d 1, 2, 3, 4, 7 and 14 (averaged across block and shelf within each treatment combination) indicated that FMC at d 7 was the best predictor of recovery ($R^2 = 0.29$; regression shown in Figure 6-6). Recovery of extra-pellet L3 at d 14 was best fitted to FMC at d 14 ($R^2 = 0.54$; Figure 6-6). For both pre-infective larvae and degenerate egg recovery at d 4, FMC at d 4 was most strongly correlated ($R^2 = 0.54$ and 0.51, respectively; Figure 6-10a & b). Success of development from pre-infective (d 4) to infective larvae (d 14) was best described by FMC at d 7 ($R^2 = 0.23$; Figure 6-10c). The linear fit to the L1 and L2 recovery data (Figure 6-10a) suggests that few pre-infective larvae are likely to develop when FMC at d 4 is less than 9%. The data shown in Figure 6-10c suggests that few pre-infective larvae developed to infective larvae when FMC at d 7 was less than 10%, however the correlation was not strong.

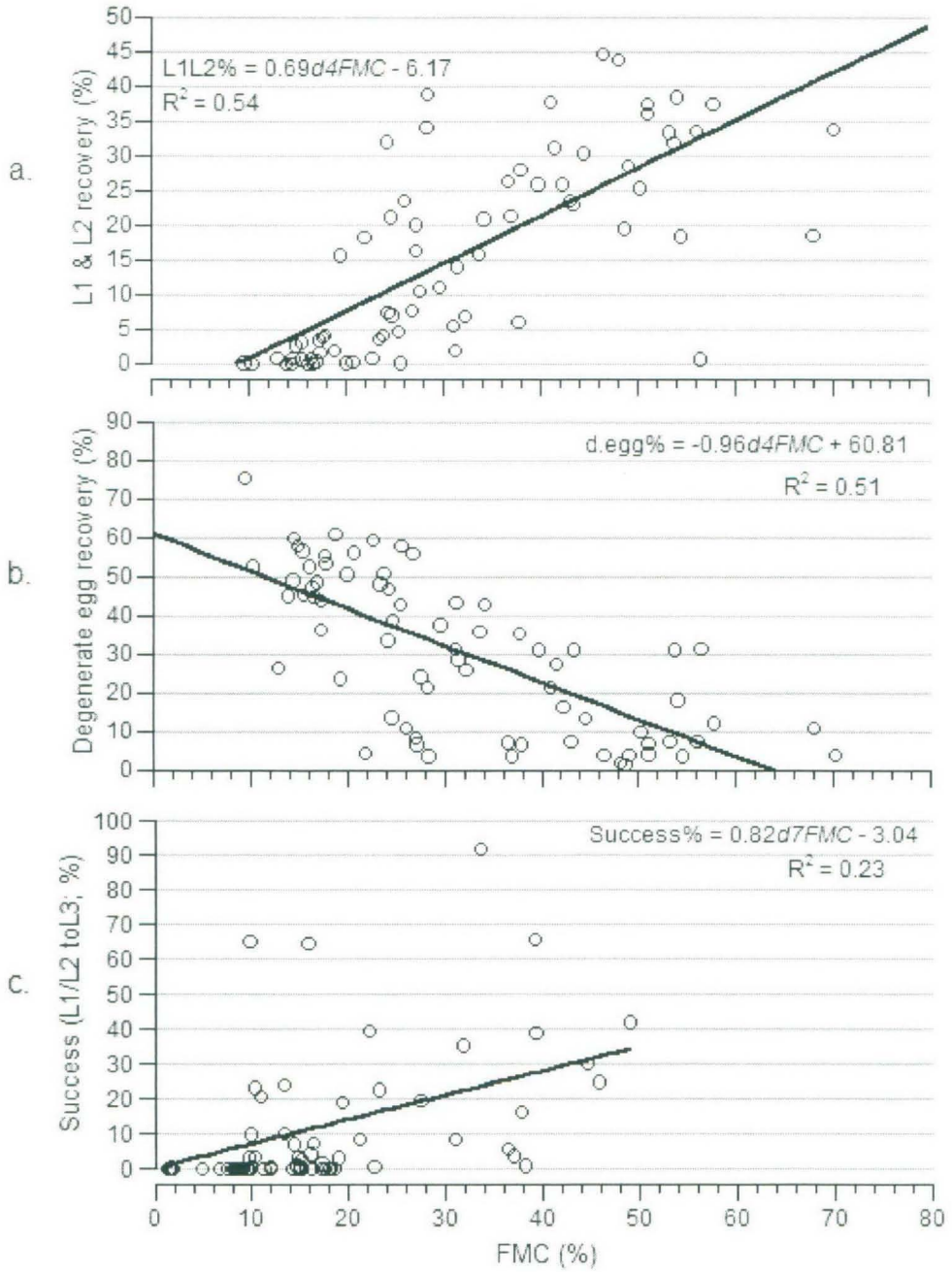

Figure 6-10: Regression of recovery of free-living stages with the most strongly correlated measure of FMC. a) L1 and L2 recovery at d 4 with FMC at d 4; b) Degenerate egg recovery at d 4 with FMC at d 4; c) Success of development from pre-infective larvae to infective larvae with FMC at d 7. Raw data and linear fits.

6.3.6 Effects on FMC

The least squares means of FMC, averaged over all treatments, decreased over time ($p<0.001$) from 32% at d 4, to 18% and 15% at d 7 and 14, respectively. The effect of evaporation rate was significant within day ($p<0.001$), with FMC declining over time across both levels of

evaporation, although there was no significant difference in FMC between d 7 and 14 under the high rate of evaporation (p=0.875; Figure 6-11). The effect of amount of simulated rainfall was significant within evaporation (p<0.001), with FMC increasing significantly with each increment of rainfall amount under the Low evaporation rate (21%, 27% and 40% for 12, 24 and 32 mm, respectively), but under the High evaporation rate only 12 and 32 mm were significantly different from each other (11% and 17%, respectively), with neither significantly different from the 24 mm value (14%). There were significant differences between simulated rainfall distributions (p=0.011), with linear contrasts indicating that FMC following 1 or 2 rainfall events (20%) was significantly lower than when rainfall was applied in 3 or 4 events (23%). Shelf was also significant (p<0.001) with lower FMC (19%) on the uppermost shelf than on the two lower shelves (mean of 23%). The changes over time in FMC (raw means; averaged across shelf and block) are shown in Figure 6-12 and Figure 6-13 for the High and Low evaporation rates, respectively, at each level of rainfall amount and distribution. Changes in FMC over time in the unwatered controls are shown in Figure 6-14. FMC was not recorded at d 1, 2 or 3 in the controls, hence no data is available for comparison with watered treatments during this period.

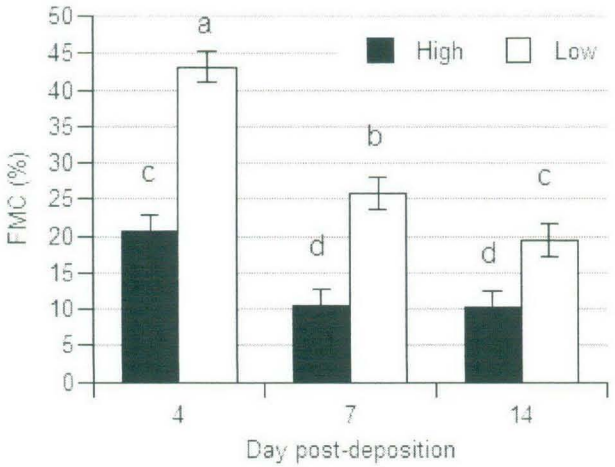

Figure 6-11: Least squares means (± 95% C.I.) of FMC over time within evaporation treatment. Means not sharing a common letter differ significantly (p<0.05).

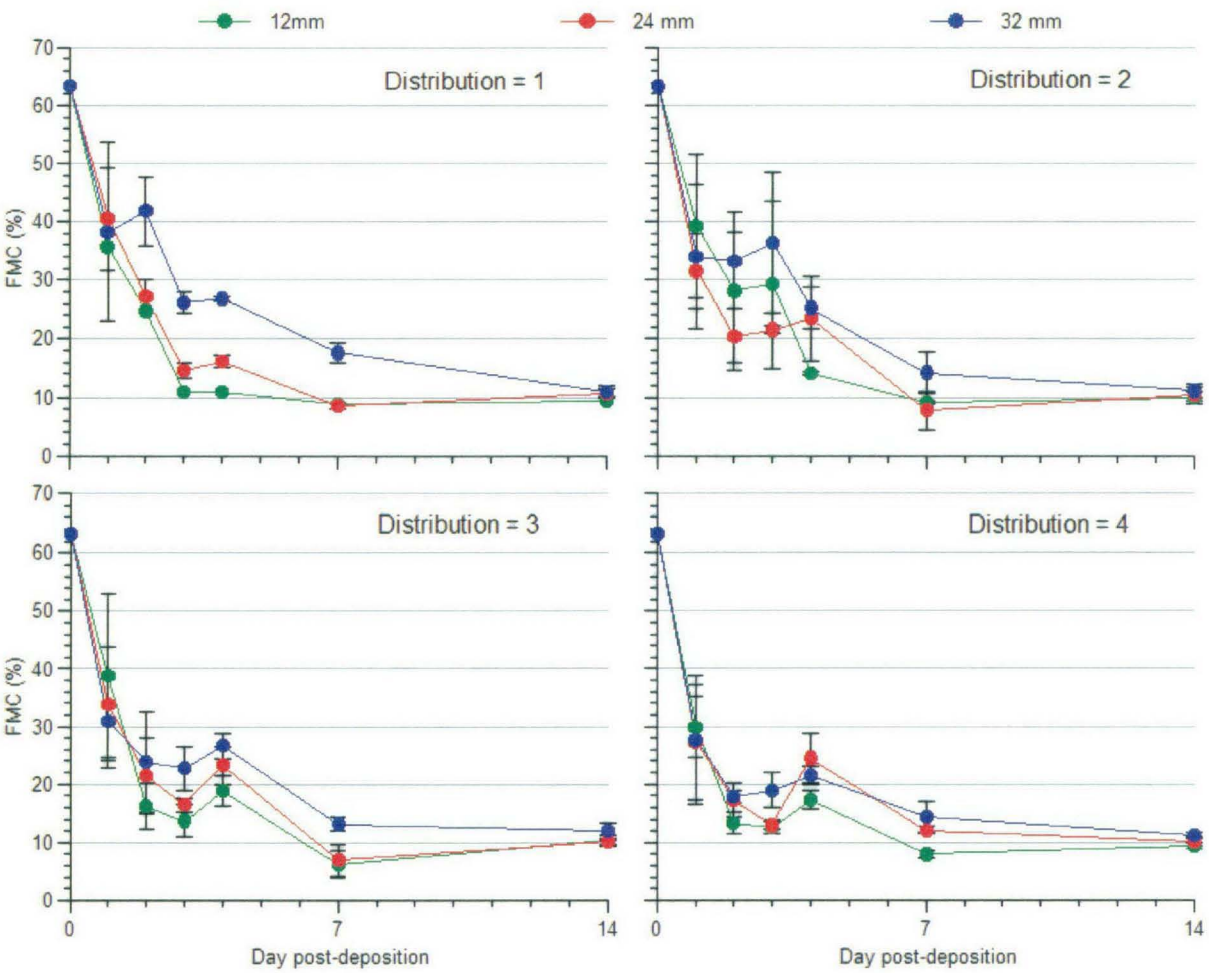

Figure 6-12: High evaporation treatment. Raw means (± s.e.) of FMC (%) over time (d 1, 2, 3, 4, 7 & 14) for each level of rainfall distribution (each amount applied in either of 1, 2, 3 or 4 events, over 1, 2, 3 or 4 days, respectively) and amount (12, 24 or 32 mm).

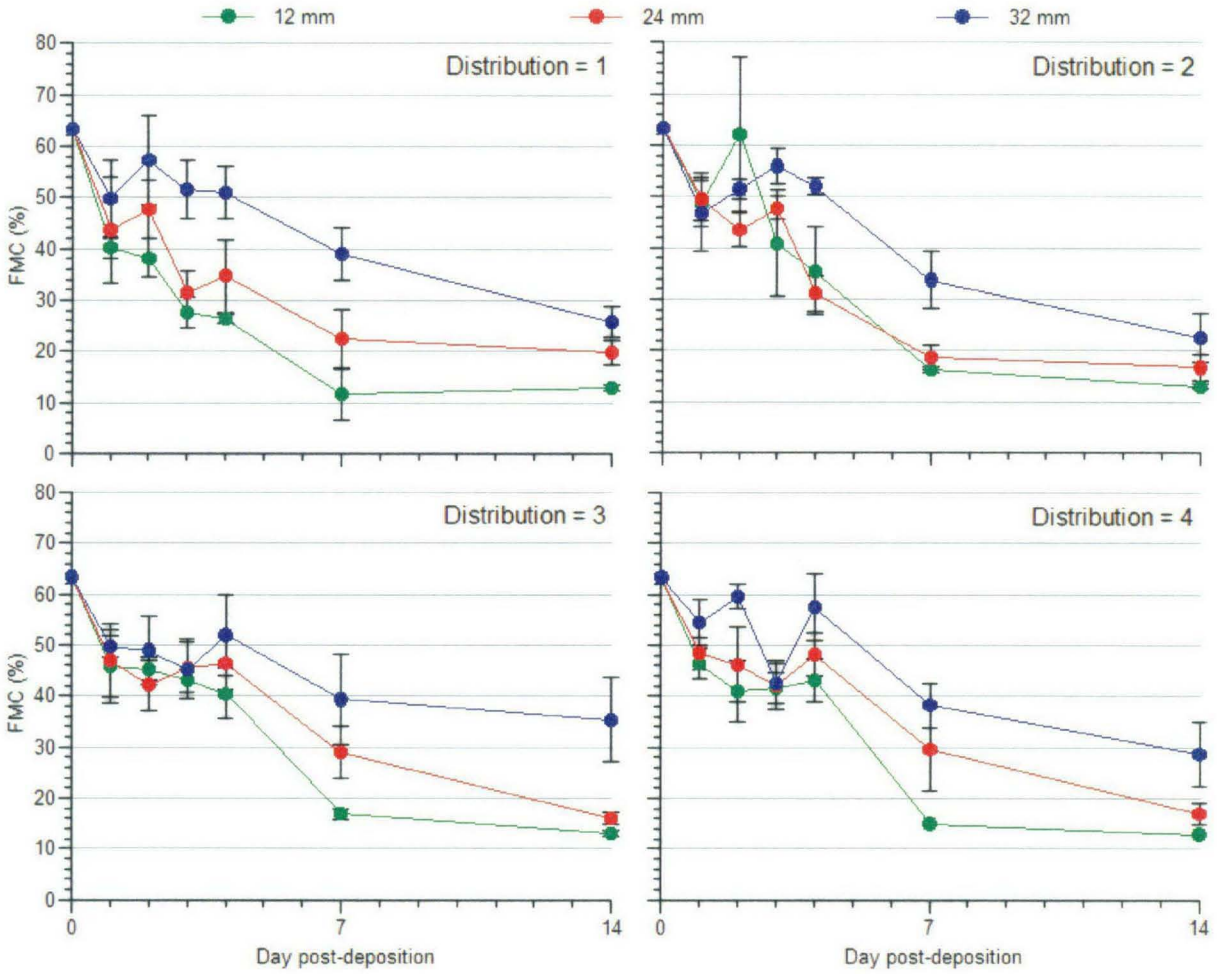

Figure 6-13: Low evaporation treatment. Raw means (± s.e.) of FMC (%) over time (d 1, 2, 3, 4, 7 & 14) for each level of rainfall distribution (each amount applied in either of 1, 2, 3 or 4 events, over 1, 2, 3 or 4 days, respectively) and amount (12, 24 or 32 mm).

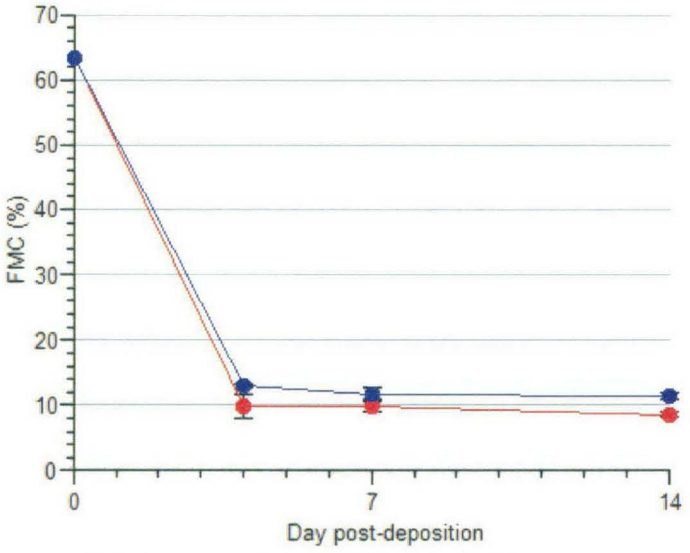

Figure 6-14: Unwatered control treatment. Raw means (± s.e.) of FMC (%) over time (d 4, 7 & 14) for both levels of evaporation rate (High and Low).

Since stepwise regression of extra-pellet L3 recovery at both d 7 and 14 with daily P/E values had indicated that d 4 P/E was strongly correlated with development (see section 6.3.5), FMC at d 4, 7 and 14 was regressed against P/E at d 4 (Figure 6-15a-c) to determine the effect of this P/E value on FMC over time. In each case, there was a strong positive linear association between the two variables, with R^2 values ranging between 0.68 and 0.74.

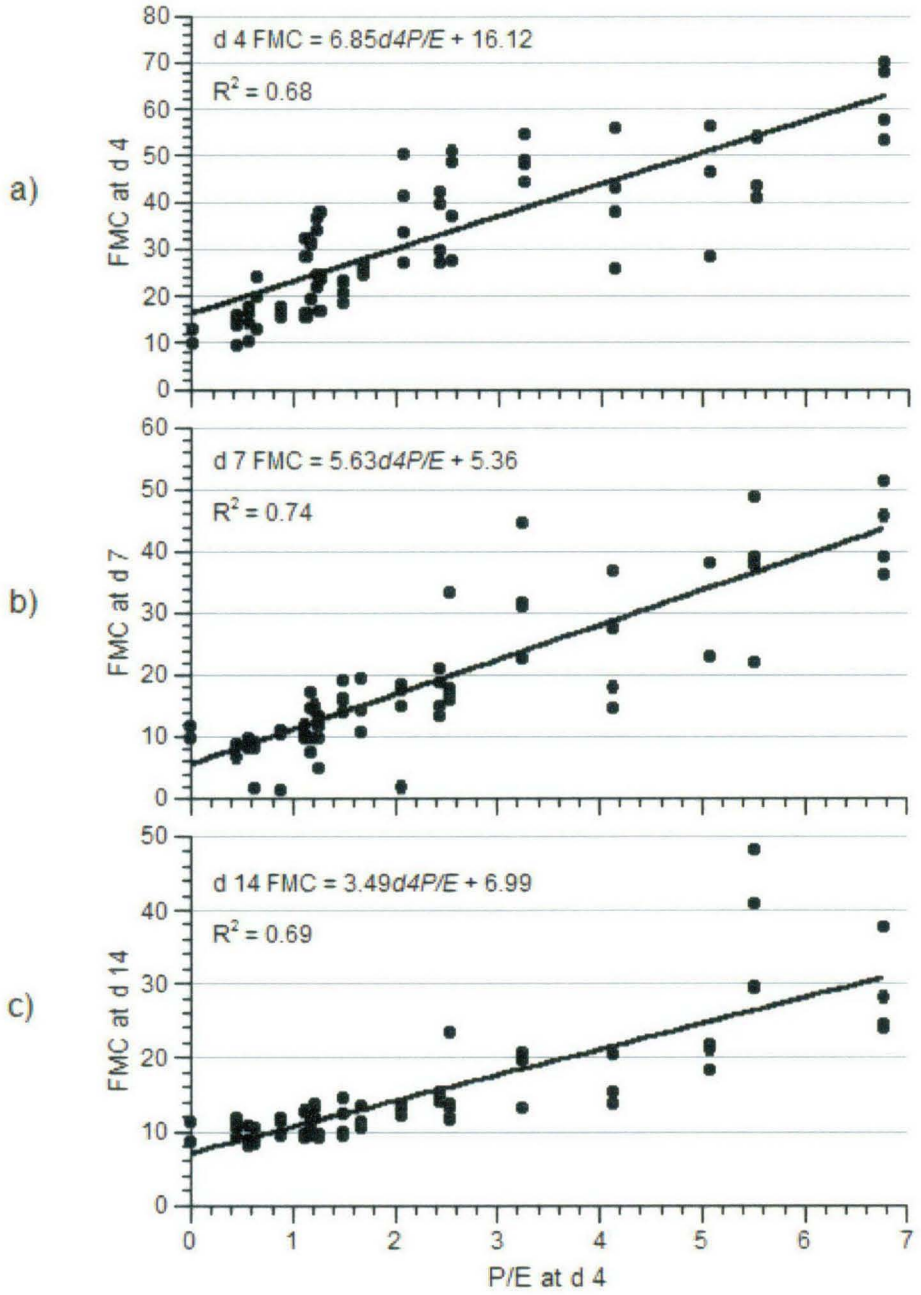

Figure 6-15: Regression of FMC (by day) with cumulative P/E at d 4. Raw means and linear fits. a) FMC at d 4 with P/E at d 4; b) FMC at d 7 with P/E at d 4; c) FMC at d 14 with P/E at d 4.

FMC at each sampling event (d 4, 7, and 14) was also regressed against the corresponding daily P/E value (ie. P/E at d 4, 7 and 14, respectively) to determine the correlation between FMC and P/E at each event (Figure 6-16). Again, there was a positive linear association between P/E and FMC on each day, with the correlation stronger at d 4 and 7 (R^2 = 0.67 and 0.75, respectively) compared to d 14 (R^2 = 0.57).

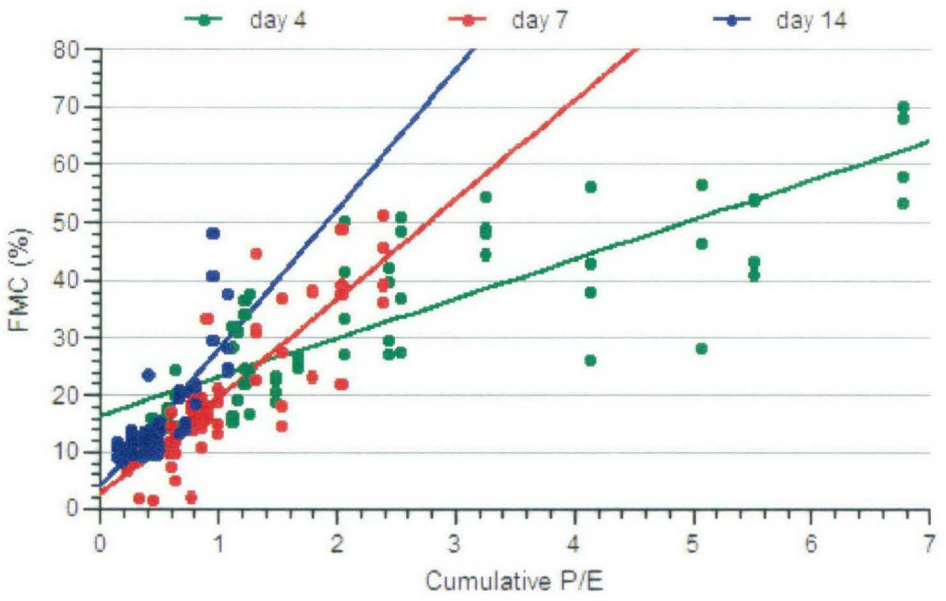

Figure 6-16: Regression of FMC with cumulative P/E within each day of sampling (d 4, 7 and 14). Raw means and linear fits. Day 4: FMC at d 4 = 6.80*d4P/E* +16.29, R^2 = 0.67; Day 7: FMC at d 7 = 17.21*d7P/E* + 2.23, R^2 = 0.75; Day 14: FMC at d 14 = 26.13*d14P/E* + 2.22, R^2 = 0.57.

6.3.7 Egg development and total recovery

Evaporation rate had a significant effect on overall embryonated egg recovery ($p<0.001$) with 9.62% recovery under the Low evaporation rate, compared to 0.92% under the High evaporation rate. Amount and distribution of simulated rainfall were also significant ($p=0.028$ and $p<0.001$, respectively). Evaporation rate was the only significant treatment effect on degenerate egg recovery ($p<0.001$), with 42.1% recovery under High evaporation and 13.3% under Low evaporation. Total recovery of free-living stages (intra- and extra-pellet) averaged 52.5% across the experiment.

6.4 Discussion

This study demonstrates that evaporation plays an important role in regulating the influence of amount of rainfall on *H. contortus* development, and that the cumulative ratio of total rainfall and evaporation in the 4 days following egg deposition offers considerable potential as a predictor of development success. The experiment has validated the qualitative relationships revealed in earlier experiments between rainfall amount and pre-infective development

(Chapter 4) and development to L3 (Chapter 5), and also confirmed that the poor recovery of L3 in the plot experiments (Chapter 4) was due largely to the inhibitive effects of the high evaporation rate, as discussed below.

One objective of the present experiment was to reconcile the results of earlier experiments conducted under the same temperature conditions, but which produced very different outcomes in terms of recovery of L3. The effects of amount of simulated rainfall observed in this study replicated those reported for the previous incubator experiment (Chapter 5), conducted under low evaporation rates, in which recovery of extra-pellet *H. contortus* L3 increased with each increment of rainfall amount regardless of the distribution of that rainfall. Similarly the low recovery rate of L3 under the high evaporation treatment in this experiment helps explain the results of the plot experiment (Chapter 4), in which evaporation averaged 4.8 mm/day. While the more rapid application of water, the presence of predatory organisms in the microenvironment (Waller & Faedo, 1996) and the potentially less sensitive recovery method may also have contributed to the poor recovery of L3 and the negligible effects of rainfall amount observed in the plot experiments, the effects of high evaporation rate, as revealed in the present experiment, are likely to have been the major limiting factor.

A second objective of the experiment was to investigate the effects of the interaction between evaporation rate and amount and distribution of rainfall on *H. contortus* development. When no rainfall was applied, as in the control treatments, faecal moisture was sufficient to allow some development to pre-infective larvae. There was an effect of evaporation rate on recovery of L1 and L2 in the absence of additional moisture, with higher recovery under the Low evaporation rate. Very low levels of translation through to intra-pellet L3 were also recorded in the controls however no L3 were recovered from outside the faeces. This may reflect an inability to escape from a drying pellet or low viability, with a trend for intra-pellet L3 recovery to decline between d 7 and 14.

Recovery of L3 increased with amount of rainfall. The effect of rainfall amount was most obvious in the latter stages of the development process, with success of development of pre-infective larvae to infective larvae increasing considerably as amount of rainfall increased. An effect of rainfall amount on egg hatching and development to pre-infective larvae was only apparent at High evaporation. This is most likely because the first simulated rainfall event began 17 h after faeces were placed in the incubators, during which time moisture would have become more limiting for hatching early development in the High than the Low evaporation treatment.

In contrast to the effect of rainfall amount, there was a substantial difference in pre-infective larval recovery between the two evaporation rates, but no effect of evaporation rate on development from pre-infective to infective larvae. It is difficult to explain the negligible effect of evaporation on development from pre-infective to infective larvae, although reductions in the difference between evaporation treatments beyond d 8 of the experiment may have contributed. It is possible, also, that the effect may be partly an artefact of the way in which success of development from pre-infective to infective was measured. Destructive sampling meant that the recovery rates on which the ratio was based – pre-infective larval recovery at d 4 and infective larval recovery at d 14 – could not be obtained from the same experimental units, and hence the ratio may not be a completely accurate reflection of actual development. It is also possible that the use of a ratio as a measurement of development may have confounded the findings, with the error associated with ratios generally greater than that associated with each the original measurements (Sokal & Rohlf, 1981).

The distribution treatment applied in the present study aimed to determine the influence of spreading rainfall events over 2–4 days post-deposition, after confirming in the earlier laboratory experiment (Chapter 5) that a single simulated rainfall event was more favourable than the same amount of rain applied in three smaller events over 6 days. That there was no

detectable difference in the current experiment in L3 recovery between rain applied in either a single event, or over either 2, 3 or 4 days, was surprising given that findings from previous experiments suggest that moisture conditions during these first 4-5 days post-deposition are key determinants of development success. However, the cumulative ratio of precipitation and evaporation may go some way towards explaining this result, as the variations in rainfall distribution failed to have any meaningful effect on the number of days post-deposition until P/E became limiting for development (ie. P/E < 1). So despite the differences in how each amount of simulated rainfall was applied over the first four days, the prevailing rates of evaporation were such that they appear to have negated any effect on moisture availability. Besier and Dunsmore (1993b) reported that more *H. contortus* L3 developed on pasture plots in southern Western Australia when 8 mm fell over 4 consecutive days compared to when 26 mm fell in a single day. However in field studies such as these, consecutive days of rain implies the presence of cloud cover and hence low evaporation rates. Separating the effects of distribution and evaporation, as was done in the present study, indicates that distribution of rainfall as measured in field studies is likely to influence the evaporation component of P/E as much as the precipitation component.

Along with influencing development potential, simulated rainfall and evaporation also appeared to affect vertical movement of L3 in the soil profile. Up to 13.5% of extra-pellet L3 recovered were found at least 25 mm below the soil surface, with higher rates of downward movement associated with low evaporation and larger amounts of simulated rainfall, suggesting that movement was not a response to unfavourable conditions at the soil surface, and possibly associated with flow of water down the soil profile. Gruner *et al.* (1982) reported that downward migration of *T. colubriformis, T. circumcincta* and *N. spathiger* to as low as 40 cm depth in the field was mainly due to water percolation through the soil. Viability of L3 below the soil surface in this study was not determined so no conclusions can be drawn as to

whether L3 recovered at depth in the soil profile may represent a source of infectivity following subsequent upward migration.

The third objective of the experiment was to quantify the threshold levels of rainfall and evaporation below which *H. contortus* free-living development is prohibited. The comparison between cumulative P/E and *H. contortus* recovery indicated that negligible L3 developed when P/E was less than 1 within 4 days of deposition, providing a quantifiable threshold of rainfall and evaporation under which development to L3 was unlikely. This finding differs from the assumptions of Barger *et al.* (1972), from which the cumulative P/E formula derives. These authors considered P/E from the opposite perspective, quantifying the success of L2-L3 transition based on how long P/E took to exceed 1, and reporting that 50% of L2 developed to L3 when P/E exceeded 1 in three weeks. Our findings indicated that if P/E was below 1 in the first few days following deposition, as was the case in each 12 mm treatment at the high evaporation rate, recovery of L3 was almost always zero. However even under the 32 mm treatment, less than 40% of pre-infective larvae reached L3 stage, indicating a substantial difference in the success of this developmental stage between this study and the data on which the modelling of Barger *et al.* (1972) is based.

A threshold level of cumulative P/E required for development between the pre-infective and infective stages was not established, as correlation between these two variables was low due to the negligible effect of evaporation on this developmental phase. The positive correlation between FMC at d 7 and success of development between pre-infective and infective larvae was also weak. As was discussed earlier, the ratio used to describe transition from pre-infective to infective larvae may have been confounded by a high degree of error, and the lack of consecutive recovery rates from the same experimental unit.

The fourth objective was to determine the moisture-related variables which best predict *H. contortus* development. As was suggested in Chapter 2, FMC may be the ultimate integrator

of moisture availability to the free-living stages, offering considerable potential as a predictor of both rate and success of development from egg to L3. This experiment suggests that under controlled conditions, where there is very little difference between micro- and macro-climate, FMC provides no advantage over the cumulative ratio of precipitation and evaporation for prediction of *H. contortus* development. FMC at d 14 showed a strong correlation with L3 recovery on the same day, although the correlation with L3 recovery averaged over time was weaker. The cumulative P/E ratio at d 3 and 4 also showed a strong correlation with L3 recovery at d 7 and 14 across all treatments, and, as discussed above, there was a strong relationship between the development to L3 and number of days P/E took to decline to less than 1. However, both variables will be useful for improving understanding of moisture effects of *H. contortus* development and prediction of paddock infectivity. As was shown by the close correlation of P/E and FMC at each sampling event, P/E was the main driver of FMC under the controlled experimental conditions. P/E is likely to remain the key influence on FMC under field conditions. However environmental factors such as aspect, slope and pasture cover are likely to modulate both the correlation between P/E and FMC, and ultimately moisture availability at the faecal pellet level. What remains to be determined is whether these modulating influences are so significant that the relationship between P/E at d 4 and L3 development established in this study requires modification in the paddock environment. The applicability of the P/E-L3 relationship to paddock conditions needs to be explored with field trials, where pasture and other microclimate factors can be accounted for.

If the association between P/E at d 4 and L3 development is shown to be less robust under field conditions (due to the modulating influence of microclimate factors on FMC), the links between P/E and FMC and between FMC and L3 provide an alternative method for prediction of development, although validation will be labour-intensive. Assuming that the correlation between FMC and L3 development is universal, determination of the association between P/E and FMC at any location will allow the effect of a given P/E on development to be determined

indirectly, given that P/E effects development largely via regulation of FMC. However this method entails a considerable volume of work in measuring FMC under various combinations of P/E, pasture cover, aspect and slope, along with other key determinants of microclimate moisture at each location. The workload, however, will be considerably smaller than measuring larval development in the field under the same combination of factors.

If the relationships established in this study do remain robust in the field, the "P/E formula" can be easily transferred to paddock conditions for prediction of pasture infectivity following deposition of *H. contortus* eggs. For example, under a rotational grazing system in which grazing events may be as short as 2-3 days, the rainfall and evaporation conditions during those days and the following 4-5 will determine the subsequent level of pasture infectivity. If evaporation is too high or rainfall too low for P/E to remain above one during that period, this work suggests that the paddock will be largely clean of *H. contortus* infective larvae.

The P/E relationship can be applied to set stocking systems to determine the developmental consequences of a given series of rainfall events on recently deposited faeces. However, pre-existing L3 on herbage from previous faecal depositions must also be accounted. Producers can obtain regional measurements of both rainfall and evaporation from meteorological services if on-site data is not recorded. However it is possible that future studies may derive estimates of evaporation from equations using wind, temperature or other weather conditions as predictors, if indirect methods such as this are found to be more accurate for prediction of *H. contortus* development. In a broader sense, the finding that a favourable moisture environment early after egg deposition is crucial for L3 development has important implications for drench decision-making in set-stocked situations. For example, in a flock where egg counts are below but approaching the drench threshold level, producers would be advised to hold off drenching in the event of continuing dry weather, but treat prior to a forecast wet weather system in order to minimise subsequent pasture contamination.

Although there is good evidence of a relationship between FMC and *H. contortus* development, it is difficult to quantify under the given conditions and further work is required to establish this relationship in a form that permits accurate prediction of L3 development. Additionally, the change in FMC over time may also have provided a good approximation of moisture availability over time, however FMC at d 0 was only measured as an average across the whole pool of faeces so no data was available for individual units. Future studies to determine the relationship between FMC and *H. contortus* development may be of greater value if changes in, rather than absolute, FMC are measured.

The regulation of evaporation rate in this study, in combination with application of simulated rainfall events, has highlighted the interactive effects of evaporation with rainfall amount in determining *H. contortus* free-living development success. Lack of development to L3 when cumulative P/E was less than 1 within 4 days of egg deposition implies a threshold level of moisture availability to free-living stages that, until now, has not been defined in terms of evaporation and rainfall. FMC also offers potential as a predictor of *H. contortus* free-living development, particularly as an integrator of both micro- and macro-climate effects on moisture availability, however further work is required to quantify its relationship with L3 development and to determine its role in predictive models based on cumulative P/E. If the relationship between cumulative P/E and *H. contortus* development success holds under field conditions, this formula will facilitate improved worm control on-farm by allowing prediction of the infective status of paddocks and therefore minimising exposure of sheep to infective larvae.