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A comparison of development times for *Protophormia terraenovae* (R-D) reared on different food substrates

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Abstract-

Experiments were conducted to determine whether a specific larval substrate impacted immature development rates. *Protophormia terraenovae* was raised on beef organs and compared with whole carcasses (rat), to determine if discrepancies in development times were observed.

The minimum development time on beef liver was the most consistent with the rat carcass but a significant difference between all substrates was found after the third instar. These differences can be explained by the differences found between heart and muscle and the other substrates.

Often length of the larvae is used to estimate insect age and so day 2 measuring of weight, length and width of the pupae was undertaken. Significant differences were found for all parameters measured on each of the substrates. As a result, the use of these measurements should not be done for *P. terraenovae* due to conflicting rearing substrates. Significant differences within substrates were only found for weight of insects developing on heart and length of insects developing on muscle.

There was no significant difference in survival from first instar to the adult stage on any of the substrates but personal observation suggested that mortality was higher in insects developing on the brain tissue. .

Key words: Rearing substrate, *Protophormia terraenovae*, Development

32 ## Introduction
33

34 Forensically important insect species including blow flies (Diptera: Calliphoridae)
35 are ubiquitous and are vital to the decomposition of remains. A decaying corpse provides
36 an ephemeral yet adequate resource for insect development. While feeding on the
37 decaying organism, the insect species in its immature stages, progresses through its
38 lifecycle. This progression is completed at a temperature dependent and predictable rate
39 which can be applied by forensic entomologists to estimate a post-mortem interval in death
40 investigations. Conflicting findings for these rates have been found (1-5). A multitude of
41 factors that result in variation in developmental rates can arise. There is a lower
42 temperature limit below which development does not occur and this, as well as rates for
43 insect development, have been found to differ for insects of the same species from
44 different geographical areas. Furthermore, the heat generated from maggot mass formation
45 can increase development rates substantially (6-9). Ambient temperature may be
46 consistent with the recorded temperature but this temperature may vary from that at which
47 the insects are developing (6, 9). If the maggots aggregate, they can create a micro
48 environment with much higher temperatures that can influence development rates (10-15).
49 Temperature fluctuations can also create varying development rates as compared to that at
50 the mean temperature (6, 9, 16).

51 Variables related to insect nutrition may also contribute to the differences in
52 development rates (17). The larvae feed on food sources that are semi-liquefied and then
53 further rely on their mouthparts to grind solid food into a pulp (18). The liquefaction can
54 result from a combination of bacteria creating an alkaline reaction during the

55 decomposition process and from the proteolytic digestion of the excreta released from the
56 larvae themselves (18). The extent to which the food source is liquefied may factor into
57 the rate of digestion or merely the ability to digest the source.

58 Often variable meat substrates including organs and sometimes something as
59 inadequate as artificial diets containing cat food (19) are used to obtain the developmental
60 data which are applied to death investigations. It is crucial to death investigations to avoid
61 any inconsistencies in development data based on any factors including rearing substrate
62 differences in developmental data collection as the consequences are too important. The
63 research completed by forensic entomologists at the Centre for Forensic Research at Simon
64 Fraser University, among many other researchers, is regularly conducted with beef liver. It
65 is important to determine whether inconsistencies in development times and rates lie
66 within the type of substrate used and whether application of the data generated with
67 substrates that are different from the specimen in question should occur. Discrepancies in
68 development times have been found among different substrates for several different blow
69 fly species. In fact, Ireland and Turner (17) found that of the three pig tissues studied,
70 brain, liver and muscle, brain was the least nourishing and most rapidly consumed by
71 *Calliphora vomitoria* Linnaeus. *Calliphora vomitoria* development was the most
72 successful on liver. This could be explained by the nutrition contribution of each tissue.
73 In comparison to muscle and liver, brain has a higher water content and fewer proteins and
74 carbohydrates to offer (17). Larvae of *Lucilia sericata* (Meigen) when fed on both cow
75 and pig liver, lung and heart were found to develop faster and to a larger size on the pig
76 tissues compared with the beef tissues (16). The authors also found that the insects

77 developed faster and to a larger size on lung tissue than on liver tissue (16). *Calliphora*
78 *vicina* (Meigen) larvae also develop at different rates on various rearing substrates (20).
79 *Calliphora vicina* was found to develop considerably faster on lung, brain, heart and
80 kidney than on pig liver (20). *Calliphora augur* (Fabricius) and *Lucilia cuprina*
81 (Wiedemann) were found to reach moult later and develop to a smaller size when
82 developing on sheep's liver compared to sheep's brain and meat (21).

83 Larval length is often used as an alternative for age estimation (1, 22, 23).
84 However, two problems arise with its use. The first of these is that an overlap between
85 lengths is often recognized for the varying stages (1). Secondly, head curling can often be
86 problematic (22) and so fixing the insects in a preservative is often done to correct this
87 (23). Under the circumstances when it is necessary to keep the insect alive or when
88 estimation is being made from a photograph, fixing insects is not an alternative. Day and
89 Wallman (22) have introduced an alternative method by measuring larval width. The
90 length and width measurements further distinguish the larval stages and perhaps can
91 distinguish the pupal stages as well. Likewise, Wells and LaMotte (24) have explored the
92 possibility of using weights to predict maggot age. A great deal of overlap of stages was
93 found between days of development extending the ranges of days beyond clear partitions.
94 Standardized measurements to distinguish age have not been explored at the pupal stage as
95 they have been with larval stages. Measurements taken at the pupal stage on day 2
96 considerably reduce the variability in measurements taken. Firstly, there is no need for
97 fixing for length measurements and this prevents a reduction in sample size. Secondly,
98 standardized measurements are possible because of the rather uncomplicated ability to

99 distinguish day 2 from any other day for each pupa as opposed to the difficulty of doing
100 the same in the larval stage.

101 The objectives of this research were to determine if significant discrepancies occur
102 with *Protophormia terraenovae* (Robineau-Desvoidy) development on various substrates
103 and to determine if it is possible to use a standardized pupal measurement for insect age
104 estimation of this species.

105 Methods and Materials

106 Four separate stock colonies were established from wild caught *P. terraenovae* and
107 have been replenished regularly each insect season from the Lower Mainland of British
108 Columbia. These insects were maintained in $(75cm)^3$ cages on a diet of milk powder,
109 sugar and water *ad libitum*. The adult flies in each cage were provided with approximately
110 200g of beef liver as an oviposition medium and when it appeared that sufficient numbers
111 of eggs had been laid (~4 hours), the medium was removed. The liver was placed into
112 rearing containers and more beef liver added.

113 Once the insects had reached first instar ~24 hours, the larvae were divided into one
114 of the 15, 4.5L wide mouthed glass rearing jars that contained approximately a 5cm depth
115 of dampened sawdust, folded industrial paper towel and the larval rearing medium. Three
116 replicates for each of the five media were conducted and a mean of 61 with a range of 50
117 to 71 first instar larvae were placed into each container. A paper towel lid was secured
118 over each jar using elastic bands to prevent the larvae from crawling out of the rearing jars.

119 First instar larvae rather than eggs were placed onto each of the rearing substrates
120 to be certain that the insects that were being used in the experiment were in fact viable.
121 Also, only 50 to 71 insects were used in each replicate to avoid overcrowding.
122 Overcrowding was avoided because of its ability to increase development rates. Maggot
123 mass formation can substantially amplify development temperatures above ambient
124 temperatures and therefore increase rates (9). Also, aggregation of maggots can cause
125 competition for resources. This too can increase development rates while decreasing insect
126 size (17).

127 Beef muscle was purchased at a butcher shop and a local abattoir was contacted for
128 beef organs including brain, heart and liver. The Animal Research Care facility at Simon
129 Fraser University provided frozen rat carcasses with abdominal incisions. The first instar
130 larvae were placed directly on the wound and other substrates. The rearing media were all
131 consistently previously frozen and thawed for the research with one exception. The veal
132 brain was never frozen. The abattoir advised against freezing as it impacts consistency of
133 the tissue.

134 The glass jars were placed in a Conviron® E/7 environmental chamber that was set
135 for continuous lighting and a relative humidity of 75%. The chamber maintained a mean
136 temperature of approximately 17˚C +/-1˚C and to account for any temperature differences
137 within the chamber the jars were rotated daily. These temperatures were recorded on ACR
138 Systems Inc. Smartbutton® data logger temperature recorders and confirmed with
139 Fisherbrand® mercury thermometers. The insects were checked approximately every 12
140 hours for stage changes in every jar. The larval stage changes were based on the number

141 of posterior spiracular slits and the crop size (25). For each stage, the minimum, mode and
142 maximum development times were determined.

143 On day 2 of the pupal stage, the insects were weighed and measured for both their
144 width and length to determine if a pattern occurs that can be used to further distinguish
145 stages within the pupal stage itself. This was done in accordance with Day and Wallman's
146 (22) larval measurements, that is; the width measurements of the pupae were made at the
147 intersection of abdominal segments five and six and the length measurements were made
148 from the outlying distances of the eighth segment of the abdomen and the anterior end of
149 the pupae. Finally the adult eclosed flies were weighed consistently on the same day, after
150 they had expired. All statistical analyses were completed using nonparametric Kruskal
151 Wallis test from the SAS statistics package JMP® version 7 and Microsoft Excel® was used
152 to plot the graphs.

153 ## Results

154 The mean temperatures for each of the three replicates in the environment chamber
155 were maintained at 17°C+/-1°C. There was no significant difference in development time
156 between replicates except the first replicate where all of the insects from each rearing jar
157 reached the adult stage on the 29th and the last day of experimentation (χ^2=5.99, df=2,
158 $p<0.0401$*).

159 Significant differences (α= 0.05) based on substrate were found for minimum
160 development to the adult stage (χ^2=9.49, df=4, $p<0.05$*). The minimum development to
161 the post feeding stage was also found to differ significantly (χ^2=9.49, df=4, $p<0.05$*). A

162 significant difference was noted for maximum development on the substrates to the post
163 feeding stage for all the food types (χ^2=9.49, df=4, p<0.03*). At alpha 0.05, significant
164 differences were not identified for mode of development to any stage (second instar,
165 χ^2=7.05 and p> 0.13; third instar, χ^2=8.26 and p>0.08; post feeding, χ^2=8.65 and p> 0.07;
166 pupal stage χ^2=2.99 and p> 0.56; and adult stage, χ^2=7.45 and p> 0.11) for any of the
167 substrates.

168 Clear differences in development rates are observed for each of the different
169 substrates and development on no one substrate reflects the development of the insects on
170 the whole rat carcasses. However; based on the mean minimum and maximum
171 development, the development on beef liver most closely resembles the development on
172 the rat carcass (Figure 1, Figure 2).

173 Mode of development is defined as when the most frequent number of insects
174 changed stage at one time. On average, insects developing in the jars that contained brain
175 tissue were the first to reach each stage. The exceptions were maximum development time
176 on heart to second instar and minimum time of development and mode of development to
177 the post feeding stage (Figure 3).

178 A significant difference was not found for the survival rates between substrates
179 however, the mortality of the insects developing on brain was highest in the third instar.
180 Also, the second liver replicate died off completely during the second instar. Percent
181 survival was greatest on muscle followed by heart which was where the insects were the

182 slowest to develop and survival was lowest on brain where the insects developed the
183 fastest (Table 1).

184 A statistical significance in weight was found between substrates for day 2 pupal
185 measurements (α=0.05, χ^2= 22.0382, df=4, p<0.0002*). The insects were weighed at both
186 the adult stage following death as well as on the second day of the pupal stage and the
187 mean weights for each replicate and each substrate are presented in Table 2. The ratio of
188 mean pupal to adult weight range from 4.83:1 for those raised on heart to 6.88:1 for those
189 raised on brain. A substantial difference in mean ratio of pupal weight compared to adult
190 weight exists for heart, liver and muscle compared to a mean ratio of 6.75:1 for the rat. A
191 consistency in size of the pupae in relation to the adult size does not appear to be a set
192 component of the insects development.

193 Also on Day 2 of the pupal stage length and width measurements were taken. For a
194 sample of 152, significant differences of the mean length and width measurements were
195 found. For length, a significant p value of less than 0.0001 was indicated (α=0.05, χ^2 of
196 23.79, df=4) and for width, a significant p value of less than 0.01 was indicated (α=0.05, χ^2
197 of 12.74, df=4) (Table3).

198 Discussion

199 Previous experiments had not been done on blow flies to compare development on
200 different rearing substrates to an entire corpse. The opportunity was taken here to compare
201 development on a wounded rat carcass to development on beef tissues including the
202 commonly used rearing substrate, liver. Beef liver is used rather extensively to determine

203 developmental data for many blow fly species and not just *P. terraenovae*. Development
204 rates were found to differ for *P. terraenovae* on the different rearing substrates but in most
205 cases not significantly. However, significant differences were found for the minimum
206 development times to the adult and post feeding stages. In all probability, the significant
207 difference to the adult stage can be explained by the significant difference between brain,
208 heart and muscle alone ($\chi2=5.99$, df=2, $p<0.05$*). Also, to further explain the significant
209 difference of the post feeding stage, although not indicated as being significant, a
210 difference is evident to the post feeding stage with heart, liver, muscle and rat ($\chi2=7.81$,
211 df=3, $p<0.050$. A significant difference was observed to the maximum development time
212 on all substrates to the post feeding stage only. This difference appears to be the result of
213 the different rates between development on brain as compared to development on muscle
214 or heart ($\chi^2=3.84$, df=1, $p<0.05$*). There were no significant differences observed for
215 mode of development for any of the rearing substrates.

216 An analysis of the mean weights of the pupae on day 2 and the adult flies after
217 death indicated that the mean weights of the insects raised on brain in the pupal stage were
218 the greatest but produced flies that were some of the smallest at a mean weight of 0.009g.
219 It can be argued that the insects that developed on muscle and heart fed the longest and
220 produced the greater sized adult flies, however, they still maintained some of the smaller
221 pupal sizes. The insects that developed on liver had a noticeably large mean weight for
222 both the pupal stage and the adult stage whereas those that were reared on the rat carcass
223 developed into small pupae and small adults. Size differences of the same species are

224 found based on which organ the insect was feeding on. If insect size was used to estimate
225 age, a strong bias would be transferred to that age determination.

226 The photoperiod which fluctuates with ambient temperature (26), was not set to
227 fluctuate between light and dark because the temperature was maintained at a constant
228 17ºC. Therefore, to maintain a consistent environment, the chambers remained in
229 continuous light as well as at continuous temperature. The replicate experiments were run
230 in an environment chamber set at 17ºC, however, the chamber did not maintain an
231 equivalent temperature throughout so it was necessary to rotate the replicates in the
232 chamber as well as rotate the jars within each replicate. A significant difference in
233 development between replicates was only found when measuring the maximum
234 development at the adult stage but may be explained by all of the third replicates reaching
235 the adult stage at the same time on the 29th day or the last day. Furthermore, the data
236 logger recordings are not representative of the jar rotations within each replicate because
237 there was only one datalogger representing all five jars at one time so the temperatures
238 exposed to each of the jars may in fact be more similar than alleged.

239 The liver development data are based predominately on two replicates since the
240 second liver replicate completely and unexpectedly died during the third instar larval stage.
241 This unanticipated result cannot be explained since the beef liver was from the same liver
242 source as the other experiments and the first instar larvae were randomly selected from the
243 four separate stock colonies and randomly placed into each of the experimental jars.

244 The weight, length and width measurements of *P. terraenovae* were taken at the
245 pupal stage to determine if a pattern occurs that can be used to further distinguish stages
246 within the pupal stage itself. The parameters measured on day 2 of the pupal stage did not
247 indicate a pattern and the use of measured parameters including weight, length and width is
248 not recommended for *P. terraenovae* pupae since a significant difference was observed for
249 the varying substrates. However significant differences within substrates were only
250 observed for weight with development on heart and length with development on muscle.
251 Therefore, the developmental substrate may determine whether these parameters can be
252 used to predict pupal age. An interesting finding that was incidentally noted was the
253 decrease in weight of the pupae as the days passed and an examination of this occurrence
254 may provide further indication of an accurate post-mortem interval for certain species of
255 blow flies and would be worth exploring.

256 The insects that developed on brain developed the fastest but had an extremely low
257 percent survival from first instar to adult emergence. Although not a statistically
258 significant difference, this is an important trend to be considered for future research. This
259 higher mortality is probably due to brain tissue having higher water content and also being
260 the least nourishing of the experimental substrates (17). Alternatively, the insects that
261 developed at the slower rates on muscle and heart had the greatest survival rates. This is
262 suggestive of an inverse relationship between survival and rate of consumption. Research
263 completed on other insects has indicated that there is an optimal balance of dietary
264 requirements and that if this balance is not met, functioning decreases (27). Perhaps this is
265 also true for *P. terraenovae* and future research should explore this phenomenon.

266 Furthermore, forthcoming research should involve comparing development rates
267 for wounded versus non-wounded substrates. The decomposition may occur at dissimilar
268 rates as the liquefaction of the rearing position may be affected differently from micro-
269 organism activity increasing alkalinity and from proteolytic digestion of the excreta from
270 the maggots themselves (18). Rates may be affected and a significant difference may be
271 observed. It is quite probable that since variation occurs between some rearing substrates,
272 it may also occur within substrates and that different development data may be required for
273 the initial stages of non-wounded substrates.

274 Conclusion

275 The research indicated that development on beef liver follows most closely with
276 that of the development on abdominal wounds of the rat carcass but it cannot be assumed
277 that data collected on liver can be applied under all circumstances. If there are no wounds,
278 the typical insect development occurs on the mucosal tissues of the face and genitalia.
279 Under such circumstances, it is probable that the brain tissue and facial muscles will be
280 consumed first and therefore, a combination of brain tissue and muscle may best represent
281 the tissues consumed during development. Development may be completed at a different
282 rate than that which is presently assumed. Nevertheless, in a death investigation, it should
283 always be noted as to where on the body the insects were collected so that the closest
284 rearing substrate development data can be applied to the tissues in question.

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367 **Figure 1 Mean minimum development times to reach each stage at**
368 **~17˚C in days**

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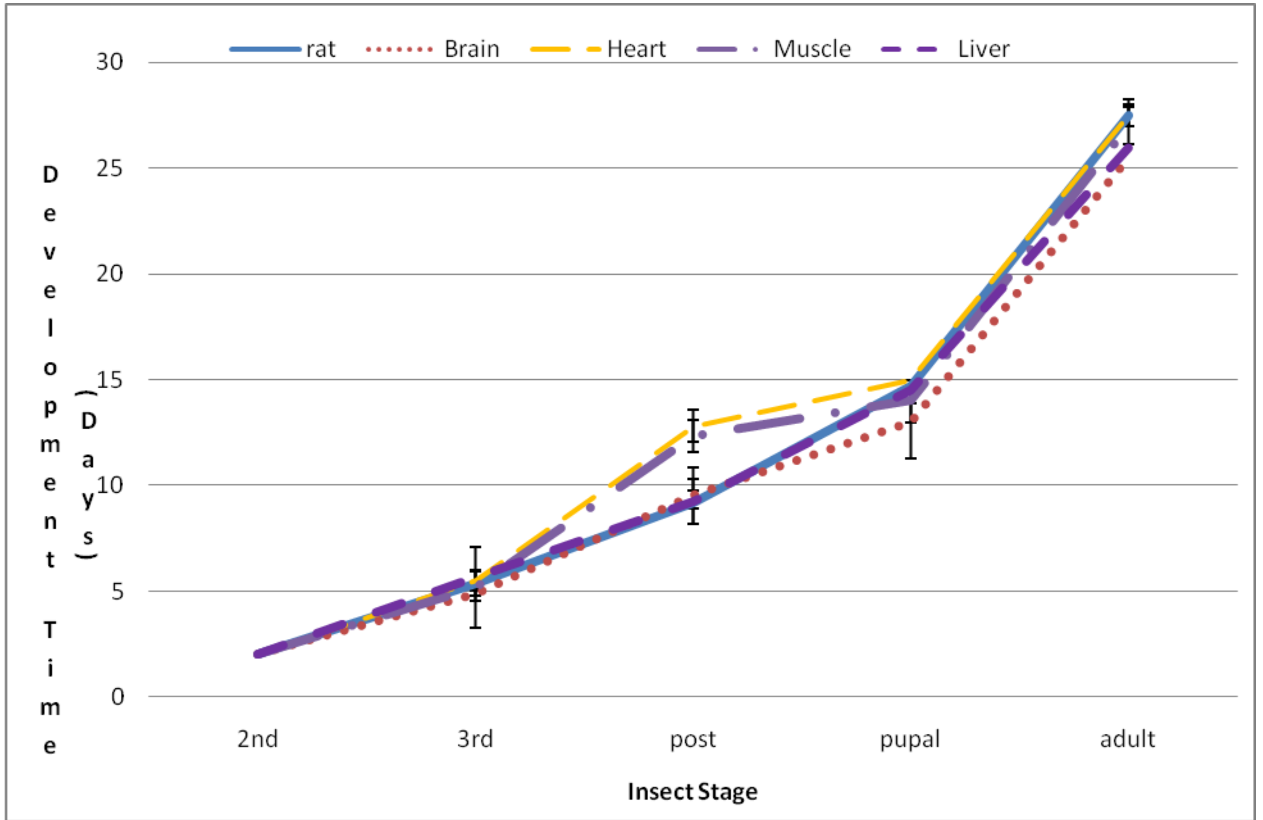

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403 **Figure 2 Mean maximum development times to reach each stage at**
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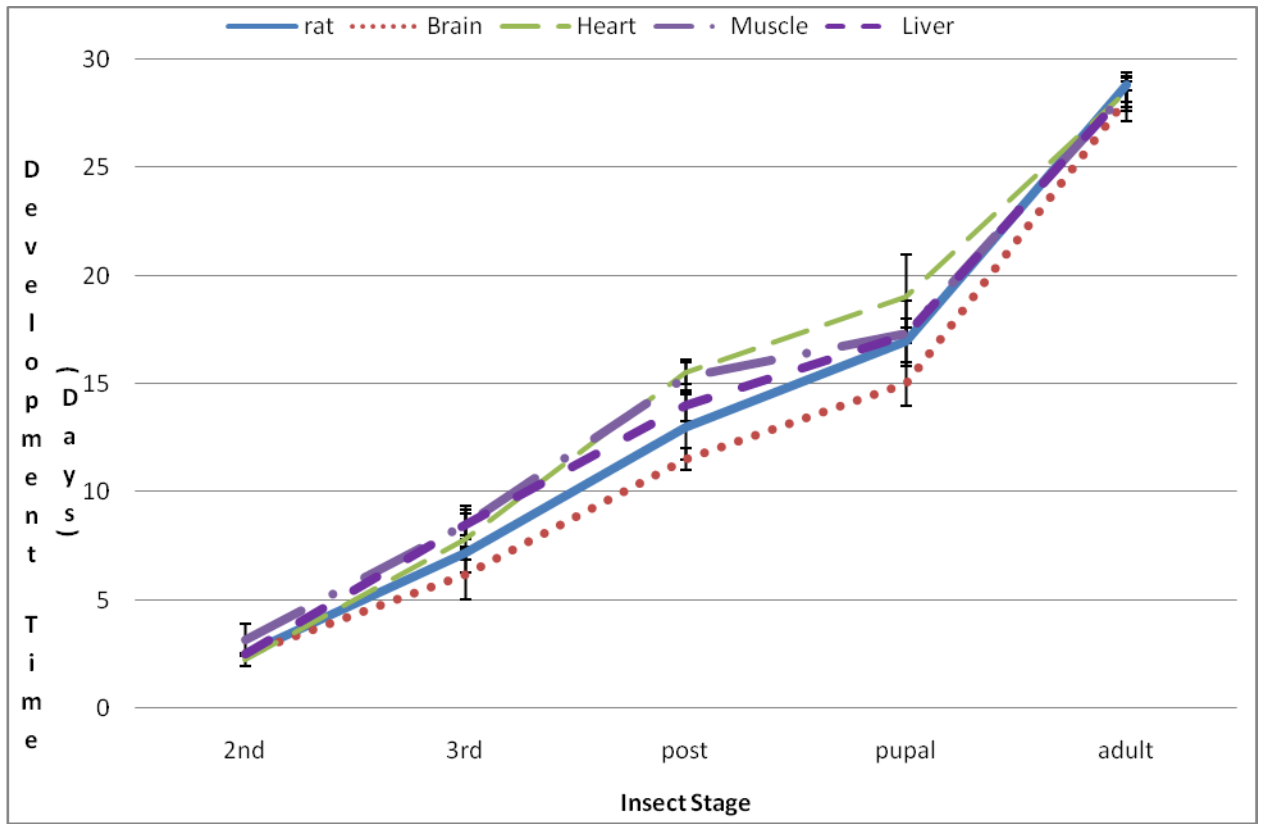

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430 **Figure 3 Mean of mode development to reach each stage at ~17°C in**
431 **days**

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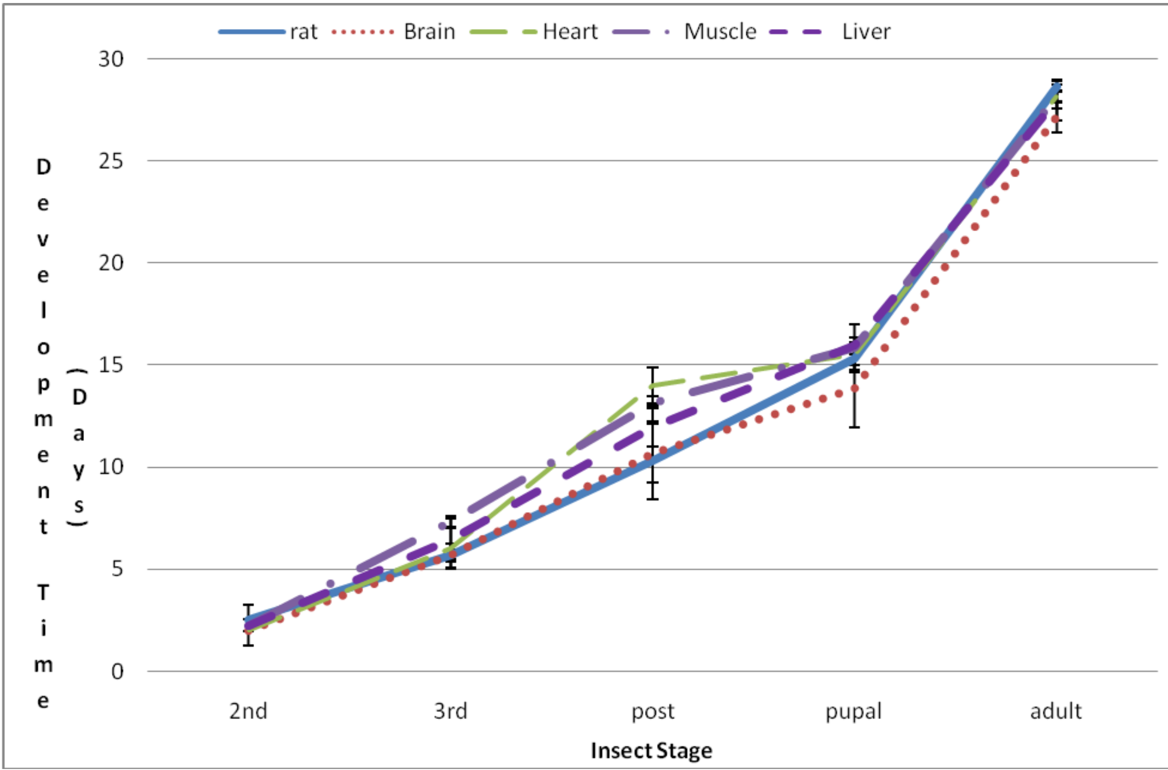

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464 **Table 1 Survival rates for each of the replicates and the mean for**
465 **each rearing substrate**
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Replicate	# Larvae	Adults Emerged	% Survival	%Mean
Rat (1)	70	11	15.7	
Rat (2)	60	12	20	
Rat (3)	65	8	12.3	16.0
Brain (1)	57	5	8.7	
Brain (2)	60	4	6.67	
Brain (3)	70	21	30	15.1
Heart (1)	70	19	27.1	
Heart (2)	68	6	8.82	
Heart (3)	58	23	39.7	25.2
Muscle (1)	51	13	25.5	
Muscle (2)	50	25	50	
Muscle (3)	52	27	51.9	42.5
Liver (1)	57	12	21	
Liver (2)	54	0	0	
Liver (3)	71	23	32.4	17.8

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491 **Table 2** **The mean weights (+/-SD) of the pupae on day 2 and the**
492 **adults two weeks following death.**
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Replicate	Mean weight of Pupae	Mean pupal weight for each substrate	Mean weight of Adults	Mean adult weight for each substrate
Rat (1)	0.053+/-0.006g		0.007+/-0.001g	
Rat (2)	0.055+/-0.006g		0.009+/-0.001g	
Rat (3)	0.054+/-0.008g	0.054+/-0.006g	0.007+/-0.002g	0.008+/-0.002g
Brain (1)	0.059+/-0.003g		0.008+/-0.001g	
Brain (2)	0.062+/-0.005g		0.008+/-0.001g	
Brain (3)	0.062+/-0.007g	0.062+/-0.006g	0.010+/-0.001g	0.009+/-0.001g
Heart (1)	0.057+/-0.002g		0.011+/-0.002g	
Heart (2)	0.049+/-0.002g		0.009+/-0.001g	
Heart (3)	0.060+/-0.007g	0.058+/-0.007g	0.013+/-0.001g	0.012+/-0.002g
Muscle (1)	0.057+/-0.005g		0.010+/-0.001g	
Muscle (2)	0.059+/-0.004g		0.011+/-0.002g	
Muscle (3)	0.055+/-0.006g	0.056+/-0.005g	0.009+/-0.002g	0.010+/-0.002g
Liver (1)	0.058+/-0.007g		0.014+/-0.002g	
Liver (2)	Nil		Nil	
Liver (3)	0.059+/-0.008g	0.059+/-0.008g	0.011+/-0.001g	0.012+/-0.002g

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513 **Table 3 The mean lengths and widths (+/-SD) of the pupae**
514 **measured on Day 2 of the Pupal stage**
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	Trial 1		Trial 2		Trial 3	
	Length (mm)	Width (mm)	Length (mm)	Width (mm)	Length (mm)	Width (mm)
rat	9.4+/-0.5	3.1+/-0.4	9.4+/-0.5	3.1+/-0.4	9.4+/-0.5	3.1+/-0.4
Brain	10.1+/-0.6	3.4+/-0.6	9.9+/-0.4	3.4+/-0.4	9.9+/-0.4	3.5+/-0.5
Heart	9.9+/-0.4	3.4+/-0.5	9.3+/-0.5	3.3+/-0.5	9.8+/-0.5	3.3+/-0.6
Muscle	9.3+/-0.3	3.1+/-0.6	9.7+/-0.3	3.5+/-0.5	9.4+/-0.5	3.2+/-0.5
liver	9.8+/-0.4	3.6+/-0.4	nil	nil	9.8+/-0.5	3.4+/-0.5

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