



Digital supplementary material to

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Detailed descriptions of all larval instars of *A. echinator*

First instar larvae: Fat vesicles (likely part of the insect fat body (ZARA & CAETANO 2004)) are always visible (Fig. 2B). Body is plump with no segmentation and gut is empty or appears yolk-like (Fig. 2B). Perimeter dorsal hairs are absent. Perimeter ventral hair number is absent for A1-10 and 0-7 for T1-3. Perimeter cephalic hair number ranges from 0-2. The overall ratio is 0:0:3:1, respectively, and the total number of hairs on the perimeter of the larvae ranged from 0-8 ($n = 10$ for all hair counts). If present, simple hairs (i.e. without obvious bifurcations) appear in short rows on the ventral surface, visible when using a stereomicroscope.

Second instar larvae: Type I and Type II 2nd instar larvae were also slightly different in head to body ratio ($p < 0.02$; Fig. 3B, Tab. S3), with Type I being more reminiscent of 3rd instar ratios and Type II resembling the ratios of 1st instar larvae. These trends are opposite the differences in body length (Fig. 3A, Tab. S3), suggesting that the two morphotypes may represent developmental programs leading to different allometric growth of body parts, but we were unable to resolve this issue further.

Type I. The head is pointed down and sometimes curled toward the anus (Fig. 2B)) and fat vesicles are always visible (Fig. 2B). Perimeter dorsal hairs are absent. Perimeter ventral hair number ranges from 0-2 for A1-10 and 1-6 for T1-3. Perimeter cephalic hair number ranges from 2-5. The overall ratio is 0:1:4:3, respectively, and the total number of hairs on the perimeter of the larvae ranged from is 5-10 ($n = 12$ for all hair counts; Tab. S2).

Type II. Larvae have a similar but less variably C-shaped dorsal profile and fat vesicles are always visible (Fig. 2B, D). Perimeter dorsal hairs are absent. Perimeter ventral hair number ranges from 0-3 for A1-10 and 9-26 for T1-3. Perimeter cephalic hair number ranges from 1-13 on the forward protruding head. The overall ratio is 0:1:16:5, respectively, and the total number of hairs on the perimeter of the larvae ranged from is 13-37 ($n = 17$ for all hair counts; Tab. S2).

Third instar worker larvae: Small and large larvae differ significantly in length ($p < 0.01$; Fig. 3A, Tab. S3). Hair patterning is distinct from the 2nd instar (Fig. 2B, D). Hairs with a bifurcating anchor tip align in triangular patches on each dorsal lateral segment matching the pattern on the 4th instar larvae (Fig. 1A, B, Fig. 4A, B, Fig. 5 A-D). The sclerotized spiracle is near the tip of the triangular patch of hairs and trachea can sometimes be viewed (Fig. 1A). Perimeter dorsal hairs of the 3rd instar small worker range from 139-233. Perimeter ventral hair number ranges from 36-61 for A1-10 and 15-41 for T1-3. Perimeter cephalic hair number ranges from 7-18. The overall ratio is 14:4:2:1, respectively, and the total number of hairs on the perimeter of the larvae ranged from is 206-350 ($n = 15$ for all hair counts; Tab. S2). Conversely, perimeter dorsal hairs of the large 3rd instar worker range from 148-203. Perimeter ventral hair number ranges from 35-53 for A1-10 and 21-25 for T1-3. Perimeter cephalic hair number ranges from 15-26. The overall ratio is 9:2:1:1, respectively, and the total number of hairs on the perimeter of the larvae ranged from is 219-296 ($n = 3$ for all hair counts; Tab. S2).

Fourth instar worker larvae (prenymph): Small and large larvae differ significantly in length ($p = 0.001$; Fig. 3A; Tab. S3). Hair patterning and type is similar to 3rd instar larvae (Fig. 1A, B, Fig. 4A, B). Perimeter dorsal hair count from a single 4th instar small worker is 192. Perimeter ventral hair number is 47 for A1-10 and 21 for T1-3. Perimeter cephalic hair number is 18. The overall ratio is 11:3:1:1, respectively, and the total number of hairs on the perimeter of the larvae is 278 ($n = 1$ for all hair counts; Tab. S2). Conversely, perimeter dorsal hairs of the 4th instar large worker range from 164-227. Perimeter ventral hair number ranges from 44-66 for A1-10 and 18-23 for T1-3. Perimeter cephalic hair number ranges from 14-20. The overall ratio is 11:3:1:1, respectively, and the total number of hairs on the perimeter of the larvae ranged from is 241-317 ($n = 7$ for all hair counts; Tab. S2).

Ventral thoracic hairs are variable, but dorsal hairs consistently have a bifurcating anchor tip that shows similarities with male hairs in the same location (Fig. 5C, I). They do remain distinct because they are shorter and stouter than the hairs of both reproductive larvae (Fig. 6). Some hairs on other parts of the body may have more than two denticles, but this level of detail can only be viewed with SEM images (Fig. 5B, C). These hairs appear short, stout and with anchor tips when viewed with a stereomicroscope (Fig. 1A-C). Cephalic hairs are shallowly branched (Fig. 7A, B, E) unlike those of the males (Fig. 7C, F) and gynés (Fig. 7D, G).

Third instar male larvae: The last abdominal segments may not yet be wider than the thoracic region (Fig. 4C). Overall body shape is plump. Fat vesicles are visible (Fig. 4C). The gut is full and there may be mycelium tufts on the body surface. The head can move freely and may be positioned more forward than downward (Fig. 4C). Hairs appear very dense all over the body (Fig. 4C). They are also aligned in triangular patches on each dorsal lateral segment similarly to the subsequent 4th and 5th instar male larvae. Perimeter dorsal hairs of the 3rd instar male larvae range from 121-242. Perimeter ventral hair number ranges from 19-53 for A1-10 and 16-29 for T1-3. Perimeter cephalic hair number ranges from 10-27. The overall ratio is 11:2:1:1, respectively, and the total number of hairs on the perimeter of the larvae ranged from is 184-326 (n = 16 for all hair counts; Tab. 2).

Fourth instar male larvae: The last abdominal segments are wider than the thoracic region (Fig. 4C). Fat vesicles are visible with a stereomicroscope (Fig. 4C). The gut is full and mycelium tufts are absent. The head can move freely, is positioned downward, and is small relative to the rest of the body area (Fig. 4C). It may be tucked in when the specimen is alive (Fig. 4C). Hairs appear less dense all over the body (Fig. 4C). They are aligned in triangular patches on each dorsal lateral segment similarly to the 3rd and 5th instar male larvae. Perimeter dorsal hairs of the 4th instar male larvae range from 154-233. Perimeter ventral hair number ranges from 34-53 for A1-10 and 20-44 for T1-3. Perimeter cephalic hair number ranges from 11-20. The overall ratio is 12:3:2:1, respectively, and the total number of hairs on the perimeter of the larvae ranged from is 224-342 (n = 5 for all hair counts; Tab. S2).

Fifth instar male larvae (prenymph): The last abdominal segments are generally wider than the thoracic region (Fig. 4C). Fat vesicles are absent or few (Fig. 4C). The gut is cleared, mycelium tufts are absent, developing legs can be clearly distinguished, and individuals are no longer moving. The head capsule is positioned downwards, empty, and very small compared to the rest of the body (Fig. 4C). Cephalic hairs are unbranched and narrow towards their simple tip (Fig. 7C, F). Hairs appear less dense than in 4th instar but continue to align in triangular patches on each dorsal lateral segment (Fig. 4C). Perimeter dorsal hairs of the 5th instar male larvae range from 128-195. Perimeter ventral hair number ranges from 26-51 for A1-10 and 26-40 for T1-3. Perimeter cephalic hair number ranges from 14-25. The overall ratio is 9:2:2:1, respectively, and the total number of hairs on the perimeter of the larvae ranged from is 194-297 (n = 5 for all hair counts; Tab. S2).

Third instar gyne larvae: The last abdominal segments are similar in width to the thoracic region (Fig. 4D). Fat vesicles are visible (Fig. 4D). The gut is full and there are zero to few mycelium tufts on the body surface. The head capsule is full and can move freely and may be positioned downward (Fig. 4D). Curled hairs appear very dense all over the body and are aligned in triangular patches similarly to the 4th and 5th instar larvae (Fig. 4D). Perimeter dorsal hairs of the 3rd instar gyne larvae range from 148-218. Perimeter ventral hair number ranges from 23-44 for A1-10 and 9-18 for T1-3. Perimeter cephalic hair number ranges from 6-12. The overall ratio is 21:4:2:1, respectively, and the total number of hairs on the perimeter of the larvae ranged from is 186-289 (n = 3 for all hair counts; Tab. S2).

Fourth instar gyne larvae: The last abdominal segments are similar in width to the thoracic region (Fig. 4D). Fat vesicles are somewhat visible (Fig. 4D). The gut is full and there are no mycelium tufts on the large body surface. The head capsule is full and can move freely and may be positioned more downward

than larger/fatter individuals (Fig. 4D). Leg development may be underway and visible through the translucent body in the oldest individuals. Hairs remain dense but less than in 3rd instar and have a similar patterning as the 3rd and 5th instar larvae. Perimeter dorsal hairs of the 4th instar gyne larvae range from 171-206. Perimeter ventral hair number ranges from 32-48 for A1-10 and 12-20 for T1-3. Perimeter cephalic hair number ranges from 7-10. The overall ratio is 21:5:2:1, respectively, and the total number of hairs on the perimeter of the larvae ranged from is 248-271 (n = 3 for all hair counts; Tab. S2).

Fifth instar gyne larvae (prenymph): The last abdominal segments are similar in width to the thoracic region (Fig. 4D). Fat vesicles are not visible, the gut is empty, and the head no longer moves and has receded from the head capsule. Developing legs and pigmented eyes may be seen through translucent body. Hairs appear similarly dense compared to 4th instar and maintain typical patterning on each dorsal lateral segment (Fig. 4D, Fig. 6D). Cephalic hairs have bulbous blunt-end simple hairs when viewed with high magnification (Fig. 7D, G). Perimeter dorsal hairs of the 5th instar gyne larvae range from 172-212. Perimeter ventral hair number ranges from 35-53 for A1-10 and 8-29 for T1-3. Perimeter cephalic hair number ranges from 9-13. The overall ratio is 16:4:2:1, respectively, and the total number of hairs on the perimeter of the larvae ranged from is 224-300 (n = 7 for all hair counts; Tab. S2).

Table S1: Microsatellite loci used to determine the sex of larvae of *A. echinator*; *k*, observed number of alleles; *T_a*, optimal annealing temperature (°C); Cycles, number of cycles in PCR; F/R primer, ratio of forward and reverse primer volumes in PCR.

Name	F/R primer sequence	Product size (bp)	<i>k</i>	<i>T_a</i> (°C)	Cycles	F/R primer (μl)
Acrins 02 (NASH, PERS. COMM.)	F: TCGCGTCCATACTATGGAAG R: AGCATTGTACCTGGGCTTTG	160-168	3	56.0	32	0.3
Acrins 05 (NASH, PERS. COMM.)	F: ACCTCTCGTACTCGGTCTGG R: GCTGAGCGTCTATGGAGCAG	114-142	11	56.0	32	0.3
Acrins 22 (NASH, PERS. COMM.)	F: CCTGACGCCTTCGAATTAC R: GATCAACATGTCTTCCAAGGTG	135-157	7	56.0	32	0.2
Acrins 29 (NASH, PERS. COMM.)	F: CACGAGTCTATCTCGTTTTACTGG R: CATGACCTTACACGCCACTG	116-122	4	56.0	32	0.3
Ech1390 (ORTIUS-LECHNER & al. 2000)	F: CTCCACATTGACACACTACTCG R: GACGAAAAGAGAGGTAATCG	106-110	3	54.0	31	0.2
Ech3385 (ORTIUS-LECHNER & al. 2000)	F: TGAAAGTAGAAACACGGATGA R: GCCCTAGAACAAATTACATCG	149-169	7	54.0	31	0.5
Ech4225 (ORTIUS-LECHNER & al. 2000)	F: CTTTTCTCCATTTCTTCGTG R: AGAGGGATAAAGTGGAAAATAA	285-338	12	54.0	31	1.4
Ech4126 (ORTIUS-LECHNER & al. 2000)	F: GTTCATTACGAGACCTGTAA R: GAACGTCAGTTTTACGACA	149-181	7	62.0	33	0.2

Table S2: Hair counts from the perimeter of the body. Hair count ratios refer to the approximate proportional distribution of hairs on the dorsal (abdomen + thorax segments), ventral abdominal (A1-10 segments), ventral thoracic (T1-3 segments) and cephalic regions, respectively (see Fig. 1).

Caste	Instar	n	Total hair-count	
			Mean (Range)	Ratio
Unknown	1	10	4 (0-8)	0:0:3:1
Type I	2	12	8 (5-10)	0:1:4:3
Type II	2	17	22 (13-37)	0:1:16:5
Small worker	3	15	266 (206-350)	14:4:2:1
Small worker	4	1	278 (na)	11:3:1:1
Large worker	3	3	258 (219-296)	9:2:1:1
Large worker	4	7	278 (241-317)	11:3:1:1
Gyne	3	3	241 (186-249)	21:4:2:1
Gyne	4	3	259 (248-271)	21:5:2:1
Gyne	5	7	270 (224-300)	16:4:2:1
Male	3	16	261 (184-326)	11:2:1:1
Male	4	5	275 (224-342)	12:3:2:1
Male	5	5	260 (194-297)	9:2:2:1

Table S3: Larval measurement data.

Caste	Instar	n	Body length (mm)	Body Area (mm ²)	Head Area (mm ²)	Inner angle (degree)	Head:body (ratio)
			Mean \pm SD (Range)	Mean \pm SD (Range)	Mean \pm SD (Range)	Mean \pm SD (Range)	Mean \pm SD (Range)
Unknown	1	16	0.75 \pm 0.09 (0.56-0.85)	0.23 \pm 0.07 (0.15-0.36)	0.02 \pm 0.01 (0.01-0.03)	70.86 \pm 5.76 (60.22-82.60)	0.08 \pm 0.02 (0.04-0.13)
Type I	2	51	1.01 \pm 0.15 (0.59-1.26)	0.48 \pm 0.13 (0.16-0.68)	0.03 \pm 0.01 (0.003-0.06)	62.78 \pm 9.73 (38.26-92.29)	0.07 \pm 0.03 (0.02-0.16)
Type II	2	24	1.39 \pm 0.21 (1.05-1.83)	0.86 \pm 0.27 (0.49-1.44)	0.07 \pm 0.02 (0.04-0.11)	75.33 \pm 8.5 (63.4-91.25)	0.08 \pm 0.02 (0.04-0.14)
Small worker	3	23	2.35 \pm 0.21 (1.99-2.8)	2.37 \pm 0.38 (1.75-3.18)	0.12 \pm 0.02 (0.06-0.16)	72.66 \pm 8.31 (50.38-86.04)	0.05 \pm 0.01 (0.04-0.07)
Small worker	4	23	2.97 \pm 0.6 (2.22-3.94)	4.01 \pm 1.67 (2.15-6.86)	0.13 \pm 0.06 (0.05-0.33)	80.36 \pm 5.91 (70.49-93.31)	0.03 \pm 0.01 (0.01-0.06)
Large worker	3	15	3.79 \pm 0.77 (2.85-5.13)	6.63 \pm 2.67 (3.34-11.95)	0.12 \pm 0.05 (0.08-0.26)	71.5 \pm 6.07 (61.05-86.69)	0.02 \pm 0.01 (0.01-0.05)
Large worker	4	23	4.65 \pm 0.76 (3.91-7.39)	9.90 \pm 3.87 (6.88-25.05)	0.12 \pm 0.04 (0.06-0.22)	74.18 \pm 6.65 (60.06-85.69)	0.01 \pm 0 (0.01-0.02)
Gyne	3	3	3.57 \pm 0.79 (2.66-4.11)	6.52 \pm 2.84 (3.28-8.60)	0.05 \pm 0.004 (0.01-0.09)	51.28 \pm 10.29 (41.79-62.22)	0.01 \pm 0 (0-0.01)
Gyne	4	3	7.39 \pm 0.4 (7.01-7.81)	25.67 \pm 2.22 (23.34-27.76)	0.17 \pm 0.04 (0.13-0.22)	56.35 \pm 6.87 (48.43-60.76)	0.01 \pm 0 (0.01-0.01)
Gyne	5	8	7.34 \pm 0.18 (7.1-7.58)	25.22 \pm 0.85 (24.26-26.37)	0.15 \pm 0.03 (0.12-0.19)	59.09 \pm 6.35 (48.31-68.15)	0.01 \pm 0 (0-0.01)
Male	3	23	3.64 \pm 0.77 (2.28-5.17)	6.76 \pm 2.81 (2.27-13.61)	0.12 \pm 0.04 (0.07-0.25)	67.26 \pm 5.48 (59.06-76.79)	0.02 \pm 0.01 (0.01-0.05)
Male	4	5	6.46 \pm 1.32 (4.93-8.32)	20.33 \pm 7.41 (12.63-31.14)	0.14 \pm 0.06 (0.07-0.24)	66.5 \pm 4.23 (61.11-71.8)	0.01 \pm 0.01 (0-0.02)
Male	5	4	7.81 \pm 0.57 (6.98-8.18)	29.50 \pm 3.99 (23.78-32.63)	0.13 \pm 0.004 (0.126-0.135)	64.78 \pm 0.4 (64.34-65.31)	0 \pm 0 (0-0.01)

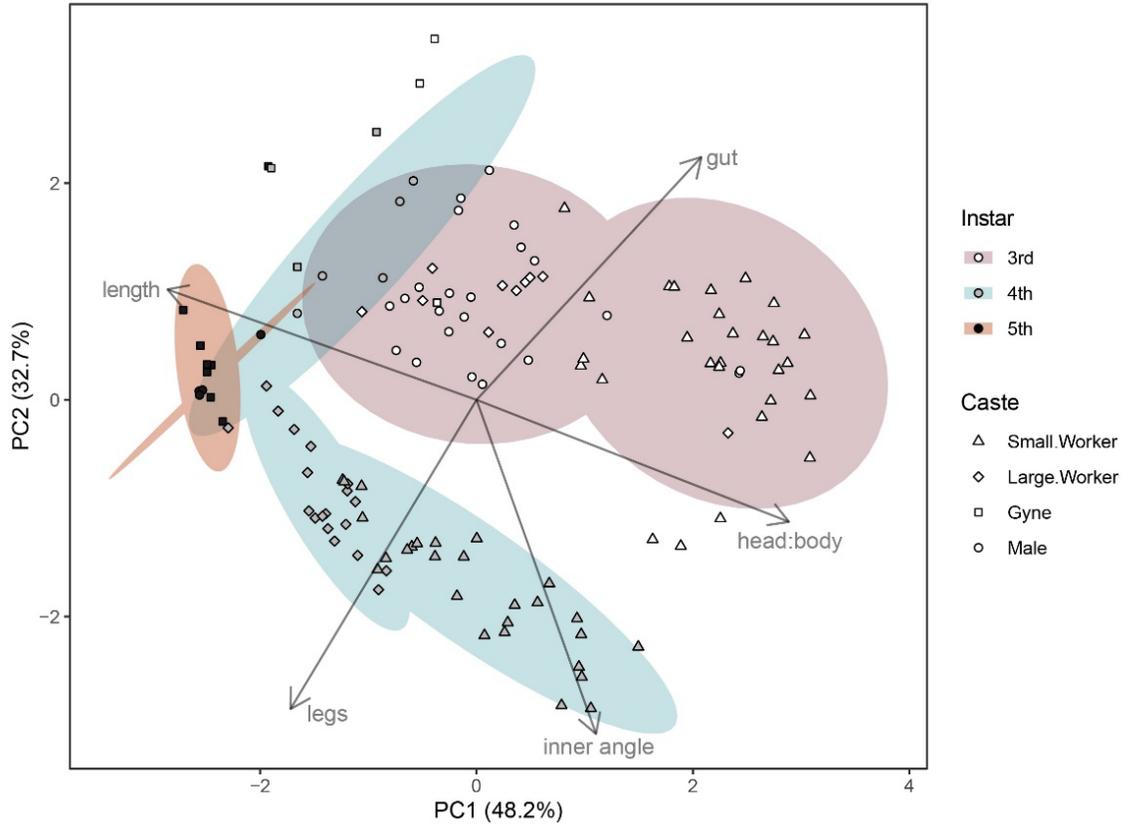


Fig. S1: PCA illustrating the combined clustering effects of five morphological traits (body length, head length and body length ratio, angle between the tip of the head and abdomen relative to the body center (i.e., inner angle) and gut and leg visibility) across castes and sexes in 3rd to 5th instar larvae. The first PC primarily separated larvae by caste (body length and head/body length ratio) whereas the second PC primarily captured differences between the final (prenymphal) and earlier instars (organ visibility and inner angle). The trends suggest that the extra instar of gynes and males is the 4th, not the 5th instar because the 4th instar is merely a slight extension along the first PC axis, while the 5th prenympal instar has a downward PC2-shift analogous to what small and large worker larvae have when they transition to their final (4th) instar. This suggests that the final instars are homologous across the sexes and female castes. Ellipses for 3rd and 4th instar gyne larvae were manually added as automatic calculations failed due to small sample sizes.

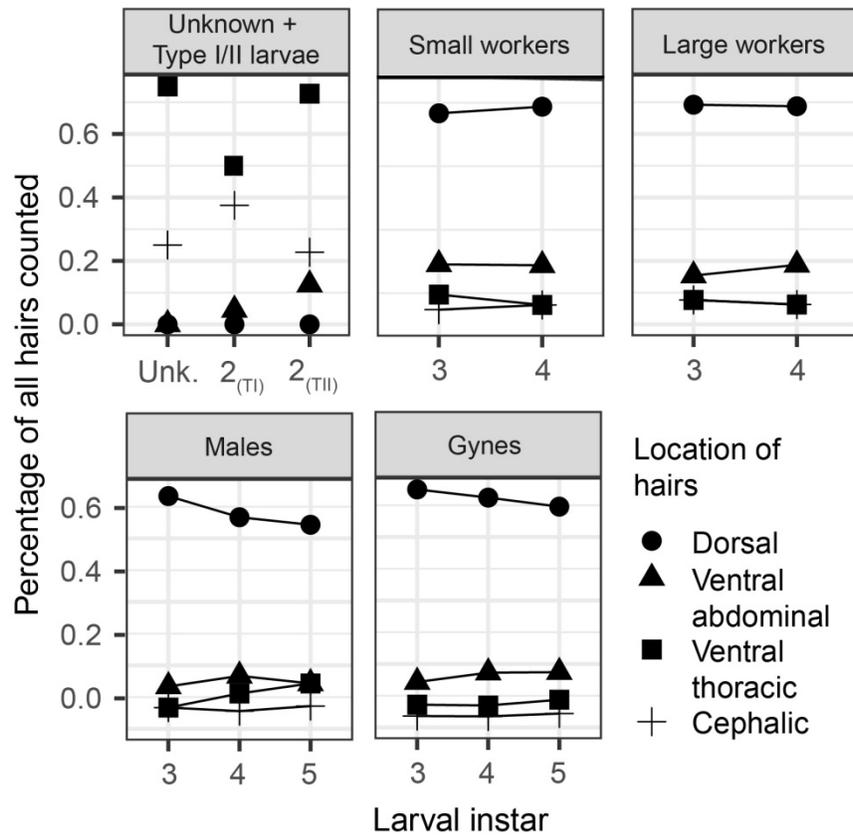


Fig. S2: Proportional representation of hair number across the four larval body parts that carry hairs during larval development separated in two early categories where caste and sex were unknown, two worker categories (large and small workers) and two reproductive categories (gynes and males), as in Tab. S2 which gives the absolute numbers. It is striking that proportions within plots are virtually constant even though the absolute numbers of hairs increase substantially as larvae grow and moult (Tab. S3), and hairs develop different morphologies across castes and sexes in the later instars (Figs. 5-7).