14 Becerra, J., and López-Sáez, J. F., in: The Role of Calcium in Biological Systems, p. 242. Eds L. J. Anghileri and A. M. Tuffet-Anghileri. CRC Press, Boca Raton 1982.
15 Tjio, J. H, and Levan, A., An Est. Exp. Aula Dei *2* (1951) 21.
16 Haas, M., Schooler, J., and Tosteson, D. C., Nature *258* (1975) 425.
17 Dhum, J., and Becker, B., Pfluegers Arch. *367* (1977) 211.
18 Bonsignore, C. L., and Hepler, P. K., Protoplasma *129* (1985) 28.

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# Amino acid and allozyme frequency changes in overwintering *Chymomyza amoena* (Diptera: Drosophilidae) larvae[1]

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*Summary:* Proline is accumulated by overwintering *C. amonea* larvae. Mortality averages over 50% by spring. $PGM^F$ in the population shifts from 70% in summer to less than 50% during winter, increasing again in summer. Product synthesis from stored glycogen may be mandated in dipteran larvae also using larval proteins for cold hardiness when completion of development is delayed until spring.
*Key words.* Drosophilid; *Chymomyza amoena;* overwintering; amino acids; proline; allozymes; phosphoglucomutase.

How drosophilids survive the winter has become of research interest[2–7]. To date, studies have been carried out principally in two groups: species in the genus *Chymomyza* which are larval overwinterers[3,4,6] and species in the genus *Drosophila*[2,5–7]. Species in neither group accumulate glycerol[8,9]. The role of sugars remains ambiguous[7]. The larval proteins have been implicated in coldhardiness in dipteran larval overwinterers[9–11]. Nevertheless, time-dependent changes affecting subzero survival have been found for both *C. amoena* and *D. auraria*[2–4]. Water loss does not appear to be a factor in overwintering success in *C. amoena*[1].

Amino acid accumulation has been implicated in successful overwintering in many insects[12–17]. Accumulation implies an active metabolic state at chilling to subzero temperatures under non-feeding conditions[14,15].

*Chymomyza amoena* is a low density, furtive species. Large numbers are not always available. In autumn 1984 there was a heavy invasion of black walnut husks, *Juglans nigra*. Studies were undertaken in winter 1984–85 to determine if amino acid changes occurred. Proline was accumulated. However, there was a 50% mortality by March despite the association of this amino acid with freeze tolerance[12,15–17].

The natural population was then monitored beginning summer 1985 to determine if allozyme polymorphisms might be implicated in the shift between warm and cold-adapted phenotypes. Significant seasonal changes in the frequencies of alleles at the *Pgm* locus were found. This locus plays a major role in the storage and retrieval of glycogen in the fat body[18]. Heat sensitive alleles have been found in other species[19]. None were detected.

Dipteran larval overwinterers delay metamorphosis to spring. Alanine increases in dead larvae; its suppression in living larvae suggests proline is actively synthesized. Proline increase also argues for conservatism in nature, possibly related to membrane stabilization at subzero temperatures[20].

*Materials and methods.* Black walnuts infested by *C. amoena* larvae were collected in November 1984 and stored in an unheated shelter. Apples with *C. amoena* larvae were kept outside at the same location. Supercooling point (SCP) changes were monitored to detect SCP declines in the walnut population following the warm autumn. Amino acid determinations were made in 10 larvae from walnut husks in January, after a significant SCP decline from the November level. Snow and ice blanketed the area from 1 January 1985 to 23 February 1985. Walnuts were again obtained at the original site, SCPs determined for 10 living and 11 dead larvae, then two groups of 20 larvae each were used for amino acid analysis for living and dead larvae. Controls were 10 larvae in January and 20 in February grown in the laboratory at 22 °C. Amino acid analysis was also made on a group of 20 *D. melanogaster* larvae in February, also grown in the laboratory. All work involved third instar size larvae only. The remaining walnuts (33) in the shelter were used to estimate the number of larvae successfully completing development per walnut husk.

SCPs were measured as before. Rapid cooling of 2–3 °C per min, is used in drosophilid work[3,4]. Freeze sensitive (FS) larvae cannot tolerate ice formation in the body fluids and die at the SCP; freeze tolerant (FT) organisms may recover. A second category of freeze tolerance has the physiological property of equivalence of SCP and freezing points (FP) and allows recognition in summer that the population will contain FT individuals in winter.

For amino acid analyses, samples were homogenized, deproteinized by filtration and amino acid content measured on a modified Dionex amino acid analyzer. Each third instar larva weighs approximately 1 mg. Samples were also coded, then decoded after analysis.

*Pgm polymorphism.* Enzymes phosphoglucomutase (PGM), phosphoglucose isomerase (PGI), malic enzyme (ME), malic dehydrogenase (MDH), α-glycerophosphate dehydrogenase (α-GPDH) and 6-phosphogluconate dehydrogenase (6PGHD), all associated with the glycolytic cycle, were screened for polymorphism via starch gel electrophoresis at Mt. Lake Biological Station in the Allegheny Mountains of Virginia in summer 1985. In June Michigan *C. amoena* from endemic crabapples, *Malus coronaria,* were allowed to oviposit on apples to provide the research material. PGM was found to be polymorphic in Michigan (MI) and Virginia (VA) populations. Alleles have the same migration rate in larvae and adults. Seasonal changes were monitored for MI *C. amoena* in natural substrates beginning in autumn 1985. Substrates sampled included black walnut husks (November), apples (January, March) from an MSU orchard and ornamental crabapples (March) from a residence. Adults from endemic crabapples supplied the *C. amoena* summer 1986 sample. Estimates of *Pgm* allele frequencies were also made for VA populations from apples obtained at four locations to eliminate possible effects of substrate: Pamplin (near Lynchburg), Danville, Blacksburg and a site along highway 700 to the Station.

Sample preparation followed established procedures. Electrophoresis[21] employed 12% starch gel (Sigma), tris maleate buffer pH 7.4 diluted 1:9 for the gel and was run at 120 volts

Table 1. Comparison of the free amino acids, in nmoles, in the hemolymph of living cold-adapted *C. amoena* larvae in late February, 1985 with warm-adapted controls at 22 °C run simultaneously. n = No. of samples of 20 larvae each

| Amino acid | Warm-adapted n = 1 | Cold-adapted n = 2 |
|---|---|---|
| Cysteic acid | 0.66 | 2.67 ± 0.95 |
| Aspartic acid | – | 0.04 ± 0.04 |
| Threonine | 0.61 | 0.48 ± 0.02 |
| Serine | 8.61 | 15.95 ± 3.95 |
| Glutamic acid | 1.70 | – |
| Proline | 9.29 | 47.98 ± 9.19 |
| Glycine | 1.44 | 0.86 ± 0.19 |
| Alanine | 12.21 | 12.20 ± 2.05 |
| Valine | 0.85 | 0.47 ± 0.06 |
| Methionine | 0.09 | 0.17 ± 0.01 |
| Isoleucine | 0.32 | 0.11 ± 0.11 |
| Leucine | 0.39 | – |
| Tyrosine | – | 0.28 ± 0.28 |
| Phenylalanine | Trace | |
| Histidine | 0.94 | 1.01 ± 0.04 |
| Lysine | 1.19 | 1.02 ± 0.34 |
| Arginine | 2.46 | 1.59 ± 0.15 |
| Total | 40.76 | 84.82 ± 15.85 |

Table 2. nmole percentage amino acid composition of the hemolymph of *C. amoena* larvae at 22 °C January and late February levels in overwintering larvae in 1985. n = No. of samples of 10 (January) or 20 (February) larvae. Controls at 22 °C also have one sample each of 10 (January) and 20 (February) larvae

| Amino acid | 22 °C | January | February living | February dead |
|---|---|---|---|---|
| Proline | 26.8 ± 4.4 | 48.7 | 56.5 ± 0.4 | 39.3 ± 2.7 |
| Serine | 14.5 ± 6.3 | 15.8 | 18.6 ± 1.2 | 14.0 ± 3.8 |
| Alanine | 27.3 ± 2.1 | 21.5 | 14.4 ± 0.3 | 34.4 ± 4.5 |
| n | 2 | 1 | 2 | 2 |

Table 3. Changes in the frequencies of $Pgm^F$ and $Pgm^M$ between summer 1985 and summer 1986 in mid-Michigan *C. amoena*. Frequencies of the slower alleles have been included, when present. n = number of adults or larvae sampled; Het. = frequency of all heterozygotes

| Season | Year | n | 2n | %F | %M | $\%S_1$ | $\%S_2$ | Het. |
|---|---|---|---|---|---|---|---|---|
| Summer | 1985 | 15 | 30 | 70 | 30 | – | – | 0.43 |
| Fall/winter | '85–'86 | 43 | 86 | 44 | 49 | 6 | 1 | 0.54 |
| March | 1986 | 35 | 70 | 38 | 56 | 3 | 3 | 0.31 |
| Summer | 1986 | 65 | 130 | 70 | 29 | 1 | – | 0.32 |
| Total | | 158 | 316 | 56 | 41 | 2 | 1 | 0.41 |

in a 4 °C refrigerated case or ice-cooled in the laboratory until the tracking dye, 1% bromophenyl blue, had migrated at least 7 cm. Stain: 50 ml 0.2 M Tris-HCl pH 8, 50 mg glucose-1-phosphate, 1 ml 1 M magnesium chloride, 25 units of glucose-6-phosphate dehydrogenase, 5 mg NADP, 1 ml 1% MTT solution; 4 ml 1% PMS solution was added 15 min after the gel had incubated in the staining solution at 37 °C. Data analysis have employed ANOVA for FP and SCP changes and are available upon request and G-statistics for allele frequency comparisons[22].

*Heat sensitive alleles.* One gel slice was heated 15 min or longer in a 60 °C waterbath prior to staining. PGM bands were compared between heat-treated and non-heat-treated gel slices.

*Results.* In November 1984 larvae in walnuts had a mean FP = −6.2 ± 0.1 °C and SCP = −7.4 ± 0.2 °C (n = 32). By December both values had declined significantly among larvae in apples: FP = −8.5 ± 0.7 °C, SCP = −9.7 ± 0.7 °C (n = 16). By January cold hardiness among larvae in walnuts had also increased significantly from November levels: FP = −7.8 ± 0.5 °C, SCP = −8.6 ± 0.5 °C (n = 18). In late February living larvae in walnuts under snow for nearly two months had a mean FP = −8.9 ± 0.8 °C and SCP = −10 ± 1.2 °C. This included four with FP = SCP = −6.5 ± 0.7 °C and six with SCP < FP (FP = −10.3 ± 0.9, SCP = −12.3 ± 1.2 °C). They did not recover. Dead larvae averaged 1 °C higher in their FP and SCP.

Table 1 compares the the hemolymph composition of free amino acids between laboratory-grown and late winter larvae. The increase in winter is as expected[14]; proline accounts for most of it. Serine doubles; the level of alanine remains the same. *D. melanogaster* larvae at 22 °C had the same amount of alanine but only half as much proline as the *C. amoena* controls.

Table 2 compares the percentage changes in nanomoles of proline, serine and alanine relative to the total free amino acid pool measured in each environment: laboratory-grown warm adapted and natural population *C. amoena* larvae in winter. Data for both samples at 22 °C and for living and dead larvae in late February are included. Among the living larvae, the increased cold hardiness in winter is paralleled by increasing amounts of proline; alanine declines. The level of serine increases only slightly. However, in the dead larvae in February there are dramatic changes in the levels of proline and alanine.

Table 3 shows the changes in allele frequencies at the *Pgm* locus between summer 1985 and summer 1986. Autumn (November) and winter (January) samples have been pooled since there was no significant difference between samples. In spring there were also no significant differences between larvae in apples and ornamental crabapples; these have been pooled. All data in table 3 refer only to living larvae. Overall there are significant differences in allele frequencies in the four periods (d.f. = 9, G = 33.4, $p < 0.005$). Changes between summer and fall are significant (d.f. = 3, G = 8.25, $p < 0.05$). Between fall and spring there is little change, then a significant shift back to summer frequencies (d.f. = 3, G = 31.5, $p < 0.005$). No heat sensitive alleles were found. Frequencies of $Pgm^F$ and $Pgm^M$ were similar in the VA *C. amoena* populations although genotypic differences among them were significant (d.f. = 9, G = 17.41, $p < 0.05$). Nevertheless, the overall frequencies of the F and M alleles were 74 ± 3% and 26 ± 3% respectively among all VA *C. amoena* in summer 1986, not significantly different from MI *C. amoena* from endemic crabapples: F = 70 ± 4%, M = 30 ± 4% also in summer, 1986.

*Discussion.* We have been using cold hardiness in *C. amoena* as a model system to compare drosophilid larval overwintering with that of other well-studied larval overwinterers. One Midwestern species has switched from FT to FS with supercooling[23]. *C. amoena* larvae continue to show a substrate-dependent cold hardiness type[3,4] that is not only not completely understood, rapid cooling methods may yield misleading results[24]. Freeze tolerance does not always depend on ice nucleators, while amino acids as cryoprotectants are more effective in FT than in FS insects; the latter usually accumulate alanine[14]. Nor are SCPs very low in the late winter larvae, and correspond well to SCP limits of winter-active insects[14]. *C. amoena* larvae may feed in winter, despite the recognized lethality of food in the gut[4].

Proline has been found to accumulate in the hemolymph of freeze tolerant insects: *Eurosta solidaginis* larvae (Diptera)[15], *Ostrinia nubilalis* larvae (Lepidoptera)[12], in *Phyllodecta laticollis* adults[16] and *Pytho americanus* adults[17], both beetles (Coleoptera). It has been found in other insect groups under stressful conditions[12] and in plants in chilling and drought conditions[25]. The biochemical conservatism may relate to membrane stability[21].

Terrestrial crane fly larvae rely on larval proteins and sorbitol[11]; *C. amoena* on larval proteins[9,10] and proline. Both the fat body and salivary glands secrete proteins into the hemolymph during development. In *C. amoena* larvae these tissues

are not as discretely separate as in well-studied *D. melanogaster* larvae where the salivary glands secrete glue proteins prior to the time of pupariation, a time gauged by the wandering stage[26]. Metamorphosis is delayed till spring. Hence other substances for cryoprotectants may have to be manufactured from stored products, as has been found for *E. solidaginis* larvae[27]. For instance, alanine is high in the dead *C. amoena* larvae, in live larvae it is depressed below control (22 °C) values while proline accounts for over 50% of the free amino acids in the hemolymph.

However, mortality remains high. One or fewer than one larva per substrate ecloses[9]. Only 27 adults completed development from the 33 walnuts held to May 1985. Reminiscent of the genetic load arguments of the 1960s[28], this suggests that seasonal changes might also involve genetic shifts between summer and winter.

Phosphoglucomutase regulates the flow of glycogen in and out of the fat body. The decline in the frequency of $PGM^F$ in late fall and its increase again after spring to 70% in summer is in agreement with findings for other drosophilid species that selection operates at this locus but that geographical clines do not exist[29]. Other studies have shown a relation between the biochemical properties of enzymes and the behavior of genes in natural and laboratory populations[30].

The fat body is reconstituted in adult *Drosophila*. From the difference between *D. melanogaster* and *C. amoena* larvae at room temperature, it is clear that proline regulation has been affected in drosophilid evolution. Only trace quantities of sorbitol have been found in adult *Drosophila*[7]. Whether or not proline functions in cold hardiness in the genus *Drosophila* remains to be determined.

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2 Higuchi, C., and Kimura, M.T., Physiol. Ent. *10* (1985) 303.
3 Band, H.T., and Band, R.N., Experientia *40* (1984) 889.
4 Band, H.T., and Band, R.N., Experientia *38* (1982) 1449.
5 David, J.R., Allemand, R., Van Herrewege, J., and Cohet, Y., in: Genetics and Biology of Drosophila, vol. 3d, p. 105. Eds M. Ashburner, H.L. Carson and J.N. Thompson Jr. Academic Press, London 1983.
6 Lumme, J., and Lakovaara, S., in: Genetics and Biology of Drosophila, vol. 3d, p. 171. Eds M. Ashburner, H.L. Carson and J.N. Thompson Jr. Academic Press, London 1983.
7 Kimura, M.T., Jap. J. Genet. *57* (1982) 575.
8 Krunic, M., Lakovaara, S., and Petrov, I., Genetika *12* (1980) 201.
9 Band, H.T., and Band, R.N., Experientia *36* (1980) 1182.
10 Band, H.T., and Band, R.N., Genetics *107* (1984) s6.
11 Duman, J.G., Neven, L.G., Beals, J.M., Olsen, K.R., and Castellino, F.J., J. Insect Physiol. *31* (1985) 1.
12 Morgan, P.D., and Chippendale, G.M., J. Insect Physiol. *29* (1983) 735.
13 Hansen, T.E., and Vijk, M.D., Zool. J. *63* (1984) 1634. In Russian, English summary.
14 Zachariassen, K.E., Phys. Rev. *65* (1985) 799.
15 Storey, K.B., Baust, J.G., and Storey, J.M., J. comp. Physiol. *144* (1981) 183.
16 Van der Laak, S., Comp. Biochem. Physiol. *73A* (1982) 613.
17 Ring, R.A., and Tesar, D.J. Insect Physiol. *26* (1980) 763.
18 Chapman, R.F., The Insects. Harvard Univ. Press, Cambridge 1982.
19 Trippa, G., Scozzari, R., and Cicchotti, R., Experientia *36* (1980) 174.
20 Rudolph, A.S., and Crowe, J.H., Cryobiology *22* (1985) 367.
21 Werth, G., Va. J. Sci. *36* (1985) 53.
22 Sokal, R.R., and Rohlf, F.J., Biometry. W.H. Freeman and Co., San Francisco 1981.
23 Duman, J.G., J. exp. Zool. *230* (1984) 355.
24 Miller, K., Comp. Biochem. Physiol. *73A* (1982) 595.
25 Yelenosky, G., Pl. Physiol. *64* (1979) 425.
26 Band, H.T., Band, R.N., and Ives, P.T., Biochem. Genet. *22* (1984) 551.
27 Storey, J.M., and Storey, K.B., J. Insect Physiol. *32* (1986) 549.
28 Band, H.T., Am. Nat. *106* (1972) 102.
29 Oakeshott, J.G., Chambers, G.K., Gibson, J.B., and Willcocks, D.A., Heredity *47* (1981) 385.
30 Alahiotis, S.N., Genetica *59* (1982) 81.

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## Ultrasound: its role in the courtship of the arctiid moth, *Cycnia tenera*

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*Summary.* Male *Cycnia tenera* (Lepidoptera: Arctiidae) were shown to produce ultrasonic clicks during courtship. The ultrasound enhances male courtship success, but only in the absence of male-produced pheromonal cues.
*Key words.* Ultrasound; pheromone; coremata; Arctiidae.

Moths were first shown to be sensitive to ultrasound in the seminal experiments of the late Kenneth Roeder. He and his collaborators showed that moths detect the ultrasonic echolocation signals produced by bats and evade bats by responding both directionally and non-directionally to these signals[1-4]. Later, arctiid moths were found to produce ultrasound using tymbal organs located on the thorax[5,6]. Whether they use these sounds to startle bats, to jam their sophisticated echolocation systems, or to 'advertize' their distastefulness (i.e. produce aposematic sound) is still under debate[7-10]. I here present evidence that ultrasound in arctiids plays yet another role: in the dogbane tiger moth, *Cycnia tenera,* it serves in the communication between the sexes.

Dogbane tiger moths are known to produce trains of ultrasonic clicks in response to bat cries and when handled[6,11,12]. Clicks are produced by the buckling and relaxation of striated, air-filled blisters of cuticle (thoracic metepisterna) called tymbal organs[5,6,13]. Each flexion/relaxation cycle produces a doublet of clicks referred to as modulation half-cycles[14]. Repeated doublets form a train. The clicks are faintly audible to humans, but most of the sound energy is ultrasonic with a peak near 50 KHz[6,9,11]. Similar trains of clicks are produced by male *C. tenera* during courtship suggesting they function as intersexual signals (fig.). The following is an investigation of their role in courtship, a role first suggested for the moth-produced clicks in 1864 by Laboulbene[15].