much by a clear determination of which populations are not affected as which ones are. Our data support previous admonitions that it may be difficult to distinguish natural population fluctuations from human-caused declines (*2*) and underscore the role of long-term surveys at numerous sites to separate the many confounding factors (*1, 34*).

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7. The 440-m drift fence is constructed of aluminum flashing, 50 cm high, buried 10 to 15 cm in well-packed soil. The pitfall traps are 40-liter buckets located every 10 m on each side of the fence. See J. W. Gibbons and R. D. Semlitsch [*Brimleyana* 7, 1 (1981)] for discussion of this technique.
8. Data are less easily interpreted for the other species because of trespass across the drift fence, apparent lack of pond philopatry, failure of adults or metamorphosed juveniles to leave the pond basin and therefore be censused at the fence, or a combination of these factors.
9. Terrestrial home ranges of *A. talpoideum* are located 12 to 280 m from their breeding pond [R. D. Semlitsch, *Can. J. Zool.* **59**, 315 (1981)] and those of *A. opacum*, 0 to 450 m [P. K. Williams, thesis, Indiana University, Bloomington, IN (1973)]. There are no paedomorphs at Rainbow Bay because the pond dries annually.
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23. Kendall's rank correlation, $n = 12$: *A. talpoideum*, $\tau_b = 0.45$, $P = 0.04$; *A. opacum*, $\tau_b = -0.26$, $P = 0.24$; *A. tigrinum*, $\tau_b = 0.78$, $P = 0.0005$; *P. ornata*, $\tau_b = 0.45$, $P = 0.04$.
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A Polypeptide from Tomato Leaves Induces Wound-Inducible Proteinase Inhibitor Proteins

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Defensive genes in plants can be activated by several different types of nonpeptide signaling molecules. An endogenous polypeptide, consisting of 18 amino acids, was isolated from tomato leaves and was able at very low concentrations to induce the synthesis of two wound-inducible proteinase inhibitor proteins when supplied to young tomato plants. The sequence of the polypeptide was determined, and an identical polypeptide was synthesized that possessed full inducing activity. These data establish that a polypeptide factor can initiate signal transduction to regulate the synthesis of defensive proteins in plant tissues.

WOUNDING OF PLANT TISSUE BY predator attacks stimulates the release of signals that activate proteinase inhibitors in both local and distant tissues (*1*). Molecules that may regulate proteinase inhibitor I and II genes in both wounded and unwounded leaves of potato and tomato plants are thought to include small pectic fragments of plant cells walls (*2, 3*), chitin and chitosan fragments from fungal cell walls (*4*), abscisic acid (*5*), auxin (*6*), salicylic acid (*7*), and methyl jasmonate (*8*). The active pectic fragments, α-1,4-galacturonic acid oligomers with degrees of polymerization (DP) of about 20 uronide units, were originally called the proteinase inhibitor inducing factor, (PIIF) (*9*). Oligogalacturonides, which elicit the synthesis of antibiotic phytoalexins in plant cells near the sites of infections (*10, 11*), were initially considered to be primary candidates as systemic signals for the wound response. However, the pectin-degrading enzymes that would be required to release plant cell wall fragments in response to wounding are not

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found in tomato leaves. In addition, labeled α-1,4-oligogalacturonides with DP above six, when applied to wounds on tomato plants, were not mobile (*12*). Thus, oligogalacturonides are probably not involved in the signal transduction pathway leading to the systemic induction of proteinase inhibitor genes in response to wounding.

A search for a systemic inducing signal for the proteinase inhibitor I and II genes in tomato leaf extracts led us to identify in tomato leaves a polypeptide that is free of carbohydrates and induces proteinase inhibitor activity when supplied to young tomato plants. The polypeptide was purified with the use of high-performance liquid chromatography (HPLC) (Fig. 1). Inducing activity was assayed by supplying young plants with eluted fractions from column separations through the cut stems for 30 min; the plants were then transferred to small vials of water, incubated under constant light for 24 hours as described (*9*), and the amount of proteinase inhibitor I and II in the leaf juice was quantified by radial immunodiffusion in agar gels that contained rabbit antiserum to inhibitor I or inhibitor II (*13, 14*). Over 30,000 young tomato plants were assayed over a 2.5 year period. With the use of this protocol, slightly more than 1 μg of the active factor was isolated from approximately 60 pounds of tomato leaves.

Step	Methodology
1.	Preliminary extraction
2.	C 18 HPLC
3.	SCX HPLC
4.	C18 HPLC
	a. pH6
	b. pH2.5
5.	SCX HPLC x 2
6.	C 18 HPLC

Fig. 1. Purification protocol for the isolation of the polypeptide inducer of proteinase inhibitor protein synthesis from tomato leaves. Step 1: Approximately 2 kg of tomato leaves *Lycopersicon esculentum* (v. Castlemart) were harvested from 20-day-old plants, grown under cycles of 17 hours light at 28°C and 7 hours dark at 18°C. Leaves were homogenized in a Waring blender for 5 min with distilled water (total volume of 4 liters) and filtered through four layers of cheesecloth. The liquid was adjusted to pH 4.5 with HCl and centrifuged at 1000*g* for 10 min. The supernatant was adjusted to pH 6.1 with 10 N NaOH, centrifuged at 10,000*g* for 10 min at 20°C, and decanted through Whatman #4 filter paper. The filtrate was chromatographed on DEAE cellulose, followed by reversed-phase C18 flash chromatography, Sephadex G25 gel filtration, and then CM Sephadex chromatography (*32*). Step 2: The active fraction (190 mg) recovered from CM Sephadex was dissolved in 10 ml 0.1% TFA, centrifuged at 20,000*g* for 5 min, filtered, and chromatographed on a reversed-phase C18 column (*33*). Step 3: The total material recovered in step 2 (2.5 mg) was subjected to strong cation exchange HPLC (*34*). Step 4: The pooled fractions from SCX-HPLC were subjected to reversed-phase C18 HPLC in 10 mM potassium phosphate, pH 6 (*35*). Step 5: The active fraction from step 4 was subjected to SCX HPLC with the same column and conditions as used in step 3, except that the gradient was shallower (*36*). Step 6: The step 5 fraction was desalted on a C18 HPLC column under the conditions of step 2. The fractions containing the activity peak eluted at 55.0 to 58.0 min and were pooled and concentrated by vacuum centrifugation to 0.5 ml. This sample contained approximately 1 μg of protein, as estimated by amino acid content after acid hydrolysis. The biological activity of the sample had the potential to induce maximal accumulation of proteinase inhibitors in 40,000 tomato plants. This sample was used for amino acid analysis and sequence determination.

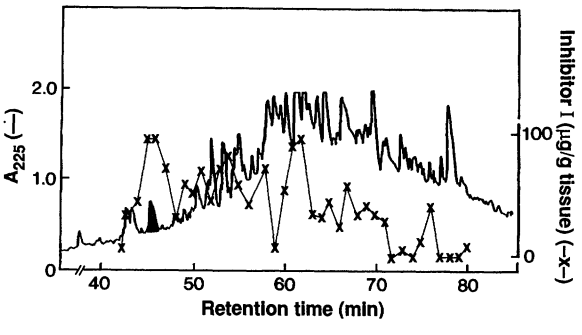

Fig. 2. Chromatography of the preliminary extract of tomato leaves on a semipreparative reversed-phase C18 column (step 2). Five microliters of each 2-ml eluted fraction was diluted to 360 μl with 15 mM sodium phosphate, pH 6.5, and assayed for proteinase inhibitor I inducing activity (x) in young excised tomato plants as described in the text. Four plants were assayed per fraction. The active fractions from the peak (in black) were pooled and further purified.

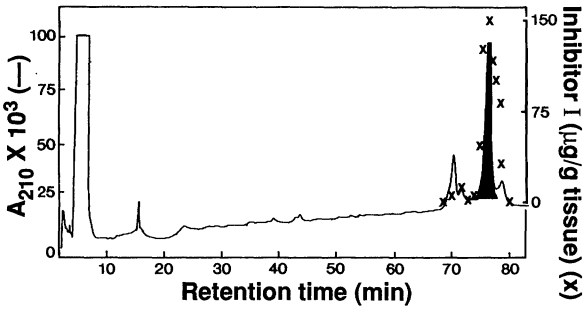

Fig. 3. Chromatography of the partially pure polypeptide on a SCX-HPLC column (step 5). Fractions (0.5 ml) were diluted as in Fig. 2 and assayed for proteinase inhibitor I inducing activity (x). The active fractions of the peak (in black) were collected and analyzed for amino acid content and sequence.

1 5 10 15 18
$^{+}H_3N$-AVQSKPPSKRDPPKMQTD-COO^{-}

Fig. 4. The amino acid sequence of the polypeptide inducer, systemin.

The elution profile of the preliminary extract of tomato leaves (Fig. 2) was complex. Several fractions exhibited proteinase inhibitor inducing activity (*15*), but one peak (Fig. 2) was selected for further purification because it contained the highest activity and was the best candidate for recovery.

After several additional purification steps, a major peak that possessed high specific activity was eluted from a strong cation exchange (SCX) HPLC column (Fig. 3). The properties of the eluted material resembled those of a polypeptide, that is, absorbance in the spectral region appropriate for peptide bonds, total loss of activity and recovery of free amino acids after acid hydrolysis, partial loss of activity in the presence of trypsin and other proteolytic enzymes, and a positive reaction to bicinchoninic acid (*16*). Total amino acid analysis (*17*) and amino acid sequence analysis (*18, 19, 20*) of the active component identified its length and determined the sequence (Fig. 4) (*20a*). No significant similarities were found to known protein sequences (*21*). This sequence is, however, a palindrome: xxQxBPPxBBxPPBxQxx (x,

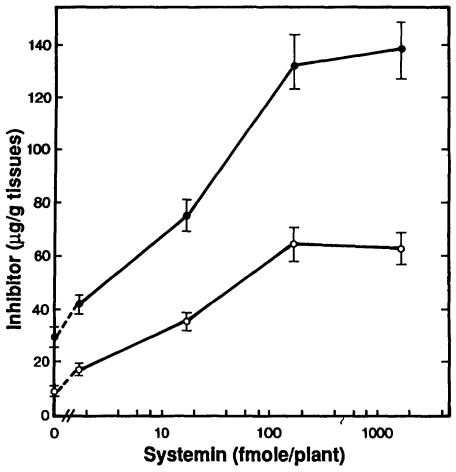

Fig. 5. Induction of the synthesis and accumulation of proteinase inhibitor I (●) and II (○). Excised leaves of young tomato plants were incubated in solutions containing the synthetic polypeptide, and the proteinase inhibitors assayed as in Fig. 2. Each data point was obtained from assays of leaves of 36 tomato plants.

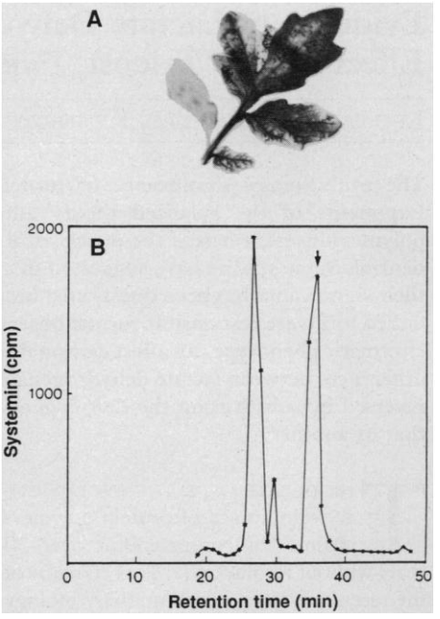

Fig. 6. (**A**) Seventeeen-day-old tomato plants were wounded across the most distal leaflet (note dark band) with a hemostat. Vaseline was applied along each side of the wound to localize applied solutions to the wounds. 100,000 cpm (5 μg) of [^{14}C]Ala-labeled polypeptide (*22*) was applied to the wound in 5 μl of H_2O. After 30 min of incubation under 200 μeinsteins $M^{-2}{\cdot}s^{-1}$, the leaf was excised and dessicated under vacuum for 2 hours at 55°C. The leaf was placed on x-ray film and the film developed after 14 days of exposure. (**B**) Polypeptide was applied to ten plants as in (A). After a 1-hour incubation, the wounded leaves were excised at their bases and then cut again about 10 mm from their ends while they were immersed in 25 mM EDTA, pH 7 (*26*). The phloem exudates were collected in 0.5 ml 25 mM EDTA, pH 7 for 1 hour. Exudates were pooled, lyophilized, and separated by C18 HPLC as in step 2 (*37*). The arrow indicates the retention time of the synthetic polypeptide. The other two peaks were not identified but were assumed to be breakdown products of the polypeptide. When wounded leaves were incubated for 3 hours, excised, and the phloem exudate collected for 2 hours, a single radioactive peak was found in the phloem exudate that eluted with the retention time of the synthetic polypeptide. [^{14}C]Ala is not retained by this column. Approximately 15 ng (0.3% of the total radioactivity applied to the wounds) was recovered in the polypeptide peak.

any residue; B, Lys or Arg; Q, Gln; P, Pro). A synthesized polypeptide of identical sequence (*22*) eluted from the C18 (step 2, Fig. 1) column with the same retention time as the native polypeptide.

The synthetic polypeptide was as effective as the native polypeptide for inducing the synthesis and accumulation of both inhibitor I and II proteins when supplied to the cut stems of young tomato plants (Fig. 5). About 40 fmol of the polypeptide per plant was required to produce half maximal accumulation of inhibitors I and II, which represents about 10^5 times more activity on a molar basis than the PIIF oligogalacturonide inducers from plant cell walls. The coordinate induction of the synthesis of inhibitor I and inhibitor II proteins in response to the polypeptide (Fig. 5) is similar to the normal wound response, which is transcriptionally regulated (*22*). This suggests that the polypeptide is activating the same signal transduction pathway as activated by wounding (*1, 23*), by oligosaccharides (*3, 4*), and by methyl jasmonate (*8*). Polypeptides that induce genes have been found in animals and yeast (*24, 25*).

The polypeptide, unlike the oligogalacturonides, is transported out of wounds to distal tissues. ^{14}C-labeled polypeptide was synthesized (*22*) and placed on fresh wounds of tomato plants. Within 30 min the radioactivity had moved throughout the leaf, and within 1 to 2 hours was identified in the phloem exudate (*26*) (Fig. 6). Because of its mobility through the phloem, we have named the inducing polypeptide "systemin."

As well as being inducible by wounding in leaves, proteinase inhibitor I and II genes are developmentally regulated in the meristems, flower tissues, and fruit of wild tomato species, and in potato tubers. Thus, some or all of these processes may be affected by systemin or similar polypeptides. Polypeptide signals may also regulate other developmental or metabolic processes in plants.

Fungal-derived proteins can elicit fensive responses in plants (*27, 28*). Glutathione, a tripeptide found throughout both the animal and plant kingdoms, activates phytoalexin genes in bean cell suspension cultures when added in micromolar to millimolar concentrations (*29*). Its mode of action was proposed to involve the reduction of disulfide bridges in receptor proteins. A 27–amino acid, cysteine-rich peptide, encoded by the avirulence gene 9 in the fungus *Cladosporium fulvum*, induces necrosis in tomato leaves (*30*) and can be isolated from the apoplastic fluid of infected tomato leaf tissue. Its biological effects appear to require quantities of peptide (*31*) that are several orders of magnitude higher than those required for systemin.

We have reported that a polypeptide, systemin, that induces the synthesis of two proteinase inhibitor proteins in tomato plants. Further analysis of systemin and its function may lead to a better understanding of signal transduction networks that regulate the expression of plant genes.

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20a. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
21. Protein Identification Resource, release 26; Pearson/Lipman FASTA program at the Molecular Biology Computer Research Resource, Harvard Medical School.
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32. The DEAE cellulose column (Whatman DE52, 5.9 by 15 cm) was equilibrated in 1 M ammonium bicarbon-

ate and washed exhaustively with distilled water. The material eluting in the void volume was collected and stored overnight at 4°C. TFA was added dropwise to the stored eluate to a final concentration of 0.2% (v/v); the solution was then clarified by centrifugation at 20,000*g* for 5 min at room temperature. The supernatant was loaded onto a reversed-phase flash column (C18, 40 μm, 3 by 25 cm) previously equilibrated with aqueous 0.1% TFA. The column was eluted with the use of compressed nitrogen at 8 psi. After the sample was loaded, the column was washed with 200 ml 0.1% TFA; the retained material was then eluted with successive washes of 20, 40, and 60% methanol in 0.1% TFA. The methanol was removed with a rotary evaporator and the remaining liquid was frozen and lyophilized. Two kilograms of leaf material yielded about 1 g of crude material containing the active factor. The procedure was repeated 15 times. Samples (approximately 4 g) of crude material dissolved in 20 ml water and adjusted to pH 7.8 with 10 M ammonium hydroxide were loaded onto a G25 Sephadex column (4 by 44 cm) that was equilibrated with 50 mM ammonium bicarbonate, pH 7.8. The material eluting at and just after the void volume was recovered and lyophilized. Four identical runs through the entire procedure produced 1.25 g of partially purified factor. The 1.25 g was dissolved in 500 ml H_2O, the pH was adjusted to 6 with 1 M NaOH, and the sample was applied to a CM Sephadex column (2 by 17 cm) and washed with 0.01 M potassium phosphate, pH 6. The activity was retained by the CM Sephadex, eluted with 250 mM ammonium bicarbonate, and lyophilized. The yield was 190 mg.

33. The material was injected, one-fifth at a time, into a semi-preparative reversed-phase C18 column (Vydac, Hesperia, CA, Column 218 TP510, 10 by 250 mm, 5-μm beads, 300 Å pores). Solvent A consisted of 0.1% TFA in water. Solvent B consisted of 0.1% TFA in acetonitrile. Samples were injected in solvent A and, after 2 min, a 90 min gradient to 30% solvent B was begun for elution. The flow rate was 2 ml/min and eluted peaks were monitored at 225 nm. Several peaks of activity were found. The major peak of activity resided in tubes 43 to 46, which were pooled and lyophilized. Total protein content of the pooled fractions was estimated at 2.5 mg.
34. Chromatography was performed on a poly-SULFOETHYL Aspartamide column (4.6 by 200 mm, 5 μM, The Nest Group, Southborough, MA) with the use of the following solvent systems: Solvent A, 5 mM potassium phosphate, pH 3, in 25% acetronitrile; solvent B, 5 mM potassium phosphate, 500 mM potassium chloride in 25% acetonitrile, pH 3. The sample was dissolved in 2 ml of solvent A, filtered, and applied to the column. After a 5-min wash with solvent A, a 60-min gradient to 50% B was applied. The flow rate was 1 ml/min, and the elution profile was monitored by absorbance at 210 nm. The active fractions, 35 to 38, were pooled and reduced in volume to 1 ml by vacuum centrifugation.
35. Chromatography was performed on a Beckman Ultrasphere Ion pair column (4.6 by 250 mm, C18, 5 μm). Solvent A was 10 mM potassium phosphate, pH 6, and solvent B was 10 mM potassium phosphate, pH 6, containing 50% acetronitrile. The active fractions, 39 to 42, were pooled and vacuum centrifuged to a final volume of 1 ml. This fraction was applied to the same column as the previous run but under the solvent and gradient conditions of step 2. The sample was adjusted to pH 3 with TFA, filtered through a 0.45-μm syringe filter and chromatographed at a flow rate of 1 ml/min. The peaks were detected at 212 nm. The fractions containing the activity, eluting at 53.5 to 56.5 min, were pooled and vacuum centrifuged to a volume of 1 ml.
36. The column was run at 0% B for 5 min at which time a gradient to 30% B in 120 min was started. The profile was detected by absorbance at 210 nm. Fractions eluting at 76 to 78.5 min were pooled and vacuum centrifuged to reduce the volume to 1 ml.
37. A 60-min gradient to 30% solvent B was employed.
38. We thank G. Munske for synthesizing the polypeptide and for helpful discussions, G. Wichelns for growing tomato plants, and B. Vallee for facilities and encouragement. Supported in part by NSF grants DCB-8702538 and DCB-8608594, and Project 1791, College of Agriculture and Home Economics, Washington State University.

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Evidence of Lactate Dehydrogenase-B Allozyme Effects in the Teleost, *Fundulus heteroclitus*

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The evolutionary significance of protein polymorphisms has long been debated. Exponents of the balanced theory advocate that selection operates to maintain polymorphisms, whereas the neoclassical school argues that most genetic variation is neutral. Some studies have suggested that protein polymorphisms are not neutral, but their significance has been questioned because one cannot eliminate the possibility that linked loci were responsible for the observed differences. Evidence is presented that an enzymatic phenotype can affect carbon flow through a metabolic pathway. Glucose flux differences between lactate dehydrogenase-B phenotypes of *Fundulus heteroclitus* were reversed by substituting the *Ldh-B* gene product of one homozygous genotype with that of another.

THE DEBATE OVER THE EVOLUTIONARY significance of protein polymorphisms has continued for over 20 years without resolution (*1, 2*). Yet, many of the recent advances in evolutionary biology depend on the implicit assumption that the majority of this genetic variation is selectively neutral. Arguments for the neutral theory are largely derived from the theoretical considerations of population genetics (*2*). They are bolstered by the view, advanced by Newsholme and Start (*3*), that metabolism is controlled by a few key regulatory or rate-limiting enzymes in a pathway, whereas other enzymes such as lactate dehydrogenase (LDH) participate in reactions that are near equilibrium and therefore of little or no importance in flux generation or metabolic control (*3*). Kascer and Burns (*4*) suggested that the control of metabolic rate is more generally shared among enzymes, including the so-called "equilibrium" enzymes like LDH. Because the neutralist hypothesis implies that polymorphic enzymes (allozymes) are functionally equivalent, some investigators have sought to resolve the controversy by examining the biochemistry of allozymes, while others have concentrated on life history correlates (*5–9*). Although these exciting studies present convincing evidence that one or more polymorphic loci are correlated with physiological or other differences between genotypes, they cannot eliminate the possibility that some unknown locus is actually responsible for the differences observed. Thus, the debate remains an important evolutionary question. We addressed this question by examining the metabolism of mummichog, *Fundulus heteroclitus* (Teleostei, Cyprinodontidae), embryos in which the native LDH-B isozyme was directly replaced with heterologous enzymes.

In the mummichog, LDH is a multilocus system that produces three gene products (LDH-A_4, LDH-B_4, and LDH-C_4): the *Ldh-A* locus is expressed in white muscle, *Ldh-B* in a variety of tissues including oocytes, and *Ldh-C* in eye and some nerve tissue (*10*). The *Ldh-B* locus has two major alleles, *Ldh-B*^{a} and *Ldh-B*^{b}, that vary clinally over the geographical range of the species (*11*). The allelic isozymes (allozymes) encoded by this locus have been purified and kinetically characterized (*12–14*). Differences have been observed in product inhibition and reaction velocities at low substrate concentrations (*14*). Within a local population, *Ldh-B*^{a} homozygotes develop faster than *Ldh-B*^{b} homozygotes (*15*). This developmental variation has been correlated with differences in oxygen consumption (*16*), lactate and glucose utilization (*17*), and survival, that is, Darwinian fitness (*6*).

Recently, we have shown that mummichog eggs contain 40 to 50 mM lactate at the time of fertilization and that a major function of LDH-B in early development is oxidation of the lactate pool (*17*). Specific activity of LDH-B is similar between genotypes (1.2×10^{-3} IU per egg), but lactate concentrations found in newly fertilized eggs are correlated with LDH-B phenotype and are essentially equal to the inhibition constant of the purified allozymes of LDH-B: 51.8 ± 5.9 mM for LDH-B_4^a and 36.6 ± 3.5 mM for LDH-B_4^b (*14*). Thus, in vivo, the *Ldh-B*^{a} homozygote accumulates more lactate and uses it at a higher rate. The *Ldh-B*^{a} homozygote also uses glucose at a higher rate even though the glucose pool size is the same for both phenotypes. Since the LDH reaction and glycolysis are both functioning to produce pyruvate in mummichog embryos during the first 24 hours, it is difficult to explain how the two LDH-B allozymes could differentially affect glucose utilization.

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