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Note

Insulin/Insulin-Like Growth Factor Signaling Controls Non-Dauer Developmental Speed in the Nematode *Caenorhabditis elegans*

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ABSTRACT

Identified as a major pathway controlling entry in the facultative dauer diapause stage, the DAF-2/**Ins**ulin **r**eceptor (InsR) signaling acts in multiple developmental and physiological regulation events in *Caenorhabditis elegans*. Here we identified a role of the insulin-like pathway in controlling developmental speed during the *C. elegans* second larval stage. This role relies on the canonical DAF-16/FOXO-dependent branch of the insulin-like signaling and is largely independent of dauer formation. Our studies provide further evidence for broad conservation of insulin/insulin-like growth factor (IGF) functions in developmental speed control.

GENETIC determinants and environmental cues interact to time animal development. Studies of the nematode *Caenorhabditis elegans* have been instrumental in our comprehension of both these processes, with the discovery of the heterochronic pathway and the signals that control entry into the facultative dauer diapause. More recently, we described a novel paradigm to investigate the interplay between genetic and environmental control of developmental timing in *C. elegans* by using the nicotinic agonist dimethylphenylpiperazinium (DMPP). We demonstrated that illegitimate activation of nicotinic acetylcholine receptors (nAChRs) by DMPP during the second larval stage induces a lethal heterochronic phenotype by disconnecting developmental speed from the molting timer, hence resulting in deadly exposure of a defective cuticle to the surrounding environment at the subsequent molt (Ruaud and Bessereau 2006). Resistance to DMPP can be achieved in mutants that delay the L2/L3 molt, such as in *catp-1*, which encodes a cation-transporting P-type ATPase (Ruaud and Bessereau 2007). Alternatively, *daf-12*/nuclear receptor (NR) mutants are insensitive to the developmental delay induced in the wild type by DMPP exposure (Ruaud and Bessereau 2006). Interestingly, *catp-1*, *daf-12*, and other DMPP-resistant mutants (our unpublished results) all interact with the genetic network controlling entry into the dauer stage. The dauer larval stage is a facultative long-lived L3 diapause induced by adverse conditions (limited food, high temperature, and high population density). These environmental cues are integrated by a complex genetic and molecular network that comprises three main branches: the DAF-12/NR, the DAF-2/Insulin receptor (InsR), and the DAF-7/TGFβ pathways (Figure 1A) (Beckstead and Thummel 2006; Fielenbach and Antebi 2008). In addition the P-type ATPase *catp-1* interacts with the DAF-2/InsR pathway to control several developmental decisions (Ruaud and Bessereau 2007). Here we further investigate the links between the pathways controlling dauer entry and L2 developmental speed. Our results indicate that the canonical DAF-16/FOXO-dependent insulin-like signaling is specifically required to implement DMPP effect and controls L2 developmental speed largely independently of its roles in dauer formation.

The DAF-2/InsR branch of the dauer pathway is required to implement DMPP developmental effects: *daf-12(0)* mutants are resistant to DMPP. To determine the relationships between DMPP-induced heterochrony and dauer formation, we tested the DMPP resistance of mutants in the insulin-like and TGFβ pathways (Figure 1A). We found that several mutants that reduce insulin signaling, including *daf-2*/InsR, *age-1*/PI3K, and *pdk-1*

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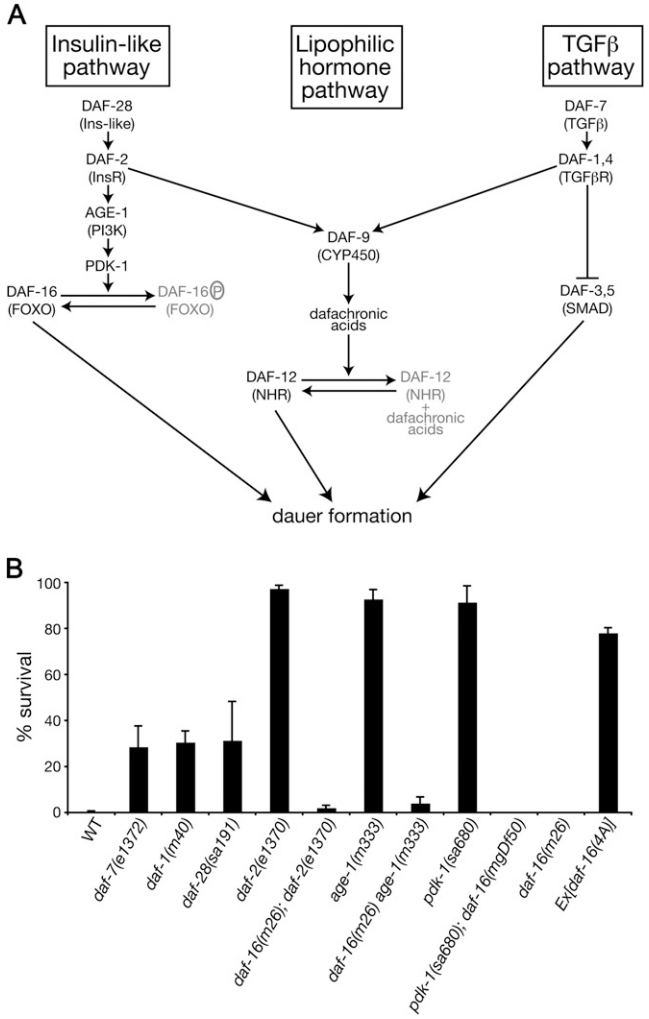

Figure 1.—The DAF-2/InsR branch of the dauer pathway is specifically required to implement DMPP toxicity. (A) Schematic representation of the dauer pathway. Three main branches (insulin/IGF pathway, lipophilic hormone, and TGFβ) control entry into the dauer diapause stage. (B) Survival of wild-type and *daf* mutant animals developing on 0.75 mM DMPP at 20°, as described in Ruaud and Bessereau (2006). *daf-28(sa191)* is a dominant-negative allele that is thought to disrupt the production of multiple insulin-like peptides (Li *et al.* 2003). Error bars represent SEM ($n \geq 3$ independent experiments).

loss-of-function mutants, are strongly DMPP resistant. In contrast, a null allele of *daf-7*/TGFβ and mutants of the TGFβ receptor subunit *daf-1* were only partially resistant (Figure 1B). All these mutants form dauer larvae regardless of environmental conditions (Daf-c phenotype). However, the selective DMPP resistance of mutants in the insulin-like signaling pathway is unlikely to be a by-product of differential Daf-c phenotypes, since *daf-2(e1370)* animals do not become dauer in replete conditions at 20° whereas 43% of *daf-7(e1372)* animals do. Rather, these results indicate that the DAF-2 branch of the dauer pathway is specifically required to implement DMPP toxicity.

DAF-16/FOXO activation is sufficient to confer DMPP resistance: Activation of the insulin receptor by its insulin ligand in vertebrates can induce activation of several different intracellular signaling pathways, including the Akt/PKB and MAP kinase pathways (Saltiel and Kahn 2001). Most of the insulin receptor transduction machinery described in vertebrates is conserved in *C. elegans*. However, the genetics of dauer formation have defined a linear pathway for insulin signaling that consists of elements both necessary and sufficient for dauer formation under the control of *daf-2*. Ultimately, DAF-2 activation induces cytoplasmic segregation of the DAF-16/FOXO transcription factor, preventing it from regulating its transcriptional targets (Figure 1A). Three lines of evidence involve the DAF-16-dependent DAF-2 signaling in DMPP sensitivity. First, strong downstream mutants of the insulin pathway such as *age-1*/PI3K and *pdk-1* are DMPP resistant. Second, the DMPP resistance of *daf-2(e1370)*, *age-1(m333)*, and *pdk-1(sa680)* is fully suppressed by *daf-16* mutations. Third, a gain-of-function mutation that renders DAF-16 insensitive to phosphorylation and thus constitutively nuclear [*daf-16(4A)*] is sufficient to confer DMPP resistance (Figure 1B) (Lee *et al.* 2001). We concluded that activation of the DAF-16 transcription factor is sufficient to confer DMPP resistance.

The DMPP resistance of *daf-2/InsR* alleles does not correlate with previously described phenotypes: Differential DMPP resistance of *daf-2(e1370)* and *daf-7(0)* mutants and our previous analysis of the DAF-12/NR branch of the dauer pathway indicate that the DMPP resistance of *daf* mutants does not correlate with their dauer phenotype (Figure 1B) (Ruaud and Bessereau 2006). Controlling dauer entry, though, is only one of the numerous activities of the DAF-2 insulin-like pathway, which also acts in non-dauer development, adult behavior, reproduction, and longevity (Gems *et al.* 1998). These multiple roles are illustrated by the phenotypic and genetic complexity of *daf-2* alleles, which have been divided into two allelic classes following extensive characterization (Gems *et al.* 1998).

Because *daf-2* null alleles are embryonic lethal, we analyzed the DMPP resistance of eight previously characterized *daf-2* hypomorphic alleles. We identified both DMPP-sensitive and DMPP-resistant *daf-2* alleles. However, the DMPP resistance of *daf-2(lf)* did not correlate with (1) allele class, which is partly defined by interactions with loss-of-function alleles of the NR-coding *daf-12* gene, (2) molecular lesions, or (3) strength of the Daf-c phenotype (Table 1, Figure 2A):

1. The DMPP-sensitive alleles *m577*, *e1371*, and *e1368* are class 1 alleles and their Daf-c phenotype is suppressed by *daf-12*/NR. Among DMPP-resistant alleles, *m41* and *m596* belong to class 1 whereas *e1370*, *e1391*, and *e979* are class 2 alleles. The Daf-c phenotype of *m41* and *m596* is suppressed by *daf-*

TABLE 1

DMPP resistance of *daf-2* alleles

Allele	Class[a]	Daf-c[b]	DMPP resistance (N)[c]	Amino acid change[d]	Mutation location (domain)[d]
+		0	1 ± 1 (405)		
e1368	1	0.3 ± 0.3	4 ± 3 (210)	S573L	Extracellular (L2)
e1371	1	0.1 ± 0.3	2 ± 1 (254)	G803E	Extracellular (FnIII2)
m41	1	14 ± 4	80 ± 9 (333)	G383E	Extracellular (Cys-rich)
m577	1	0	9 ± 5 (351)	C1045Y	Extracellular (FnIII2)
m596	1	0.4 ± 0.4	90 ± 3 (235)	G547S	Extracellular (L2)
e1370	2	0.5 ± 0.7	99 ± 8 (156)	P1465S	Intracellular (kinase)
e1391	2	66 ± 7	92 ± 4 (182)	P1434L	Intracellular (kinase)
e979	2	98 ± 0.8	72 ± 6 (174)	C146Y	Extracellular (L1)

[a] Classes are defined in Gems *et al.* (1998). Class 1 mutants are Daf-c, increased **a**dult lon**ge**vity (Age), and **i**ntrinsic **t**hermo**t**olerance (Itt) and exhibit low levels of L1 arrest at 25°. Class 2 mutants exhibit these defects as well as some or all of the following: reduced adult motility, abnormal adult body and gonad morphology, high levels of embryonic and L1 arrest, production of progeny late in life, and reduced brood size. Class 1 and class 2 alleles also differ in their genetic interactions with *daf-12* loss-of-function alleles.

[b] Dauer formation at 20° as reported in Gems *et al.* (1998), for comparison with DMPP resistance.

[c] Percentage survival on 0.75 mM DMPP at 20° (average ± SEM, $n \geq 3$ independent experiments).

[d] Adapted from Patel *et al.* (2008).

12(m20) while *e1370*, *e1391*, and *e979* arrest development when combined with *daf-12(m20)*.

2. Mutations in the extracellular domains (*m41*, *m596*, and *e979*), as well as the intracellular region (*e1370* and *e1391*), can confer DMPP resistance, while two mutations separated by only 26 amino acids in the L2 extracellular region of the DAF-2 receptor (*m596* and *e1368*) have opposite DMPP phenotypes (Patel *et al.* 2008).
3. Alleles that did not form any dauers at 20° in replete conditions such as *m596* or *e1370* are as DMPP resistant as strong Daf-c alleles such as *e1391* or *e979*. In addition, we could not detect any correlation between any other previously described phenotype and the DMPP resistance of *daf-2* alleles (data not shown).

Tissue-specific requirement of the DAF-2/InsR pathway for L2 developmental speed: The DAF-2/InsR pathway is a major regulator of dauer formation, metabolism, and life span of *C. elegans*. The downstream components (Hertweck *et al.* 2004) as well as the tissues (Apfeld and Kenyon 1998; Wolkow *et al.* 2000; Libina *et al.* 2003) involved in these different functions are overlapping but distinct. We used two approaches to identify tissues in which DAF-2 signaling was required to control L2 developmental speed. First, we designed a strategy for conditional tissue-specific expression of *daf-2* on the basis of a FLP/FRT approach (Davis *et al.* 2008) because ectopic expression of the *daf-2* cDNA using standard strategies was highly toxic and prevented reliable scoring of dauer formation and DMPP resistance. In the absence of FLP expression, we observed marginal but significant rescue of irreversible dauer formation (Table 2), suggesting that this conditional expression is not as tightly regulated as previously anticipated on the basis of GFP detection (Davis *et al.* 2008). In agreement with previously published results, however, expression in the intestine, the epidermis, or the nervous system was sufficient to rescue irreversible

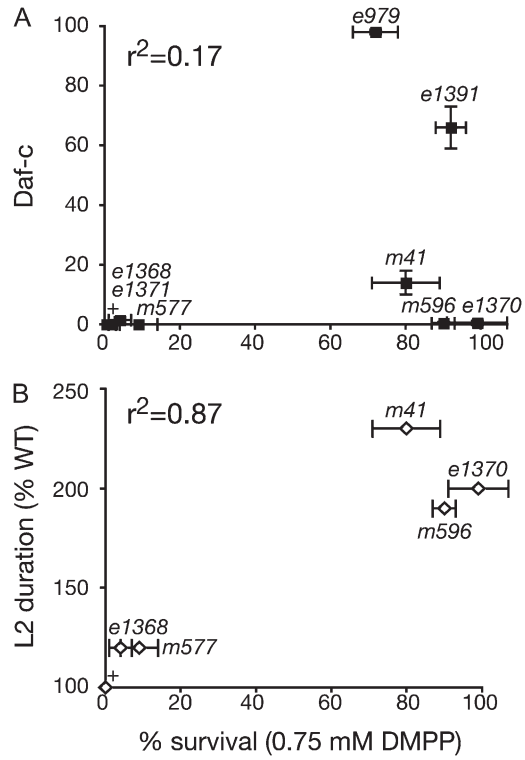

Figure 2.—Correlation between DMPP resistance, Daf-c phenotype, and L2 developmental speed of *daf-2/InsR* alleles. (A) A low Pearson's coefficient ($r^2 = 0.17$) indicates lack of correlation between DMPP resistance and Daf-c phenotype of *daf-2* alleles. (B) A high Pearson's coefficient ($r^2 = 0.87$) shows that DMPP resistance correlates well with L2 developmental speed.

TABLE 2

DMPP resistance and developmental speed in animals with induced tissue-specific *daf-2* expression

Genotype	% dauer[a] (no. of animals, experiments)	DMPP^{R}[b] (no. of animals, experiments)	L2 duration in hr (no. of lines scored)[c]
+	0 ± 0 (66, 3)	0 ± 0 (130, 5)	11
daf-2(m41)	100 ± 0 (236, 10)	98 ± 2 (87, 6)	23
	Control (P*dpy-30*-FRT-*mCherry*-terminator-FRT-*gfp*-SL2-*daf-2*)		
daf-2(m41); krEx837-840 (four lines)	85 ± 5 (169, 6)	97 ± 1 (214, 5)	22 (2)
	Induced panneuronal *daf-2* (P*dpy-30*-FRT-*mCherry*-terminator-FRT-*gfp*-SL2-*daf-2*; P*rab-3-flp*)		
daf-2(m41); krEx705,707 (two lines)	65 ± 8[d] (277, 11)	99 ± 1 (147, 3)	22 (2)
	Induced intestinal *daf-2* (P*dpy-30*-FRT-*mCherry*-terminator-FRT-*gfp*-SL2-*daf-2*; P*vha-6-flp*)		
daf-2(m41); krEx691,693 (two lines)	64 ± 7[d] (278, 12)	99 ± 1 (140, 5)	25 (1)
	Induced hypodermal *daf-2* (P*dpy-30*-FRT-*mCherry*-terminator-FRT-*gfp*-SL2-*daf-2*; P*dpy-7-flp*)		
daf-2(m41); krEx704,746 (two lines)	43 ± 6[d] (268, 10)	99 ± 1 (177, 3)	24 (2)
	Induced panneuronal, intestinal, and hypodermal *daf-2* (P*dpy-30*-FRT-*mCherry*-terminator-FRT-*gfp*-SL2-*daf-2*; P*rab-3-flp*; P*vha-6-flp*; P*dpy-7-flp*)		
daf-2(m41); krEx841-844 (four lines)	38 ± 5[d] (241, 3)	99 ± 0 (313, 4)	22 (2)

FLP-inducible *daf-2* constructs were designed essentially as in Davis *et al.* (2008), using the Multisite Gateway system from Invitrogen (Carlsbad, CA). The transgenes in the "off" configuration express the mCherry reporter under the control of ubiquitous *dpy-30* promoter, and the *let-858* transcriptional terminator prevents transcription of the downstream elements. Downstream of the transcriptional terminator is an artificial operon leading to the expression of *gfp* and *daf-2* mRNA in the same cells, where the GFP coding sequence is fused to the SL2 splice leader acceptor site (Gendrel *et al.* 2009), followed by the *daf-2* cDNA. FLP expression under one of the tissue-specific promoters brings the *gfp* and *daf-2* sequences under the control of the tissue-specific promoter. In all the strains above, the P*dpy-30*-FRT-*mCherry*-terminator-FRT-*gfp*-SL2-*daf-2* construct was injected at 10 ng/μl and the FLP-expressing constructs were injected at 5 ng/μl into *daf-2(m41)* animals. These low concentrations were used because of high toxicity observed with transgenes expressing DAF-2 both conditionally and nonconditionally (data not shown).

[a] Eggs were laid at 15° overnight. Adults were removed and plates were shifted to 25°. Percentage of non-dauer L4 or adult animals was scored 72 hr postshift by visual inspection.

[b] Percentage of survival on 0.75 mM DMPP at 20° (average ± SEM).

[c] L2 stage duration at 20° was determined as in Ruaud and Bessereau (2007).

[d] $P \leq 0.05$ when compared to the P*dpy-30*-FRT-*mCherry*-terminator-FRT-*gfp*-SL2-*daf-2* control lines (Student's *t*-test).

dauer formation in *daf-2(m41)* animals at 25°. In contrast, it failed to rescue their DMPP resistance (Table 2). Expressing the FLP concomitantly in the intestine, epidermis, and neurons further improved rescue of the Daf-c phenotype but failed to rescue the resistance to DMPP (Table 2). Second, we used previously characterized transgenic lines to express wild-type DAF-16/FOXO in *daf-2(e1370); daf-16(mu86)* double mutants (Libina *et al.* 2003). DAF-16 expression under its own promoter or in neurons is sufficient to restore the Daf-c phenotype of the double-mutant combination. In contrast, clear rescue of the DMPP sensitivity of the double mutant was observed only in transgenic strains expressing DAF-16 under the control of its own promoter (Table 3). Animals expressing DAF-16 in the intestine or muscle, but not in neurons, were significantly more resistant than control transgenic lines. However, these data must be interpreted with caution because overexpression of ROL-6(gf) and DAF-16 is known to slow down the overall development, which was previously demonstrated to confer partial resistance to DMPP by itself (Ruaud and Bessereau 2006). Together, these results indicate that the requirements of the insulin-like pathway for DMPP sensitivity and dauer formation are different, suggesting that DAF-2 functions in distinct combinations of tissues or requires additional temporal regulation to control developmental speed at the second larval stage.

DAF-2/InsR signaling controls non-dauer developmental timing: We previously identified two developmental properties that could induce DMPP resistance: an insensitivity to the DMPP-induced developmental delay [in the case of *daf-12(0)* mutants] and a delayed L2/L3 molt [in the case of *catp-1(lf)* mutants]. To identify the mechanism that renders *daf-2* mutants DMPP resistant, we monitored their molting cycle. We observed that the duration of the L2 stage was only slightly increased in the DMPP-sensitive alleles *e1368* and *m577*, while it was extended to approximately twice the wild-type duration in the DMPP-resistant alleles *m41*, *m596*, and *e1370* (Figure 3, Table 4). The DMPP resistance of *daf-2* alleles strongly correlates with the duration of their L2 stage, as indicated by the high Pearson correlation coefficient between those two

TABLE 3

DMPP resistance and developmental speed in animals with tissue-specific *daf-16* expression

Genotype	% dauer[a]	DMPP[R] (no. of animals, experiments)[b]	L2 duration in hr (no. of lines scored)[d]
daf-2(e1370)	90.2 ± 7.2	94 ± 2 (643, 9)	20
daf-16(mu86); daf-2(e1370)	0.1 ± 0.6	2 ± 1 (793, 9)	10
daf-16(mu86); daf-2(e1370); krEx841-843[rol-6(gf)]	0	17 ± 8 (634, 12)	13 (3)
daf-16(mu86); daf-2(e1370); muEx176[Pdaf-16::gfp::daf-16]	35.1	100 ± 13[c] (118, 5)	30 (1)
daf-16(mu86); daf-2(e1370); muEx169[Punc-119::gfp::daf-16]	63	34 ± 8 (196, 10)	15 (1)
daf-16(mu86); daf-2(e1370); muEx211[Pges-1::gfp::daf-16]	0	45 ± 8[c] (188, 6)	ND
daf-16(mu86); daf-2(e1370); muEx212[Pmyo-3::gfp::daf-16]	0	37 ± 5[c] (213, 6)	15 (1)

daf-16 tissue-specific rescue lines are described in Libina *et al.* (2003). Three independent *rol-6(gf)* lines were generated as a control by injecting pRF4 at 100 ng/μL, together with L3570 (P*myo-3::GFP*) at 10 ng/μL.

[a] Dauer formation at 25.5° as reported in Libina *et al.* (2003). In *rol-6(gf)* control lines, dauer formation was scored at 25.5° (three lines, six experiments, 535 total individuals).

[b] Percentage of survival on 0.75 mM DMPP at 20° (average ± SEM).

[c] $P \leq 0.05$ when compared to the *rol-6(gf)* co-injection controls (Student's *t*-test).

[d] L2 stage duration was determined at 20°. ND, not determined.

variables ($r^2 = 0.87$, Figure 2B). Moreover, the developmental delay observed in *m41*, *m596*, and *e1370* animals was suppressed by mutations in *daf-16*/FOXO, paralleling the suppression of DMPP resistance (Table 4). Similarly, L2 duration in DAF-2 and DAF-16 tissue-specific expression lines correlates well with their DMPP resistance (Tables 2 and 3). Finally, this developmental delay is stage specific, as very little difference was observed in the duration of L1 and L3 stages between DMPP-sensitive and DMPP-resistant *daf-2* mutants. It takes only 9 hr for *daf-2(e1368)* and *daf-2(e1370)*, for example, to complete the L3 stage—a value very similar to the 8-hr L3 stage of wild type. Previous studies reported that the L2 stage of animals that will enter the dauer diapause (L2d) is longer by a few hours when compared to the reproductive L2 stage observed under

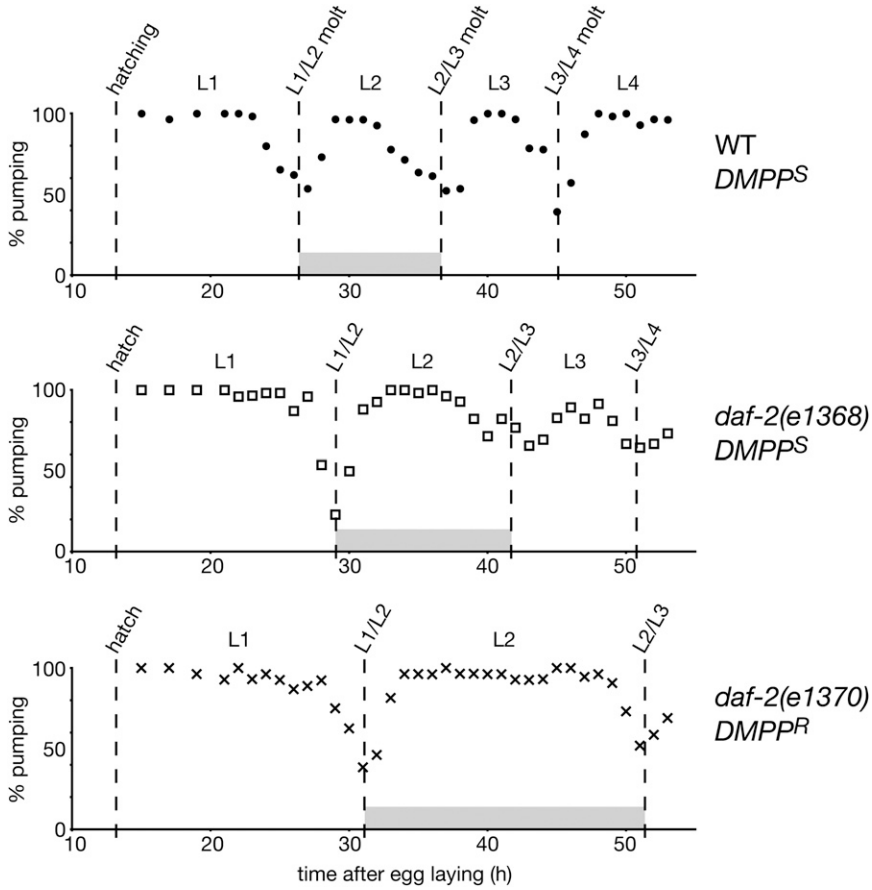

Figure 3.—Global developmental rate of some *daf-2/InsR* mutant alleles. During the lethargus period that precedes molting, rhythmic contractions of the pharynx (also called pharyngeal pumping) cease. Each dot, open square and x represents the percentage of worms pumping at a given time ($n > 25$ individuals). The results of similar experiments performed for additional allelic combinations are presented in Table 4.

TABLE 4

Effects of *daf-2* and *daf-16* on developmental speed and DMPP resistance

	Interacting mutation				
	+			*daf-16(0)*[a]	
daf-2 allele	L1 duration (hr)	L2 duration (hr)	DMPP^R	L2 duration (hr)	DMPP^R
+	14	10	−	11	−
e1368	17	12	−	ND	ND
m41	17	23	+	13	−
m577	16	12	−	ND	ND
m596	15	19	+	14	−
e1370	19	20	+	11	−

For DMPP resistance (DMPP^R), − refers to DMPP sensitivity as seen in the wild type and + indicates strong resistance on 0.75 mM DMPP. Developmental speed and DMPP^R were determined at 20°. ND, not determined. $N \geq 25$ individuals.

[a] The *daf-16(0)* allele used is *mgDf50*, except in the double mutant *daf-16(m26); daf-2(m41)*.

favorable developmental conditions (Swanson and Riddle 1981; Golden and Riddle 1984). Although we cannot rule out that the developmental delay observed in DMPP-resistant *daf-2* mutants is due to a transient entry into L2d, the clear uncoupling between DMPP resistance and dauer phenotypes suggests that the insulin-like pathway specifically regulates non-dauer developmental speed during the reproductive L2 stage.

Conclusions: Our previous studies indicated an overlap between the pathways controlling dauer formation and L2 developmental speed. Here we show that the canonical DAF-16-dependent insulin-like signaling is required to implement DMPP toxicity and control L2 developmental speed. This function is most probably independent of its role in dauer formation since most of the mutants tested formed very few dauer larvae in our culture conditions. Moreover, the tissues in which DAF-2 signaling is required to control dauer formation and L2 developmental speed are not identical. Taken together, our results identify a novel function of insulin/insulin-like growth factor (IGF) signaling in controlling non-dauer developmental speed in *C. elegans*.

Insulin and IGF-1 signaling are major determinants of development and growth speed in vertebrates and invertebrates. IGF-1 mutant mice develop slowly and have a small adult size (Liu *et al.* 1993). Similarly, reduction of insulin/IGF signaling in Drosophila induces small-sized adults that develop slowly under favorable nutritional conditions (Bohni *et al.* 1999; Colombani *et al.* 2005; Shingleton *et al.* 2005). These phenotypes are reminiscent of animals that have been deprived of food during their development. Insulin/IGF signaling could therefore control adult size depending on food availability, thus maximizing reproductive success in a variable environment. The DAF-2 pathway was originally identified as a major regulator of a nematode-specific developmental arrest, the dauer diapause. By revealing this additional function in controlling non-dauer development, our study extends and strengthens the conservation of insulin/IGF signaling roles in the environmental control of developmental speed. Furthermore, because of the tractability of the *C. elegans* system, this new phenotype might represent an interesting paradigm to investigate the cellular and molecular mechanisms underlying such regulation, which are still poorly understood.

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