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Lipophilic regulator of a developmental switch in *Caenorhabditis elegans*

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Abstract

In *Caenorhabditis elegans*, the decision to develop into a reproductive adult or arrest as a dauer larva is influenced by multiple pathways including insulin-like and transforming growth factor β (TGFβ)-like signalling pathways. It has been proposed that lipophilic hormones act downstream of these pathways to regulate dauer formation. One likely target for such a hormone is DAF-12, an orphan nuclear hormone receptor that mediates these developmental decisions and also influences adult lifespan. In order to find lipophilic hormones we have generated lipophilic extracts from mass cultures of *C. elegans* and shown that they rescue the dauer constitutive phenotype of class 1 *daf-2* insulin signalling mutants and the TGFβ signalling mutant *daf-7*. These extracts are also able to rescue the lethal dauer phenotype of *daf-9* mutants, which lack a P450 steroid hydroxylase thought to be involved in the synthesis of the DAF-12 ligand; extracts, however, have no effect on a DAF-12 ligand binding domain mutant that is predicted to be ligand insensitive. The production of this hormone appears to be DAF-9 dependent as extracts from a *daf-9;daf-12* double mutant do not exhibit this activity. Preliminary fractionation of the lipophilic extracts shows that the activity is hydrophobic with some polar properties, consistent with a small lipophilic hormone. We propose that the dauer rescuing activity is a hormone synthesized by DAF-9 that acts through DAF-12.

Introduction

Under conditions of poor nutrition or overcrowding the nematode *Caenorhabditis elegans* is able to enter an alternative developmental stage called the dauer larva, in which the animal is non-feeding, non-reproducing and stress resistant (Riddle, 1988). Two parallel endocrine pathways, activated by insulin and transforming growth factor β (TGFβ), transduce environmental signals of pheromone, food availability and temperature from sensory neurons to mediate the decision to proceed with normal reproductive growth or initiate dauer formation (Kimura *et al*., 1997; Riddle & Albert, 1997; Patterson & Padgett, 2000; Tatar *et al*., 2003). These two pathways are thought to converge on a secondary endocrine pathway that involves a lipophilic hormone acting through the nuclear hormone receptor (NHR) DAF-12, which then effects the switch from the normal developmental programme to the diapause programme (Fig. 1) (Antebi *et al*., 1998, 2000; Gerisch & Antebi, 2004; Mak & Ruvkun, 2004).

Genetic epistasis analysis with dauer formation mutants places *daf-12* at the terminal end of the pathway because *daf-12* dauer defective (Daf-d) mutations are able to suppress the dauer constitutive (Daf-c) mutations of the insulin and TGFβ pathways (Larsen *et al*., 1995). A large number of *daf-12* mutants have been identified and grouped into six classes, which vary with respect to their dauer formation and heterochronic phenotypes (Antebi *et al*., 1998, 2000). Mutations in the putative DNA binding domain of DAF-12 generate Daf-d phenotypes, indicating that DAF-12 is required for dauer formation. However, *daf-12* is unique among *daf* genes in that both Daf-d and Daf-c alleles have been isolated (Antebi *et al*., 1998, 2000).

A ligand for DAF-12 is thought to be synthesized by DAF-9, a cytochrome P450 with homology to steroid and fatty acid hydroxylases, which lies upstream of *daf-12* by genetic epistasis (Gerisch *et al*., 2001; Jia *et al*., 2002). *daf-9* mutants are unconditionally Daf-c but only form partial dauers, defined by the lack of radial constriction of the pharynx and their continued pharyngeal pumping. Weak *daf-9* mutants arrest transiently as partial dauers and then develop into fertile adults that have a gonadal migration defect, whereby the distal tip cells of the developing gonad fail to migrate dorsally (Gerisch *et al*., 2001; Jia *et al*., 2002). This phenotype is also seen in Daf-c *daf-12* mutants, suggesting that *daf-9(–)* animals are deficient for a DAF-12 ligand, whereas *daf-12* Daf-c mutants are ligand insensitive (Gerisch *et al*., 2001).

Further evidence for the existence of an endogenous lipophilic ligand for DAF-12 comes from studies of the cholesterol dependence of *C. elegans*. Under laboratory conditions *C. elegans* must be supplemented with exogenous cholesterol because nematodes are unable to synthesize their own sterols. Cholesterol deprivation in wild-type animals leads to a variety of phenotypes, including developmental arrest and reduced fertility (Shim *et al*., 2002; Merris *et al*., 2003). Gerisch *et al*. noted that wild-type nematodes in cholesterol-deficient media often display the same non-reflexed gonad phenotype as *daf-9* mutants, while the Daf-c phenotype of some weak *daf-9*

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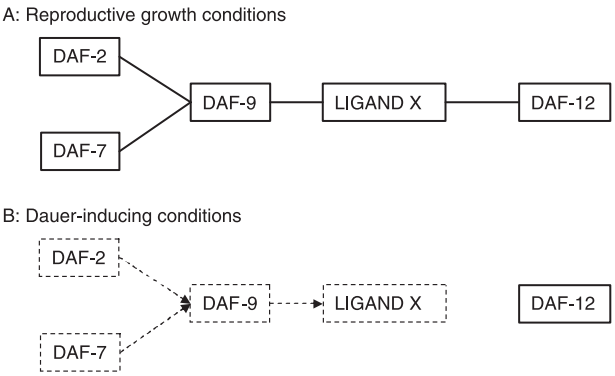

Fig. 1 Model for dauer formation and predicted action of lipophilic hormone produced by DAF-9 and acting on DAF-12. (A) Reproductive growth conditions. Signalling through the insulin (DAF-2) and TGFβ (DAF-7) pathways increases DAF-9 activity to produce a lipophilic hormone, ligand X. Ligand X binds to the nuclear hormone receptor DAF-12 to allow reproductive growth and development to proceed. (B) Dauer-inducing conditions. Reduced signalling through DAF-2 and DAF-7 reduces the activity of DAF-9, leading to a reduction in the synthesis of ligand X. Reduced levels of ligand X result in the suppression of programmes of normal growth and lead to dauer formation Solid lines indicate up-regulation and dashed lines indicate down-regulation.

alleles is enhanced under conditions of cholesterol deprivation (Gerisch *et al*., 2001; Jia *et al*., 2002). Finally, mutation of the *npc-1* and *npc-2* genes, which affect lysosomal transport of sterols, causes development delay as single mutants but the double mutant exhibits a Daf-c phenotype (Sym *et al*., 2000).

In order to identify the hormonal regulator of dauer formation we have generated lipophilic extracts from nematodes and tested their effects. We describe an extract that can prevent dauer formation in *daf-2* and *daf-9* mutants but has no effect on a Daf-c *daf-12* mutant that is predicted to be ligand insensitive. Moreover, extracts generated from worms with a *daf-9* mutant background fail to rescue either *daf-2* or *daf-9* mutants. These findings are consistent with the predicted activity of a DAF-12 ligand.

Results

We generated ether extracts from 1-, 2- and 3-day-old N2 and *daf-12(m20)* cultures and tested their ability to rescue the Daf-c phenotype of *daf-2(e1368)*. A number of lines of evidence suggest that the lipophilic dauer-regulating hormone may be a steroid or sterol, and the ether extraction used in this work has previously been shown to be optimal for the extraction of unconjugated steroids (Chatman *et al*., 1999). Between 1 and 5 g of worms was extracted for each condition and resuspended in 20 μL DMSO per gram of starting material. Extracts were tested in duplicate and were applied to the bacterial lawn as a 0.5-g equivalent of worms in 10 μL DMSO.

Extracts from 1-day-old N2 and *daf-12(m20)* worms prevented dauer formation in approximately 25% of *daf-2(e1368)* animals at 25 °C (Table 1). A small fraction of animals were rescued following exposure to extracts from 2-day-old N2 but in

Table 1 Effect of extracts from N2 and *daf-12(m20)* from different development stages on constitutive dauer formation of *daf-2(e1368)* at 25 °C

Extract genotype and development stage		Dauers*	L3-young adult	Gravid adult	*N*†
N2	L1/L2	75	11	14	358
	L3/L4	94	3	3	365
	Adult	100	–	–	357
daf-12(m20)	L1/L2	76	6	18	377
	L3/L4	12	16	72	513
	Adult	97	1	2	306

Plates were scored after 3 days and results are presented as per cent.
*Dauers were scored on morphology alone, and includes dauer-like larvae.
†Total number of worms scored across three independent extracts.

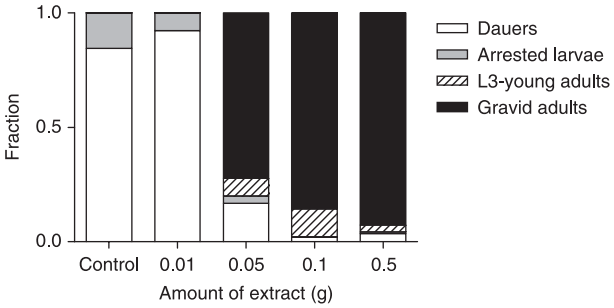

Fig. 2 Ether extract from 2-day-old (L3/L4) *daf-12(m20)* worms prevents dauer formation in *daf-2(e1368)* at 25 °C in a dose-dependent manner. Plates were scored for the presence of dauers (resistant to 1% SDS), arrested larvae (dauer-like but sensitive to 1% SDS), developing larvae (L3, L4 and young adults) and gravid adults. All extracts were applied to plates in a total volume of 10 μL DMSO. Total number of animals scored per condition: control, $n = 51$; 0.01 g, $n = 52$; 0.05 g, $n = 60$; 0.1 g, $n = 96$; 0.5 g, $n = 136$.

the presence of extracts from 2-day-old *daf-12(m20)*, the majority of *daf-2(e1368)* animals bypassed diapause, with 72% developing into gravid adults (Table 1). Extracts from adults of either strain had no effect on dauer formation (Table 1). As an additional control we generated an ether extract from 5 g of the concentrated bacteria that had been used as food. Bacteria generate a food signal that promotes normal growth and development as well as exit from the dauer stage in wild-type animals (Golden & Riddle, 1982), and thus it was possible that we were simply purifying and treating worms with a concentrated food signal. However, the bacterial extract failed to have any effect on dauer formation in *daf-2(e1368)* (data not shown).

Given that we saw the most rescue with extracts from 2-day-old *daf-12(m20)* all subsequent experiments were carried out with these extracts. Dose–response experiments with the *daf-12(m20)* ether extract demonstrated that there was still > 70% rescue when the amount of extract was reduced to 0.05 g per plate but rescue was lost at 0.01 g per plate (Fig. 2). The rescue of the Daf-c phenotype of *daf-2(e1368)* was seen consistently in three independent experiments using extracts generated from worms grown at different times (Table 2). Of two other *daf-2* class 1 mutants tested we found that the extracts rescued

Table 2 Effect of extracts from L3/L4 *daf-12(m20)* on constitutive dauer formation in different genotypes at 25 °C

Treated genotype	Treatment	Dauers*	Arrested larvae†	L3-young adult	Gravid adult	*N*‡
daf-2(e1368)	–	71	29	–	–	189
	+	2	4	27	67	341
daf-2(e1371)§	–	90	10	–	–	134
	+	2	–	8	90	215
daf-2(m41)§	–	91	9	–	–	131
	+	91	7	1	1	125
daf-2(e1370)§	–	86	14	–	–	132
	+	72	28	–	–	138
daf-7(e1372)	–	100	–	–	–	248
	+	99	1	–	–	176
daf-9(gk160)	–	–	100	–	–	119
	+	–	13	24	63	106
daf-12(rh273)	–	–	74	22	4	258
	+	–	62	37	1	281

Results are presented as per cent.
*Resistant to 1% SDS.
†Dauer-like animals that were sensitive to 1% SDS.
‡Total number of worms scored across three independent extracts.
§Tested with two independent extracts.

Table 3 Effect of L3/L4 *daf-12(m20)* extract treatment on dauer formation in *daf-2(m41)* and *daf-7(1372)* at 20 °C and 22.5 °C

Genotype	Temperature	Treatment	Dauers*	L3-young adult	Gravid adult	*N*†
daf-2(m41)	20 °C	–	100‡	–	–	199
		+	–	45	55	227
	22.5 °C	–	100	–	–	155
		+	50	10	40	193
daf-7(e1372)	20 °C	–	89	11	–	120
		+	7	42	51	102
	22.5 °C	–	100	–	–	182
		+	97	–	3	114

Plates were scored after 3 days and results are presented as per cent.
*Dauers were scored on morphology alone, and includes dauer-like larvae.
†Total number of worms scored across two independent extracts.
‡*daf-2(m41)* grows slowly at 20 °C and takes 5 days to develop into gravid adults. At day 5 in controls 189/199 animals were gravid adults.

daf-2(e1371) to a similar extent to *daf-2(e1368)*, but failed to rescue *daf-2(m41)* or the class 2 allele *daf-2(e1370)* at 25 °C (Table 2). Furthermore the extracts failed to rescue dauer formation in a Daf-c mutant from the TGFβ signalling pathway, *daf-7(e1372)* at 25 °C (Table 2). However, at semipermissive temperatures the extracts were able to prevent dauer formation in both *daf-2(m41)* and *daf-7(e1372)* (Table 3).

We then examined the ability of these extracts to rescue the lethal dauer phenotype of *daf-9(gk160)* homozygotes. *daf-9* mutants are unconditionally Daf-c but do not undergo all the morphological changes associated with true dauers. In particular there is no radial constriction of the pharynx, which continues to pump. In contrast to *daf-2* and *daf-7*, in which extracts prevented diapause entry, these experiments tested the ability of extracts to promote dauer exit. We found a dose-dependent rescue of 3-day-old *daf-9* partial dauers after 2 days of exposure to the extract, which was strengthened by day 3 (Fig. 3). On average, 63% of *daf-9* partial dauers developed into gravid adults in the presence of the extract, whereas 24% of animals exited the partial dauer stage to become L4 or young adults (Table 2). A proportion of worms in this latter group exhibited abnormal morphology, such as vulval protrusion, often accompanied by a distended gut, and some demonstrated a moulting defect in which the old cuticle was incompletely shed. It is of note that these phenotypes are similar to those observed in *let-767* mutants that are defective for a putative sterol-modifying enzyme (Kuervers *et al*., 2003).

Alleles of *daf-9* that are able to break through or bypass dauer arrest have been shown to have a non-reflexed gonad due to the aberrant migration of the distal tip cells of the developing gonad (Gerisch *et al*., 2001; Jia *et al*., 2002). We found that rescued *daf-9(gk160)* homozygotes that developed into reproductive adults all exhibited a normally reflexed gonad (Fig. 3D). To confirm their identity as *daf-9* homozygotes, fertile adults were transferred to normal NGM plates and allowed to lay eggs. Three days later all eggs developed into partial *daf-9* dauers,

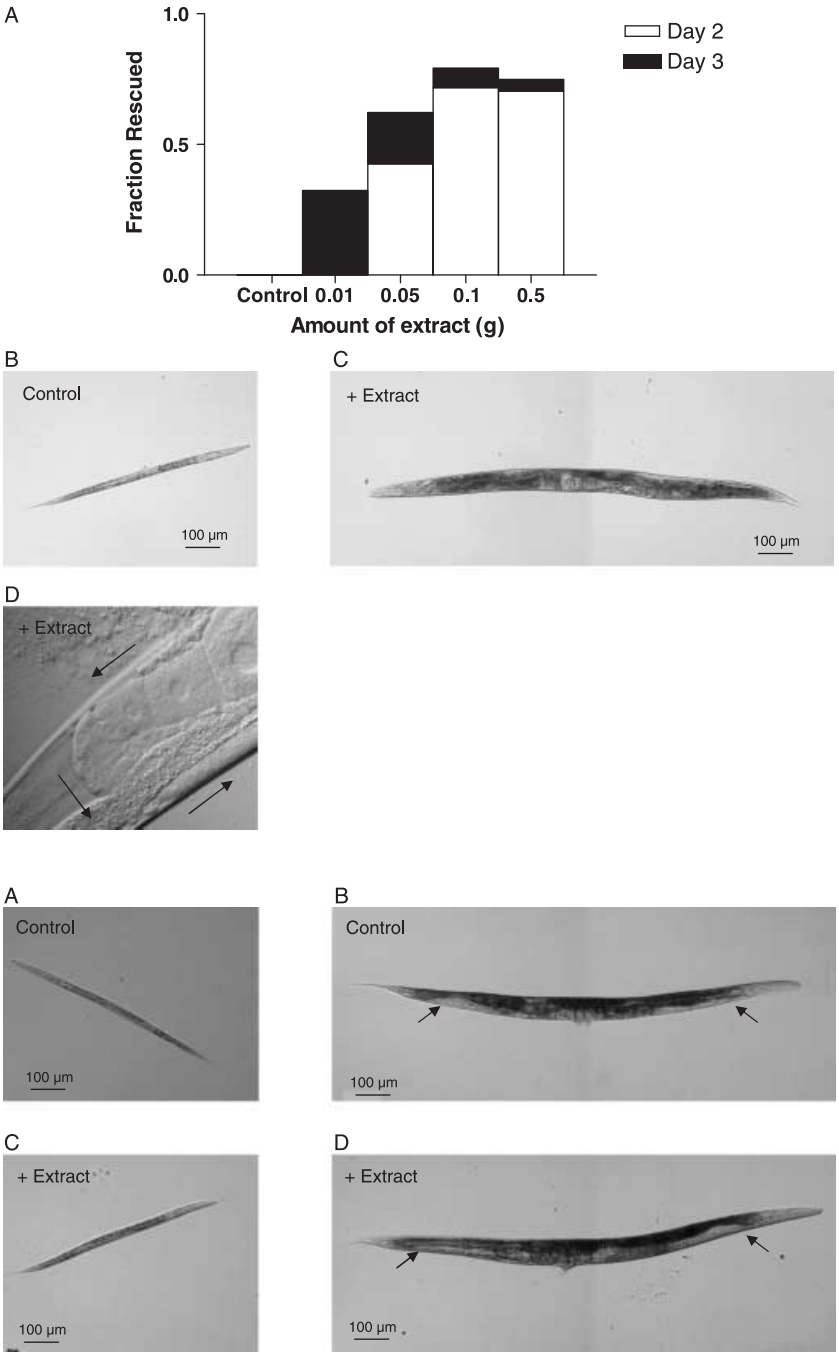

Fig. 3 Ether extract from 2-day-old (L3/L4) *daf-12*(*m20*) worms rescues the partial dauer phenotype of *daf-9*(*gk160*). (A) Dose-dependent rescue of *daf-9*(*gk160*). Three-day-old homozygous partial dauers were transferred to plates containing extracts and incubated at 25 °C. Plates were scored for the presence of L4, young adults and gravid adults on days 2 and 3 after exposure to extract. Total number of animals scored per condition: control, $n = 35$; 0.01 g, $n = 54$; 0.05 g, $n = 31$; 0.1 g, $n = 46$; 0.5 g, $n = 44$. (B) *daf-9*(*gk160*) homozygote after 3 days in the absence of extract. (C) *daf-9*(*gk160*) homozygote after 3 days exposure to extract. (D) Reflexed gonad in an extract-treated *daf-9*(*gk160*) homozygote. The arrows indicate the direction of cell migrations during gonadal development.

Fig. 4 Ether extracts from *daf-12*(*m20*) fail to rescue dauer and gonad phenotypes of *daf-12*(*rh273*). *daf-12*(*rh273*) eggs were transferred to plates containing ether extracts from 0.5 g of 2-day-old *daf-12*(*m20*) worms and incubated for 3 days at 25 °C. Control *daf-12*(*rh273*) worms are shown in A (partial dauer) and B (gravid adult) and extract-treated worms in C (partial dauer) and D (gravid adult). The arrows on B and D indicate the gonadal migration defect, in which the gonad fails to migrate and extends along the length of the animal.

confirming the genotype of the rescued parent and indicating an absence of maternal rescue by the extract (data not shown).

The ability of the lipophilic extract to rescue *daf-9* suggested that the active component may be a ligand for DAF-12. We therefore examined the effect of this extract on *daf-12(rh273)* mutants, which carry a mutation in the putative ligand binding domain of DAF-12 that is predicted to reduce affinity for ligand. Under normal growth conditions *daf-12(rh273)* mutants transiently arrest as partial dauers before continuing on to develop into gravid adults that display the same gonadal migration defect as *daf-9* mutants. After 3 days at 25 °C there was no difference in the number of arrested partial dauers in control or extract-treated populations (Table 2). In addition, those animals that developed into adults exhibited the same non-reflexed gonad as control animals (Fig. 4). These data suggest that *daf-12(rh273)* animals are insensitive to the dauer-rescuing activity in the extracts. To test further the hypothesis that we had identified a DAF-12 ligand, we generated extracts from *daf-12(m20)* animals in a *daf-9(–)* background. Although extracts from *daf-12(m20)* were able to rescue both *daf-2(e1368)* and *daf-9(gk160)*, extracts from 2-day-old cultures of *daf-9(gk160);daf-12(m20)* had no effect on either strain (Fig. 5). A mixture of

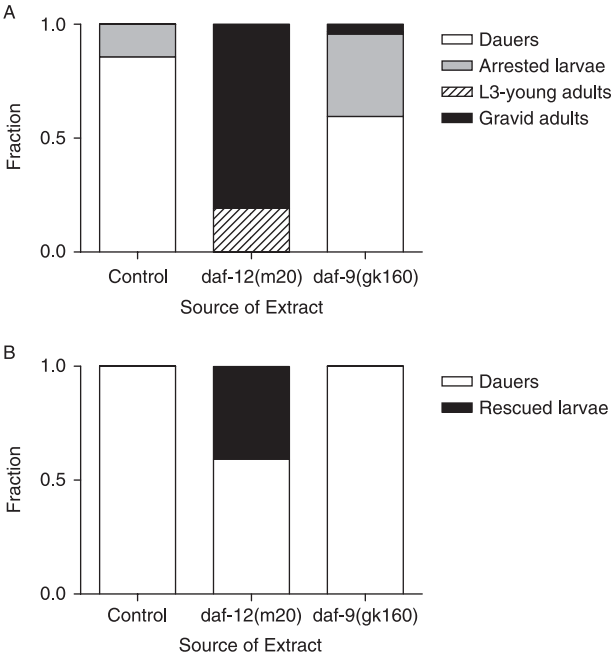

Fig. 5 Ether extracts from *daf-9(gk160);daf-12(m20)* fail to rescue dauer constitutive phenotypes of *daf-2(e1368)* and *daf-9(gk160)*. Ether extracts were generated from 2-day-old cultures of *daf-12(m20)* and *daf-9(gk160);daf-12(m20)* and applied to plates as 0.5 g extracted worms in 10 μL DMSO. (A) *daf-2(e1368)* eggs were transferred to plates containing extract from either *daf-12(m20)* or *daf-9(gk160);daf-12(m20)* and incubated at 25 °C for 3 days. Plates were scored for the presence of dauers (SDS resistant), arrested larvae (SDS-sensitive dauer-like animals), developing larvae (L3, L4 and young adults) and gravid adults. Total number of animals scored per condition: control, $n = 42$; *daf-12(m20)*, $n = 99$; *daf-9(gk160);daf-12(m20)*, $n = 94$. Similar results were obtained in two replicate experiments with independent extracts. (B) Three-day-old *daf-9(gk160)* partial dauers were transferred to plates containing extract from either *daf-12(m20)* or *daf-9(gk160);daf-12(m20)* and incubated at 25 °C for 2 days. Plates were scored for the presence of partial dauers, developing larvae (L4 and young adults) and gravid adults 2 days later. Total number of animals scored per condition: control, $n = 29$; *daf-12(m20)*, $n = 44$; *daf-9(gk160);daf-12(m20)*, $n = 51$. Similar results were obtained in a replicate experiment with an independent extract.

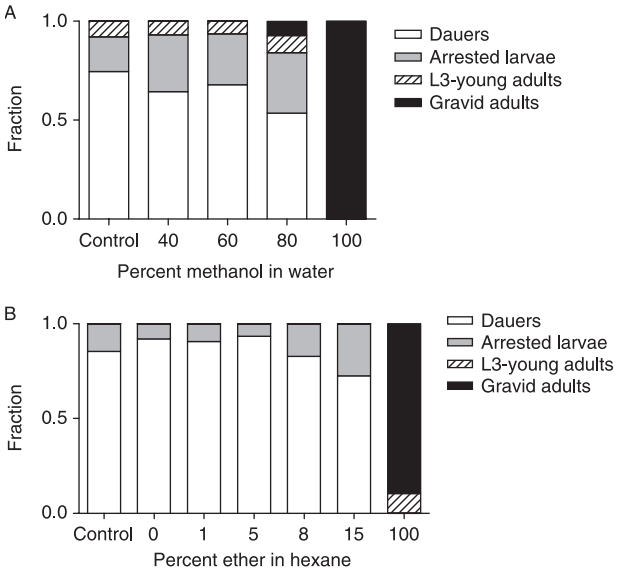

Fig. 6 Fractionated ether extracts rescue the dauer constitutive phenotype of *daf-2(e1368)* at 25 °C. (A) Reverse phase fractionation of ether extracts from 2-day-old cultures of *daf-12(m20)* on Sep – Pak C18 solid-phase extraction columns. Extracts were applied to the column in 20% methanol in water and eluted with increasing amounts of methanol. Total number of animals scored per condition: control, $n = 63$; 40%, $n = 87$; 60%, $n = 78$; 80%, $n = 69$; 100%, $n = 100$. (B) Normal phase fractionation of ether extracts from 2-day-old cultures of *daf-12(m20)* on Sep – Pak silica solid-phase extraction columns. Extracts were applied to the column in hexane and eluted with increasing amounts of ether in hexane. Total number of animals scored per condition: control, $n = 76$; 0%, $n = 76$; 1%, $n = 64$; 5%, $n = 92$; 8%, $n = 93$; 15%, $n = 135$; 100%, $n = 202$.

daf-12(m20) and *daf-9;daf-12* extracts was able to rescue dauer formation in *daf-2(e1368)* to the same degree as *daf-12(m20)* extracts alone (data not shown).

In order to begin characterization of the active component of these extracts we performed some simple fractionation using solid-phase extraction columns. The fractionated extracts were then tested for their ability to rescue *daf-2(e1368)* dauer formation at 25 °C. We first assessed hydrophobicity by fractionating the extract using a C18 reverse-phase solid-phase column. Extracts were applied to the column in 20% methanol in water and then eluted off with increasing methanol/water mixtures from 40% to 100%. We found that the dauer-rescuing activity eluted in the most hydrophobic fraction (Fig. 6A). We next performed normal phase fractionation using a silica solid-phase extraction column. Extracts were applied to the column in hexane and elution was performed using ether/hexane mixtures. Dauer-rescuing activity localized to the 100% ether fraction, indicating polar properties (Fig. 6B). Further fractionation of the extract showed that activity could be eluted off in 65% ether (data not shown).

Discussion

Genetic and molecular analysis of dauer formation in *C. elegans* has defined two endocrine pathways that are thought to modulate the levels of a lipophilic hormone that regulates the decision to proceed with normal programmes of reproductive development or to enter diapause. We have identified a lipophilic nematode extract that influences dauer formation in a manner that is consistent with a hormone produced by the cytochrome P450, DAF-9, and acts on the NHR, DAF-12.

Identification of a lipophilic regulator of dauer formation

The decision to commit to programmes of normal reproductive development or to enter the diapause programme is made early in nematode development. Commitment to reproductive growth programmes is made by the end of the L1 moult or during the early part of L2, while commitment to dauer formation is not made until the end of L2d (Golden & Riddle, 1984). Thus we hypothesized that the hormone that promotes normal reproductive

growth would be most abundant in extracts from L1/L2 larvae. Consistent with this hypothesis we found that extracts made from 24-h nematode cultures were able to prevent dauer formation in *daf-2(e1368)*. However, extracts from 2-day-old *daf-12(m20)* (L3/L4) showed the strongest activity, which was not present in extracts from age-matched N2 cultures.

The *daf-12(m20)* L3/L4 extracts were most effective in rescuing the Daf-c phenotype of weak class I alleles of *daf-2*, but did not prevent dauer formation in stronger Daf-c mutants of *daf-2* or in *daf-7* mutants at 25 °C. However, at lower temperatures where both *daf-2(m41)* and *daf-7(e1372)* still exhibit a high proportion of dauer formation (Swanson & Riddle, 1981; Gems *et al.*, 1998), we found that the extracts could reverse the Daf-c phenotype. These observations suggest that the level of the endogenous lipophilic hormone in Daf-c mutants correlates with the strength of the Daf phenotype. We therefore suggest that the amount of hormone that needs to be replaced in the weak *daf-2* alleles is lower than in the more severe alleles. This is supported by studies of *daf-9* over-expression. Expression of a functional *daf-9*::GFP construct in the hypodermis under the control of the *dpy-7* promoter was required to prevent dauer formation in *daf-2* or *daf-7* mutants, whereas over-expression driven by the *sdf-9* promoter in two neuron-like cells, designated as XXXL/R, had no effect (Gerisch & Antebi, 2004; Mak & Ruvkun, 2004). Given the relative sizes of these two tissues it is likely that the amount of hormone produced in the hypodermis under the control of the *dpy-7* promoter is much higher and more available to target tissues than that produced in the XXXL/R neurons. It is also possible that the ability of the lipophilic extracts to rescue dauer formation is limited by the mode of administration, as feeding the hormone to worms may result in limited availability of hormone at the appropriate tissues.

Lipophilic regulator of dauer formation is a candidate DAF-12 ligand

We have hypothesized that the active hormone in these dauer-rescuing extracts is a candidate DAF-12 ligand, based on its ability to rescue the dauer arrest of *daf-2*, *daf-7* and *daf-9* mutants and its failure to alter the phenotype of a ligand-insensitive *daf-12* mutant. Furthermore the activity of extracts derived from *daf-12(m20)* larvae is lost when DAF-9 activity is removed by way of a *daf-9;daf-12* double mutant. These observations provide biochemical evidence for the existence of a lipophilic hormone involved in diapause and confirm the genetic epistasis experiments that place *daf-9* upstream of *daf-12* (Gerisch *et al.*, 2001; Jia *et al.*, 2002). DAF-9 is a cytochrome P450 with similarity to steroid and fatty acid hydroxylases, and two models for its mode of action have been proposed (Gerisch *et al.*, 2001; Jia *et al.*, 2002; Gerisch & Antebi, 2004; Mak & Ruvkun, 2004). In the first, DAF-9 is involved in the synthesis of a ligand that when bound to DAF-12 allows progression of programmes of normal reproductive development. According to this model, loss of DAF-9 activity in a *daf-9* mutant would result in loss of the dauer-rescuing activity. In the second model, DAF-9 is responsible for metabolism and removal of the DAF-12 ligand and thus the levels of DAF-12 ligand would be expected to be elevated in *daf-9* mutants. Our observation that extracts from *daf-9* homozygotes failed to exhibit any dauer-rescuing activity is consistent with a role for DAF-9 in the synthesis of a DAF-12 ligand rather than its removal.

Spatial and temporal expression of the lipophilic regulator

DAF-9 is expressed in three locations in the worm. Expression in the XXXL/R cells in the head is evident from late embryos, throughout all larval stages and into the adult. Hypodermal expression begins in mid-L2 and diminishes by the end of L4, while spermathecal expression is only detected in adults. Given the temporal and spatial pattern of expression of DAF-9 in these tissues, XXXL/R expression could be important for mediating the initial commitment to reproductive growth programmes, which has usually occurred by the L1 moult or by early L2 (Golden & Riddle, 1984). Hypodermal expression is likely to strengthen this commitment, particularly in the face of mildly unfavourable environments (Gerisch & Antebi, 2004).

The hypodermis appears to be a major site of DAF-9 expression, and by implication hormone synthesis, yet hypodermal DAF-9 expression is absent in *daf-12* mutants (Gerisch & Antebi, 2004; Mak & Ruvkun, 2004). It is therefore intriguing that we observed significant dauer-rescuing activity in extracts derived from *daf-12* mutants. Despite the loss of hypodermal DAF-9 in *daf-12* mutants, expression of DAF-9 in the XXXL/R neurons is maintained and thus we assume that this is the source of activity in our extracts. There may also be a feedback loop between the XXXL/R neurons and the hypodermis that regulates the amount of DAF-9-dependent hormone. In this respect, observations from expression mosaics indicate that XXX cells normally inhibit *daf-9* expression in the hypodermis (Gerisch & Antebi, 2004) and thus it is possible that hypodermal *daf-9* may normally inhibit XXXL/R *daf-9*. Because DAF-9 expression in XXXL/R neurons in *daf-12* mutants is not elevated (Gerisch & Antebi, 2004; Mak & Ruvkun, 2004), an increase in the production of the DAF-9-dependent hormone would need to occur independently of the level of protein, through post-translational modification or changes in enzyme activity. An example of this is found in humans, whereby steroid hormone biosynthesis is acutely regulated, within minutes, by the StAR (steroidogenic acute regulatory) protein, which increases substrate availability for P450 enzymes by delivering cholesterol to the inner mitochondrial membrane while chronic regulation is achieved through changes in transcription (Stocco, 2001).

The hormone activity in extracts from 1-day-old cultures from N2 prevented dauer formation in approximately 25% of *daf-2(e1368)* animals, whereas we saw very little activity in extracts from 2-day-old worms grown at 25 °C. Hypodermal *daf-9* has been shown to be elevated at this temperature in wild-type L3 larvae and so it is surprising that we saw no activity, particularly as similar extracts from *daf-12* mutants showed significant

rescue. The lack of activity in these extracts may relate to the temporal pattern of hypodermal *daf-9* expression, whereby expression begins in mid-L2 and ends in L4. Our mass culture conditions may generate a different pattern of *daf-9* expression to that previously reported, resulting in extracts being generated at a time when *daf-9* is not active. Equally, it is difficult to compare the level of activity between extracts in the absence of a truly quantitative assay for this putative hormone. Our dose–response experiments indicate that a critical level of activity is required to prevent dauer formation in our bioassay. Thus the lack of rescue using N2 extracts does not necessarily indicate that there is no activity present but that it may be below this threshold. At present the active component of these extracts remains to be determined but preliminary purification indicates that it is strongly hydrophobic with significant polar properties. These data are consistent with the chemical properties of a small lipophilic molecule but we are unable to say at this stage to which general class of molecule it belongs.

Lipophilic hormone signalling in *C. elegans*

Despite the abundance of genes encoding NHRs in the genome (Sluder *et al*., 1999) and the evidence for steroid hormone activity, no lipophilic hormones have yet been identified in *C. elegans*. We have shown that lipophilic extracts from worms possess the activity predicted of a hormone produced by DAF-9 and acting on DAF-12. This demonstrates an endogenous hormonal activity in *C. elegans* and confirms that lipophilic hormones are likely to play a major role in intercellular signalling in nematodes. Additionally, in a recent examination of eight previously uncharacterized NHR genes, four were found to have roles in dauer formation (Gissendanner *et al*., 2004) suggesting that DAF-12 may not be the only target for lipophilic hormones during dauer formation. As DAF-9 and DAF-12 have also been implicated in the control of adult lifespan in *C. elegans* (Larsen *et al*., 1995; Gems *et al*., 1998), this hormone is also a potential target for intervention in nematode aging.

Experimental procedures

Nematode culture

Bristol N2 (wild-type), DR1572[*daf-2(e1368) III*], DR1568[*daf-2(e1371) III*], DR1564[*daf-2(m41) III*], CB1370[*daf-2(e1370) III*], CB1372[*daf-7(e1372) III*], VC305 *[+/szT1[lon-2(e678)] I; daf-9(gk160)/szT1 X*], DR20[*daf-12(m20) X*], CB5584[*mIs 12 II*], DR205[*lon-2(e678); daf-12(m20) X*], AA87[*daf-12(rh273) X*] and *E. coli* OP50 were obtained from the *Caenorhabditis* Genetic Center at the University of Minnesota.

Homozygous *daf-9(gk160)* worms segregating from VC305 [*+/szT1[lon-2(e678)] I; daf-9(gk160)/szT1 X*], are lethal partial dauers and never become fertile adults (http://elegans.swmed.edu/perl/CGCStrainSearch.pl?terms=VC305). We generated a *daf-9(gk160);daf-12(m20)* double mutant, in which the Daf-c phenotype of *daf-9(gk160)* was suppressed by the Daf-d phenotype of *daf-12(m20). daf-12;lon-2* males expressing GFP were generated from a cross of CB5584[*mIs12 II*] males with DR205[*lon-2(e678); daf-12(m20) X*] hermaphrodites. These males were then mated with VC305 [*+/szT1[lon-2(e678)] I; daf-9(gk160)/szT1 X*] hermaphrodites. *daf-12;lon-2/daf-9* heterozygotes were selected away from VC305 hermaphrodites by the presence of GFP, and *daf-12;lon-2/szT1* by size, and were allowed to self-fertilize on *daf-2* RNAi plates at 27 °C. This allowed the selection of *daf-12(m20)* homozygous animals by virtue of their Daf-d phenotype. *daf-12, daf-9* and *lon-2* are all found on the X chromosome at positions +2.39, –3.49 and –6.72, respectively, and thus a recombination event was required to generate *daf-12;daf-9* homozygotes. *daf-12;lon-2* homozygotes were identified and removed on the basis of their length. *daf-12;daf-9* recombinants were identified as Daf-d, non-Lon progeny and were confirmed after self-fertilization by generation of clonal lines, which were checked for the absence of lon mutants in their progeny and Daf-d phenotypes. The presence of the *daf-9* mutation was confirmed in these clonal lines by PCR on multiple individuals, as described by The *C. elegans* Knockout Consortium (http://celeganskoconsortium.omrf.org/). The resulting strain was designated GL216[*daf-12(m20); daf-9(gk160) X*].

For routine culture worms were maintained at 20 °C on 5-cm nematode growth medium (NGM) agar plates carrying a lawn of *E. coli* OP50 (Sulston & Hodgkin, 1988). For generation of lipophilic extracts, worms were initially grown by mass culture on 10-cm plates at 25 °C (Fabian & Johnson, 1994). Gravid 3-day-old worms were washed off approximately 20 large plates and subjected to sodium hypochlorite treatment to generate eggs. The eggs were inoculated into 2-L glass flasks containing 90 mL S-medium (+ cholesterol, final concentration 5 μg mL^{-1}) and 10 mL of concentrated *E. coli*. The liquid cultures were then incubated in a shaking incubator at 25 °C, 150 r.p.m. for 1, 2 or 3 days to generate L1/L2, L3/L4 and adults, respectively. Worms were harvested by low-speed centrifugation and washed three times with S-basal before the worm pellet was snap frozen in liquid nitrogen. By this method, approximately 1 g of L1/L2, 3 g L3/L4 and 5 g gravid adults could be harvested from each 100 mL of culture.

Preparation of lipophilic extracts

Frozen worm pellets were thawed at room temperature into an equal volume of TBS pH 7.5 (~5 g worms + 5 mL TBS) and then subjected to four 1-min sonications on ice. Worm lysates were transferred to 50-mL glass centrifuge tubes. Ten millilitres of worm lysate and 30 mL ether were vortexed vigorously until the two phases became miscible and viscous. Following centrifugation for 10 min at 1700 g, the ether phase was transferred to a glass tube. This process was repeated once more and the ether extracts were then evaporated under nitrogen and resuspended in 1 mL hexane. The hexane was then transferred to a 1-mL reactivial, evaporated under nitrogen and resuspended in dimethyl sulphoxide (DMSO).

Reverse phase fractionation

Ether extracts were prepared from 2 g (wet weight) of worms as described above and the dry extract was resuspended in 10 mL 20% methanol in water. Reverse phase fractionation was performed using Sep – Pak C18 columns (Waters, MA, USA) under gravity flow. In brief, the column was activated with 5 mL methanol and then equilibrated with 10 mL 20% methanol. The extract was applied to the column and the flow-through collected. Sequential elutions were performed using 5 mL of 40, 60, 80 and 100% methanol in water. The eluates were then dried using an evaporative centrifuge and resuspended in DMSO for bioassays.

Normal phase fractionation

Extracts were prepared as described above and the dried-down extract was resuspended in 5 mL hexane. Normal phase fractionation was performed using Sep – Pak silica columns (Waters, MA, USA) under gravity flow. The column was equilibrated with 5 mL hexane prior to sample loading, followed by a wash with 5 mL hexane. The extract was eluted with 10 mL ether in hexane at ratios of 0, 1, 5, 8, 15 and 100%. Fractions were collected in glass tubes, evaporated under nitrogen and resuspended in DMSO.

Dauer rescue assays

All dauer rescue assays were carried out on 3-cm Petri dishes containing 2 mL NGM and spotted with a lawn of 50 μL *E. coli* OP50. Lipophilic extracts were applied to the bacterial lawn in DMSO at least 30 min before the transfer of worms. We found that at low concentrations of DMSO (< 1%) there was an increase in the number of sodium dodecyl sulphate (SDS)-sensitive animals that retained a dauer-like appearance, whereas higher concentrations of DMSO (> 1%) resulted in a number of unhatched eggs and L1 arrest. Based on these results extracts were added to plates in 10 μL DMSO to give a final plate concentration of 0.5%.

Dauers were identified by resistance to 1% SDS for 30 min. Any dauer-like larvae that were SDS sensitive were scored as arrested larvae. In the presence of food, *daf-12(rh273)* and *daf-9(gk160)* homozygotes do not form true dauers, but are considered to be partial dauers (Gerisch *et al.*, 2001; Jia *et al.*, 2002). They possess much of the dauer-like morphology but there is no radial constriction of the pharynx and the animals continue to pump, and as a consequence are SDS sensitive. These animals were scored as arrested larvae.

For rescue of Daf-c mutants (*daf-2*, *daf-7* and *daf-12*) approximately 25–40 eggs were transferred to extract plates and incubated at 25 °C for 3 days. Plates were then scored for the number of animals at each developmental stage. To assess the ability of extracts to rescue the Daf-c phenotype of *daf-9(gk160)*, the parental strain, VC305, was grown at 25 °C for 3 days and *daf-9(gk160)* partial dauers were transferred to extract plates and incubated at 25 °C. Plates were then examined on days 2 and 3 after transfer for rescue of the partial dauer phenotype.

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