Activity of cGMP-Dependent Protein Kinase (PKG) Affects Sucrose Responsiveness and Habituation in *Drosophila melanogaster*

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The cGMP-dependent protein kinase (PKG) has many cellular functions in vertebrates and insects that affect complex behaviors such as locomotion and foraging. The *foraging* (*for*) gene encodes a PKG in *Drosophila melanogaster*. Here, we demonstrate a function for the *for* gene in sensory responsiveness and nonassociative learning. Larvae of the natural variant sitter (for^s) show less locomotor activity during feeding and have a lower PKG activity than rover (for^R) larvae. We used rover and sitter adult flies to test whether PKG activity affects (1) responsiveness to sucrose stimuli applied to the front tarsi, and (2) habituation of proboscis extension after repeated sucrose stimulation. To determine whether the differences observed resulted from variation in the *for* gene, we also tested for^{s2}, a sitter mutant produced on a rover genetic background. We found that rovers (for^R) were more responsive to sucrose than sitters (for^s and for^{s2}) at 1-, 2-, and 3-wk old. This was true for both sexes. Differences in sucrose responsiveness between rovers and sitters were greater after 2 h of food deprivation than after 24 h. Of flies with similar sucrose responsiveness, for^R rovers showed less habituation and generalization of habituation than for^s and for^{s2} sitters. These results show that the PKG encoded by *for* independently affects sensory responsiveness and habituation in *Drosophila melanogaster*.

Cyclic guanosine monophosphate (cGMP) plays diverse roles in both vertebrates and invertebrates. In vertebrates, changes in cGMP levels affect olfaction (Breer and Shepherd 1993; Kroner et al. 1996), taste (Rosenzweig et al. 1999; Krizhanovsky et al. 2000), the regulation of gene expression, and the activation of immediate early genes. But cGMP is also implicated in long-term potentiation, long-term depression, and early stages of memory consolidation (for review, see Wang and Robinson 1997). In invertebrates, cGMP plays a role in olfaction, vision, mechanosensation, hypoxia, and memory formation (for review, see Müller 1997; Bicker 2001). cGMP acts by regulating different receptor proteins, cGMP-regulated ion channels, cGMP-regulated phosphodiesterases, and cGMP-dependent protein kinases (PKG; for review, see Müller 1997; Wang and Robinson 1997; Bicker 2001). This study focuses on the role of a PKG encoded by the *foraging* (*for*) gene in sensory responsiveness and habituation to sucrose stimuli in the adult fruit fly, *Drosophila melanogaster*.

Natural variation in *for* affects larval and adult food-search behavior (Sokolowski 2001). In the presence of food, *Drosophila* larvae behave as rovers or sitters (Sokolowski 1998). Rover larvae have long foraging paths on food and have a greater tendency to leave a patch of food. In contrast, sitter larvae have short foraging trails and are more likely to stay within a patch of food. In the absence of food, both natural variants have long paths and do not differ in their locomotor behavior (Pereira et al. 1995; Sokolowski et al. 1997; Sokolowski and Riedl 1999). This behavioral polymorphism is also found in adult flies. Adult rovers walk further away from a drop of sucrose than sitters (Pereira and Sokolowski 1993). Detailed analysis of the *for* gene revealed that the behavioral differences found between rovers and sitters result from differences in the expression levels of *for*, which encodes one of the two cGMP-dependent protein kinases in fruit flies (Osborne et al. 1997). In *Drosophila*, two genes encode cGMP-dependent protein kinases, *dg1* and *dg2* (Kalderon and Rubin 1989). *dg2* is synonymous with the *for* gene. Higher expression of *for*-RNA and PKG levels are found in adult heads of for^R rovers compared with for^s and for^{s2} sitters (Osborne et al. 1997). Sitters and rovers are therefore excellent variants to study the role of PKG activity in *Drosophila* behavior.

Recently, *Amfor*, an ortholog of the *Drosophila for* gene was cloned in the honey bee (Ben-Shahar et al. 2002). This gene affects the foraging behavior in honey bees. Sensory responsiveness in honey bees correlates with different aspects of foraging behavior and nonassociative and associative learning, as discussed below. We hypothesized that the *Drosophila for* gene, which affects the foraging behavior in *Drosophila*, might also influence sensory responsiveness and nonassociative learning in this fly. In this study, we used tests in *Drosophila* for sucrose responsiveness and for habituation of the proboscis extension response (PER), which were originally developed for honey bees.

In *Drosophila*, the PER can be elicited by stimulating sucrose receptors on the tarsus, the terminal segment of the fore leg (Fig. 1). Habituation of the PER occurs after repeated tarsal stimulation with a low sucrose concentration without feeding the fly (Le Bourg 1983; Fois et al. 1991; Minois and Le Bourg 1997). These paradigms are very similar to those frequently used in honey bees. The only major difference is that in bees, usually the comparatively large antennae are stimulated with sucrose solution to elicit proboscis extension. Numerous experiments with honey bees (*Apis mellifera*) demonstrate that individual antennal responsiveness to sucrose significantly correlates with a number of different behaviors. Bees with high antennal sucrose responsiveness show less habituation of PER elicited by antennal sucrose stimulation (Scheiner 2001). Bees with high sucrose responsiveness also show better performance in tactile and olfactory asso-

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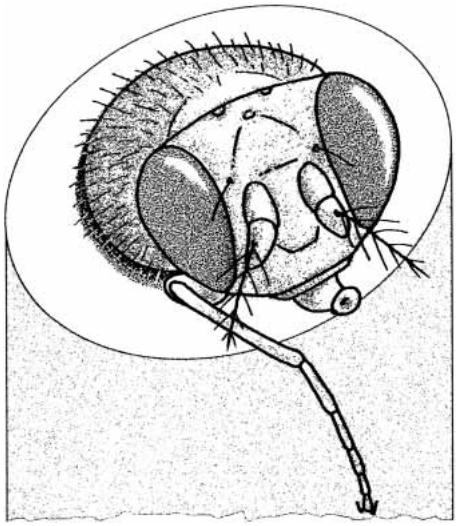

Figure 1 Adult *Drosophila* mounted for measuring sucrose responsiveness. Each fly was individually fixed in a pipette tip whose end was cut off. One leg protruded from the brink of the pipette tip. The tarsus of this leg was stimulated with a toothpick moistened with sucrose solution of a certain concentration. It was noted which sucrose concentrations elicited proboscis extension.

ciative learning paradigms (Scheiner et al. 1999, 2001a,b, 2003a) than bees with low responsiveness. The proboscis extension responses of bees to antennal stimulation with pollen, the choice preference for odors in an olfactometer, and the phototactic behavior of bees also correlate with their sucrose responsiveness (Scheiner et al. 2004), suggesting that animals with high sucrose responsiveness are also very responsive to other sensory stimulus modalities. In addition, sucrose responsiveness of bees correlates with division of labor for foraging. Whereas water collectors and pollen foragers display very high sucrose responsiveness, nectar foragers are less responsive to sucrose (Page et al. 1998; Pankiw and Page 1999; Scheiner et al. 1999, 2001b, 2003a; Pankiw and Page 2000). This shows that the sucrose responsiveness of a bee correlates with different forms of behavior in the animal. Understanding the molecular basis of variation in sucrose responsiveness may in part help us understand mechanisms underlying both the division of labor and learning in insects.

The cellular processes controlling sucrose responsiveness in bees are only partially known. The biogenic amines octopamine, tyramine, and dopamine can modulate sucrose responsiveness in the range of minutes (Scheiner et al. 2002). Activity of the cAMP-dependent protein kinase (PKA) in the antennal lobes correlates with sucrose responsiveness, and feeding the PKA activator 8-Br-cAMP (adenosine 3′5′-cyclic monophosphate 8-Bromo-sodium salt) increases sucrose responsiveness (Scheiner et al. 2003b). The effect of differences in PKG activity on sucrose responsiveness has never been studied. We hypothesise that the cGMP/PKG pathway is involved in the regulation of sucrose responsiveness in honey bees, because both PKG activity (Ben-Shahar et al. 2002) and sucrose responsiveness (Pankiw et al. 2002) correlate with the initiation of foraging behavior. cGMP has been shown to play a role in habituation of the proboscis extension response in honey bees, although this process does not involve changes in PKG activity (Müller and Hildebrandt 2002).

Our experiments investigate a role for PKG in *Drosophila melanogaster* behavior. The *Drosophila* model provides us with the means to investigate the genetic and molecular underpinnings of sucrose responsiveness and habituation of the proboscis extension response. Here, we identify a role for PKG in these behaviors.

RESULTS

Sucrose Responsiveness of for^s Sitters and for^R Rovers After 24 Hours of Food Deprivation

Sucrose responsiveness of 1-, 2-, and 3-wk-old for^s sitters and for^R rovers was tested in males and females after 24 h of food deprivation. Figure 2 shows an example of the sucrose-concentration response curves for 1-wk-old sitters and rovers. The concentration-response curves of the other age groups were very similar to those in Figure 2 and, therefore, are not shown. In both genotypes, responsiveness increased with increasing sucrose concentrations. Significant differences between the responses of for^s sitters and for^R rovers to specific sucrose concentrations are indicated for males (Fig. 2A) and females (Fig. 2B). Mean sucrose response scores (Fig. 3) are a compound measure of concentration-dependent sucrose responsiveness, which can be used to compare different experimental groups (for review, see Scheiner et al. 2004). Analysis of variance demonstrated that sucrose re-

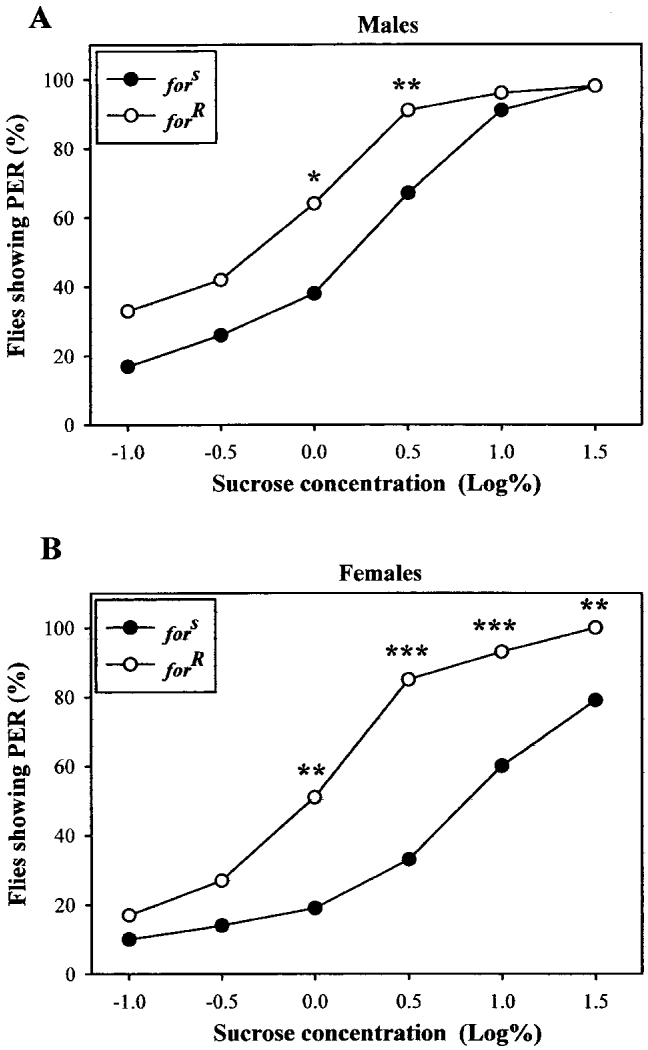

Figure 2 Sucrose-concentration response curves of 1-wk-old for^s sitters and for^R rovers that were food deprived for 24 h. The abscissae show the logarithm of the different sucrose concentrations tested. The ordinates show the percentage of flies responding with proboscis extension. (*A*) Male for^s sitters ($n = 42$) and for^R rovers ($n = 45$). (*B*) Female for^s sitters ($n = 42$) and for^R rovers ($n = 41$). (*) $P \leq 0.05$; (**) $P \leq 0.01$; (***) $P \leq 0.001$, two-tailed Fisher Exact Probability Test.

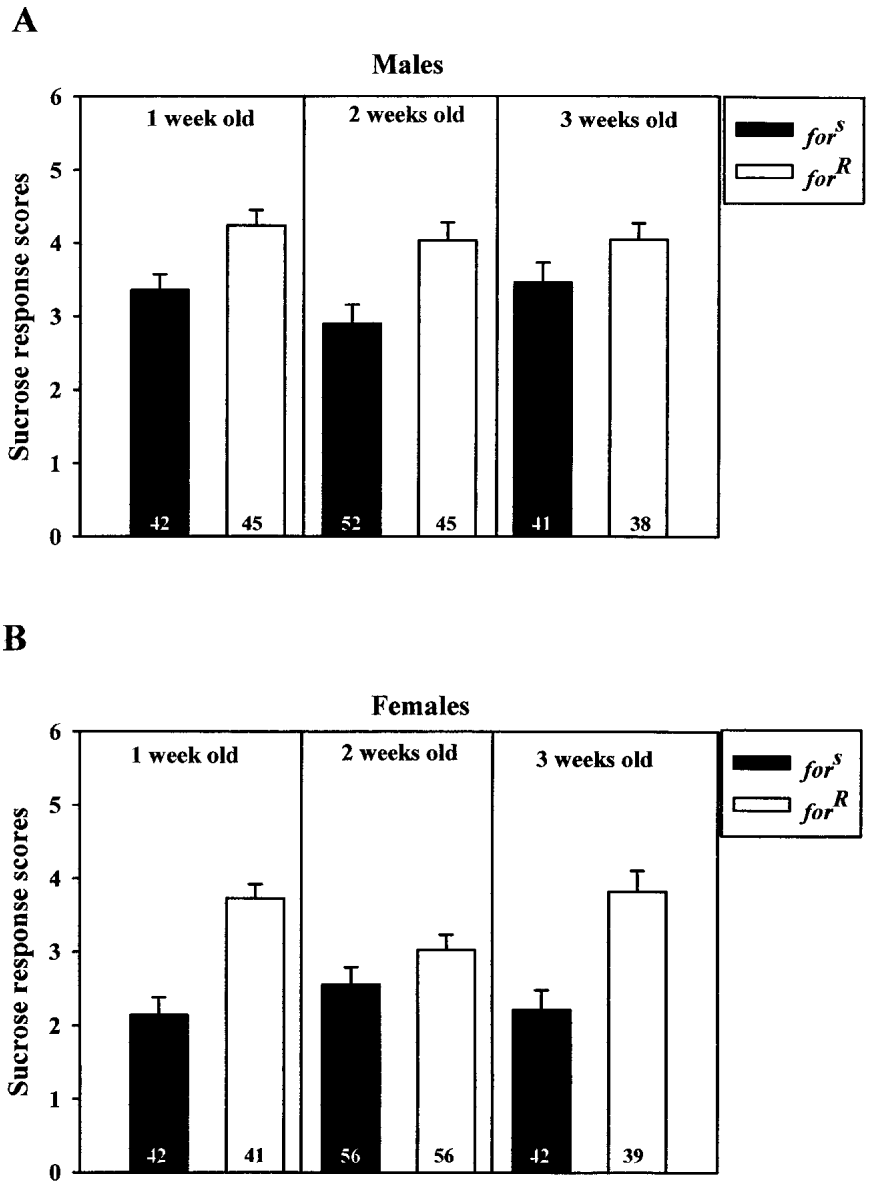

Figure 3 Mean sucrose response scores and SEM of 1-, 2-, and 3-wk-old *for*^{s} sitters and *for*^{R} rovers that were food deprived for 24 h. (*A*) Males; (*B*) females. Number of flies tested in each group is indicated.

sponse scores were strongly affected by genotype [$F_{(1,538)} = 55.43$, $P \leq 0.001$, ANOVA]. *for*^{R} rovers were significantly more responsive to sucrose than *for*^{s} sitters (Fig. 3; $P \leq 0.001$, Scheffé Test). Gender also affected sucrose response scores, but to a smaller extent [$F_{(1,538)} = 29.50$, $P \leq 0.001$, ANOVA]. Males generally displayed higher sucrose responsiveness than females (Fig. 3, $P \leq 0.001$, Scheffé Test). Age had no significant effect on sucrose responsiveness [$F_{(2,537)} = 1.59$, $P > 0.01$, ANOVA], and there were no interaction effects of genotype, gender, and age on sucrose responsiveness. Overall, rovers were more responsive to sucrose than sitters.

Sucrose Responsiveness of *for*^{s} Sitters, *for*^{s2} Sitters, and *for*^{R} Rovers After 2 Hours of Food Deprivation

We compared the sucrose responsiveness of sitters and rovers that had only been food deprived for 2 h, because the foraging behavior of sitters and rovers becomes more similar with long food deprivation (Sokolowski and Riedl 1999). We also measured the sucrose responsiveness of the sitter mutant *for*^{s2} (see Materials and Methods). Figure 4 shows the sucrose-concentration response curves of 1-wk-old *for*^{s} sitters, *for*^{s2} sitters, and *for*^{R} rovers food deprived for 2 h. The sucrose-concentration response curves of the other age groups look very similar to those shown in Figure 4 (data not shown). Male (Fig. 4A) and female (Fig. 4B) rovers responded significantly more often than sitters to most of the sucrose concentrations tested. Sucrose response scores were strongly affected by genotype [Fig. 5; $F_{(2,841)} = 144.14$, $P \leq 0.001$, ANOVA]. *for*^{R} rovers displayed a significantly higher sucrose responsiveness than *for*^{s} sitters ($P \leq 0.001$, Scheffé Test) or *for*^{s2} sitters ($P \leq 0.001$, Scheffé Test). The mutant *for*^{s2} sitters did not differ in their sucrose responsiveness from the natural *for*^{s} sitters ($P > 0.01$, Scheffé Test). Thus variation in *for* correlated with differences in sucrose responsiveness between rovers and sitters. Gender also affected sucrose response scores, but less than genotype [Fig. 5; $F_{(1,842)} = 16.36$, $P \leq 0.001$, ANOVA]. Males were more responsive to sucrose than females ($P \leq 0.001$, Scheffé Test). Age did not affect sucrose responsiveness [$F_{(2,841)} = 3.71$, $P > 0.01$, ANOVA], and there were no interaction effects of genotype, gender, and age on sucrose responsiveness.

Responsiveness to the different sucrose concentrations in sitters and rovers was strongly affected by food deprivation time [$F_{(1,1089)} = 86.71$, $P \leq 0.001$, ANOVA]. After 2 h of food deprivation, sucrose responsiveness was lower than after 24 h of food deprivation (cf. Fig. 4 with Fig. 2, $P \leq 0.001$, Scheffé Test). In addition, we found a significant interaction effect of food deprivation time by genotype [$F_{(1,1089)} = 157.93$, $P \leq 0.001$, ANOVA].

The distributions of sucrose response scores for the different experimental groups can be used to better illustrate the variation in sucrose responsiveness. Figure 6 shows examples of the distributions of sucrose response scores for 1-wk-old *for*^{s} sitters, *for*^{s2} sitters, and *for*^{R} rovers after 2 h of food deprivation. Most of the *for*^{s} and *for*^{s2} sitters show low or no responsiveness to sucrose. Consequently, the frequency of flies in SRS class 0, which indicates animals that do not respond to sucrose, is very high. Sitters with high sucrose response scores (SRS classes >3) are almost absent. In *for*^{R} rovers, most animals respond to sucrose, which is indicated by the low frequency of nonresponders (SRS class 0). Many *for*^{R} rovers show intermediate sucrose response scores (SRS classes 2, 3). Some *for*^{R} rovers show high sucrose responsiveness (SRS classes >3). These data show that the differences in sucrose responsiveness of sitters and rovers are mainly due to differences in the distribution of flies with high and low sucrose response scores.

A detailed statistical analysis of the differences between sucrose response score distributions for the different experimental

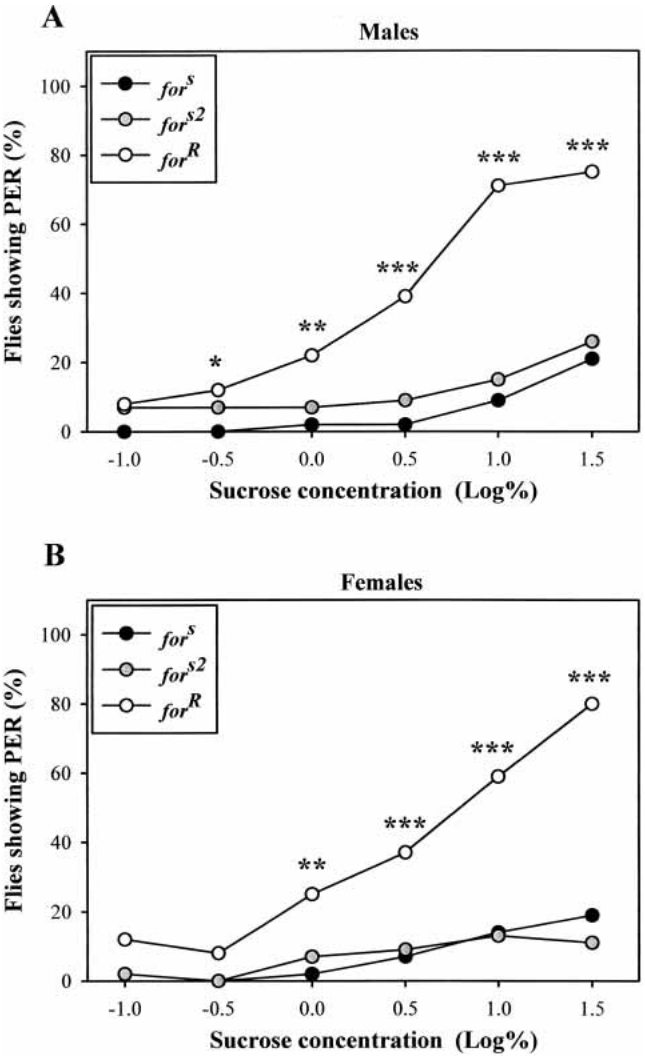

Figure 4 Sucrose-concentration response curves of 1-wk-old *for*^{s} sitters, *for*^{s2} sitters, and *for*^{R} rovers that were food deprived for 2 h. The abscissae show the logarithm of the different sucrose concentrations tested. The ordinates show the percentage of flies responding with proboscis extension. (*A*) Male *for*^{s} sitters ($n = 47$), *for*^{s2} sitters ($n = 46$), and *for*^{R} rovers ($n = 51$). (*B*) Female *for*^{s} sitters ($n = 43$), *for*^{s2} sitters ($n = 45$), and *for*^{R} rovers ($n = 49$). (*) $P \leq 0.05$; (**) $P \leq 0.01$; (***) $P \leq 0.001$, two-tailed Fisher Exact Probability Test.

groups after 2 and 24 h of food deprivation is shown in Table 1. For 2 h of food deprivation, the distributions of flies with high sucrose response scores and those with low sucrose response scores differed significantly between rovers and the two sitter strains in all age groups. After 24 h of food deprivation, when the differences between sitters and rovers were less pronounced, the distribution of sucrose response scores of sitters differed significantly from that of rovers in three of six groups. With longer food-deprivation time, sitters became more like rovers and showed increased sucrose responsiveness. However, rovers also displayed enhanced sucrose responsiveness after a long period of food deprivation.

Habituation in *for*^{s} Sitters, *for*^{s2} Sitters, and *for*^{R} Rovers

The intensity of the habituating stimulus determines the rate of habituation (Thompson and Spencer 1966). It was shown that honey bees do not show significant habituation of the proboscis extension response for sucrose concentrations of 50%, whereas habituation to a water stimulus is very pronounced (Braun and Bicker 1992). Honey bees with high sucrose responsiveness need more trials for habituation than bees with low sucrose responsiveness (Scheiner 2001). As *for*^{s} sitters and *for*^{s2} sitters differed from *for*^{R} rovers in their responsiveness to sucrose, differences in their habituation could be related to differences in their sucrose responsiveness. For the habituation experiment, we therefore selected flies of the three genotypes that did not differ in their sucrose responsiveness. If the course of habituation depended only on sucrose responsiveness, we would not expect to find differences in the habituation of the three genotypes. Possible differences in the habituation of sitters and rovers would indicate effects of the *for* gene independent of sucrose responsiveness.

Habituation experiments were performed with flies that did not respond to water, that responded to 10% sucrose, and that showed proboscis extension in the first habituation trial. Because sitters were much less responsive to sucrose than rovers, only 39 *for*^{s} sitters and 36 *for*^{s2} sitters compared with 108 *for*^{R} rovers fulfilled these criteria. The sucrose-concentration response curves of these flies (Fig. 7A) show that *for*^{s} sitters, *for*^{s2} sitters, and *for*^{R} rovers that were tested for habituation did not differ in their responses for different sucrose concentrations. The mean sucrose response scores of these three groups did not differ significantly [$F_{(2,184)} = 3.37$, $P > 0.01$, ANOVA). As all flies in this experiment had to respond to 10% sucrose, the responsiveness is 100% at this concentration (Fig. 7A).

Habituation scores were calculated to analyze the overall degree of habituation in the different genotypes (see Materials and Methods). Gender and age groups were pooled for these calculations, because these factors did not affect habituation scores [gender: $F_{(1,185)} = 3.28$, $P > 0.01$; age: $F_{(2,184)} = 0.97$, $P > 0.01$, ANOVA]. Genotype showed a strong effect on habituation scores [Fig. 7B, $F_{(2,184)} = 10.89$, $P \leq 0.001$, ANOVA]. *for*^{R} rovers had significantly higher habituation scores than *for*^{s} sitters ($P \leq 0.001$, Scheffé Test) or *for*^{s2} sitters ($P \leq 0.01$, Scheffé Test), which indicates less habituation in rovers than in sitters. The two sitter strains did not differ in their habituation scores ($P > 0.01$; Scheffé Test).

The course of habituation (Fig. 7C) was analyzed by using the pooled data of flies of different genders and age groups. The habituation curves of all three genetic variants could be fit to decaying exponential functions [*for*^{s}: $f(x) = 94.7 * e^{-0.05*x}$, $R^2 = 0.93$, $P \leq 0.001$; *for*^{s2}: $f(x) = 90.7 * e^{-0.04*x}$, $R^2 = 0.84$, $P \leq 0.001$; *for*^{R}: $f(x) = 98.2 * e^{-0.02*x}$, $R^2 = 0.93$, $P \leq 0.001$]. The exponential decay rate of *for*^{s} and *for*^{s2} sitters was significantly greater than that of *for*^{R} rovers (*for*^{s} vs. *for*^{R}: $T = 6.26$, $df = 27$, $P \leq 0.001$; *for*^{s2} vs. *for*^{R}: $T = 2.12$, $df = 38$, $P \leq 0.05$; two-tailed Welch's approximate T Test). The decay of *for*^{s} sitters was significantly greater than that of *for*^{s2} sitters ($T = 3.58$, $df = 27$, $P \leq 0.01$; two-tailed Welch's approximate T Test). This demonstrates that the naturally occurring *for*^{s} sitters displayed the most rapid habituation, followed by the mutant *for*^{s2} sitters and, finally, *for*^{R} rovers, which habituated more slowly.

Both naturally occurring sitters (*for*^{s}) and mutant sitters (*for*^{s2}) showed significantly more generalization of habituation than *for*^{R} rovers and also responded less frequently to the high sucrose concentration applied in the generalization test (Fig. 7C; $P \leq 0.05$, two-tailed Fisher Exact Probability Test).

DISCUSSION

Sucrose Responsiveness and Foraging Strategies

Our data identify a new function of the *for* gene in sucrose responsiveness of *Drosophila*. In tests involving different genders, different age groups, and different food-deprivation intervals,

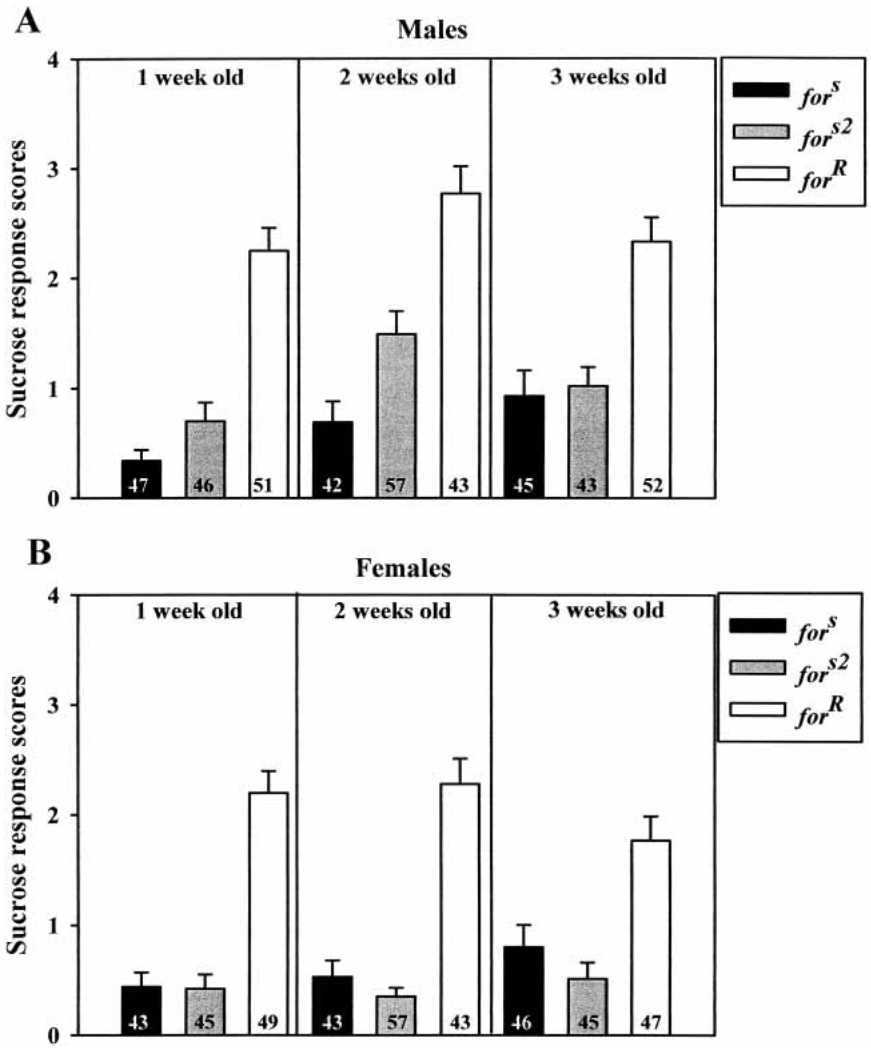

Figure 5 Mean sucrose response scores and SEM of 1-, 2-, and 3-wk-old *for*^{s} sitters, *for*^{s2} sitters, and *for*^{R} rovers that were food deprived for 2 h. (*A*) Males; (*B*) females. Number of flies tested in each group is indicated.

naturally occurring sitters (*for*^{s}) and mutant sitters (*for*^{s2}) were significantly less responsive to sucrose than rovers. These data suggest that variation in *for*-PKG affects sucrose responsiveness. The differences in the sucrose responsiveness of adult sitters and rovers correlate with differences in their foraging strategies. Adult rovers move further away from a droplet of sucrose after feeding than adult sitters. In addition, adult rovers travel in a straight line away from the droplet of sucrose solution on which they had fed, whereas sitters stay close to the sucrose resource (Pereira and Sokolowski 1993). Our measures of sucrose responsiveness directly support the hypothesis that sitters might differ from rovers in their evaluation of the sucrose droplet and may therefore use a different foraging strategy. Taken together, these findings suggest a correlation between PKG activity, foraging behavior, and sensory responsiveness in *Drosophila*.

A similar correlation between sucrose responsiveness and foraging behavior has been observed in the honey bee. Individuals with high sucrose responsiveness early in life will collect more dilute nectar later in life, whereas those with lower sucrose responsiveness early in life will accept high-concentrated nectar later in life (Pankiw and Page 2000). Quantitative trait locus mapping has shown that the quality of the nectar collected and the sucrose responsiveness map in part to the same locus (Page and Erber 2002). Whether the behavioral correlation between sucrose responsiveness and foraging behavior in honey bees is linked to PKG activity has to be tested. If we extend our results from *Drosophila* to the honey bee, we expect that bees with high sucrose responsiveness should have higher PKG activities than bees with low sucrose responsiveness.

The foraging behavior of sitter and rover *Drosophila* and of honey bee foragers appears to be independent of their general locomotor behavior. Sitters do not differ in walking behavior from rovers in the absence of food (Pereira and Sokolowski 1993; Sokolowski et al. 1997). On a non-nutritive agar plate, both adult sitters and rovers display long locomotion paths. Similarly, honey bee foragers with high sucrose responsiveness do not differ in their locomotor activity from foragers with low sucrose responsiveness (Scheiner et al. 2004).

A correlation between food-searching behavior, PKG activity, and sensory responses has also been demonstrated in the nematode *Caenorhabditis elegans* (Fujiwara et al. 2002). Variation in *C. elegans* PKG causes them to behave as dwellers or roamers while feeding. Dwellers restrict their locomotor behavior on food to a confined region (like sitters), whereas roamers travel widely across the plate (like rovers). *che-2* mutants of *C. elegans* have a morphological defect in their sensory cilia and increased PKG activity. These mutants show both a defect in sensory perception (possibly related to the defects in cilium structure) and reduced roaming (possibly related to increased PKG activity). Worms of a *che-2* suppressor strain retain the defect in sensory cilium structure, but show a decrease in PKG compared with the *che-2* mutants. Interestingly, worms of this *che-2* suppressor strain show increased roaming and display normal sensory perception despite their defects in cilium structure (Fujiwara et al. 2002). These data suggest that in *C. elegans*, PKG activity is also involved in sensory perception and food-searching behavior. However, in contrast to bees and fruit flies, high PKG activity appears to correlate with dwelling behavior and low sensory sensitivity in *C. elegans*.

In the blowfly, *Phormia regina*, food-searching behavior also correlates with chemosensory responsiveness. McGuire and Tully (1986) found a correlation between what they called dancing behavior and the central excitatory state (CES). The CES describes the sensitization of flies to tarsal stimulation with water after they had briefly been stimulated with a high sucrose concentration. It can be assumed that flies that have high CES scores (and thus show strong sensitization) place a higher value on the sensitizing sucrose solution than flies with low CES scores. A direct correlation between sucrose responsiveness and sensitization has been shown for the honey bee (Scheiner 2001). McGuire and Tully (1986) show that flies selected for high CES scores move further away from a drop of sucrose after feeding (dancing) than flies selected for low CES scores. This behavior is comparable to the foraging behavior of sitters and rovers. In both species, flies

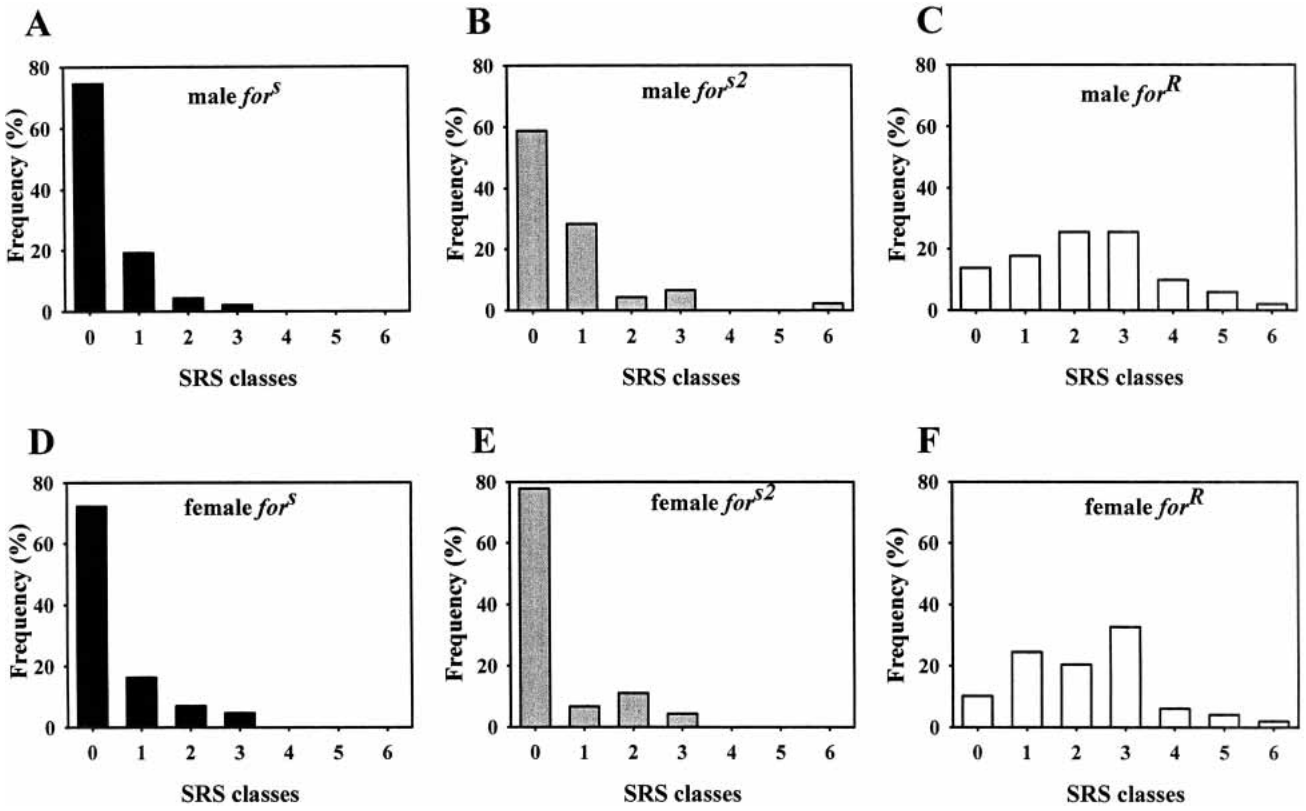

Figure 6 Frequency distributions of sucrose response scores in male (*A,B,C*) and female (*D,E,F*) *for*^{s} sitters, *for*^{s2} sitters, and *for*^{R} rovers. All flies were 1-wk old and had been food deprived for 2 h. The abscissae show the different classes of sucrose response scores (SRS). The ordinates give the percentage of flies belonging to a certain SRS class. The total number of flies tested in each group is shown in Figure 5.

with high sucrose sensitivity walk further away from a drop of sucrose than flies with low sucrose responsiveness.

Thus, data from *Drosophila*, *Apis*, *C. elegans*, and *Phormia* all show a correlation between gustatory responses and food-searching behavior. This relationship appears to involve different levels of PKG activity in *Drosophila* and *C. elegans*. It is of great interest to study the function of the PKG pathway in gustatory responses and food-search behavior in *Apis* and *Phormia*. These studies might reveal common conserved underlying mechanisms tying sensory responsiveness to foraging behavior in the evolution of food-search behavior.

Table 1. Kolmogorov-Smirnov Test of Distributions of Sucrose Response Scores Between *for*^{s} Sitters and *for*^{R} Rovers After 2 or 24 h of Food Deprivation and Between *for*^{s2} Sitters and *for*^{R} Rovers After 2 h of Food Deprivation

	24 h food deprivation	2 h food deprivation	
	for^{s} vs. *for*^{R}	*for*^{s} vs. *for*^{R}	*for*^{s2} vs. *for*^{R}
1-week-old males	$P \leq 0.05$	$P \leq 0.001$	$P \leq 0.001$
1-week-old females	$P \leq 0.001$	$P \leq 0.001$	$P \leq 0.001$
2-week-old males	$P > 0.05$	$P \leq 0.001$	$P \leq 0.01$
2-week-old females	$P > 0.05$	$P \leq 0.001$	$P \leq 0.001$
3-week-old males	$P > 0.05$	$P \leq 0.001$	$P \leq 0.01$
3-week-old females	$P \leq 0.01$	$P \leq 0.001$	$P \leq 0.001$

The numbers of flies tested are the same as those shown in Figure 3 (for 24 h of food deprivation) and Figure 5 (for 2 h of food deprivation). The significance level *P* is given.

Effects of Gender and Age on Sucrose Responsiveness

We found that male flies were more responsive to sucrose than females, as has been reported in previous *Drosophila* studies (Fujishiro and Kijima 1988). One reason for this behavioral difference between the sexes may lie in the fact that the tarsi of male front legs contain nearly 30% more taste bristles than those of females (Nayak and Singh 1983). Whether the number of taste bristles or their sensitivity contributes to sucrose responsiveness in males and females remains to be determined.

Our study showed that age had no effect on sucrose responsiveness. We showed that 7-, 14-, and 21-day-old flies do not differ in their sucrose responsiveness. This is in agreement with Brigui et al. (1990) and Le Bourg (1996), who, in addition, found that 30-day-old flies were more responsive to sugar than 50-day-old flies. Together, these studies suggest that sucrose responsiveness is stable during the first ~30 days of life, but then decreases as flies age. It would be interesting to study whether the decrease in sucrose responsiveness in very old flies correlates with a decrease in PKG levels or enzyme activities.

Habituation in Sitters and Rovers

When stimulated with a 10% sucrose solution delivered at a 3-sec interstimulus interval, naturally occurring sitters (*for*^{s}) and mutant sitters (*for*^{s2}) displayed a higher degree of habituation and showed stronger generalization of habituation than rovers (*for*^{R}). This phenomenon should not have been related to a lower responsiveness to the habituating sucrose stimulus in sitters, because the sucrose responsiveness of the sitters and rovers tested for habituation did not differ. Therefore, our results suggest a

direct link between PKG activity and nonassociative learning in *Drosophila*. Low PKG activity correlates with strong habituation, independent of sucrose responsiveness. In honey bees, individuals with low sucrose responsiveness display stronger habituation than bees with high sucrose responsiveness (Scheiner 2001). If PKG plays a similar role in the habituation of honey bees, we expect that bees that need many trials for habituation should have a higher PKG activity than bees that need few trials for habituation. Feeding of the PKG activator 8-Br-cGMP should decrease the rate of habituation in honey bees. Unfortunately, genetic rescue experiments to manipulate enzyme levels are not feasible in bees.

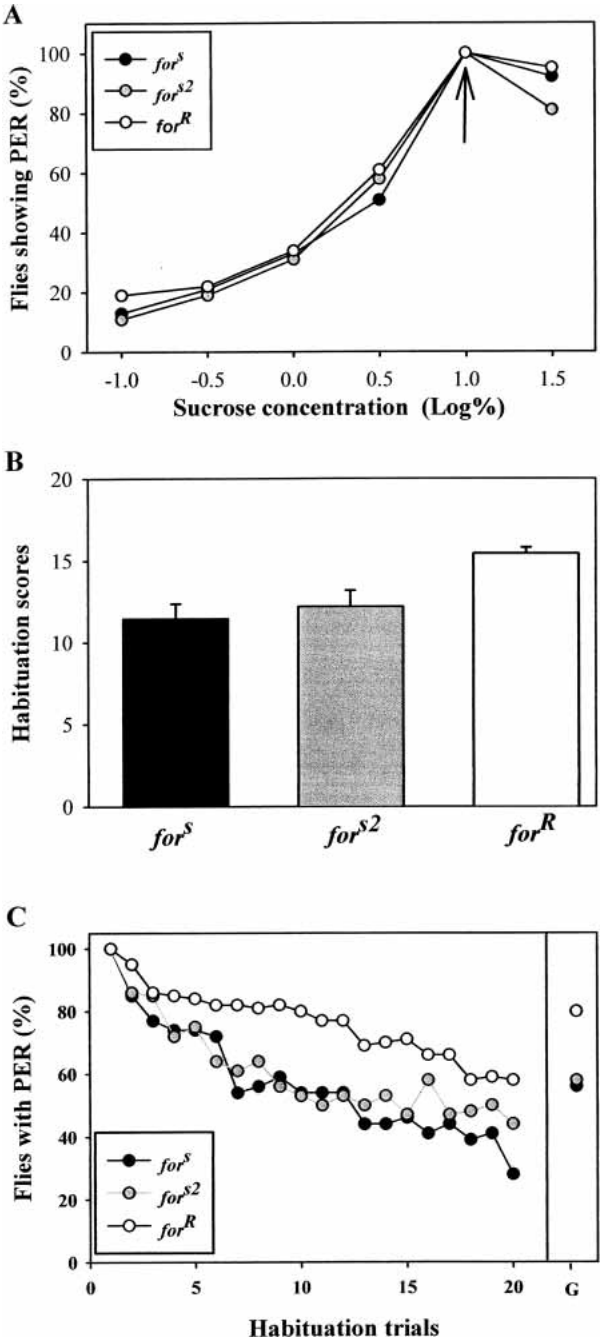

Figure 7 Sucrose responsiveness and habituation in adult for^S sitters, for^{S2} sitters, and for^R rovers. (*A*) Sucrose-concentration response curves of those for^S sitters, for^{S2} sitters, and for^R rovers that fulfilled the three criteria for habituation; no response to water stimulation, PER to 10% sucrose, and PER in the first habituation trial. The arrow indicates responsiveness at 10% sucrose. This concentration was used as habituating stimulus. (*B*) Mean habituation scores (the total number of responses of 20 stimulations) and SEM of natural for^S sitters ($n = 39$), mutant for^{S2} sitters ($n = 36$), and for^R rovers ($n = 108$). (*C*) Course of habituation and test for generalization of habituation. The abscissa shows the different habituation trials and the generalization test (G). The ordinate gives the percentage of flies showing proboscis extension.

Interestingly, habituation of the giant fibre escape circuit is also affected by the *for* gene in *Drosophila* (Engel et al. 2000). The giant fibre pathway mediates a jump-and-flight response normally induced by a visual startle reflex. This response can be elicited by electrical brain stimulation and shows typical characteristics of habituation after repeated electrical stimulation (Engel and Wu 1996, 1998). In this paradigm, which uses a completely different stimulus modality, sitters once again showed faster habituation than rovers (Engel et al. 2000). This demonstrates that *for*-PKG also affects habituation at the neurophysiological level. Interestingly, two independent measures of habituation involving different stimulus modalities show the same patterns. *for*-PKG determines the degree of habituation both at the behavioral level and at the neurophysiological level. It should now be tested whether an increase in the PKG activity of *Drosophila*, for example by using transgenic flies or by feeding the PKG activator 8-Br-cGMP, induces a decrease in habituation.

Working Mechanisms of PKG

How the cGMP/PKG-signaling pathway acts to set the levels for sucrose responsiveness and habituation in sitters and rovers is unclear. PKG is expressed in various neuronal tissues (Wang and Robinson 1997; Sokolowski 1998), and we are still far from understanding the different roles of the cGMP/PKG-signaling pathway. Most probably, the cGMP/PKG pathway acts at various levels of neuronal signal processing by affecting protein phosphatases, intracellular calcium levels, ion channels, and receptors for neurotransmitters (Wang and Robinson 1997). PKG could affect the sensitivity of tarsal gustatory receptors. It has been shown that an intracellular increase in cGMP can increase the excitability of neurons (Gammie and Truman 1997). Renger et al. (1999) demonstrated an effect of *for*-PKG on neuronal excitability in sitters and rovers in the giant neuron culture system and in recordings from the larval neuromuscular junction. But PKG could also affect connectivity to the proboscis muscles. Immunolabeling of the neuromuscular junctions showed that sitter larval muscles have different motor axon terminal projections than rover larval muscles (Renger et al. 1999). The role of cGMP in smooth muscle contraction has been well characterized in mammals (for review, see Lincoln et al. 1996). PKG may also affect the processing of sucrose stimuli in the thoraco-abdominal ganglion or the suboesophageal ganglion. In *Drosophila*, the axons of gustatory sensilla of the legs terminate in the thoraco-abdominal ganglion (Stocker 1994; Singh 1997). This is the first stage of processing gustatory stimuli from the tarsi. The second stage of processing is the suboesophageal ganglion, which receives direct and indirect input from gustatory sensilla from the tarsi and other parts of the body (Mitchell et al. 1999). In the ventral region of the suboesophageal ganglion, the motoneurons innervating proboscis muscles have their dendritic arborizations and partially overlap with sensory gustatory projections (Singh 1997). PKG could affect both of these centers of gustatory processing. Targeted expression of *for* to different neuronal tissues will help elucidate the role of PKG in sucrose responsiveness and habituation in these tissues.

MATERIALS AND METHODS

Strains

We used the *for*^{R} rover strain, the *for*^{s} sitter strain, and the *for*^{s2} mutant sitter strain of *Drosophila melanogaster* from Marla B. Sokolowski's laboratory at the University of Toronto. Sitters and rovers are naturally occurring variants of the *for* gene. The *for*^{s2} strain is a sitter behavioral mutant produced on a rover genetic background and has a significantly lower PKG activity than rovers (Osborne et al. 1997). Experiments were conducted at the Technical University of Berlin. Flies were cultured in 60-mL plastic culture vials on 15 mL of standard *Drosophila* food at 25° C in a 12-h light/12-h darkness photocycle. Newly emerged flies were collected daily and stored in separate vials. From then on, flies were placed in new vials every week, before new flies could hatch from pupae. Flies were tested at 1-, 2-, and 3-wk old. Males and females were tested and evaluated separately.

Food Deprivation Time

The foraging behavior of sitters and rovers is strongly affected by the length of the food deprivation time. In adult flies, the differences between sitters and rovers in the length of the foraging paths are smaller after longer food-deprivation periods than after shorter ones (Sokolowski and Riedl 1999). For that reason, we decided to study sucrose responsiveness in sitters and rovers after two different food-deprivation periods, 24 and 2 h. We expected that possible differences in sucrose responsiveness should be greater after 2 h of food deprivation than after 24 h of starvation. The flies spent their food deprivation time in vials containing ~5 mL of 1% agar to prevent drying out.

Measuring Sucrose Responsiveness

After the starvation period of either 24 ± 0.5 h or 2 ± 0.5 h, each fly was caught individually and placed in a pipette tip (0.5–20 µL, Th. Geyer, Berlin) whose end was cut off. One leg of the fly protruded out of the pipette tip (Fig. 1). The mounting and testing of individual flies requires great skill and long practice. Therefore, the same person had to mount and test the flies. After mounting, it was impossible to arrange the flies in a different order, because the flies could easily escape from their holding pipette tips during handling. However, the persons doing the experiments were ignorant of the behavioral background of the three genotypes and blind to any prior hypotheses.

The tarsus was touched with a toothpick moistened with water or one of the following sucrose concentrations: 0.1%, 0.3%, 1%, 3%, 10%, 30% (w/v). These concentrations correspond to the logarithmic series that was used for similar tests in honey bees (Scheiner 2001; Scheiner et al. 2001c, 2002, 2003a). For each experiment, one of seven different sequences of applying water and the different sucrose concentrations was selected. This pseudo-randomized order was chosen to minimize experimental bias by the sequence of applied stimuli. Each stimulus (water or sucrose concentration) was presented once to each individual. For each individual, we recorded whether a specific stimulus concentration elicited proboscis extension. Only flies that did not respond to stimulation with water were analyzed to prevent experimental bias by thirst. The total number of responses to the six different sucrose concentrations represents the sucrose response score (SRS) of a fly and is a measure of its sucrose responsiveness (Scheiner et al. 1999, 2001a,b, 2002, 2003a). With these experimental conditions, an individual fly's SRS could range between 0 and 6. Flies not responding to any of the sucrose stimuli received an SRS of 0. Individuals responding to all sucrose concentrations had an SRS of 6.

Habituation of Proboscis Extension

For habituation of the proboscis extension response using a 10% sucrose solution, only flies that fulfilled the following criteria were tested: (1) flies did not respond to water, (2) flies responded to 10% sucrose when sucrose responsiveness was determined, and (3) flies responded in the first habituation trial with a proboscis extension.

Only some of the flies shown in Figures 4–6 fulfilled these criteria for habituation. Particularly *for*^{s} and *for*^{s2} flies fulfilling the criteria for habituation were very rare. Therefore, additional flies, particularly of the two sitter strains, had to be tested for their sucrose responsiveness in order to gain sufficient numbers of individuals for the habituation test. The numbers of flies tested for habituation in each of the three genotypes were the following: *for*^{R}: males: 1-wk-old: 20, 2-wk-old: 17, 3-wk-old: 26; *for*^{R}: females: 1-wk-old: 19, 2-wk-old: 14, 3-wk-old: 12; *for*^{s}: males: 1-wk-old: 1, 2-wk-old: 7, 3-wk-old: 14; *for*^{s}: females: 1-wk-old: 3, 2-wk-old: 2, 3-wk-old: 12; *for*^{s2}: males: 1-wk-old: 3, 2-wk-old: 8, 3-wk-old: 15; *for*^{s2}: females: 1-wk-old: 5, 2-wk-old: 1, 3-wk-old: 4.

The first habituation trial started 2 min after testing the responsiveness to different sucrose concentrations at the earliest. With an inter-trial interval of 3 sec, each fly was stimulated at its protruding front tarsus with 10% sucrose 20 times. Generalization of habituation was tested 3 sec after the last habituation trial by applying a 30% sucrose stimulus. In each habituation trial and during the generalization test, it was recorded whether an animal responded with PER. The proboscis was never stimulated. Flies that accidentally licked sucrose were discarded. As in previous experiments with bees, habituation was quantified by calculating the habituation score for each animal (Scheiner 2001). The habituation score of a fly constitutes the total number of responses to the 20 sucrose stimulations.

Statistics

For the graphic display of sucrose-concentration response curves (Figs. 2, 4, and 7A), the percentage of flies showing proboscis extension at stimulation with the different sucrose concentrations was calculated. The number of responding flies at each sucrose concentration was compared between naturally occurring sitters (*for*^{s}), rovers (*for*^{R}), and *for*^{s2} mutant sitters using two-tailed Fisher Exact Probability Tests (GraphPad Instat 2.05a). Sucrose response scores (SRS) are a reliable measure of sucrose responsiveness (for review, see Scheiner et al. 2004). The effect of genotype, gender, and age on SRS was tested using analysis of variance (ANOVA, SPSS 10.0). The SRS of a few groups did not follow normal distribution. Nevertheless, we used ANOVA, because it is very robust against departures from normality (Lunney 1970; Zar 1999; Bortz et al. 2000). We used a significance threshold of $P \leq 1\%$ for analysis of variance in all post hoc tests. Pairwise comparisons were made using Scheffé Tests. The distributions of SRS were compared between different groups using two-tailed Kolmogorov-Smirnov Tests (SPSS 10.0).

The habituation scores of the three different genotypes (Fig. 7B) did not differ from normal distribution. To test for effects of genotype on habituation, analysis of variance (ANOVA) was performed on the habituation scores (SPSS 10.0). The habituation scores were compared between natural sitters (*for*^{s}), mutant sitters (*for*^{s2}), and rovers (*for*^{R}) using two-tailed Scheffé Tests (SPSS 10.0).

For the graphic display of the habituation curves, the percentage of flies showing proboscis extension in the different habituation trials was calculated (Fig. 7C). In addition, the percentage of flies showing proboscis extension in the generalization test is shown. The number of flies showing proboscis extension in the generalization test was compared between naturally occurring sitters (*for*^{s}), mutant sitters (*for*^{s2}), and rovers (*for*^{R}) using two-tailed Fisher Exact Probability Tests (GraphPad Instat 2.05a). The habituation curves of all three genetic variants could be fit by decaying exponential functions with two parameters, a and b, $f(x) = a^*e^{-b^*x}$ (Sigma Plot 2001). The parameter b, which is a measure for the decay rate of the e-function, was compared between groups with two-tailed Welch's approximate T Tests (GraphPad Instat 2.05a).

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