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Persistent one-way walking in a circular arena in *Drosophila melanogaster* Canton-S strain

Running title: *Drosophila* one-way walking

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Abstract

We describe persistent one-way walking of *Drosophila melanogaster* in a circular arena. Wild-type Canton-S adult flies walked in one direction, counter-clockwise or clockwise, for minutes, whereas white-eyed mutant w^{1118} changed directions frequently. Computational analysis of locomotor behavior showed that counter-clockwise walking and clockwise walking were the two main components of locomotion in circular arenas. Genetic analysis revealed that while wild-type genetic background suppressed the number of counter-clockwise and clockwise walks during five minutes of locomotion, the *white* (w^+) gene promoted persistent one-way walking by increasing the maximal duration of one-way walking. These results support a pleiotropic function of w^+ in promoting persistent one-way walking in addition to eye pigmentation.

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Introduction

Walking locomotion in *Drosophila melanogaster* (fruitfly) displays many distinctive features. Negative geotaxis and positive phototaxis are the classical stereotypes of locomotion in adult flies (Carpenter 1905; McEwen 1918; Cole 1922). Directional persistence and local wall attraction are the two main features of walking in open field arenas (Soibam *et al*. 2012). Wing-clipped flies walk back and forth towards inaccessible visual targets in Buridan's paradigm (Götz 1980; Colomb *et al*. 2012; Colomb & Brembs 2014). In addition, adult flies are unwilling to walk through confined spaces, a phenomenon termed claustrophobia (Ewing 1963). While restricted in small arenas, flies perform relentless walking for hours (Cole 1995; Xiao & Robertson 2015).

A common interest is to understand the genetic basis for walking behavior in *Drosophila*. Two widely used strains, wild-type Canton-S and white-eyed mutant w^{1118}, have different walking performance observed in several experimental settings. Canton-S flies walk towards light more often than w^{1118} flies (Kain *et al*. 2012). In circular arenas, Canton-S flies have higher boundary preference than w^{1118} flies (Liu *et al*. 2007; Xiao & Robertson 2015; Qiu, Xiao & Robertson 2017). Canton-S recover walking after anoxia faster and more consistently than w^{1118} flies (Xiao & Robertson 2016). When rigidly restrained, Canton-S flies show spontaneous and rhythmic motor activities that can be recorded extracellularly from the brain, whereas w^{1118} flies have greatly reduced rhythmic motor activities (Qiu *et al*. 2016). These findings raise a concern: either the *white* (w^+) gene, which is null-mutated in w^{1118} flies, or its genetic background, is responsible for the walking performance of Canton-S different from w^{1118} flies.

w^+ is a classic eye-color gene discovered by Thomas Hunt Morgan in 1910 (Morgan 1910). The product of w^+ is a subunit of transmembrane ATP-binding cassette (ABC) transporter, which loads vesicles/granules with biogenic amines (Borycz *et al*. 2008), second messenger (Evans *et al*. 2008), metal ion (Tejeda-Guzmán *et al*. 2017), metabolic intermediates (Sullivan & Sullivan 1975; Anaka *et al*. 2008) and pigment precursors (Sullivan & Sullivan 1975; O'Hare *et al*. 1984; Dreesen *et al*. 1988; Tearle *et al*. 1989). Increasing evidence has supported the proposal that w^+ possesses pleiotropic housekeeping functions in addition to eye pigmentation (Zhang & Odenwald 1995; Hing & Carlson 1996; Campbell & Nash 2001; Evans *et al*. 2008; Borycz *et al*. 2008; Anaka *et al*. 2008; Xiao & Robertson 2016; Hersh 2016; Xiao & Robertson 2017; Xiao *et al*. 2017). We hypothesized that w^+ modulates locomotor behavior and promotes persistent walking performance.

In this study, we describe persistent one-way walking of Canton-S flies in circular arenas. Preliminary observations show that Canton-S flies walk in one direction, counter-clockwise or clockwise, for minutes in circular arenas, whereas w^{1118} flies change directions frequently. We extracted the behavioral features of walking in the arena, and show that counter-clockwise walking and clockwise walking are the two main locomotor components, and that sporadic pausing is associated with directional persistence but not directional change. We further show that while wild-type genetic background primarily suppresses the number of counter-clockwise and clockwise walks, w^+ promotes persistent one-way walking by increasing the maximal duration of counter-clockwise or clockwise walking.

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Results

Persistent one-way walking of wild-type flies in circular arenas

A male, wild-type Canton-S fly walked in one direction, counter-clockwise or clockwise, for minutes in a circular arena (1.27 cm diameter 0.3 cm depth). This persistent one-way walking was consistent between individuals (video S1). Male flies of a white-eyed mutant w^{1118}, however, changed directions frequently and failed to maintain the walking direction for at least a minute (video S2). 3D walking trajectories of Canton-S flies, represented as a time-series of connected X-Y positions per 0.2 s, showed a regular, coil-like shape during 60 s locomotion (Figure 1a). The trajectories of w^{1118} flies displayed a shape of collapsed coil with visually increased irregularity (Figure 1b). The 2D path of Canton-S showed a strong preference for the perimeter of arena, whereas the 2D path of w^{1118} flies displayed reduced preference for perimeter and frequent crossing of the central region for the 60 s period. The persistent one-way walking was also observed in Canton-S females compared with w^{1118} females (Figure S1). Several different wild-types, including Oregon-R, Hikone-AS and Florida-9, showed a similar performance of persistent one-way walking in circular arenas. Several white-eyed mutants (w^{1}, w^{a} and w^{cf}) displayed irregular trajectories similar to w^{1118} flies (Figure S2). Therefore, wild-type flies showed persistent one-way walking in the circular arenas.

Counter-clockwise walking and clockwise walking were the main components of locomotion in a circular arena

Using a fly-tracking protocol (Xiao & Robertson 2015) and software R (R Core Team 2014), we computed the parameters of locomotor performance and extracted the components of walking behavior.

Four walking components could be identified in a fly. They were counter-clockwise walking, clockwise walking, pausing and unclassified activities. Canton-S showed a walking pattern that was clearly recognizable. Several flies walked in one direction, interspaced with a few pauses, throughout 300 s. In contrast, w^{1118} fly displayed a complicated pattern with frequent switch between walking structures (Figure 2a).

Counter-clockwise walking and clockwise walking were the two main locomotor components, comprising the most time proportion in Canton-S (median 0.92, interquartile range (IQR) 0.87 - 0.96, n = 31) (Friedman test with Dunn's multiple comparison) as well as w^{1118} flies (median 0.82, IQR 0.78 - 0.86, n = 48) (Friedman test with Dunn's multiple comparison) compared respectively with other locomotor components. Pausing comprised a relative small proportion of time in Canton-S (median 0.06, IQR 0.03 - 0.12, n = 31) and w^{1118} flies (median 0.07, IQR 0.5 - 0.09, n = 48). Time proportions for unclassified activities in Canton-S (median 0.009, IQR 0.005 - 0.013, n = 31) and w^{1118} flies (median 0.10, IQR 0.08 - 0.11, n = 48) were also small (Figure 2b). The unclassified activities mainly fast transitions between components which failed to meet a criteria for "classified" activities: a minimum of five consecutive steps in the same category.

During 300 s locomotion, the time for counter-clockwise walking (median 187.6 s, IQR 65.4 - 244.8 s, n = 31) and the time for clockwise walking (median 60.6 s, IQR 9.0 - 207.4 s, n = 31) were statistically the same in Canton-S flies (P = 0.1470, Wilcoxon matched pairs test). The time

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for counter-clockwise walking (median 123.1 s, IQR 105.4 - 134.2 s, n = 48) and the time for clockwise walking (median 129.2 s, IQR 111.7 - 138.9 s, n = 48) were also the same in w^{1118} flies ($P = 0.4327$, Wilcoxon matched pairs test) (Figure 2c). There was no preference for counter-clockwise or clockwise direction in circular arenas in either strain.

Pausing was associated with directional persistence

It was observed that flies paused sporadically in the arenas (see Figure 2a). It is possible that a fly pauses and changes walking direction. We examined whether the pausing was associated with directional change or directional persistence of flies in the arenas.

Canton-S flies paused several times during a period of 100 s. There was no substantial difference of walking direction before and after a pause. Similarly, w^{1118} flies showed no apparent change of walking direction before and after a pause (Figure 3a). Within 300 s, the number of pauses with directional persistence (median 4, IQR 3 - 6, n = 31) was higher than that with directional change (median 0, IQR 0 - 1, n = 31) in Canton-S ($P < 0.0001$, Wilcoxon matched pairs test) (Figure 3b). The number of pauses with directional persistence (median 8, IQR 5 - 10, n = 48) was also higher than that with directional change (median 1, IQR 0 - 1, n = 48) in w^{1118} flies ($P < 0.0001$, Wilcoxon matched pairs test) (Figure 3c). Thus, pausing was associated with directional persistence of walking in the arena.

Wild-type genetic background suppressed the number of counter-clockwise and clockwise walks

Canton-S and w^{1118} flies have different alleles of *w* and genetic background. We examined the contributions of w^{+} and its genetic background to persistent one-way walking.

We first compared the numbers of counter-clockwise and clockwise walks in w^{+} F1 and w^{1118} F1 male flies. w^{+} F1 was the progeny of w^{1118} males and Canton-S females, and reciprocally, w^{1118} F1 the progeny of Canton-S males and w^{1118} females. These two types of males had different X chromosomes (including *w* allele) and the same combinations on the second, third and fourth chromosome pairs. Both w^{+} F1 and w^{1118} F1 flies changed directions frequently in circular arenas during 300 s locomotion (Figure 4a). The number of counter-clockwise and clockwise walks in w^{+} F1 (median 34.5, IQR 29.3 - 46.0, n = 24) was comparable with that in w^{1118} F1 (median 43.0, IQR 31.5 - 47.5, n = 32) ($P = 0.1234$, Mann-Whitney test) (Figure 4b). Likely, wild-type genetic background but not w^{+} was responsible for the number of counter-clockwise and clockwise walks in the arena.

Female F1 flies were also examined. The number of counter-clockwise and clockwise walks in w^{+}/w^{1118} flies (progeny of Canton-S males and w^{1118} females, median 31.0, IQR 28.0 - 38.8, n = 24) was statistically the same as that in w^{1118}/w^{+} (progeny of w^{1118} males and Canton-S females, median 34.5, IQR 28.0 - 40.5, n = 16) ($P = 0.5067$, Mann-Whitney test) (Figure 4b). Thus, female F1 also changed directions frequently in circular arenas. These two types of females carried the same genomic contents (heterozygous *w* alleles and 1:1 mixed genetic background). Data supported that wild-type genetic background was associated with the number of counter-clockwise and clockwise walks. Additionally, these data suggested that the effect of cytoplasmic background (or maternal effect) is negligible.

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We further examined the numbers of counter-clockwise and clockwise walks in Canton-S, w^{1118}F10, w^{+}F10 and w^{1118} male flies. w^{1118}F10 and w^{+}F10 were generated by a serial back-crossing between Canton-S and w^{1118} for ten generations (Xiao & Robertson 2016). w^{1118}F10 flies, which carried w^{1118} allele in wild-type genetic background, had the number of counter-clockwise and clockwise walks (median 8.0, IQR 4.0 - 10.0, n = 43) slightly higher than Canton-S (median 5.0, IQR 2.0 - 8.0, n = 31) with non-significant difference ($P > 0.05$, Kruskal-Wallis test with Dunn's multiple comparison). w^{+}F10 flies, which carried w^{+} in isogenic background, however, had the number of counter-clockwise and clockwise walks (median 44.0, IQR 34.0 - 53.0, n = 47) markedly greater than Canton-S ($P < 0.001$, Kruskal-Wallis test with Dunn's multiple comparison) (Figure 4c). Therefore, a replacement of wild-type genetic background with isogenic beckground increased the number of counter-clockwise and clockwise walks, whereas a replacement of w^{+} locus with w^{1118} locus had no effect. Data indicated that the wild-type genetic background potently suppressed the number of counter-clockwise and clockwise walks.

w^{+} locus suppressed the number of counter-clockwise and clockwise walks in flies with isogenic background

The effect of w^{+} locus on the number of counter-clockwise and clockwise walks was unobservable in flies containing wild-type genetic background. This effect could be prominent if w^{+} was accompanied with w^{1118} isogenic background. The number of counter-clockwise and clockwise walks in w^{+}F10 flies was lower than that in w^{1118} flies (median 61.0, IQR 50.0 - 70.8, n = 48) ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple comparison) (Figure 4c). Thus, w^{+} locus suppressed the number of counter-clockwise and clockwise walks in flies carrying isogenic background.

w^{+} locus promoted pausing during locomotion in the circular arena

Preliminary observations indicated that w^{+}-carrying flies (including Canton-S) paused more often than w^{1118}-carrying flies (including w^{1118} strain) (see Figure 2 and 4). We examined the effect of w^{+} locus on the number of pauses during 300 s locomotion.

The number of pauses in w^{+} F10 (median 13.0, IQR 10.0 - 26.0, n = 47) was greater than that in w^{1118} (median 9.0, IQR 6.0 - 11.0, n = 48) ($P < 0.0001$, Mann-Whitney test) (Figure 5a). The number of pauses in Canton-S (median 7.0, IQR 6.0 - 8.0, n = 31) was also greater than that in w^{1118} F10 (median 3.0, IQR 2.0 - 4.0, n = 43) ($P < 0.0001$, Mann-Whitney test) (Figure 5a). w^{+} F10 and w^{1118} flies carried isogenic background, whereas Canton-S and w^{1118} F10 flies carried wild-type genetic background. Therefore, w^{+} locus promoted pausing during locomotion in the arena. This effect was observed consistently in flies with either isogenic or wild-type genetic background.

w^{+} locus increased maximal duration of one-way walking

The maximal duration of one-way walking (including both directions of counter-clockwise and clockwise) is more indicative of the persistence of one-way walking than the number of counter-clockwise and clockwise walks. We explored the effect of w^{+} locus on the maximal duration of

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one-way walking within 300 s locomotion. Flies carrying different *w* alleles with the same genetic background were compared.

The maximal duration of one-way walking in w^+ F10 flies (median 21.4 s, IQR 17.6 - 32.8 s, n = 47) was greater than that in w^{1118} flies (median 18.6 s, IQR 14.9 - 22.3 s, n = 48) ($P = 0.0030$, Mann-Whitney test). The maximal duration of one-way walking in Canton-S (median 184.6 s, IQR 129.2 - 235.4 s, n = 31) was markedly longer than that in w^{1118} F10 flies (median 97.8 s, IQR 76.4 - 156.2 s, n = 43) ($P < 0.0001$, Mann-Whitney test) (Figure 5b).

Clearly, Canton-S flies had the ability to walk in one direction at a median around 185 s in circular arenas. These data indicated that w^+ locus increased the maximal duration of one-way walking in the arena.

w^+ duplicated to the Y chromosome increased the maximal duration of counter-clockwise or clockwise walking

A direct evidence is required to support the hypothesis that the w^+ gene promotes persistent one-way walking in circular arena. We examined flies with w^+ duplicated to the Y chromosome. Two duplication lines (w^{1118}/Dp(1;Y)$B^S w^+ y^+$ and w^{1118}/Dp(1;Y)$w^+ y^+$) have been previously established (Xiao & Robertson 2016; Xiao *et al*. 2017).

The maximal duration of one-way walking in w^{1118}/Dp(1;Y)$B^S w^+ y^+$ flies (median 32.3 s, IQR 24.2 - 41.0, n = 42) was longer than w^{1118} ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple comparison). The maximal duration of one-way walking in w^{1118}/Dp(1;Y)$w^+ y^+$ flies (median 38.2 s, IQR 29.0 - 49.6 s, n = 47) was also longer than w^{1118} ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple comparison) (Figure 6a). Data supported that w^+ promoted persistent one-way walking in circular arena.

Four copies of mini-white in the genome promoted persistent one-way walking

Drosophila mini-white, a miniature form of w^+, has been integrated into the genome of many transgenic lines (e.g. UAS lines and Gal4 drives). These flies are ideal resources to test whether w^+ promotes persistent one-way walking in circular arenas.

We examined male flies carrying a *mini-white* on the X chromosome. All selected fly lines have been generated in w^{1118} isogenic background. Additionally, the *mini-white* on the X chromosome is subject to a dosage compensation (Qian & Pirrotta 1995; Arkhipova *et al*. 1997). The maximal duration of one-way walking was at a median of 28.5 s (IQR 19.5 - 43.8 s, n = 24) in UAS-bPDE5/Y, 19.6 s (IQR 15.8 - 28.8 s, n = 19) in UAS-*white* (D4)/Y, 21.3 s (IQR 17.1 - 26.5 s, n = 30) in UAS-Httex1-Q23-eGFP/Y, 30.6 s (IQR 22.0 - 36.4 s, n = 23) in UAS-Httex1-Q72-eGFP/Y and 24.6 s (IQR 15.7 - 45.2 s, n = 21) in UAS-Httex1-Q103-eGFP/Y. There was no significant difference between any of the tested UAS flies and w^{1118} flies (Kruskal-Wallis test with Dunn's multiple comparison) (Figure 6a). Therefore, a *mini-white* on the X chromosome was insufficient to increase the maximal duration of one-way walking of flies in circular arenas.

Flies carrying one or multiple *mini-white* on the autosomes were tested. We chose UAS lines with *mini-white* integrated into site-specific recombination sites (i.e. attP40 and attP2), and UAS lines with *mini-white* randomly inserted into the second or third chromosome. The selected UAS flies have been backcrossed into isogenic w^{1118} lines for ten generations. In addition, several

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lines containing two or four genomic copies of *mini-white* were examined. The maximal duration of one-way walking was 23.5 s (IQR 20.1 - 32.1 s, n = 16) in 10×UAS-IVS-mCD8::GFP (attP40)/+ flies, 26.4 s (IQR 20.5 - 38.2 s, n = 14) in 10×UAS-IVS-mCD8::GFP (attP2)/+ flies, 20.6 s (IQR 17.7 - 27.6 s, n = 16) in UAS-mito-HA-GFP.AP (II)/+ flies and 25.3 s (IQR 21.3 - 47.4 s, n = 16) in UAS-mito-HA-GFP.AP (III)/+ flies. There was no statistical difference between any line of tested UAS flies and w^{1118} flies (Kruskal-Wallis test with Dunn's multiple comparison). Thus, flies with one genomic copy of *mini-white* on the autosome showed unaffected maximal duration of one-way walking (Figure 6b). The maximal duration of one-way walking in flies carrying two genomic copies of *mini-white* (10×UAS-IVS-mCD8::GFP (attP40)/+;10×UAS-IVS-mCD8::GFP (attP2)/+) was at a median of 21.1 s (IQR 17.1 - 25.7 s, n = 16), which was statistically the same as that in w^{1118} flies ($P > 0.05$, Kruskal-Wallis test with Dunn's multiple comparison).

Interestingly, the maximal duration of one-way walking in flies carrying four genomic copies of *mini-white* (10×UAS-IVS-mCD8::GFP (attP40);10×UAS-IVS-mCD8::GFP (attP2) homozygotes) was at a median of 40.0 s (IQR 20.1 - 71.1 s, n = 16), a level higher than that in w^{1118} flies ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple comparison). Hence, four genomic copies of *mini-white* increased the maximal duration of one-way walking.

Consistently, the maximal duration of one-way walking in another line carrying four genomic copies of *mini-white* (tubP-Gal80^{ts};NP6520-Gal4 homozygotes, median 34.5 s, IQR 24.2 - 53.6 s, n = 24) was higher than that in w^{1118} flies ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple comparison), although the maximal duration of one-way walking in flies with two genomic copies of *mini-white* (tubP-Gal80^{ts}/+;NP6520-Gal4/+, median 24.2 s, IQR 18.0 - 32.6 s, n = 24) was the same as w^{1118} flies ($P > 0.05$, Kruskal-Wallis test with Dunn's multiple comparison) (Figure 6b).

Together, two homozygotes (or four genomic copies) of *mini-white* increased the maximal duration of one-way walking, whereas one copy of *mini-white* on the X chromosome, or one or two copies on the autosomes had no effect on persistent one-way walking. These data provided additional evidence that w^{+} promoted persistent one-way walking of flies in circular arenas.

Pan-neuronal overexpression of white increased the maximal duration of one-way walking

Often, the Gal4/UAS expression system introduces two genomic copies of *mini-white*, which was observed to be insufficient to promote persistent one-way walking. This validated the application of *mini-white*-carrying Gal4/UAS system to explore the effect of tissue-specific expression of *white*. Presumably, overexpression of the White protein in targeted tissues, for example, the central nervous system, could have an effect on the maximal duration of one-way walking. We examined flies with targeted expression of *white* using pan-neuronal driver elav-Gal4.

Flies expressing White in the neurons (elav-Gal4/+;UAS-*white* (H8)/+) had the maximal duration of one-way walking (median 40.5 s, IQR 23.2 - 53.3 s, n = 12) longer than elav-Gal4/+ (median 19.8 s, IQR 17.9 - 22.6 s, n = 16) ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple comparison) or UAS-*white* (H8)/+ (median 25.0 s, IQR 17.7 - 27.4 s, n = 16) ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple comparison). Flies expressing a green fluorescent protein (GFP) in the neurons (elav-Gal4/+; 10×UAS-IVS-GFP-WPRE (attP2)/+) had the maximal duration of one-way walking (median 26.8 s, IQR 18.6 - 41.3 s, n = 8) the same as controls 10×UAS-IVS-GFP-WPRE (attP2)/+ (median 24.3 s, IQR 21.3 - 28.8 s, n = 8) ($P > 0.05$, Kruskal-Wallis test

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with Dunn's multiple comparison) (Figure 7a). Thus, pan-neuronal overexpression of White but not GFP increased the maximal duration of one-way walking.

RNAi knockdown of w^+ decreased the maximal duration of one-way walking

Downregulation of *white* through RNA interference (RNAi) provided another approach to examine the role for *white* in promoting persistent one-way walking. After a recombination of wild-type X chromosome (including target gene w^+) into elav-Gal4 and UAS-*white*-RNAi flies, we examined the effect of RNAi knockdown of w^+ on the maximal duration of one-way walking.

The maximal duration of one-way walking in *white-RNAi* flies (w^+; elav-Gal4/+; UAS-*white*-RNAi (attP2)/+, median 30.8 s, IQR 17.6 - 51.3 s, n = 22) was decreased compared with controls w^+; elav-Gal4/+ (median 64.2 s, IQR 41.6 - 103.7 s, n = 33) ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple comparison) or controls w^+; UAS-*white*-RNAi (attP2)/+ (median 53.5 s, IQR 33.1 - 91.7 s, n = 26) ($P < 0.05$, Kruskal-Wallis test with Dunn's multiple comparison). The maximal duration of one-way walking in flies expressing a GFP (w^+; elav-Gal4/+; 10×UAS-IVS-GFP-WPRE (attP2)/+, median 48.2 s, IQR 38.2 - 66.0 s, n = 16) was statistically the same as controls w^+; 10×UAS-IVS-GFP-WPRE (attP2)/+ (median 60.4 s, IQR 35.7 - 94.2 s, n = 16) ($P > 0.05$, Kruskal-Wallis test with Dunn's multiple comparison) (Figure 7b).

Therefore, RNAi knockdown of w^+ decreased the maximal duration of one-way walking. Without RNAi to w^+, targeted expression of GFP using the same expression system had no effect on persistent one-way walking.

Discussion

Drosophila persistent one-way walking in a circular arena for minutes is a phenomenon that has not been previously reported. Using the techniques of fly tracking, behavioral computation and genetic manipulation, we show that wild-type Canton-S male flies are able to walk in one direction, counter-clockwise or clockwise, for around 185 s in the circular arenas. Whereas the genetic background of Canton-S flies suppresses the number of counter-clockwise and clockwise walks, w^+ promotes persistent one-way walking by increasing the maximal duration of counter-clockwise or clockwise walking.

Features of persistent one-way walking

The extraction of locomotor components of walking behavior provides rich information about the behavioral elements, the frequency of each element, and how they transit from one to another over time. Canton-S and w^{1118} males increase locomotion in the circular arenas as a response to spatial restriction, and maintain the active walking for at least an hour (Xiao & Robertson 2015). Here we demonstrate further details of walking behavior in addition to the increased locomotion. We summarize our observations that are common to Canton-S and w^{1118} flies as three aspects.

First, counter-clockwise walking and clockwise walking are the two main locomotor components. There is no preference for counter-clockwise or clockwise direction in the arena. Second, the intermittent pausing is clearly associated with directional persistence but not directional

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change. Thus, pausing is indicative of a state that flies rest and retain a memory of walking direction, rather than a state in which flies are unsure of their directions. Cocaine-treated Canton-S flies show a general tendency to rotate in one direction both before and after an immobility (Gomez-Marin *et al*. 2016), a finding similar to our observation of the association between pausing and directional persistence in a circular arena. Third, during counter-clockwise or clockwise walking flies move forward. We did not observe flies walking backward persistently in the arena. This walking performance is different from the phenotype of walking backward consistently (Bidaye *et al*. 2014).

Wild-type genetic background suppresses the number of counter-clockwise and clockwise walks

Strain-specific walking performance indicates the genetic basis for persistent one-way walking. Canton-S and w^{1118} flies have two major genetic differences: the *w* allele and its genetic background. We have ruled out the effect of cytoplasmic background on this phenotype, because female progenies from reciprocal crosses have the same performance of one-way walking. A ten-generation backcrossing leads to an exchange of genetic background between two strains, allowing us to compare the effects of wild-type genetic background with the presence of different *w* loci. That the low number of counter-clockwise or clockwise walks is transferable, from Canton-S to w^{1118}-carrying flies with wild-type genetic background, indicates a strong association between genetic background and persistent one-way walking. Additionally, heterozygous female flies with a 1:1 mixture of genomic chromosomes show no difference of persistent one-way walking. And even males with a half-half mixture of autosomes, regardless of the presence of wild-type or isogenic X chromosome, have the same performance of one-way walking. These findings highlight that genetic background is strongly associated with persistent one-way walking. Thus, to examine the contribution of w^+ to this phenotype, the genetic background should be carefully controlled.

w^+ promotes persistent one-way walking

w^+ locus suppresses the number of counter-clockwise and clockwise walks in flies carrying isogenic background. However, this effect becomes unobservable or masked in flies carrying wild-type genetic background, or a background heterozygous between isogenic and wild-type.

In such a scenario, the *mini-white* gene, carried in many transgenic lines with isogenic background, offers a great opportunity to examine the contribution of w^+ to persistent one-way walking. We find that two homozygous alleles (or four genomic copies) of *mini-white* promote persistent one-way walking in flies with isogenic background, while one copy on the X chromosome, or one or two copies on the autosomes, is insufficient. This copy-number-dependent phenomenon has been observed elsewhere. It has been reported that *mini-white* is responsible for another complex behavior, the male-female copulation success, in a manner that is copy-number-dependent (Xiao *et al*. 2017). These findings support the hypothesis that w^+ allele promotes persistent one-way walking.

The effect of w^+ is further evident by three additional observations. First, w^+ duplicated to the Y chromosome has a promoting effect on persistent one-way walking in flies with isogenic background. Second, pan-neuronal expression of the White protein promotes persistent one-way

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walking. There is, however, no effect through the expression of GFP instead of White using the same Gal4/UAS system. This observation confirms that the two genomic copies of *mini-white*, introduced by the Gal4/UAS expression system, is insufficient to promote persistent one-way walking. The finding also indicates that the effect of w^+ on persistent one-way walking is tissue-specific. Presumably, this effect becomes observable in flies with sufficient expression of White in the central nervous system. Third, RNAi knockdown of w^+ reduces the maximal duration of one-way walking. Targeted expression of GFP without knockdown of w^+ using the same expression system, with the presence of w^+, has no effect.

It is intriguing that in general, a Canton-S fly is able to walk in one direction in a circular arena for around 185 seconds, while a w^{1118} fly only 19 seconds. We have previously reported that Canton-S flies have spontaneously rhythmic motor activities with a periodicity around 19 s, but this periodicity is greatly reduced in w^{1118} flies (Qiu *et al*. 2016). Whether rhythmic motor activities promote persistent one-way walking in Canton-S flies is currently unclear.

For over a hundred years, it has been believed that w^+ controls eye color in *Drosophila*. This has led to an application of mini-*white* as a marker gene indicating successful transformation of a transgene. This application heavily relies on the function of w^+ in eye pigmentation. However, mini-*white* causes abnormal courtship in male flies (Zhang & Odenwald 1995; Hing & Carlson 1996), and confers male-female copulation success (Xiao *et al*. 2017). Additionally, w^+ promotes fast and consistent locomotor recovery from anoxia (Xiao & Robertson 2016; Hersh 2016). Wild-type flies have enhanced memory of thermal stimulus (Sitaraman *et al*. 2008), and increased vesicular content of biogenic amines (Borycz *et al*. 2008) compared with *w* mutant flies. The extra-retinal functions that the White protein transports cyclic guanosine monophosphate (cGMP) and zinc ion have been documented (Evans *et al*. 2008; Xiao & Robertson 2017; Tejeda-Guzmán *et al*. 2017). These findings suggest that w^+ has pleiotropic functions in housekeeping rather than a function responsible for eye color. We provide further evidence in the current study. That Canton-S flies are able to walk in one direction in circular arenas for around 185 seconds, and that w^+ increases the maximal duration of one-way walking firmly support a pleiotropic effect of w^+ in promoting persistent one-way walking.

Materials and Methods

Flies

Fly strains used in this study and their sources were: Canton-S (Bloomington *Drosophila* Stock Center (BDSC) # 1); w^{1118} (L. Seroude laboratory); Oregon-R (BDSC # 2376); Hikone-AS (BDSC # 3); Florida-9 (BDSC # 2374); w^1 (BDSC # 145); w^a (BDSC # 148); w^{cf} (BDSC # 4450); UAS-bPDE5 (Vermehren-Schmaedick *et al*. 2010); UAS-*white* (D4) and UAS-*white* (H8) (Evans *et al*. 2008); UAS-Httex1-Qn-eGFP (n = 23, 72 or 103) (Zhang *et al*. 2010); 10×UAS-IVS-mCD8::GFP (attP40) (BDSC # 32186); 10×UAS-IVS-mCD8::GFP (attP2) (BDSC # 32185); 10×UAS-IVS-GFP-WPRE (attP2) (BDSC # 32202); UAS-mito-HA-GFP.AP (BDSC # 8442); UAS-mito-HA-GFP.AP (BDSC # 8443); tubP-Gal80^{ts} (BDSC # 7019); NP6520-Gal4 (Awasaki *et al*. 2008); elav-Gal4 (BDSC # 8765) and UAS-*white*-RNAi (BDSC # 31088). w^+ F10 and w^{1118} F10 were the fly lines generated by a serial backcrossing between Canton-S and w^{1118} flies for ten generations (Xiao & Robertson 2015). w^+ duplication lines (w^{1118}/Dp(1;Y)$B^S w^+ y^+$ and

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w^{1118}/Dp(1;Y)$w^{+}y^{+}$) were generated previously (Xiao & Robertson 2016; Xiao *et al*. 2017). Flies were maintained with standard medium (cornmeal, agar, molasses and yeast) at 21-23 °C with 60-70 % relative humidity. An illumination of light/dark (12/12 h) cycle was provided with three light bulbs (Philips 13 W compact fluorescent energy saver) in a room around 133 square feet. Flies were collected within 0 - 2 days after emergence. We used pure nitrogen gas to anesthetize flies during collection time. Collected flies were raised in food vials at a density of 20 - 25 flies per vial for at least three additional days. A minimum of three days free of nitrogen exposure was guaranteed before the test. The ages of tested flies were 4 - 9 days old. Unless otherwise indicated, male flies were used for experiments. To avoid natural peak activities in the mornings and evenings (Grima *et al*. 2004), experiments were performed during the light time with three hours away from light on/off switch.

Locomotor assay

Locomotor assay was performed by following a reported protocol (Xiao & Robertson 2015). In general, flies were loaded into circular arenas (1.27 cm diameter and 0.3 cm depth) with one fly per arena. The depth of 0.3 cm was considered to allow flies to turn around but suppress vertical movement. We machined 128 arenas (8 $\times$ 16) in an area of 31 $\times$ 16 cm^2 Plexiglas. The bottom side of arena was covered with thick filter paper allowing air circulation. The top was covered by a slidable Plexiglas with holes (0.3 cm diameter) at one end for fly loading. The Plexiglas with arenas was secured in a large chamber (48.0 $\times$ 41.5 $\times$ 0.6 cm^3). A flow of room air (2 L/min) was provided to remove the effect of dead space (Bouhuys 1964). A time of 5-min was allowed for flies to adapt to the experimental settings. Locomotor activities were video-captured at 15 frames per second, and stored for post analysis. Fly positions (the locations of center of mass) with 0.2 s interval were computed by custom-written fly tracking software (Xiao & Robertson 2015). The positions were used for subsequent movement analysis, including 3D trajectory construction and structural extraction of walking behavior.

Construction of time-series of 3D trajectory

Time-series of 3D trajectory was constructed by using the X-Y positions over a period. Briefly, for each fly, a data containing 1500 position information, corresponding to 300 s locomotion, was used for trajectory construction. The function Cloud() from an R package "Lattice" (Sarkar 2008) was used for 3D data visualization. The arena size was remained unchanged throughout this study. Therefore, for simplification, we omitted the *x*, *y* axises (representing position coordinates) and *z* axis (representing time), and provided a color key as an indicator of time. 3D trajectory and walking directions were judged according to the camera view.

Computation of counter-clockwise and clockwise walking directions

Computation of walking direction (counter-clockwise or clockwise) was performed by following a reported method (Qiu, Robertson & Xiao 2017). There were three main procedures: (1) Compute angular coordinates of fly positions by a trigonometric function atan2(y, x). (2) Calculate parameter ω - the angular displacement per 0.2 s. To avoid the big jump of ω value due to radian rotation,

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we calculated ω twice using radian interval (0, 2π] and (-π, π], and chose one with smaller absolute value. (3) Determine the walking direction as counter-clockwise walk ($\omega > 0$) or clockwise walk ($\omega < 0$).

We defined a "counter-clockwise walk" as at least five consecutive displacements with $\omega > 0$, and a "clockwise walk" at least five consecutive displacements with $\omega < 0$. Classified data were further treated to allow 1-2 steps of pause or backward walk (with radian < 0.21) without an apparent change of direction. To improve the estimation, we separated pause steps from counter-clockwise or clockwise walk. A "pause" was defined as at least five consecutive steps with step size < 0.28 mm (Qiu, Robertson & Xiao 2017). The activities that were not categorized to "counter-clockwise walk", "clockwise walk" or "pause" were assigned as "unclassified".

Statistics

Data processing and visualization were conducted by using software R (R Core Team 2014) and these supplementary packages: gdata, lattice (Sarkar 2008) and adehabitatLT (Calenge 2006). Data normality was examined by D'Agostino $\&$ Pearson omnibus normality test. Nonparametric tests (Mann-Whitney test, Wilcoxon matched pairs test and Friedman test with Dunn's multiple comparison) were performed for the comparison of medians between groups. Data were presented as scattered dots and median. A $P < 0.05$ was considered significant difference.

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Supporting Information

Video S1: Walking activities of Canton-S flies in the circular arenas
Video S2: Walking activities of w^{1118} flies in the circular arenas

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Figure legends

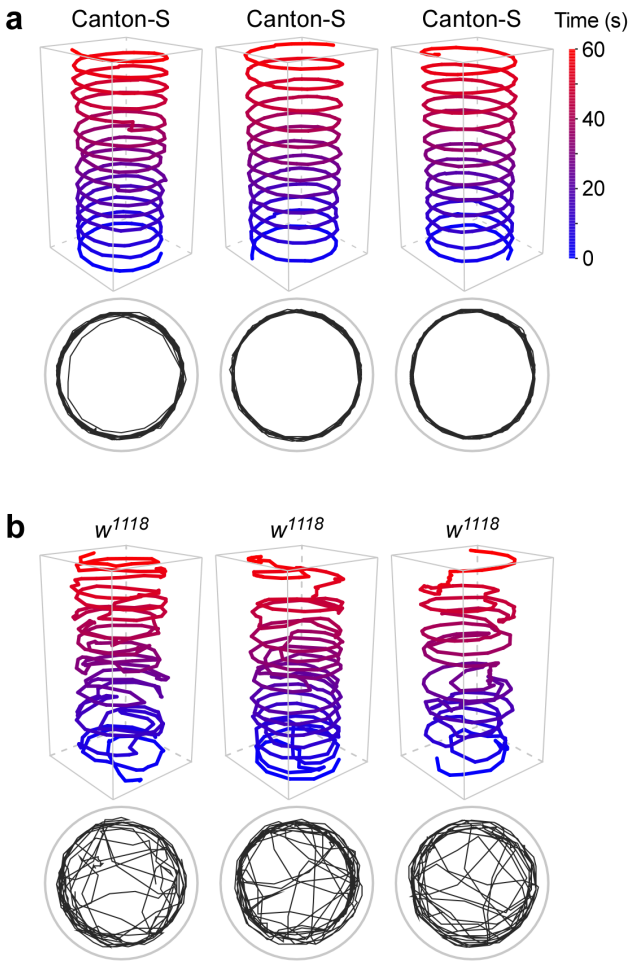

Figure 1: **Persistent one-way walking of Canton-S flies in the circular arenas.** (**a**) 3D walking trajectories and 2D path of Canton-S flies in the circular arenas (1.27 cm diameter 0.3 cm depth) during 60 s locomotion. There is only one fly in each arena. Color key indicates the time. (**b**) 3D walking trajectories and 2D path of w^{1118} flies during 60 s locomotion.

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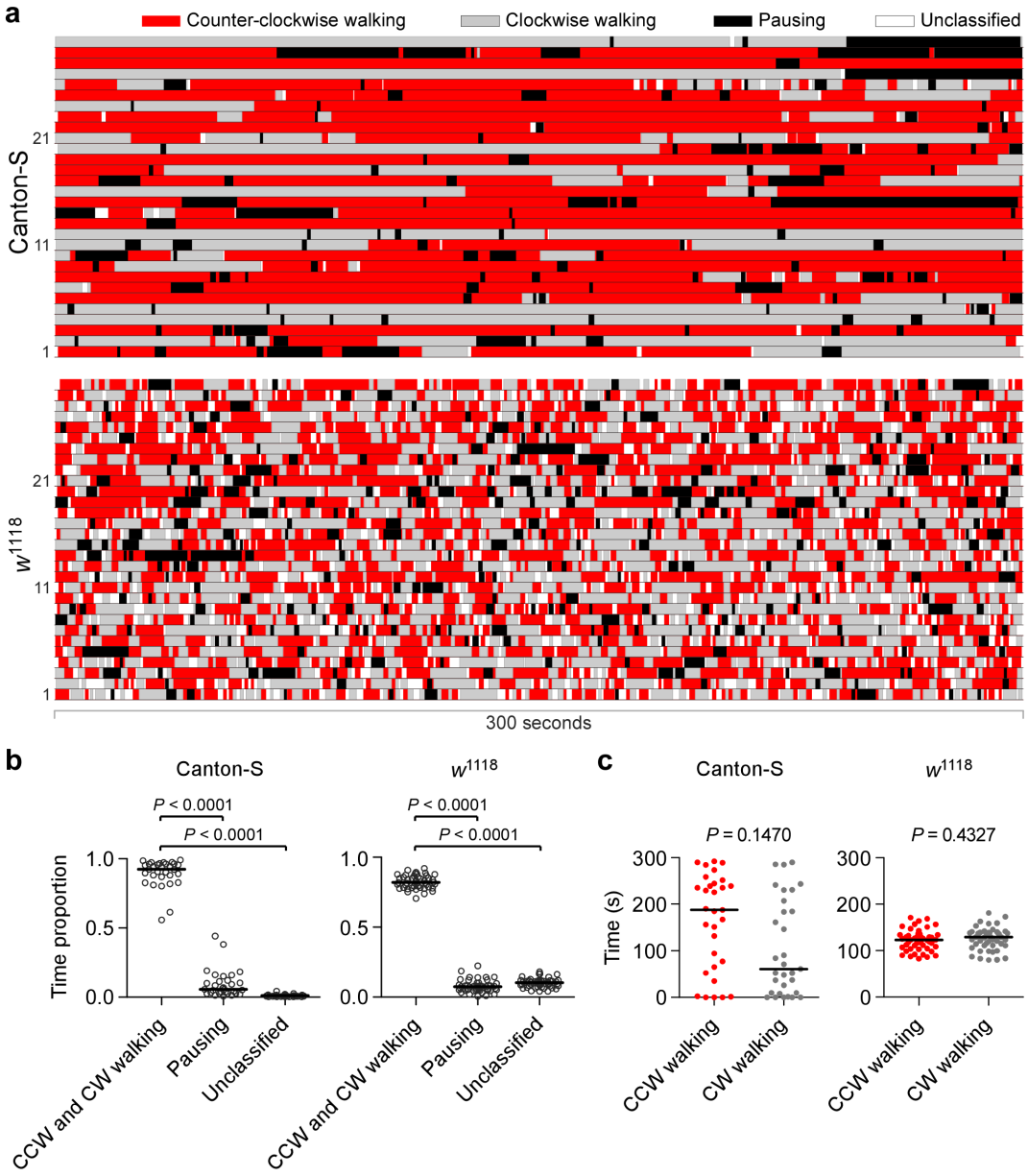

Figure 2: **Walking structures of flies in the circular arenas.** (**a**) Schematic structures of walk during 300 s locomotion. Shown are the counter-clockwise walk (CCW, in red), clockwise walk (CW, grey), pause (black) and unclassified (white) activities from 30 Canton-S and 30 w^{1118} flies. (**b**) Time proportion for CCW and CW in Canton-S (left) and w^{1118} flies (right). Data are presented as scattered dots and median (black line). *P* values are from Friedman test with Dunn's multiple comparison. (**c**) Time for CCW (red) and CW (grey) during 300 s locomotion in Canton-S (left) and w^{1118} flies (right). *P* values are from Wilcoxon matched pairs test.

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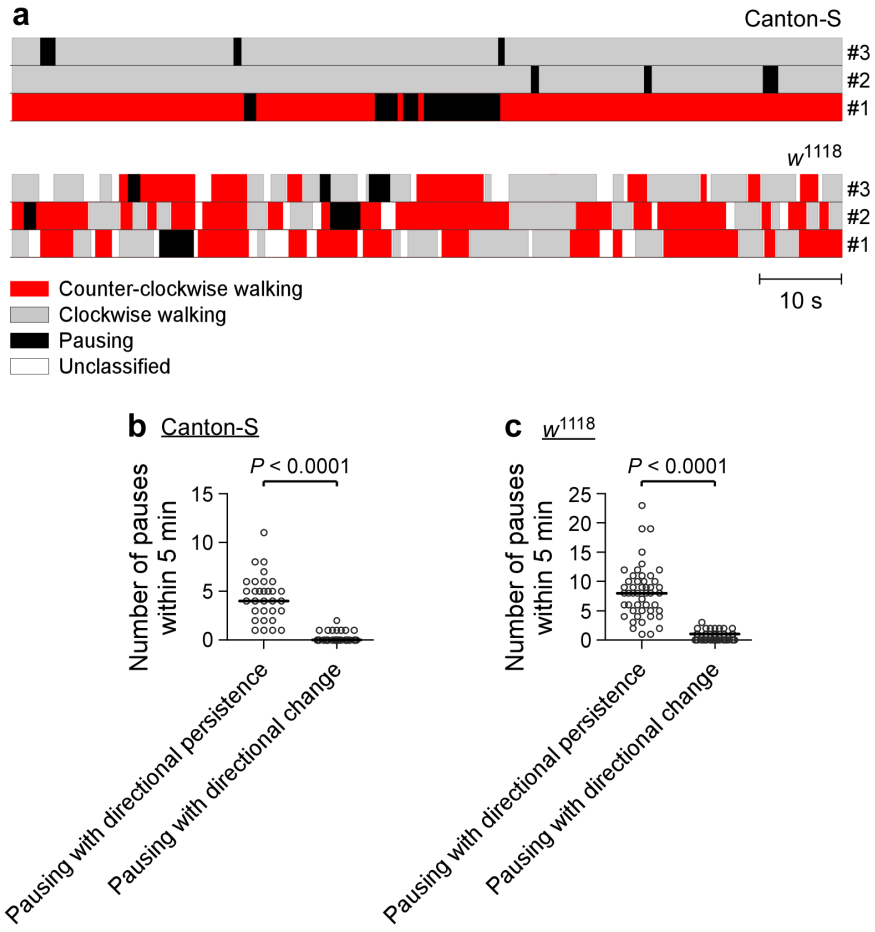

Figure 3: **Pause associated with directional persistence.** (**a**) Representations of pause and several walking structures in Canton-S and w^{1118} flies. Shown are the 100 s activities of flies in the circular arenas. Walking structures are counter-clockwise walk (red), clockwise walk (grey), pause (black) and unclassified activities (white). (**b**) Association between pause and directional persistence in Canton-S flies. *P* value from Wilcoxon matched pairs test. (**c**) Association between pause and directional persistence in w^{1118} flies. *P* value from Wilcoxon matched pairs test.

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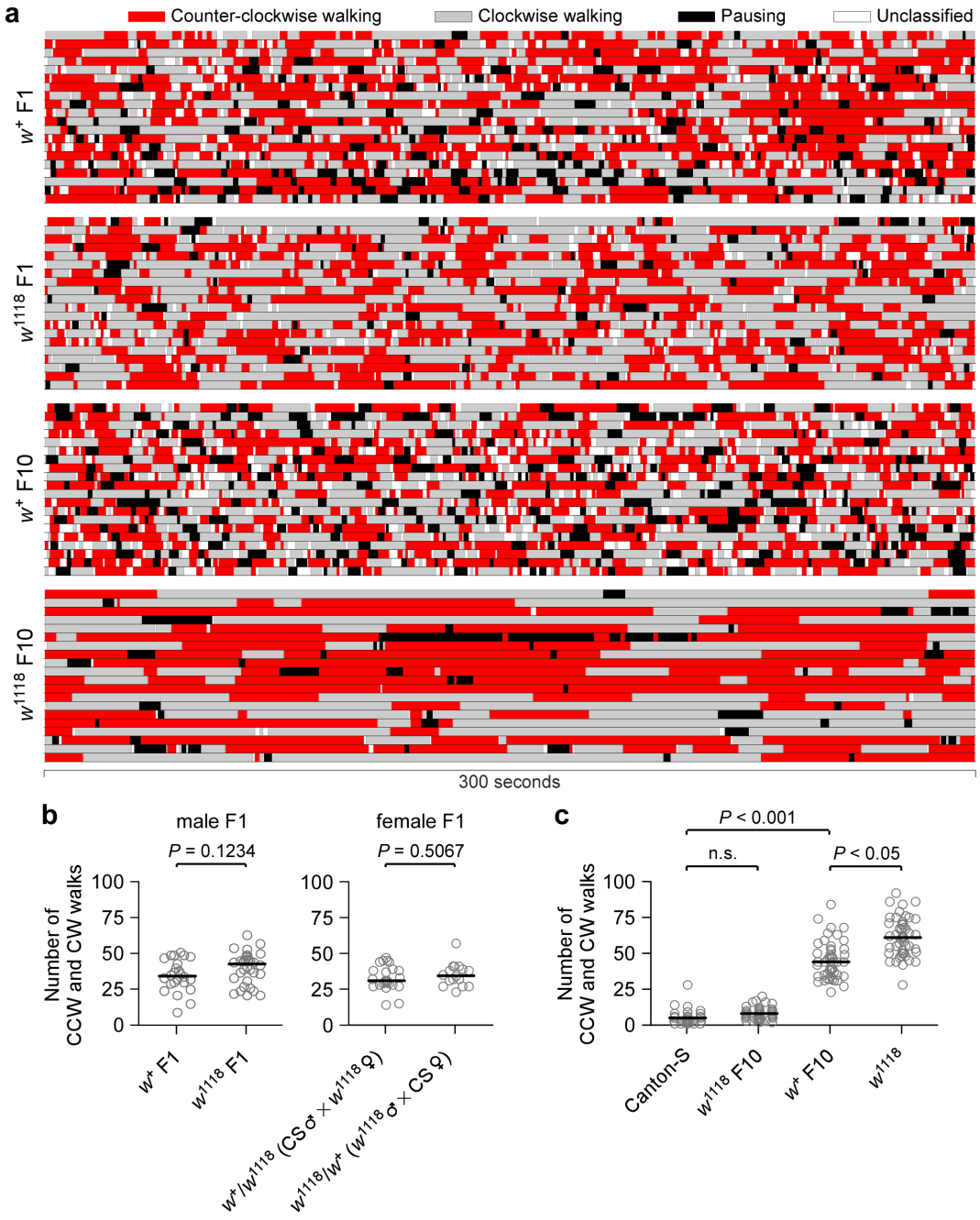

Figure 4: **Contributions of wild-type genetic background and w^+ locus to the number of counter-clockwise and clockwise walks.** (**a**) Schematic structures of walking during 300 s locomotion in w^+ F1, w^{1118} F1, w^+ F10 and w^{1118} F10 flies. Shown are the counter-clockwise walking (red), clockwise walking (grey), pausing (black) and unclassified (white) activities from 20 flies of each genotype. (**b**) Number of CCW (counter-clockwise) and CW (clockwise) walks in male and female F1 flies. Parental crosses are indicated for female F1. Data are presented as scattered dots and median (black line). *P* values from Mann-Whitney test. (**c**) Number of CCW and CW walks in Canton-S, w^{1118} F10, w^+ F10 and w^{1118} flies. *P* values from Kruska-Wallis test with Dunn's multiple comparison.

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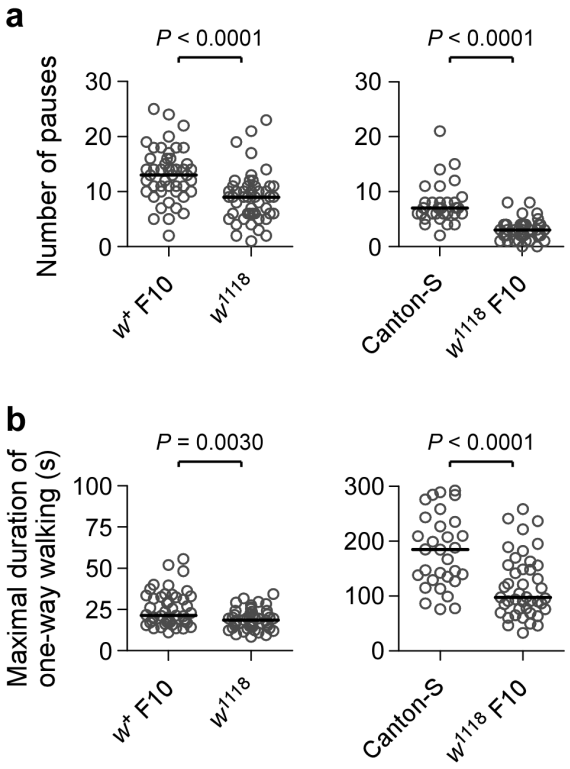

Figure 5: ***w*$^{+}$ promoted pausing and persistent one-way walking.** (**a**) Number of pauses during 300 s locomotion in flies with the same genetic background. (**b**) Maximal duration of CCW or CW walks within 300 s locomotion in different flies. The genotypes are indicated. Data are presented as scattered dots and median (black line). CCW, counter-clockwise; CW, clockwise. *P* values from Mann-Whitney test.

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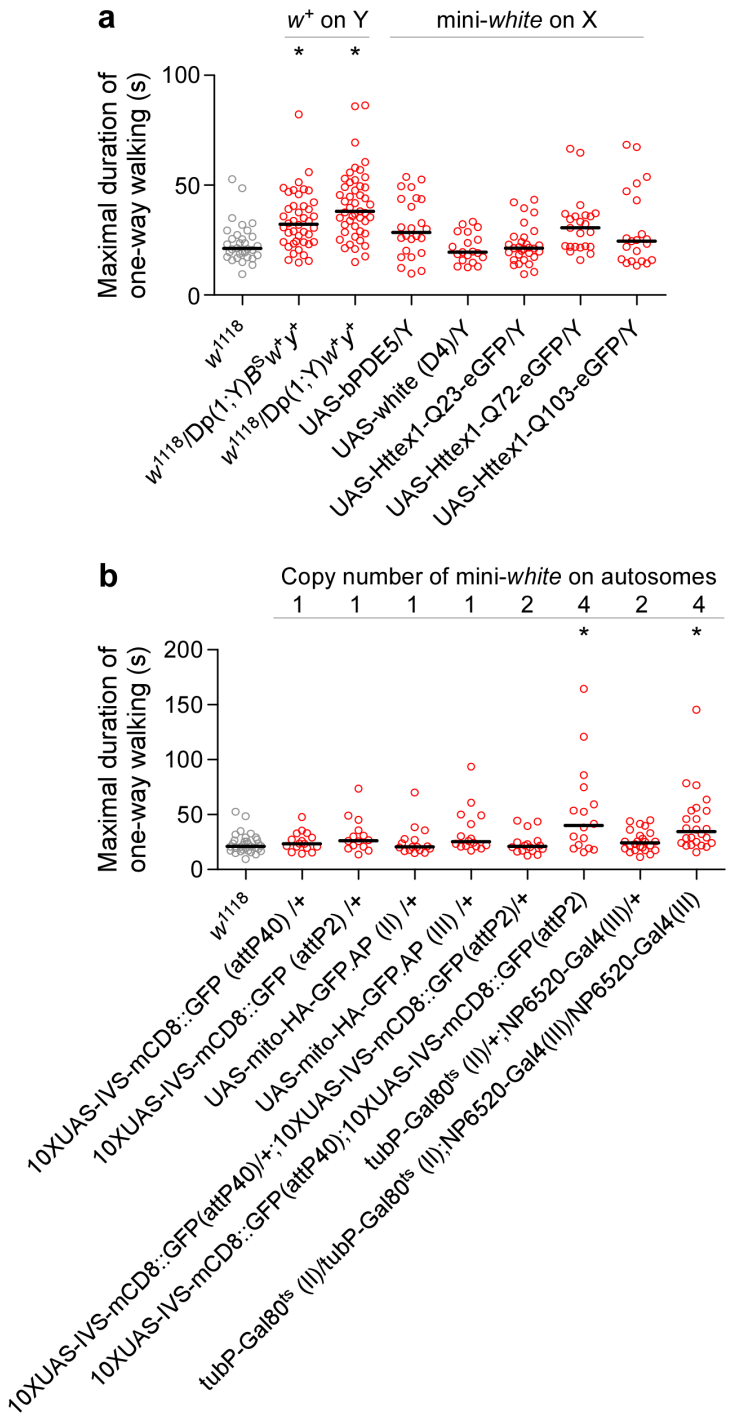

Figure 6: ***w*$^+$ duplicated to the Y chromosome or four genomic copies of mini-*white* increased the maximal duration of one-way walking.** (**a**) Effect of w^+ duplicated to the Y chromosome or *mini-white* inserted to the X chromosome on the maximal duration of one-way walking. (**b**) Effects of different copies of mini-*white* inserted to the autosomes on the maximal duration of one-way walking. *, $P < 0.05$ from Kruskal-Wallis test with Dunn's multiple comparison. Data of w^{1118} flies (grey) are duplicated here from Fig.5 for comparison. Data of mini-*white*-carrying flies are labeled with red. See Materials and Methods for the information of fly genotypes.

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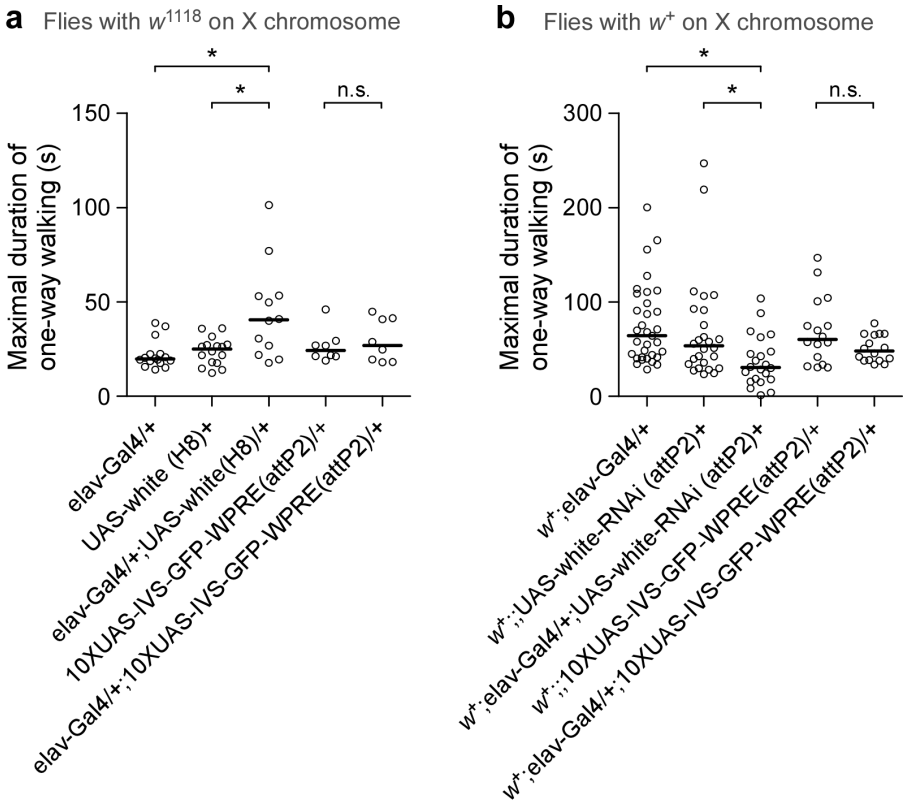

Figure 7: **Pan-neuronal overexpression or downregulation of *white* affects the maximal duration of one-way walking.** (**a**) Pan-neuronal overexpression of *white* increases the maximal duration of one-way walking. There is no effect of Gal4/UAS-driven overexpression of GFP. Tested flies carry a w^{1118} allele on the X chromosome. (**b**) Pan-neuronal knockdown of w^{+} through RNAi decreases the maximal duration of one-way walking. There is no effect of Gal4/UAS-driven overexpression of GFP. Tested flies carry a w^{+} on the X chromosome. *, $P < 0.05$ from Kruskal-Wallis test with Dunn's multiple comparison. n.s., non-significance.

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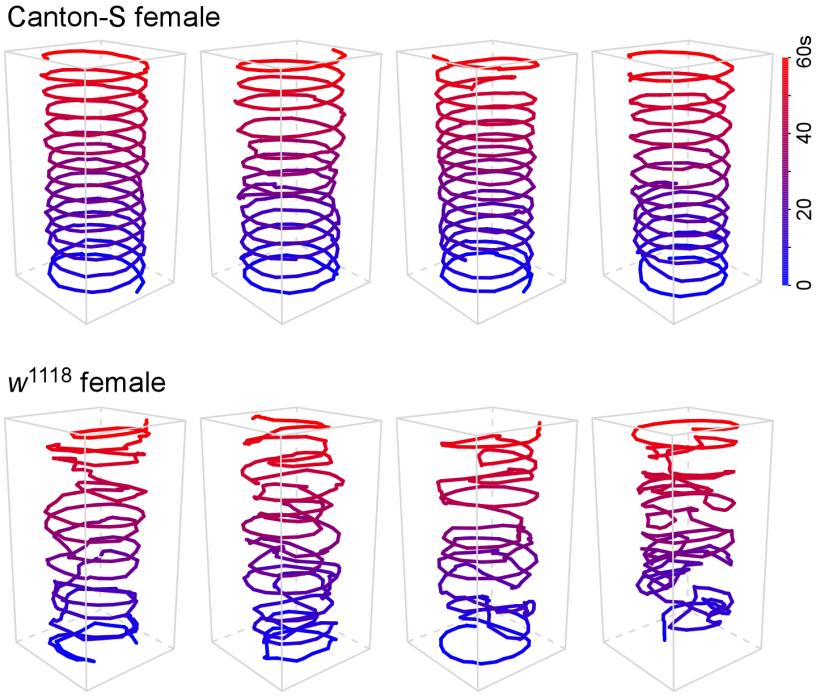

Figure S1: **Walking trajectories in Canton-S and w^{1118} female flies.** Persistent one-way walking was visually observed in Canton-S (upper panel), and it was likely lost in w^{1118} (lower panel) flies.

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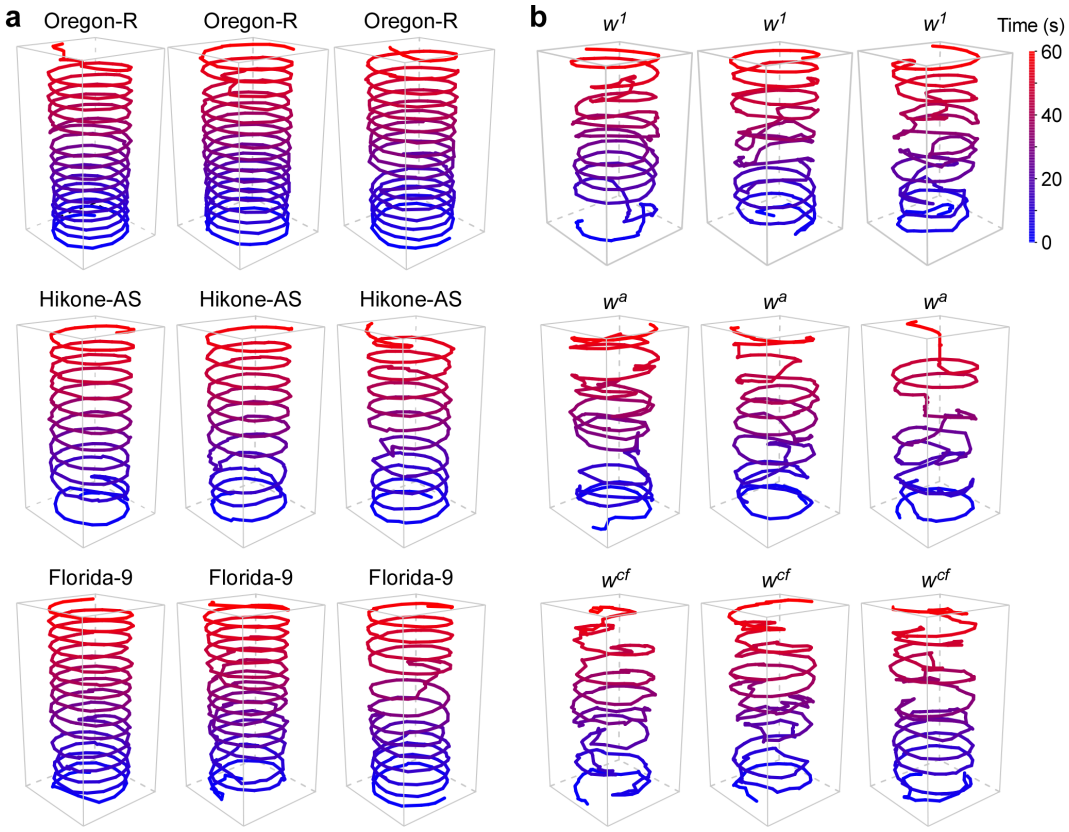

Figure S2: **Walking trajectories of several wild-types and *w* mutants.** (**a**) Walking trajectories of Oregon-R, Hikone-AS and Florida-9 in the circular arenas during 60 s locomotion. (**b**) Walking trajectories of w^1, w^a and w^{cf} in the circular arenas during 60 s locomotion. Color key indicates the time.