File S1

SUPPLEMENTARY MATERIAL AND METHODS

Creation of *Akh* gRNA transgenic flies: A gRNA target site in the mature AKH coding sequence was identified using the target prediction tool available on www.shigen.nig.ac.jp/fly/nigfly/cas9. For construction of the *Akh*-targeting transgene construct (pBFv.U6.2-*Akh*; MG457), oligonucleotides 5` CTTCGTTGACCTTCTCGCCGGATT 3` (RKO895) and 5` AAACAATCCGGCGAGAAGGTCAAC 3` (RKO896) were annealed and the resulting gRNA–coding DNA was cloned via *Bbs*I into the pBFv.U6.2 vector (KONDO and UEDA 2013). The pBFv.U6.2-*Akh* plasmid was injected into the attP40 strain (y^1 w^{67c23}; *P{CaryP}attP40*) by BestGene, the F1 generation was crossed to y^1 *sc** v^1; *In(2LR)Gla,* $wg^{Gla\text{-}1}$ Bc^1 / CyO (BDSC35781), and the following transgenic line with ubiquitous expression of *Akh* gRNA was generated by BestGene: y^1 *sc** v^1; *P{v*; BFv-U6.2-Akh.gRNA}attP40*.

Generation of the *Akh* mutant flies: For the induction of *Akh* mutations, y^1 *sc** v^1; *P{v*; BFv-U6.2-Akh.gRNA}attP40* was crossed to *P{*$ry^{+t7.2}$*=hsFLP}1,* y^1 w^{1118}; *P{*$y^{+t7.7}$ w^{+mC}*=UAS*-Cas9.P*}attP2, P{*w^{+mC}=GAL4::VP16-*nos.UTR}CG6325*^{MVD1} to generate founder males that expressed both CAS9 and *Akh* gRNA in the germline. Founder males were crossed to *w*;* Kr^{If1} / CyO*;* D^1 / TM3*,* Ser^1 females. Male *Akh* candidate mutants were subsequently selected for the presence of both balancers and against the presence of w^{+mC} (genotype: *w*; + /* CyO*;* $Akh^{*?}$ / TM3*,* Ser^1). Selection for the absence of w^{+mC} guaranteed absence of *nos*-GAL4 and *UAS*-CAS9 constructs, whereas selection for the presence of CyO ensured absence of the *Akh* gRNA in the male genome. These males were mated to the *w*;* TM3*,* Sb^1*, P{2xTb1*-RFP*}*TM3*/In(3L)* D^1 females in a single male crosses, and after mating, candidate mutant males were individually genotyped for the presence of the mutations within the *Akh* region by T7 endonuclease assay.

T7 endonuclease assay: Primers 5`TGTACATGTCCCCAGTCGGA3` (MGO921) and 5` CTATCTACTCGCGGTGCTT3` (MGO922) were used to PCR-amplify *Akh* coding region of heterozygous (over TM3 balancer) *Akh* candidate mutant males. PCR amplification of *Akh* mutations resulted in heteroduplex formation, which was detected by T7 endonuclease assay (New England Biolabs). The assays were done with 2 µl of the PCR reaction in a 10 µl reaction volume (NEBuffer2), with incubation time 15 min at 37°C. Cleavage products were subsequently analyzed by agarose gel electrophoresis.

Creation of Akh^A, Akh^{AP} and Akh^{SAP} stable stocks and backcrossing of the mutant alleles to a common genetic background: The following primer pairs were used to track the mutations during the backcrossing:

5`ACCTTCTCGCCGGGCAAG3`(MGO944) and 5`ATTGGCACGATCGGTTGGGT3` (MGO945) for Akh^A,

5`ATTACCAATCGTGGCTCGCA3`(MGO946) and 5`CACCGAACGCTTGTCAGCT3` (MGO947) for Akh^{AP}, 5`TGTACATGTCCCCAGTCGGA3` (MGO921) and 5`GCTATCTACTCGCGGTGCTT3` (MGO922) for Akh^{SAP} and 5`ACGCATTCAGGTGTATAGTCC3` (RKO377) and 5`TCAATCCCGAAACATGCTTAC 3` (RKO438) for $AkhR^{1}$.

Wing area measurement

Wing area of female flies used for the fecundity assay was measured using a Zeiss Axiophot microscope equipped with a digital camera AxioCam HRc and ZEN 2011 software. The left wing of each female was removed and fixed between two microscope slides. Wing outlines were traced as described by Klepsatel and colleagues (KLEPSATEL *et al.* 2013) and wing area was calculated using ZEN 2011 software. Wing area of 25 to 26 females was measured per genotype. Data were analyzed by one-way ANOVA.

Lipid determination by coupled colorimetric assay

Lipid measurements were done as described in Hildebrandt et al. (HILDEBRANDT *et al.* 2011). Lipid data were normalized to protein content. For each genotype, 4-6 replicas of 5 flies each were tested per developmental stage or starvation time point. Experiments were repeated at least three times. Lipid data were normalized to protein levels. Data were analyzed by one-way ANOVA to test the effect of genotype, or by two-way ANOVA to test the effects of genotype and starvation exposure or developmental time point, and their interactions.

Glycogen determination

Glycogen measurements in the 96 well plate format were based on the conversion of glycogen to glucose by amyloglucosidase (Sigma) and on its subsequent measurement by the glucose assay (GO) kit (Sigma) as described in Tennessen et al. (TENNESSEN *et al.* 2014). Fly homogenates were diluted with 0.05% Tween-20, in order to retain the sugar amounts within the linear range of the assay (0 to 0.16 μg of glycogen or glucose per μl of the analyzed homogenate). Each reaction was run with 30 μl of the fly homogenate. For each sample blank absorbance measurements at 540 nm were performed on two 30μl aliquots of diluted homogenates to which 100 μl of the GO reagent supplemented with 1 μl of amyloglucosidase (for total glucose determination) or no amyloglucosidase (for free glucose determination) was added. Samples were incubated for 30 min at 37°C with mild shaking before the reaction was stopped by adding 100 μl of 12N H_2SO_4. Final absorbance was measured at 540 nm. Initial absorbance of the homogenates was subtracted from the final absorbance, and glycogen and glucose levels were determined based on the absorbance of glucose and bovine liver glycogen standards (both from Sigma). The glycogen content was calculated from the difference between the total and the free glucose values. Obtained glycogen data were normalized to the protein content. For each genotype, 4-6 replicas of 5 flies

each were tested per developmental stage or starvation time point. Experiments were repeated at least three times. Data were analyzed by one-way ANOVA followed by Tukey´s HSD post-hoc test to detect the effect of genotype, or by two-way ANOVA to test the effects of genotype and starvation exposure or developmental time point, and their interactions.

Determination of circulating sugars

Hemolymph samples (three replicates of 30 flies each per genotype) were collected by centrifugation (6 min, 9.000 rcf at 4°C) of decapitated flies in a holder tube (0.2 ml tube with 5 holes of 0.6 mm diameter) placed in a 0.5 ml collection tube. One μl of the collected hemolymph was diluted with 99 μl of 0.05% Tween-20 and immediately heat inactivated at 70°C for 5 min. The homogenate was further diluted 1:6 prior to the sugar measurements. Measurements of circulating sugars were performed using a modification of the Tennessen method (TENNESSEN *et al.* 2014). Briefly, the assay was based on conversion of trehalose to glucose by porcine trehalase (Sigma), and subsequent measurement of glucose by the glucose assay (GO) kit (Sigma). Measurements were done in 96 well plate format. Per sample, 30 μl of the diluted homogenate was used. First, absorbance of the samples and standards was measured at 540 nm, then 100 μl of the GO reagent (pH adjusted to 6.8 by 1M phosphoric acid) + 0.3 μl of porcine trehalase (Sigma, T8778-1U) were added. Samples were incubated overnight at 37°C with mild shaking. The reaction was stopped by adding 100 μl of 12N H_2SO_4. Samples were mixed and final OD was measured at 540 nm. Initial absorbance of the homogenates was subtracted from the final absorbance, and sugar levels were determined based on the standard curves. The experiment was repeated three times. Data were expressed as circulating sugars per 1 μl of hemolymph and analyzed by one-way ANOVA followed by Tukey´s HSD post-hoc test.

Thin layer chromatography (TLC)

The TLC analysis was performed as described by Baumbach et al (BAUMBACH *et al.* 2014a), with minor modifications. In detail, lipids were extracted according to Blight and Dyer (BLIGH and DYER 1959). Two biological replicates of five flies each were homogenized in 150 μl methanol, 75 μl chloroform and 60 μl water by ten 1.4 mm ceramic cylinders (Peqlab) using a Peqlab Precellys 24 instrument (10 sec, 5000 rpm). Samples were incubated for one hour in a water bath at 37°C. Afterwards, first 75 μl chloroform and then 75 μl 1M KCl were added and vigorously mixed. Separation of phases was achieved by centrifugation (1000 x g; 2 min) and the chloroform phase was transferred into a new 1.5 ml tube. Solvent was evaporated in a SpeedVac concentrator. Lipid pellets were resuspended in 100 μl chloroform / methanol (1:1) for the control genotype. The re-suspension volume for the Akh^{A}, Akh^{AP} and $AkhR^{1}$ was adjusted to the equivalent protein amount of the control.

Fly protein content of all genotypes was determined by the protein assay (see section 2.10) using independent sibling flies. Finally, 20 µl of the mix was separated on high performance thin layer chromatography (HPTLC) plates (Merck, 105633) using n-hexane / diethylether / acetic acid (70:30:1, v/v/v; Merck) for unpolar lipids (TG, DG, MG and FA). As lipid standard, we used 40 µg of glyceryltrioleate, 40 µg of 1,3-diolein, 40 µg of 1,2-dioleoyl-*rac*-glycerol, 40 µg of monoolein (all as mix in SUPELCO Mono-, Di-, Triglyceride Mix, SIGMA 1787-1AMP) supplemented with 4 µg oleic acid (FA; CALBIOCHEM #4954). Plates were air-dried, immersed in 8% (w/v) H_3PO_4 containing 10% (w/v) copper (II) sulfate pentahydrate, charred for 5 min at 180 °C and imaged on a Canon LiDE220 scanner.

Paraquat resistance assay - application of paraquat on the nerve cord

The assay was done according to Cassar and colleagues (CASSAR *et al.* 2015) with minor modifications. The assay is based on survival of flies after direct exposure to paraquat applied on the nerve cord of decapitated flies. In accordance to the literature our pilot experiment showed that decapitation and application of the vehicle on the nerve cord does not affect viability of flies for at least 24 h after decapitation, given that flies are housed in humid chambers (data not shown). Paraquat resistance was assayed by dipping the neck of cold anesthetized decapitated flies into PBS with or without 40mM paraquat, housing the decapitated flies in humid chambers at 25°C, and recording the survival rate 5 h after the paraquat application. Flies that did not recover from cold anesthesia were excluded from the analysis. Statistical significance of differences in the survival among the genotypes was tested by two-tailed Fischer exact test.

Startle-induced vertical climbing

The climbing assay is based on the 'countercurrent distribution' method described by Benzer (BENZER 1967) with modifications. Flies were CO_2-anesthetised and sorted not later then 24 h prior to the assay. The climbing assay device consisted of two detachable tubes (upper and lower tube; Polystyrene, 17 x 95 mm) connected by a funnel in vertical head-to-head position (50 x 40 mm part was attached to bottom tube, stem part 10 x 12 mm, was connected to the upper tube). Flies were transferred to the lower tube and the device assembled as described. After 30 sec of adaption, flies were tapped down to induce climbing. Flies that climbed into the upper tube within 60 sec (with every 15 sec gentle tapping for startle induction) were flipped into a new lower tube for another round of climbing as described. After six rounds of the assay, flies from the upper tube were transferred to a new tube, which was considered as 7^{th} lower tube for the calculation of the climbing index (CI). Then, the number of flies in the lower tubes (number 1 to 7) was counted and the climbing index was calculated according to (SHCHERBATA *et al.* 2007):

$$CI = \frac{\sum_{n=1}^{7} \text{number of flies in each lower tube} * n}{\text{total number of flies in replica} * 7}$$

Assay was done on ≥8 replicates per genotype per trial. Each replica consisted of 13 to 25 male 7-day-old flies. The climbing index of these replicas was used to calculate the averaged climbing index. The experiment was repeated at least twice. Statistical significance of the genotype effect on climbing ability was analyzed by one-way ANOVA followed by Tukey´s HSD post-hoc test.

Flight performance assay

Flight performance analysis was based on the assay developed by Benzer (BENZER 1973) and modified by Babcock and Ganetzky (BABCOCK and GANETZKY 2014). Briefly, flight performance was measurement as landing position of flies ejected into the flight tester. Flight tester consisted of 45 cm long plastic cylinder with inner diameter of 9 cm, a short adapter connecting the cylinder to a plastic funnel, and of a 21 cm long drop tube (diameter 4.5 cm) attached to the funnel. The walls of the plastic cylinder were covered with paper sheet with sticky substance (beet syrup), that ensured capturing flies at their first attempt to land. Bottom of the cylinder was covered with a layer of water, in order to capture also the fraction of flies that did not start flying in the tester. An unplugged vial (diameter 3.5 cm) with at least 25 flies was dropped into the drop tube, and as the vial fell down and hit the funnel, flies were ejected into the flight tester. Measurement was based on the assumption that the attempt to land corresponds to the moment when the fly starts the flight. Flies were captured at their first attempt to land, as they stuck to the tester sheet of paper. After the trial, the paper sheet with flies was removed and photographed. Landing high was calculated as the distance from the bottom of the tester. Measurements were done in ImageJ. Assay was done on male flies of 4-5 days of age. The experiment was repeated twice. Quantitative data were analyzed by one-way ANOVA followed by Tukey´s HSD post-hoc test.

Statistical analyses

Measurement variables were analyzed by two-tailed Student´s t-test, by one-way analysis of variance (ANOVA) with genotype as fixed effect, followed by Tukey's HSD (honest significant difference) post-hoc tests, or by two-way ANOVA if the effect of genotype and other nominal category and their interactions were tested. The only exception was lipid droplet area measurement, where Mann-Whitney-test was used. Nominal variables were analyzed by two-tailed Fischer exact test, starvation survival data by log-rank test.

Hatchability and larva-to-adult survival data were arcsine square root transformed before performing one-way ANOVA. Developmental rate data were log transformed before performing one-way ANOVA.

Samples that did not differ significantly from each other ($P > 0.05$) were labeled with identical letter codes in the

figures; samples that differ significantly ($P < 0.05$) from each other were labeled with different letter codes. In case where only two genotypes were compared, *P* values were indicated by star symbols (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). In panels aiming to illustrate effects of two nominal variables (effect of genotype and effect of the developmental stage or starvation exposure), letter code was not used, and the reader is directed to consult the corresponding figure legend for *F* and *P* values.

Unless stated otherwise, data on measurement variables and survival (log rank tests) were analyzed using PAST (HAMMER *et al.* 2001): http://palaeo- electronica.org/2001_1/past/issue1_01.htm. Data on nominal variables were analyzed by Graphpad QuickCalcs.

Fly stocks

Genotype	**Reference / stock number**	**Internal stock number**
y^1 sc v^1; P{v*; BFv-U6.2_Akh_gRNA}attP40*	this study	MGF1492
w; Kr^{If-1}*/CyO; D^1*/TM3, *Ser^1*	BDSC7198	RKF1365
P{ry$^{+t7.2}$=hsFLP}1, y^1 w^{1118}; P{y$^{+t7.7}$ w^{+mC}=UAS-Cas9.*P}attP2, P{w^{+mC}*=GAL4::VP16-*nos.UTR}CG6325^{MVD1}*	(PORT *et al.* 2014), BDSC54593	MGF1476
w;* TM3*, Sb^1, P{2xTb1*-RFP*}*TM3*/ln(3L)D D^1*	BDSC36338	
w; Akh^A*	this study	MGF1594
w; Akh^{AP}*	this study	MGF1595
w; Akh^{SAP}*	this study	MGF1596
y**w*; $AkhR^1$*	(GRÖNKE *et al.* 2007)	RKF639
w^{1118}; Akh^A/ TM3*, Ser^1* floating	this study	MGF1629
w^{1118}; Akh^{AP}/ TM3*, Ser^1* floating	this study	MGF1630
w^{1118}; $AkhR^1$	this study	MGF1634
w; akhp-GAL4, UAS-mCD8 GFP; akhp-GAL4/* SM5a-TM6 *Tb*	(KIM and RULIFSON 2004)	RKF694
w^{1118}; Akh RNAi	VDRC11352	RKF1054
w^{1118}; akhp-GAL4, UAS-mCD8 GFP/CyO	this study	MGF1632
w^{1118}; akhp-GAL4, UAS-mCD8 GFP/CyO; Akh^A* / TM3 *Ser^1*	this study	MGF1639
w^{1118}; akhp-GAL4, UAS-mCD8 GFP/CyO; Akh^{AP}*/TM3 *Ser^1*	this study	MGF1640
w^{1118}; akhp-GAL4, UAS-mCD8 GFP/CyO; Akh^{SAP}*/TM3 *Ser^1*	this study	MGF1641
Canton S		
w^{1118}	VDRC60000	RKF1084

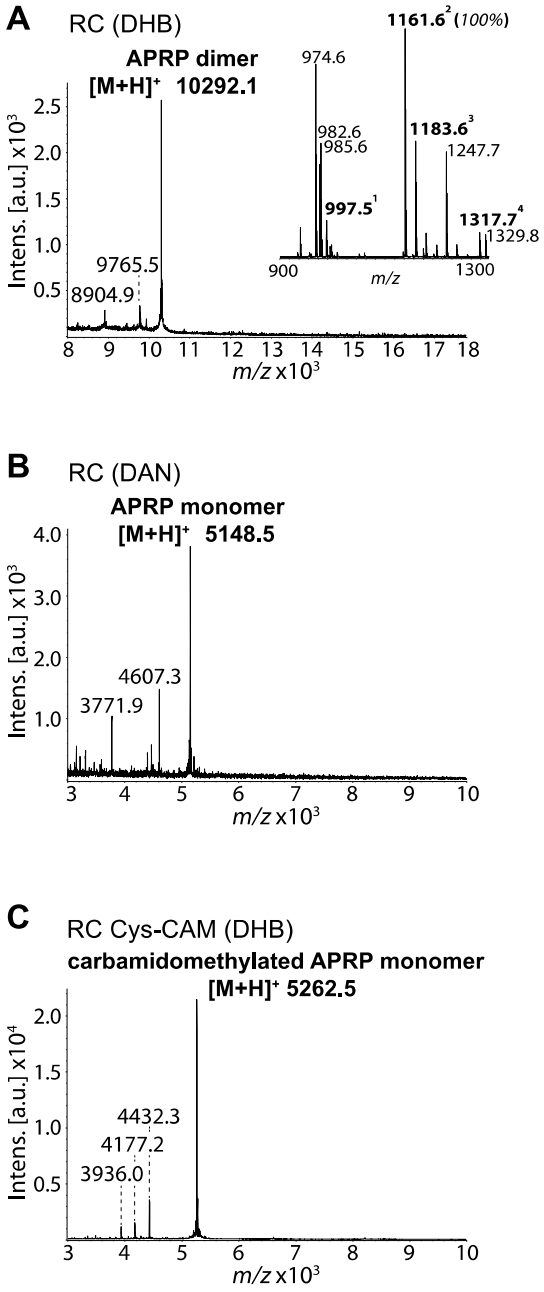

Figure S1 Mass spectrometry characterization of APRP dimers and monomers. MALDI-TOF mass spectra in positive reflectron mode from single *Drosophila* RC preparations before (**A**) and after (**B**) reduction of disulfide bonds. (**C**) Carbamidomethylated APRP monomer in a pooled extract of 20 RCs. (**A**) Direct tissue profiling of a single RC showed mass matches for all known AKH products in the mass range *m/z* 900-1300 (inset, 1 pQLTFSPDWa, 997.5 $[M+Na]^+$; 2 pQLTFSPDWGK-OH, 1161.6 $[M+H]^+$, 3 1183.6 $[M+Na]^+$; 4 pQLTFSPDWGKR-OH, 1317.7 $[M+H]^+$). The ion signal recorded at *m/z* 10292.1 matched a putative APRP dimer. (**B**) Reduction of cysteine bonds by direct profiling of a single RC with DAN matrix revealed a mass match for a possible APRP monomer (*m/z* 5148.5) and confirmed a dimeric structure. The ion signal was not recorded prior to reduction of disulfide bonds. (**C**) Carbamidomethylation of cysteines resulted in a mass shift of *m/z* +114 and confirmed the presence of two cysteines. Subsequent fragmentation of the precursor ion *m/z* 5262.5 confirmed the predicted APRP monomer sequence (see Fig. S2). All ion signals are labeled with monoisotopic masses. Cys-CAM, carbamidomethylation of cysteines.

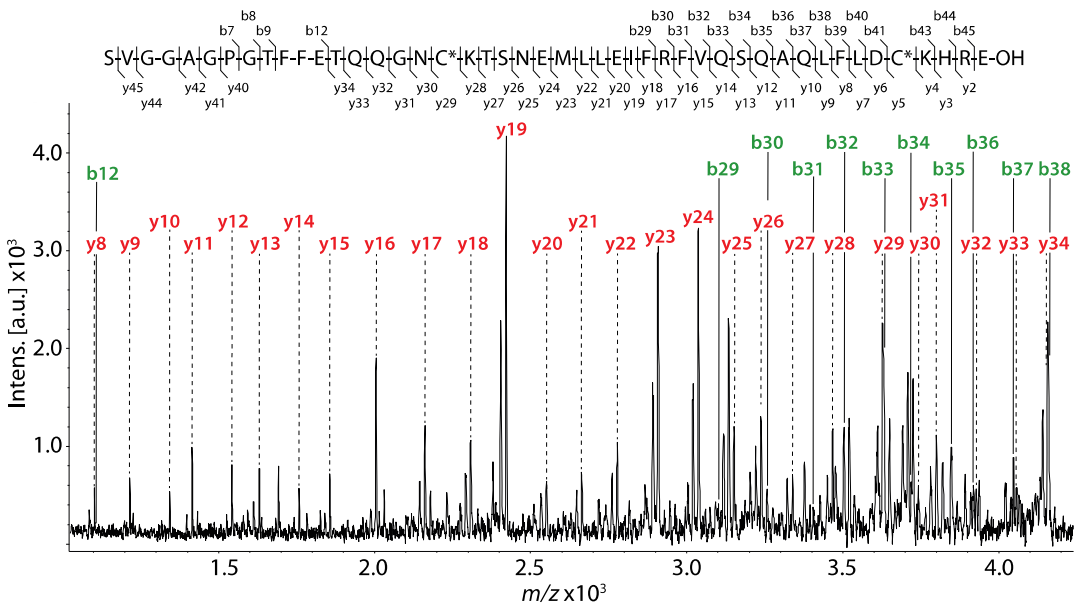

Figure S2 Tandem mass spectrometry characterization of the APRP monomer. Detail of MALDI-TOF MS^2 fragment ion spectrum of the precursor ion at *m/z* 5262.5 $[M+H]^+$ (see Fig. S1). Ion signals of b-type fragments are labeled in green, y-type fragments in red. Identified fragments confirmed the predicted APRP sequence with sequence coverage of 64.4%. C* carbamidomethylated cysteine.

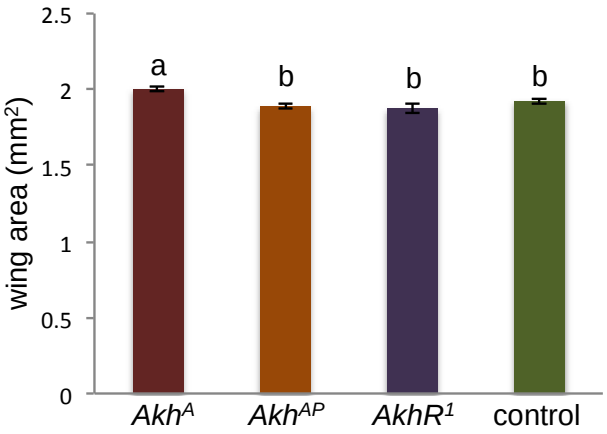

Figure S3 AKH signaling and APRP are dispensable for regulation of body size. Wing area measurement showed slightly increased wing size in *Akh*^{A}, however, the effect was most likely independent of AKH signaling, as *Akh*^{AP} and *AkhR*^{1} had normal size (one-way ANOVA, $F_{3,94}$ = 7.47, P < 0.001).

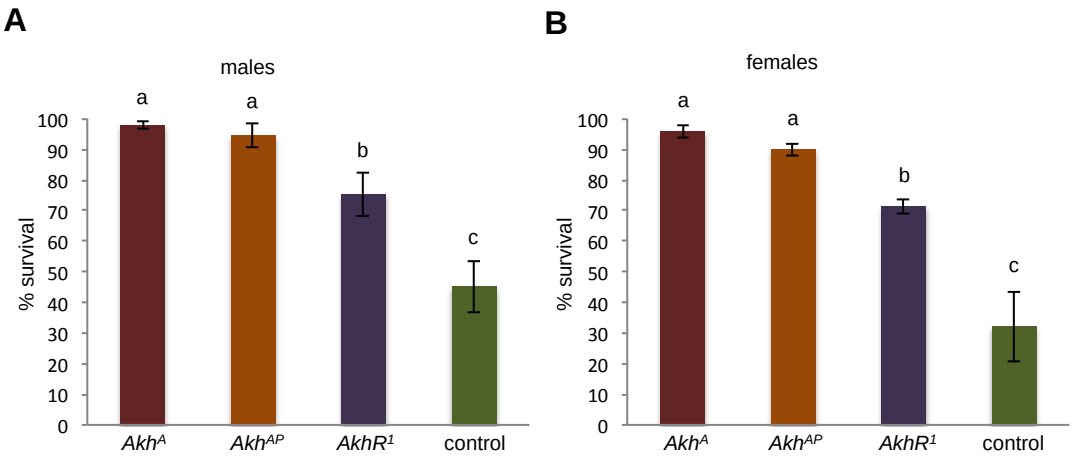

Figure S4 Standard paraquat resistance assay based on drug feeding revealed increased oxidative stress resistance of Akh^{A}, Akh^{AP} and $AkhR^{1}$ mutants when compared to controls. **(A)** Assay conducted on males. Fischer exact test: Akh^{A} vs. control: $P < 0.001$; Akh^{AP} vs. control: $P < 0.001$; $AkhR^{1}$ vs. control: $P < 0.001$; Akh^{A} vs. Akh^{AP}: $P > 0.05$; Akh^{A} vs. $AkhR^{1}$: $P < 0.001$; Akh^{AP} vs. $AkhR^{1}$: $P < 0.001$ (n[Akh^{A}] = 97, n[Akh^{AP}] = 110, n[$AkhR^{1}$] = 81, n[control] = 98). **(B)** Assay conducted on females. Fischer exact test: Akh^{A} vs. control: $P < 0.001$; Akh^{AP} vs. control: $P < 0.001$; $AkhR^{1}$ vs. control: $P < 0.001$; Akh^{A} vs. Akh^{AP}: $P > 0.05$; Akh^{A} vs. $AkhR^{1}$: $P < 0.001$; Akh^{AP} vs. $AkhR^{1}$: $P < 0.001$ (n[Akh^{A}] = 109, n[Akh^{AP}] = 81, n[$AkhR^{1}$] = 92, n[control] = 116).

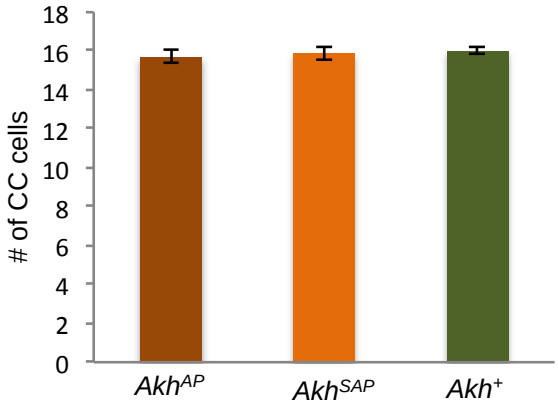

Figure S5 Corpora cardiaca cell number is independent of APRP. Represented are cell counts of CC cells tagged by fluorescence under indirect control of the *Akh* promoter (*akhp*-Gal4 > *UAS*-mCD8 GFP) in Akh^{AP} and Akh^{SAP} mutants compared to Akh^{+} controls (one-way ANOVA, $F_{2,27}$ = 0.3, P =0.74). Note that the Akh^{+} control is identical to the one used in Figure 9A.

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(if not represented in the main text references)

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