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Period-regulated feeding behavior and TOR signaling modulate survival of infection

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SUMMARY

Most metazoans undergo dynamic, circadian-regulated changes in behavior and physiology. Currently it is unknown how circadian-regulated behavior impacts immunity against infection. Two broad categories of defense against bacterial infection are resistance, control of microbial growth, and tolerance, control of the pathogenic effects of infection. Our study of behaviorally arrhythmic *Drosophila* circadian *Period* mutants identified a novel link between nutrient intake and tolerance of infection with *B. cepacia*, a bacterial pathogen of rising importance in hospital-acquired infections. We found that infection tolerance in wild-type animals is stimulated by acute exposure to dietary glucose and amino acids. Glucose-stimulated tolerance was induced by feeding or direct injection; injections revealed a narrow window for glucose-stimulated tolerance. In contrast, amino acids stimulated tolerance only when ingested. We investigated the role of a known amino acid-sensing pathway, the TOR (Target of Rapamycin) pathway, in immunity. TORC1 is circadian-regulated and inhibition of TORC1 decreased resistance, as in vertebrates. Surprisingly, inhibition of the less well-characterized TOR complex 2 (TORC2) dramatically increased survival, through both resistance and tolerance mechanisms. This work suggests that dietary intake on the day of infection by *B. cepacia* can make a significant difference in long-term survival. We further demonstrate that TOR signaling mediates both resistance and tolerance of infection and identify TORC2 as a novel potential therapeutic target for increasing survival of infection.

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AUTHOR CONTRIBUTIONS

MSH, VWA, RMO, and MJU designed experiments. Experiments were performed by: VWA, RMO, and CGZ (survival, bacterial load); VWA (AMPs, starvation); RMO (metabolic storage); MJU (Western blots); EFS (phagocytosis); VMH (melanization); KRM and WWJ (feeding). MSH, JCC, VWA, and RMO produced the manuscript.

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INTRODUCTION

Evolutionarily conserved circadian mechanisms regulate daily, dynamic changes in animal behavior and physiology [1]. The core circadian clock is composed of four transcriptional regulators paired as two heterodimers in an auto-regulatory transcriptional negative feedback loop [2]. In *Drosophila*, Clock and Cycle form one heterodimer and Timeless (Tim) and Period (Per) form the other. Clock and Cycle are transcriptional activators, promoting the expression of *Tim* and *Per* as well as hundreds of tissue-specific target genes [1, 3, 4]. Circadian oscillations in gene expression are thought to cause circadian oscillations in physiological function and ultimately organismal behavior.

We previously found that *Drosophila* innate immunity against *S. pneumoniae* infection is circadian-regulated [5, 6]. For both flies and vertebrates, innate immunity is the first line of defense against infection. *Drosophila* lack adaptive immune components such as T cells and B cells and rely on innate immune responses to survive infection [7]. Evolutionary conservation extends to the two primary *Drosophila* immune signaling pathways, the Toll and Imd pathways [8]. Flies and vertebrates employ several similar innate immune mechanisms to kill bacteria, including phagocytosis by immune cells, reactive oxygen species generation (melanization in flies), and secretion of antimicrobial peptides (AMPs).

Resistance is only one type of defense against bacterial infection. Resistance mechanisms such as the immune functions listed above control bacterial proliferation, reducing pathogenesis by decreasing the host's pathogen burden. A second distinct, complementary type of defense is termed tolerance [9, 10]. Tolerance physiologies allow the organism to survive the pathological effects of infection—caused by microbes or the host immune response—without necessarily decreasing bacterial load [11, 12].

Tolerance physiologies are not well understood, but include feeding and metabolism. In *Drosophila*, decreased survival of infection for two bacterial pathogens, *M. marinum* or *L. monocytogenes*, is associated with decreased metabolic stores [13, 14]. The effect of feeding behavior on infection is pathogen-specific: decreased feeding increases survival of *S. typhimurium*, *E. coli*, and *E. caratova* infections, but decreases survival of *L. monocytogenes* infection [15, 16]. In most cases, the precise nutrients important for survival and underlying molecular signaling pathways have not been identified.

Both feeding behavior and metabolic gene expression are circadian-regulated, and both fly and mouse circadian mutants exhibit metabolic disorders and altered feeding behavior [17, 18]. While we and others have shown previously that host resistance against specific pathogens is circadian-regulated, it is not clear whether loss of circadian-regulated metabolism and feeding behavior affect immunity against infection [5, 6, 19].

Here we exploit a rapid, lethal infection of *Drosophila* with the human pathogen *Burkholderia cepacia* to examine how acute differences in feeding behavior and diet impact infection tolerance. *B. cepacia* is a significant cause of hospital-acquired infection and tolerance mechanisms increasing survival of this infection are currently unknown [20]. We found that Per^{01} circadian mutants have increased tolerance to infection with *B. cepacia* and that increased tolerance is dependent on increased nutrient intake. In wild-type flies,

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infection tolerance is stimulated by influx of dietary glucose and amino acids at the time of infection. Because the TOR pathway is a classic amino-acid sensor, we asked whether TOR kinase mediates infection tolerance [21]. TOR associates with two related but distinct complexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC2), which in some contexts have opposite effects [22, 23]. We found that TORC1 activity is circadian-regulated and that TORC1 activates resistance, as observed in vertebrates [24]. In contrast, the less well-characterized TORC2 had the opposite effect on survival and inhibits both resistance and tolerance. This work suggests that specific pharmacological TORC2 inhibitors could provide novel host-directed therapeutics for survival of infection.

RESULTS

Period (Per^{01}) mutants are more tolerant of *B. cepacia* infection than wild type

We found that arrhythmic *Per^{01} Drosophila* mutants survived longer than isogenic wild-type controls when infected with the human pathogen *Burkholderia cepacia*, a previously described infection model [25–27] (Figure 1A–B, $p<0.0001$). To determine whether this increased survival was due to altered resistance or tolerance, we measured bacterial loads of individual flies during infection. Whether the kinetics of survival were slow (over days, 18°C) or fast (over hours, 29°C), wild type and *Per^{01}* mutants carried equivalent bacterial loads (Figure1C–D, $p>0.05$ for each time point). This result suggests that the enhanced survival of *Per^{01}* mutants is not due to greater resistance, but due to greater host tolerance.

Known resistance mechanisms do not explain increased survival of infection

To confirm that *Per^{01}* mutants are more tolerant of *B. cepacia* infection, we analyzed three well-characterized resistance mechanisms following infection: antimicrobial peptide (AMP) induction, melanization, and phagocytosis. We found no significant differences between wild type and *Per^{01}* mutants in *B. cepacia*-induced AMP expression (Figure 1E–F, Figure S1A–E) or systemic melanization, typically not induced by *B. cepacia* (Figure 1G) [28]. While inhibition of phagocytosis by bead pre-injection decreased survival of both *Per^{01}* and wild-type controls (both $p<0.0001$), *Per^{01}* mutants still survived significantly longer than wild type (Figure 1H, $p<0.0001$), suggesting that phagocytosis is not responsible for the increased survival of *Per^{01}* mutants. Taken together, these results suggest that *Per^{01}* mutants have increased tolerance, not resistance, during *B. cepacia* infection.

Per mutants have decreased energy storage

We hypothesized that increased metabolic stores underlie the increased tolerance of *Per^{01}* mutants. Metabolic gene expression is circadian-regulated [17, 18], and increased metabolic stores underlie increased survival during infection with two other facultative intracellular bacterial pathogens, *M. marinum* and *L. monocytogenes* [13, 14]. If *Per^{01}* mutants have increased metabolic reserves, they should be less susceptible to starvation. In contrast, we found that *Per^{01}* mutants starve more quickly than wild-type controls (Figure 2A, $p<0.0001$), suggesting that *Per^{01}* mutants have fewer metabolic reserves than wild type. To test this, we measured three major types of energy storage: fat (triglycerides), glycogen, and circulating sugars (trehalose and glucose). Consistent with sensitivity to starvation, uninfected *Per^{01}* mutants had significantly lower levels of triglycerides ($p=0.0004$) and

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glycogen (p=0.0007), while trehalose and glucose levels were similar to wild type (p=0.7065) (Figure 2B).

Although *Per*^{01} mutants have lower metabolic reserves than wild type before infection, *Per*^{01} mutants may have higher metabolic reserves during infection. To test this, we measured metabolic reserves during *B. cepacia* infection. Both *Per*^{01} mutants and wild type lost energy stores during infection, but *Per*^{01} mutants maintained the same or lower energy stores than wild type (Figure 2C). At 16 hours post-infection, just before flies begin to die, triglyceride levels in *Per*^{01} mutants were still lower than wild type (~70% of wild type, p=0.0001), with levels of circulating sugars and glycogen similar to wild type (p=0.9314 and 0.4804, respectively). These data indicate that the increased tolerance of *Per*^{01} mutants is not due to greater energy stores up until the lethal phase of infection.

Per^{01} mutants exhibit increased feeding behavior

Because *Per*^{01} mutants have low metabolic reserves, we hypothesized that they eat more than wild type and that this increased feeding itself enhances infection tolerance. To test this, we measured the consumption of ^{32}P-labeled, solid food (Figure 2D) [29, 30] and liquid food using the Capillary Feeder (CAFE) assay (Figure 2E) [29, 31]. In the ^{32}P-labeled food assay, *Per*^{01} mutants ate 14% more than wild type; in the CAFE assay, *Per*^{01} mutants ate 23% more than wild type (Figure 2D, p=0.016; Figure 2E, p=0.034). These results resemble those of Xu et al. with flies expressing a dominant-negative form of Clock (another core circadian regulator) in metabolic tissues [17]. Thus *Per*^{01} mutants exhibit significantly greater food intake than wild type.

Nutrient availability enhances infection tolerance of *Per*^{01} mutants

If the increased survival of *Per*^{01} mutants is due to increased feeding, then decreasing nutrient intake by dietary restriction should abolish the enhanced survival time of *Per*^{01} mutants after *B. cepacia* infection. To restrict dietary intake, flies were fed a low sugar, protein-free diet containing only water, agar, and 1% glucose ~24 hours before and during infection and compared to flies on standard diet (Figure 3A). We found that this restricted diet decreased survival time after high-dose infection for both wild type (Figure 3B, p<0.0001) and *Per*^{01} mutants (Figure 3D, p<0.0001). *Per*^{01} mutants survived significantly longer than wild type flies when fed standard food (20/20 experiments), with an average of 22% increased median survival time. In contrast, diet-restricted *Per*^{01} mutants either had no survival advantage over wild type (4/12 experiments), survived significantly less well than wild type (2/12 experiments), or survived an average of only 7% longer than wild type (6/12 experiments) (Figure 3F). Bacterial loads remained unchanged under all feeding conditions (Figure 3C,E,G; p>0.05 for all time points). Thus dietary restriction decreases host tolerance of infection. While we cannot exclude the possibility that dietary restriction overrides differences between *Per*^{01} mutants and wild type by a different mechanism than that causing increased tolerance in *Per*^{01} mutants, these results suggest that the increased feeding behavior of *Per*^{01} mutants on the day of infection contributes to their increased tolerance of *B. cepacia* infection.

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Dietary glucose and amino acids enhance infection tolerance in wild type flies

To identify specific dietary components contributing to tolerance of infection, we supplemented the restricted diet with defined nutrients (Figure 4A). Because Per^{01} mutants display pleiotropic defects in metabolism and other circadian-regulated physiologies [3], we focused on wild-type flies. We first tested if increased dietary glucose complements the restricted diet, which contains 1% glucose, by comparing the effects of titrating dietary glucose (1%, 5%, 10%, or 15% glucose, no protein) with standard food (5–10% sugar, plus yeast extract). Wild-type flies exhibited shortest survival time when switched to 1% dietary glucose 24 hours before infection and survived longest on standard food (Figure 4B, $p<0.0001$ comparing standard food or 1% glucose with any other condition). While increasing dietary glucose from 1% to 5% increased survival time (Figure 4B, $p<0.0001$), further increases in dietary glucose did not (4B, $p>0.05$ for any pair-wise comparison of 5%, 10%, and 15% glucose). Despite the survival benefit conferred by 5% glucose relative to 1% glucose, bacterial load was unchanged (Figure 4D, $p>0.05$ for all time points). Moreover, no glucose-only diets increased survival time to that observed on standard food ($p<0.0001$). Thus glucose enhances infection tolerance, but glucose alone is not sufficient for optimal survival of infection. This result suggests that other components in standard food also contribute to survival of *B. cepacia* infection.

In addition to sugar, standard food contains a complex mixture of lipids, proteins, vitamins, and other nutrients derived from yeast and cornmeal ingredients. We tested whether 5% glucose supplemented with amino acids was sufficient to substitute for standard food. A diet of 5% glucose plus amino acids 24 hours before infection significantly increased survival time relative to 5% glucose alone (Figure 4C, $p<0.0001$, Figure S2A), with no change in bacterial load (Figure 4E, all time points $p>0.05$). In fact, 5% glucose plus amino acids was sufficient to increase survival time to that observed with standard food (Figure 4C, $p>0.05$). The survival benefit of amino acids was not dependent on high glucose and was also observed with 1% glucose diet (Figure S2B). Thus both dietary glucose and amino acids contribute to tolerance of infection, and acute exposure to both nutrients ~24 hours before *B. cepacia* infection is necessary for optimal survival.

Glucose is required at the time of infection for increased host tolerance

We set out to more precisely characterize the required timing of the glucose contribution to infection tolerance. We found that a 50 nL injection of 5% glucose administered into the circulatory system of diet-restricted flies could significantly increase infection survival time relative to buffer injection (Figure 4F, $p=0.0007$). This dose of glucose is equivalent to the quantity ingested by a single fly in 1 hour (calculated from feeding experiments; Figure 2D – E). Glucose injection most often promoted survival when administered within 2 hours before or at the time of infection (Figure 4F, Figure 5/8 experiments). In contrast, glucose injected more than 2 hours before infection or after infection rarely provided any survival benefit (Figure S2C–D, 1/11 experiments). Thus, with our infection protocol, the effective time window for glucose-induced survival is unexpectedly narrow, consistent with an acute rather than chronic effect of diet upon infection tolerance. These results suggest that acute glucose intake stimulates specific signaling pathways that increase immune tolerance when activated around the time of infection.

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Injection of amino acids at two different concentrations at different time points before or during infection did not improve survival time (Figure 4G, amino acids vs. buffer injection, $p>0.05$; also Figure S2E–G). Flies injected with buffer were still able to respond to dietary amino acids (Figure 4G, $p<0.0001$). Thus, in contrast to glucose, amino acids appear to stimulate infection tolerance only when ingested and not when injected.

Increased TORC1 signaling correlates with increased survival for *Per*^{01} mutants and flies with greater nutrient availability

Since transient exposure to nutrients enhances infection tolerance, we next wanted to determine whether molecular pathways stimulated by these nutrients play a role in survival of *B. cepacia* infection. The role of insulin-like signaling during infection has been characterized in *Drosophila* [13, 32–35]. We focused instead on the less well-characterized role of the kinase TOR in innate immunity, as TOR complex 1 (TORC1) is the canonical sensor of amino acid availability [21].

We first set out to determine if TORC1 kinase activity is circadian-regulated by monitoring phosphorylation of its downstream target S6K over the circadian cycle in wild type and *Per*^{01} mutants. We found that TORC1 activity oscillates over the circadian cycle in wild-type flies, with a peak of activity at ZT9–13 (Figure 5A). This peak of TORC1 activity correlates with low Per protein levels in wild type [36]. Consistent with this, TORC1 activity did not oscillate in *Per*^{01} mutants and exhibited high, equivalent levels at both ZT9 and ZT21 (Figure 5B). Thus TORC1 activation is circadian-regulated and increased in *Per*^{01} mutants during the time course of infection, suggesting that increased TORC1 activation may contribute to *Per*^{01} mutants' increased survival of infection.

We next tested TORC1 activity of wild-type flies in dietary conditions associated with increased survival of infection. We found that TORC1 activity was higher in flies fed food containing amino acids than in flies fed food without amino acids (Figure 5C, all $p\leq0.0163$). Thus both wild-type flies on nutrient-rich diets and *Per*^{01} mutants exhibit increased TORC1 kinase activity. Interestingly, TORC1 activity is higher in flies fed 5% glucose plus amino acids than those fed standard food ($p=0.0014$), suggesting that TORC1 activity may not solely mediate differences in survival.

Decreased TORC1 signaling causes decreased resistance

To directly test the role of TORC1 in survival of infection, we inhibited TORC1 activity in two ways. First, we injected flies with rapamycin, a TORC1-specific inhibitor (9.6 ng per fly, equivalent to the mammalian dose of 16 mg/kg [37]) [38]. Injection of rapamycin inhibited survival of infection relative to injection of buffer alone (Figure 5D, $p<0.0001$). Unexpectedly, we found that rapamycin-injected flies had increased bacterial load, indicating decreased resistance (Figure 5E, $p>0.05$, $p=0.0049$, $p=0.0198$). Second, we inhibited TORC1 activity using a temperature-driven system to over-express Tsc1 and Tsc2, proteins forming a TORC1-inhibitory complex [39]. Tsc1/2 over-expression was confirmed by qRT-PCR (Figure S3A – B). Similar to rapamycin injection, genetic inhibition of TORC1 reduced survival after *B. cepacia* infection (Figure 5F, $p<0.0001$ for both controls) and caused increased bacterial loads (Figure 5G, $p>0.05$, $p=0.0367$, $p=0.0022$). Taken together,

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these results suggest that in flies, as in vertebrates [40, 41], TORC1 mediates resistance against *B. cepacia* infection. While inhibition of TORC1 in Per^{01} mutants with rapamycin injection decreased their survival after infection (Figure S3C), rapamycin injection did not abolish Per^{01} mutants' survival advantage over wild-type controls (Figure S3D), suggesting that increased TORC1 activity is not solely responsible for their increased survival.

Increased resistance is correlated with decreased TORC2 signaling

TOR kinase associates with another, less well-understood complex, TORC2. Since TORC1 and TORC2 might compete for limited TOR kinase and these complexes appear to have opposing roles in cell growth and T cell differentiation [22, 23], we next asked whether TORC2 activity underlies infection tolerance. TORC2 is not known to play a role in survival of infection. To test this, we reduced TORC2 signaling in two ways.

First, we examined the survival of mutants lacking RicTOR, an essential molecular component of TORC2 but not TORC1, after *B. cepacia* infection [42]. $RicTOR^{\Delta 1/\Delta 2}$ mutants had the opposite survival phenotype as that seen with TORC1 inhibition: they lived dramatically longer than isogenic controls (Figure 6A, $p<0.0001$). We also found that $RicTOR^{\Delta 1/\Delta 2}$ mutants carried decreased bacterial load relative to wild type (Figure 6B, $p>0.05$, $p=0.0087$, $p=0.0022$). These results suggest that, while TORC1 activates resistance, TORC2 inhibits resistance.

To confirm this, we examined mutants lacking Sin1, another TORC2-specific component [43]. Similar to $RicTOR^{\Delta 1/\Delta 2}$ mutants, $Sin1^{e03756}$ mutants exhibited increased survival time after infection and decreased bacterial load relative to wild type (Figure 6C, $p<0.0001$, Figure 6D, $p>0.05$, $p>0.05$, $p=0.0043$). Thus, inhibition of TORC2 by loss of either RicTOR or Sin1 increased both survival and resistance against *B. cepacia* infection.

Because increased tolerance is defined functionally as increased survival without decreased bacterial load, increased resistance due to dietary TORC1 activation might mask increased tolerance due to genetic TORC2 inhibition. We therefore tested $Sin1^{e03756}$ mutants for survival of infection and bacterial load in the absence of dietary amino acids. Consistent with TORC2 inhibition of tolerance, $Sin1^{e03756}$ mutants survived infection longer than wild type with no decrease in bacterial load (Figure 6E, $p=0.0051$, Figure 6F, all $p>0.05$). Interestingly, $Sin1^{e03756}$ mutants without amino acids had identical survival kinetics and bacterial load as wild type flies fed amino acids, suggesting that amino acids had an equivalent effect on tolerance as loss of Sin1 (Figure S4A – B). These results suggest that Sin1, an essential component of TORC2, inhibits both resistance and tolerance of *B. cepacia* infection.

DISCUSSION

By examining a circadian mutant with increased infection tolerance against *B. cepacia*, we identified increased feeding as a circadian-regulated behavior contributing to increased tolerance. Increased feeding by Per^{01} mutants was not associated with increased energy stores, suggesting that their increased tolerance does not depend on metabolic reserves. Two specific nutrients, glucose and amino acids, fully substitute for standard food in promoting

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optimal tolerance after *B. cepacia* infection. Our data suggest a narrow window for glucose's contribution to survival—with this rapid infection, an increase in circulating glucose in the two hours before infection can increase overall survival time. This is consistent with the hypothesis that nutrient sensing leads to an acute activation of infection tolerance (Figure 7). Thus what and how much a fly ingests near the time of infection has a significant effect on its survival of infection.

To explore the effects of dietary amino acids on survival of infection, we investigated the role of TORC1 signaling, a canonical amino acid sensing pathway. We found that TORC1 kinase activity oscillates with circadian rhythm, likely through circadian-regulated feeding behavior as seen in vertebrates [44, 45]. We also uncovered a role for TORC1 in resistance against infection in *Drosophila*. In vertebrates, TORC1 is known to mediate resistance and rapamycin is a well-characterized immunosuppressant; however, these immunosuppressive effects are thought to result primarily from inhibiting the growth and maturation of dendritic cells and T-cells [24], adaptive immune cell types with no clear functional analogs in *Drosophila*. Our data now suggest a role for TORC1 in innate immunity against infection (Figure 7). It remains to be seen whether rapamycin acts as an immuno-suppressant for *Drosophila* infected with other pathogens besides *B. cepacia*. These results potentially open the genetically tractable system of *Drosophila* to investigating TORC1 interactions with innate immune components.

We further found a novel role for the less well-known TOR complex 2 as a potent inhibitor of immunity—that is, loss of TORC2-specific components RicTOR or Sin1 caused dramatic increases in survival time after infection and impacted both resistance and tolerance (Figure 7). Loss of Sin1 increases resistance in the presence of amino acids and increases tolerance in the absence of amino acids. Because there exists a resistance phenotype, possibly due to amino acids-stimulation of TORC1, we cannot say whether loss of Sin1 increases tolerance in the presence of amino acids, as host tolerance is functionally defined as changes in survival in the absence of correlated changes in bacterial load. $RicTOR^{\Delta 1/\Delta 2}$ mutants in the presence or absence of amino acids exhibit increased resistance to infection (Figure S4C–D). The disparity between $Sin1^{e03756}$ and $RicTOR^{\Delta 1/\Delta 2}$ mutants could be due to differences in the distribution of TOR between TORC1 and TORC2 lacking one component or the other. Our results suggest that TORC1 and TORC2 act in opposition during immunity and we speculate that these complexes may be oppositely circadian-regulated--that is, *Per* mutants have high TORC1 and low TORC2 activity.

The finding that TORC2 inhibition increases survival of infection is surprising but not completely without precedent. TORC2 is mainly thought to play a role in tissue-specific morphology, stimulated by growth factors and PI3K and acting on downstream targets such as cytoskeletal components, Akt, and SGK1 [38, 46, 47]. In *Drosophila*, TORC2 has been implicated in tolerance of heat stress [48], cell and tissue growth [49, 50], and neuronal outgrowth [51, 52]. While most immune effects of TOR are thought to act through TORC1, recent evidence suggests that, in mouse embryonic fibroblasts, RicTOR inhibits Toll-like receptor-stimulated cytokine expression [53]. Thus RicTOR may have conserved immune-suppressive effects in both vertebrates and invertebrates. While the direct targets of TORC2

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relevant for infection resistance and tolerance remain unknown, their identification will be an important goal of future studies.

The cellular and molecular mechanisms that promote host tolerance of infection are not well-understood [10, 54]. *B. cepacia* is a significant opportunistic bacterial pathogen, particularly in hospital settings with susceptible patients [20]. This hospital-acquired infection can be associated with high rates of mortality, up to 50% for severe strains, and is often antibiotic-resistant [55, 56]. Understanding the tolerance mechanisms stimulated by acute glucose and dietary amino acids will help to identify targets for pharmacological treatments. Here we have identified TORC2 as a potential pharmacological target to increase host survival time after infection, as TORC2 mutants are able to survive infection up to 59% longer than wild type. The potential therapeutic value of TORC2 inhibition has not been explored, as there are currently no known small molecule inhibitors specific to TORC2 and not TORC1. The *Drosophila* model of infection described here may therefore prove useful in screening for such TORC2-specific inhibitors and for further dissection of acute, nutrient-stimulated, TOR-mediated host defenses against bacterial infections such as *B. cepacia*.

EXPERIMENTAL PROCEDURES

(See Supplemental Information for details.)

Fly strains

$w^{1118}per^{01}$ (null) mutants [36] were outcrossed with a w^{1118} Canton S strain, used as isogenic controls [57]. Wild-type Oregon R flies were used to test effects of dietary components and rapamycin. *UAS-Tsc1/Tsc2* (from Marc Tatar [58]) homozygous males were crossed to w^{1118}*;tub>Gal80-ts;tub>Gal4/TM6c* virgins and maintained at 18°C until 29°C transgene induction 24 or 48 hours before infection. *RicTOR* null mutants (imprecise p-element excision alleles $RicTOR^{01}$ and $RicTOR^{02}$) and precise excision controls were obtained from Stephen Cohen [50]. Experiments used trans-heterozygous $RicTOR^{01}$/ $RicTOR^{02}$ flies. $Sin1^{e03756}$ (SAPK-interacting protein 1) mutants are null piggyBac transposon insertion mutants from Bloomington *Drosophila* Stock Center, stock #18188 [50]. 5–10 day-old males raised on standard molasses food were used for all experiments.

Infections

Infections were performed as described [26] with *Burkholderia cepacia* (ATCC strain #25416). Death was assayed visually the next day every hour or more frequently as needed. Survival curves are plotted as Kaplan-Meier graphs and log-rank analysis performed using GraphPad Prism. All infection experiments were performed with a minimum of 3 independent trials and yielded statistically similar results, except where noted. Graphs and p-values in figures are representative trials.

Bacterial load quantitation

Bacterial load was quantified as described [26] and analyzed by unpaired t-tests for 0 hour time points; subsequent time points were analyzed with non-parametric Mann-Whitney tests,

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which does not assume normal distribution as bacteria grow exponentially. Data are plotted with SEM.

qRT-PCR, melanization, and phagocytosis assays

Assays were performed as described, using *B. cepacia* for infection [6, 59]. p-values for AMP induction and melanization were obtained by t-tests for three independent trials; data are represented as mean ± SEM. p-values for phagocytosis assays were obtained by log-rank analysis. See Supplement for primer sequences.

Starvation assay

Using the DAM5 system (TriKinetics), 5–7 day old male flies were incubated on agar alone. Time of death was determined by complete loss of movement. p-values were obtained by log-rank analysis.

Metabolic storage assays

Samples consisted of 8 male flies (5–10 days old) homogenized in buffer. Metabolic storage levels were measured by enzyme-based colorimetric assays as described [13, 60]. Values were normalized to the average weight for that genotype and to the mean value for wild type, then plotted with the normalized SEM. p-values were obtained by unpaired t-test.

Feeding assays

CAFE assays and ^{32}P feeding assays were performed as described [29–31]. p-values were obtained by unpaired t-test; data are represented as mean ± SEM.

Protein extraction and Western blotting

Western blot analysis of whole-fly homogenates was performed by standard methods using 1:1000 anti-phospho-S6K (Thr398) (Cell Signaling #9209), 1:10,000 anti-Actin-HRP (Sigma A3854), and 1:2000 anti-rabbit-HRP (Cell Signaling #7074). p-values were obtained by unpaired t-test; data are represented as mean ± SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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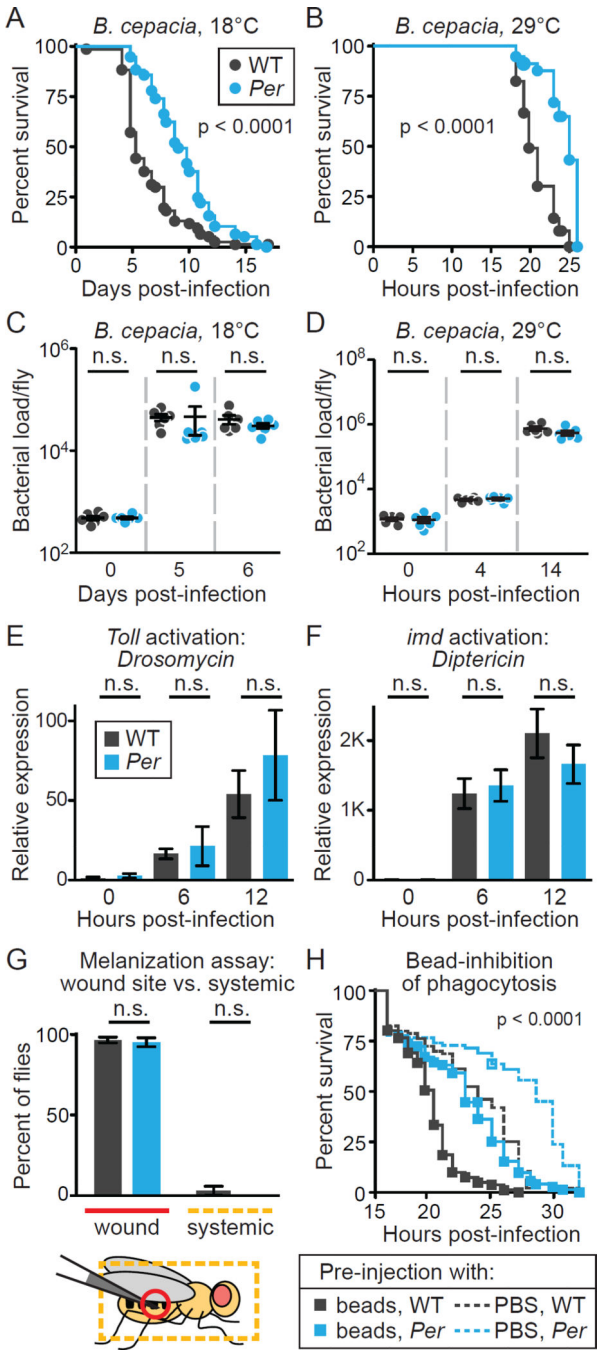

Figure 1. *Period* mutants exhibit greater tolerance than isogenic controls during infection with *B. cepacia*

Per^{01} mutants (blue) survived longer than wild type (dark grey) during A) a long infection (low dose at low temperature, 18°C; Per^{01}, n=78; WT, n=77, p<0.0001) and B) a short infection (high dose at high temperature, 29°C; Per^{01}, n=57; WT, n=64, p<0.0001) with *B. cepacia*. Per^{01} mutants and wild type flies had similar bacterial loads over time following a C) long infection (n≥4 flies/time point, all n.s.) and D) short infection (n=6 flies/time point, all n.s.) with *B. cepacia*. Consistent with a tolerance phenotype, antimicrobial peptide (AMP) induction via the *Toll* and *imd* pathways did not differ between Per^{01} mutants and

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wild type flies after *B. cepacia* infection as shown by: E) *Drosomycin* and F) *Diptericin* (n=3 samples of 6 flies each, all n.s.). Other AMPs are shown in Figure S1 (n=3 samples of 6 flies each, all n.s.). G) Per^{01} mutants and wild type flies did not exhibit differences in systemic and injection wound site melanization after *B. cepacia* infection (3 trials, n=17 – 22 flies/trial/genotype, all n.s.). H) Inhibition of phagocytosis by bead pre-injection did not block the Per^{01} mutant survival advantage over wild type after *B. cepacia* infection (Per^{01}, n=76 with beads, n=81 with buffer; wild type, n=81 with beads, n=80 with buffer; $p<0.0001$ for all pair-wise curve comparisons except WT buffer vs. Per^{01} with beads, n.s.). p-values for survival curve comparisons were obtained by log-rank analysis; p-values for bacterial load comparisons were obtained using unpaired t tests for 0 hour time points, while subsequent time points were tested with non-parametric Mann-Whitney U tests; p-values for AMP and melanization comparisons were obtained using unpaired t tests; error bars represent the mean ± S.E.M.; n.s.=not significant ($p>0.05$).

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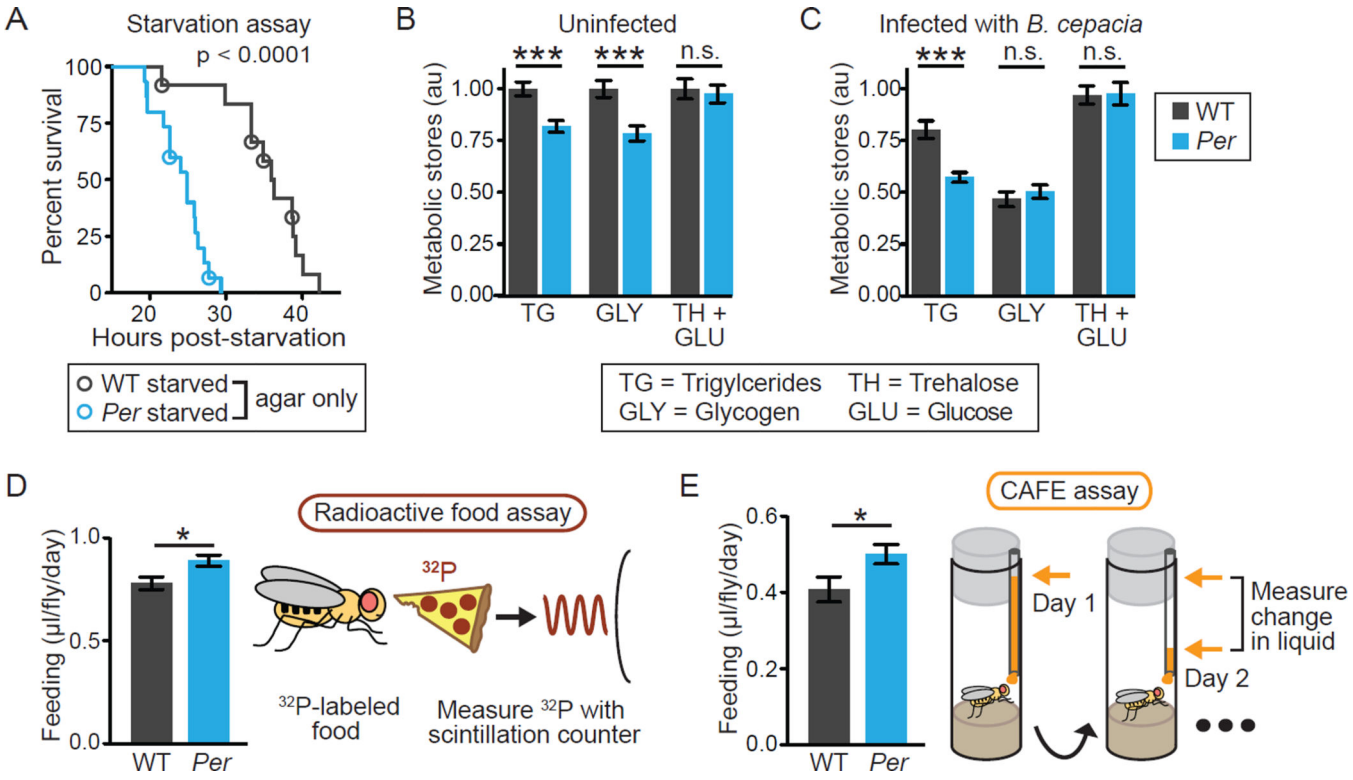

Figure 2. *Per*^{01} mutants have lower metabolic resources and eat more than wild type flies

A) Uninfected *Per*^{01} mutants were more sensitive to starvation than uninfected wild type flies (*Per*^{01} n=15; WT n=12; p<0.0001). B) Quantification of metabolic storage levels comparing uninfected *Per*^{01} mutants and wild type flies (n=12 for both) revealed that *Per*^{01} mutants had lower levels of triglycerides (p=0.0004) and glycogen (p=0.0007) and similar levels of primary circulating sugars (n.s.). C) 16 hours after infection with *B. cepacia*, *Per*^{01} mutants relative to wild type (n=12 for both) had lower levels of triglycerides (p=0.0001) and similar levels of glycogen and primary circulating sugars (both n.s.). D) In the radioactive food assay, *Per*^{01} mutants ate ~14% more than wild type (*Per*^{01} n=9; WT, n=9, p=0.016). E) In the CApillary FEeder (CAFE) assay, *Per*^{01} mutants ate ~23% more than wild type (*Per*^{01}, n=24; WT, n=21; p=0.034). p-values were obtained by unpaired t-test; error bars represent the mean ± S.E.M.; n.s.=not significant (p>0.05); *=p≤0.05; ***=p≤0.001.

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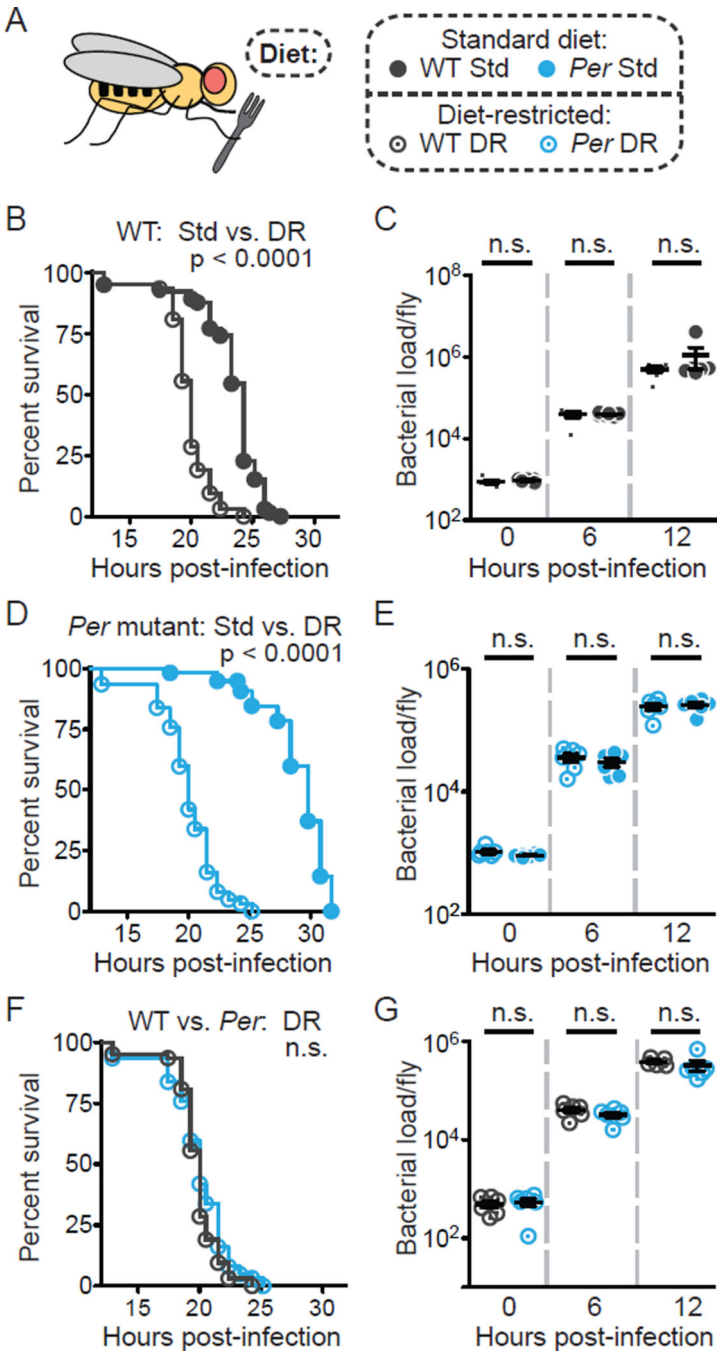

Figure 3. Dietary restriction does not increase infection tolerance of either *Per*^{01} mutants or wild type

A) Schematic of dietary conditions: wild-type flies and *Per*^{01} mutants were raised on standard food (Std) and then transferred to fresh Std food or subjected to dietary restriction on 1% glucose (DR) for 24 hours prior to and during *B. cepacia* infection. Dietary restriction decreased survival time after infection for both B) wild type (Std food n=66, DR n=63, p<0.0001) and D) *Per*^{01} mutants (Std food n=59, DR n=62, p<0.0001). F) Dietary restriction eliminated the consistent survival advantage of *Per*^{01} mutants over wild-type flies (*Per*^{01} n=62, WT n=63, n.s.). Dietary restriction did not alter bacterial load for C) wild type (n≥5

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flies/time point) or E) *Per*^{01} mutants (n≥5 flies/time point, n.s., all time points); moreover, G) diet-restricted wild type and *Per*^{01} mutants had similar bacterial loads (n≥5 flies/time point, n.s.). p-values were obtained by unpaired t-test (0h) and non-parametric Mann-Whitney test (other time points). p-values for survival curve comparisons were obtained by log-rank analysis; p-values for bacterial load comparisons were obtained using unpaired t tests for 0 hour time points, while subsequent time points were tested with non-parametric Mann-Whitney U tests. Error bars represent the mean ± S.E.M.; n.s.=not significant ($p>0.05$).

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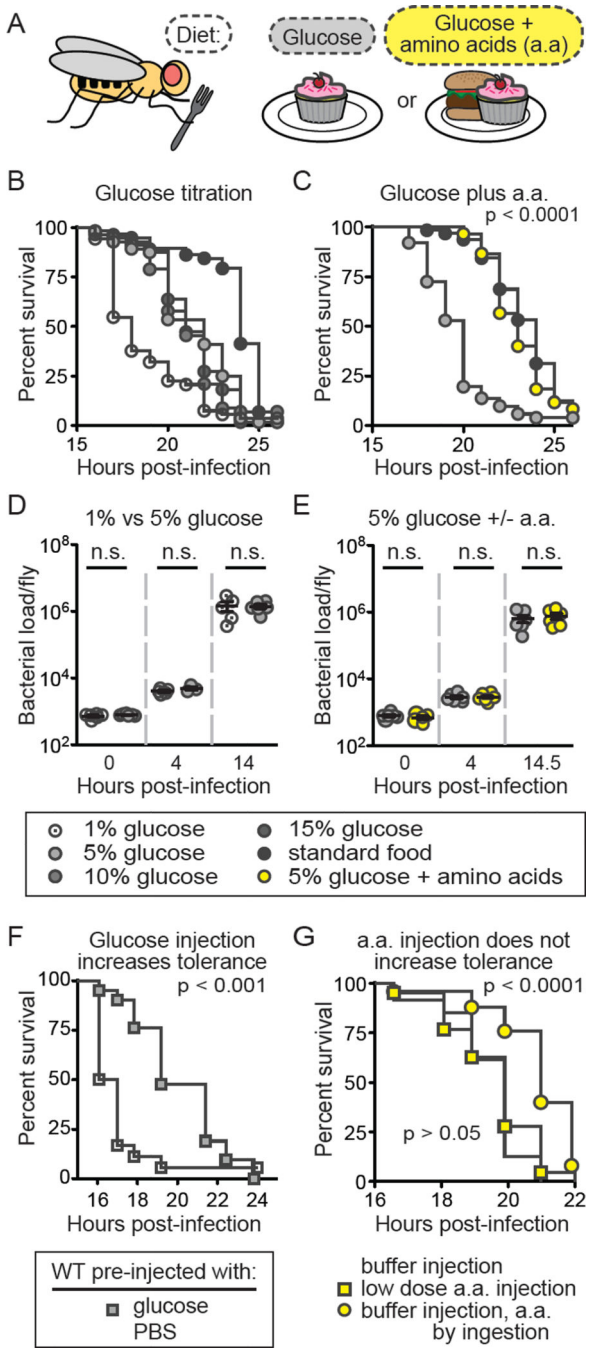

Figure 4. Glucose and amino acids increase tolerance of *B. cepacia* infection

A) Schematic of dietary conditions: wild-type flies were raised on standard food and switched 24 hours before *B. cepacia* infection to fresh standard food, glucose diets (B,D) or glucose diet plus amino acids (C,E). B) Increasing glucose concentration (5%, 10%, or 15%) increased survival time relative to 1% glucose diet ($n \geq 53$, $p < 0.0001$ in all cases) and caused similar survival kinetics compared to each other ($n \geq 55$, n.s. in all cases). Flies on standard food ($n = 58$) survived longer than flies on any glucose diet ($p < 0.0001$ for all). C) Supplementing 5% glucose with amino acids ($n = 60$) increased survival time significantly

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longer than 5% glucose alone (n=51, p<0.0001 in all cases) and was sufficient for survival kinetics similar to standard food (n=64, n.s.). There was no difference in bacterial load comparing flies fed D) 1% vs. 5% glucose (n=6 flies/time point, n.s. for all) or E) 5% glucose vs. 5% glucose plus amino acids (n=6 flies/time point, n.s. for all). F) Wild-type flies survived longer when injected 1.5 hours before infection with 50 nL of 5% glucose (n=21) than with PBS control (n=18, p=0.0007). G) Injection of amino acids prior to infection (n=43) does not increase the survival advantage relative to buffer alone (n=47, n.s.), and buffer injection does not eliminate the survival advantage provided by amino acid ingestion (n=25, p<0.0001). Additional examples of nutrient injections are shown in Figure S2. p-values for survival curve comparisons were obtained by log-rank analysis; p-values for bacterial load comparisons were obtained by unpaired t-test (0h) and non-parametric Mann-Whitney test (later time points); error bars represent the mean ± S.E.M.; a.a.=amino acids; n.s.=not significant (p>0.05).

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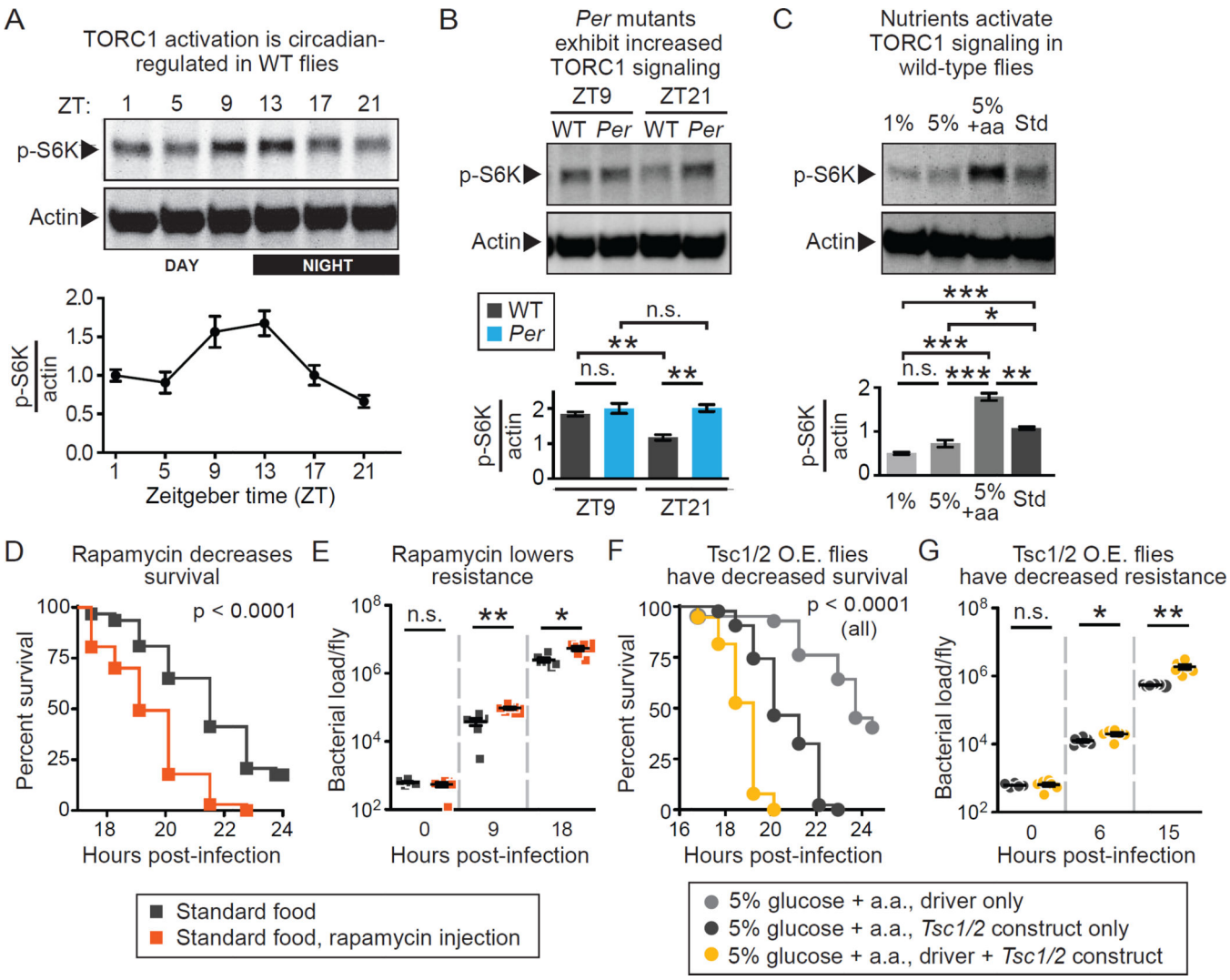

Figure 5. TORC1 signaling increases tolerance of infection

A) TORC1 activation is circadian regulated in wild-type flies: Western blot analysis (upper panel) and quantification (lower panel) showing phospho-S6K levels peak in the evening and trough in the morning. B) *Per*^{01} mutants exhibit increased levels of phospho-S6K at ZT21, as determined by Western blot analysis (n=10, ZT21 p=0.0027, ZT9 n.s.). WT flies exhibit reduced levels of phospho-S6K at ZT21 compared to ZT9 (n=10, p=0.0026). *Per*^{01} mutants do not show this difference (n.s.). C) Nutrients activate TORC1 signaling in wild type flies, as evidenced by increased levels of phospho-S6K (n=10, p≤0.0163 for all comparisons except 1% glucose vs. 5% glucose, n.s.). D – E) Inhibition of TORC1 by co-injection of rapamycin at the time of infection reduces resistance, as shown by: D) reduced survival (n=67) compared to co-injection of buffer (n=70, p<0.0001) and E) increased bacterial load after infection (n=6, 0 hrs n.s., 9 hrs p=0.0049, 18 hrs p=0.0198). See also Figure S3C–D for infections of *Per*^{01} mutants co-injected with rapamycin. F–G) Inhibition of TORC1 by over-expression of Tsc1/2 (see also Figure S3A–B) also reduces resistance: Tsc1/2 overexpression (O.E.) mutants (n=38) exhibit F) decreased survival time relative to flies containing the driver alone (n=42, p<0.0001) or the construct alone (n=43, p<0.0001)

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and G) increased bacterial load after infection (n=6 for both mutant and construct alone, 0 hrs n.s., 6 hrs p=0.0367, 15 hrs p=0.0022). p-values for survival curve comparisons were obtained by log-rank analysis; p-values for bacterial load comparisons were obtained using unpaired t tests for 0 hour time points, while subsequent time points were tested with non-parametric Mann-Whitney U tests; error bars represent the mean ± S.E.M; ZT=zeitgeber; aa=amino acids; n.s.=not significant ($p>0.05$); *=$p\leq0.05$; **=$p\leq0.01$; ***=$p\leq0.001$.

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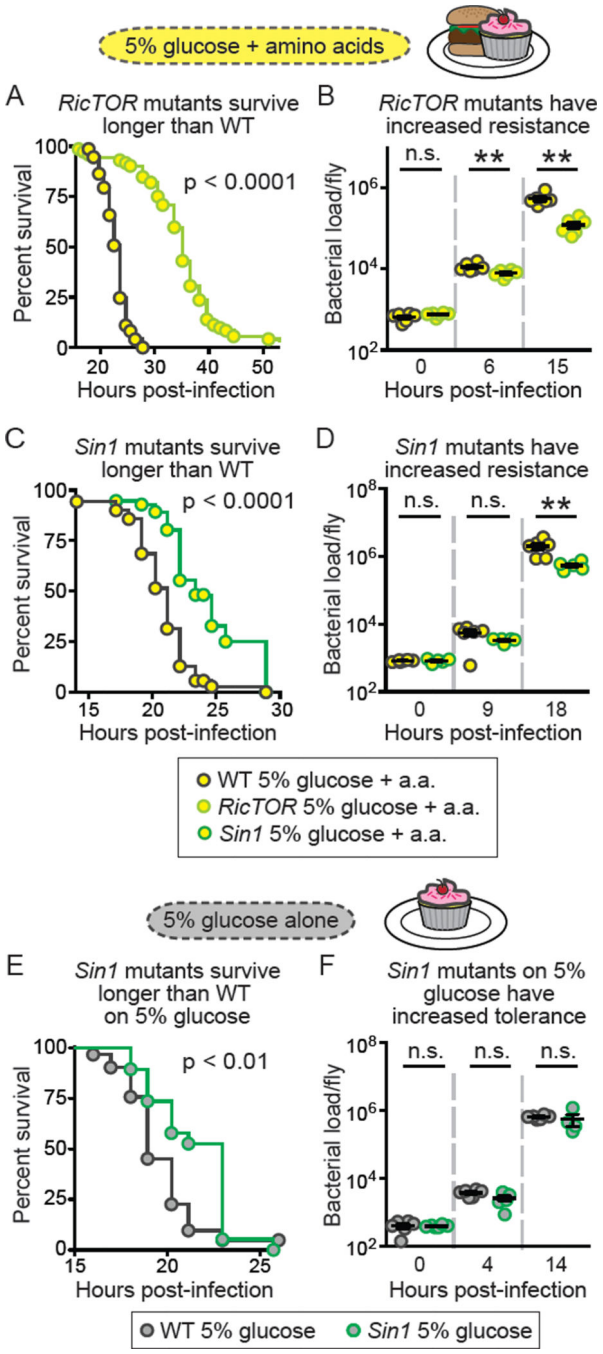

Figure 6. TORC2 activity decreases both resistance and tolerance of infection

RicTOR and Sin1 are two components of TORC2. A–B) Loss of RicTOR increases resistance in the presence of dietary amino acids, as *RicTOR*$^{A1/A2}$ mutants: A) (n=72) survive infection longer than wild type flies (n=73, p<0.0001) and B) exhibit decreased bacterial load after infection (n=6, all groups, 0 hr n.s., 6 hrs p=0.0087, 15 hrs p=0.0022). C–D) Loss of Sin1 also increases resistance in the presence of dietary amino acids, as *Sin1*^{e03756} mutants: C) (n=56) survive infection longer than wild type flies (n=70, p<0.0001) and D) exhibit decreased bacterial load after infection (n=6, all groups, 0 hr n.s., 9 hrs n.s.,

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18 hrs p=0.0043). E–F) In the absence of dietary amino acids (5% glucose alone), *Sin1*^{e03756} mutants exhibit increased tolerance: E) *Sin1*^{e03756} mutants (n=24) survive infection longer than wild type (n=19, p=0.0051) and F) have similar bacterial load after infection (all n≥4, n.s.). See also Figure S4 for additional infection data for *Sin1*^{e03756} and *RicTOR*$^{\Delta 1/\Delta 2}$. p-values for survival curve comparisons were obtained by log-rank analysis; p-values for bacterial load comparisons were obtained using unpaired t-tests for 0 hour time points, while subsequent time points were tested with non-parametric Mann-Whitney U tests; error bars represent the mean ± S.E.M.; n.s.=not significant (p>0.05); **=p≤0.01.

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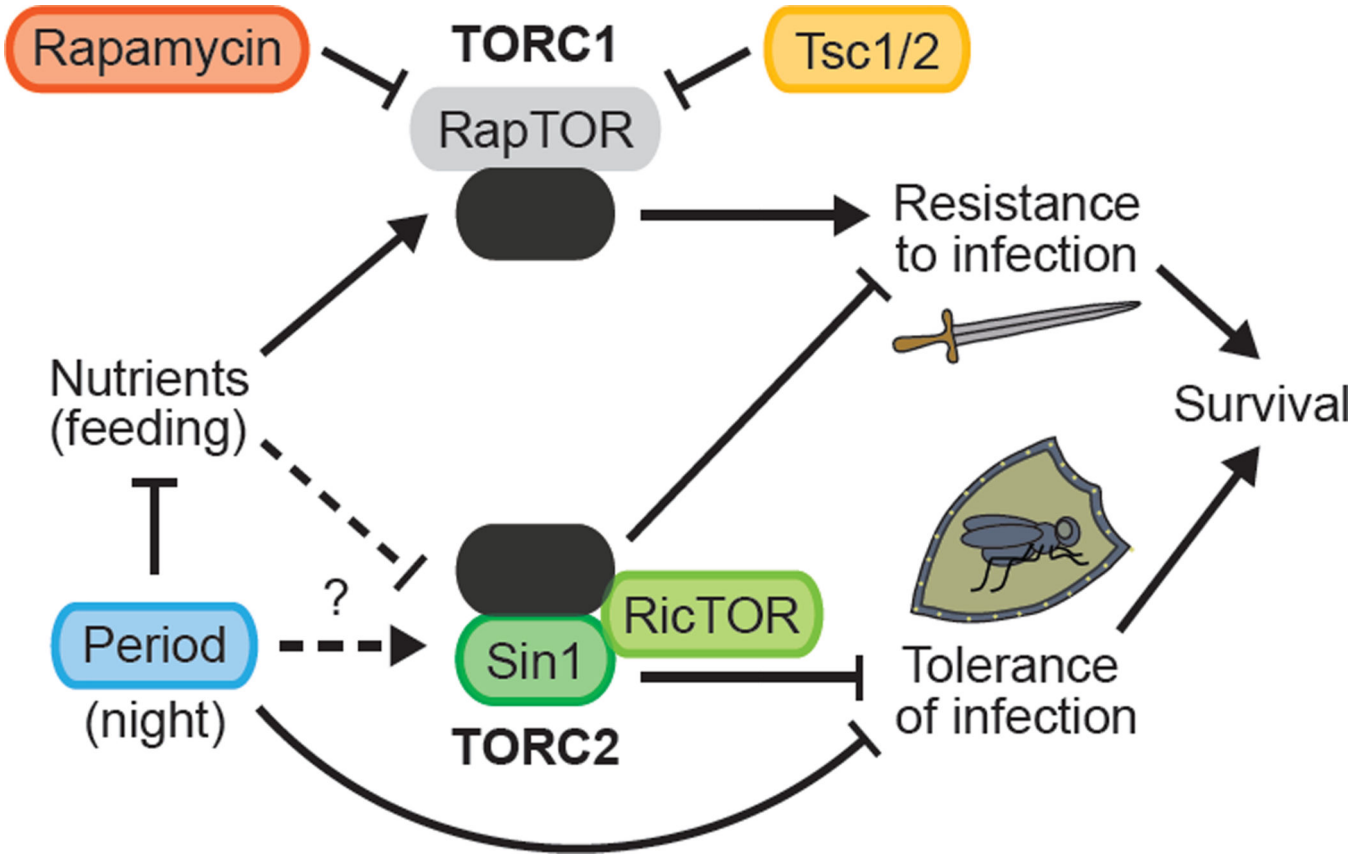

Figure 7. Schematic for nutrient-dependent and TOR signaling effects on survival from infection

Period activity decreases food (nutrient) consumption and reduces both resistance and tolerance to infection. Period and nutrients both regulate TORC1 signaling to modulate resistance to infection. Period also inhibits tolerance from infection, perhaps via promoting the tolerance inhibitory function of TORC2.