

RESEARCH ARTICLE

Mosquitocidal properties of IgG targeting the glutamate-gated chloride channel in three mosquito disease vectors (Diptera: Culicidae)

Jacob I. Meyers^{1,*}, Meg Gray² and Brian D. Foy²

ABSTRACT

The glutamate-gated chloride channel (GluCl) is a highly sensitive insecticide target of the avermectin class of insecticides. As an alternative to using chemical insecticides to kill mosquitoes, we tested the effects of purified immunoglobulin G (IgG) targeting the extracellular domain of GluCl from *Anopheles gambiae* (AgGluCl) on the survivorship of three key mosquito disease vectors: *Anopheles gambiae* s.s., *Aedes aegypti* and *Culex tarsalis*. When administered through a single blood meal, anti-AgGluCl IgG reduced the survivorship of *A. gambiae* in a dose-dependent manner (LC₅₀: 2.82 mg ml⁻¹, range 2.68–2.96 mg ml⁻¹) but not *A. aegypti* or *C. tarsalis*. We previously demonstrated that AgGluCl is only located in tissues of the head and thorax of *A. gambiae*. To verify that AgGluCl IgG is affecting target antigens found outside the midgut, we injected it directly into the hemocoel via intrathoracic injection. A single, physiologically relevant concentration of anti-AgGluCl IgG injected into the hemocoel equally reduced mosquito survivorship of all three species. To test whether anti-AgGluCl IgG was entering the hemocoel of each of these mosquitoes, we fed mosquitoes a blood meal containing anti-AgGluCl IgG and subsequently extracted their hemolymph. We only detected IgG in the hemolymph of *A. gambiae*, suggesting that resistance of *A. aegypti* and *C. tarsalis* to anti-AgGluCl IgG found in blood meals is due to deficient IgG translocation across the midgut. We predicted that anti-AgGluCl IgG's mode of action is by antagonizing GluCl activity. To test this hypothesis, we fed *A. gambiae* blood meals containing anti-AgGluCl IgG and the GluCl agonist ivermectin (IVM). Anti-AgGluCl IgG attenuated the mosquitocidal effects of IVM, suggesting that anti-AgGluCl IgG antagonizes IVM-induced activation of GluCl. Lastly, we stained adult, female *A. aegypti* and *C. tarsalis* for GluCl expression. Neuronal GluCl expression in these mosquitoes was similar to previously reported *A. gambiae* GluCl expression; however, we also discovered GluCl staining on the basolateral surface of their midgut epithelial cells, suggesting important physiological differences in Culicine and Anopheline mosquitoes.

KEY WORDS: *Aedes aegypti*, *Anopheles gambiae*, *Culex tarsalis*, GluCl, Mosquitocidal antibody

INTRODUCTION

Mosquito-borne diseases, such as malaria, dengue and West Nile virus, account for an estimated 1,434,000 deaths annually and 60,056,000

disability adjusted life years (World Health Report, 2012, available at http://www.who.int/gho/publications/world_health_statistics/2012/en/). These diseases are transmitted by Anopheline and Culicine mosquitoes. Historically, the most successful examples of vector-borne disease control have involved targeting mosquito vectors through the use of chemical insecticides. However, cross-cutting strategies that affect multiple mosquito disease vectors are currently limited to broad spectrum insecticides, to which resistance has become widespread (Corbel et al., 2007; Saavedra-Rodriguez et al., 2007; Norris and Norris, 2011; Ranson et al., 2011). Development of novel control strategies is necessary to maintain control of, and further eliminate, the transmission of mosquito-borne diseases.

Current insecticides target critical proteins involved in neuronal signaling. These include voltage-gated sodium channels (targeted by pyrethroids and organochlorines) (Zlotkin, 1999), acetylcholinesterases (organophosphates and carbamates) (Casida, 1963; Fukuto, 1990) and glutamate-gated chloride channels (avermectins) (Campbell et al., 1983; Cully et al., 1996; Kobylinski et al., 2010, 2011; Sylla et al., 2010). The glutamate-gated chloride channel of *Anopheles gambiae* (AgGluCl) and other Anopheline mosquitoes has proven to be an exceptionally sensitive target for the insecticidal drug ivermectin (IVM), when introduced through a blood meal (Jones et al., 1992; Gardner et al., 1993; Foley et al., 2000; Fritz et al., 2009; Chaccour et al., 2010; Kobylinski et al., 2010; Sylla et al., 2010). GluCl is a member of the Cys-loop family of ligand-gated ion channels. This channel is only expressed in invertebrates, where it gates an inhibitory chloride current on the post-synaptic membranes of neurons and muscle fibers (Cull-Candy, 1976; Fritz et al., 1979; Janssen et al., 2007, 2010).

Given that GluCl can be targeted by drugs found in a blood meal and that GluCl is not expressed in mammals, we wanted to test the efficacy of AgGluCl as a candidate mosquitocidal vaccine antigen. The concept of using vaccines to kill blood-feeding arthropods gained validity with the success of TickGARD^{PLUS}, an anti-tick vaccine targeting the midgut antigen Bm86 (Jonsson et al., 2000), and has continued with the more recent development of the subolesin/akirin antigens in ticks and also recently in mosquitoes (de la Fuente et al., 2011, 2013; da Costa et al., 2014). Research into mosquitocidal vaccines has been conducted since the 1940s, but with much less success, with most strategies focusing on targeting midgut antigens (Dubin et al., 1948; Hatfield, 1988a; Lal et al., 2001; Foy et al., 2003). In most experiments, animals were immunized against heterogeneous mosquito tissue homogenates, which led to variable reductions in survival and fecundity in multiple mosquito species, including animals immunized against head tissue homogenates where we would expect AgGluCl expression to occur (Almeida and Billingsley, 1998; Foy et al., 2002). Consequently, an efficacious anti-mosquito vaccine has never been developed despite decades of intermittent research (Jacobs-Lorena and Lemos, 1995; Willadsen and Billingsley, 1996; Billingsley et al., 2008).

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As GluCl is found outside of the midgut, it is important to understand which mosquito species permit IgG to translocate from the blood meal into the hemolymph. Previous literature shows that antibody translocation is not uniform across mosquito species (Vaughan and Azad, 1988; Jeffers and Michael Roe, 2008). Antibodies have been shown to translocate across the midgut of *A. gambiae* and other *Anopheles* spp. for up to 48 h post-ingestion (Vaughan and Azad, 1988; Beier et al., 1989). There are conflicting reports concerning antibody translocation across the midgut of *Aedes aegypti*, though most reports show that there are barriers to this process (Hatfield, 1988a,b; Ramasamy et al., 1988; Vaughan and Azad, 1988; Jacobs-Lorena and Lemos, 1995). *Culex tarsalis* has never been tested for this process, but *Culex pipiens* has been shown to have little to no antibody translocation directly following blood feeding (Vaughan and Azad, 1988).

We administered a polyclonal anti-AgGluCl immunoglobulin G (anti-AgGluCl IgG) to *A. gambiae* Giles 1902, *A. aegypti* (Linnaeus) and *C. tarsalis* Linnaeus 1758 through a blood meal or directly into the hemocoel by intrathoracic injection to determine and quantify its broad mosquitocidal activity across these diverse mosquito species. We also examined the effects on survivorship of co-administering anti-AgGluCl IgG with a known GluCl agonist, IVM, to study the mechanism of action of anti-AgGluCl IgG. In parallel, we compared GluCl tissue expression and antibody translocation into the hemolymph of these three diverse mosquito vectors.

RESULTS

Production and verification of anti-AgGluCl IgG specificity

Polyclonal anti-AgGluCl IgG was generated in rabbits against the bacterially expressed N-terminal extracellular domain of AgGluCl. GluCl is highly conserved across mosquito species, including *A. gambiae* and *A. aegypti* (Fig. 1A). Antibody titer and specificity against the recombinant immunized protein was verified by ELISA (1:512,000) and western blot against the recombinant, immunized AgGluCl extracellular antigen, and immunolabeling of C6/36 cells (derived from *Aedes albopictus*) expressing native, full-length AgGluCl (Fig. 1B,C).

Anti-AgGluCl IgG kills *A. gambiae* when administered through a blood meal or intrathoracic injection

To measure the effects of anti-AgGluCl IgG on *A. gambiae* survivorship, we fed mosquitoes on blood meals containing anti-AgGluCl IgG ranging from 1.0 to 3.0 mg ml⁻¹. Blood feeding of five different anti-AgGluCl IgG concentrations showed that anti-AgGluCl IgG induces a strong, dose-dependent mosquitocidal effect (Fig. 2A). Non-specific polyclonal (control) rabbit IgG, when blood fed at the highest concentration tested, did not affect mosquito mortality. These results were consistent across three replicates. We calculated the LC₅₀ (the concentration of anti-AgGluCl IgG that was lethal to 50% of the mosquitoes) of anti-AgGluCl IgG to be 2.82 mg ml⁻¹ (2.68, 2.96 mg ml⁻¹; *N*=1499) (Fig. 2A).

To verify that anti-AgGluCl IgG was affecting survivorship by translocating across the midgut and binding to AgGluCl, which we found was only expressed in the hemocoel (see the companion paper, Meyers et al., 2015), we injected a physiologically relevant concentration (958 ng ml⁻¹) of anti-AgGluCl IgG (Vaughan et al., 1990) or control rabbit IgG directly into the hemocoel by intrathoracic injection. A single injection of anti-AgGluCl IgG significantly reduced *A. gambiae* survivorship over 4 days [*P*=0.0083; hazard ratio: 2.322 (1.242, 4.30), *N*=141] compared with control (Fig. 2B).

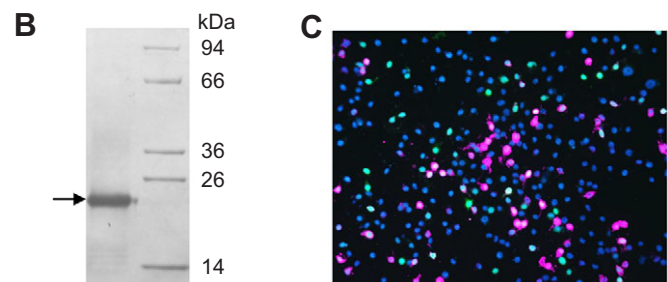
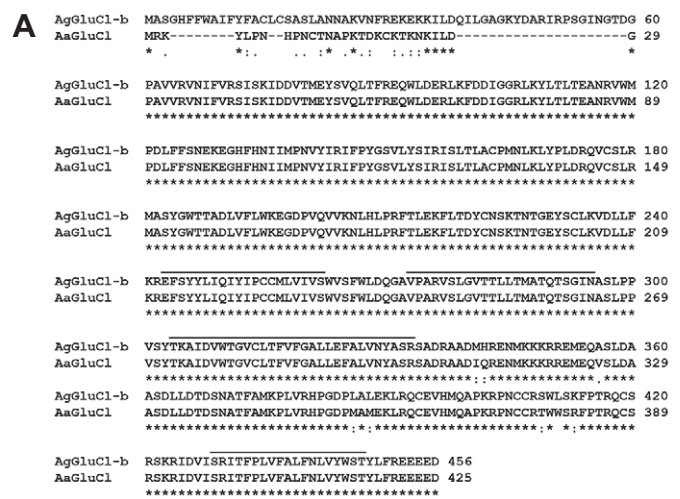


Fig. 1. Mosquito GluCl alignment and anti-AgGluCl IgG specificity. (A) ClustalW alignment of predicted amino acid sequences for the glutamate-gated chloride channels AgGluCl-b (AGAP001434) from *Anopheles gambiae* and AaGluCl (AAEL003003) from *Aedes aegypti*. Black bars denote transmembrane domains, asterisks indicate identical residues, and colons and full-stops indicate conservation between groups of strongly and weakly similar properties, respectively. (B) Western blot of anti-AgGluCl IgG against the AgGluCl extracellular domain. (C) C6/36 cells transfected with AgGluCl and eGFP (green). Cells were immunostained for anti-AgGluCl IgG (pink) and DAPI (blue) (Habluetzel et al., 1997).

Anti-AgGluCl IgG antagonizes AgGluCl activity

We hypothesized that the mode of action of anti-AgGluCl IgG is to antagonize channel function by binding to regions on the extracellular domain of AgGluCl that are critical for channel opening. To test this, we co-administered anti-AgGluCl IgG with IVM, a known GluCl agonist. We fed *A. gambiae* a single blood meal containing the LC₇₅ of IVM (Kobylinski et al., 2010) and a physiologically relevant concentration (282 µg ml⁻¹) of anti-AgGluCl IgG (Stoute et al., 1997), and monitored survivorship over 4 days (Fig. 3). IVM alone and IVM mixed with control non-specific rabbit IgG produced significant mosquitocidal effects relative to control groups [IVM versus PBS: *P*<0.0001, hazard ratio: 8.930 (5.765, 11.99), *N*=208; IVM+control IgG versus PBS: *P*<0.0001, hazard ratio: 8.641 (6.576, 14.31), *N*=193]. Control IgG had no effect on IVM toxicity [IVM versus IVM+control IgG: *P*=0.9213, hazard ratio: 0.9890 (0.7497, 1.290), *N*=225]. A single blood meal containing a physiologically relevant concentration of anti-AgGluCl IgG did not significantly reduce mosquito survivorship though the data were trending towards significance [anti-AgGluCl IgG versus PBS: *P*=0.0621, hazard ratio: 1.837 (0.9894, 3.531), *N*=183]. However, the combination of IVM and anti-AgGluCl IgG attenuated the mosquitocidal effects of IVM alone [IVM versus IVM+anti-AgGluCl IgG: *P*<0.0001, hazard ratio: 2.022 (1.842, 3.471), *N*=117], suggesting anti-AgGluCl IgG partially blocks IVM-induced activation of AgGluCl.

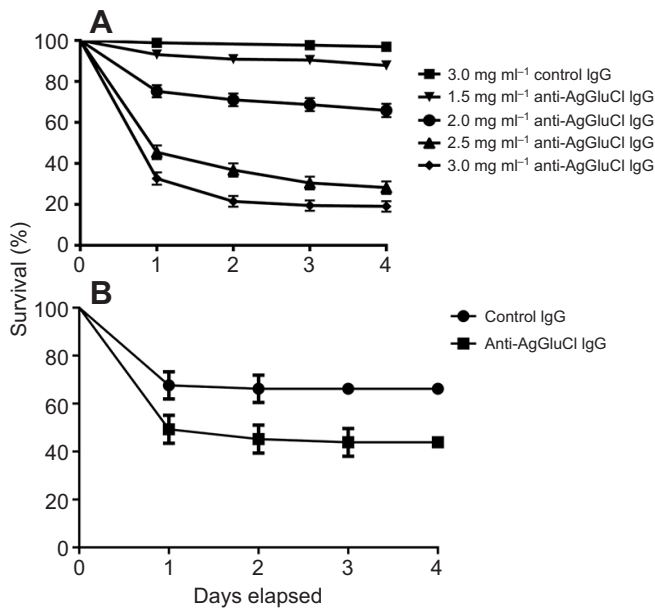


Fig. 2. Anti-AgGluCl IgG kills *A. gambiae* when administered through a blood meal or directly into the hemocoel. (A) Survival curves of *A. gambiae* after feeding on a single blood meal containing anti-AgGluCl IgG or non-specific polyclonal (control) IgG. (B) Survival curves of *A. gambiae* after a single intrathoracic injection of anti-AgGluCl IgG. Data are presented as means and s.e.

Anti-AgGluCl IgG kills *A. aegypti* and *C. tarsalis* when administered through intrathoracic injection but not through a blood meal

As GluCl is highly conserved across multiple mosquito species (percentage homology *A. gambiae* versus *A. aegypti*: 92.47%) (Megy et al., 2012), we tested to see whether anti-AgGluCl IgG had the same mosquitocidal properties in *A. aegypti* and *C. tarsalis* as we observed in *A. gambiae*. When introduced through a blood meal, anti-AgGluCl IgG had no effect on survivorship in *A. aegypti* up to a concentration of 4.0 mg ml⁻¹ [$P=0.6124$, hazard ratio: 0.4526 (0.06213, 3.297), $N=80$] (Fig. 4A). *Culex tarsalis* blood fed on anti-AgGluCl IgG up to 3.5 mg ml⁻¹ also showed no effect on survivorship [$P=0.0867$, hazard ratio: 2.131 (0.8966, 5.066), $N=161$] (Fig. 4C).

To test whether anti-AgGluCl IgG could affect mosquito survivorship if it was artificially made to cross the midgut, we intrathoracically injected *A. aegypti* and *C. tarsalis* with anti-AgGluCl IgG. When a physiological relevant concentration of anti-AgGluCl

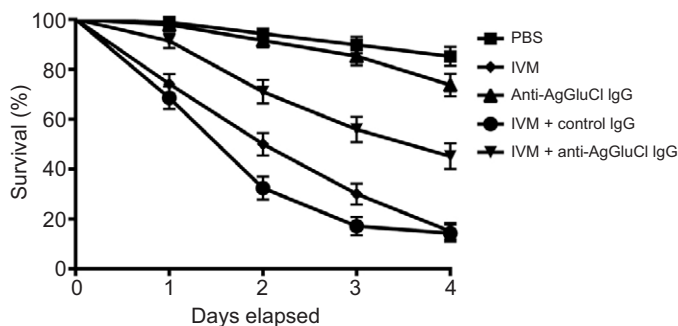


Fig. 3. Survival curves of *A. gambiae* after feeding on a single blood meal containing anti-AgGluCl IgG plus the GluCl agonist ivermectin (IVM). Data are presented as means and s.e.

IgG (958 ng ml⁻¹) (Vaughan et al., 1990) was administered through a single intrathoracic injection, it reduced survivorship in both *A. aegypti* [$P=0.0221$, hazard ratio: 2.969 (1.169, 7.537), $N=122$] and *C. tarsalis* [$P=0.0237$, hazard ratio: 2.536 (1.132, 5.682), $N=124$] over 4 days, equal to the reduction in survivorship of *A. gambiae* that underwent the same treatment (Fig. 4B,D and Fig. 2B).

IgG is found in the hemolymph of *A. gambiae*, but not *A. aegypti* or *C. tarsalis*, following a blood meal containing anti-AgGluCl IgG

To determine whether IgG introduced through a blood meal is able to traverse the midgut into the hemocoel of these three mosquito disease vectors, we fed mosquitoes a blood meal containing anti-AgGluCl IgG and extracted their hemolymph at 3 h post-blood feeding. Hemolymph, diluted in PBS from the extraction procedure, was blotted for the rabbit-derived anti-AgGluCl IgG. Anti-AgGluCl IgG was detected in the diluted hemolymph of *A. gambiae* that had fed on anti-AgGluCl IgG-containing blood meals, but not in *A. aegypti* or *C. tarsalis* (Fig. 5A). A standard curve of anti-AgGluCl IgG was created from 3 ng ml⁻¹ to 3 μ g ml⁻¹ to estimate the amount of anti-AgGluCl IgG that had diffused into the *A. gambiae* hemolymph. From this standard, we calculated 12.92 ng ml⁻¹ anti-AgGluCl IgG in the hemolymph of *A. gambiae* following a blood meal containing anti-AgGluCl IgG (Fig. 5B). This calculation compensated for the dilution of hemolymph in PBS.

GluCl expression in *A. aegypti* and *C. tarsalis*

Given that anti-AgGluCl IgG reduces the survivorship of *A. gambiae*, *A. aegypti* and *C. tarsalis* when introduced into the hemocoel, we wanted to examine GluCl tissue expression to understand what physiological systems anti-AgGluCl IgG could be affecting. In the accompanying study, we found that AgGluCl is expressed in antenna segments, the Johnston's organ, the head and the thoracic ganglia of adult, female *A. gambiae* (Table 1) (Meyers et al., 2015). We stained sagittal sections of whole adult female mosquitoes for GluCl and a neuronal marker (Jan and Jan, 1982) to distinguish neuronal tissue. Immunolabeling of *A. aegypti* and *C. tarsalis* showed GluCl expression in the antennal segments, Johnston's organ, optic lobe, supraesophageal ganglion and thoracic ganglia, just as we found in *A. gambiae* (Table 1) (Meyers et al., 2015). There were small puncta of GluCl staining on antennal segments localized at the base of hair sensillae (supplementary material Fig. S1A, Fig. S2A). The Johnston's organ, a mechanosensory organ in the pedicel associated with audition and flight coordination, had specific GluCl staining in scolopidea (supplementary material Fig. S1B, Fig. S2B). GluCl staining was present in neuronal tissues found in the head (supplementary material Fig. S1C, Fig. S2C). Lastly, there was GluCl staining on neuronal cell bodies found in all three thoracic ganglia (supplementary material Fig. S1D, Fig. S2D). Surprisingly, *A. aegypti* and *C. tarsalis* exhibited unique GluCl staining on the midgut epithelium, which was not present in *A. gambiae*. Midguts were dissected and individually stained to visualize the precise location of GluCl in the midgut. In *A. aegypti*, GluCl expression was restricted to the anterior midgut and was not present in the posterior midgut (Fig. 6). In *C. tarsalis*, GluCl expression was found throughout the anterior and posterior midgut (Fig. 7). In both *A. aegypti* and *C. tarsalis*, GluCl expression was confined to the basolateral side of the midgut epithelial cells and was not found on the epithelial microvilli that project into the midgut lumen (Fig. 6D and Fig. 7D).

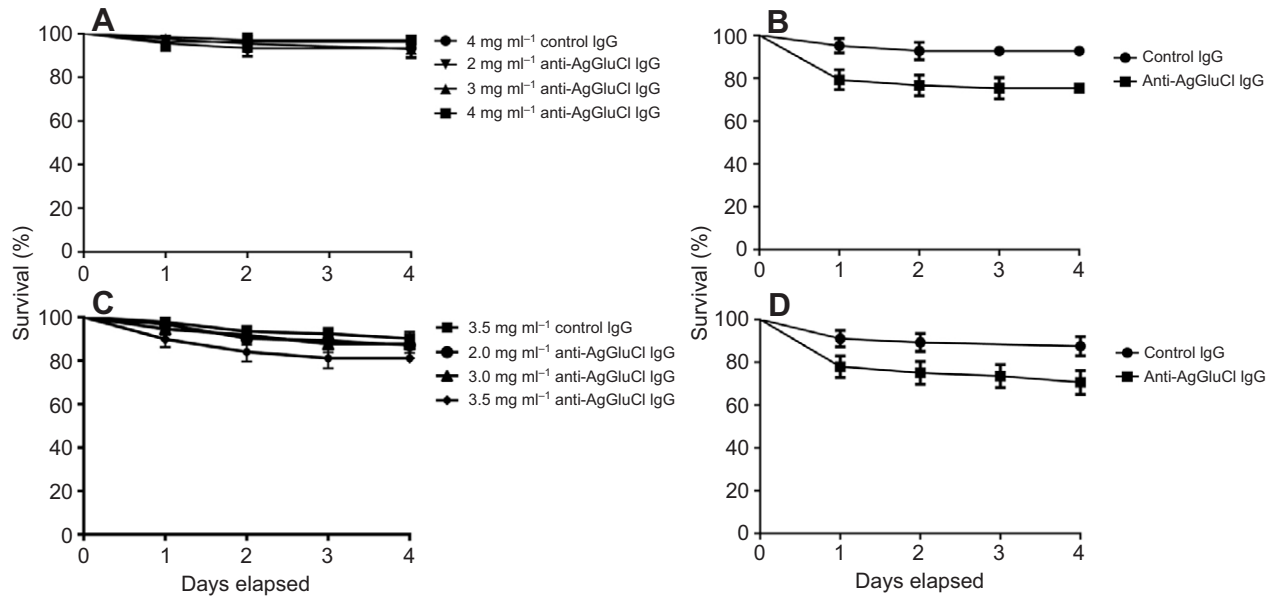


Fig. 4. Anti-AgGluCl IgG kills *Aedes aegypti* and *Culex tarsalis* when administered directly into the hemocoel, but not through a blood meal. Survival curves of *A. aegypti* (A) and *C. tarsalis* (C) after feeding on a single blood meal containing anti-AgGluCl IgG and of *A. aegypti* (B) and *C. tarsalis* (D) after a single intrathoracic injection of anti-AgGluCl IgG. Data are presented as means and s.e.

DISCUSSION

In the present study, we have shown that IgG targeting the N-terminal extracellular domain of AgGluCl reduces *A. gambiae* survivorship when mixed with calf blood and administered through an artificial membrane feeder. To our knowledge, this is the first example of a single antigen that can be targeted by IgG in a mosquito blood meal to consistently and significantly reduce mosquito survivorship. Previous vaccine development strategies targeting mosquitoes have immunized animals with whole mosquito tissues, which could never be employed as a vaccine antigen (Alger and Cabrera, 1972; Almeida and Billingsley, 1998), or against a single midgut antigen. These resulted

in small and highly variable reductions in survivorship and fecundity and differed between mosquito species, immunized hosts and trials (Dubin et al., 1948; Alger and Cabrera, 1972; Hatfield, 1988a,b; Ramasamy et al., 1988; Vaughan and Azad, 1988; Srikrishnaraj et al., 1993; Noden et al., 1995; Almeida and Billingsley, 1998, 2002; Lal et al., 2001; Foy et al., 2003; Billingsley et al., 2008; da Costa et al., 2014). In contrast, blood meals containing anti-AgGluCl IgG consistently reduced *A. gambiae* survivorship across all replicates in a dose-dependent manner.

The LC₅₀ concentration of anti-AgGluCl IgG in a blood meal and the range of anti-AgGluCl IgG concentrations that reduced

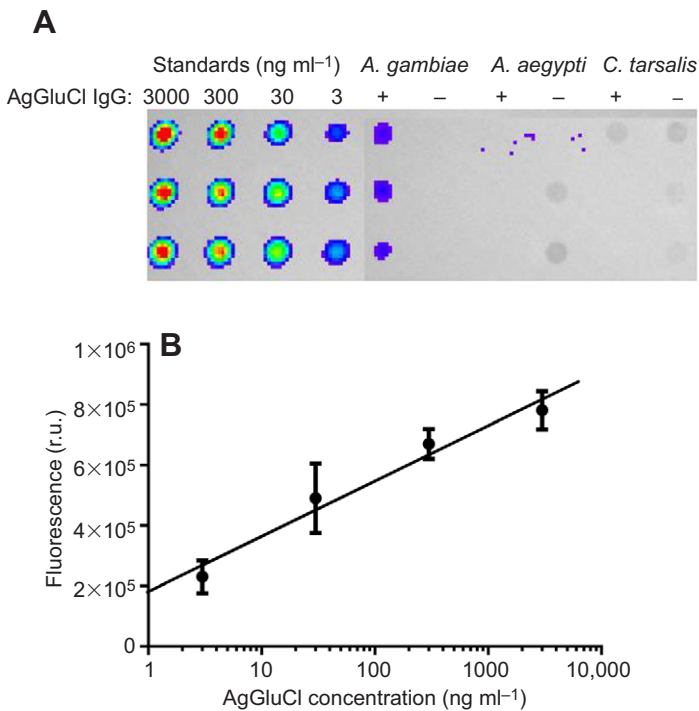


Fig. 5. Dot blot of mosquito hemolymph. (A) Dot blot of anti-AgGluCl IgG standards and hemolymph extracted from *A. gambiae*, *A. aegypti* and *C. tarsalis* after feeding on an anti-AgGluCl IgG-containing blood meal (+) or a normal blood meal (-). (B) Anti-AgGluCl IgG standard curve: $y=183,300x+180,344$; $r^2=0.8816$. r.u., relative units.

Table 1. GluCl expression in *Anopheles gambiae*, *Aedes aegypti* and *Culex tarsalis*

	<i>A. gambiae</i>	<i>A. aegypti</i>	<i>C. tarsalis</i>
Antenna	+	+	+
Johnston's organ	+	+	+
Head	+	+	+
Thoracic ganglia	+	+	+
Anterior midgut	–	+	+
Posterior midgut	–	–	+

A. gambiae survivorship are greater than IgG concentrations found in an immunized human host (Shawler et al., 1985; Stoute et al., 1997). Such a vaccine would not benefit from natural boosting as the host is not naturally exposed to AgGluCl epitopes, so any vaccine would depend on relatively high antibody titers. Our anti-AgGluCl IgG was created against the large 244 amino acid extracellular domain. AgGluCl epitope optimization targeting highly antigenic regions of this domain or passive immunization of monoclonal antibodies against targeted extracellular AgGluCl epitopes could improve anti-AgGluCl IgG mosquito toxicity. Also, serial blood feeding might result in accumulation of anti-AgGluCl IgG, causing an increase in mortality over time. Though IgG in the mosquito hemolymph has been shown to be cleared by 48 h post-feeding, those experiments were done with IgG that had no hemocoelic targets (Vaughan and Azad, 1988). If anti-AgGluCl IgG persists in the hemocoel by binding to GluCl-expressing tissues, there could be aggregate effects from serial blood feeding over time on a physiological concentration of anti-AgGluCl IgG on *A. gambiae* survivorship and fecundity. Furthermore, evidence from the field suggests very frequent biting by *A. gambiae*, approximately every 1–2 days, providing multiple opportunities for anti-AgGluCl IgG ingestion (Beier, 1996; Scott et al., 2006). The concentration of anti-AgGluCl IgG used for intrathoracic injections is within the range of IgG concentrations found in *Anopheles stephensi* hemolymph 3 h

after feeding on rats immunized against *Plasmodium falciparum* circumsporozoite protein (Vaughan et al., 1990). This suggests that *A. gambiae* survivorship might be affected when fed directly on an AgGluCl-immunized host. The mosquitocidal effects of anti-AgGluCl IgG injected into the hemocoel also suggest that anti-AgGluCl IgG is binding to antigens found outside of the midgut to affect *A. gambiae* survivorship.

When we co-administered anti-AgGluCl IgG with the potent GluCl agonist IVM, we found an attenuation of IVM-induced mortality. This suggests that anti-AgGluCl IgG works by antagonizing AgGluCl activity. AgGluCl antagonism would disrupt inhibitory glutamatergic synaptic communication and potentially cause neuronal hyper-excitation. It is still unclear how anti-AgGluCl IgG antagonizes AgGluCl activity; it could directly block channel activity or indirectly affect activity by causing AgGluCl to be internalized into intracellular vesicles. The likely explanation is that anti-AgGluCl IgG binding to multiple extracellular epitopes of AgGluCl antagonizes the channel and prevents IVM-induced opening of the channel. Though the conformation of AgGluCl would change with IVM binding, it seems unlikely that this would affect the binding of the polyclonal anti-AgGluCl IgG because the antibodies likely bind to multiple epitopes on the extracellular domain of AgGluCl, while IVM binding occurs in between subunits on residues found on the AgGluCl transmembrane domain (Hibbs and Gouaux, 2011). Auto-immune antibodies against other channels have been shown to antagonize channel activity in mammals, which is likely the same mechanism of action of anti-AgGluCl IgG (Weber et al., 2000; Wang et al., 2007; Briani et al., 2008; Vernino et al., 2008). However, GluCl is not expressed in any mammalian species, making it unlikely that anti-AgGluCl IgG will have auto-immune effects in humans or livestock. The mammalian receptors most closely related to the insect GluCl are the GABA-gated chloride channel and the glycine-gated chloride channel (Lynagh and Lynch,

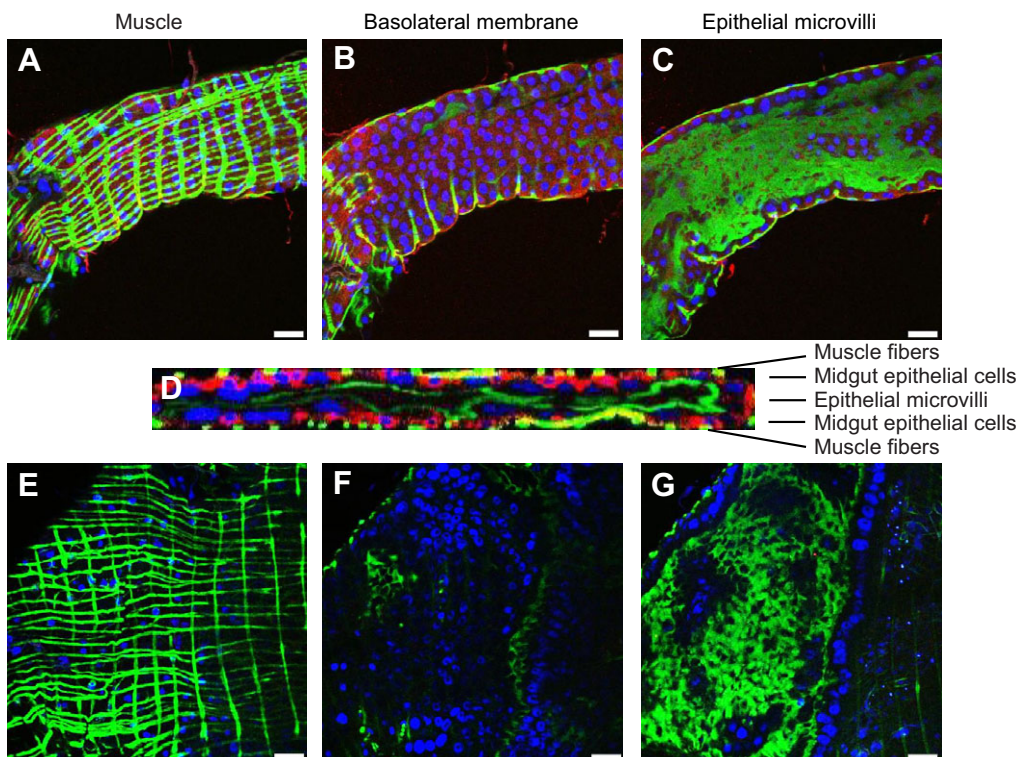


Fig. 6. Immunostaining of dissected *A. aegypti* anterior and posterior midgut. Confocal slices of the muscle, basolateral membrane and epithelial microvilli of the anterior (A–C) and posterior (E–G) midgut. (D) Confocal stack through the thickness of the anterior midgut. Red: GluCl; green: actin; blue: DAPI. Scale bars: 50 μm.

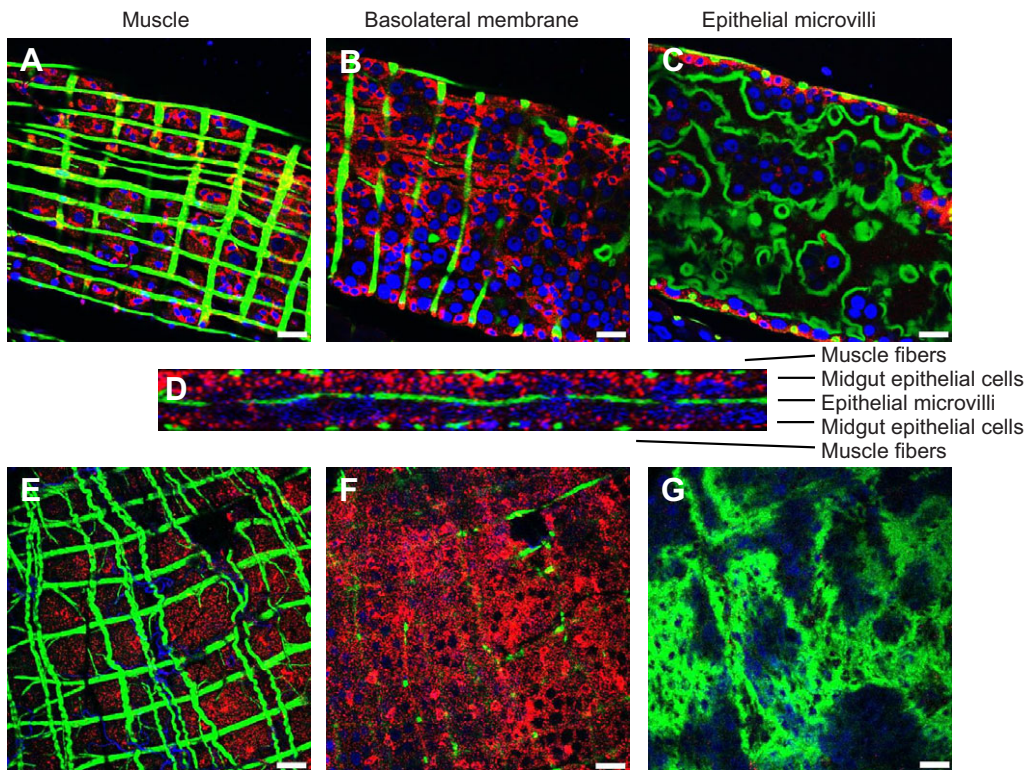


Fig. 7. Immunostaining of dissected *C. tarsalis* anterior and posterior midgut. Confocal slices of the muscle, basolateral membrane and epithelial microvilli of the anterior (A–C) and posterior (E–G) midgut. (D) Confocal stack through the thickness of the posterior midgut. Red: GluCl; green: actin; blue: DAPI. Scale bars: 50 μm.

2012) but their amino acid similarity with AgGluCl is <35%. Nevertheless, anti-AgGluCl IgG reactivity to these channels and other neurotransmitter receptors should be tested in the design of any vaccine that would use AgGluCl epitopes as antigens.

As GluCl is highly conserved across multiple mosquito species, we tested to see whether blood meals containing anti-AgGluCl IgG could affect the survivorship of two other important disease vectors, *A. aegypti* and *C. tarsalis*. Anti-AgGluCl IgG did not affect *A. aegypti* or *C. tarsalis* survivorship when administered through a blood meal. When a physiologically relevant concentration of anti-AgGluCl IgG was directly injected into the hemocoel, it reduced the survivorship in both *A. aegypti* and *C. tarsalis*. Surprisingly, all three mosquito species had the same reduction in survivorship to intrathoracic injections of anti-AgGluCl IgG. This suggests that anti-AgGluCl IgG has a similar affinity for the GluCl across these mosquito species and that GluCl plays a similar, critical physiological role in all three species.

Previous research has shown that whole IgG passes through the midgut of *A. gambiae* (Vaughan and Azad, 1988). There are conflicting reports on antibody translocation in *A. aegypti*, but most work has shown that this process does not occur (Vaughan and Azad, 1988; Jacobs-Lorena and Lemos, 1995). There have been no reports on antibody passage across the midgut of *C. tarsalis*, though other *Culex* species have been shown to have little to no antibody passage across the midgut (Vaughan and Azad, 1988). In agreement with previous work, we only detected anti-AgGluCl IgG in the diluted hemolymph of *A. gambiae* but not *A. aegypti*, nor did we find anti-AgGluCl IgG in the diluted hemolymph of *C. tarsalis* post-blood feeding. These results provide an explanation for the sensitivity of these three mosquito species to blood meals containing anti-AgGluCl IgG. Using our standard curve, we detected 12.92 ng ml⁻¹ of anti-AgGluCl IgG in *A. gambiae* hemolymph. This is well below the concentration found in *A. stephensi* following blood feeding on an immunized rat (900–958 ng ml⁻¹) (Vaughan et al., 1990). However,

in our experiment we added rabbit-derived anti-AgGluCl IgG to calf blood that already contained calf IgG. We believe the anti-AgGluCl IgG concentration we detected was low because the rabbit-derived anti-AgGluCl IgG comprises only a small portion of the total IgG in the blood meal. It is also possible that a major portion of anti-AgGluCl IgG remained bound to target antigens throughout the hemocoel, sequestering it from our hemolymph extraction. Though the concentration of anti-AgGluCl IgG in the hemolymph may not reflect what would be present in the hemolymph following a blood meal from an AgGluCl-immunized host, it still verifies the presence of IgG in the hemolymph and confirms that IgG is able to translocate across the *A. gambiae* midgut. It is not clear how IgG in a blood meal translocates into the hemocoel of *A. gambiae*, nor is it known what the barriers are to this process in *A. aegypti* or *C. tarsalis*. Further research is required to examine this key divergence in midgut physiology between Anopheline and Culicine mosquitoes.

To begin to understand what physiological role GluCl has in *A. aegypti* and *C. tarsalis*, we stained sagittal slices of whole, adult female mosquitoes for GluCl and found a similar neuronal GluCl expression pattern to that in *A. gambiae* (Meyers et al., 2015). There was only one tissue that had disparate GluCl expression between these three mosquito species; this was the midgut, which exhibited GluCl staining in *A. aegypti* and *C. tarsalis* but not in *A. gambiae* (Meyers et al., 2015). GluCl expression in the midgut came as a surprise because *A. aegypti* and *C. tarsalis* were not sensitive to anti-AgGluCl IgG when administered through a blood meal. Closer examination of GluCl midgut expression through direct immunostaining of dissected midguts showed that GluCl expression only occurred on the basolateral surface of the midgut epithelial cells and was not present on the epithelial microvilli found in the midgut lumen of both *A. aegypti* and *C. tarsalis*. This means that GluCl antigens are not directly exposed to the blood meal and that IgG would need to translocate across midgut epithelial cells to bind to extracellular epitopes of GluCl found on the basolateral surface of the midgut

epithelium. Our data show that IgG is unable to translocate into the hemocoel in *A. aegypti* and *C. tarsalis*, which is why GluCl on the basolateral surface of the midgut is not affected by anti-AgGluCl IgG-laden blood meals. At present, there is no evidence that anti-AgGluCl IgG insensitivity in *A. aegypti* and *C. tarsalis* is related to GluCl expression on the midgut. Though GluCl on the midgut could be acting as a ‘sink’ and binding anti-AgGluCl IgG from a blood meal before it could reach neuronally expressed GluCl in the head and thorax, it has been previously shown that anti-*Rickettsia typhi* antibodies also do not translocate from the blood meal to the hemolymph in *A. aegypti* and *C. pipiens*, giving further evidence that this refractory phenotype to anti-AgGluCl IgG-laden blood meals is due to a barrier in antibody translocation (Vaughan and Azad, 1988). GluCl expression is confined to the anterior midgut of *A. aegypti* and not present in the posterior midgut, whereas GluCl expression was detected in both the anterior and posterior midgut of *C. tarsalis*. It is unclear what role GluCl has on the midgut epithelium or why it is differentially expressed across these three mosquito species, but its role in chloride ion transport suggests it might be involved in maintaining ionic balance during midgut alkanization and digestion (del Pilar Corena et al., 2005).

Targeting GluCl through a vaccine strategy will not be effective at killing Culicine mosquitoes because IgG is unable to translocate into the hemocoel following a blood meal. However, a mosquitocidal vaccine targeting the highly conserved AgGluCl has major potential against multiple *Anopheles* spp. which have previously been shown to pass IgG through their midgut, including *Anopheles arabiensis*, *Anopheles stephensi*, *Anopheles funestus* and *Anopheles albimanus* (Vaughan and Azad, 1988; Beier et al., 1989). Further research testing the effects of anti-AgGluCl IgG on mosquito survivorship and the fecundity of *A. gambiae* and other *Anopheles* spp. after feeding on animals immunized against extracellular AgGluCl epitopes will be required to discern the potential of AgGluCl as a target antigen of a pan-*Anopheles* vaccine and as an intervention to control malaria parasite transmission.

MATERIALS AND METHODS

Mosquitoes

Anopheles gambiae s.s. G3 strain (origin The Gambia), *Aedes aegypti* (Vergel) and *Culex tarsalis* (California) were raised at 28–31°C, 80% relative humidity on a 14 h:10 h light:dark cycle. Larvae were fed ground Tetramin® fish food daily. Adults were provided with water and 10% sucrose solution *ad libitum*. Colony mosquitoes were blood fed every 3–4 days on defibrinated calf blood.

Production and verification of anti-AgGluCl IgG specificity

Polyclonal anti-AgGluCl IgG was prepared by GenScript USA, Inc. (Piscataway, NJ, USA). Briefly, two rabbits were immunized against the recombinant N-terminal extracellular domain of AgGluCl produced in *Escherichia coli* (244 amino acids in length). Rabbit serum was collected and affinity purified to isolate polyclonal rabbit IgG. Antibody specificity to AgGluCl was verified via ELISA, western blot and immunostaining of C6/36 cells (derived from *Aedes albopictus*) (Singh and Paul, 1969) transfected with AgGluCl cloned into the pIB/V5-His TOPO® plasmid (Life Technologies™, Grand Island, NY, USA) (Fig. 1B,C).

Blood feed with artificial membrane feeder

Two to 4 day post-emergence mosquitoes were fed defibrinated calf blood spiked with purified rabbit anti-AgGluCl IgG (GenScript), purified rabbit control IgG (GenScript), IVM or vehicle via glass bell feeders (Lillie Glass Feeders, Symrna, GA, USA) sealed with pig sausage casing and heated to 37°C. Mosquitoes were given 30–45 min to feed, after which they were knocked down briefly at 4°C for sorting and removal of unfed and partially

fed mosquitoes. Fully engorged mosquitoes were monitored for survivorship for 4 days following the blood meal.

Intrathoracic injection of anti-AgGluCl IgG

Adult female mosquitoes were briefly knocked down at 4°C for intrathoracic injection. Glass capillaries (3-00-203-G/XL, Drummond Scientific, Broomall, PA, USA) were pulled using a Flaming/Brown Micropipette Puller Model P-87 (Sutter Instruments, Novato, CA, USA) to create microcapillary injection needles. Mosquitoes were injected with 69 nl of either 958 ng ml⁻¹ anti-AgGluCl IgG or control IgG using a Drummond Nanoject II Automatic Injector (3-000-204, Drummond Scientific) and glass microcapillary injection needles. Following injection, mosquitoes were maintained for 4 days and survivorship was monitored.

Survivorship analysis and anti-AgGluCl IgG LC₅₀ determination

Replicates were pooled and analyzed by log-rank test (Mantel-Haenszel method; proportioned hazards model) with 95% confidence intervals using Prism software (GraphPad, La Jolla, CA, USA). LC₅₀ determination followed previously described analysis (Kobylinski et al., 2010). Briefly, survivorship replicates were pooled into a non-linear mixed model with probit analysis, which accounted for background mortality in the control group and assessed replicate effects, to calculate the lethal concentration that killed 50% of mosquitoes (LC₅₀). Wing lengths from 20 mosquitoes from each group were measured and compared across groups to ensure mosquito size did not affect survivorship (Lounibos et al., 1995).

Hemolymph extraction and dot blot

Hemolymph samples were taken from *A. gambiae*, *A. aegypti* and *C. tarsalis* 3 h following blood feeding on de-fibrinated calf blood (control) or de-fibrinated calf blood containing 3.0 mg ml⁻¹ rabbit-derived anti-AgGluCl IgG. Hemolymph was extracted by hemocoel perfusion as previously described (Vaughan et al., 1990). Briefly, a small perforation was made on the dorsal portion of the abdomen between the 7th and 8th segment. Mosquitoes were intrathoracically injected with PBS, using glass microcapillary injection needles, until a drop (~0.5 µl) of hemolymph protruded from the previously made abdominal tear. This hemolymph was collected into a glass microcapillary, extruded into a microcentrifuge tube and stored at -80°C. Each sample contained pools of 10 mosquito hemolymph extractions.

Fifty microliters of anti-AgGluCl IgG standards (3 ng ml⁻¹ to 3 µg ml⁻¹) and extracted mosquito hemolymph were blotted onto nitrocellulose paper using the Bio-Rad Bio-Dot SF Microfiltration Apparatus (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's protocol. As mosquito hemolymph extractions yielded very little volume, we diluted 5 µl of hemolymph with 45 µl PBS for blotting onto the nitrocellulose paper. Blots were blocked with 3% BSA in 0.05% Tween-20 Tris-based buffer solution (TTBS) for 1 h on a rocker. Blots were treated with a 1:50,000 dilution of goat anti-rabbit IgG (H+L) HRP conjugate (Thermo-Scientific, Waltham, MA, USA) in 1% BSA TTBS solution for 3 h on a shaker and then washed for 15 min in TTBS. Antibodies were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo-Scientific) as per the manufacturer's protocol. Fluorescence was measured using IVIS-200 Optical Imaging System (Xenogen, St Hopkinton, MA, USA) and quantified using Living Image 3.0 software (Caliper Life Science, Waltham, MA, USA). Anti-AgGluCl IgG standards were fitted with a linear regression curve, which was used to estimate the concentration of anti-AgGluCl IgG in the mosquito hemolymph.

Immunohistochemistry (IHC)

Whole mosquito specimens were prepared from female blood-fed mosquitoes aged 2–4 days post-emergence. After blood feeding, engorged females were injected intrathoracically (Drummond Nanoject) with 46 nl of 4% paraformaldehyde. Specimens were briefly washed in 70% ethanol before overnight fixation in 4% paraformaldehyde at 4°C. After fixation, specimens were paraffin embedded, cut into 5 µm thick slices and mounted onto slides (Colorado HistoPrep, Fort Collins, CO, USA). Slides were heated at 65°C for 10 min and treated with xylene to remove the paraffin layer and then

re-hydrated with graded washes in ethanol and PBS. Specimens were treated with graded washes of methanol and PBS to reduce autofluorescence. Slides were blocked for 2 h with 10% non-fat dry milk and 0.1% Triton-X in PBS. Primary antibody staining consisted of 1:500 anti-AgGluCl IgG (prepared by GenScript) and 1:500 goat anti-HRP (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) incubated overnight at 4°C. Specimens were washed with 0.05% TTBS and then incubated with 1:1000 donkey anti-rabbit Alexa 555 (Invitrogen, Waltham, MA, USA) and 1:1000 donkey anti-goat Alexa 488 (Invitrogen) for 3 h at room temperature. For midgut staining, midguts were dissected from 2–4 day old females and fixed in 4% paraformaldehyde overnight. Midguts were blocked with 3% bovine serum albumin (BSA) in TTBS for 1 h and stained with 1:500 anti-AgGluCl IgG for 3 h. Specimens were subsequently washed in TTBS and stained with 1:1000 donkey anti-rabbit Alexa 555 (Invitrogen) and Acti-stain 488 phalloidin (Cytoskeleton Inc., Denver, CO, USA) for 1 h. Slides from either preparation were mounted with VectaShield® containing DAPI (Vector Laboratories, Burlingame, CA, USA). Negative controls underwent the same procedure, but substituting histidine affinity-purified polyclonal rabbit IgG from non-immunized rabbits as the primary antibody.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

J.I.M. and B.D.F. conceived the study and designed the experiments. J.I.M. and M.G. performed the experiments. J.I.M. and B.D.F. analyzed the data and wrote the manuscript.

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Supplementary material

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