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ELUCIDATION OF SEROTONIN-RELATED PROFILES IN AEDES AEGYPTI DIGESTIVE TRACT PREDICTS NOVEL PUTATIVE ROLES OF SEROTONIN

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ABSTRACT

Despite its biological importance, understanding of serotonin roles in insect physiology is limited. To address this, the current study aimed to answer basic questions associated with serotonin-related distribution patterns in the *Aedes aegypti* (yellow fever mosquito) digestive tract. The rationale behind such analyses is based on the fact that serotonin effects are determined by its distribution and availability. Therefore, investigating such patterns is a pivotal step towards elucidating the serotonergic functions and significance. Two stages of the mosquito development cycle, those of larva and adult (female), were the focus of investigations. Their differential modes of feeding (continuous vs. discontinuous), diets, gut morphology and physiology make them a useful system to study and understand the fundamentals of the serotonergic mechanisms that regulate food processing in insects. Immunolocalization studies were the main approach carried out, and have not only identified differential aspects of the serotonergic network between the two stages but, importantly, have provided evidence assigning novel putative roles for serotonin in *Aedes aegypti* physiology. Overall, the midgut of both mosquito stages is innervated with serotonergic axons. However, while in the larval midgut, axon patterns vary significantly between the anterior and posterior section, in the female adult midgut, such differences were not observed. The posterior midgut (pMG) in both mosquito stages is richly innervated with serotonergic axons containing multiple but small release sites that, apart from regulating the gut epithelial cells, may mediate the Malpighian tubules (MT) functions. This statement is corroborated by AaSer-1 (a putative serotonin receptor) expression in the MT of both larvae and adults. Since MT are resting on the pMG surface, it is likely that the serotonergic source affecting them originates from the pMG. Thus, a new model has been proposed which attests (previously questioned) serotonin roles in diuresis. To further elaborate on the serotonergic-related profiles and significance during blood digestion, the expression of AaSer-1 in the adult female gut was investigated. While AaSer-1-specific immunofluorescence in sucrose-fed female adults is rather 'unremarkable' and mainly limited to the MT, in the blood-fed mosquitoes, expression patterns suggest receptor involvement in the nuclear, tracheal, muscular, and MT functions during blood digestion and absorption.

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Associations of AaSer-1 with tracheoblast formations and mediation of nuclear functions during blood digestion indicate novel putative roles of serotonin in mosquito (insect) physiology. Taken together, results provide strong evidence for the serotonin significance and complexity in the regulation of mosquito feeding-related processes.

Keywords

Serotonin, Serotonin receptors, Immunolocalization, Mosquitoes, *Aedes aegypti*, Digestive tract, Midgut, Malpighian tubules

1. INTRODUCTION

Serotonin (5-hydroxytryptamine; 5-HT) is a biogenic amine whose mode of action mediates a wide range of biological processes in both vertebrate and invertebrate phyla [1-3]. Its synthesis requires two enzymatic steps to convert tryptophan to 5-HT (Fig 1). 5-HT is present in the central nervous system (CNS), as well as in many peripheral tissues. However, in humans, the majority of 5-HT is present in the gastrointestinal tract (GI) to transduce various effects, including gut peristalsis [1, 2]. The enormously complex plethora of serotonergic effects are translated by an array of 5-HT receptors whose modes regulate diverse behavioral and physiological processes. Based on pharmacological data in mammalian systems, these receptors have been classified into seven families and several subfamilies [4, 5].

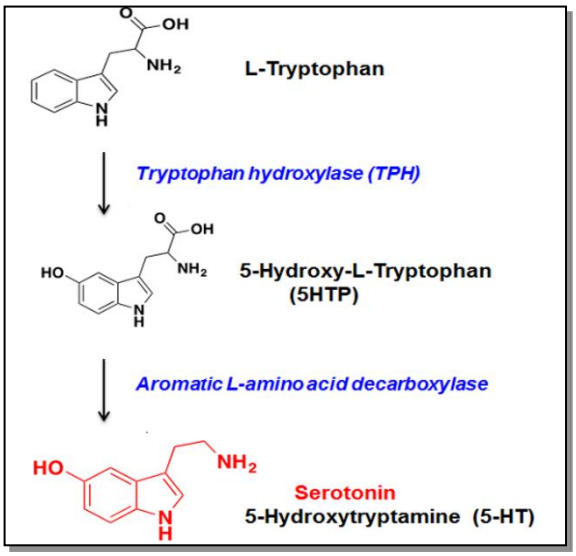

Figure 1. Serotonin (5-HT) biosynthesis pathway. 5-HT is synthesized from the amino acid L-tryptophan in two step-enzymatic reactions.

While elucidation of the serotonergic functions in mammalian systems has greatly advanced, current knowledge on this topic in insects is rather modest. However, research to date points out that serotonergic roles and functions in insects are equally diverse and significant [3]. Similar to vertebrates, 5-TH is found in the CNS [6-8] and peripheral organs [9-13] to regulate an array of processes [14-18], including feeding-related [19-21]. Studies in*Rhodniusprolixus* are a classical example of the serotonergic regulation of homeostasis through coordination of several feeding-related processes [22]. While in the early stages of feeding and nutrient processing, 5-HT mediates saliva secretion and promotes cuticle plasticization to allow expansion, in the later stages, 5-HT regulates diuresis and urine secretion.

Knowledge of the serotonergic roles in mosquito molecular physiology is limited [11, 12, 23]. However, previous studies on cloning and immunolocalization of AaSeR-1, a putative 5-HT receptor in the alimentary canal of *Aedes aegypti* (*A. aegypti*)larvae depicted rather intriguing and complex expression patterns, indicating receptor and thus, 5-HT diverse roles in mosquito physiology [24].

A. aegypti is a vector of several pathogens causing life-threatening diseases such as yellow fever, chikungunya, dengue fever. This mosquito also transmits the Zika virus. While in the past Zika virus did not trigger any serious effects on human health, its latest mutations are thought to cause neurological disorders, including disruption of brain development in fetuses, a condition also known as microcephaly. The story of Zika virus underlines the urgent need for the development of sustainable and safe novel control measures for mosquitoes and other arthropod vectors.

A. aegypti belongs to the order Diptera, whose life cycle undergoes full metamorphosis (holometabolous) through a four-stage development – egg, larva, pupa and adult (Fig 2). While larvae and pupae are adapted to aquatic conditions, adult mosquitoes require terrestrial habitat with close proximity to water. Only adults and larvae consume food, meaning that their feeding apparatus, digestive tract, and feeding physiology have evolved in accordance with their environment. Larvae are characterized by a continuous mode of feeding whose alimentary canal has three morphologically distinct zones – the foregut, midgut, and hindgut [25].

However, nutrient digestion and absorption occur mainly in the midgut, which is rather prominent with anterior and posterior sub-regions [25]. In contrast, adult mosquitoes feed intermittently on nectar, with only females requiring blood diet to enable egg

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development. While nectar juices are directed into the crop (a separate diverticular from the midgut), blood is digested in the midgut [26]. 5-HT biological effects are determined by its availability and distribution patterns. Thus, establishing 5-HT patterns will enable the prediction of putative roles and physiological significance of the serotonergic regulation in *A. aegypti* gut function. Although previous study has shown some elements of the *A. aegypti* serotonergic network [11], the current investigations have provided an elaborate and detailed analyses suggesting novel putative roles and complex effects of the serotonergic regulation in *A. aegypti* gut.

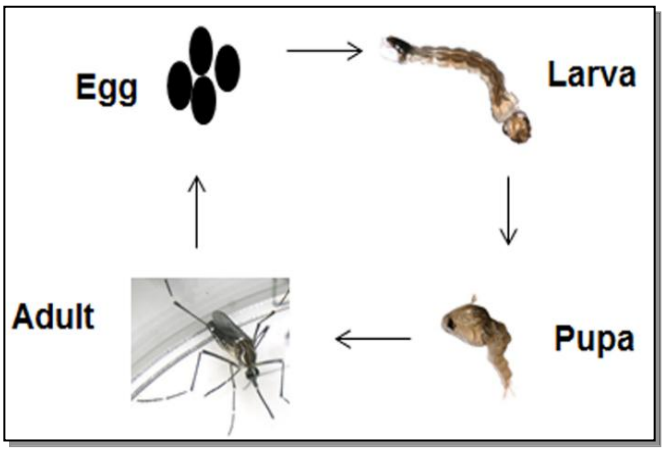

Figure 2. *Aedes aegypti* life cycle. The mosquito development is characterized by four stages – egg, larva, pupa, and adult (full metamorphosis). At approximately 72 h post-blood feeding, the female mosquito will initiate egg laying. Eggs (1mm long) are usually laid on damp surfaces in close proximity to water. After hatching, larvae reside in water pools and feed on algae and other microscopic organisms. The larval development undergoes four instars where the latest instar in the end of its development is transformed into a pupal stage. Pupae are active but do not feed. The cycle is complete when adults are emerging from pupae.

The first objective of this study was to investigate and establish 5-HT-related profiles in the *A. aegypti* digestive tract. Since larvae and adult females have different feeding modes, diets, and gut physiology, the second objective focused on identifying specific 5-HT trends that may correlate with the larval or adult feeding and digestive physiology. The third objective aimed to elucidate the serotonergic involvement in blood digestion, through investigating the expression of AaSeR-1. Understanding such mechanisms will not only provide answers related to basic science but, importantly, pinpoint key sites for targeting and development of sustainable pest control strategies. Therefore, elucidating the role of 5-HT in this process is of paramount importance. The following findings have created a valuable platform for the development of a new paradigm that assigns novel putative roles of 5-HT in *A. aegypti*, as well as underlining the serotonergic physiological significance in *A. aegypti* digestion and absorption mechanisms: (A) Larval and female adult gut of *A. aegypti* have different serotonergic profiles. (A-1) While in larvae, differential 5-HT axon patterns between the anterior midgut (aMG) and posterior midgut (pMG) were observed, in the female adult midgut, no significant differences between the two sub-regions were recorded. (A-2) The type of 5-HT innervation in the larval midgut suggests that 5-HT is released from multiple sites but in small quantities. Therefore, this mode of serotonergic regulation is likely to mediate local (acting on neighboring cells) or semi-systemic effects (released in the hemolymph but affecting cells that are close to 5-HT released sites, such as Malpighian tubules (MT)). In the female adult gut, however, in addition to the larval type 5-HT innervation, a larger 5-HT release sites (ganglia-like structures) are found which have the potential to deliver larger quantities, and thus, mediate stronger effects. (B) In the larval gut, most of the serotonergic innervation is observed in areas (caeca and pMG) of active digestion and enzyme production, suggesting the significant involvement of 5-HT in these processes. (C) The expression of AaSer-1 in the MT, as well as the elaborate 5-HT axon innervation in the pMG, and MT resting on the pMG surface, are facts that combined together form a basis for a new model that justifies 5-HT roles in the modulation of the MT functions. (D) The serotonergic presence in the GC cells, tracheoles in the MT, as well as the expression of AaSer-1 in the nucleus, trachea and especially during tracheoblast formations, are findings that break the limitations of the current perception for the serotonergic functions in insect physiology and attest novel putative 5-HT roles. (E) The final, but very important point emphasizes on the physiological significance and diverse putative roles of 5-HT during blood digestion, absorption, and secretion of water and ions. This was demonstrated by the differential expression patterns of AaSeR-1 in the gut of sucrose- and blood-fed *A. aegypti* females.

2. MATERIALS AND METHODS

2.1 Insects

Eggs of *A. aegypti* were hatched in a 50:50 mixture of tap and deionized water to which a small amount of baker's yeast was added to provide anoxic conditions. Larvae were maintained in 50:50 mixture of tap and deionized water at 27-30^{o}C and fed each day with ground fish food (TetraColor). Newly emerged adults were kept in cages in a controlled environment chamber (27-30^{o}C, 55-70% humidity). Prior blood feeding, adults were sucrose-fed (10% sucrose diet) for 10-15 days. For mosquito blood-feeding, sedated rats

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were exposed to the mosquito colony by placing them on mesh-covered cage window to allow contact between. Animal procedures and care are performed under protocol IACUC-04432-002, at the Eastlick Vivarium, Washington State University, Pullman.

2.2 5-HT immunohistochemical analyses

For 5-HT immunolocalization 3-4 instar larvae and 10-15 days old sucrose-fed female adult mosquito were used. Following dissection, mosquito guts (larvae and female adults) were fixed in 100 mM Phosphate buffer pH 7.4 (100 mM Na4HPO4, 100 mM NaH2PO4; J.T. Baker) containing 4 % Paraformaldehyde (Ted Pella Inc) for 2 h at room temperature (RT). After fixation, samples were washed (6 x 10 min) with 1 x PBS pH 7.4, and then blocked (3 h) in 1 x PBS (pH 7.4) containing 5 % goat serum (Gibco) and 5% Triton (Sigma). This was followed by incubation (3-4 h, at RT) in antiserum diluent solution (1 x PBS with 5 % goat serum and 0.5 % Triton) containing either: (i) anti-serotonin antiserum (1:300; Sigma), for 5-HT detection, (ii) pre-absorbed (BSA-conjugated 5-HT) anti-5-HT antiserum (1:300), for specificity analyses; (iii) Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) secondary antibodies (1:300; Molecular Probes-Life technologies), for negative control. The subsequent washing steps (10 x 10 min) were carried out to ensure complete removal of unbound primary antibodies. To visualize specific binding of anti-AaSeR-1 antibodies, samples from (i) and (ii) were incubated with Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) secondary antibodies (1:300) for 2 h at RT. Final wash (1 x PBS, pH 7.4) for (i), (ii) and (iii) was carried out overnight with minimum of 15 changes of buffer. Followingthe final wash, samples were transferred onto glass slides and immersed into VectaShield (Vector Labs) mounting medium with 4', 6-diamidino-2-phenylindole staining (DAPI; blue color), which is a DNA-specific marker. Imaging analyses were carried out on Zeiss 510 META Confocal Laser Scanning Microscope at the Franceschi Microscopy and Imaging Center, Washington State University, Pullman.

2.3 AaSeR-1 immunohistochemical analyses

AaSeR-1 cloning information and immunolocalization procedures are described in [24]. Mosquito female adults used for these analyses were either sucrose-fed or blood-fed (24 h post-blood feeding).

3. RESULTS AND DISCUSSION

3.1 5-HT in the *A. aegypti* larval digestive tract

The larval midgut has four morphologically distinct regions – cardia, gastric caeca (GC), anterior (aMG) and posterior midgut (pMG) [25]. The cardia (Figure 3A) is a dome-shaped structure that connects the midgut with the esophagus and envelops the cardiac valve, whose primary role is to direct ingested food toward the midgut [25]. The specific immunofluorescence observed in the cardia-GC region suggest ubiquitous 5-HT presence, highlighting the significant physiological role of 5-HT in this region of the digestive tract (Fig 3A). The serotonergic axon/neuron network covering the cardia is characterized by complex distribution patterns (Fig 3B). Particularly noticeable is the concentration of serotonergic axons on the border region between the cardia and esophagus, possibly related to the regulation of muscle functions (Fig 3B). The posterior end of cardia evolves into eight pouch-like formations, also called gastric caeca (GC). Emanating from the border-line between the cardia and anterior midgut, GC not only connect these two regions, but play a prominent role in nutrient digestion and absorption (Fig 4A) [25]. Each caecum consists of cells that carry diverse functions, and have been classified into (i) reabsorbing/secreting, (ii) ion-transporting, (iii) membrane-secreting, and (iv) imaginal cells [27]. Apparently, functional cell specialization has a spatial profile, where cells at the proximal site of the GC may participate in digestion, and those at the distal sites could mediate ion transport [25, 27]. The elaborate serotonergic axon innervation in the GC region suggest that 5-HT coordinate functions between the proximal and distal part of the caecum, since serotonergic axons are linking these sites both longitudinally and transversely (Fig 4A and 4B). Although still under investigation, it appears that some GC cells are positive for 5-HT (Fig 4B). These cells do not have the typical shape of the enteroendocrine cells (vase- or triangular-like shapes) and therefore, at present, they will be referred as 5-HT positive cells. Further co-localization studies, particularly with anti-Tryptophan hydroxylase, will elaborate on current findings and provide a conclusive answer as to whether these cells have endocrine functions and participate in 5-HT biosynthesis. The expression of AaSeR-1 in some GC cells [24] supports current results and strengthens the conclusion that the serotonergic network in the GC region is ubiquitous and it is likely to be a key regulator of GC functions.

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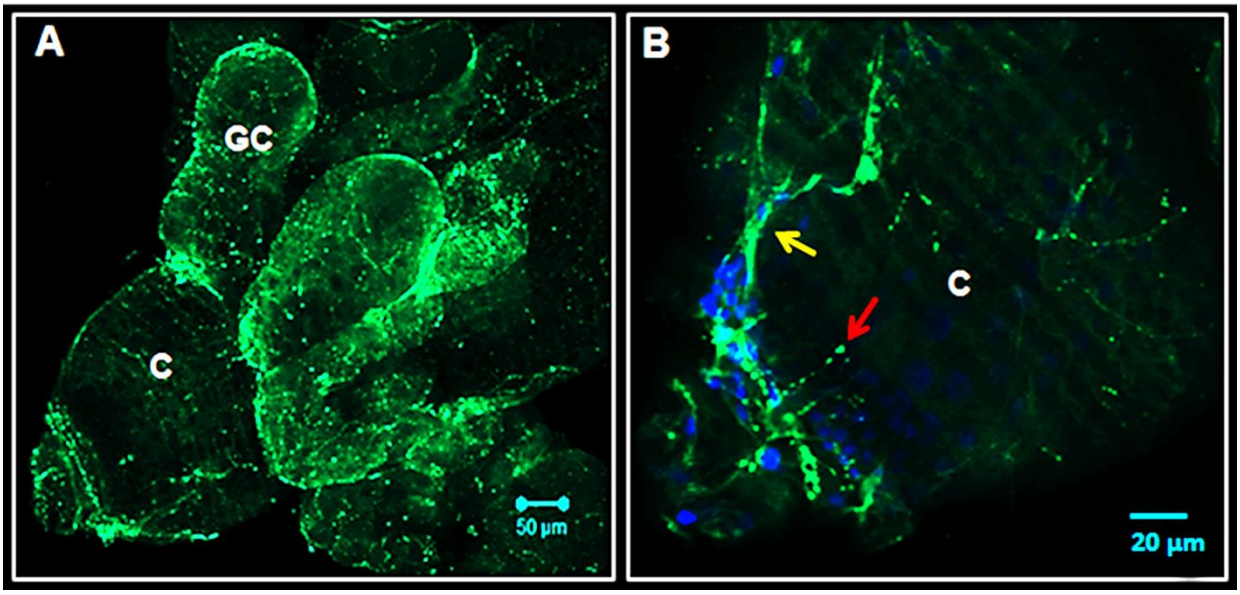

Figure 3. 5-HT-specific immunofluorescence in the cardia and gastric caeca of *Aedes aegypti* larval gut. (A) 5-HT-specific immunofluorescence patterns (green, Ex. 488) are suggesting ubiquitous serotonergic presence in the cardia (C) and gastric caeca (GC) and thus, underlying the biological significance of 5-HT regulation in these regions. (B) A higher magnification image is depicting both axons (red arrow) and neurons (yellow arrow) in the cardia. Nuclear DNA is stained with DAPI in blue (Ex. 405). Scale bars (µm) are included in each image.

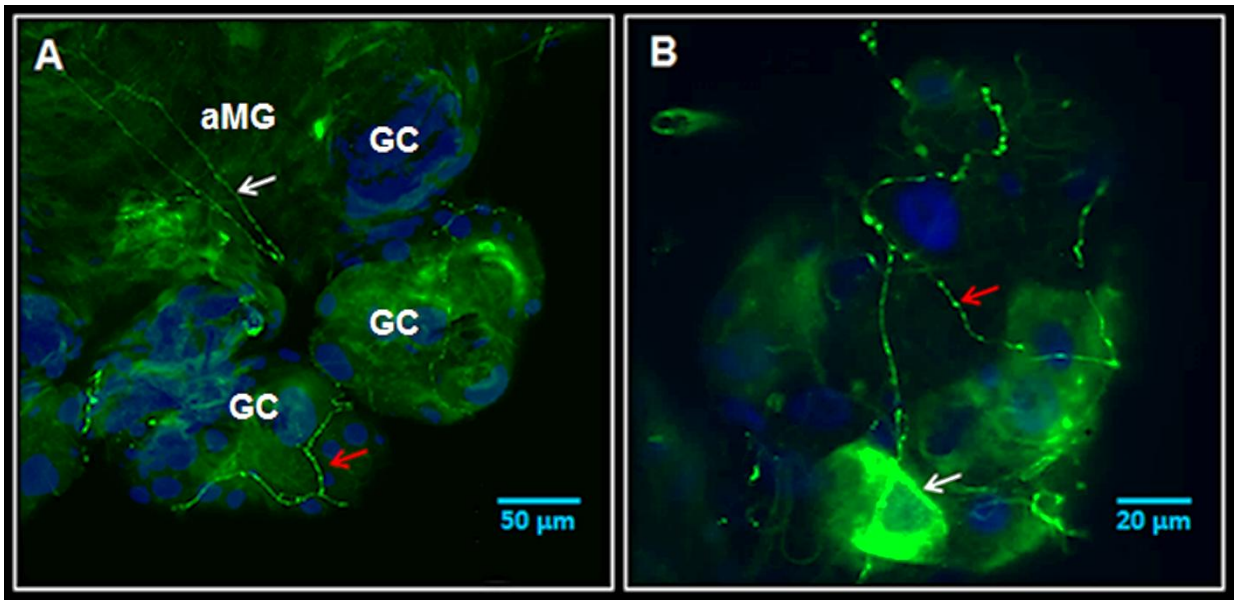

Figure 4. Detailed imaging analyses of 5-HT-specific immunofluorescence in the gastric caeca of the *Aedes aegypti* larval gut. (A) A superimposed image with 5-HT-specific (green, Ex. 488) and nuclear-stained fluorescence (blue; DAPI, Ex. 405) is showing serotonergic axons with their position in relation to the nuclei in the anterior midgut (aMG, white arrow) and gastric caeca (GC, red arrow). (B) A higher magnification image of a single cecum covered with 5-HT axons (red arrow) demonstrating that some caecal cells are 5-HT-positive (white arrow). The image is superimposed with blue nuclear-specific (DAPI stained) fluorescence to show nuclei position. Scale bars (µm) are included in each image.

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In contrast to cardia-GC region, 5-HT-specific immunofluorescent patterns in the aMG are limited to two serotonergic axons that project along the aMG with no visible branching (Fig 5A). It must be noted that these analyses do not have an absolute quantitative nature, since preparation procedures such as dissecting (stretching gut tissue) or numerous washes, may affect axon integrity. Therefore, caution must be exercised with the interpretation of these results. However, analyses from several samples support the above, and furthermore, point out that the serotonergic axon network varies between the aMG and pMG. Unlike aMG, the 5-TH innervation in the pMG is more complex and ubiquitous, with serotonergic axons projecting longitudinally and transversely to form an elaborate neurohemal plexus with multiple release sites (Fig 5B). The differential serotonergic profiles between the aMG and pMG reflect the uniqueness of the *A. aegypti* larval midgut, which has remarkable ability to maintain highly alkaline pH (>10) in the aMG via basally expressed vacuolar-type ATPase (V-ATPase) [28], and almost neutral in the pMG via apical V-ATPase [29, 30]. Both alkalization in the aMG and acidification in the pMG have been shown to be mediated by 5-HT, at least in part [30, 31]. Knowledge of serotonergic signaling cascades occurring in the *A. aegypti* midgut are yet to be established, but it is emerging that intracellular Ca^{2+} (Ca^{2+}i) fluxes are involved in the translation of some 5-HT effects in the midgut of *A. aegypti* larvae [32]. It was shown that following 5-HT treatment, the aMG and pMG respond with different Ca^{2+}i patterns [32], which corroborate with the above. Further evidence of 5-HT differential effects in aMG and pMG is provided by AaSeR-1-specific immunofluorescence in the larval midgut [24]. Whilst in the aMG, AaSeR-1 was localized in the nucleus and nucleolus of some principal cells, in the pMG, AaSeR-1 was associated with the plasma membrane [24]. In addition, receptor-specific immunofluorescence was found in some enteroendocrine cells (EECs) [24]. The expression of AaSeR-1 in the nucleus and nucleolus in the aMG not only suggest novel 5-HT roles, such as regulation of nuclear functions, but also provides evidence challenging contemporary views of the serotonergic roles in insect physiology. Furthermore, receptor nuclear expression postulates questions related to 5-HT origin.

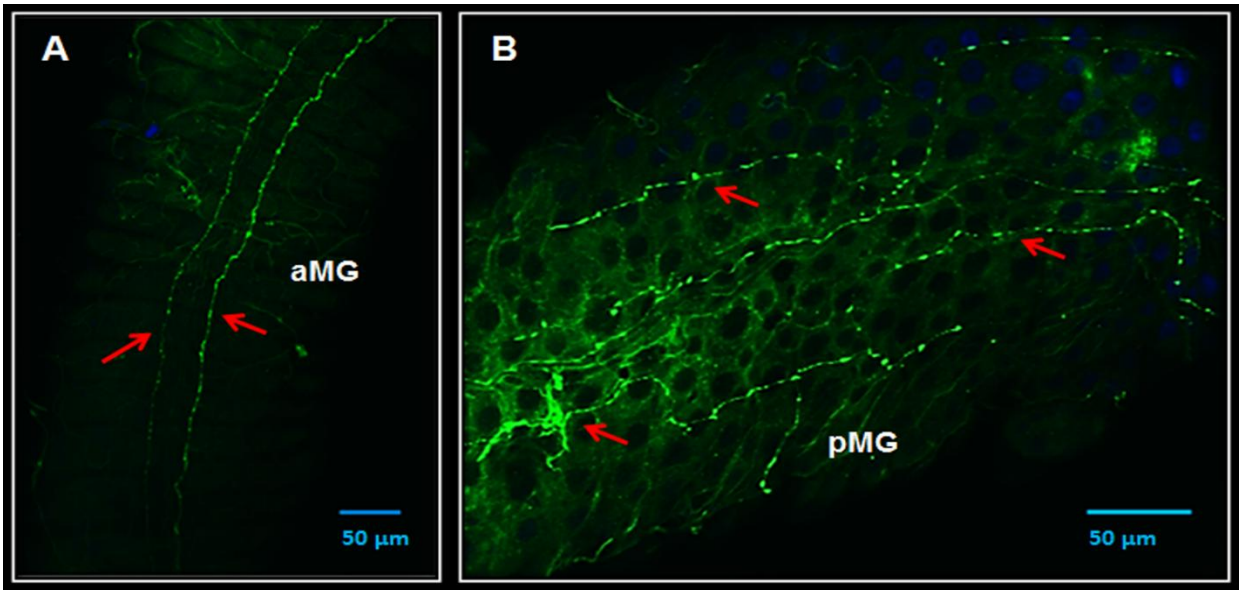

Figure 5. A representation of the serotonergic axon networks in the anterior (aMG) and posterior (pMG) midgut of *Aedes aegypti* larvae. (A) 5-HT-specific immunofluorescence (green, Ex. 488) in the aMG is suggesting the presence of only two axons (red arrow). (B) In contrast to the aMG, the pMG is characterized with an elaborate serotonergic axon plexus (red arrows) containing many release sites. Both images are superimposed with blue nuclear-specific fluorescence (DAPI-stained, Ex. 405) to show the nuclei position. Scale bars (µm) are included in each image.

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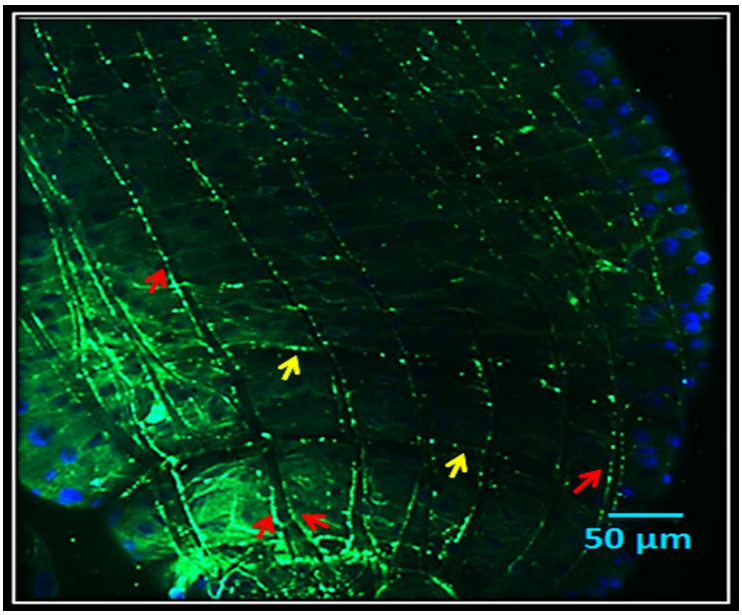

Figure 6. The 5-HT axon plexus is projecting along the visceral muscle network in the pMG of *Aedes aegypti* larvae. 5-HT-specific immunofluorescence (green, Ex. 488) in the pMG suggests that there are 'muscle-specific' serotonergic axons, since they are stretching along the circular (yellow arrows) and the longitudinal (red arrows) muscles. The image is superimposed with blue nuclear-specific fluorescence (DAPI-stained, Ex. 405) to show nuclei position. Scale bar (µm) is included in the image.

The 5-HT regulatory roles of muscle functions are well documented [20, 33]. Results in this study also suggest such roles where 5-HT axons were observed along gut muscles, corroborating with previous data on AaSer-1 expression in both circular and longitudinal muscles [24]. It appears that the longitudinal muscles are characterized by elaborate serotonergic axon innervations found to stretch along both sides of each muscle (Fig 6). Such axon projections are less visible on the circular muscles. This may be due to the fact that the circular muscles are positioned under the longitudinal muscles, which would ultimately affect the visibility and accuracy of imaging analyses.

Altogether, analyses on the serotonergic plexus in the midgut of *A. aegypti* larvae depict specific trends. First, the primary sites for nutrient processing and synthesis of digestive enzymes, GC and pMG, are richly innervated with 5-HT axons. This correlation not only demonstrates 5-HT physiological significance in the regulation of these sub-regions of the midgut, but importantly, connects 5-HT with processes related to nutrient digestion and absorption, production and secretion of digestive enzymes. In addition, only in these two regions there are EECs whose role is to

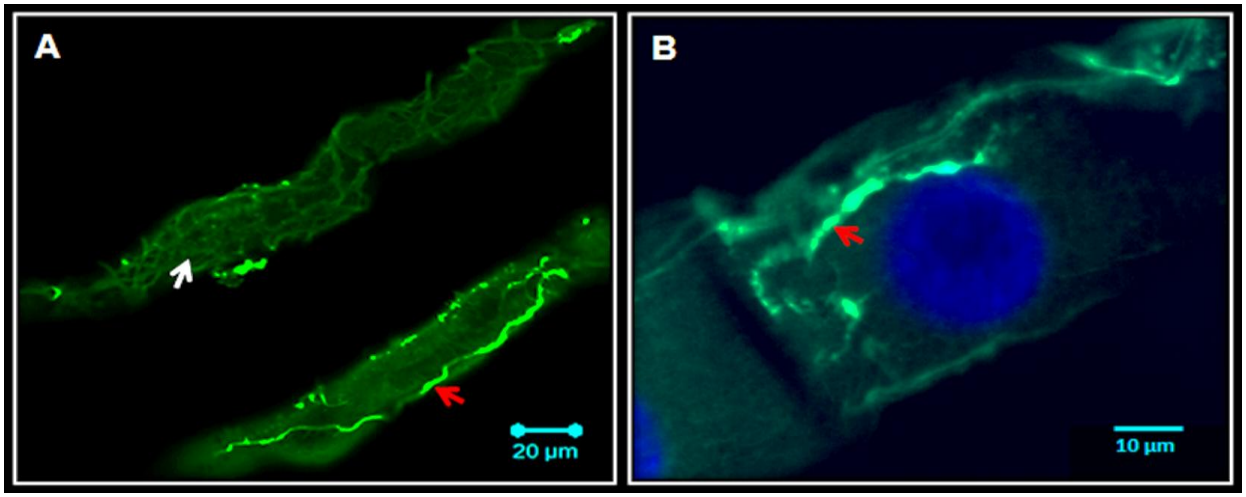

Figure 7. 5-HT-specific immunofluorescence was detected in the tracheolar system of the MT of *Aedes aegypti* larvae. (A) Surprisingly, serotonergic immunofluorescence (green, Ex. 488) was found in the extracellular tracheolar network (red arrow) of the MT. The tracheal system in insects is providing oxygen to tissues and cells, where the larger trachea diverged into smaller and finer formations called tracheoles. The MT are richly innervated with tracheoles (white arrow). (B) A rare image is depicting 5-HT specific immunofluorescence associated with 'intracellular' positioned tracheolar. 'Intracellular' tracheoles are technically outside the cell since they dent the plasma membrane to position nearby mitochondria and deliver O2. The image is superimposed with blue nuclear-specific fluorescence (DAPI-stained, Ex. 405) to show the nucleus position. Scale bars (µm) are included in each image.

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synthesize and secrete hormones. The fact that AaSer-1 was localized in some EECs in the pMG [24] not only demonstrates 5-HT regulatory functions in these cells but also shows the complexity and plasticity of the serotonergic system in insect physiology. Thus, 5-HT not only regulate post-feeding-related processes directly, but may have indirect effects as well by affecting the biosynthesis of other hormones. Second, in the aMG, 5-HT presence is limited to two axons, which not only demonstrates the differential profile between aMG and pMG, but importantly, based on its presence it may be

concluded that 5-HT impact is limited on aMG functions. In contrast to the midgut, 5-HT innervation in the hindgut (HG) region does not appear to be present. However, surprisingly, 5-HT-specific immunofluorescence was detected in the tracheolar network of the MT (Fig 7). Unlike mammalian systems, where O_2 is carried out by the blood, gas exchange functions in insects are performed by the tracheal system.

Tracheae arise from spiracles which are openings usually positioned on the sides of insect body [34]. To ensure sufficient O_2 supply to all cells, the tracheal system branches into an elaborate network where larger tracheae diverge into smaller ones, and those under 1 µm in diameter are called tracheoles [34]. Tracheoles may rest on the cell surface or indent the plasma membrane to position in close proximity with the mitochondria and deliver O_2. MT are richly innervated by tracheoles whose 'extracellular' (Fig 7A) and 'intracellular' (Fig 7B) networks have shown specific 5-HT- immunofluorescence. This is a rather surprising result, requiring further investigations to provide a deeper understanding of the serotonergic origin and roles in the MT tracheoles. However, at present, data not only point out novel serotonergic sites but importantly provides a basis for questioning the traditional views of the 5-HT origin and physiological roles in insects.

3.2 5-HT and AaSeR-1 *in A. aegypti* female adult

In contrast to larvae, *A. aegypti* adults are discontinuous feeders, meaning that the periods of feeding and non-feeding determine specific molecular mechanisms and phases of digestion [35, 36]. As indicated earlier, adult mosquitoes feed on a nectar-based diet for nutritional sustenance [35]. Only female adults consume blood to enable egg development. Blood digestion and absorption takes place mainly in the midgut [36]. Since feeding-related processes in adult mosquitoes are different (e.g. discontinuous feeding, discontinuous synthesis and secretion of digestive enzymes; different diet type, etc.) from those of larvae (continuous feeding), it is important to establish whether such differences are also present in 5-HT distribution profiles.

The aMG and pMG sections of the *A. aegypti* adult female midgut do not have a clear differentiation as what is observed in the larval midgut. There are no obvious differences between the 5-HT profiles in these sub-regions as well (Fig 8A and Fig 8B). Overall, the serotonergic innervation is ubiquitous, which points out the physiological importance of 5-HT regulatory functions in the female adult midgut. It appears that while longitudinally positioned serotonergic axons have no particular pattern, the transversely orientated axons are spaced between four cell rows (Fig 8A and 8B). In addition to the serotonergic axon network, bigger neurohemal structures (ganglia-like) are observed, suggesting that these may release a larger quantity of 5-HT (compared to one axon release site) and produce stronger effects (Fig 8A and 8B). This finding indicates that 5-HT plexus in the gut of adult mosquitoes have two modes of serotonergic release to possibly facilitate the stressful process of blood digestion. These type of neurohemal structures have not been found in the larval gut. Furthermore, different confocal optical sections suggest that the serotonergic plexus in the gut of female adult mosquito is much more complex and elaborate (Fig 8C). The area around the pylorus (pMG) is richly innervated as well (Fig 9). Specific immunofluorescence was not found in the HG, including the MT.

Mosquito adaptation of processing large volumes of blood is a remarkable mechanism, controlled by an array of hormones, including 5-HT. To elaborate further on the serotonergic involvement in the regulation of blood-related post-feeding processes, the elucidation of AaSeR-1 expression patterns in the *A. aegypti* female adult gut were carried out. Prior to blood-feeding, the receptor is predominantly found in the MT (Fig 10A). There are also some indications of the AaSeR-1 association with tracheae (Fig 10B). However, this must be interpreted with caution, since tracheal structures (decaying matter, taenidia formations, etc.) may trap antibodies within. While the AaSer-1 expression in the gut of sucrose-fed mosquito adults is rather 'unremarkable', in blood-fed mosquitoes, the strong and ubiquitous receptor-specific immunofluorescence demonstrates the receptor expression at multiple sites in the midgut. This suggests receptor involvement in the regulation of diverse molecular mechanisms in different types of tissues and cells, during blood processing (Fig 11A). First, AaSeR-1-specific immunofluorescence was found in the nucleus of some gut epithelial cells (Fig 11A-11C) indicating that the receptor activation is correlated with blood digestion and absorption mechanisms and the overall maintenance of mosquito homeostasis. This phase of feeding is characterized by high metabolic rates, including the transcriptome network being at its activity peak to facilitate rapid blood digestion and processing [37, 38], as well as, the biosynthesis

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of *de novo* enzymes and the maintenance of cell homeostasis [39-42]. The nuclear-related patterns of AaSeR-1 highlights two important aspects: (i) the receptor-specific temporal expression patterns in the nuclei are associated with the regulation of blood digestion and absorption; (ii) since the receptor is activated by 5-HT to translate specific effects, it must be noted the significant role of 5-HT in the regulation of some nuclear/transcriptome mechanisms occurring during blood processing. Nuclear association of AaSeR-1 was previously reported in *A. aegypti* larval gut [24], which strengthens the above and provides further understanding of the serotonergic functions in mosquito and insect physiology.

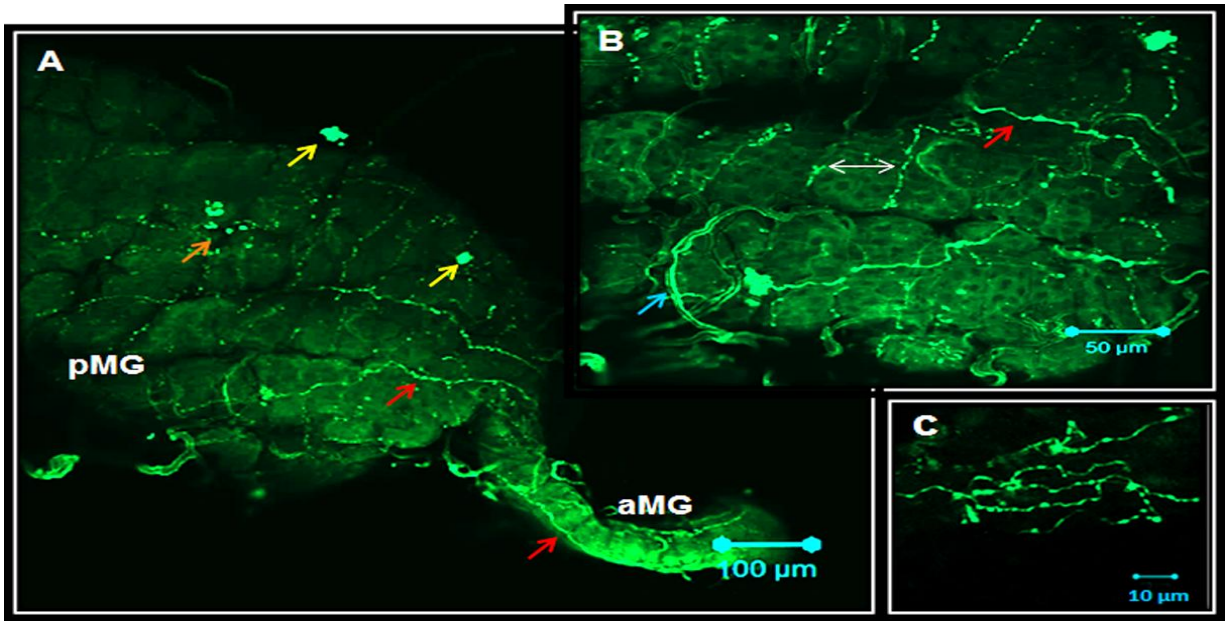

Figure 8. The midgut of *Aedes aegypti* female adults is richly innervated by serotonergic axons network. (A)5-HT-specific immunofluorescence (green, Ex. 488; red arrow) in the anterior midgut (aMG) and posterior midgut (pMG) of female adults suggests the presence of the serotonergic plexus in this regions (red arrow). In addition, smaller (brown arrow) and larger (yellow arrow) 5-HT–positive neurohemal (ganglia-like) structures are observed as well. (B) Higher magnification image depicts both longitudinal (red arrow) and transverse innervation patterns (white arrow). The transverse type innervation is positioned between 4 cells (white arrow). There are some indications of serotonergic presence in the tracheal system (blue arrow). (C) Image from a different optical section is suggesting that the serotonergic plexus in the midgut is multilayered and complex. Scale bars (µm) are included in each image.

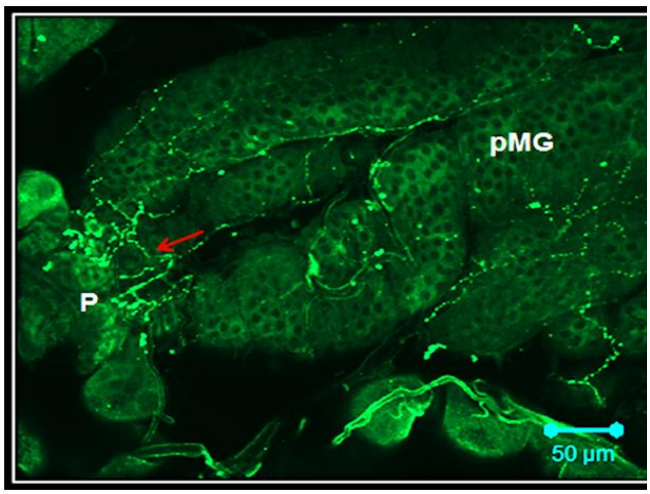

Figure 9. A dense serotonergic axon network is observed near the pylorus (P) area of the *Aedes aegypti* female adult midgut. 5-HT-specific immunofluorescence patterns (green, Ex. 488, red arrow) in the rear end of the posterior midgut (pMG) showing an elaborate 5-HT axons network that may participate in the regulation of the pyloric valve and the Malpighian tubules. Scale bar (µm) is included in the image.

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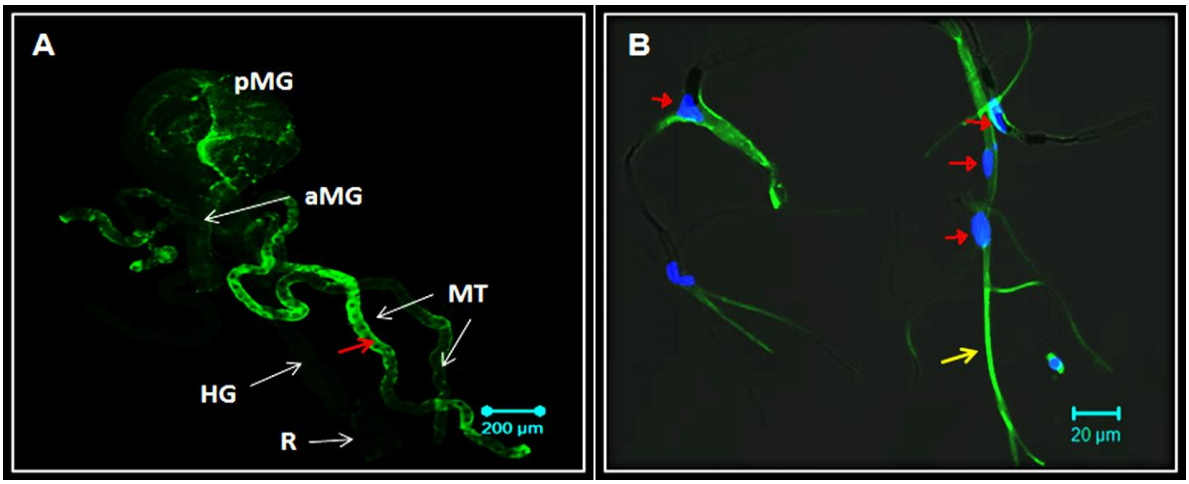

Figure 10. AaSeR-1-specific immunofluorescence in the midgut of sucrose-fed *Aedes aegypti* female. (A)In sucrose-fed adult mosquitoes, the AaSeR-1-specific immunofluorescence (green, Ex. 488; red arrow) is mainly observed in the MT. (B) A bright field image superimposed with green (specific for AaSeR-1, yellow arrow) and blue nuclear-specific (DAPI-stained, Ex. 405, red arrow) fluorescence demonstrates the receptor presence in the tracheal system. Scale bars (µm) are included in each image.

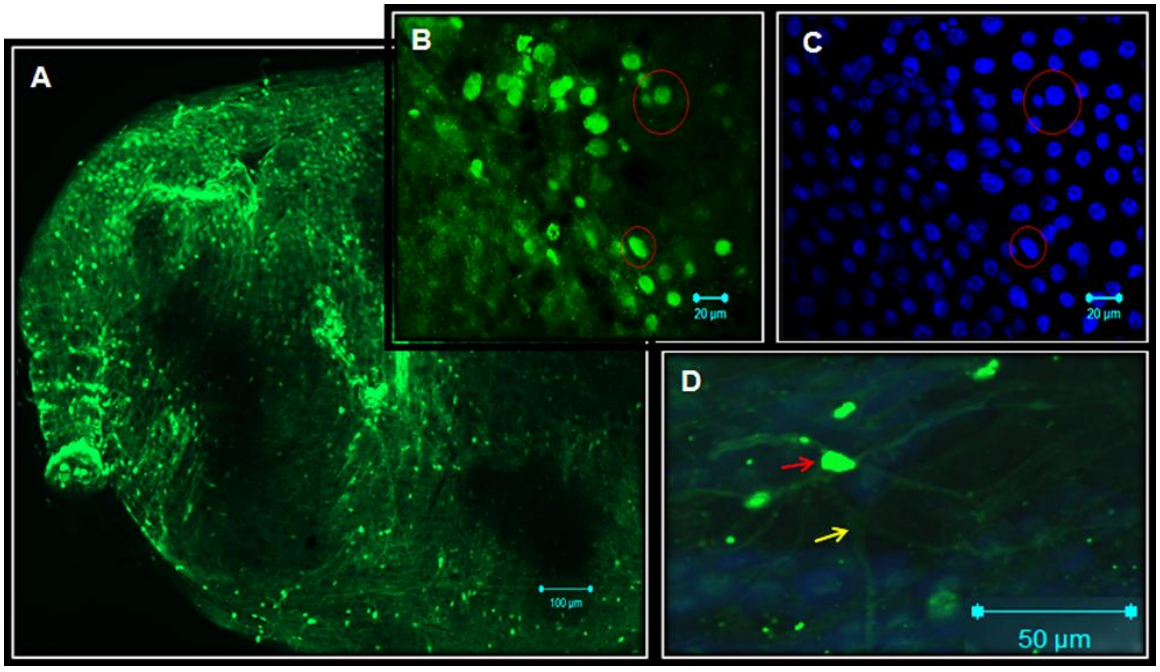

Figure 11. AaSeR-1-specific immunofluorescence in the midgut of blood-fed *Aedes aegypti* adult female at 24 h post-blood feeding. (A) In contrast to sucrose-fed female adult mosquitoes, AaSeR-1-specific immunofluorescence (green, Ex. 488) in the gut of blood-fed mosquitoes is ubiquitous. (B) Higher magnification image depicts specific immunofluorescence in the nucleus (examples circled in red, red arrows). (C) Co-localization with DNA-specific (DAPI-stained Ex. 405) blue fluorescence confirms AaSeR-1 nuclear expression (examples circled in red, red arrows). (D) A rare image of tracheoblast with AaSeR-1-specific immunofluorescence (red arrow) is indicating that the receptor is involved in the formation of a new trachea. Fully developed trachea are shown with yellow arrow. Scale bars (µm) are included in each image.

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AaSeR-1 specific immunofluorescence was detected in some tracheal epithelial cells at 24 h post-blood feeding period, suggesting that gas exchange mechanisms during phases of high metabolic rates, such as processing of the blood meal, are regulated in part by 5-HT (Fig 11D). The tracheal epithelial cells are involved in the development of new tracheoles [43]. The process is initiated by the growth of the tracheal epithelial cells towards the principal cells that require extra O_2[43]. Usually, in the first phase of enlargement, the tracheal epithelial cells assume a triangular shape as seen in Fig 11D, often called tracheoblast. The fact that receptor-specific immunofluorescence was observed during this phase suggests that the serotonergic regulation is a part of the molecular machinery orchestrating the development of new tracheae and tracheoles. This finding not only highlights a novel serotonergic role in mosquito and possibly insect physiology, but also provides a rare imaging of a tracheoblast formation (Fig 11D).

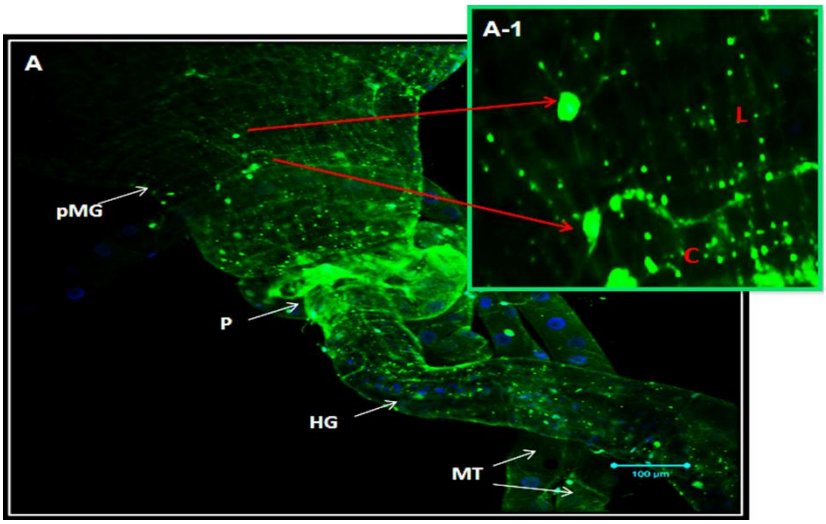

Figure 12. AaSeR-1 expression in the posterior midgut (pMG) and the hindgut (HG) of blood-fed *Aedes aegypti* female at 24 h post-blood feeding. (A)A lower magnification image of pMG-HG region is demonstrating AaSeR-1-specific immunofluorescence (green, Ex. 488) at 24 h post-blood feeding. While in the pMG AaSeR-1 is associated with the circular (C) and longitudinal (L) muscles and with larger formations (ganglia-like or soma-like), in the HG, receptor expression does not have specific patterns. The image is superimposed with blue fluorescence, resulting in of DNA-specific DAPI staining (Ex. 405) to show nuclei position. Legend: P – pylorus; MT - Malpighian tubules. (A-1) Enlargement of a small section from pMG is depicting AaSeR-1-specific immunofluorescence in the circular (C) and longitudinal (L) muscles. Furthermore, ganglia-like (or soma-like) formations with receptor-specific immunofluorescence (red arrows) are also shown. Scale bar (µm) is included in the image.

Further evidence for the complexity of the serotonergic regulatory functions is the AaSeR-1 expression in the gut muscles (Fig 12). The receptor-specific immunofluorescence was demonstrated in the circular and longitudinal muscles of the pMG in *A. aegypti* adults at 24 h post-blood feeding. Currently, it is unknown if the receptor is involved in the mediation of peristaltic movements or other muscle functions. However, at this stage of investigations is evident that its expression in the gut muscles is correlated with the post-blood feeding mechanisms, including the support of the rather fragile gut cell monolayer during the rapid expansion that occurs after blood ingestion. In addition, the receptor-specific immunofluorescence was demonstrated in ganglia-like (or soma-like) formations in the pMG, as well as in the hindgut (HG) but without specific patterns (Fig 12). The AaSeR-1 multiple functionality were further demonstrated by its expression in the MT, a key organ for excretion of water and solutes. The strong AaSeR-1-specific immunofluorescence observed in the MT of blood-fed mosquitoes (Fig 13A) emphasize the importance of the receptor-mediated serotonergic effects on the MT function during blood digestion and absorption. The ubiquitous presence of AaSer-1 in the MT of both larvae and adults is a pivotal point of a new model which proposes that 5-HT released from the serotonergic plexus of the pMG regulates gut epithelial cells, as well as the MT by local or semi-systemic effects. The type of serotonergic innervation in the pMG, which is ubiquitous, mesh-like axon network with numerous, but small release sites indicates that 5-HT is dispensed at multiple sites in small quantities. Such amounts of 5-HT are likely to impact cells and tissues at close proximity, either through direct delivery to the nearby cells or semi-systemically via the hemolymph at small distances. Since MT are positioned on pMG surface, it is likely that the primary serotonergic source that regulates MT functions originates from the 5-HT plexus of the pMG. The proposed model challenges previous views, where 5-HT role as a diuretic hormone in *A. aegypti* adult gut has been questioned [44].

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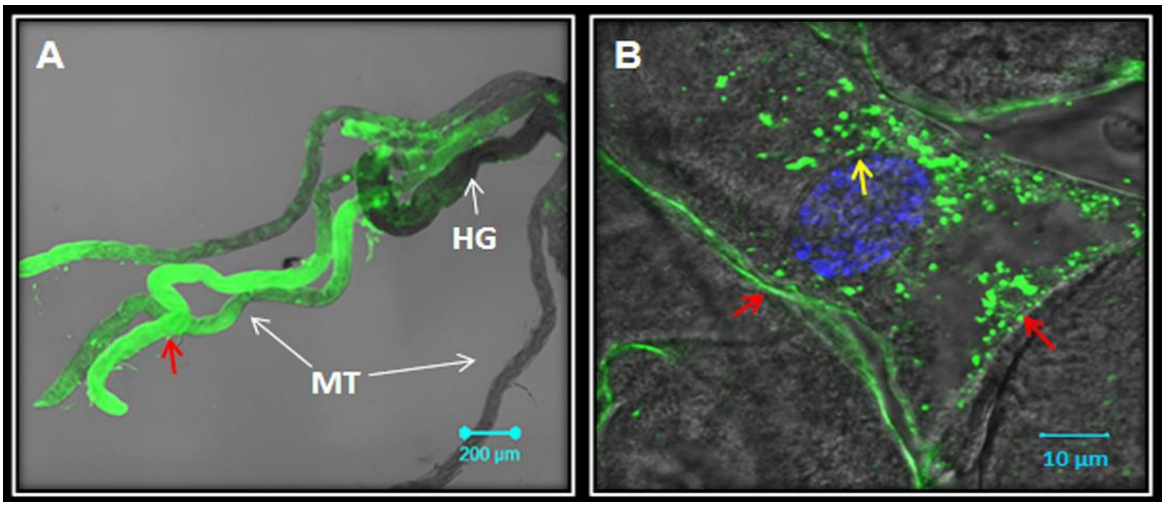

Figure 13. AaSeR-1 expression in the Malpighian tubules (MT) of blood-fed *Aedes aegypti* female at 24 h post-blood feeding. (A)A bright field image superimposed with AaSeR-1-specific green immunofluorescence (Ex. 488) in the MT, indicating receptor high level of expression at 24 h post-blood feeding. The variable receptor expression patterns between different MT and between regions of each MT suggest complex physiological roles of AaSer-1. Legend: HG – hindgut. (B) A bright field image superimposed with AaSeR-1-specific (green, Ex. 488) and nuclear-specific (blue, DAPI stained, Ex. 405) fluorescence is demonstrating receptor expression in a principal cell of the MT. Receptor-specific immunofluorescence is associated possibly with the plasma membrane (red arrows) and with the intracellular domain of some principle cells of the MT. The latter may suggest that the receptor is a part of an intracellular membrane network (e.g. nuclear membrane, yellow arrow). Scale bars (µm) are included in each image.

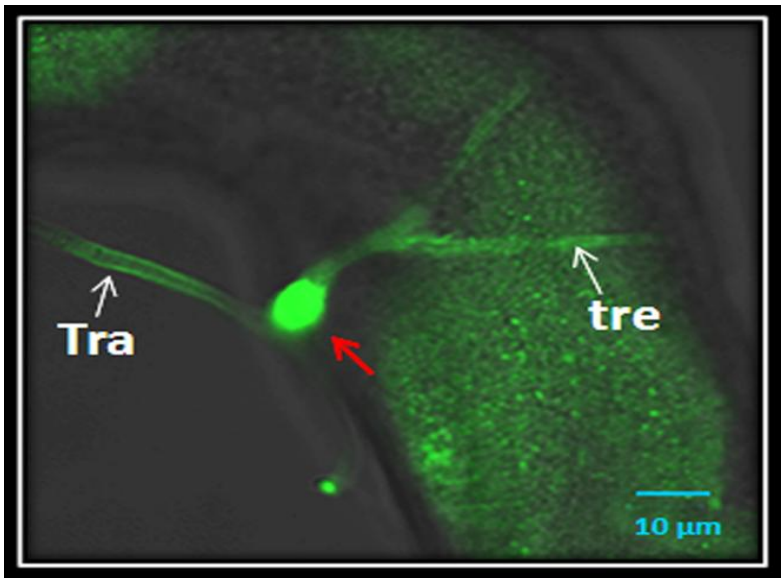

Figure 14. AaSeR-1 expression in a tracheolar cell in the Malpighian tubules (MT) of *Aedes aegypti* female at 24 h post-blood feeding. Both type trachea (Tra) and tracheoles (tre) are providing oxygen supply to the MT. A bright-field image superimposed with green fluorescence is depicting AaSeR-1-specific expression (green, Ex. 488; red arrow) in a tracheolar cell in the MT at 24 h post-blood feeding. Scale bar (µm) is included in the image.

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However, in the light of the current data,Cady and Hagedorn's findings [44] may be interpreted differently by suggesting that 5-HT does not have a systemic mode of action (high concentrations in the hemolymph) on the regulation of diuresis during blood processing, but rather local or semi-systemic effects. The proposed model also highlights another important point, which emphasizes that the experimental design and interpretation of results must include as many aspects of the biological system as possible, in order to understand data in its biological context, relation, and significance. Further to the AaSer-1 presence in the MT, the receptor-specific immunofluorescence is associated with the plasma and intracellular membrane networks of the principal, and not the stellate cells (Fig 13B). An additional receptor function is suggested by AaSeR-1-specific immunofluorescence found in some tracheolar cells from the tracheolar system of the MT (Fig 14). As described above, (similar to the AaSeR-1 in tracheal cells) the receptor may mediate some regulatory functions in the formation of new tracheoles, since receptor activation correlates with post-blood feeding where respiration rates are at their highest level. Serotonergic mediated effects in tracheolar cells are also confirmed by previously studied 5-HT7-like receptor [23].

As a part of the comprehensive and rigorous investigations, negative controls (Fig 15 and Fig 16) were carried out in parallel to all immunolocalization analyses to validate above results. No specific immunofluorescence was demonstrated in all negative controls, confirming the validity of 5-HT and AaSer-1 immunolocalization data.

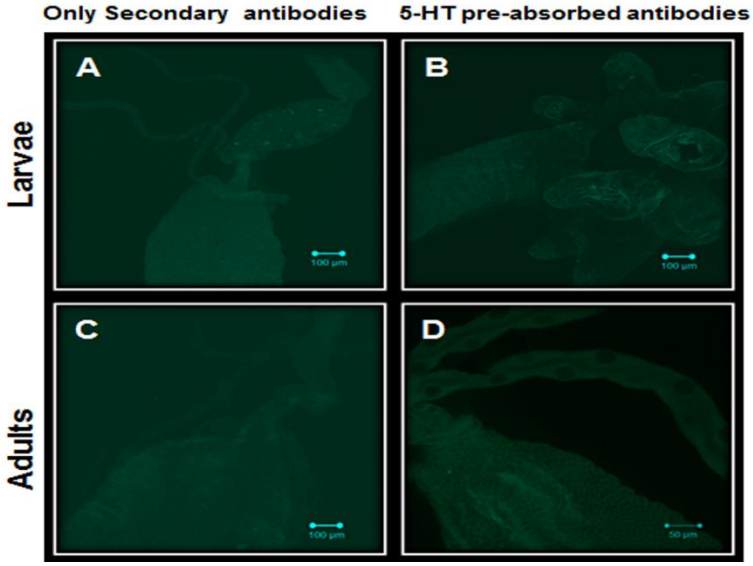

Figure 15. 5-HT-specific immunofluorescence was not observed in the *Aedes aegypti* gut when samples were subjected to negative control treatments. Images of larval guts treated with (A) secondary antibodies only and (B) 5-HT pre-absorbed antibodies are not showing specific fluorescence. Similar result were obtained for the female adult gut treated with either (C) secondary antibodies or (D) 5-HT pre-absorbed antibodies. Altogether, negative controls have confirmed the validity of 5-HT-specific immunofluorescence patterns in the gut of larvae and female adult mosquitoes.

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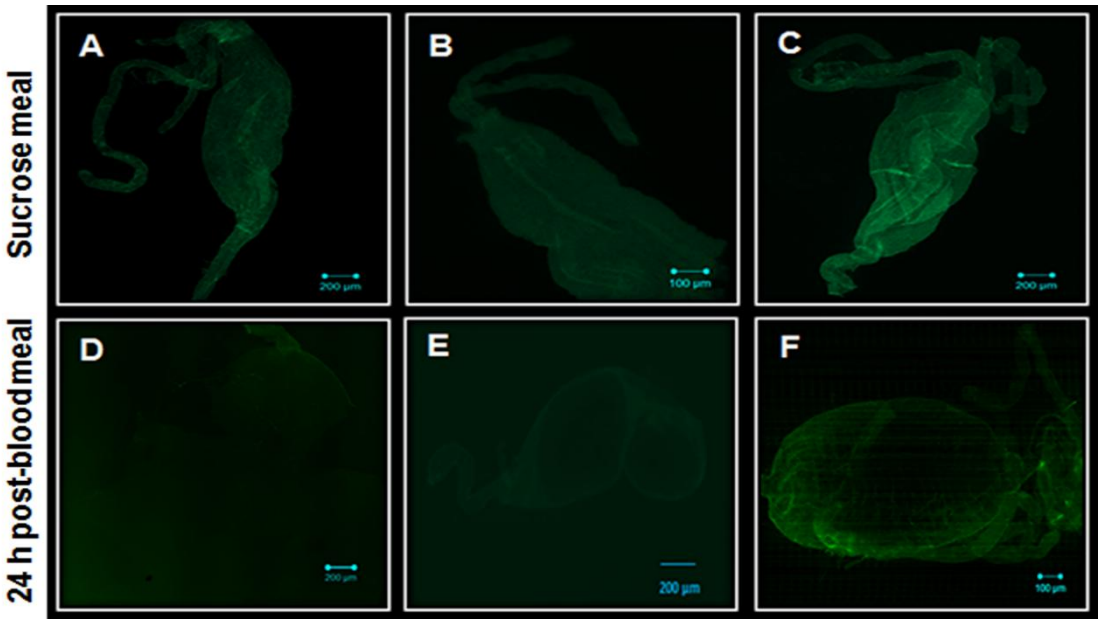

Figure 16. No specific expression of AaSeR-1 was observed in the *Aedes aegypti* gut following negative control treatments. Images depicting the gut of sucrose-fed mosquitoes treated with: (A) pre-immune serum, (B) secondary antibodies and (C) peptide pre-absorbed antiserum, did not demonstrate AaSeR-1-specific immunofluorescence. Similar results were obtained for blood-fed mosquitoes when samples were treated with either (D) pre-immune serum (E) secondary antibodies or (F) peptide pre-absorbed antibodies. Lack of specific immunofluorescence in all negative control groups is a confirmation for the credibility of AaSer-1 expression patterns.

In summary, the study demonstrated three important points from the serotonergic system in the *A. aegypti* digestive tract. First, the study identified serotonergic distribution patterns and specific trends, which provide a basis for predicting 5-HT putative roles. Second, the study demonstrated the complexity and remarkable diversity of putative serotonergic roles, through the expression analyses of AaSer-1. Such an array of roles is achieved through AaSer-1 spatial (eg. different cell compartments, different types of cells and tissues) and temporal expression profiles (eg. sucrose-fed vs. blood-fed), resulting in multiple physiological effects. Third, the elaborate serotonergic plexus in the gut underlines the significant role of 5-HT in the regulation of food digestion and absorption, as well as secretion of water and ions.

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