Investigating the Role of a Circadian Neuropeptide as a Prominent Neuroendocrine Signal in *Drosophila melanogaster*

By: Aaron Talsma, Shafer Lab, University of Michigan

Abstract:

In *Drosophila melanogaster*, circadian rhythms are governed by a network of pacemaker neurons that communicate largely with neuropeptides. One of the most important signaling peptides in this circuit is pigment dispersing factor (PDF) due to its vital role in synchronizing the cellular pacemakers of the network and coordinating behavioral output. The circadian functions of PDF are carried out by 16 of the 24 PDF expressing neurons located in the central nervous system of the fly. These circadian clock neurons are located in the central brain. The function of the remaining eight PDF neurons, which are located in the ventral nerve cord of the fly and do not have a circadian role, is still unknown. Interestingly, PDF has a functional homologue in the mammalian circadian network called vasoactive intestinal peptide (VIP). Along with its circadian role, VIP is also important in many signaling pathways in the mammalian viscera. Here, we show the function of PDF in its control of non-circadian functions in *Drosophila melanogaster*, including the regulations of body size and mass, intestinal and renal function, and reproduction. We also provide support for the hypothesis that the eight PDF neurons in the ventral nerve cord are the source of the PDF used in these visceral signaling functions.

Introduction:

  Drosophila pigment dispersing factor (PDF) is a member of a large family of octadecapeptides which were originally found to affect pigment dispersal in the eyes of crustaceans (Rao et al, 1985). This family of neuropeptides is highly conserved between crustaceans and insects (Rao and Rhiem, 1993). PDF is most similar to the crustacean β-pigment dispersing hormone (β-PDH) (Rao and Rhiem, 1993), and was discovered due to the ability of β-PDH antibodies to recognize PDF (Nässel et al, 1993). However, PDF has not been found to affect pigment dispersal in *Drosophila*. Instead, PDF is expressed in a set of 24 neurons in the *Drosophila* central nervous system (CNS), 16 of which were implicated in circadian time keeping (Helfrich-Förster, 1995).

  Nearly every organism experiences a daily cycle of light and darkness due to the rotation of the Earth. In order to better adapt to these cycles, nearly every organism, from bacteria to mammals, has developed a mechanism to keep time. In *Drosophila,* time is kept by a network of about 150 neurons in the brain, which rely on a cell autonomous time keeping mechanism as well as network connections to maintain a functional circadian clock. PDF has been shown to be an important signaling peptide in the circadian clock (Renn et al, 1999; Shafer and Taghert, 2009). It is expressed in 16 neurons in the brain, eight per hemisphere: four small ventrolateral neurons (s-LNv) and four large ventrolateral neurons (l-LNv) (Nässel et al, 1993; Helfrich-Förster, 1995). These LNv's have been implicated as key pacemakers in the circadian network. When they are ablated (Renn et al, 1999), or when the expression of PDF is knocked down using GAL4 driven RNAi in only these neurons (Shafer and Taghert, 2009), flies tend to have daily rhythms that cycle faster (a shorter period) than those of the wild-type flies in a light-dark cycle and cannot maintain strong circadian rhythms in constant darkness. The other eight PDF neurons in the CNS of the fly are located at the tip of the abdominal ganglia of the ventral nerve cord (VNC), which is essentially the spinal cord of *Drosophila* (Nässel et al, 1993). These neurons do not express the cellular circadian machinery, nor do they play any role in keeping time or the behavioral output of the clock in *Drosophila* (Shafer and Taghert, 2009; Nässel and Winther, 2010).

  The only known PDF receptor is a seven trans-membrane GPCR encoded by the CG13758 gene. There are two established lines of flies that have large deletions of the PDFR, known as $PdfR^{5304}$ and $PdfR^{3369}$ (Hyun et al, 2005). Both of these lines show behavioral

phenotypes similar to that of PDF null flies: they have faster periods and cannot keep time in constant darkness. PDF binds selectively to PDFR and causes an intracellular increase in cAMP (Hyun et al 2005). Live imaging studies have shown that many neurons in the circadian network respond to PDF with an increase in cAMP. These studies have also shown that PDFR can be activated by another neuropeptide, DH31. However, the binding coefficient for DH31 is much lower than for PDF (Mertens et al 2005; Shafer et al. 2008).

The extent of PDF's role in signaling to various organ systems in *Drosophila* is not yet known due to difficulties in determining the expression of PDFR. When PDFR was discovered, three different labs attempted to map PDFR in the fly brain by creating their own antibodies for PDFR. However, all of the antibodies displayed different staining patterns (Hyun et al 2005; Mertens et al 2005; Lear et al 2005), each of which were found to persist in the *PdfR^{5304}* deletion mutant and thereby shown to be spurious (Shafer et al. 2008). Recently, a transgenic approach was taken to determine PDFR expression patterns. In this study, a large piece of genomic DNA, which theoretically containing all of the *PdfR* regulatory elements, was cloned and a *myc* tag was added to the end of the *PdfR* gene. This new gene construct was then inserted into the genome of PDFR null flies and antibodies for the *myc* tag were used to map expression. From this study, it has been determined that PDFR is expressed in many of the *Drosophila* clock neurons and in some other non-circadian neurons that may contribute to output for the circadian clock (Im and Taghert, 2010). However, despite these discoveries, there is still no easy way to determine the complete expression of PDFR throughout the body of *Drosophila*.

The mammalian circadian clock bears many similarities to the fly circadian clock. Both clocks use many of the same proteins in the cellular clockwork and both also depend on network interactions among clock neurons (Vosko et al, 2007). In the case of the mouse, this network consists of about 20,000 neurons located in the suprachiasmatic nuclei (SCN). Within the SCN, there are two major regions: the ventral (core) region and the dorsal (shell) region. The core region expresses a neuropeptide called vasoactive intestinal peptide (VIP) and sends VIP projections to the dorsal region and to other neurons within the ventral region (Vosko et al, 2007). Though its sequence has no clear homology to PDF, VIP and PDF play remarkably similar timekeeping roles. VIP knockout mice have accelerated rhythms in a normal light-dark cycle and are arrhythmic in constant darkness, very much like PDF null flies (Colwell et al, 2003). VIP is also believed to synchronize the cellular time keeping machinery in the neurons of

the SCN, much like PDF (Aton et al, 2006). VIP has two receptors, $VPAC_1R$ and $VPAC_2R$, which both signal by increasing intracellular cAMP synthesis and which also have a high similarity to *PdfR* in the fly (for a review of VPAC/PACAP receptors see Vaudry et al, 2000). $VPAC_2R$ is highly expressed in the SCN and $VPAC_2R$ knockout mice show similar phenotypes to PDFR null flies (Harmar et al 2002). These findings illustrate the many similarities between the *Drosophila* and mammalian circadian clock.

VIP is also involved in signaling in the periphery. In the urogenital tract of mice, VIP positive neurons make a small but distinct percentage of afferent neurons innervating the bladder, urethra, and penis (Keast and deGroat, 1992). It has also been demonstrated that VIP and pituitary adenylate cyclase-activating protein (PACAP) neurons send axonal projections throughout the bladder and to smooth muscles in the urethra. The effect of VIP on the bladder varies greatly among species. In some species, such as cat, rabbit pig, and human, it tends to inhibit bladder emptying while in other species, such as rat and guinea pig, it helps to facilitate it (Yoshiyama and de Groat, 2008). In the intestine, VIP positive neurons make up a significant portion of the enteric neurons. Many vagal efferent neurons, hypothesized to be "command neurons," synapse specifically onto VIP positive neurons in the small and large intestines. These VIP neurons can be electrically stimulated to release VIP, which causes intestinal smooth muscles to relax and thus decreased intestinal motility (Fujimiya and Inui, 2000). These functions of VIP as well as the similarities between VIP and PDF in the circadian clock led us to investigate the effects of PDF on the digestive system, the renal system, and the reproductive viscera in *Drosophila.*

The digestive system of *Drosophila* is generally divided into five parts. From anterior to posterior they are the esophagus, the cardia, the midgut (ventriculus), the hindgut (anterior intestine), and the rectal ampulla (rectum) (see Fig. 1C for a diagram). The renal organs of the fly, known as the malpighian tubules (MPT), connect to the gut at the midgut-hindgut junction through two muscular stalks, which we refer to as ureters, on opposite sides of the intestine. The midgut consists of an outer layer of longitudinal and circular smooth muscle and an inner layer of epithelia (Miller, 1994). The midgut is divided anatomically into two parts: the shorter straight section contained in the thorax and the longer coiled portion contained in the abdomen (Miller, 1994). The hindgut is similar in diameter to the midgut but it is much shorter. It contains a thick layer of epithelia surrounded by a comparatively denser coating of circular muscles (Miller,

1994). Due to the nature of our dissection, we examined only the portion of the viscera contained in the abdomen along with the MPT and hindgut.

The renal system of the fly is separated into the anterior and posterior halves. Each half of the renal system consists of two long tubules, called the distal renal tubules, which meet and flow into one short muscular tube called the ureter, which then flows into the midgut as described above (Miller, 1994). The distal renal tubules consist of two cell types, which are the more prolific principal cells and the less common stellate cells (Denholm et al, 2003). These tubules are responsible for most of the filtration of the hemolymph (Dow and Romero, 2010). The tubules of the anterior MPT associate themselves with the anterior portion of the midgut while the renal tubules of the posterior MPT associate with the hindgut and the reproductive viscera (Miller, 1994). Like the renal tubules, the ureter also contains principal and stellate cells, but it has an additional layer of visceral muscle that contains both circular and longitudinal muscles which surround the epithelia (Denholm et al, 2003).

All organisms must be able to extract nutrients from the environment in order to survive. Animals have progressively had to evolve and develop more complex mechanisms for these processes because the cells that are directly exposed to the environment are designed to protect the body from deleterious environmental factors and cannot absorb or process nutrients. The solution to the problem of maintaining metabolic balance, taking in nutrients, and processing them was the digestive system. Due to its crucial role in maintaining homeostasis, the digestive system is regulated by a multitude of pathways and in turn can also regulate the activity of many other systems in the body. One of the most important centers for the communication between the digestive system and the rest of the body is the enteric nervous system. In humans, it comprises a staggering 500 million neurons (Miguel-Aliaga, 2012). Despite the fact that the enteric nervous system plays a critical role in regulating metabolism and likely contributes to a multitude of metabolic disorders, it is still poorly understood.

One of the biggest hurdles to understanding the function of the mammalian enteric nervous system is its sheer size and complexity. *Drosophila,* on the other hand, has a much simpler neuroanatomy by comparison. Furthermore, the genetic power of techniques used in *Drosophila* is remarkable. Despite the differences between *Drosophila* and mammals, there are still many similarities (VIP for example) which could allow discoveries in *Drosophila* to further

our understanding of mammalian systems. Here, we investigate the novel role of a canonical circadian neuropeptide in signaling to the peripheral organs in *Drosophila*.

Materials and Methods:

Fly Stocks: Flies were reared on cornmeal and yeast food at 25 °C. The recipe for 1 L of food is as follows: 1 L water, 16.9 g Yeast, 71.2 g cornmeal, 12 g Agar, 75 mL corn syrup, and 4.7 mL propionic acid. The transgenic stocks used were w^{33} and w^{15} (Renn et al, 1999), $PdfR^{5304}$ and $PdfR^{3369}$ (Hyun et al, 2005), w^{1118};; *pdf+ (iso #2)* and w^{1118};;*pdf(01) (iso #8)* (a generous gift from Ravi Allada), *UAS-PdfR^{16L}* (Mertens et al, 2005), *Mef2-Gal4* (Ranganayakulu et al, 1996), *24B-Gal4* (Brand and Perrimon, 1993; Fyrberg et al, 1997), *Myola-Gal4* (Jiang and Edgar, 2009), *UAS-Epac1camps(50A)* (Shafer et al, 2008), and *UAS-eGFP* (Stevaux et al, 2002). Canton S (CS) and/or w^{1118} lines were used as wild-type controls.

Fly Weight Assay: To control population density, a consistent number of eggs was gathered and placed into a vial of food by a process called purping (described below). For a given experiment, flies from different lines were always assayed in parallel and thus raised on the same batches of food, in the same environmental conditions, and were weighed on the same day. Between 10 and 11 days after purping the flies would eclose. Flies were anesthetized with CO_2, sorted into groups of ten males or females, placed on ice, and weighed shortly thereafter (within an hour) on a microgram balance (Sartorius biotech, Göttingen, Germany). Generally, weighing occurred on day 14 or 15 after purping, by which time all the flies in a vial had emerged and mated. When flies were weighed earlier than 14 days after purping, newly eclosed flies, which are bloated and much heavier than slightly older flies, were not included in the assay.

Purping: Between 100 and 200 flies were placed in a screened cage with a 135 mm grape juice agar plate for a bottom with a small amount of active yeast paste on the center of the plate. The flies were allowed to lay eggs for either eight or sixteen hours on the agar. After this period the agar plate was exchanged for a fresh plate. The eggs were collected by filling the agar plate with phosphate buffered saline (PBS) and gently loosening the eggs with a cotton tipped swab. The eggs were then moved into a 15 ml Falcon tube (Falcon, Franklin Lakes, NJ) by

washing the agar plate with PBS and pouring it into the tube. The eggs were allowed to settle to the bottom of the tube and then ten microliters of eggs suspended in PBS were pipetted into each vial of cornmeal food where the flies then developed. This technique effectively controls population density in each vial. (Thank you to the Pletcher lab at the University of Michigan for the protocol.)

Extraction of Viscera: Flies were anesthetized with either ice or CO_2. Flies anesthetized on ice were then dissected in cold HL3(hemolymph-like saline 3) (70 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM D(+)-Trehalose, 115 mM Sucrose, 5 mM HEPES) whereas flies anesthetized with CO_2 were dissected in room temperature HL3. The dissection was adapted from one used previously (Dow et al, 1994). Briefly, anesthetized flies were placed in saline and then abdomens were separated from heads and thoraxes with microscissors (Fine Science Tools, Foster City, CA). To remove the viscera, forceps were used to gently pry apart the abdominal cuticle. After the cuticle was partially removed, the MPT were dissociated from the gut by grabbing them as distally as possible and pulling them away from the midgut or hindgut gently. The gut was unraveled by first removing the trachea and other connective tissue and then pulling gently on both ends. After the viscera were removed they were placed in a new 35mm petri dish containing 1.8 mL of room temperature HL3. The midgut, hindgut and MPT were spread out and stuck gently to the bottom of the dish, to which they readily adhered.

Contraction Recordings: After guts were placed in the 35 mm Petri dish (Falcon, Franklin Lakes, NJ) with 1.8 mL HL3, the dish was placed under an Olympus SZX7 stereoscope with an Olympus DP 21 CCD camera (Olympus, Center Valley, PA). A six minute and twelve second (the maximum recording time on our system) or a three-minute video of the basal contraction rate was taken. Next, 0.2 mL of a 10x treatment was added to the dish via a micropipette and the dish was allowed to sit for three minutes. Then another video was taken, again either six minutes and twelve seconds or three minutes. The videos were then scored blindly for the number of contractions in each of the ureters, the midgut and the hindgut.

Extraction of reproductive viscera: Flies were anesthetized with ice and dissected in cold HL3. This dissection is very similar to the visceral dissection described above. The abdomen was cut from the fly with microscissors (Fine Science Tools, Foster City, CA) and the cuticle was peeled apart to expose the organs. The different pieces of the intestine were removed

whenever possible to make access to the reproductive viscera easier. After most of the cuticle, all except for the portion around the genitals, and the intestine were removed, the trachea was gently pulled off of the reproductive viscera and the various sections were gently spread out. The reproductive viscera were then placed into a fresh 35 mm petri dish with 1.8 mL of room temperature HL3. The reproductive viscera were gently spread out attached to the bottom of the dish as described above so that all the parts could be seen clearly. They were then allowed to sit covered for one hour. The reason for this was that there was an extremely high rate of basal contraction after the reproductive viscera were placed in the fresh HL3, and this decreased somewhat after an hour. After the hour had passed, a three-minute video of basal contractions was taken with an Olympus SZX7 stereoscope and a DP 21 CCD camera. Then, 0.2 mL of a 10x treatment was added to the dish. The genitals were allowed to sit for another three minutes and then a second three-minute video was taken. The videos were scored blindly for contractions in each accessory gland and in the ejaculatory duct.

FRET Imaging: The ratiometric cAMP sensor, Epac1camps, was expressed using a UAS promoter, driven by *Mef2-Gal4*, which is a muscle driver. Guts were imaged using an Olympus FV 1000 scanning laser confocal microscope, through a LUMPL 20x/0.50 water objective with immersion cone and correction collar (Olympus, Center Valley, PA). Flies were anesthetized on ice and dissected in cold Ringer's saline (Cold Spring Harbor Protocols). After dissection they were placed immediately into a new 35 mm petri dish containing 1.8 mL room temperature HL3. The dish was then imaged with the confocal microscope by scanning the sample every five seconds with a 440nm laser using a DM405-440/151 dichroic mirror and CFP/YFP emission was separated with a SDM510 dichroic mirror. The treatment of peptide or vehicle was added between 40 and 45 seconds of the recording as described above for the contraction assay. Regions of interest were selected using Fluoview software to contain circular muscle in parts of the ureter that did not move significantly within the frame during the recording. This was done to minimize any changes in FRET due to contractions of the ureter. Confocal apertures for CFP and YFP were increased to increase the thickness of the optical section as another way to compensate for ureter contractions. The ratio of YFP/CFP was calculated using the formula:
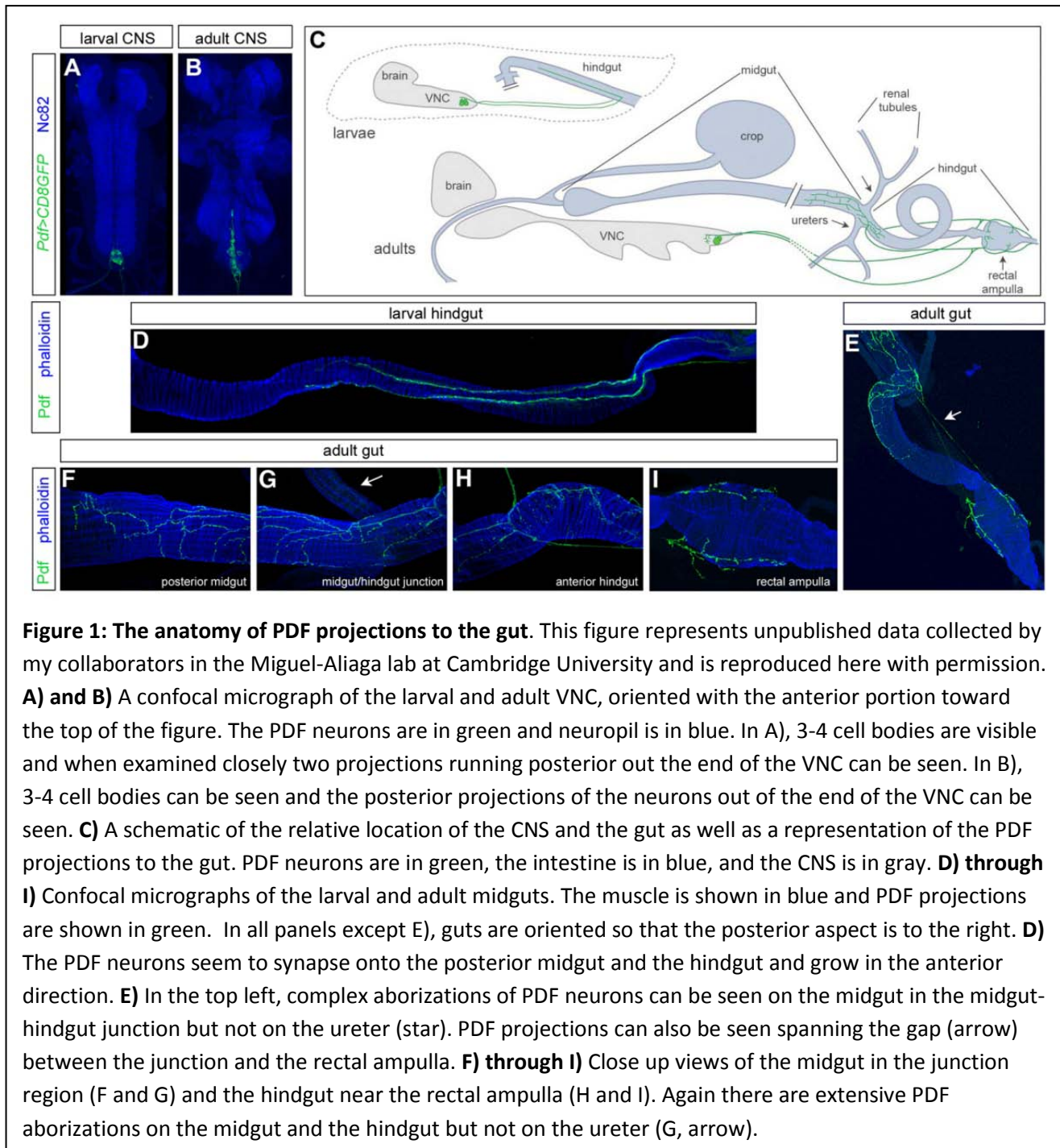
$$\text{Spillover Corrected FRET} = \frac{(\text{YFP} - (\text{CFP} * 0.444))}{\text{CFP}}$$

to adjust for the bleed-through of CFP emission. Individual traces were then created using a 6 value running average and normalized to the initial FRET ratio of each ROI. This data analysis was carried out in Microsoft Excel (Redmond, WA). Statistical calculations were made using Prism 5 (Graphpad, La Jolla, CA).

Results:

Abdominal PDF neurons send anterior projections that synapse on the midgut at the midgut-hindgut junction. The VNC of drosophila is analogous to the spinal cord of mammals. Neurons located in the abdominal ganglia of the VNC send projections to the periphery, including the viscera (Cognigni, Bailey and Miguel-Aliaga 2011). To investigate the targets of the PDF neurons in the VNC, we removed and stained both the ventral nerve cord and the intestine for PDF. This work was done by our collaborators in the Miguel-Aliaga lab at Cambridge University and reproduced here with permission. Figure 1C shows a schematic of the connection between the abdominal ganglion and the viscera in both the larva and the adult. Both the larval and adult VNC have PDF neurons present, and the projections of these neurons can be seen leaving through the posterior tip of the VNC (Fig. 1 A, B). The confocal micrographs of the larval gut show PDF neurons growing anteriorly along the midgut but without any aborizations (Fig. 1 D). The micrographs of the adult gut show that the PDF projections are located on the midgut and hindgut almost entirely in the midgut-hindgut junction region, with a few projections going to the rectal ampulla (Fig. 1 E-I). This pattern of innervations led us to hypothesize that these PDF neurons would signal primarily to the midgut and hindgut. Surprisingly, this was not the case.

PDF causes dose-dependent and PDFR dependent contractions in the ureters of the fly renal system, but does not cause contractions in the intestine. *Drosophila* guts, including the midgut, hindgut, rectal ampulla, and MPT, were explanted and placed in a dish. Upon bath application of various concentrations of PDF, muscle contraction rates were scored in the ureters, the midgut, and the hindgut. *CS* ureters showed a well defined dose response in both room temperature and cold saline. For males in cold saline there was a noticeable increase in contractions at 1 nM PDF and a significant increase in contractions at concentrations greater than

Figure 1: The anatomy of PDF projections to the gut. This figure represents unpublished data collected by my collaborators in the Miguel-Aliaga lab at Cambridge University and is reproduced here with permission. **A) and B)** A confocal micrograph of the larval and adult VNC, oriented with the anterior portion toward the top of the figure. The PDF neurons are in green and neuropil is in blue. In A), 3-4 cell bodies are visible and when examined closely two projections running posterior out the end of the VNC can be seen. In B), 3-4 cell bodies can be seen and the posterior projections of the neurons out of the end of the VNC can be seen. **C)** A schematic of the relative location of the CNS and the gut as well as a representation of the PDF projections to the gut. PDF neurons are in green, the intestine is in blue, and the CNS is in gray. **D) through I)** Confocal micrographs of the larval and adult midguts. The muscle is shown in blue and PDF projections are shown in green. In all panels except E), guts are oriented so that the posterior aspect is to the right. **D)** The PDF neurons seem to synapse onto the posterior midgut and the hindgut and grow in the anterior direction. **E)** In the top left, complex aborizations of PDF neurons can be seen on the midgut in the midgut-hindgut junction but not on the ureter (star). PDF projections can also be seen spanning the gap (arrow) between the junction and the rectal ampulla. **F) through I)** Close up views of the midgut in the junction region (F and G) and the hindgut near the rectal ampulla (H and I). Again there are extensive PDF aborizations on the midgut and the hindgut but not on the ureter (G, arrow).

10 nM PDF (10^{-8} p < 0.0001 vs. basal; 10^{-7} p = 0.0003 vs. basal; 10^{-6} p < 0.0001 vs. basal; with the student's t-test) (Fig. 2A). This was also the case for females in warm saline (10^{-8} p = 0.0004 vs. basal; 10^{-7} p = 0.0088 vs. basal; 10^{-6} p = 0.0055 vs. basal; with a paired t-test) (Fig. 2C). For females in cold saline, there was a detectable increase in contractions at 10 nM PDF and a significant increase in contractions at concentrations greater than 100 nM PDF (10^{-7} p < 0.0001

vs. basal; 10^{-6} p = 0.0027 vs. basal; with the student's t-test) (Fig. 2B). The EC50 is $2.406*10^{-9}$ M for males in cold saline, $8.729*10^{-9}$ M for females in cold saline, and $2.709*10^{-10}$ M for females in warm saline. These results agree very well with the binding properties of PDFR in S2 cells, which also showed an increase in cAMP at 1 nM PDF and had an EC50 of 1.81 nM (Hyun 2005). The midgut (light gray line, Fig. 2 A, B, C) never responded significantly or consistently to PDF and the hindgut (data not shown) did not respond to PDF at all.

The response of ureters to PDF depends on PDFR. Two strains of flies lacking PDFR ($PdfR^{5304}$ and $PdfR^{3369}$) were examined to see if bath applied PDF increased the contraction rate of ureters significantly compared to their basal rate. Only wild-type flies (w^{1118}) responded to PDF with a large increase in contraction rate, which was significantly higher than all other contraction rates (vs. w^{1118} basal, p < 0.0001 [paired t-test]; vs. $PdfR^{5304}$ treated, p < 0.0001
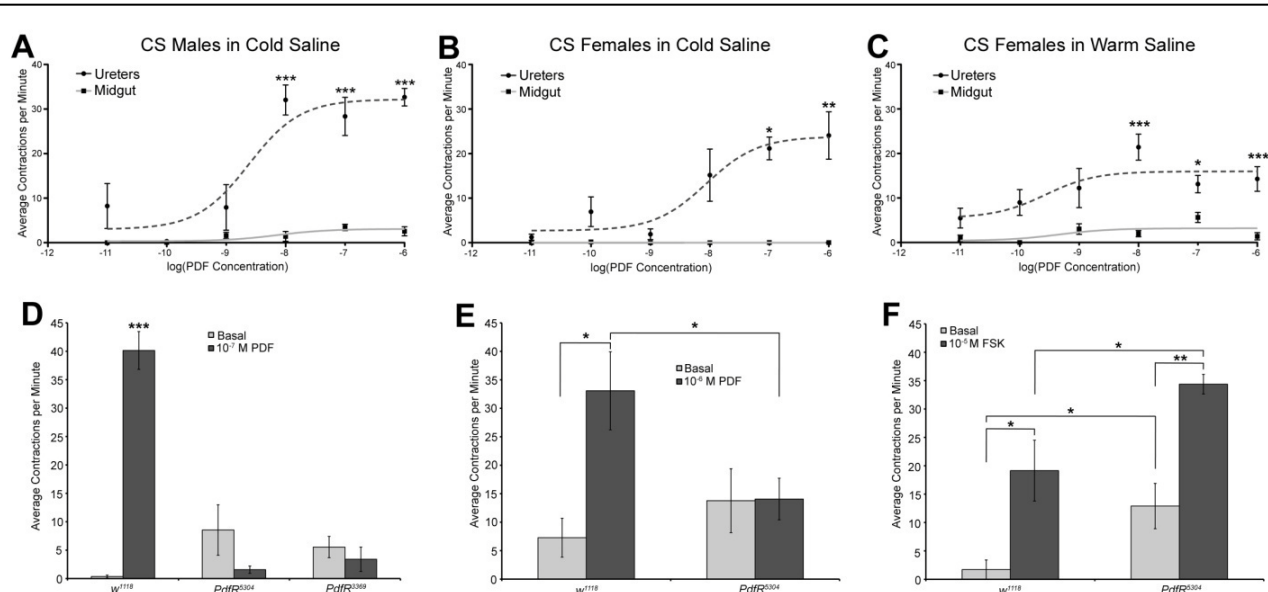
Figure 2: The ureters respond to PDF in a dose-dependent and PDFR dependent manner. A) through C) Dose response curves for wild-type flies under various conditions. Responses represent the actual contraction rate after PDF application. Significance was determined by comparing the response of each gut to its basal rate (data not shown) using a paired t-test (*** p < 0.001, ** p < 0.01, * p < 0.05). **A)** Males anesthetized on ice and dissected in cold saline. **B)** Females anesthetized on ice and dissected in cold saline. **C)** Females anesthetized with CO_2 and dissected in room temperature saline. **D)** Comparison of the PDF response of w^{1118} and PdfR null ($PdfR^{5304}$ and $PdfR^{3369}$) ureters in cold saline. w^{1118} ureters showed a significant increase in contraction rate relative to all other treatment groups while PDFR null flies did not respond. **E)** Comparison of the PDF response of w^{1118} and $PdfR^{5304}$ ureters in warm saline. Again, w^{1118} ureters showed a significant response to PDF but PDFR flies did not. **F)** w^{1118} and $PdfR^{5304}$ ureters treated with FSK in warm saline to articificially increase intracellular cAMP. For all panels, error bars represent the mean plus or minus the standard error of the mean (SEM).

[student's t-test]; vs. *PdfR³³⁶⁹* treated, $p < 0.0001$ [student's t-test]) (Fig. 2D). This response was not significantly changed by the temperature of the saline, as flies treated in warm saline showed a similar PDF response (*w¹¹¹⁸* treated vs. *w¹¹¹⁸* basal, $p = 0.0165$ [paired t-test]; *w¹¹¹⁸* treated vs. *PdfR⁵³⁰⁴* treated, $p = 0.0230$ [student's t-test]) (Fig. 2E). Adding vehicle (0.1% DMSO in HL3) to the bath did not increase the contraction rate above basal for any of the lines (data not shown). To confirm that PDFR null flies did in fact fail to respond to PDF due to a lack of PDFR rather than damage sustained during dissection, guts were explanted and treated with forskolin (FSK). FSK is an activator of adenylyl cyclase and thus should activate the same pathway as PDFR. Indeed both wild-type and PDFR null responded to FSK with an increase in ureter contraction (*w¹¹¹⁸* basal vs. FSK, $p = 0.0128$ [paired t-test]; *PdfR⁵³⁰⁴* basal vs. FSK, $p = 0.0015$ [paired t-test]) (Fig. 2F). Both the basal ($p = 0.0192$) and FSK treated ($p = 0.0145$) contraction rates of *PdfR⁵³⁰⁴* were significantly greater than *w¹¹¹⁸* contraction rates (with the student's t-test).
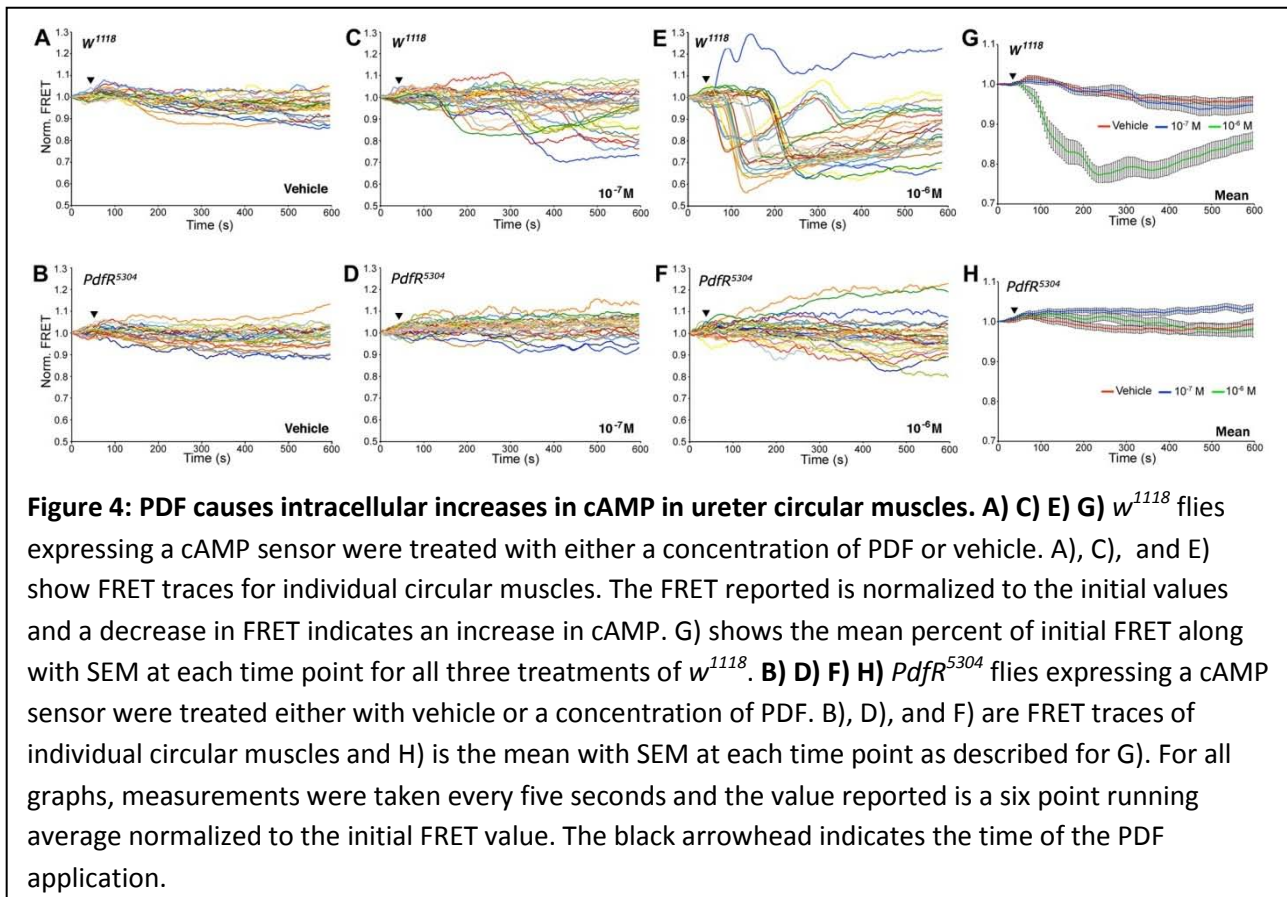
cAMP may increase midgut contractions but PDF does not. Midguts from wild-type and PDFR null flies were first explanted and treated with PDF in cold saline. Under these conditions, no significant response to PDF was seen (Fig. 3A). However, several labs that study the viscera of larger insects noted that cold temperatures inactivate the gut. Therefore, we examined the guts in warm saline as well and again observed no significant response to PDF (Fig. 3B). As a positive control, to ensure that the midgut could still respond in the explant assay, the midgut was also treated with FSK, which has been shown to elicit midgut contractions in larger insects (Blake et al, 1996). In wild-type flies, a significant increase in contraction rates
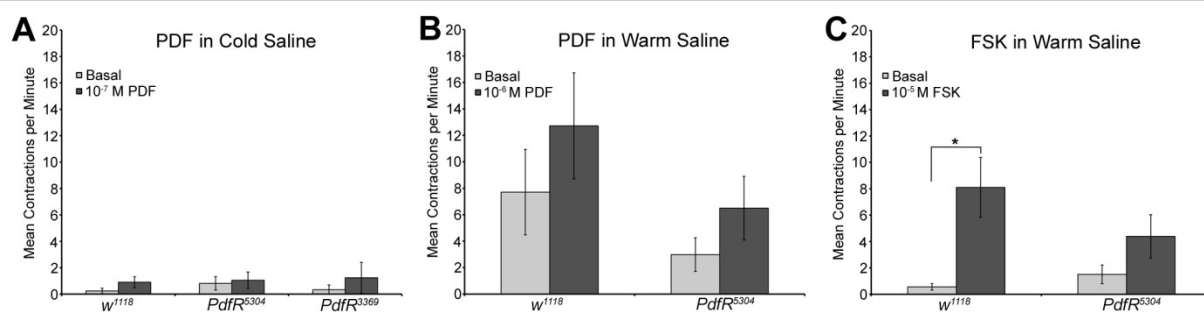
Figure 3: Midgut contractions can be induced by forskolin driven cAMP increases but not by PDF. Midguts were assayed to determine if they could respond to a PDF or forskolin under various conditions. **A)** Wild-type and PDFR null midguts were assayed in cold HL3 for a response to PDF. **B)** Wild-type and PDFR null midguts were assayed in room temperature HL3 for a PDF response. **C)** Midguts were assayed in room temperature HL3 for a response to FSK and only wild-type guts were found to respond. For all panels, error bars indicate the mean ± SEM. Significance was determined with either a paired t-test (tests among a genotype) or a student's t-test (tests between genotypes) and is represented by stars (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

was observed (p = 0.0151, paired t-test) and in PDFR null flies, there was a qualitative but not significant increase in contractions (p = 0.1138, paired t-test) (Fig. 3C). This indicates that PDF alone is not sufficient to cause midgut contractions under our experimental conditions, and that increases in cAMP likely stimulate midgut contraction. Therefore, midgut muscle contraction is likely regulated by a different GPCR.

PDF causes an increase in intracellular cAMP in the circular muscles of the ureters of flies with a wild-type background, but not in PDFR null flies. Either *UAS-Epac1camps(50A)* or *PdfR^{5304};UAS-Epac1camps(50A)* virgin females were crossed to *Mef2-Gal4* males. The resulting flies expressed Epac1camps, a FRET based cAMP sensor, in the musculature of the MPT and the gut (see Fig. 5E for Mef2 expression), either with (Fig. 4, w^{1118}) or without (Fig. 4, *PdfR^{5304}*) PDFR. Wild-type flies showed a significant decrease in FRET in response to µM concentrations of PDF, which indicates an increase in cAMP (analyzed with a 2-way ANOVA and Bonferroni post tests to compare every pair of means at each time point). The FRET decrease became significantly different from vehicle at 110 s (p < 0.05, Bonferroni post-test) and remained significantly lower for the entire recording. Interestingly, a 0.1 µM concentration of PDF did not elicit a significant FRET response in wild-type despite the fact that

Figure 4: PDF causes intracellular increases in cAMP in ureter circular muscles. A) C) E) G) w^{1118} flies expressing a cAMP sensor were treated with either a concentration of PDF or vehicle. A), C), and E) show FRET traces for individual circular muscles. The FRET reported is normalized to the initial values and a decrease in FRET indicates an increase in cAMP. G) shows the mean percent of initial FRET along with SEM at each time point for all three treatments of w^{1118}. **B) D) F) H)** *PdfR^{5304}* flies expressing a cAMP sensor were treated either with vehicle or a concentration of PDF. B), D), and F) are FRET traces of individual circular muscles and H) is the mean with SEM at each time point as described for G). For all graphs, measurements were taken every five seconds and the value reported is a six point running average normalized to the initial FRET value. The black arrowhead indicates the time of the PDF application.

ureters always responded to this concentration with a significant increase in contraction rate. This likely is a due to the sensitivity of the Epac sensor. In fact, many of the individual traces do show a cAMP response. However, these responses were not as long lived or as great in magnitude as the responses to 10^{-6} M PDF. Flies lacking PDFR did not show any FRET response to PDF, indicating that PDF does indeed signal through the known PDFR and that this response is mediated by an increase in cAMP.

The response of ureters to PDF is recued by driving a transgenic PDFR element in the circular muscles of the ureters. Both *Mef2-Gal4* and *24B-Gal4* are expressed in the musculature of the ureter (Fig. 5 E and C, respectively), while *Myola-Gal4* is expressed in a subset of the epithelial cells of the ureter (Fig. 5 A). All three of these lines were used to drive expression of PDFR under the control of the UAS promoter in the ureters in order to rescue the ureter PDF response. Rescue flies are the progeny of *PdfR^{5304}; UAS-PdfR* and Gal4 males yielding *PdfR^{5304}; X-Gal4>PdfR* males. The UAS and Gal4 controls are the progeny of *PdfR^{5304}; UAS-PdfR* virgin females crossed with w^{1118} males, yielding *PdfR^{5304}; UAS-PdfR* flies, and *PdfR^{5304}* virgin females crossed with Gal4 males, yielding *PdfR^{5304}; Gal4* flies, respectively. Again, in both cases only males were tested.

As expected, *Myola>PdfR* flies showed no rescue of PDF activity on the ureters (Fig. 5 B). There was no significant difference between the rescue and Gal4 control (p = 0.1608) or the rescue and UAS control (p = 0.0535). *Mef2>PdfR* flies showed a qualitative increase in PDF response that returned the contraction rate to near wild-type levels (indicated by the black arrowhead). The Mef2 rescue response was significantly increased compared to the UAS control (p = 0.0006) but not compared to the Gal4 control (p = 0.1012). We conclude that *Mef2>PdfR* gave a partial rescue of PDF signaling. *24B>PdfR* showed an increase in contractions as well. The 24B rescue showed significantly more contractions than both the Gal4 control (p = 0.0061) and the UAS control (p = 0.0003). All statistical tests were unpaired t-tests in this analysis. These results support the hypothesis that PdfR is required in the ureter muscles for PDF signaling.

PDF induces contractions in the accessory glands of the male reproductive viscera. Several labs that work with PDF null and PDFR null flies, including our own, have noted anecdotally that these flies seem to have trouble reproducing. To that end we examined the reproductive viscera of male flies. Male wild-type and PDFR null flies were dissected and the response of the reproductive viscera to PDF treatment was assessed. The accessory glands of
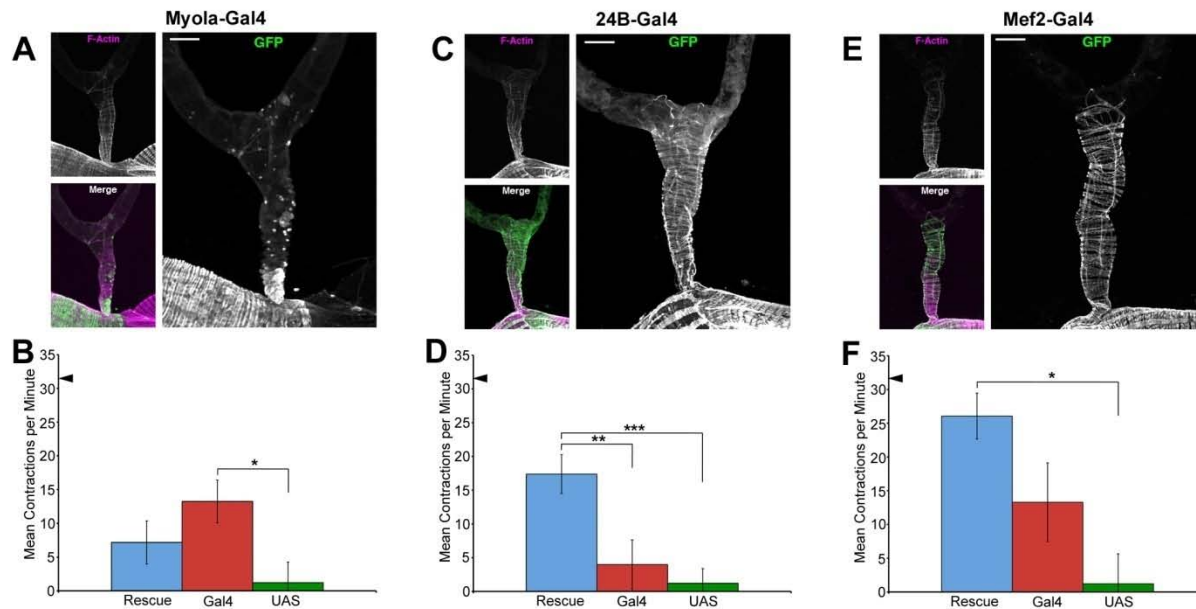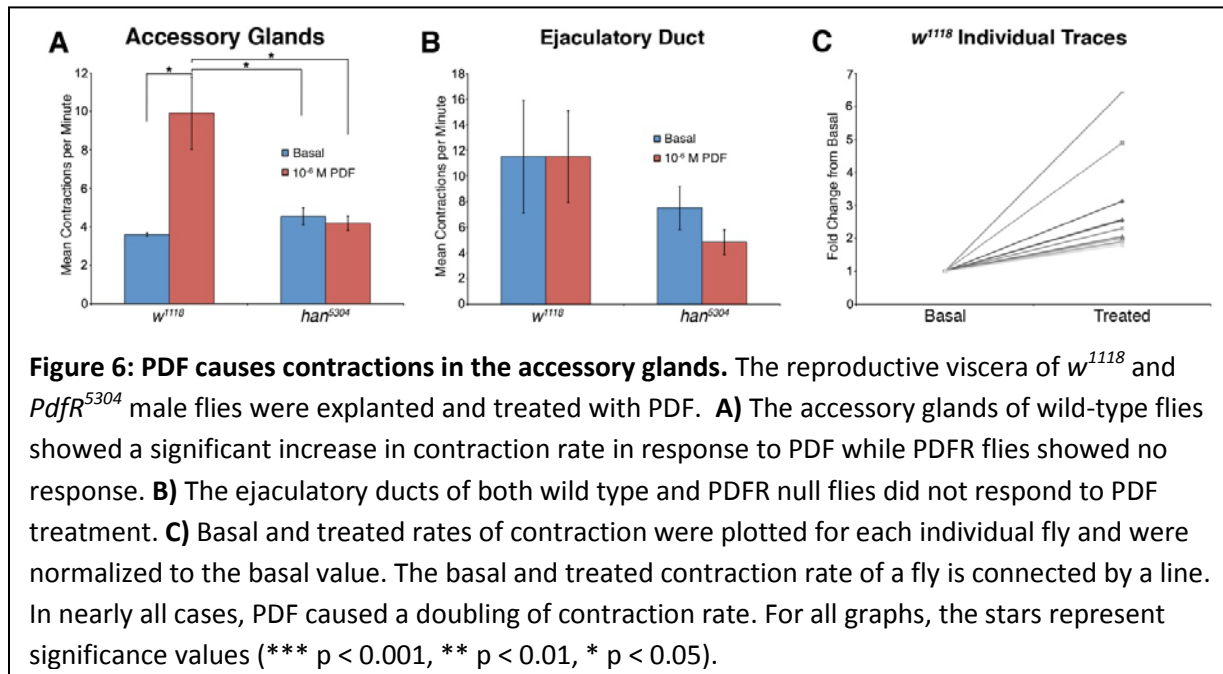
Figure 5: Driving expression of PDFR in the circular muscle of the ureter rescues the PDF induced contractions. A rescue experiment was performed by crossing various Gal4 lines with *PdfR^{5304}; UAS-PdfR* to drive PDFR expression in PDFR null flies. The lines tested had the general genotypes of: "Rescue" is *PdfR^{5304}; X-Gal4>PDFR*, "Gal4" is *PdfR^{5304}; X-Gal4*, "UAS" is *PdfR^{5304}; UAS-PdfR*, where X-Gal4 is designated above the micrographs. On all charts displaying the contraction rates, the black arrowhead indicates the *w^{1118}* contraction rate. **A) and B)** *Myola-Gal4*. **A)** Micrographs of *Myola>GFP* ureters. The top left micrograph is phalloidin marking the circular muscles, the large micrograph is *Myola>GFP*, and the bottom left micrograph is a merge to show colocalization. Note that Myola does not drive expression in the muscles. **B)** Contraction rates of the three lines in response to 10^{-6} PDF. **C) and D)** *24B-Gal4* **C)** Micrographs of *24B>GFP* ureters. Panels are laid out as in A). Note that in the merge view, there is colocalization indicating the 24B drive expression in the circular muscles. **D)** Quantification of PDF response as described in B) and above. The stars indicate a significant increase in contractions of the 24B rescue over both of the controls. **E) and F)** *Mef2-Gal4* **E)** Micrographs of *Mef2>GFP* ureters. Panels are laid out as in A). Note that Mef2 also colocalizes strongly with phalloidin. **F)** Quantification of PDF response as described in B) and above. Note the significant increase in contractions of the Mef2 rescue over the UAS control but not in the Gal4 control. However, the Mef2 rescue did restore contraction rates almost back to wild-type levels. For all graphs, the stars represent significance values (*** p < 0.001, ** p < 0.01, * p < 0.05).

wild-type flies showed a significant increase in contraction rate from basal (p = 0.0125, paired t-test) while other organs in the genital tract did not respond (Fig. 6 A, B). PDFR null flies did not show any increases in contraction rate in response to PDF, suggesting that the PDF response is regulated by the known PDF receptor (Fig. 6 A, B). Interestingly, in wild-type flies, addition of PDF caused the rate of contractions in each accessory gland to double in nearly all cases (Fig. 6 C).

Figure 6: PDF causes contractions in the accessory glands. The reproductive viscera of w^{1118} and $PdfR^{5304}$ male flies were explanted and treated with PDF. **A)** The accessory glands of wild-type flies showed a significant increase in contraction rate in response to PDF while PDFR flies showed no response. **B)** The ejaculatory ducts of both wild type and PDFR null flies did not respond to PDF treatment. **C)** Basal and treated rates of contraction were plotted for each individual fly and were normalized to the basal value. The basal and treated contraction rate of a fly is connected by a line. In nearly all cases, PDF caused a doubling of contraction rate. For all graphs, the stars represent significance values (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

Loss of PDF and PDFR has opposing effects on body weight. Due to the striking effects of PDF on the ureters and the accessory glands of *Drosophila*, we attempted to find a whole body effect of PDF loss. Several PDF null and PDFR null lines were tested to see if loss of PDF signaling caused a change in the weight of the flies. w^{15} are PDF null flies and w^{33} flies are their wild-type control. These lines were created by mobilizing a p-element that had previously inserted near the *pdf* locus and then screening for gene function that was lost during transposition (Renn et al, 1999). Lines *iso #2* and *iso #8* are isogenized lines that were made to decrease the genetic variability in the stocks. w^{15} and w^{33} flies showed significant differences in weight between both males and females throughout their life time (Fig. 7 A, B). In these lines, flies lacking PDF were always heavier than those with PDF. However, for w^{1118} flies versus PDFR null flies ($PdfR^{5304}$ and $PdfR^{3369}$), the wild-type flies were always significantly heavier (Fig. 7 C, D). This result seems to contradict the result in w^{15}/w^{33} flies: in one set of flies, loss of PDF signaling increases weight while in the other set of flies, loss of PDF signaling decreases weight. It should also be noted that in PDFR null flies, $PdfR^{5304}$ has a larger deletion of *pdfr* than $PdfR^{3369}$ (Hyun et. al. 2005) suggesting that a larger deletion of *pdfr* caused a larger decrease in weight. The isogenized flies do not clarify the effects of PDF signaling on weight. Once again, loss of PDF causes a significant increase in weight, but this effect is only seen in females (Fig. 7 E, F).
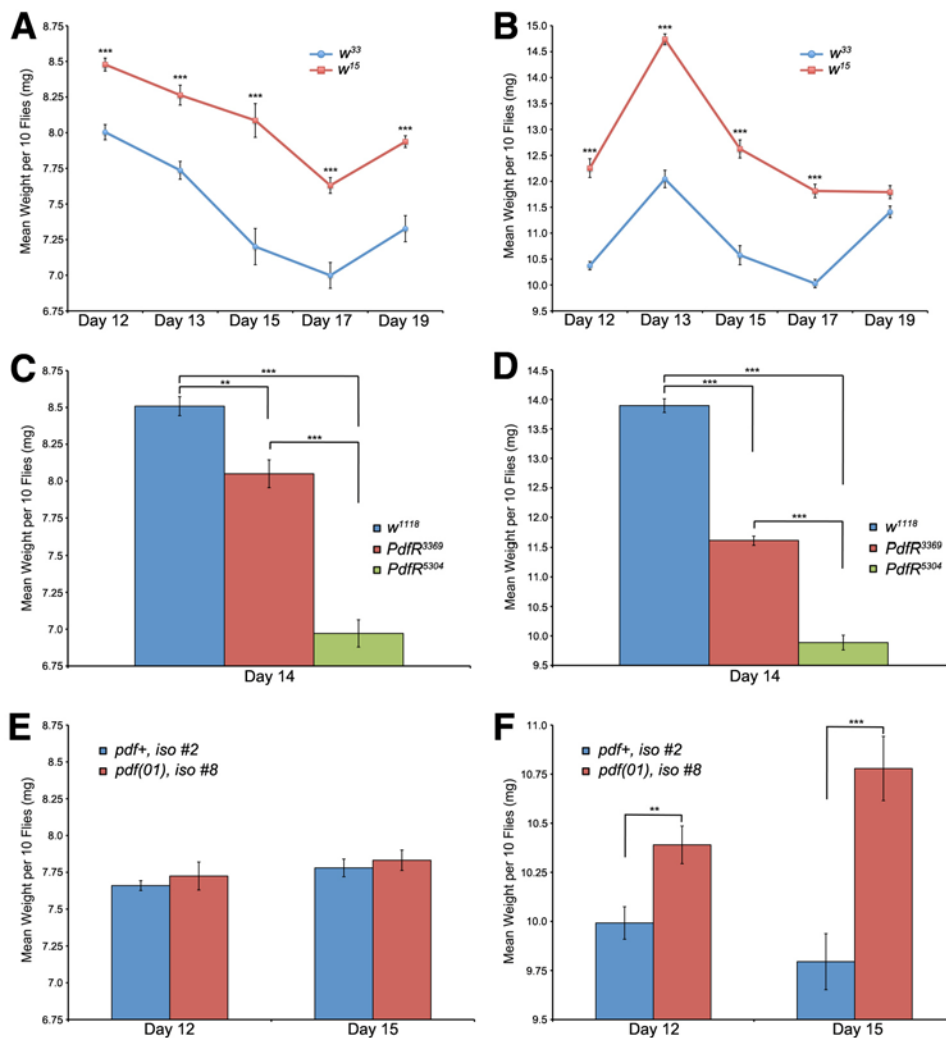
Figure 7: Loss of PDF and loss of PDFR have different effects on the weight of Drosophila. Males and females of the genotypes indicated above were weighed and analyzed separately. Significance is indicated by stars (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) **A)** Week long time course of w^{33} and w^{15} male weights starting from one day after eclosion (Day 12). All comparisons were done between males of the same age and significance was determined with a student's t-test. **B)** Week long time course of w^{33} and w^{15} females as described in A). **C)** Comparison of w^{1118} and PdFR null males on Day 14 (3 days after eclosion). Significance was determined as in A). **D)** Comparison of w^{1118} and PdFR null females on Day 14 (3 days after eclosion). Significance was determined as in A). **E)** Comparison of *pdf+* and *pdf(01)* isogenized males on Day 12 and Day 15. Significance was determined as in A). **F)** Comparison of *pdf+* and *pdf(01)* isogenized females on Day 12 and Day 15. Significance was determined as in A).

Discussion:

The intestinal, renal, hepatic, and nervous systems all work together to maintain metabolic homeostasis in mammals. In order to keep this balance, these systems must communicate with each other. In vertebrates, the enteric, sympathetic, and parasympathetic nervous systems play an important role in communication among these systems. As the prevalence of metabolic disorders grows, so does the need to understand the interplay between the nervous, intestinal, renal, and hepatic systems (Miguel-Aliaga, 2012). However, due to the complexity of these systems, they remain poorly understood.

Drosophila shares many functional and genetic similarities with mammals. In addition, it has the advantage of being a much simpler and more genetically tractable model for studying physiological phenomena. Many of the general organizational principles of mammalian systems are also present in *Drosophila* intestines (Cognigni et al, 2011). The study of the visceral regulation in *Drosophila*, however, is still a relatively young field. Our study makes new contributions to understanding the interplay between the nervous system and the viscera in the fly.

PDF extends the understanding of the *Drosophila* renal system and provides another similarity between *Drosophila* and mammals. The MPT of *Drosophila* have been studied extensively for their role in maintaining salt and water balance as well as for filtering toxins (O'Donnell and Rheault, 2005; Bijelic and O'Donnell, 2005; see Dow et al, 2010 for review). These studies focused largely on the distal renal tubules in *Drosophila* renal function. Due to these studies, the mechanisms through which *Drosophila* exchanges ions and water with the lumen of the renal tubules are well understood and several peptides that increase or reduce secretion rates have been discovered (Dow et al, 1994). However, the role and function of the ureters in renal processes have not been studied. We explored the function of PDF in control of the ureters. Our anatomical data in Figure 1 show that the PDF projections from the ventral nerve cord terminate on the midgut-hindgut junction. We show that bath applied PDF is sufficient to cause an increase in contractions in the ureters, with no apparent effects on gut motility. Furthermore we show this PDF response is mediated by the canonical G-protein coupled PDF receptor pathway and that PDFR is both necessary and sufficient for a PDF response in the ureters. This is the first described peptide in *Drosophila* that signals to the ureter and it may function as an excretory signal for the renal system.

These data also extend the similarities between mammalian VIP and *Drosophila* PDF. Both peptides are required for normal circadian rhythms (Colwell et al, 2003; Renn et al, 1999) and play a critical role in synchronizing the cellular oscillators of the neurons circadian clock network in mice (Aton et al, 2005) and *Drosophila* (Nitabach et al, 2006). VIP was also shown to have species dependent myotropic effects on the sphincter muscles in the bladder and urethra (Yoshiyama and de Groat, 2008). Our data show that PDF also has a myotropic effect on the ureters of *Drosophila*, which is the last segment of the renal organ and thus may be analogous to the mammalian urinary tract. These data also suggest a selective pressure in both mammals and insects by which the same peptide was co-opted as both a circadian neuropeptide and an enteric hormone. The fact that VIP and PDF share no obvious sequence similarities indicates that this pressure likely occurred in parallel and not on the same peptide in a common ancestor. Further study of the evolution of these peptides and their receptors may provide some insight into the origin of the enteroendocrine system and enteric nervous system.

The PDF neurons are also unique in the way that they signal with PDF. Our anatomical data show that the PDF projections are tightly associated with the midgut and not the ureters. However, it seems that only the ureters respond to PDF myotropically. This indicates that PDF must diffuse a substantial distance to reach its target, and therefore, the neurons must maintain relatively high concentrations of PDF in the local hemolymph. This implies a novel signaling mechanism in which a neuropeptide is released in high concentrations onto the midgut and then diffuses long distances through the hemolymph to its targets. Thus, our data indicate that PDF may act as a circulating hormone (long range signaling) in addition to its role as a neurotransmitter (short range signaling) in the circadian network.

PDF and its receptor may signal in multiple pathways to regulate the size and weight of *Drosophila*. Our data show that PDF null flies tend to weigh more than their wild-type controls and that this phenomenon is most consistently observed in females. We also show that PDFR null flies tend to weigh less than wild-type flies (Figure 7). These results seem to be contradictory because in both PDFR null and PDF null flies, PDF signaling is lost, and yet the flies have opposite phenotypes. However, PDFR is not receptive only to PDF; it also can respond somewhat potently to DH31 (Shafer et al, 2008). This suggests that PDFR may be important in two separate signaling pathways that affect weight: one that communicates via PDF and one that communicates with DH31. A recent study has suggested that there is a subset of larval midgut

endothelial cells that express DH31 and that these cells release DH31 to control gut motility (LaJeunesse et al, 2010). This study did not investigate how this signal is transduced, and PDFR may in fact be responsible for this function of DH31. In this model, a loss of PDFR would cause a dysregulation of gut motility which may decrease weight by several mechanisms, including a decrease in feeding or a decrease in nutrient absorption.

It is also possible that DH31 can compensate for loss of PDF in some, but not all, of the PDF signaling pathways in a PDF null mutant. In this model, the non-compensated PDF pathways signal to prevent an increase in weight in one body system, while the compensated pathways signal to promote an increase in weight in another body system. In PDF null flies, only the non-compensated pathways would become dysregulated and thus the average weight of the flies increases. However, in PDFR null flies, signaling in both pathways would be lost and thus both pathways would be dysregulated, causing a new set point for weight depending on the magnitude of the effect of each pathway on weight. The fact that we see a decrease in weight rather than an increase would indicate that the compensated pathway has a greater effect on weight than the non-compensated pathway. This is what we would expect in general because pathways that have more redundancies tend to have larger effects on physiology.

Loss of PDF signaling to the male accessory glands may be responsible for the increase in weight seen in the PDF null females. The accessory glands are responsible for making a set of peptides that become components of the semen. These seminal peptides are responsible for changing the physiology of females to promote sperm storage, decrease mating receptivity (Kalb, DiBenedetto, and Wolfner, 1993), increase egg laying (Herndon and Wolfner, 1995), and modify digestive function (Cognigni, Bailey, and Miguel-Aliaga, 2011). Our data suggest that PDF causes the accessory glands to contract, which may be responsible for moving the signaling peptides into the seminal fluid. Without PDF signaling in their mates, the female flies do not receive the sex peptides, and thus would still behave like unmated flies, which lay fewer eggs. We suspect that this egg retention is responsible for the increase in weight seen in PDF null females. Based on this idea and the suggestion that DH31 may signal to regulate gut motility (LaJeunesse et al, 2010), it would seem that in either of the models proposed above, signaling to the midgut through PDFR works to increase fly weight and signaling to the reproductive viscera works to decrease weight.

In conclusion, we have shown that PDF has a myotropic effect on both the ureter and the male accessory gland. In the ureter, this effect is mediated by PDFR and causes an increase in intracellular cAMP in the circular muscles. The PDF neurons in the abdominal ganglia project to the midgut and likely release PDF into the hemolymph to signal to the ureters and possibly the accessory glands. Our data on the weight of PDF and PDFR flies shows that the physiological role of PDF and PDFR are complex and suggest that both are important in several signaling pathways that may regulate functions as diverse as nutrient uptake, content of seminal fluid, and water balance. Taken together, these data show that PDF is an important signaling peptide in the viscera as well as the circadian clock.

References:

1. Aton S.J., Colwell C.S., Harmar A.J., Waschek J., Herzog E.D. (2005). Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons. *Nature Neuroscience,* 8, 476–483.

2. Bijelic G. and O'Donnell M. J. (2005) Diuretic factors and second messengers stimulate secretion of the organic cation TEA by the Malpighian tubules of *Drosophila melanogaster*. *Journal of Insect Physiology*, 51, 267-275.

3. Blake P. D., Kay I., and Coast G. M. (1996) Myotropic activity of *Acheta* diuretic peptide of the foregut of the House Cricket, *Acheta domesticus* (L.). *Journal of Insect Physiology*, 42(11-12), 1053-1059.

4. Brand A. H. and Perrimon N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, 118(2), 401-415.

5. Chapman T., Liddle L. F., Kalb J. M., Wolfner M. F., and Partridge L. (1995). Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. *Nature*, 373, 241-4.

6. Cognigni P., Bailey A. P., and Miguel-Aliaga I. (2011). Enteric Neurons and Systemic Signals Couple Nutritional and Reproductive Status with Intestinal Homeostasis. *Cell Metabolism,* 13, 92-104.

7. Colwell C. S., Michel S., Itri J., Rodriguez W., Tam J., Lelievre V., Hu Z., Liu X., and Waschek J. A. (2003). Disrupted circadian rhythms in VIP- and PHI- deficient mice. *Am J Physiol Regul Integr Comp Physiol*, 285, R939-49.

8. Denholm B., Sudarsan V., Pasalodos-Sanchez S., Artero R., Lawrence P., Maddrell S., Baylies M., and Skaer H. (2003). Dual Origin of the Renal Tubules in *Drosophila*: Mesodermal Cells Integrate and Polarize to Establish Secretory Function. *Current Biology*, 13, 1052-7.

9. Dow J. A. T. and Romero M. F. (2010). Drosophila provides rapid modeling of renal development, function, and disease. Am J Physiol Renal Phsyiol, 299, F1237-44.

10. Dow J. A. T., Maddrell S. H. P., Gortz A., Skaer N. J. V., Brogan S., Kaiser K. (1994). The malpighian tubules of *Drosophila melanogaster*: a novel phenotype for studies of fluid secretion and its control. *Journal of Experimental biology*, 197, 421-428.

11. Duffy, J. B. (2002). GAL4 System in *Drosophila*: A Fly Geneticist's Swiss Army Knife. *Genesis,* 34, 1-15.

12. Fujiyama M. and Inui A. (2000). Peptidergic regulation of gastrointestinal motility in rodents. Peptides, 21, 1565-82.

13. Fyrberg C., Becker J., Barthmaier P., Mahaffey J. and Fyrberg E. (1997) A Drosophila muscle-specific gene related to mouse quaking locus. *Gene*, 197(1-2), 315-323.

14. Harmar A. J., Marston H. M., Shen S., Spratt C., West K. M., Sheward W. J., Morrison C. F., Dorin J. R., Piggins H. D., Reubi J-C., Kelly J. S., Maywood E. S., and Hastings M. H. (2002). The VPAC Receptor Is Essential for Circadian Function in the Mouse Suprachiasmatic Nuclei. *Cell*, 109, 497-508.

15. Heifetz Y., Lung O., Frongillo Jr. E. A., and Wolfner M. F. (2000). The *Drosophila* seminal fluid protein Acp26Aa stimulates release of oocytes by the ovary. *Current Biology*, 10(2), 99-102.

16. Helfrich-Forster C. (1995). The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA,* 92, 612-6.

17. Herndon L. A. and Wolfner M. F. (1995). A *Drosophila* seminal fluid protein, Acp26Aa, stimulates egg laying in females for 1 day after mating. *Proc. Natl. Acad. Sci. USA*, 92, 10114-8.

18. Hyun S., Lee, Y., Hon S-T., Bang S., Paik D., Kang J., Shin J., Lee J., Jeon K., Hwang S., Bae E., and Kim J. (2005). *Drosophila* GPCR Han Is a Receptor for the Circadian Clock Neuropeptide PDF. *Neuron,* 48, 267-78.

19. Im S. H. and Taghert P. (2010). PDF Receptor Expression Reveals Direct Interactions Between Circadian Oscillators in *Drosophila*. *The Journal of Comparative Neurology,* 518, 1925-45.

20. Jiang H. and Edgar B. A. (2009) EGFR signaling regulates the proliferation of Drosophila adult midgut progenitors. *Development*, 136(3), 483-493

21. Kalb J. M., DiBenedetto A. J., and Wolfner M. F. (1993) Probing the function of *Drosophila melanogaster* accessory glands by direct cell ablation. *Procedings of the National Academy of Sciences*, 90, 8093-97.

22. Keast J. R. and De Groat W. C. (1992). Segmental Distribution and Peptide Content of Primary Afferent Neurons Innervating the Urogenital Organs and Colon of Male Rats. *The Journal of Comparative Neurology*, 319, 615-23.

23. LaJeunesse D.R., Johnson B., Presnell J. S., Catignas K. K., and Zapotoczny G. (2010). Peristalsis in the junction region of the *Drosophila* larval midgut is modulated by DH31 expressing enteroendocrine cells. *BMC Physiology,* 10(14).

24. Lear B. C., Merrill E., Lin J-M., Schroeder A., Zhang L., and Allada R. (2005). A G Protein-Coupled Receptor, *groom of PDF*, Is Required for PDF Neuron Action in Circadian Behavior. *Neuron*, 48, 221-227.

25. Mertens I., Vandingenen A., Johnson E. C., Shafer O. T., Li W., Trigg J. S., De Loof A., Schoofs L., and Taghert P. H. (2005). PDF Receptor Signaling in *Drosophila* Contributes to Both Circadian and Geotactic Behaviors. *Neuron,* 48, 213-219.

26. Miguel-Aliaga I. (2012). Nerveless and gutsy: intestinal nutrient sensing from invertebrates to humans. *Semin Cell Dev Biol*, doi:10.1016/j.semcdb.2012.01.002.

27. Miller A. (1994) The internal anatomy and histology of the imago of *Drosophila melanogaster.* in Demerec M., ed., *Biology of* Drosophila, Cold Spring Harbor Laboratory Press, New York, 420-534.

28. Nassel D. R., Shiga S., Mohrherr C. J., and Rao K. R. (1993) Pigment-dispersing hormone-like peptide in the nervous system of the flies Phormia and Drosophila: immunocytochemistry and partial characterization. *Journal of Comparative Neurology,* 331(2), 183-198.

29. Nassel D. R. and Winther A. M. E. (2010) *Drosophila* neuropeptides in regulation of physiology and behavior. *Progress in Neurobiology*, 92(1), 42-104.

30. Nitabach M. N., Wu Y., Sheeba V., Lemon W. C., Strumbos J., Zelensky P. K., White B. H. and Holmes T. C. (2006). Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. *The Journal of Neuroscience*, 26(2), 479-489.

31. O'Donnell M. J. and Rheault M. R. (2005) Ion-selective microelectrode analysis of salicylate transport by the Malpighian tubules of *Drosophila melanogaster*. *The Journal of Experimental Biology*, 208, 93-104.

32. Ranganayakulu G., Schulz R. A., and Olsen E. N. (1996) Wingless signaling induces nautilus expression in the ventral mesoderm of the *Drosophila* embryo. *Developmental Biology*, 176(1), 143-148.

33. Shafer O. T., Kim D. J., Dunbar-Yaffe R., Nikolaev V. O., Lohse M. J., and Taghert P. H. (2008). Widespread Receptivity to Neuropeptide PDF throughout the Neuronal

Circadian Clock Network of *Drosophila* Revealed by Real-Time Cyclic AMP Imaging. *Neuron*, 58, 223-37.

34. Stevaux O., Dimova D., Frolov M. V., Taylor-Harding B., Morris E. and Dyson N. (2002) Distinct mechanisms of E2F regulation by *Drosophila* RBF1 and RBF2. *EMBO J,* 21(18), 4927-4937.

35. Shafer O. T. and Taghert P. H. (2009). RNA-Interference Knockdown of *Drosophila* Pigment Dispersing Factor in Neuronal Subsets: The Anatomical Basis of a Neuropeptide's Circadian Functions. *PLoS ONE,* 4(12), e8298.

36. Siviter R. J., Coast G. M., Winther A. M. E., Nachman R. J., Taylor C. A. M., Shirras A. D., Coates D., Isaac R. E., and Nassel, D. R. (2000). Expression and Functional Characterization of a *Drosophila* Neuropeptide Precursor with Homology to Mammalian Prepprotachykinin A. *The Journal of Biological Chemistry*, 275(30), 23273-80.

37. Rao K. R., and Riehm J. P. (1993). Pigment-Dispersing Hormones. *Annals New York Academy of Sciences*, p 78-88.

38. Rao K. R., Riehm J. P., Zahnow C. A., Kleinholz L. H., Tarr G. E., Johnson L., Norton S., Landau M., Semmes O. J., Sattelberg R. M., Jorenby W. H., and Hintz M. F. (1985). Characterization of a pigment-dispersing hormone in eyestalks of the fiddler crab *Uca pugilator*. *Proc. Natl. Acad. Sci. USA,* 82, 5319-22.

39. Renn S. C. P., Park J. H., Rosbash M., Hall J. C., and Taghert P. H. (1999). A *pdf* Neuropeptide Gene Mutilation and Ablation of PDF Neurons Each Cause Severe Abnormalities of Behavioral Circadian Rhythms in *Drosophila*. *Cell*, 99, 791-802.

40. Vaudry D., Gonzalez B. J., Basille M., Yon L., Fournier A., and Vaudry H. (2000). Pituitary Adenylate Cyclase-Activating Polypeptide and Its Receptors: From Structure to Functions. *Pharmacological Reviews*, 52(2), 269-324.

41. Vosko A. M., Schroeder A., Loh D. H., Colwell C. S. (2007). Vasoactive intestinal peptide and the mammalian circadian system. *General and Comparative Endocrinology*, 152, 165-75.

42. Yoshiyama M., and De Groat W. C. (2008). The Role of Vasoactive Intestinal Polypeptide and Pituitary Adenylate Cyclase-Activating Polypeptide in the Neural Pathways Controlling the Lower Urinary Tract. *J Mol Neurosci*, 36, 227-40.