Temporal Progression of *Herpetomonas muscarum* Leidy (Kinetoplastida: Trypanosomatidae) in the Midgut of *Musca domestica* L. (Diptera: Muscidae)[1]

Dana Nayduch[2]

Department of Biology, Georgia Southern University, Statesboro, Georgia 30460 USA

J. Entomol. Sci. 44(2): 141-148 (April 2009)

Abstract *Herpetomonas muscarum* Leidy (Kinetoplastida: Trypanosomatidae) is a protistan symbiont that colonizes the hindgut of the housefly, *Musca domestica* L. (Diptera: Muscidae). The temporal location and transition of this symbiont within the fly has been understudied. In this study, the progression of *Herpetomonas* within the mid and hindgut with reference to the peritrophic matrix (PM) was examined microscopically and was compared with the fate of a bacterium (GFP-tagged *E. coli*). The housefly PM is a double-layered, open-ended physical barrier that separates ingested substances from the midgut epithelium and terminates near the hindgut. In the midgut, bacteria were confined within the inner PM, lysed by digestive enzymes, and compacted into fecal pellets within 12 h. In contrast, *Herpetomonas* initially resided within the inner PM, but many protists moved to the interPM space within a few hours. Additionally, protists rapidly progressed to the open end of the PM at the midgut/hindgut junction, in as little as 4-6 h postingestion, entering the ectoperitrophic space and attaching to the hindgut epithelium. Unlike bacteria, *Herpetomonas* demonstrated a hastened progression to the hindgut and avoided being immobilized, lysed and enclosed in a fecal pellet by the inner PM. Thus, whereas flies do not have permanent bacterial "flora" (because bacteria cannot escape the PM and are trapped and lysed), this protist has found a way to circumvent this fate and establish as a permanent hindgut symbiont. These results have applicable relevance to human-parasitic trypanosomatids that use stercorarian (posterior station) transmission from vectoring insects, such as *Trypanosoma cruzi* Chagas in triatomine bugs.

Key Words alimentary canal, peritrophic matrix (PM), protist, symbiont

The midgut of the housefly (*Musca domestica* L.; Diptera: Muscidae) is entirely lined by a double-layered type II peritrophic matrix (PM) which prevents large ingested materials, including microbes, from breaching the midgut epithelium (Lehane 1997). The housefly PM is noncellular and is continuously-extruded from a specialized region of cells in the anterior midgut called the cardia, proximal to the proventriculus (Lehane 1997). Digestion occurs within the lumen of the PM, and the inner PM layer then envelops and compresses residual waste into fecal pellets (Nayduch et al. 2005). The PM and any materials contained within continuously progress posteriorly in a conveyor-belt like fashion, and waste products are released at the hindgut/rectum junction where the PM terminates to an open end (Richards and Richards 1977). Thus, ingested microorganisms must have a mechanism for evading this

[1]Received 14 July 2008; accepted for publication 21 September 2008.
[2]Address inquiries (email: dnayduch@georgiasouthern.edu).

entrapment by moving posteriorly in a timely manner to avoid being digested and excreted as feces.

The temporal progression of symbiotic microbes, especially protists, within the PM of dipteran hosts has been understudied. Previously, Nayduch et al. (2005) showed that *Aeromonas caviae* bacteria cannot escape the housefly PM (in particular the inner PM) and are subject to immobilization, lysis and excretion. In contrast, symbiotic protists, such as *Herpetomonas* in houseflies (Hupperich et al. 1992) and trypanosomes in tsetse (Welburn and Maudlin 1999, Gibson and Bailey 2003), apparently escape into the ectoperitrophic space of their dipteran hosts by swimming out the terminal free end of the PM, thereby truly colonizing the gut epithelium. Gibson and Bailey (2003) examined the temporal location and fate of *Trypanosoma brucei* (Plimmer and Bradford, 1899) in the midgut of tsetse flies (*Glossina morsitans* Westwood) and determined that trypanosomes were able to establish permanent infections if they (1) were able to differentiate into procyclic forms and (2) concurrently escaped the PM before the blood food bolus was excreted as waste. Trypanosomes that established were subsequently able to swim via flagella to the anterior midgut (proventriculus), where they continued to the salivary glands for transmission to the definitive host (anterior stage transmission).

Parasitic kintetoplastids are heteroxenous and are transferred to the definitive host via saliva during feeding (e.g., brucei group *Trypanosoma* spp. and *Leishmania* spp.) or via feces (e.g., lewisi group trypanosomes such as *T. cruzi*). In contrast, *Herpetomonas muscarum* Leidy is monoxenous, being transmitted from fly to fly via ingestion of feces containing symbionts that were shed from the hindgut (Becker 1923). Nearly all orders of insects are hosts for various monoxenous kinetoplastid symbionts, where some are clearly commensals and others parasites. Interestingly, monoxenous kinetoplastids are believed to be the ancestors of the vertebrate-parasitic trypanosomatids based on the "insect-first" model of kinetoplastid evolution. This model suggests that *Trypanosoma* and *Leishmania* are descended from monoxenous symbionts of hematophagous insects which survived after being incidentally transmitted to the vertebrate host during feeding (Simpson et al. 2006). Thus, understanding the relationship between monoxenous protists and their insect hosts could be of considerable interest as a model system for elucidating vector/parasite relationships.

The relationship between houseflies and *Herpetomonas* spp. mainly has been investigated from a morphological standpoint. As such, most studies have focused on the cellular and ultrastructural interactions of this symbiont with hindgut cells. For instance, Hupperich et al. (1992) described the formation of hemidesmosomes integral to the ultrastructural attachment of *H. samuelpessoai* to the housefly hindgut. In this study, the temporal progression of *H. muscarum* within the housefly midgut was investigated, and its fate was compared with that of GFP-expressing *Escherichia coli* bacteria at similar time points with particular reference to the PM.

Materials and Methods

Housefly rearing. Houseflies were obtained from a stable colony, first established at Georgia Southern University in 2004, where they are given food and water ad libitum and maintained at ~25°C with a 14 h light:10 h dark photoperiod. Houseflies were confirmed to be free of natural infection with *H. muscarum* by microscopic examination of the hindgut. Adult houseflies that had recently eclosed were used for all infections.

Infections with GFP-expressing *E. coli*. GFP-expressing *E. coli* were obtained from Brian Weiss, Yale University, and had been transformed with eGFPuv plasmid (BD Bioscience, Palo Alto, CA) as previously described (Weiss et al. 2006). Houseflies (n = 16) were individually housed, fasted for 8 h, and fed a 2 μL droplet of approximately 1×10^6 cells of recombinant bacteria.

Infections with *H. muscarum*. *Herpetomonas muscarum* was purchased from American Type Culture Collection (ATCC 30,260) and maintained in liver infusion tryptose medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO-BRL). Houseflies (n = 16) were individually housed, fasted for 8 h, and fed a 2 μL droplet of log phase *H. muscarum*. The feeding experiment was replicated twice because infection rates were not always 100% (i.e., 4 of 4 per time point, below), presumably because some flies did not eat the droplet before it evaporated.

Microscopic examination of the housefly alimentary canal. Four flies were sacrificed for each treatment at 1, 4, 6, 12 h postingestion, and the crop, proventriculus, stomach, midgut and hindgut/rectum were removed intact by microdissection. In some specimens, to clearly view the position of the microbes relative to the inner and outer PM (IPM and OPM, respectively), the gut was perforated applying light pressure to the cover slip which resulted in extrusion of the PM through the gut wall. Further, although all flies were dissected and examined microscopically, images were captured only from specimens (n = 4) best representing key phenomena occurring at the time-points listed. All specimens were examined with a Leica Leitz Laborlux-12 microscope (Leica Microsystems Inc., USA) under bright field illumination. Additionally, UV light was used to illuminate GFP-expressing *E. coli*, and images were viewed using a Chroma 41,001 filter (Chroma Technology Corp., Rockingham, VT). Bright field, dark field and phase contrast illumination were used for the microscopical examination of *H. muscarum*. Digital photographs were taken with a Leica DFC420 digital camera.

Results and Discussion

The temporal fate of both *H. muscarum* and *E. coli* within the alimentary canal of flies was examined by microscopy. Within all treatment groups (i.e., times that flies were examined after ingesting the droplet of either protists or bacteria), microbes showed similar progression within the alimentary canals. Because there was little variability within these treatments, figures representing key events at these time points are shown rather than individual images of each fly.

Fate of GFP-expressing *E. coli*. At 1 h postingestion free-swimming bacteria and clumps of adhered bacteria were observed in the anterior midgut (Fig. 1) of infected flies. Adhered bacteria were sometimes immobilized and attached to the inner (luminal) surface of the PM as previously reported for other bacterial species by Nayduch et al. (2005). All bacteria were contained within the inner PM and were neither observed in the interPM space nor in contact with the gut epithelium. At 4 h, flies contained some bacteria in their crop (not shown), whereas in most flies bacteria had progressed further within the midgut, and many were apparently being lysed as evidenced by the release of free GFP (Fig. 2). The inner PM began to envelop bacteria and free GFP in the initial formation of fecal pellets (FP; Fig. 2). At 6 h postingestion, clumps of immobilized bacteria were observed in the crop, along with free GFP (not shown) and most bacteria in the midgut had been trapped within the inner PM and lysed (as in Fig. 2). At 12 h, some flies still contained intact, but immobilized, cells of *E. coli* in the crop. However, the midguts of all flies were mostly devoid of live bacteria.

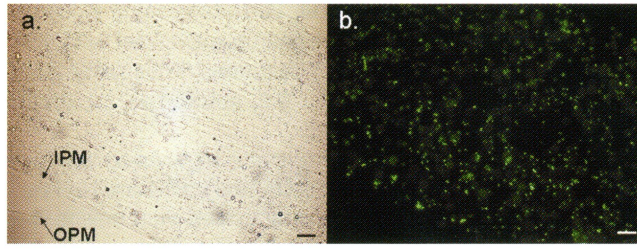

Fig. 1. GFP-expressing *E. coli* in the peritrophic membrane of the housefly midgut, 1 h postingestion, viewed with (a) bright field and (b) UV light microscopy. OPM = outer peritrophic matrix; IPM = inner peritrophic matrix. Scale bar = 10 μm.

In many flies, fecal pellets had apparently been excreted as they were not present in the distal midgut or the hindgut. If fecal pellets were present, they consisted of free GFP from lysed bacteria. At no time points within these 24 h were bacteria observed outside of the PM lumen, i.e., bacteria were contained within the inner PM, were lysed, and were compressed into fecal pellets and excreted.

Fate of *H. muscarum*. The fate of the protist symbiont *H. muscarum* differed significantly from that observed for *E. coli*. At 1 h, *H. muscarum* was highly motile and active, populating all areas of the anterior alimentary canal, including the crop (not shown) and anterior midgut (Fig. 3). In the midgut, *H. muscarum* was restricted to the PM lumen,

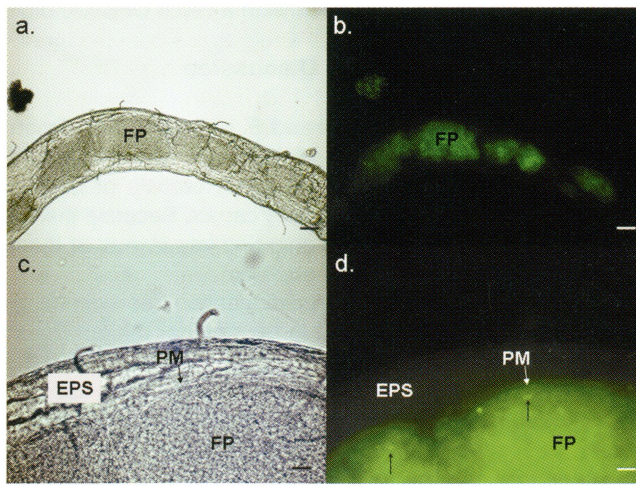

Fig. 2. Fecal pellets (FP) in the distal midgut of the housefly, 4 h after ingesting a bolus of bacteria. GFP-expressing *E. coli* was lysed and enclosed within the peritrophic membrane (PM), as evidenced by free GFP (b, d); none were present in the ectoperitrophic space (EPS). Specimens were viewed with bright field (a, c) and UV (b, d) light microscopy. Scale bars: 100 μm (a, b) and10 μm (c, d).

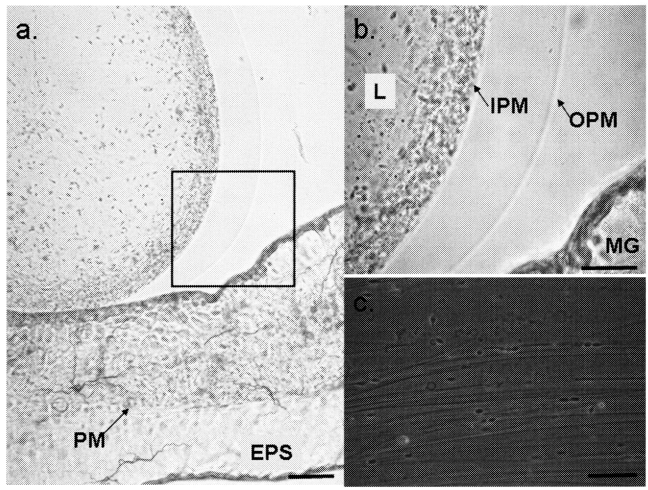

Fig. 3. *Herpetomonas muscarum* in the housefly midgut (MG), 1 h postingestion. Specimens were viewed with bright-field (a, b), and phase-contrast (c) light microscopy. Peritrophic matrix (PM) was extruded from midgut to show detail of inner PM (IPM) and outer PM (OPM), indicated by box (a) and further magnified (b). All protists were restricted to the IPM lumen, and none were present in the ectoperitrophic space (EPS). Scale bars: 100 μm (a) and 50 μm (b, c).

i.e., entrapped within the inner PM (IPM; Fig. 3), as was seen for bacteria (above). However, as early as 4 h but more evident at 6 h postingestion, *H. muscarum* was observed within the interPM space in all flies (IPS; Fig. 4). Further, at 6 h, *H. muscarum* had progressed to the hindgut and rectum of the flies, an area where the PM is not present. Protists were observed attaching via their flagella to the epithelial surface of these areas at 12 h (Fig. 5). It was necessary to tease apart the hindgut to view the protists attaching to the epithelium, as viewing through an intact specimen proved difficult due to the presence of dark-refracting fecal matter. At these later time points (6 and 12 h), *H. muscarum* was observed in all areas of the alimentary canal (crop, midgut, hindgut, rectum), with highest numbers in the distal areas of the gut. Apparently, there may be a timely tropism toward the caudal area of the gut in an effort to escape the open end of the PM. It is likely that protists observed within the interPM space had migrated to that area after swimming out the caudal end of the PM, possibly in search of the gut epithelium. This is supported by the fact that protists were not observed penetrating and crossing the PM in any part of the midgut. Similar observations have been made for trypanosomes in tsetse (Gibson and Bailey 2003), i.e., the protists in that study also did not traverse the PM. Most notably, the ability to escape PM entrapment is unique to this protist and is in stark contrast to the fate of bacteria. In houseflies, *E. coli* (observed here) and other bacterial species (Nayduch et al. 2005) adhered to the PM, were lysed, and were not observed outside of the PM lumen at any time or location.

Bacteria are routinely ingested by flies during their coprophagic feeding habits and lifestyle of living and breeding in decaying substrates. There has been no evidence that bacterial flora truly exist in flies in the sense that bacteria have never been shown

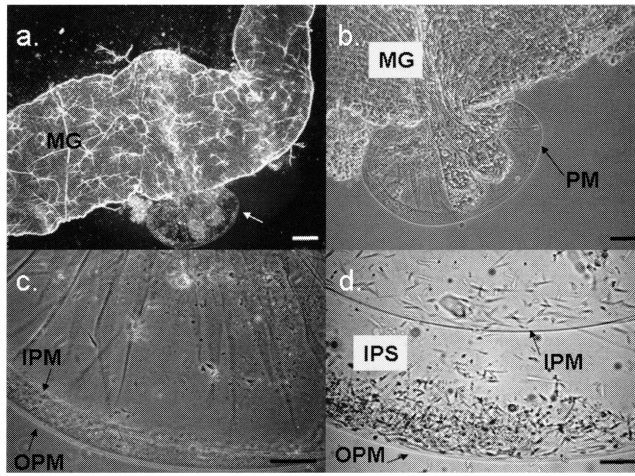

Fig. 4. *Herpetomonas muscarum* in the housefly midgut (MG), 4 h postingestion. Specimens were viewed with dark-field (a), phase-contrast (b, c), and bright-field (d) light microscopy. Peritrophic matrix (PM) was extruded from midgut to show detail of inner PM (IPM) and outer PM (OPM), indicated by arrow (a) and further magnified (b-d). Protists were found in the IPM lumen, the interPM space between the IPM and OPM (IPS) and the ectoperitrophic space (not visible in this figure). Scale bars: 150 μm (a), 75 μm (b), 50 μm (c), 25 μm (d).

to exit the PM and colonize the midgut epithelium. Thus, bacteria are ephemeral residents that only exist within flies for a short period of time after ingestion. In this study, *E. coli* was immobilized by the inner PM and lysed within 12 h. In contrast, the protist (and life-long symbiont) *H. muscarum* was not observed being immobilized, and was able to rapidly progress to the distal open end of the PM and attach to the hindgut and rectal epithelium. Because the PM of flies is continuously extruded from the cardia, moving posteriorly like a conveyor belt in conjunction with peristalsis, microbes must avoid entrapment by the PM to establish as permanent residents in the fly alimentary canal. It is interesting that both microbes used in this study were actively motile, yet only *H. muscarum* was apparently able to use this behavior (flagellar motion) to move out of the PM. Perhaps the immobilization of *E. coli* (the observed adherence to the inner PM wall) was critical in hindering its progress. Adherence of bacteria to the PM has been demonstrated in blow flies (*Calliphora erythrocephala* L.) and was mediated by mannose-specific lectins (Peters et al. 1983). The housefly PM also may have lectins on its luminal surface to bind and immobilize bacteria, rendering them unable to escape lysis by digestive enzymes.

Whether *H. muscarum* is lysed by midgut digestive enzymes was not investigated. However, the epithelial surfaces of the hindgut and rectum do not secrete such factors, because the terminal process of digestion in dipterans concludes in the distal part of the midgut (Terra et al. 1988). Further, the PM may actually serve to initially protect protists by providing for the slow diffusion of midgut enzymes across its pores. In the sandfly *Phlebotomus papatasi* Scopoli, flies that were fed infective bloodmeal along with chitinase (used to dissolve the PM), showed greater killing of developing

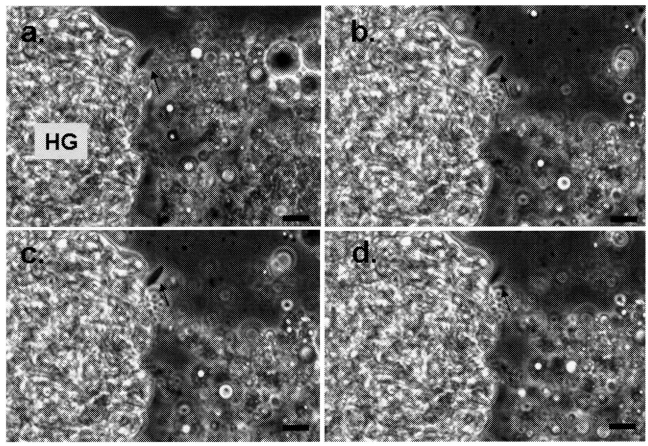

Fig. 5. *Herpetomonas muscarum* in the housefly hindgut (HG), 12 h postingestion. Specimens were viewed with phase-contrast microscopy. The hindgut was teased apart to more easily view the attached protist (arrow), which was captured in four frames 1 sec apart (a-d). Scale bar = 10 µm.

Leishmania parasites than in flies fed infective blood without the enzyme (Pimenta et al. 1997). The authors suggested that the PM served as a barrier hindering the rapid diffusion of digestive enzymes, thereby protecting the parasites from proteolytic digestion during early infection stages. *Leishmania,* like *H. muscarum*, would be allowed additional time to escape from the PM before digestive enzymes accumulated in the PM lumen, or before the PM itself became a physical barrier to development. In other words, establishment can occur if microbes can withstand small doses of lytic enzymes while concurrently progressing out of the digestive regions of the midgut and moving to the safe haven of the hindgut. Resistance to lysis may also be mediated by protective factors on the protist cell surface. Such a mechanism has been suggested for African trypanosomes that are coated with a dense layer of procyclin glycoproteins when they reside in the tsetse midgut (Güther et al. 2006). Removal of these glycoproteins by genetic knockout rendered the protists unable to establish infection. Finally, attachment via flagella (Hupperich et al. 1991) further assists in *H. muscarum* establishment in the fly by physically anchoring the protist to the gut lining while feces continue to pass via peristaltic action. The combined effects of these counter-defenses and activities are putative adaptations of this protist which allow for life in the inhospitable alimentary canal of the housefly.

The nature of the housefly-*Herpetomonas* symbiosis remains unclear in that it has not been determined whether the protist is indeed a parasite, commensal or mutual of its host. Irrespectively, this system could serve as a model for other insect-protist symbioses, particularly those between vectors and human parasitic protists. Most applicable for comparison could be that of protists also transmitted via vector feces, such as *Trypanosoma cruzi* Chagas in triatomine bugs. Further studies are needed to more closely examine the role of the insect physiological factors in this symbiosis, as the

PM is only a physical barrier and other defenses such as the immune response were not examined here.

Acknowledgments

Juli Sergi, Matt Kennedy and Rob Simone are thanked for help with the initial phases of this project and for fly rearing. Virginia Wiggins and Muaz Ibrahim provided assistance with digital photography. Special thanks to Brian Weiss at Yale University for the GFP-expressing *E. coli* and Michael Yabsley at the University of Georgia for LIT medium. Funding for this research was provided by NSF 0634955 "REU Site in Chemistry and Biology at Georgia Southern University: Focus on Underrepresented Minorities" summer grant.

References Cited

Becker, E. R. 1923. Observations on the morphology and life history of *Herpetomonas muscae-domesticae* in North American muscoid flies. J. Parasitol. 9: 199-214.

Gibson, W. and M. Bailey. 2003. The development of *Trypanosoma brucei* within the tsetse fly midgut observed using green fluorescent trypanosomes. Kinetoplastid Biol. Dis. 2: 1-13.

Güther, M. L., S. Lee, L. Tetley, A. Acosta-Serrano and M. A. Ferguson. 2006. GPI-anchored proteins and free GPI glycolipids of procyclic form *Trypanosoma brucei* are nonessential for growth, are required for colonization of the tsetse fly, and are not the only components of the surface coat. Mol. Biol. Cell 17: 5265-5274.

Hupperich, K., E. P. Camargo and R. Milder. 1991. Ultrastructural study of the host-parasite relationship of trypanosomatids in the housefly. Parasitol. Res. 78: 48-55.

Lehane, M. J. 1997. Peritrophic matrix structure and function. Annu. Rev. Entomol. 42: 525-550.

Nayduch, D., G. P. Noblet and F. Stutzenberger. 2005. The fate of *Aeromonas caviae* in the midgut of the housefly *Musca domestica*. Invertebr. Biol. 124: 75-79.

Peters, W., H. Kolb and V. Kolb-Bachofen. 1983. Evidence for a sugar receptor (lectin) in the peritrophic membrane of the blowfly larva, *Calliphora erythrocephala* Mg. (Diptera). J. Insect Physiol. 29: 275-280.

Pimenta, P. F., G. B. Modi, S. T. Pereira, M. Shahabuddin and D. L. Sacks. 1997. A novel role for the peritrophic matrix in protecting *Leishmania* from the hydrolytic activities of the sand fly midgut. Parasitol. 115: 359-369.

Richards, A. G. and P. A. Richards. 1977. The peritrophic membranes of insects. Annu. Rev. Entomol. 22: 219-240.

Simpson, A. G., J. R. Stevens and J. Lukes. 2006. The evolution and diversity of kinetoplastid flagellates. Trends Parasitol. 22: 168-174.

Terra, W. R., F. P. Espinoza-Fuentes and C. Ferreira. 1988. Midgut amylase, lysozyme, aminopeptidase, and trehalase from larvae and adults of *Musca domestica*. Arch. Insect Biochem. Physiol. 9: 283-297.

Weiss, B. L., R. Mouchotte, R. V. M. Rio, Y.-N. Wu, Z. Wu, A. Heddi and S. Aksoy. 2006. Interspecific transfer of bacterial endosymbionts between tsetse fly species: infection establishment and effect on host fitness. Appl. Environ. Microbiol. 72: 7013-7021.

Welburn, S. C. and I. Maudlin. 1999. Tsetse-trypanosome interactions: rites of passage. Parasitol. Today 15: 399-403.