Universidade Estadual de Campinas

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Termotolerância e efeito do jejum, realimentação e infecção por *Trypanosoma cruzi* na resposta a choques de temperatura em *Panstrongylus megistus* (Burmeister).

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- Investigação imunocitoquímica de HSP70 em túbulos de Malpighi de *Panstrongylus megistus* submetidos a choques de temperatura (em preparação).

Índice

I- RESUMO

I - Resumo

O efeito dos choques térmicos, quentes e frios, foi estudado buscando-se investigar a ocorrência de tolerância termal em *Panstrongylus megistus*. A taxa de sobrevivência e mudas foi acompanhada em ninfas de 5o. estádio e alados de ambos os sexos de *P. megistus* após choques térmicos seqüenciais nos quais o primeiro foi de 35°C ou 40°C (1 h) (hipertérmico) ou de 5°C ou 0°C (1 h) (hipotérmico) precedendo um choque mais severo de 40°C ou 0°C (12 h), respectivamente. Os choques foram separados por intervalos de 8, 18, 24 ou 72 h a 28°C. A fim de se investigar se a tolerância termal nessa espécie era acompanhada por alterações nucleares, ninfas foram dissecadas após intervalos de 24 h, 10 e 30 dias seguindo-se o segundo choque, para determinação e contagem de fenótipos nucleares em túbulos de Malpighi. Demonstrou-se tolerância aos choques quentes e frios sendo que dependendo da temperatura do choque, do intervalo entre os tratamentos à 28°C, fase do desenvolvimento e sexo, a intensidade de tolerância desenvolvida diferiu. Enquanto a tolerância ao calor nas ninfas se verificou quando o período entre os choques foi de até 24 h (choque inicial a 35°C) ou mais (choque inicial a 40°C), taxa expressiva de tolerância nos alados foi verificada apenas em condições de 8 h de intervalo entre os choques, sendo o choque inicial dado a 40°C. Também diferindo do que acontece nas ninfas, cuja tolerância ao choque frio se expressou de modo semelhante em todas as situações analisadas, nos alados foi demonstrada expressiva tolerância ao choque frio apenas quando o choque inicial foi dado a 0°C e o tempo entre choques seguidos ultrapassou 18 h. As fêmeas apresentaram maior tolerância termal. Os resultados de tolerância termal foram os primeiros reportados em triatomíneos e sugerem o envolvimento de proteínas de choque térmico (HSP), embora outros mecanismos possam também estar envolvidos. Essa ação protetora, no entanto, parece perder a eficácia com o desenvolvimento do inseto.

Igualmente foram encontrados modelos diferentes de alterações nos fenótipos nucleares induzidos pelos choques quentes e frios e identificados após reação de Feulgen. Tolerância ao choque quente envolveu alterações morfológicas representadas por um decréscimo na forma de morte celular por apoptose simultaneamente a um aumento nas respostas de sobrevivência celular. Choques frios seqüenciais não envolveram fusão celular/nuclear e ainda elicitaram aumento de necrose com a avanço do tempo pós-choque. As temperaturas de 40 e 0° C

foram mais efetivas que as temperaturas de 35 e 5°C em elicitar tolerância ao calor e ao frio, respectivamente, como foi demonstrado pela análise dos fenótipos nucleares. Diferentes choques térmicos podem ativar diferentes mecanismos de proteção celular contra o estresse em *P. megistus*, favorecendo uma adaptação do inseto a vários ecótopos.

O efeito do jejum seguido por realimentação e choque térmico nos fenótipos nucleares de *P. megistus* foi também estudado citologicamente em túbulos de Malpighi de ninfas de 4o.estádio. Os insetos foram mantidos em jejum até 100 dias a 28°C e sua sobrevivência examinada diariamente. Parte dos insetos em jejum foi realimentada e parte dos insetos em jejum, ou realimentados após jejum, recebeu um choque de 40°C por 1 h. A análise dos fenótipos foi procedida 1 e 7 dias após tais procedimentos. Detectou-se que após 40 dias de jejum, a taxa de sobrevivência dos espécimes foi alta (90%), decrescendo logo após. Com o avanço do tempo de jejum, a frequência de necroses aumentou. Realimentação após jejum agiu como um estresse médio, aumentando a frequência de apoptose. Ausência de núcleos gigantes foi observada em todos os insetos. Embora o choque térmico tenha promovido um aumento na frequência de núcleos necróticos, não afetou a resposta ao jejum e à realimentação, já que não se verificou uma associação entre os fatores. Espécimes bem nutridos apresentaram uma menor taxa de fenótipos alterados quando comparados a espécimes mantidos em jejum ou realimentados após o jejum. Uma vez que períodos de estresse nutricional podem influenciar a distribuição geográfica e temporal das espécies, as alterações celulares, de morte ou proteção, podem contribuir para o conhecimento do comportamento de *P. megistus* em condições naturais e em laboratório.

O efeito dos choques térmicos em espécimes infectados com *Trypanosoma cruzi* sobre a incidência de sobrevivência e mudas, fenótipos nucleares, prevalência e intensidade de infecção por *Trypanosoma cruzi* bem como multiplicação e metaciclogênese, inclusive após choque quente (40°C, 1 h), foi investigado em *P. megistus*. Ninfas de 4o. instar foram submetidas a infecção com a cepa Y de *T. cruzi* e acompanhadas durante 45 dias. O número de epimastigotas e tripomastigotas foi determinado por exame do conteúdo da glândula retal e os fenótipos nucleares de *P. megistus* estudados em seus túbulos de Malpighi. Demonstrou-se que o choque térmico não promoveu diferenças na sobrevivência e na incidência de mudas em espécimes infectados e não infectados. A infecção promoveu aumento na taxa de sobrevivência e decréscimo na ocorrência de

mudas. Não houve diferença significante quanto à prevalência e à magnitude de infecção em espécimes submetidos ou não a choque térmico. Os espécimes submetidos a choque tiveram um decréscimo no número de epimastigotas e tripomastigotas, indicando que alterações na multiplicação do parasita e na metaciclogênese foram induzidas pelo choque térmico. Somente para núcleos com suspeita de apoptose, os fatores infecção e estresse, isoladamente, foram significantes, sugerindo que a presença do parasita possa ser um estímulo para indução do programa de morte celular. As alterações induzidas pela infecção em associação ao choque térmico, especialmente com relação à multiplicação e metaciclogênese, poderão vir a contribuir para nossa compreensão do sistema vetor-parasita, dos modelos epidemiológicos envolvidos e criação adequada desses insetos em laboratório.

II- ABSTRACT

II- Abstract

The effects of heat and cold shocks on thermal tolerance in *Panstrongylus megistus* were studied. The incidence of survival and molting in fifth-instar nymphs and adults was determined following treatments in which a mild heat shock (35°C or 40°C, 1 h) or cold shock (5°C or 0°C, 1 h) preceded a more drastic shock (40°C or 0°C, 12 h), respectively. Sequential shocks were separated by intervals of 8, 18, 24 or 72 h at 28°C (control temperature). To investigate whether insect survival was accompanied by nuclear changes, fifth-instar nymphs were dissected 1, 10 and 30 days after the second shock and the frequency of nuclear phenotypes present in the Malpighian tubules was determined.

Heat and cold shock tolerance was induced but varied according to shock temperature, interval between shocks at 28°C, developmental stage and sex. In contrast to the response in nymphs, for which higher values of heat tolerance were sustained for up to 24 h (preliminary shock at 35°C) or even longer (preliminary shock at 40°C) between sequential shocks, in adults marked tolerance was seen only when the intervals between shocks was ≤ 8 h (preliminary shock at 40°C). Also in contrast to nymphs, which exhibited cold-tolerance is all conditions, adult insects acquired tolerance to more severe shocks only when the preliminary shock was given at 0°C and the time between shocks was greater than 18 h. Females showed greater tolerance than males. These results are the first to show thermal tolerance in a blood-sucking hemipteran and suggest the involvement of heat shock proteins, although other protective mechanisms may also be operating. This cellular protection may become less effective as the insect develops.

Heat and cold shocks produced different patterns and frequencies of nuclear phenotypes as shown by the Feulgen reaction. Heat shock tolerance involved a decrease in apoptosis and a simultaneous increase in cell survival responses. Sequential cold shocks did not involve cell/nuclear fusion and even elicited an increase in necrosis with increasing time after the shock. Temperatures of 40° and 0°C were more effective than 35° and 5°C in eliciting heat and cold shock tolerance, respectively, as shown by cytological analysis of the nuclear phenotypes. Different sequential thermal shocks can tigger different mechanisms of cellular protection against stress in *P. megistus* and may allow the insect to adapt to various ecotopes.

The effect of fasting in association with refeeding and heat shock on the nuclear phenotypes of *P. megistus* was studied cytologically in Malpighian tubules of fourth-instar nymphs. The insects were fasted up to 100 days at 28°C and their survival followed daily. Groups of nymphs were separated each month, with part of the group being refed and the other part kept fasting. Insects in each of these subgroups received either a heat shock at 40°C for 1 h or where maintained at 28°C (no heat shock). The Malpighian tubules were removed 1 and 7 days after each assay and subjected to the Feulgen reaction for identification and counting of the nuclear. Insect survival was high (90%) after 40 days of starvation but decreased thereafter. Only necrosis increased with the duration of fasting. Feeding after fasting acted as a mild stressing agent and increased the frequency of apoptosis. No giant nuclei was observed in the insects. Although heat shock increased the frequency of necrotic nuclei it did not affect the starvation and refeeding responses since there was significant interaction between factors. Well-nourished specimens showed a smaller frequency of altered phenotypes than starved or refed insects. Since periods of nutritional stress influence the geographic and temporal distribution of a species, cellular changes such as death or protective mechanisms can contribute to our understanding of the biological behaviour in *P. megistus* in natural or laboratory conditions.

The incidence of survival and molting, the frequency of nuclear phenotypes, the frequency of infection by *T. cruzi*, and the grades of metacyclogenesis, including after heat shock (40°C, 1 h), were also investigated. Fourth-instar nymphs infected with the strain Y of *T. cruzi* and monitored for 45 days thereafter. The number of epimastigotes and trypomastigotes was determined by examining the rectal gland contents. The Malpighian tubules were used to determine the nuclear phenotypes. Heat shock did not alter differences in the survival and molting rates in infected, compared to non-infected insects. However, infection increased the survival and decreased the molting rates. There were no significant changes in the prevalence and magnitude of infectivity following heat shock. Shocked insects showed reduced number of epimastigotes and trypomastigotes, indicating that changes in the multiplication of the parasite and metacyclogenesis were affected by heat shock. Infection and stress increased the frequency of nuclei suspected of apoptosis and suggest that the presence parasite could induce apoptosis. The changes induced by infection in association with heat shock, especially the multiplication and metacyclogenesis of *T.*

cruzi and the mechanisms of resistance developed in the vector, may contribute to our understanding of the vector-parasite system, of the epidemiological patterns involved and adequate rearing of *P. megistus* in the laboratory.

III- INTRODUÇÃO

III- Introdução

1. Considerações gerais

Panstrongylus megistus (Burmeister) é um dos mais importantes vetores de doença de Chagas no Brasil, devido à sua ampla distribuição, altos índices de infecção com *Trypanosoma cruzi* e comportamento variado de domiciliação dependendo da área em que se encontra (Forattini, 1980). Atualmente é considerado também o principal vetor no Leste, Sudeste e de alguns estados do Nordeste do Brasil (Barbosa et al., 2001). É uma espécie nativa do país, que ocorre em regiões silváticas e extra-Amazônia e tornou-se adaptada ao modo de vida domiciliar como conseqüência das modificações antrópicas ocorridas na paisagem local, que de coberta (florestas do domínio tropical atlântico), vem sendo transformada em paisagem aberta (Forattini, 1980; Silveira et al., 1984). Assim, o processo de domiciliação dependeu de um grau de desagregação do ambiente com o deslocamento de vetores de ecótopos naturais pela eliminação ou redução de fontes alimentares primitivas (Silveira, 2000).

Panstrongylus megistus é intradomiciliar, adquirindo grande valor epidemiológico ao longo da zona da mata do Nordeste, enquanto em áreas da Serra do Mar apresenta hábito marcantemente silvestre, invadindo casas esporadicamente e raramente construindo colônias (Forattini et al., 1978; Sherlock, 1979; Silveira et al., 1984; Silveira & Rezende, 1994). Já na região Centro-Oeste de São Paulo, essa espécie é encontrada tanto em biótopos naturais como artificiais e caracteristicamente em peridomicílios, com frequente invasão do domicílio (Barreto, 1979). Ao lado de *Triatoma sordida*, tem sido a espécie mais importante do Estado de São Paulo, cujos habitats são frequentemente invadidos pelo homem (Wanderley, 1991). Na região de Campinas, foi verificado que *P. megistus* é a espécie predominante (89,28%), cujas formas aladas são encontradas com frequência infectadas por *T. cruzi* (12,08%), apresentando dispersão principalmente no último semestre do ano (Rodrigues et al., 1992).

Embora uma diminuição no número de casos e na taxa de mortalidade por doença de Chagas tenha sido observada a partir de 1980, com a virtual eliminação de *Triatoma infestans* em várias áreas, ela ainda é a terceira maior causa de óbitos ocasionada por doença infecto-parasitária no Brasil (13,5%), afetando, segundo a Organização Mundial de Saúde, 20 milhões de pessoas, principalmente na América Central e do Sul (Silveira & Rezende, 1994; Dos Reis., 1997). Na América Latina, a doença de Chagas produziu o maior ônus de enfermidade entre as denominadas doenças tropicais

representando uma perda econômica anual de 6,5 bilhões de dólares (Dias, 2000). A malária, esquistossomose, leishmaniose e hanseníase produzem, conjuntamente, um gasto de aproximadamente a quarta parte do produzido pela doença de Chagas. Somente as infecções respiratórias agudas, as doenças diarréicas e a AIDS produzem um ônus maior (Schumuñis, 2000). Em 1987, estimou-se que os custos com marcapassos e cirurgias devidos à doença de Chagas no Brasil foram de 250 milhões de dólares, enquanto a perda de horas de trabalho por absenteísmo representou outros 625 milhões de dólares (Dias, 1987).

O caráter crônico da doença, o grande número de reservatórios, a inexistência de drogas em larga escala para a terapêutica e o tipo de população afetada ou sob risco, predominantemente rural, têm ocasionado uma prioridade instável e precária ao controle da endemia chagásica (Silveira & Rezende, 1994). Em 1994, estimava-se que mais de 80% dos casos novos de infecção seriam produzidos por transmissão vetorial, da qual dependiam outras formas de veiculação da doença, como a transfusional que contribuía com 17% dos casos, a congênita com 2% e outras vias com 1% (Silveira, 2000). Embora a diminuição da população rural favoreça a menor transmissão vetorial da infecção, essa via é a rota mais importante de transmissão da doença de Chagas até o momento, explicando a importância do estudo da biologia dos triatomíneos, inclusive como estes insetos reagem frente a diversos agentes estressores, principalmente para planejamentos de controle e criação dos insetos em laboratório (Rodrigues et al., 1991; Silva & Silva, 1993; Garcia et al., 1999; Schmuñis, 2000).

Dado o interesse crescente no estudo das espécies de triatomíneos outras que *Triatoma infestans*, que no passado eram consideradas secundárias, e as prioridades epidemiológicas para o peridomicílio bem como para novas frentes de ocupação agrícola (Dias, 2000), estudos com *P. megistus* se revestem de fundamental interesse.

2. Biologia celular e resposta ao estresse

Estudos sobre o efeito de diferentes estressores têm sido realizados em diversos tipos de organismos. Em triatomíneos, várias alterações a nível de sobrevivência, ocorrência de mudas e fenótipos nucleares são induzidas com o estresse. Nesses insetos, pesquisas sobre o efeito de estressores, a nível celular, têm sido efetuadas em túbulos de Malpighi. Estes órgãos apresentam células poliplóides, com alto e crescente grau de ploidia, ao longo do desenvolvimento ninfal, atingindo um máximo no 5o. estádio ninfal (classes Feulgen-DNA 32C e 64C) (Mello, 1971, 1975, 1987, 1989, Mello & Raymundo, 1980). Suas células são binucleadas, contendo estruturas de cromatina condensada

do tipo heterocromatina constitutiva que se associam, formando corpos maiores denominados cromocentros, os quais, sob certas condições de estresse podem sofrer descondensação e possivelmente transcrição (Mello, 1983, 1989). Em *P. megistus*, os cromocentros são menores do que os de *T. infestans*, e representam apenas o cromossomo Y duplicado diversas vezes durante o processo de poliploidização (Mello et al., 1986).

Alterações nucleares, na forma e tamanho, no estado de compactação da heterocromatina e eucromatina, ou mesmo em morte celular têm sido descritas em *T. infestans* após ação de vários estressores como radiação ionizante gama (Álvares-Garcia, 1988), metais pesados (Kubrusly, 1984; Mello et al., 1995), jejum (Andrade & Mello, 1987; Mello & Raymundo, 1980; Mello, 1978, 1983; 1989) e choque térmico (Dantas & Mello, 1992; Mello et al., 2001). Alguns desses novos fenótipos aparecem como uma possível tentativa de ativação de genes silentes (núcleos com heterocromatina em descompactação). Outros, decorrentes de fusão nuclear e/ou celular (núcleos gigantes) atuam como um mecanismo de sobrevivência celular ou do órgão sob condições desfavoráveis (Wigglesworth, 1967; Mello e Raymundo, 1980; Mello, 1989; Dantas & Mello, 1992). Fenótipos como apoptose e necrose, tipos distintos de morte celular (Kerr, 1971; Kerr et al., 1972), têm sido definidos em *T. infestans* principalmente em termos de suas características morfológicas (Dantas & Mello, 1992; Mello et al., 2001).

Dentre os estressores, o choque térmico é um dos melhores modelos de estudo da análise de processos celulares básicos envolvidos no controle da expressão gênica (Burdon, 1986; Bonato & Juliano, 1987; Amaral et al., 1988). O choque quente tem sido estudado em uma ampla variedade de células e organismos e consiste em uma rápida mudança para temperaturas elevadas, acima da temperatura ótima de crescimento (Lindquist, 1986; Welch, 1993). Choque frio é um outro tipo de choque térmico, o qual é definido como injúria celular observada imediatamente após rápido resfriamento, mas na ausência de formação de gelo nos fluidos extracelulares (Chen et al.,1987).

Em *P. megistus*, como em *T. infestans*, tem sido encontrada uma variabilidade de respostas aos choques de temperatura (Garcia, 1997; Garcia et al., 1999; 2000 a, b). Essa variabilidade é dependente da temperatura a que os insetos são submetidos, duração do tratamento, fase do desenvolvimento e sexo dos espécimes. Alterações na resposta de sobrevivência ou comportamento de muda, bem como na resistência celular são observadas após choques de temperatura (Rodrigues et al., 1991; Dantas & Mello, 1992; Tavares, 1998; Garcia et al., 1999, 2000 a, b; Mello et al., 2001). Não foram observadas grandes diferenças entre espécimes de diferentes hábitos frente aos choques de temperatura (Garcia et al., 1999), embora diferenças de comportamento entre espécimes domiciliares e

silvestres sejam bem documentadas em condições naturais (Forattini, 1980).

Embora a tolerância aos choques quentes e frios tenha sido observada em várias espécies de animais, não foi investigada em Triatominae. Em vista de trabalhos anteriores com choques térmicos terem mostrado uma variabilidade de resposta, a compreensão de efeitos de estresses consecutivos sobre a sobrevivência de *Panstrongylus megistus* seja em ninfas de 5o. estádio ou em adultos é também matéria de interesse.

É bem conhecido que todos os organismos respondem a diversos tipos de estresse por ativação de um número limitado de genes específicos para a produção de proteínas de choque térmico ou de estresse (HSP), os quais são previamente silentes ou ativos em baixo nível (Lindquist, 1986; Komatsu et al., 1996). Tem sido sugerido que a presença aumentada de proteínas de choque térmico (HSP) seja, portanto, o principal fator de proteção celular contra os efeitos do estresse. As proteínas de choque térmico podem ser divididas em constitutivas (normalmente produzidas nas células) podendo aumentar ou não sua concentração com o estresse e proteínas induzidas somente em condições de estresse (Welch, 1993). Entre as várias HSP, as mais estudadas têm sido as da família da HSP70, proteínas altamente conservadas durante a evolução, sendo reportadas em uma ampla variedade de células, desde arqueobactérias, várias espécies de algas e todas as classes de cordados (Nover & Scharf, 1997; Lewis et al., 1999). Em organismos superiores, HSP 70 inclui formas sintetizadas sob condições de estresse ou não que atuam como importantes componentes de vários modelos celulares, por exemplo, participando nas translocações dentro de organelas intracelulares, facilitando a proteólise em lisossomos ou dentro da mitocôndria, ou exercendo papel na função receptora de esteróides e possivelmente na ativação de fosfatases (Rybcznski & Gilbert, 1995).

Alguns membros dessa família também funcionam como chaperonas moleculares (Ellis, 1991). Estas proteínas podem se ligar às cadeias nascentes de polipeptídeos, participando no arranjo conformacional da cadeia protéica, permanecendo associadas até o dobramento correto das proteínas (Welch, 1993). Com a hipertermia, ocorre um aumento de proteínas denaturadas que expõem resíduos internos, os quais podem interagir para formar agregados danosos à célula (Krebs & Feder, 1998). Consequentemente, ocorre a ativação do sistema de degradação das células a qual é dependente da ubiquitina, pequeno polipeptídeo que se liga ao grupo amino terminal das proteínas aberrantes, transformando-as em substrato para as proteases, que as degradam (Bond & Schlesinger, 1985). Com a diminuição no pool de ubiquitina livre na célula, o fator de transcrição do choque (HSTF), que normalmente está associado à ubiquitina, seria ativado (Munro & Pelham, 1985). Esse fator se ligaria,

então, ao elemento do choque térmico (HSE) liderando a formação de um complexo de transcrição estável, que permite o reconhecimento do sítio promotor para a transcrição e mensagem para produção de HSP (Burdon, 1986). Essas HSP podem então reconhecer e ligar-se a essas proteínas não dobradas ou não nativas e minimizar os agregados (Lindquist & Craig, 1988; Parsell & Lindquist, 1993) ou então torná-los mais acessíveis à ação de proteases, diminuindo assim a carga do sistema dependente de ubiquitina. Esse mecanismo é auto-regulável; quando a concentração de ubiquitina aumenta novamente, tornando-se disponível, o fator de transcrição é novamente inativado (Bienz & Pelham, 1987).

A concentração das HSP varia conforme o tipo celular considerado. Em insetos, por exemplo, HSP 70 é virtualmente ausente em células não estressadas mas após um fator de estresse como incubação em altas temperaturas, sua síntese é facilmente detectada (Velazquez et al., 1983). A amplitude de temperatura para indução de HSP difere consideravelmente com o organismo e correlaciona-se com o alcance normal de exposição no ambiente, sendo a resposta ao choque térmico, transitória em alguns organismos e tipos celulares e sustentada em outros, podendo também variar na razão de síntese protéica (Lindquist, 1986). Em adição ao importante papel contra insulto de temperatura, essas proteínas poderiam estar envolvidas também no desenvolvimento de termotolerância, que é uma resistência transitória a temperaturas elevadas após um condicionamento prévio a temperaturas moderadas ou tratamentos amenos com outros estressores (Lindquist & Craig, 1988; Sanchez & Lindquist, 1990; Carretero et al., 1991; Komatsu et al., 1996).

Constituindo uma reação à agressão, trata-se de um fenômeno transitório, ao contrário da resistência ao calor herdada geneticamente (Lindquist, 1986). Diversos autores reportaram que o tempo de retenção de termotolerância após choque térmico varia de diversas horas a diversos dias em células de plantas, fungos e animais. Quando um organismo, após ter sido submetido à hipertermia, retorna à temperatura normal, sua capacidade de sobreviver em temperaturas extremas decai (Lindquist, 1986).

Segundo De Carvalho et al (1994), em *Trypanosoma cruzi*, as proteínas sintetizadas à temperatura de 37°C por 1 hora são estáveis pelo menos 24h, sendo o parasita capaz de utilizar o estoque de mRNA durante o primeiro choque para responder a um choque subsequente. Em *Sarcophaga crassipalpis*, a exposição a choques de 40°C por 2-3 horas dobra a tolerância dessas moscas à temperatura de 45°C (Yocum & Denlinger, 1994).

A termotolerância pode ser alcançada através de agentes diversos, os quais podem ter efeitos opostos em propriedades físico-químicas. Dois processos são responsáveis pelo aumento da estabilidade celular induzida por estresse: resistência aumentada de componentes da célula, por ex, um

aumento em sua resistência primária e habilidade reparatória das células (Alexandrov, 1994). No caso da resistência primária, a resposta das células a temperaturas extremas seria baseada em distúrbios da estrutura nativa espacial de macromoléculas protéicas (Munro & Pelham, 1985; Ananthan et al., 1986; Lepock et al., 1988; Mackey et al., 1991). O aumento na termoestabilidade primária de macromoléculas refletiria na queda de sua mobilidade conformacional. Consequentemente elas se tornariam mais estáveis ao calor e a outros agentes cuja ação fosse dependente da habilidade do sistema de enfraquecer as ligações que mantém a estrutura espacial destas macromoléculas (Alexandrov, 1994).

Quanto ao mecanismo estimulador da habilidade reparatória, mais progresso tem sido alcançado relacionado com as HSP, sendo muitos os pesquisadores que aceitam o envolvimento dessas proteínas (principalmente chaperonas) em tal processo. Essas proteínas se acumulam nas células durante o choque, em resposta ao aparecimento de proteínas anormais, e podem assegurar uma recuperação mais rápida e mais completa durante estresses repetitivos do que o estresse inicial, possivelmente através de dois mecanismos, a eliminação de proteínas anômalas com o uso de ubiquitina e/ou dobramento correto das proteínas para um formato biologicamente ativo ou, alternativamente, na solubilização de aglomerados de proteínas anormais. Após o choque, a habilidade reparatória permanece por vários dias. Entretanto não está claro se as HSP, ubiquitinas e as chaperonas permanecem em altos níveis durante todo o período. Poderia haver recuperação sem a síntese de novas proteínas, talvez devido a uma reserva suficiente de proteínas constitutivas relacionadas às HSP (Alexandrov, 1994).

Termotolerância deve promover também uma capacidade aumentada de resistência à apoptose. O mecanismo para resistência a esse processo ativo de morte celular, entretanto, é diferente da resistência geral, caracterizada por uma habilidade aumentada de resistir ou reparar danos termo-induzidos (Mosser & Martin, 1992). É possível que o choque térmico, inibindo a síntese da maioria das proteínas celulares, mas aumentando a síntese de HSP, proteja as células contra apoptose por inibição da síntese ou modificação de uma ou mais proteínas relacionadas à morte celular (Samali & Cotter, 1996).

Segundo Alexandrov (1994), o desenvolvimento da termotolerância pode ser observado durante ou logo após o aquecimento, e em outros casos uma resistência aumentada é detectada somente algumas horas após o retorno das células às temperaturas normais. Parece que este último caso ocorre após choques de curta duração, enquanto um aumento de termorresistência é observado imediatamente após aquecimento prolongado (Li et al., 1982; Lin et al., 1984).

Embora o mecanismo de termotolerância não seja bem conhecido, vários autores têm sugerido que a cinética de indução de termotolerância, sob muitas condições diferentes, correlaciona-se com a cinética de síntese de proteínas de choque térmico, cronológica e quantitativamente. (Lindquist, 1986; Lindquist & Craig, 1988; Sanchez & Lindquist, 1990, Komatsu et al., 1996). Em *Chironomus thummi*, por exemplo, a proteção gerada por exposição a temperaturas moderadas começa após 3 horas, alcança o máximo 8 – 24 h e decai 72 h após o tratamento condicionante. Essa proteção ótima alcançada correlaciona-se com o máximo nível de HSP induzida (Carretero et al., 1996).

Apesar do grande número de dados na literatura favorecerem o envolvimento das HSP no desenvolvimento de resistência aumentada das células para injúrias repetidas, somente recentemente tem sido demonstrado seu requerimento para o estabelecimento de termotolerância (Sanchez & Lindquist, 1990). Entretanto, há autores que restringem essa aplicação, sugerindo que existam vários mecanismos para o choque, que induzem aumento na estabilidade das células, alguns com o envolvimento das HSP, enquanto outros não (Cavicchioli & Watson, 1986 ; Amaral et al., 1988; Carretero et al., 1991).

Exposições ao choque frio também estimulam várias alterações fisiológicas que aumentam a capacidade dos mesmos de sobreviverem a um choque mais severo. O aumento na concentração de glicerol pode ser considerado um dos principais mecanismos de proteção celular contra a segregação e redistribuição dos componentes da membrana ocasionadas pelo resfriamento (Quinn, 1985; Chen et al., 1987). Tem sido constatado que HSP são também crioprotetoras, o que está em concordância com a observação geral de que crioprotetores são macromoléculas estabilizadoras (Komatsu et al., 1990, 1996). Inversamente, estudos também demonstram que agentes crioprotetores como o glicerol podem agir como agentes protetores durante choques quentes, estabilizando macromoléculas celulares (Komatsu et al., 1990). Entretanto, em alguns aspectos a resposta ao choque frio parece diferir daquela promovida pelo choque quente, sendo que outros mecanismos protetores podem também estar envolvidos (Burton et al., 1988; Chen et al., 1991; Denlinger et al., 1991; Lopez-Garcia & Forterre, 2000).

Em *Sarcophaga crassipalpis*, por exemplo, proteção gerada por resfriamento ocorre 10 min após exposição a 0°C enquanto 30 min são requeridos para induzir proteção a altas temperaturas. (Chen et al., 1991). Segundo esses mesmos autores, temperaturas elevadas protegem contra injúria pelo frio mas não existem evidências de que baixas temperaturas possam proteger esses insetos contra injúria pelo calor. Minois (2001), estudando expressão de HSP 70 em *Drosophila melanogaster*, constatou que exposição a choque hipertérmico médio aumenta a resistência ao frio, mas não a outros

estressores. Outra observação é de que a síntese de HSP em resposta ao choque frio parece ser diferente da observada em temperaturas elevadas. No choque quente, a síntese de proteínas normais é reprimida enquanto a síntese de HSP aumenta; já no choque frio, ocorrem concomitantemente (Chen et al., 1991).

3. Resposta ao estresse nutricional

Vários organismos são capazes de sobreviver por longos períodos sem alimento, aproximadamente 1 ano em alguns invertebrados (Hervant et al., 1997, 1999; Hervant et al., 2001). A resistência ao jejum envolve vários mecanismos fisiológicos e comportamentais, como habilidade em manter e/ou rapidamente utilizar estoques metabólicos, redução na atividade locomotora e no consumo de oxigênio, que aumentam o potencial de sobrevivência e reprodutivo de espécies com privação alimentar prolongada (Hoffmann & Harshman, 1999; Hervant et al., 2001).

Na maioria dos insetos, durante o jejum, primeiro o glicogênio é consumido, seguido por proteínas e gorduras (Wigglesworth, 1984). Uma característica importante dos triatomíneos é que embora capazes de ingerir grande quantidade de sangue, na maioria das vezes muito superior ao seu peso, podem também suportar jejum por tempo prolongado (Sherlock, 1979). Entretanto, o excessivo estado de jejum aumenta a mortalidade dos insetos (Garcia & Azambuja, 2000). A frequência da alimentação dos triatomíneos depende da combinação entre temperatura e a umidade relativa onde são mantidos, bem como da espécie e período necessário para as mudas (Garcia & Azambuja, 2000).

Ninfas do 1o. ao 5o. estádio devem ser alimentadas a cada 3 a 4 semanas, período este suficiente para que os insetos degradem seu alimento, processem as ecdises e estejam prontos para uma nova alimentação. Os adultos devem ser alimentados a cada 15 dias (Garcia & Azambuja, 2000). É através do repasto sanguíneo e consequente distensão abdominal que se inicia a liberação do hormônio cerebral, o qual estimula as glândulas protorácicas que secretam a ecdisona, hormônio de muda (Wigglesworth, 1984).

Em diversos organismos, a presença aumentada de proteínas de estresse tem sido induzida após vários fatores de estresse, entre eles, o jejum (Lindquist, 1986). Alterações fenotípicas indicando mecanismos de sobrevivência celular e de morte celular também têm sido descritas em triatomíneos quando submetidos a este fator de estresse (Mello & Raymundo, 1980; Mello, 1978, 1983, 1989). Em ninfas de 5o. estadio de *T. infestans* submetidas a 5 meses de jejum, ocorre aumento nos graus de ploidia somática resultantes de fusão nuclear. (Andrade e Mello, 1987; Mello, 1978; 1989).

Esse fenômeno afeta células nas quais o grau de ploidia ótimo havia sido alcançado antes do jejum (classes de conteúdo de DNA 32C e 64C) não sendo, portanto, um mecanismo usual no qual a poliploidização é estabelecida em núcleos somáticos de espécimes bem nutridos (Andrade & Mello, 1987). Além de fusão nuclear, o jejum promove aumento na frequência de núcleos com aparente descompactação da heterocromatina bem como de núcleos necróticos e apoptóticos (Andrade & Mello, 1987; Mello, 1989; Mello et al., 2001).

A habilidade de recuperação de períodos de nutrição pobre ou inadequada é também uma adaptação muito importante para o potencial de sobrevivência e reprodutivo de qualquer espécie com privação alimentar prolongada (Hervant et al., 2001). Essa resposta depende da presença de estoques de nutrientes apropriados, junto com respostas adaptativas, por exemplo, ajustes de comportamento e aumento do consumo de oxigênio como resultado da normalização do metabolismo digestivo (Hervant et al., 2001).

Existem também sugestões de que a realimentação, após um período prolongado de jejum, seja ela própria um fator de estresse, estimulando também o aumento da produção de proteínas de estresse (HSP) (Burdon, 1986). Em *T. infestans*, entretanto, demonstrou-se que insetos em jejum aos quais era oferecida realimentação não apresentavam melhoria na habilidade de sobrevivência, havendo inclusive escassez de núcleos gigantes, possivelmente porque os mesmos poderiam ter sofrido necrose (Mello, 1989). No insetário da SUCEN em Mogi-Guaçu, os triatomíneos, inclusive *P. megistus*, costumam ser submetidos a períodos de jejum, a fim de se prolongar o seu ciclo evolutivo, já que esses insetos só mudam de um estádio para outro tendo completado uma alimentação, a qual promove a distensão do estômago e liberação do hormônio de muda (Wigglesworth, 1984). Pelo fato dos espécimes de *P. megistus* serem realimentados após certos períodos de jejum, existe, portanto, a possibilidade de que sobre eles incida também um segundo fator de estresse.

Dentre os poucos estudos de jejum em *P. megistus*, Braga & Lima (2001) constataram que com a privação alimentar, o potencial reprodutivo dos espécimes era diminuído mas ainda assim eram capazes de manter a colonização. É de interesse, portanto, investigar o efeito do jejum, em associação à realimentação e ao choque de temperatura, sobre os fenótipos nucleares em espécimes de *Panstrongylus megistus,* bem como em comparação a resultados já obtidos na literatura para *Triatoma infestans* (Campos et al., 2002).

4. A infecção por *Trypanosoma cruzi*

Trypanosoma cruzi, o agente etiológico da doença de Chagas, é um protozoário cuja heterogeneidade genética pode resultar em diferentes modelos de virulência, patogenicidade e infectividade aos animais (Contreras et al., 1998). Representa, portanto, um complexo de populações de parasitos circulando entre humanos, vetores, reservatórios silvestres e animais domésticos, apresentando diferentes formas fisiológicas e funcionais relacionadas a seu complexo ciclo de vida, com ultraestrutura modificada de acordo com o estágio evolutivo (Souza, 2000). Estudos também demonstram que populações de *T. cruzi* diferem em sua habilidade para sobreviverem, multiplicarem-se e diferenciarem-se no inseto vetor (Lana et al., 1998).

O ciclo de vida desse protozoário alterna-se entre vertebrados e insetos. Nos hemípteros vetores, o parasita é encontrado no trato digestivo, invadindo também os túbulos de Malpighi. Na natureza, a infecção por esse parasita é iniciada quando tripomastigotas metacíclicos são eliminados junto com as excreções dos vetores infectados, invadem as células dos mamíferos, escapando do vacúolo endocítico, e transformando-se em amastigotas. Estas formas replicam-se várias vezes no citoplasma da célula hospedeira e diferenciam-se em tripomastigotas. Após divisões sucessivas, tripomastigotas presentes no interior do vacúolo parasítico, iniciam um intenso movimento, ocorrendo então, a ruptura da célula com liberação de formas tripomastigotas, formas de transição e amastigotas no espaço intercelular. Assim, nas células de mamíferos encontram-se formas amastigotas (não infectantes) e tripomastigotas sanguíneos (infectantes). Dentro do intestino do vetor, os tripomastigotas sanguíneos ingeridos durante o repasto sanguíneo sofrem diferenciação em epimastigotas proliferativos que se transformam em tripomastigotas metacíclicos no reto (Brener & Alvarenga, 1975).

A compreensão da relação vetor-tripanosoma é limitada, sendo que os estudos de metaciclogênese dentro de triatomíneos têm sido principalmente descritivos (Piesman & Sherlock, 1985). Apesar da importância das razões envolvidas na virulência das formas metacíclicas, poucos estudos têm sido realizados, principalmente porque essa forma de desenvolvimento é eliminada pelas fezes do vetor em quantidades pequenas (Contreras et al., 1998). Estudos quantitativos do desenvolvimento de *T. cruzi* no vetor são também de difícil comparação devido ao fato da densidade populacional ser influenciada por vários fatores, entre eles, o sistema vetor-parasita (Garcia & Azambuja, 1991).

Apesar das dificuldades já apontadas na compreensão da relação vetor-*T. cruzi*, os tripanossomas apresentam características especiais que os fazem excelentes modelos para o estudo de questões biológicas básicas (Souza, 2000). Alguns fatores parecem estar envolvidos nas interações entre vetor e o parasito como número de parasitas ingeridos pelo vetor, cepa do parasita, fatores intrínsecos relacionados às espécies vetoras envolvidas, bem como suas características genéticas e estágios de desenvolvimento (Garcia & Azambuja, 1991).

Doses mínimas de parasitos parecem suficientes para o estabelecimento de uma infecção. Garcia & Dvorak (1982), por exemplo, constataram que tripomastigotas de *T. cruzi* de duas cepas diferentes necessitam de uma dose mínima de 5 parasitas para infectar *Dipelatogaster maximus* enquanto Alvarenga & Leite (1982) observaram que inoculação de 3 parasitas em *Triatoma infestans* resultaram em pelo menos 12% de infecção no 20o. dia, enquanto 10 parasitas infectaram 63% dos espécimes dentro de 15 dias.

Apesar de alguns trabalhos terem demonstrado uma correlação entre a quantidade de tripanossomas ingeridos e a proporção de insetos infectados (Almeida, 1973; Neal, 1977), vários autores não encontraram uma uniformidade na resposta obtida, sugerindo que o número de parasitos presentes na fonte alimentar não parece ser um fator determinante da infecção dos triatomíneos, o que implicaria que a quantidade de sangue ingerida pelo inseto também não é (Miles et al., 1975; Neal & Miles, 1977; Mello & Chiarini, 1980; Silva et al., 1994). A densidade parasítica aumentada em espécimes adultos, com relação aos demais estádios ninfais, segundo Piesman & Sherlock (1985), deve-se provavelmente à rápida capacidade de digestão nessa fase, podendo acelerar a velocidade de metaciclogênese. Entretanto, os resultados discordantes com relação a susceptibilidade dos triatomíneos à infecção, sugerem a presença de vários parâmetros relacionados ao parasita e ao inseto (Mello & Chiarini, 1980).

Entre os vários fatores que influenciam o curso das manifestações durante a infecção, a cepa do parasita têm sido considerada o mais importante (Andrade, 1990). O grande número de hospedeiros diferentes e vetores é aparentemente um dos fatores responsáveis pela existência de muitas cepas diferentes de *T. cruzi*, as quais variam em virulência, tropismo do tecido, patogenicidade e perfil isoenzimático (Bice & Zeledon, 1970). Dias (1940) foi um dos primeiros pesquisadores a relacionar os diferentes níveis de infecção encontrados em triatomíneos com diferentes cepas de *T. cruzi* (Mello & Chiarini, 1980). Sabe-se que o *T. cruzi* não representa uma população homogênea de parasitas, mas um grupo heterogêneo e complexo de populações que diferem entre si segundo critérios morfológicos, fisiológicos, bioquímicos e clínicos (Silveira, 2000). A ausência de reprodução sexuada e de

recombinação genética clássica seria fator determinante para que um isolado do parasita evoluísse independentemente dos outros, sendo que o elevado grau de diversidade do parasita poderia estar associado à sua necessidade de adaptação e sobrevivência em diferentes hospedeiros (Silveira, 2000).

Chagas, quando descreveu o *T. cruzi* em 1909, verificou que formas diferentes apareciam no sangue circulante. Mais tarde, a existência de diferentes modelos morfológicos das formas sanguíneas foi relacionada às características biológicas do parasita. Brener et al. (1974) verificaram que em algumas populações havia o predomínio de formas delgadas durante todo o curso da infecção experimental em camundongos. Em algumas cepas, à medida que a infecção se prolongava, as formas largas passavam a predominar. Foi então sugerido que as formas delgadas desapareceriam rapidamente da circulação para cumprir um ciclo intracelular, enquanto as formas largas permaneceriam na circulação (Souza, 2000). Segundo Brener (1969), as formas finas penetram mais rapidamente nas células do hospedeiro vertebrado e parecem ser mais sensíveis à ação dos mecanismos imunitários do que as formas largas (Albuquerque, 1998). Assim podemos encontrar cepas que apresentam um predomínio de formas delgadas, como por exemplo, a cepa Y. Já em outras, como a cepa CL, predomina a forma larga. A cepa Y foi isolada em 1950 por xenodiagnóstico de um caso agudo humano de doença de Chagas em São Paulo (Silva & Nussenzweig, 1953) e a cepa CL, isolada por Brener & Chiari (1963) de *T. infestans* naturalmente infectados coletados no Rio Grande do Sul. Aparentemente estas formas largas seriam menos infectivas e mais adaptadas ao desenvolvimento no vetor (Souza, 2000).

Outros fatores intrínsecos do vetor que interferem no desenvolvimento do parasito têm sido descritos, embora não se conheçam os mecanismos que regulam a metaciclogênese no inseto, nem os elementos que mantêm altas razões de formas metacíclicas em algumas espécies. Poucos avanços têm ocorrido quanto à compreensão de fatores moleculares no trato digestivo do vetor, o qual é considerado um ambiente hostil e pode alterar a infecção pelo *T. cruzi*. É provável que o desenvolvimento do parasito possa ser dependente e controlado pelo ambiente químico e bioquímico no intestino do vetor (Garcia & Azambuja, 1991; Garcia & Dvorak, 1992). Uma vez que os parasitas são ingeridos durante o repasto sanguíneo, fatores do sangue podem ser requeridos para desenvolvimento e diferenciação do parasita (Garcia & Azambuja, 1991). As condições no intestino do vetor se alteram após alimentação ou durante o jejum (Shaub & Losch, 1988; Garcia & Azambuja, 1991; Kollien & Schaub, 1997). Imediatamente após a alimentação, a população estabelecida de epimastigotas no estômago do vetor reage com os fatores imunocompetentes do sangue dos vertebrados, por exemplo, fatores de lise do complemento, sendo que na segunda parte do intestino médio, a população dos

flagelados cresce após a alimentação (Schaub, 1988). Devido ao fato de espécimes de *T. infestans* se infectarem experimentalmente em preparados livres de sangue (Alvarenga & Brener, 1978), a liberação de secreções no trato digestivo, parece ser de maior importância para o desenvolvimento e diferenciação dos parasitas do que qualquer papel do sangue e de proteases digestivas induzidas pela alimentação (Garcia & Azambuja, 1991).

A excreção também afeta a população de flagelados no reto do barbeiro, reduzindo o número de flagelados, ou alterando a distribuição de diferentes estágios desenvolvimentais (Garcia & Azamubuja, 1991). Há autores, inclusive que consideram que a diurese, preferencialmente a outros fatores da hemolinfa ou produtos digestivos, induz o desenvolvimento de tripomastigotas metacíclicos de *T. cruzi* (Kollien & Schaub, 1997).

O atracamento dos tripomastigotas à superfície do epitélio das glândulas retais tem sido considerado um fator questionável, sendo o processo de adesão, provavelmente decorrente de um reconhecimento celular em que glicoproteínas e proteínas do tipo lectina, presentes tanto na superfície da célula hospedeira como do parasita estão envolvidas (Garcia & Azambuja, 1991; Souza, 2000). Todas as formas do ciclo evolutivo aderem à célula hospedeira, porém o grau de adesão varia de acordo com a cepa do parasita, com a forma evolutiva e com a célula hospedeira (Souza, 2000).

Entre os fatores que influenciam a relação vetor-parasito a espécie vetora é de extrema importância. Mais de 100 espécies de insetos hematófagos da família Reduviidae e subfamília Triatominae agem como vetoras da doença de Chagas. Além de *T. cruzi*, esses insetos podem também agir como vetores de outros tripanosomatídeos, tais como *Trypanosoma rangeli* e *Blastocrithidia triatominae*, os quais são de interesse por causa de sua patogenicidade no vetor (Garcia & Azambuja, 1991).

Para estudo de infecção com *T. cruzi*, deve-se lembrar que algumas espécies de vetores podem limitar a infecção em humanos e animais devido à incapacidade de favorecer o processo de metaciclogênese (Szumlewicz & Moreira, 1994). O principal critério para identificar o inseto vetor como modelo experimental é sua habilidade para manter uma alta proporção de sua população infectada após alimentação nos hospedeiros chagásicos (Szumlewicz et al., 1990).

O estudo do processo de infecção por *T. cruzi* nos triatomíneos é particularmente importante devido ao uso desses insetos no xenodiagnóstico. A experiência tem demonstrado que, mesmo quando o nível de parasitemia é baixo, não se detectando facilmente o parasita em observações microscópicas, é possível recuperar o parasita no vetor. É com base nesse fato que se utiliza o xenodiagnóstico (Souza, 2000). Este é um método eficiente de diagnóstico na corrente sanguínea,

particularmente útil na fase crônica da doença de Chagas (Castro et al., 1983; Moreira & Szumlewicz, 1997). A aplicação do xenodiagnóstico, tanto para diagnosticar quanto para acompanhar experimentações com drogas tripanosomicidas, tem estimulado estudos para se conhecer as espécies de triatomíneos mais susceptíveis ao *T. cruzi*, a fim de selecioná-las como modelo experimental (Silva & Silva, 1993). A susceptibilidade dos triatomíneos é um fator extremamente importante na epidemiologia da doença de Chagas (Mello & Chiarini, 1980).

A sensibilidade de diferentes espécies de triatomíneos à infecção por *T. cruzi* vem sendo estudada tanto em seres humanos como em animais de laboratório. Na literatura, há poucos estudos comparativos quanto às sensibilidades de diferentes espécies no uso em xenodiagnóstico. Pereira et al. (1996) constataram que ninfas de *P. megistus* apresentam maior sensibilidade à infecção por *T. cruzi* e que a presença de positividade mais exclusivamente em *P. megistus* ou *T. infestans* indica a necessidade de se empregar mais de uma espécie de triatomíneo com diferentes sensibilidades à infecção pelo *T. cruzi*. Tal observação sugere a circulação de subpopulações dos parasitas com potenciais de desenvolvimento nos diferentes triatomíneos (Pereira et al., 1996).

Alvarenga & Bronfen (1984) estudaram em *T. infestans*, *P. megistus* e *Dipetalogaster maximus*, a capacidade de manutenção e de metaciclogênese de 2 cepas de *T. cruzi*: Y e CL. Os resultados demonstraram existir diferenças de interação entre tais cepas e os diferentes vetores e haver uma resistência à infecção manifestada a partir do repertório nutricional existente no trato digestivo que permite ou não um maior desenvolvimento do parasita. *Panstrongylus megistus* mostrou-se apto a receber as duas amostras do *T. cruzi*, sugerindo que o fator regulador de diferenciação se apresenta com características semelhantes às observadas em *T. infestans*, mas com o fator de resistência à infecção presente de forma a permitir a implantação de diferentes cepas. Essa espécie também foi considerada a mais promissora por Szumlewicz & Muller (1987), ao constatarem um elevado índice de positividade (91,2%) e elevada carga parasitária.

Embora *T. cruzi* seja altamente patogênico para o homem e outros mamíferos, não há evidência concreta de que seja prejudicial ao triatomíneo (Lima et al., 1992). Segundo Tobie (1961), o critério para determinar a patogenicidade em artrópodos é limitado e os efeitos patogênicos são avaliados com base na mortalidade aumentada. No caso de *T. cruzi*, informações são mais limitadas e os efeitos da infecção do parasita no ciclo reprodutivo dos triatomíneos necessitariam de estudos adicionais (Lima et al., 1992).

Vários trabalhos têm demonstrado a susceptibilidade do *P. megistus* ao *T. cruzi*. Lima et al (1992), estudando ninfas infectadas de 1o. instar de *Panstrongylus megistus*, verificaram que o

número de ecdises, o desenvolvimento e a mortalidade permanecem constantes, enquanto a reprodução significantemente se torna reduzida (produção de ovos, fertilidade, número de ovos férteis e longevidade das fêmeas). Os espécimes de *P. megistus* revelaram-se altamente suscetíveis, com 100% de infecção até a fase adulta. A eficiência dessa espécie também já havia sido verificada por Szumlewicz &Muller (1987) ao constatarem uma positividade de 90%, quando os espécimes eram alimentados em animais com infecção crônica pela cepa Y (Szumlewicz et al., 1988). Outra vantagem é a ocorrência dessa espécie em muitas partes do Brasil, facilidade para alimentação sob alterações das condições do ambiente, rapidez no desenvolvimento, lentidão na locomoção e baixa mortalidade Destaca-se também sua extraordinária capacidade de sustentar a rápida evolução e boa multiplicação de poucos parasitas ingeridos na fase crônica. (Szumblewicz et al., 1988).

Como alterações a nível de sobrevivência, ocorrência de mudas e fenótipos nucleares têm sido verificados em *P. megistus* após choque térmico (Garcia et al. 1999; 2000 a , b), o efeito desse estressor em espécimes infectados por *T. cruzi*, requereria estudos com o objetivo de se investigar possíveis alterações nos parâmetros citados, bem como no percentual de positividade ao *T. cruzi*, intensidade de infecção, multiplicação e metaciclogênese. A verificação do aumento de mecanismos protetores ou de morte celular utilizados pelo vetor durante a infecção, também mereceria estudo.

A compreensão do efeito de estressores, particularmente do choque térmico em associação à infecção pelo *T. cruzi* poderiam contribuir para criação mais adequada de insetos nos laboratórios, bem como para a compreensão das alterações biológicas em condições naturais, já que essa espécie tem se revelado um dos modelos mais convenientes para uso em xenodiagnóstico (Szumlewicz et al., 1988; Lima et al., 1992).

IV- OBJETIVOS DO TRABALHO

IV- Objetivos

Considerando-se os dados anteriormente levantados relativos a choques de temperatura em *P. megistus* (Garcia, 1997; Garcia et al., 1998) em que algumas importantes questões permaneceram em aberto, o trabalho visou:

a) a compreensão do efeito de choques térmicos, quentes e frios, consecutivos, buscando-se investigar a ocorrência de termotolerância na espécie;

b) o conhecimento sobre o efeito do jejum em associação com a realimentação e o choque de temperatura sobre os fenótipos nucleares, particularmente em termos de mecanismos de sobrevivência e morte celular;

c) o efeito dos choques térmicos em espécimes infectados com *Trypanosoma cruzi* sobre os mesmos parâmetros do ítem b, bem como no percentual de positividade, intensidade da infecção parasitária, multiplicação e metaciclogênese.

V- ARTIGOS ANEXOS

ARTICLE

Experimentally Induced Heat-Shock Tolerance in *Panstrongylus megistus* (Hemiptera: Reduviidae)

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ABSTRACT The survival and molting incidence of fifth-instar nymphs of *Panstrongylus megistus* (Burmeister, 1835), a vector of Chagas' disease, were investigated following sequential heat shocks in which a mild shock (35 or 40°C, 1 h) preceded a more drastic one (40°C, 12 h). The shocks were separated by 8-, 18- 24-, or 72-h periods at 28°C. The heat-shock tolerance response was more effective when the first shock was given at 40°C. When the period between shocks was 18 h, the tolerance to sequential shocks (in terms of specimen survival) weakened, which suggested a transient control of the process that enables the organism to circumvent the unfavorable effects of severe shock. In terms of molting incidence, the heat-shock tolerance was only demonstrated when the period between the first shock at 40°C for 1 h and the second shock at 40°C for 12 h was ≥24 h. These results are the first to demonstrate the induction of heat-shock tolerance in a blood-sucking hemipteran.

KEY WORDS *Panstrongylus megistus*, heat-shock tolerance, heat shock, survival, molting incidence

Panstrongylus megistus (BURMEISTER, 1835) is a blood-sucking insect and an important vector of Chagas' disease in Brazil because of its wide distribution, high rates of infection with *Trypanosoma cruzi* (Chagas, 1909), and geographically different capacities for invading artificial ecotopes (Forattini 1980). *P. megistus* occurs in sylvatic areas outside of the Amazon and has adapted to domestic environments as a result of human action that has changed natural forest into open land (Forattini et al. 1978, Silveira et al. 1984, Silveira and Rezende 1994).

The survival rate and cell physiology of blood-sucking insects are affected by stressing agents, including high temperature. The response to thermal shocks in *P. megistus* varies as a function of the temperature and duration of the shock, the developmental phase, the sex of the specimens, and, in certain cases, the insect's habit and nutritional condition (Garcia et al. 1999). A shock at 40°C for 1 h has little effect on nymph survival, whereas a shock of 12 h duration at the same temperature markedly increases mortality (Garcia et al. 1999, 2000).

The involvement of heat shock proteins in the survival of *P. megistus* after short heat shock has been suggested, although no evidence of a role for these proteins has been provided for the Triatominae (Garcia et al. 1999). Should heat shock proteins be involved in the survival of these insects after heat shock, an acquired tolerance to more drastic heat shock conditions could be induced experimentally after conditioning the insects to milder stress conditions, as reported for other species (Lindquist 1986, Lindquist and Craig 1988, Sanchez and Lindquist 1990, Komatsu et al. 1996).

An understanding of how *P. megistus* responds to stress is important to establish the best conditions for rearing specimens in the laboratory because this species is used for research and xenodiagnosis. Such knowledge could also help to explain the biological characteristics of specimens found in natural ecotopes.

In this study, survival and the incidence of molting following sequential heat shocks were investigated in *P. megistus* to evaluate the acquired heat-shock tolerance responses typical of the protective action induced by heat shock proteins in this species.

Materials and Methods

Fifth-instar nymphs of a domestic population of *P. megistus* reared at 28°C and 80% RH in the laboratory at Sucen (Mogi-Guaçu, SP) were used.

The specimens were fasted for 15 d at 28°C and then subjected to heat shock at 35 or 40°C for 1 h. The choice of a moderate fasting condition at this stage of the experiment was based on the observation that only after such treatment were some fifth-instar nymphs capable of surviving prolonged heat shock (Garcia et al. 1999). Immediately after the shock, the nymphs

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Table 1. Survival and molting incidence (%) in *P. megistus* nymphs 30 d after sequential heat shocks

Experimental conditions	*n*	Survival	Molting incidence
Control #1, 28°C	100	87.0	68.0
Control #2, a single shock at 35°C (1 h)	53	84.9	52.8
Control #3, a single shock at 40°C (1 h)	53	90.5	45.2
Control #4, a single shock at 40°C (12 h)	100	18.0	1.0
Time (h) at 28°C between shocks at 35°C (1 h) and 40°C (12 h)			
8	53	83.0	22.6
18	53	62.2	7.5
24	53	71.6	9.4
72	53	9.4	5.6
Time (h) at 28°C between two shocks at 40°C (1 and 12 h)			
8	53	90.5	16.9
18	53	56.6	18.8
24	53	79.2	58.4
72	53	84.9	39.6

Table 2. Comparison of survival rates using the Mantel–Hantzel test (Kalbfleish and Prentice 1980)

Compared conditions			W	df	P
Control groups	Test: 1st shock (°C, 1h)[a]	Test: Interval (h at 28°C) between shocks			
#1 vs #2			0.2	1	0.6683
#1 vs #3			0.7	1	0.4159
#1 vs #4			14.2	1	4.56e-7*
#1 vs	35	8	0.5	1	0.4790
#1 vs	40	8, 18, 24, 72	35.0	4	4.56e-7*
		8	0.005	1	0.9351
		18	23.0	1	1.58e-6*
		24	1.0	1	0.3295
		72	0.007	1	0.9291
#2 vs	35	8, 18, 24, 72	103.1	4	0.0000*
		8	0.1	1	0.7611
		18	7.7	1	0.0056*
		24	3.2	1	0.0741
		72	72.4	1	0.0000*
#3	40	8, 18, 24, 72	35.4	4	4.04e-7*
		8	0.9	1	0.3359
		18	19.2	1	1.17e-5*
		24	2.4	1	0.1121
		72	1.5	1	0.2189
#4 vs	35	8, 18, 24, 72	189.6	4	0.0000*
		8	75.0	1	0.0000*
		18	33.4	1	7.31e-9*
		24	70.7	1	0.0000*
		72	77.9	1	0.0000*
#4 vs	40	8, 18, 24, 72	97.1	4	0.0000*
		8	58.2	1	2.38e-14*
		18	52.9	1	3.44e-13*
		24	70.8	1	6.01e-13*
		72	80.2	1	1.00e-15*

#1, 28°C; #2, one shock at 35°C (1 h); #3, one shock at 40°C (1 h); #4, one shock at 40°C (12 h); *, highly significant.
[a] Second shock at 40°C (12 h).

were returned to control conditions and then subjected to a further shock at 40°C for 12 h. The second shock was started 8, 18, 24, and 72 h after the first one. The temperature of 35°C was chosen as a mild shock temperature, whereas that of 40°C was chosen based on previous results (Garcia et al. 1999).

There were four control groups: #1- insects maintained at 28°C, a temperature used to rear *P. megistus* in the laboratory of Sucen since 1980; #2 - insects exposed to a single shock at 35°C for 1 h; #3 - insects exposed to a single shock at 40°C for 1 h; #4 - insects exposed to a single shock at 40°C for 12 h.

After the second shock, the insects were returned to the control temperature (28°C) and monitored daily for 30 d to score changes in survival rate and molting incidence. During this period, the insects were fed once a week. The survival and molting rates were compared statistically using Mantel–Hantzel's nonparametric procedure (Kalbfleish and Prentice 1980).

Results

The survival rate of insects subjected to a single short heat shock at 35°C (#2) or 40°C (#3) did not differ from that of insects reared at 28°C. However, the survival rate of insects subjected to a 12-h period at 40°C (#4) decreased significantly compared with that of insects maintained at 28°C (Tables 1 and 2).

After the sequential shocks, the survival rate increased significantly compared with the results for insects subjected to a 12 h shock at 40°C, when the time between shocks was extended from 8 to 24 h. This recovery also occurred when the time between the shocks was extended to 72 h, provided the first shock was given at 40°C. When the first shock was at 35°C and the period between shocks longer than 24 h, the survival rate was lower than after a single long shock at 40°C (Tables 1 and 2).

The survival rate after sequential shocks was similar to that of insects reared at 28°C (#1), provided the first shock was at 40°C; the exception was when the period between shocks was 18 h (Table 2). When the first shock was at 35°C, the survival rate was not statistically different from that of insects at 28°C (#1), provided the period between shocks was 8 h (Tables 1 and 2).

The survival rate when the first shock at 35°C (#2) was followed by a long shock at 40°C and the time between shocks was 18 h and, especially, 72 h, was lower than after a single shock at 35°C. In all the other cases the survival rate did not exceed the control value (#2) (Tables 1 and 2).

Comparison of the response to a single shock at 40°C (#3) with that to a similar shock followed by a long shock at the same temperature, with only 18 h between shocks showed that the survival rate was smaller in the latter group. In addition, the survival rate did not exceed that for a single shock at 35°C (Tables 1 and 2).

The molting rate of insects subjected to a short heat shock at 35°C (#2) or 40°C (# 3) differed significantly

Table 3. Comparison of molting rates using the Mantel-Haenszel test (Kalbfleish and Prentice 1980)

Compared conditions			W	df	P
Control group	Test: 1st shock (°C, 1h)[a]	Test: Interval (h at 28°C) between shocks			
#1 vs #2			17.8	1	2.42e-5**
#1 vs #3			6.8	1	0.0090**
#1 vs	40	8, 18, 24, 72	20.0	4	5.00e-4**
		8	18.7	1	1.54e-5**
		24	0.2	1	0.8692
		72	1.2	1	0.2728
#2 vs	35	8, 18, 24, 72	123.4	4	0.0000**
		8	65.6	1	<e-15**
		18	41.8	1	<e-9**
		24	53.8	1	<e-13**
		72	16.2	1	0.0000**
#3 vs	40	8, 18, 24, 72	14.8	4	0.0051*
		8	1.5	1	0.2259
		18	0.4	1	0.5084
		24	5.9	1	0.0152*
		72	2.2	1	0.1377

#1, 28°C; #2, one shock at 35°C (1 h); #3, one shock at 40°C (1 h); *, significant; **, highly significant.
[a] Second shock at 40°C (12 h).

from that of insects reared at 28°C (Tables 1 and 3). In nymphs subjected to a long heat shock at 40°C, the molting rate during the period studied was close to zero (Table 1). The molting rate after sequential shocks increased compared with that for nymphs subjected to a single 12-h shock at 40°C (#4), regardless of the time between the shocks. No statistical comparison was done in this case because the value for control #4 was close to zero (Table 1). This increase was similar to the rate in nymphs reared at 28°C (#1) when the first shock was at 40°C (1 h) and the time between shocks was ≥24 h. The molting rate when a shock at 35°C was followed by a long one at 40°C was always smaller than after a single shock at 35°C (#2), regardless of the time between shocks. The same was valid for comparisons in which the first shock was given at 40°C, except when the time between shocks was ≥24 h. When both heat shocks were given at 40°C (1 and 12 h), the molting rate surpassed that of a single short shock at 40°C (#3) only for the 24-h period between shocks (Tables 1 and 3).

Discussion

A long shock at 40°C significantly decreased survival in *P. megistus* nymphs (Garcia et al. 1999). However, as shown here, the survival and molting rates increased after sequential shocks in which a gentler shock preceded a more severe one, thus demonstrating that heat-shock tolerance can be induced in this species. Heat-shock tolerance has been demonstrated in several animal species (Lindquist 1986), but this is the first report showing such a phenomenon in a blood-sucking hemipteran. Although heat shock proteins have not yet been investigated in hemipteran reduviids, the heat-shock tolerance seen here is typical of heat shock proteins involvement (Lindquist and Craig 1988, Sanchez and Lindquist 1990, Vogel et al. 1997, Krebs and Bettencourt 1999).

In terms of survival rate, the induced heat-shock tolerance tended to weaken with increasing time between shocks when the first shock was at 35°C, the mildest of the temperatures tested here. In contrast, when the first shock was carried out at 40°C, the acquired heat-shock tolerance induced survival rates similar to those of *P. megistus* nymphs reared at 28°C. This survival rate was the same for shorter (8 h) and longer (72 h) intervals between shocks, but decreased at an interval of 18 h. These results suggest that a shock at 40°C is a rather compelling condition in comparison with the one at 35°C, because the former induces a more effective survival response in *P. megistus*. However, even when the first shock was conducted at 40°C, the recovery process mediated by sequential shocks was only transiently controlled.

The incidence of molting was affected in a manner similar to survival rate only when the first shock was at 40°C and the intervals between shocks were long. This finding indicates that the hormonal paths involved in the last molt of *P. megistus* may require a greater period at 28°C to circumvent the effects caused by the longer shock at 40°C.

Because heat shock induces nuclear phenotypic changes in *P. megistus* cells, including responses typical of cell death and survival (Garcia et al. 2000), we are currently examining whether heat-shock tolerance in this species is accompanied by cell nuclear changes.

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BRIEF COMMUNICATION

Effect of Sequential Cold Shocks on Survival and Molting Incidence in *Panstrongylus megistus* (Burmeister) (Hemiptera, Reduviidae)

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The survival and molting incidence were studied in the insect, *Panstrongylus megistus*, following sequential cold shocks in which a milder shock at 0 or 5°C for 1 h preceded a more severe shock (0°C, 12 h). The shocks were separated by intervals of 8, 18, 24, and 72 h at 28°C. The survival rate after sequential shocks was identical to that of unshocked controls. Cold-shock tolerance differed from heat-shock tolerance since the latter varied with the time between shocks and was much more transient. Sequential cold shocks produced a higher molting incidence when the first shock was given at 0 compared to 5°C. This response was more rapid than that to sequential heat shocks. Cold-shock tolerance in *P. megistus* may involve heat-shock proteins, although other protective mechanisms may also occur concurrently. This is the first report of cold-shock tolerance in a blood-sucking hemipteran.

Key Words: Panstrongylus megistus; cold-shock tolerance; survival; molting incidence.

The blood-sucking hemipteran, *Panstrongylus megistus*, is an important vector of Chagas' disease because of its wide geographical distribution, high rates of infection with *Trypanosoma cruzi*, and adaptability in invading artificial ecotopes (5). The survival rate and the molting incidence of *P. megistus* nymphs with domestic habit in response to heat and cold shocks vary as a function of the temperature and duration of the shock and, in certain cases, with the state of nourishment conditions (6). Molting in fifth instar nymphs was drastically affected by a short cold shock compared to the much gentler effect produced by a short heat shock (6).

Prolonged cold and heat shocks are deleterious to *P. megistus* nymphs (6), but tolerance to prolonged heat shock can be elicited after sequential shocks, probably as a result of heat shock protein (hsp) activation (7). Cold tolerance in *P. megistus* may result if hsp can protect against cold as reported for other insect species or if short cold shocks can stimulate physiological changes that increase the ability to survive more severe cold shocks (2, 4).

In this study, the influence of sequential cold shocks on survival and molting incidence in fifth instar nymphs of a domestic population of *P. megistus* (Burmeister) (Hemiptera, Reduviidae) reared at 28°C in the laboratory was investigated and compared with the response to sequential heat shocks.

Specimens fasted for 15 days at 28°C underwent a cold shock at 5 or 0°C for 1 h. The choice of a moderate fasting condition was based on the higher mortality of fifth instar nymphs in this state of nourishment (6). Immediately after the shock, the nymphs were returned to control conditions and then subjected

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to a shock at 0°C for 12 h. The second shock was started 8, 18, 24, and 72 h after the first one. The temperature of 0°C was chosen based on previous results (6), and that of 5°C was chosen as a milder shock temperature.

Four groups were used as controls: 1, nymphs maintained at 28°C, a temperature used to rear *P. megistus* in the insect facilities at Sucen (Mogi-Guaçu, SP) since 1980; 2, nymphs subjected to a single shock at 5°C for 1 h; 3, nymphs subjected to a single shock at 0°C for 1 h; and 4, nymphs subjected to a single shock at 0°C for 12 h. After the shocks, the nymphs were maintained at 28°C and monitored daily for 30 days to score changes in survival rates and molting incidence. During this period, the insects were fed once a week. The survival and molting rates were compared statistically using Mantel–Hantzel's nonparametric procedure (8).

The survival rate of nymphs subjected to a short cold shock at 5°C (control No. 2) or 0°C (control No. 3) did not differ statistically (P value = 0.20) from that of the nymphs reared at 28°C (control No. 1). However, the shock at 0°C for 12 h (control No. 4) killed nearly all of the specimens (Table 1), which is in agreement with previous data (6).

TABLE 1
Survival and Molting Incidence (%) in *P. megistus* Nymphs 30 Days after Sequential Cold Shocks

Experimental conditions	*n*	Survival (%)	Molting incidence (%)
Control No. 1, 28°C	100	87.0	68.0
Control No. 2, one shock at 5°C (1 h)	53	96.1	29.4
Control No. 3, one shock at 0°C (1 h)	53	86.8	41.5
Control No. 4, one shock at 0°C (12 h)	30	3.3	0.0
Time (h at 28°C) between shocks at 5°C (1 h) and 0°C (12 h)			
8	53	96.2	26.4
18	53	98.0	28.8
24	53	94.7	24.5
72	53	98.0	35.3
Time (h at 28°C) between two shocks at 0°C (1 h and 12 h)			
8	53	81.1	41.5
18	53	85.9	47.3
24	53	82.4	50.9
72	53	72.2	40.7

When the severe cold shock was preceded by a milder shock, interspersed with a period in which the insects were kept at the usual rearing temperature, tolerance was induced, since the survival rates and molting incidence increased (Table 1). When the temperature of the first shock was 5°C, the survival rate after the second shock was the same as that of specimens reared at 28°C or subjected to a single shock at 5°C; a similar response was seen when the first shock was at 0°C. The cold-shock tolerance in this case did not change when the interval between shocks was 8–72 h. This response differs from the heat-shock tolerance response in *P. megistus* after sequential shocks separated by the same intervals used here (7). Heat-shock tolerance tended to weaken with increasing time between shocks (first shock, 35°C) or varied with this time (first shock, 40°C), being thus transiently controlled (7). The present data agree with the observation in flies that the thermoprotective response to low temperature is more rapid than to high temperature (4).

With short cold shock at 0°C the incidence of molting was lower than that at control 28°C but higher than that at 5°C (Tables 1 and 2). The tolerance seen when the first shock was at 5°C was equal to that of nymphs given a single shock at 5°C (Tables 1 and 2). However, the tolerance acquired when the first shock was at 0°C was more effective than when the first shock was at 5°C, with the molting incidences surpassing those obtained after a single shock at 0°C (1 h) and reaching the value seen in nymphs reared at 28°C (Table 2).

Induced cold-shock tolerance was thus demonstrated in *P. megistus*, a species which also develops heat-shock tolerance (7). Prevention of cold-shock injury by brief exposure to moderately low temperature has been reported for other insect species, including the hemipteran *Oncopeltus fasciatus* (9), which has a low supercooling point

TABLE 2
Comparison of Molting Rates Using the Mantel–Hantzel Test

Conditions compared					
	Test				
Control	First shock (°C, 1 h)[a]	Interval (h at 28°C) between the shocks	W	*df*	*P* value
No. 1 vs No. 2 vs No. 3			24.4	2	<0.0001*
No. 1 vs	0	8, 18, 24, 72	6.1	4	0.19
No. 1 vs	5	8, 18, 24, 72	47.0	4	<0.0001*
No. 2 vs	5	8, 18, 24, 72	2.1	4	0.71
No. 3 vs	5	8, 18, 24, 72	7.3	4	0.12

Note. No. 1, 28°C; No. 2, one shock at 5°C (1 h); No. 3, one shock at 0°C (1 h).
[a]2nd shock, 0°C, 12 h.
*Highly significant.

(−15°C) (11). However, this is the first report of induced cold-shock tolerance or a cold-hardening response in a blood-sucking hemipteran species (for other insect species with responses to low temperature, see the list published by Lee (9)).

The cold-shock tolerance induced by sequential shocks in *P. megistus* was more effective in increasing the molting incidence of nymphs to levels reached by nonshocked controls when the first shock was at 0 than at 5°C. The observation that with as little as the 8 h between shocks the molting incidence was equal to that of nymphs subjected to a single shock suggested that a prompter recovery of hormonal balance (6) may be involved in the last molt of *P. megistus* subjected to sequential cold shocks in comparison to sequential heat shocks (7). Thermal shocks have been suggested to sustain the juvenile hormonal production in *Rhodnius prolixus*, another blood-sucking hemipteran (12). The heat-shock tolerance response in *P. megistus* required a longer period between shocks (28°C) to circumvent the unfavorable effects caused by a longer shock at 40°C (7).

The heat-shock tolerance response induced in *P. megistus* is typical of hsp involvement (7). These proteins may also be involved in the induction of cold-shock tolerance since the synthesis of hsp during recovery from cold stress has been reported in other insect species (2, 4). If this is also the case in *P. megistus*, it does not necessarily mean that the proteins synthesized in response to heat and cold shocks are the same (4), particularly since there are differences in the tolerance patterns. Other protective mechanisms may also operate concurrently during the cold-shock tolerance response (4), including the modulation of DNA topoisomerase activity, the control of DNA topology, and the expression of stress genes (10) unrelated to the heat-shock response. Selection based on the ability to survive low temperatures (3) may also be possible.

The tolerance to sequential cold shocks in *P. megistus* may be relevant to survivors of this species making that they could well succeed in areas of southern Brazil where there is a transient seasonal drop in temperature, close to 0°C. Since active domestic invasion by flying *P. megistus* has been reported to occur during warmer months in these areas (1), the capability of a cold-hardening response in some of their specimens would provide them with an adaptive advantage.

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EXPERIMENTALLY INDUCED HEAT- AND COLD-SHOCK TOLERANCE IN ADULT *Panstrongylus megistus* (BURMEISTER) (HEMIPTERA, REDUVIIDAE)

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(With 4 figures)

Running title: Heat and cold shock tolerance in *P. megistus* adults

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ABSTRACT

The survival rate of domestic male and female adult *Panstrongylus megistus* was studied after sequential heat and cold shocks in order to investigate shock tolerance in comparison to that previously reported for nymphs. Sequential shocks were given such that a milder shock (0°C, 5°C, 35°C or 40°C for 1 h) preceded a severe one (0°C or 40°C for 12 h), separated by intervals of 8, 18, 24 and 72 h at 28°C (control temperature). The preliminary thermal shock induced tolerance to the more severe shock, although the intensity of the tolerance depended on the initial shock temperature and on the interval between treatments. Despite the observed tolerance, the survival rate for insects subjected to both shocks decreased when compared to that of individuals subjected to a single mild shock. When tolerance differed with sex, females showed greater values than males. In contrast to the response detected in nymphs, for which higher values of heat tolerance were sustained for intervals up to 24 h (preliminary shock, 35°C) or even longer (preliminary shock, 40°C) between sequential shocks, expressive values were verified in adults only for intervals between shocks up to 8 h (preliminary shock, 40°C). Also differing from findings for nymphs, which exhibited expressive cold-shock tolerance under conditions in which preliminary shocks were given at 5°C or 0°C and the periods between shocks were up to 72 h long, the adults were capable of acquiring an expressive tolerance response to a more severe cold shock only when the preliminary shock was given at 0°C and time between shocks surpassed 18 h. It is assumed that the mechanisms that play a role in the cellular protection of *P. megistus* under sequential temperature shocks (heat shock protein action?) may loose effectiveness with insect development.

Key words: *Panstrongylus megistus* – adults - heat shock - cold shock - survival – tolerance.

RESUMO

Tolerância ao calor e ao frio experimentalmente induzida em alados de *Panstrongylus megistus* (Burmeister) (Hemíptera, Reduviidae)

A taxa de sobrevivência de alados domiciliares machos e fêmeas de *Panstrongylus megistus* foi investigada após choques seqüenciais quentes e frios a fim de se investigar aquisição de termotolerância em comparação a dados já reportados para ninfas. Os insetos foram submetidos a choques térmicos seqüenciais sendo que um choque mais suave (0°C, 5°C, 35°C ou 40°C por 1 h) precedeu o choque mais severo (0°C ou 40°C por 12 h). Estes foram separados por intervalos de 8, 18, 24 e 72 h a 28°C (temperatura controle). Demonstrou-se que o choque térmico preliminar pode induzir tolerância ao mais severo, embora a intensidade de tolerância seja dependente da temperatura inicial do choque e do intervalo entre os tratamentos. Apesar da tolerância observada, a taxa de sobrevivência diminuiu em insetos submetidos a ambos os choques quando comparados àqueles espécimes submetidos a um único choque suave. Quando a tolerância diferiu com o sexo, as fêmeas apresentaram uma tolerância maior. Diferente da resposta observada em ninfas, para as quais a tolerância a choques quentes se estende até quando o período entre os choques é de 24 h (choque inicial a 35°C) ou mais (choque inicial a 40°C), taxa expressiva de tolerância nos adultos foi verificada apenas em condições de até 8 h de intervalo entre os choques e sendo o choque inicial dado a 40°C. Também diferindo do que acontece nas ninfas, cuja tolerância ao choque frio se expressa em todas as condições semelhantemente estudadas, nos adultos se demonstrou expressiva tolerância ao choque frio apenas quando o choque inicial foi dado a 0°C e o tempo entre choques seguidos ultrapassou 18 h. Presume-se que em *P. megistus* os mecanismos que desempenham papel na proteção celular a choques seqüenciais de temperatura (proteínas de choque?) possam perder a eficácia com o desenvolvimento do inseto.

Palavras-chave: *Panstrongylus megistus* – adultos - choque quente – choque frio - sobrevivência – tolerância.

INTRODUCTION

According to the World Health Organization, Chagas' disease affects 20 million people, mostly in Central and South America. In Brazil, Chagas' disease is the third greatest cause of death by infectious parasitic diseases (Silveira & Rezende, 1994; Dos Reis, 1997), with vectorial transmission being the principal route of infection. Studies of the responses of blood-sucking insects to various stressing factors represent an important approach in understanding how to control these species (Rodrigues *et al.*, 1991; Silva & Silva, 1993; Garcia *et al.*, 1999; Schmuñis, 2000).

Panstrongylus megistus (Burmeister), a blood-sucking hemipteran, is an important vector of Chagas' disease in Brazil because of its wide geographical distribution, high rates of infection and its ability to adapt to artificial ecotopes (Forattini, 1980). *P. megistus* is native to Brazil but is increasingly associated with human habitations because of extensive destruction of its natural habitats (Silveira, 2000).

Changes in the survival rate, molting incidence and nuclear phenotypes of *P. megistus* have been observed following exposure to stress agents (Mello, 1978; Mello & Raymundo, 1980; Garcia *et al.*, 1999; 2000a, b). Similarly, heat and cold shock tolerance has been reported for *P. megistus* nymphs when they are subjected to sequential thermal shocks (Garcia *et al.*, 2001a, b). However, cold-shock tolerance differed from heat-shock tolerance in nymphs of *P. megistus*. Heat-shock tolerance varied with the time between sequential shocks, being thus transiently controlled, whereas cold-shock tolerance did not change when intervals of 8-72 h between shocks were considered. In addition, sequential cold shocks produced a higher molting incidence when compared to sequential heat shocks (Garcia *et al.*, 2001a, b).

Considering that differences in survival rates after single heat or cold shocks have been reported when adults are compared to nymphs and that the thermal shock response in adults vary with sex (Garcia *et al.*, 1999), the survival rate of *P. megistus* adults after sequential heat and cold shocks may also differ. In the present study, survival rates after sequential heat and cold shocks, in which a mild shock precedes a severe one, were thus investigated in male and female adults of *P. megistus*.

MATERIAL AND METHODS

Domestic adult male and female *P. megistus* specimens with domestic habit, and reared in the laboratory at Sucen (Mogi-Guaçu, SP) were used. The insects were separated into groups of 50 specimens each whivh were put into glass cylinders (total: 21) covered with cheesecloth and fastened with elastic bands. The insects were fasted for 15 days before being subjected to heat shock (35°C or 40°C for 1 h) or cold shock (5°C or 0°C for 1 h) followed by a return to their optimal rearing temperature (28°C). After various intervals (8 h, 18 h, 24 h, 72 h) following the mild shock, the insects were subjected to a more severe treatment (12 h at 40°C or 0°C).

The temperatures of 40°C and 0°C were chosen because of the need to use relatively extreme temperatures in comparison to the control (28°C), operational facilities, and comparison with previous data on *P. megistus* (Garcia *et al.*, 1999; 2001a, b). The temperatures of 35°C and 5°C were chosen as milder shock temperatures, based on previous results (Garcia *et al.*, 2001a, b). A single shock at 40°C for 1 h has been reported to induce fall in the survival of adult specimens not so severe as that promoted by a single shock at 0°C for the same period (Garcia *et al.*, 1999). Single shocks at 40°C and 0°C for 12 h are lethal to both male and female *P. megistus* adults (Garcia *et al.*, 1999).

The choice of a moderate fasting condition prior to the shock assays was based on previous data which demonstrate a slight better opportunity for survival of males with a domestic habit under cold-shock conditions in this state of nourishment (Garcia *et al.*, 1999). Immediately after the shock assays the insects were returned to the temperature of 28°C, fed once a week on hen's blood, and monitored daily for 30 days to investigate changes in survival rates.

Five control groups were used: #1: insects maintained at 28°C but not subjected to heat or cold shock, #2: insects subjected to a single cold shock at 0°C (1 h), #3: insects subjected to a single cold shock at 5°C (1 h), #4: insects subjected to a single heat shock at 35°C (1 h), and #5: insects subjected to a single heat shock at 40°C (1 h).

The survival rates were compared using Mantel-Hantzel non-parametric test, the Cox proportional hazard semi-parametric test and a Weibull parametric test (Kalbfleish & Prentice, 1980).

RESULTS

For consistency with previous papers (Garcia *et al.*, 2001 a, b), only results obtained with Mantel-Hantzel non-parametric test are presented, although all three statistical tests gave similar results.

Total sample (1050 specimens)

There was strong evidence of a difference among treatments, when all the subgroups ($W = 69.1$, $p = 0.000$), and both sexes ($W = 9.6$, $p = 0.002$) were considered.

Control samples of each subgroup (250 specimens)

When control groups #1 to #5 were compared among themselves, there was strong evidence of a difference in survival ($W = 14.4$, $p = 0.006$). However, there was no difference with sex ($W = 1.2$, $p = 0.280$).

The insect survival in each control group was 70% for 1 h at 0°C, 68% for 1 h at 5°C, 96% for 1 h at 35°C, 76% for 1 h at 40°C and 80% for unshocked insects. Each shock (heat or cold, short or long) decreased the survival of the insects (Figures 1.2, 2.2, 3.2 and 4.2). The groups in which most insects survived were those which received no shock or which were subjected to a shock at 35°C, followed by those which received shocks at 40°C, 0°C, and 5°C.

Insects subjected to a single heat (35°C and 40°C) or cold (0°C and 5°C) shock

Insects subjected to one single shock at 35°C (group #4) differed significantly from those subjected to a single shock at 40°C (group #5) ($W = 394.8$, $p = 0.000$). A similar result was obtained with cold shock at 0°C (group #2) compared with 5°C (group #3) ($W = 307.5$, $p = 0.000$). There was also a difference when the survival of insects subjected to single heat shocks (groups #4 and #5) was compared with that of insects subjected to single cold shocks (groups #2 and #3).

The survival for males plus females subjected to sequential heat shocks was 37%, whereas the survival for those subjected to cold shocks was 67%. However, when considering control groups that received single heat shocks (#4 + #5) in comparison with control groups that received single cold shocks (#2 + #3), the survival was greater for heat-shocked insects (86%; cold-shocked insects, 69%).

Insects subjected to an initial shock at 0°C for 1h

Since the survival curves obtained after all treatments differed significantly at $p \leq 0.05$, multiple comparisons were necessary to identify the different curves. To guarantee an overall

significance level of 0.05, a p value of 0.01 was used for subsequent tests which compared the curves.

There was significant difference when a single shock at 0°C (group #2) was compared with sequential shocks at this temperature (W = 248.2, p = 0,000). The insects subjected to sequential cold shocks at 0°C differed significantly from each other (W = 15.9, p = 0.001). When the shocks were separated by intervals of 18, 24 or 72 h at 28°C these insects survived longer than those subjected to shocks separated by an 8-h interval. The survival rates of insects subjected to sequential cold shocks with intervals of 18 h and 24 h at 28°C between shocks were similar to each other (W = 0.8, p = 0.359). The survival curve of insects whose interval between shocks was 72 h and that of insects subjected to a single shock at 0°C were similar (Figure 1.2; W = 0.7, p = 0.397). Survival was not affected by sex (W = 2.8, p = 0.092; Figure 1.1). The relative frequency of survival confirmed the difference between control group #2 and that of the insects subjected to two shocks.

Insects subjected to an initial shock at 5°C for 1h

Although the survival rate of insects subjected to cold shock 18 h after the first shock was apparently higher than in the remaining groups, including control group #3, the difference was not statistically consistent (W = 7.8, p = 0.100; Figure 2.2).

Comparison of control group #3 with insects subjected to sequential shocks showed that the survival rate of females was greater than that of males (W = 7.8, p = 0.005; Figure 2.1).

Insects subjected to an initial shock at 35°C for 1h

There was a significant difference between survival of control group #4 and that of insects subjected to sequential shocks separated by various periods at 28°C (W = 55.2, p = 0.000), with

control group #4 surviving longer than insects subjected to sequential shocks. There was no difference among insects subjected to sequential shocks separated by 8, 18, 24 and 72 h at 28°C ($W = 1.0$, $p = 0.801$; Figure 3.2), although females always survived longer than males (Figure 3.1).

Insects subjected to an initial shock at 40°C for 1h

There was strong evidence of a difference in the survival of control group #5 compared to insects subjected to sequential heat shocks ($W = 164.8$, $p = 0.000$). Only the results for insects subjected to a second shock 8 h after the first one were similar to those of the control ($W = 2.6$, $p = 0.105$). These two conditions produced a survival rate greater than the remaining treatments (control, 76%; 8h, 60%; 18h, 2%; 24h, 6%; 72h, 8%) (Fig. 4.2). The survival of insects subjected to a second shock after an interval of 24 and 72 h at 28°C was similar in both groups ($W = 1.3$, $p = 0.247$). Although there was a significant difference among all groups of insects subjected to sequential heat shocks, the results were not affected by sex ($W = 0.0$, $p = 0.993$; Figure 4.1).

Control group at 28°C

There was no difference in the survival of males and females in control group #1 ($W = 0.0$, $p = 0.954$). Comparison of the groups that received one or two shocks with group #1 showed that the insects in group #2 survived less than those in group #1 whereas insects of almost all groups subjected to sequential cold shocks had survival curves similar to that of control group #1 (Figure 1.2). The insects in group #3 and all samples subjected to sequential cold shocks had survival curves similar to that of control group #1. However, control #3 insects survived less than insects belonging to control #1 (Figure 2.2). Control group #4 insects survived more than control group #1 insects, although the survival curves of both were similar (Figure 3.2). Control group #5 and insects subjected to heat sequential shocks in which the shocks were separated by 18, 24, and 72 h at 28°C, survived less than control group #1 insects (Figure 4.2).

DISCUSSION

Present results indicated induced tolerance to heat and cold shocks in adults of *P. megistus* in terms of their survival to sequential shocks in which a mild shock preceded a more severe one. Shocks at 40°C or 0°C for 12 h are both lethal to these insects when given isolatedly (GARCIA *et al.*, 1999). The tolerance rate was found to be predominantly a function of the temperature of the preliminary shock and time elapsed between sequential shocks. The different responses to sequential thermal shocks probably elicit different mechanisms of cellular protection against stress.

Expressive values of heat-shock tolerance were elicited in adults only when they were subjected to sequential treatments in which the preliminary shock was given at 40°C and the second one 8 h after the first shock, a situation which differs from findings reported for nymphs (GARCIA *et al.*, 2001b). In the case of nymphs, higher values of heat-shock tolerance were sustained for intervals between shocks extending up to 24 h (preliminary shock, 35°C) or even longer (preliminary shock, 40°C) (GARCIA *et al.*, 2001b).

The mechanism(s) involved with heat-shock tolerance in adults probably require(s) that the shock being given at a temperature much drastically shifting from 28°C to 40°C in order to elicit an efficient protective action. This is possibly required to activate significant changes in genomic expression, with specialized metabolic responses thus being triggered (GASCH *et al.*, 2000). Even so, this action is very transient, differing from the situation reported for nymphs (GARCIA *et al.*, 2001b).

The cold-shock tolerance response in adults of *P. megistus* also differed from the response described for nymphs of the same species under same experimental conditions (GARCIA *et al.*, 2001a). *P. megistus* nymphs exhibit expressive cold-shock tolerance under various sequential

shock conditions (preliminary shock at 5°C or 0°C; periods at 28°C extending from 8 to 72 h long between shocks) (GARCIA et al., 2001a). In the present study, adults were found to acquire expressive tolerance to a severe cold shock at 0°C for 12 h only when this shock was preceded by a mild shock at 0°C for 1 h and the time between shocks surpassed 18 h. Maybe in this case, for protective mechanisms being elicited effectively, adults require a period longer than that in nymphs. Even so, the tolerance expressed in adults was much lower than that elicited in nymphs. The temperature of 5°C was found not to be a good elicitor of protective mechanisms in adults, a situation completely different from that verified for nymphs subjected to the same experimental conditions (GARCIA *et al.*, 2001a).

It is concluded that the mechanisms that play a role in the cellular protection against sequential temperature shocks in *P. megistus* may loose effectiveness with insect development. A certain disadvantage was also found for males under adult phase.

Acquisition of heat-shock tolerance is assigned to involvement in a wide sense with heat shock proteins (HSP) (WELCH, 1993). These proteins may also be involved with cold-shock tolerance in some insects (DENLINGER et al., 1991). A preliminary immunocytochemical investigation of HSP70 in Malpighian tubules of *P. megistus* subjected to heat shock at 40°C for 1 h, has not succeeded in identifying increase in this protein type (GARCIA, 2002), similarly to findings for Malpighian tubules of *Drosophila* (SINGH & LAKHOTIA, 1995). For the case of *Drosophila*, it has been assumed that a rapid fall in concentration of HSP70 as mediated by proteolytic action may jeopardize the immunocytochemical results and does not mean absence of this protein (SINGH & LAKHOTIA, 1995). Additional investigation on HSP is this required for *P. megistus* subjected to thermal shocks.

Anyway, although not discarding a differential participation of HSP types in the heat- and cold-shock tolerance responses in *P. megistus*, other mechanisms should also be considered, among which modulation of DNA topoisomerase activity, and even expression of stress genes unrelated to the heat-shock response and genetic variation in metabolic traits (CLARK & FUCITO, 1998; LOPES-GARCIA & FORTERRE, 2000).

The different patterns of heat and cold shock tolerance of *P. megistus* as a function of its different developmental phases may have favored this species' adaptation to different environments, especially when considering its nymphal phases, and could explain its success and wide distribution throughout Brazil. Considering the marked susceptibility of *P. megistus* to *Trypanosoma cruzi*, present results may be relevant to our understanding of the biological responses to stress in these insects under their natural environment or in the laboratory.

ACKNOWLEDGMENTS

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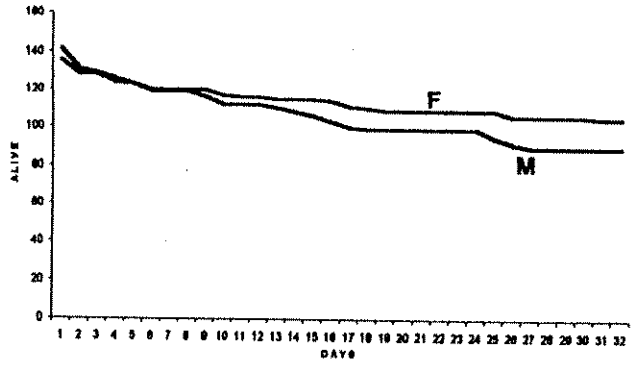
Figures 1.1 - 2.2. Survival curves for cold-shocked adult *P. megistus.*

1.1. A single shock at 0°C (1 h) in males (M) and females (F). 1.2. Sequential shocks at 0°C (first one for 1 h; second one for 12 h) separated by different periods at 28°C. Controls: 28°C (C) and a single shock at 0°C for 1 h (S). 2.1. A single shock at 5°C (1 h) in males (M) and females (F). 2.2. Shock at 5°C (1 h) followed by a shock at 0°C (12 h), separated by different periods at 28°C. Controls: 28°C (C) and a single shock at 5°C for 1 h (S).

Figures 3.1 - 4.2. Survival curves for heat-shocked adult *P. megistus.*

3.1 A single shock at 35°C (1 h) in males (M) and females (F). 3.2. Preliminary shock at 35°C (1 h) followed by a shock at 40°C (12 h), separated by different periods at 28°C. Controls: 28°C (C) and a single shock at 35°C for 1 h (S). 4.1. A single shock at 40°C (1 h) in males (M) and females (F). 4.2. Sequential shocks at 40°C (first one for 1 h; second one for 12 h) separated by different periods at 28°C. Controls: 28°C (C) and a single shock at 40°C for 1 h (S).

1.1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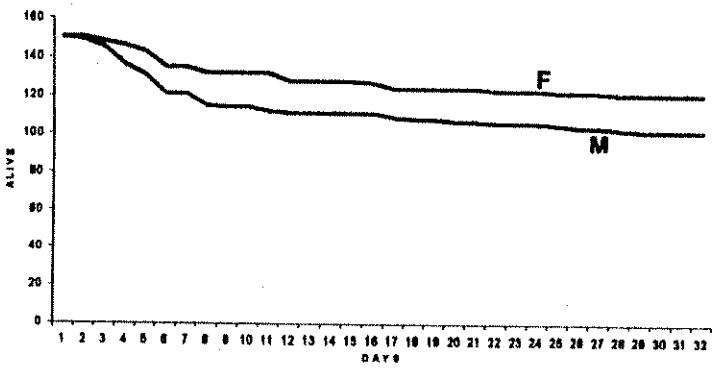
2.1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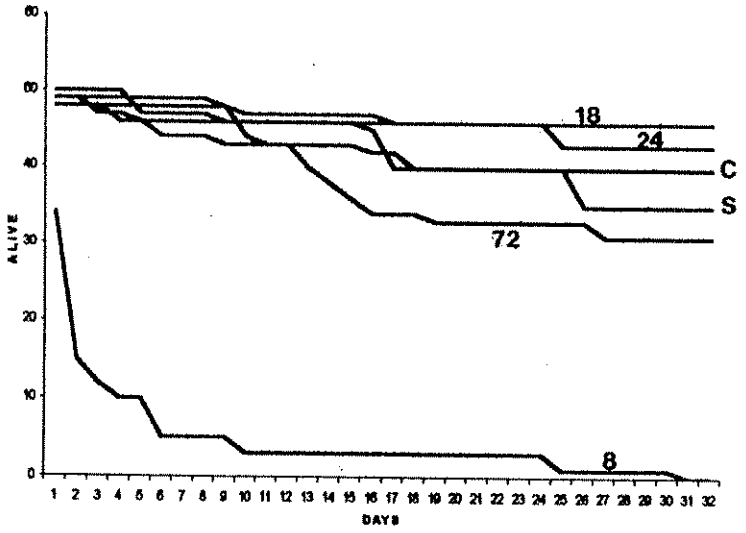
1.2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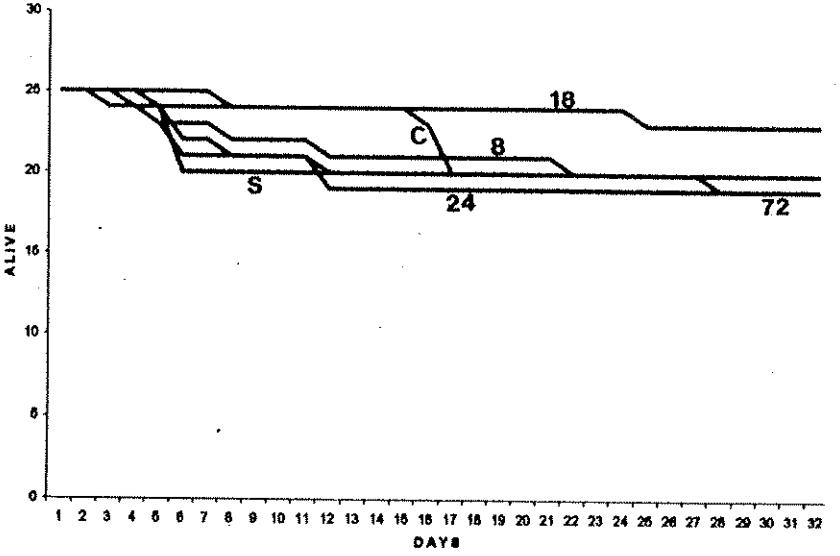
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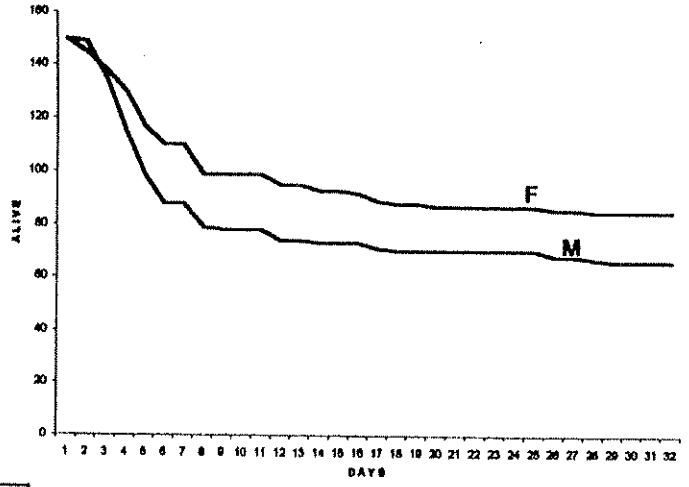
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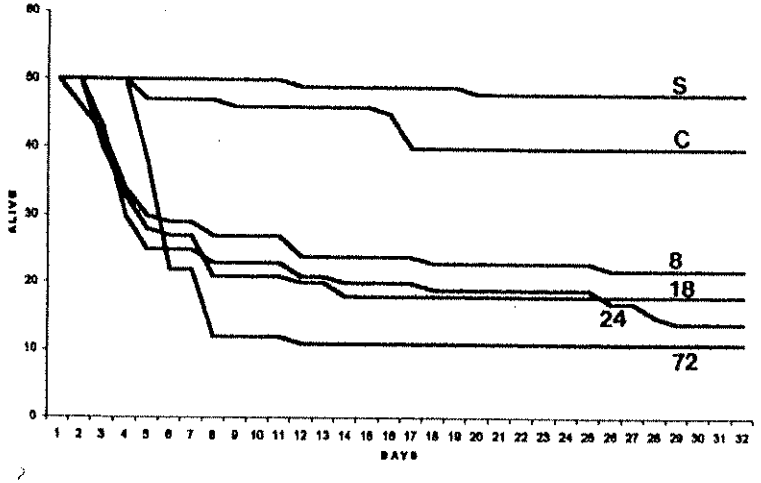
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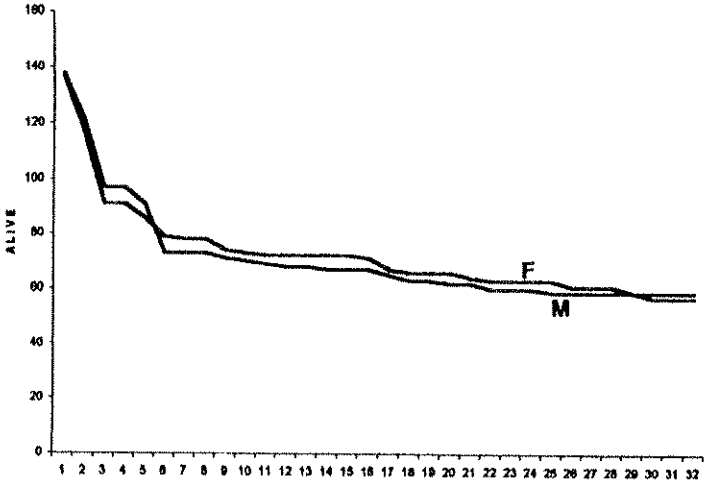
4.1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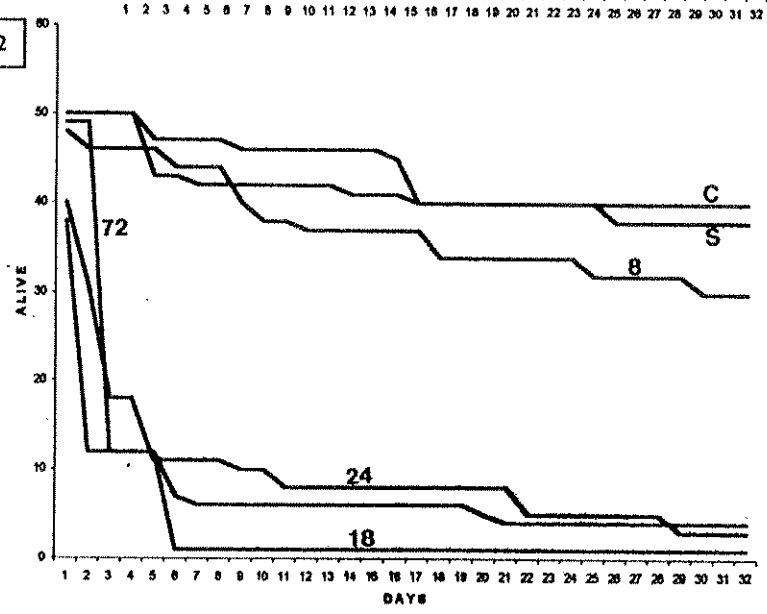
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Effect of Sequential Heat and Cold Shocks on Nuclear Phenotypes of the Blood-sucking Insect, *Panstrongylus megistus* (Burmeister) (Hemiptera, Reduviidae)

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Thermal shocks induce changes in the nuclear phenotypes that correspond to survival (heterochromatin decondensation, nuclear fusion) or death (apoptosis, necrosis) responses in the Malpighian tubules of Panstrongylus megistus. *Since thermal tolerance increased survival and molting rate in this species following sequential shocks, we investigated whether changes in nuclear phenotypes accompanied the insect survival response to sequential thermal shocks. Fifth instar nymphs were subjected to a single heat (35 or 40°C, 1 h) or cold (5 or 0°C, 1 h) shock and then subjected to a second shock for 12 h at 40 or 0°C, respectively, after 8, 18, 24 and 72 h at 28°C (control temperature). As with specimen survival, sequential heat and cold shocks induced changes in frequency of the mentioned nuclear phenotypes although their patterns differed. The heat shock tolerance involved decrease in apoptosis simultaneous to increase in cell survival responses. Sequential cold shocks did not involve cell/nuclear fusion and even elicited increase in necrosis with advancing time after shocks. The temperatures of 40 and 0°C were more effective than the temperatures of 35 and 5°C in eliciting the heat and cold shock tolerances, respectively, as shown by cytological analysis of the nuclear phenotypes. It is concluded that different sequential thermal shocks can trigger different mechanisms of cellular protection against stress in* P. megistus, *favoring the insect to adapt to various ecotopes.*

Key words: heat shock - cold shock - sequential shocks - nuclear phenotypes - *Panstrongylus megistus*

Panstrongylus megistus, one of the most important vectors of Chagas disease, is a blood-sucking insect with a wide geographical distribution in Brazil, high rates of infection with *Trypanosoma cruzi*, and an adaptability for invading artificial ecotopes (Forattini 1980). Prolonged cold and heat shocks are deleterious to *P. megistus* nymphs (Garcia et al. 1999), but resistance to these stressing conditions can be elicited following sequential shocks in which a mild shock precedes a severe one (Garcia et al. 2001a, b). Heat-shock tolerance follows a pattern typical of heat shock protein activation (Garcia et al. 2001a). Cold-shock tolerance also presumably involves heat shock proteins, although consideration of other protective mechanisms has not been eliminated (Garcia et al. 2001b).

~~Single heat and cold shocks can change the nuclear phenotypes in the Malpighian tubules of *P. megistus* (Garcia et al. 2000a, b).~~ The normal nuclear phenotype in males has a small heterochromatic body which contains several copies of the Y chromosome (Mello et al. 1986), but phenotypes indicative of survival (decondensed heterochromatin, nuclear fusion) and death (apoptosis, necrosis) responses have also been found in male nymphs and adults. Nuclei with heterochromatin loosening may represent an attempt to activate silent genes during stress (Mello 1983, 1989). Giant nuclei are characterized by a bigger size when compared with normal nuclei, and originate from nuclear and/or cellular fusion (Wigglesworth 1967, Mello & Raymundo 1980). This phenotype has been described as a possible mechanism of cellular and organ protection under unfavourable conditions in blood-sucking insects (Wigglesworth 1967, Mello & Raymundo 1980, Dantas & Mello 1992, Garcia et al. 2000a, b). Apoptosis and necrosis are defined in terms of their morphological characteristics (Kerr 1971, Kerr et al. 1972, Garcia et al. 2000a, b). With the exception of the normal phenotype, the other phenotypes are more frequent in specimens subjected to heat and cold shocks (Garcia et al. 2000a, b). The frequency of fused nuclei and of nuclei with heterochromatin decondensation compared with that of other phenotypes was considered insufficient to cope with the deleterious effects of a severe heat shock (Garcia et al. 2000a). For the response to cold shock, the activation of nuclear and cell fusion which generates giant nuclei was found to be less effective or necessary than that elicited by heat shock (Garcia et al. 2000b).

Considering the cell survival and cell death responses expressed by nuclear phenotype changes in *P. megistus* subjected to single heat and cold shocks (Garcia et al. 2000a, b) and the increase in specimen survival and molting rates in this species under sequential heat and cold shocks (Garcia et al. 2001a, b), we suspected that changes

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in nuclear phenotypes may also occur under sequential temperature shocks. In the present study, the influence of sequential heat and cold shocks on nuclear phenotypes was investigated in Malpighian tubule epithelial cells of *P. megistus*.

MATERIALS AND METHODS

Insects and shock assays - Male fifth instar nymphs of *P. megistus* (Burmeister) (Hemiptera, Reduviidae) were reared at 28°C and 80% relative humidity and fasted for 15 days prior to the temperature shock experiments, since only with moderate fasting do some nymphs survive prolonged heat and cold shocks (Garcia et al. 1999).

The insects were subjected to a shock at 35 or 40°C for 1 h, returned to 28°C and then subjected to a further shock at 40°C for 12 h. Other groups of insects were subjected to a shock at 5 or 0°C for 1 h, returned to 28°C and then subjected to a further shock at 0°C for 12 h. The choice of the shock temperatures was based on previous reports (Garcia et al. 2001a, b). For both the heat and cold shock assays, the second shock started 8, 18, 24 and 72 h after the first one. After the second shock, the insects were returned to 28°C and fed on hen blood once a week. Control specimens were maintained at 28°C, the temperature used to rear *P. megistus* in Sucen's laboratory since 1980, or subjected to a single shock at 0, 5, 35 and 40°C, for 1 h, and 0 or 40°C for 12 h.

Organ preparations - Malpighian tubule preparations were obtained immediately after the shocks or 10 and 30 days later. Organs from at least three specimens were used for each experimental condition and the corresponding control. The whole organs were mounted on glass slides, immediately fixed in ethanol: acetic acid solution (3:1, v/v) for 1 min, rinsed in 70% ethanol for 5 min, and air dried at room temperature. The tubules were then subjected to the Feulgen reaction, with acid hydrolysis in 4 M HCl at 25°C for 65 min. The Feulgen-stained material was rinsed in sulfurous and distilled water, air dried, cleared in xylene and mounted in Canada balsam.

Nuclear counting and statistics - A Nikon light microscope was used to count the total number of Malpighian tubule epithelial cell nuclei per specimen and to determine the frequency of the different nuclear phenotypes present.

To assess the relationship between stress conditions and the various nuclear phenotypes, analysis of variance (ANOVA) with two factors (interval between shocks and number of days following shocks) was used, considering an unbalanced number of nuclei with specific phenotypes for different specimens (Kalbfleish & Prentice 1980).

The same tests used for the heat shock results were applied to cold shock data, except that severe cold shocks (12 h) were not tested because in this case almost all insects died.

RESULTS

The same phenotypes observed in this work have been described and reported elsewhere (Mello 1978, Mello et al. 1986, Garcia et al. 2000a, b). The most frequent phenotype observed in the control groups and in the groups subjected to single or sequential shocks was the normal one. Among the changed phenotypes, there were nuclei with heterochromatin decondensation, as well as giant nuclei and nuclei with morphological aspects of typical and suspected apoptosis and necrosis.

The frequencies of the nuclear phenotypes in the various experimental conditions are shown in Tables I and II.

Apoptosis - The frequency of apoptosis in the insects that received sequential heat shocks did not differ from that of the unshocked group (p: 0.317), but did increase in comparison to that of the insects that received the single mild shock (p: 0.036) and did decrease in comparison to that of the specimens that received the single severe shock (p: 0.015). In general, with advancing time after the heat shock, the frequency of this phenotype increased (p: 0.004).

When considering cold sequential shocks, the frequency of apoptosis decreased as compared with the unshocked group (p: 0.003), but did not differ from that of the specimens that received the single mild shock (p: 0.510).

Change in shock temperature from 35 to 40°C or 5 to 0°C did not affect significantly the frequency of apoptosis in single heat and cold shock assays, respectively (Tables III, IV). However, it caused a decrease in frequency of this phenotype when a second heat shock was applied (Table III).

Nuclei suspected of apoptosis - The frequency of this phenotype in the specimens subjected to sequential heat shocks decreased as compared with the unshocked control (p: 0.000) and the group that received the single severe shock (p: 0.000), but did not differ from that of the specimens that received the single mild shock (p: 0.791). Thirty days after the sequential shocks, the frequency of the nuclei suspected of apoptosis was lower than the average of the frequencies detected for 1 and 10 days after the shocks (p: 0.001). When the time between shocks was 24 h and the insects were analyzed 1 day after the shocks, the frequency of this phenotype was smaller than the average of the frequencies detected for the other periods between shocks (p: 0.001). When the time between shocks was 72 h and the insects were analyzed 10 days after shocks, the frequency of this phenotype increased (p: 0.004).

Under cold shock conditions, the frequency of the nuclei suspected of apoptosis was generally similar to that verified after heat shocks.

When a shock at 40 or 0°C was applied, irrespective of a second shock being given, there was a decrease in the frequency of nuclei suspected of apoptosis in comparison with shocks at 35 or 5°C, respectively (Tables III, IV).

Necrosis - In general, sequential heat shocks did not affect the frequency of necrosis when compared with the unshocked group (p: 0.091) and the groups subjected to the single mild (p: 0.073) or severe (p: 0.904) shocks. When the period between shocks was equal to 18 h and the insects were dissected 30 days after the shocks, the frequency of necrosis was lower than the average of the frequencies obtained for longer periods between shocks (p: 0.002).

No change in the frequency of necrosis was verified 1 day after sequential cold shocks. However, with advanc-

TABLE I

Frequency of nuclear phenotypes after sequential heat shocks in *Panstrongylus megistus* (2nd shock: 40°C, 12 h)

1st shock (°C)	Interval between shocks (h)	D	n	Nuclear phenotypes (absolute arithmetic means) A	As	NE	G	HD	N	Total
Unshocked	Control	1	3	5[a]	1672	828[a]	11[a]	79[a]	8196	10800
		10	3	11	2488	390[a]	2[a]	147[a]	8982	12019
		30	4	17[a]	2214	1710	8[a]	264	9089	13100
Single	Shock	1	3	8[a]	2619	1244	34[a]	437[a]	13474	17843
(40°C, 12h)		30	3	79[a]	3387	2097[a]	126[a]	159	10702	16550
35	-	1	3	1[a]	1960	901	0	366[a]	14041	17269
		10	3	5[a]	1443[a]	1672	0	169[a]	12872	16162
		30	4	2[a]	666[a]	773[a]	1[a]	199[a]	10641	12282
	8	1	3	5	2045	1993[a]	0	659	11723	16424
		10	3	24[a]	1385[a]	2509	0	166[a]	11890	15974
		30	3	10[a]	654	1601	85	668[a]	8735	11752
	18	1	2	1[a]	2020	2474[a]	0	1598	12295	18387
		10	4	83[a]	929[a]	2399[a]	0	766	10776	14953
		30	3	5[a]	1045[a]	456[a]	54[a]	49	9580	11189
	24	1	3	4[a]	681[a]	565	0	450[a]	11697	13396
		10	3	3[a]	590[a]	1094	15[a]	779	10863	13343
		30	4	18[a]	646[a]	1177[a]	79[a]	149[a]	10772	12842
	72	1	3	5[a]	1565	2037	0	437[a]	9854	13888
		10	2	7	3362	1529[a]	22[a]	236	10402	15563
		30	3	7[a]	380	1353[a]	434	20[a]	6732	8926
40	-	1	3	0	11[a]	464	0	168[a]	8584	9227
		10	3	1[a]	4	176[a]	0	232[a]	7804	8217
		30	2	7[a]	41	321	0	38	8089	8495
	8	1	3	4[a]	3[a]	733	0	65[a]	9428	10233
		10	2	1[a]	115	429[a]	0	12[a]	11010	11566
		30	4	6[a]	30[a]	705	0	31	7019	7791
	18	1	4	13[a]	61[a]	682	0	106[a]	8488	9349
		10	4	3[a]	125[a]	590	0	125	8338	9180
		30	3	3[a]	15[a]	1207	0	87	8669	9989
	24	1	3	3[a]	33[a]	1198	0	133	9612	10979
		10	3	4[a]	82	934[a]	0	46	9666	10732
		30	4	8[a]	61	2832	1[a]	151	9191	12243
	72	1	3	0	23	292[a]	0	129	8080	8524
		10	3	0	27[a]	353	0	3	9402	9785
		30	3	4	22	488[a]	16[a]	150	8079	8759

a: nonsignificant mean since it was neither equal nor over twice its standard deviation; A: apoptosis, As: nuclei suspected of apoptosis, D: days after shocks, G: giant nuclei, HD: heterochromatin decondensation, n: number of insects, N: normal nuclei, NE: necrosis

ing time after shock, the frequency of this phenotype increased significantly.

Change in shock temperature from 35 to 40°C or 5 to 0°C did not affect the frequency of necrosis in single heat and cold shock assays, respectively (Tables III, IV). However, it promoted a decrease in frequency of necrosis when a second heat shock was given (Table III).

Giant nuclei - Sequential shocks increased the frequency of giant nuclei in comparison with the group subjected to the single severe shock (p: 0.001), but did not differ from that of the insects that received the single mild shock (p: 0.612). The frequency of giant nuclei 30 days after heat shocks was greater than for the other times after shocks. In addition, sequential shocks decreased the frequency of giant nuclei relative to the unshocked group 1 day after shocks (p: 0.005), but increased the frequency of this phenotype 30 days after shocks (p: 0.002).

When considering the application of cold shocks, the occurrence of giant nuclei decreased in comparison with the unshocked group (p: 0.000).

Increasing the temperature of the preliminary heat shock from 35 to 40°C decreased the frequency of giant nuclei in sequential shock assays (Table III). No change was promoted in the frequency of this phenotype when the temperature of the preliminary shock in sequential cold shock assays increased from 0 to 5°C (Table IV).

Heterochromatin decondensation - The frequency of this phenotype increased after sequential heat shocks in comparison with the unshocked group (p: 0.027) or with the groups subjected to the single mild or severe shock (p: 0.030 and 0.011, respectively).

Under cold shock conditions, heterochromatin decondensation was more effective at short periods after sequential shocks.

TABLE II

Frequency of nuclear phenotypes after sequential cold shocks in *Panstrongylus megistus* (2nd shock: 0°C, 12 h)

1st shock (°C)	Interval between shocks (h)	D	n	Nuclear phenotypes (absolute arithmetic means) A	As	NE	G	HD	N	Total
0	-	1	3	7[a]	124[a]	1659	1[a]	85	8485	10361
		10	3	3[a]	185[a]	1778	0	31[a]	10177	12175
		30	3	1[a]	35	565	0	55[a]	10124	10780
	8	1	3	3[a]	336	1584	1[a]	194[a]	10935	13053
		10	3	21[a]	225	3438	0	50	11906	15641
		30	3	30	88	1396	0	89[a]	8248	9851
	18	1	3	12[a]	220[a]	1620	0	85	11852	13790
		10	3	3[a]	169[a]	1926	0	170[a]	10917	13185
		30	3	7[a]	78	1482	1[a]	55	8431	10053
	24	1	3	5[a]	74[a]	1050	0	45	8950	10124
		10	4	1[a]	59[a]	386	0	11[a]	8558	9014
		30	2	9	81	2605	1[a]	235[a]	8915	11845
	72	1	3	34[a]	124	3171	0	231[a]	8647	12209
		10	3	0	95[a]	821[a]	0	28[a]	11246	12189
		30	3	14	151	303	0	11[a]	11446	11924
5	-	1	3	2[a]	1427	944	0	462	11858	14692
		10	3	4[a]	957	2534	0	313	12091	15900
		30	2	2[a]	128	1123	0	141	10170	11564
	8	1	3	8[a]	108	1313	0	196[a]	15966	18567
		10	3	0	505	1424	0	246	11318	13494
		30	2	17	239	4194	1[a]	251	9940	14611
	18	1	3	24[a]	1015	1424	0	381	11986	14831
		10	3	1	770	1821	0	219	13230	16042
		30	3	2[a]	860[a]	1926[a]	1[a]	90	14066	16945
	24	1	3	0	337	1180	0	558	11864	13940
		10	3	0	364	1006	0	80	9816	11266
		30	3	10[a]	428	1343	1[a]	170	8916	10868
	72	1	3	6[a]	487[a]	411	0	545	8310	9759
		10	3	0	632[a]	1118	0	255	11751	13757
		30	3	8	361	2956	0	179	11153	14657

Controls were the same as in Table I; *a*: nonsignificant mean since it was neither equal nor over twice its standard deviation; A: apoptosis, As: nuclei suspected of apoptosis, D: days after shocks, G: giant nuclei, HD: heterochromatin decondensation, n: number of insects, N: normal nuclei, NE: necrosis

TABLE III

Statistical significance of contrasts for each nuclear phenotype under heat shock conditions

Nuclear phenotype	Contrast C1a x C1b	T x T'
A	1.000	0.012
As	0.000	0.000
NE	0.906	0.022
G	1.000	0.000
HD	1.000	0.000
N	0.006	0.000

C1: single heat shock at 35 (a) or 40°C (b) for 1 h. T and T': sequential shocks irrespective of time between them; preliminary shock done at 35 (T) or 40°C (T'). The factors with a significant effect for $\alpha = 0.05$ are underlined. A: apoptosis, As: nuclei suspected of apoptosis, G: giant nuclei, HD: heterochromatin decondensation, N: normal nuclei, NE: necrosis

TABLE IV

Statistical significance of contrasts for each nuclear phenotype under cold shock conditions

Nuclear phenotype	Contrast C1a x C1b	T x T'
A	0.999	0.244
As	0.000	0.000
NE	1.000	0.545
G	0.999	0.908
HD	0.087	0.000
N	0.836	0.069

C1: single cold shock at 5 (a) or 0°C (b) for 1 h. T and T': sequential shocks irrespective of time between them; preliminary shock done at 5 (T) or 0°C (T'). The factors with a significant effect for $\alpha = 0.05$ are underlined.. A: apoptosis, As: nuclei suspected of apoptosis, G: giant nuclei, HD: heterochromatin decondensation, N: normal nuclei, NE: necrosis

Change in shock temperature from 35 to 40°C or from 5 to 0°C had no effect on the frequency of heterochromatin decondensation in single heat and cold shock assays, respectively (Tables III, IV). However, it promoted a decrease in frequency of this phenotype when sequential shocks were given (Tables III, IV).

Normal nuclear phenotype - The frequency of this phenotype did not differ when heat (p: 0.179) or cold (p: 0.187) sequential shocks were compared to the unshocked group, but decreased relative to that of insects subjected to the single mild shock (p: 0.018 - heat shock, p: 0.011 - cold shock). On the other hand, the frequency of normal nuclei in the groups subjected to sequential shocks was larger than in the group subjected to a severe shock only (p: 0.036).

Change in frequency of normal nuclei with the temperature of the preliminary or the single shock was only verified in the heat shock assay (Table III). The frequency of normal nuclei was larger when the temperature of the preliminary heat shock was 40°C.

DISCUSSION

Nuclei with normal phenotype as well as with phenotypes indicative of cell survival (nuclear fusion and heterochromatin decondensation) and cell death (apoptosis and necrosis) were found concomitantly in the Malpighian tubules of *P. megistus* under most of the experimental conditions used. These phenotypes had been previously reported not only after single heat and cold shocks in *P. megistus* (Garcia et al. 2000a, b), but also after single heat and cold shocks and sequential cold shocks in *Triatoma infestans* (Dantas & Mello 1992, Mello et al. 2001, Campos et al. 2002).

Heat shock tolerance has been demonstrated for *P. megistus* in terms of increased specimen survival and molting rates elicited by sequential heat shocks in comparison with single heat shocks (Garcia et al. 2001b). Present results indicate that heat shock tolerance in *P. megistus* also involves morphological changes at the cellular level. The expression of these changes is characterized by decrease in a cell death form (apoptosis) simultaneously to an increase in cell survival responses (Garcia et al. 2000b, Mello et al. 2001), especially when comparisons are made with results obtained after a single severe shock.

The heat shock tolerance in terms of protecting cell mechanisms elicited by sequential heat shocks being more efficient and longer lasting was verified when the temperature of the 1st shock was 40°C in comparison with the shock at 35°C. A more drastic change in temperature from 28 (control) to 40°C is possibly required to activate massive alterations in genomic expression, with numerous specialized metabolic responses thus being triggered (Gasch et al. 2000).

Cold shock tolerance, also demonstrated in *P. megistus* in terms of increased specimen survival and molting rates after sequential cold shock assays (Garcia et al. 2001a), was equally accompanied by changes in frequency of specific cell nuclear phenotypes. However, the frequency of these changes were not always the same as those observed after sequential heat shocks. Apoptosis, for instance, decreased significantly after sequential cold shocks only in comparison with unshocked controls. While cell/nuclear fusion increased with sequential heat shocks, this form of cell survival (Wigglesworth 1967, Mello 1989, Mello et al. 2001) decreased with sequential cold shocks.

As regards the cellular response to sequential cold shocks when the temperature of the 1st shock was 0°C in comparison to 5°C, it differed from the situation considered in the sequential heat shock assays. A more drastic change in temperature from 28 to 0°C revealed a reduced ability or need of the cells to cope with this condition when examining the decrease in frequency of heterochromatin decondensation in relation to that promoted when the 1st shock was given at 5°C. However, this difference in frequency of nuclear phenotypes did not affect the insect survival rate (Garcia et al. 2001a).

The frequency of necrosis was differently affected when heat and cold shock assays were compared to each other. It increased significantly under sequential cold shock conditions. As regards the response to this experimental condition, the same agents which induce decrease in frequency of apoptosis may be responsible for increase in frequency of necrosis with advancing time after shocks. Among these factors, change in the cellular ATP/AMP ratio promoted by the stress might be causing a sudden decrease in cellular ATP concentrations (Hardie 1999, Gasch et al. 2000) thus favoring metabolic conditions to death through necrosis.

In conclusion, as with specimen survival (Garcia et al. 2001a,b), different patterns of changes in the nuclear phenotypes were induced by sequential heat and cold shocks in *P. megistus*. These results demonstrate that different sequential thermal shocks can elicit different mechanisms of cellular protection against stress in this insect. This is in agreement with observations in other cell models showing that although a large set of genes (~ 900 in yeast cells) shows a similar response to almost all environmental changes, additional features of the genomic responses are specialized for specific conditions (Gasch et al. 2000). This variable capacity may allow *P. megistus* to adapt to various ecotopes over a wide geographic range

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Effect of starvation in association with refeeding and heat shock on nuclear phenotype changes in *Panstrongylus megistus* (Burmeister)

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Summary

The effect of fasting in association with refeeding and heat shock on nuclear phenotypes of the blood-sucking hemipteran, *Panstrongylus megistus*, was studied cytologically in Malpighian tubules of fourth instar nymphs. The insects were fasted for up to 100 days at 28°C and their survival was followed daily. Groups of nymphs were separated each month, with part of the group being refed and the other part kept fasting. Insects in each of these subgroups received either a heat shock at 40°C for 1 h or where maintained at 28°C (no heat shock). The Malpighian tubules were removed 1 and 7 days after each assay and subjected to the Feulgen reaction for identification and counting of the nuclear phenotypes. Insect survival was high (90%) after 40 days but decreased of starvation thereafter. Necrosis, rather than apoptosis, increased with fasting, suggesting that this species may not have developed efficient mechanisms for dealing with this stress factor. Feeding after fasting acted as a mild stressing agent and increased the frequency of apoptosis but not of necrosis. Heat shock did not affect responses to the starvation and refeeding. A decrease in the frequency of apoptosis, of necrotic nuclei and of nuclei with heterochromatin decondensation was shown in well-fed compared with fasted or fasted then refed insects.

Panstrongylus megistus, a blood-sucking hemipteran, is one of the most important vectors of Chagas' disease, because of its wide geographical distribution, high rates of infection with *Trypanosoma cruzi* and varied domiciliar behaviour (Forattini, 1980). *P. megistus* is a native Brazilian species the domiciliation of has increased markedly as a result of habitat destruction which has resulted in the elimination or reduction of natural food resources (Silveira, 2000). Chagas' disease affects 20 million people, mostly in Central and South America, and is the third largest cause of death by infectoparasitary diseases in Brazil (Silveira & Rezende, 1994; dos Reis, 1997). Although a decrease in the rural human population favors a reduction in the vectorial transmission of Chagas' disease in Brazil, this route of transmission is still the most frequent, thus reinforcing the relevance of studies on the biology of blood-sucking hemipterans and their responses to several stressing agents, as part of control programs and for rearing these insects in the laboratory (Rodrigues et al., 1991; Silva & Silva, 1993; Garcia et al., 1999; Schmunis, 2000).

The 1st to 5th nymphal instars of blood-sucking hemipterans require blood meals every 3 - 4 weeks whereas adults need to feed every two weeks (Garcia & Azambuja, 2000). These periods are sufficient for the insects to digest their meals. In the case of nymphs, the indicated period above is necessary for distension of the insect abdomen and stimulation of the prothoracic glands as well as secretion of the molting hormone ecdysone (Wigglesworth, 1984). The frequency of feeding in these hemipterans depends on the temperature and relative humidity of the environment, as well as on the species considered, and the time required for each moult (Garcia & Azambuja, 2000). In addition to ingesting an amount of blood many times greater than their body weight, blood-sucking hemipterans may also fast for long periods (Sherlock, 1979).

Changes in the nuclear phenotypes indicative of cell survival and cell death have been described in blood-sucking hemipterans subjected to several stressing agents, including fasting (Mello & Raymundo, 1980; Mello, 1978, 1989; Mello et al., 1995, 2001). Cell and nuclear fusions may occur during fasting to produce degrees of somatic ploidy normally seen with DNA endoreplication (Mello, 1978; 1989; Mello & Raymundo, 1980; Andrade & Mello, 1987). In addition to nuclear fusion, fasting also results in an increase in the

frequency of heterochromatin unraveling as a form of cell survival, as well as in the frequency of apoptosis and necrosis (Andrade & Mello, 1987; Mello, 1989; Mello et al., 2001).

Refeeding has been suggested to be a stressing factor when practiced after prolonged starvation (Burdon, 1986). However, the survival of starved specimens of the blood-sucking hemipteran *Triatoma infestans* was unaltered by subsequent feeding (Mello, 1989). In the laboratory at Sucen (Mogi-Guaçu-SP), *P. megistus* specimens are often subjected to short periods of fasting in order to extend their developmental cycle (V.L.C.C. Rodrigues umpublished data). This routine procedure provides a good model for investigating whether refeeding acts as an additional stressing factor in fasted insects.

In the present study, the frequency of nuclear phenotypes was investigated in Malpighian tubule cells of *P. megistus* nymphs subjected to fasting with or without refeeding. Since heat shock also affects the frequency of nuclear phenotypes in *P. megistus* (Garcia et al., 2000a), the effect of associating this stressing agent with fasting and refeeding was also examined.

Materials and Methods

Insects, experimental conditions and cell preparations

Fourth instar nymphs of a domestic population of *P. megistus* (Hemiptera, Reduviidae) reared at 28°C and 80% relative humidity in the laboratory at Sucen (Mogi-Guaçu, SP) were used.

The insects were fasted for up to 100 days and monitored daily to score changes in their survival rate. Groups of nymphs were separated each month, with part of the group being refed, while the other part was kept fasting. Insects in each of these subgroups subsequently received a heat shock at 40°C for 1 h before being returned immediately to the control temperature (28°C) or were maintained at 28°C all the time (no heat shock). One and 7 days later, the nymphs were dissected to remove their Malpighian tubules which were mounted *in toto* on glass slides, fixed in ethanol-acetic acid (3:1, v/v) for 1 min, rinsed in

70% ethanol for up to 5 min and then subjected to the Feulgen reaction with hydrolysis for processed for 65 min in 4 M HCl. The Malpighian tubules were subsequently rinsed with three washes in sulfurous water (5 min each) and one wash in distilled water before being air dried. The preparations were cleared in xylene and mounted in Canada balsam. As a general control group, insects fed once a week were also examined.

The total number of Feulgen-stained Malpighian tubule epithelial cell nuclei and the number of different nuclear phenotypes were counted in each specimen. Photomicrographs were obtained using a Zeiss Axiophot 2 microscope (Oberkochen, Germany).

Statistics

The survival rates of fully-nourished and fasted insects were compared using the Kaplan-Meyer test (Kaplan & Meyer 1958). The frequencies of nuclear phenotypes in Malpighian tubules under the various experimental conditions were compared using ANOVA after convenient stabilization and normalization of the data using an arc sin transformation (Little & Rubin 1987).

Results

The survival rate in fasted insects was high (90%) after up to 40 days drastically thereafter. After 100 days of fasting, the survival rate of the fasted nymphs was approximated 30% (Fig. 1). Comparison of the survival rates of fasted and well-nourished nymphs using the Mantel-Hantzel test (Kalbfleish & Prentice, 1980) showed that there was a significant difference between the survival curves of these two groups with a decreased survival in fasted specimens ($p = 8.35e^{-14}$).

Normal nuclei containing one small heterochromatic body predominated in nymph Malpighian tubules under the various conditions. The phenotypk alterations consisted of apoptotic nuclei (A), nuclei suspected of apoptosis (As), necrotic nuclei (NE), and nuclei with heterochromatin decondensation (HD) (Fig. 2). Giant nuclei were not observed.

The frequencies of the nuclear phenotypes in fasted and refed specimens with or without heat shock are shown in Tables I and II. The effect of fasting time, refeeding, heat shock and time after heat shock on the various phenotypes was analysed statistically, as shown in Table III.

Apoptosis – Apoptosis was not elicited by starvation time of starvation (Table I). However, refeeding induced apoptosis, especially after fasting for one month (Tables II and III). Heat shock decreased the frequency of apoptosis, especially shortly after its application.

Nuclei suspected of apoptosis – The frequency of this phenotype decreased significantly with the duration of fasting (Tables I and III), but was affected variably by heat shock in fasted specimens. Heat shock increased suspected apoptosis only a one month fast. Refeeding either increased the frequency of suspected apoptosis shortly after a one month fast or reduced it in refed specimens subjected to heat shock (Table II). Refeeding with or without subsequent heat shock had aviable effect on the frequency of nuclei suspected of apoptosis in nymphs fasted two and three months (Table II). In specimens refed after the a three month fast, the frequency of nuclei suspected of apoptosis increased seven days after the heat shock. The unpredictable change in the frequency of this phenotype according to

the stress conditions meant the effect of "feeding" was not significant in establishing cause-effect relationships (Table III).

Necrosis – Necrosis was observed with increasing duration of fast (Table I). Refeeding with or without subsequent heat shock increased the frequency of necrosis only in specimens fasted for three months (Table II). However, the effect of "refeeding" was geneerally not significant for this nuclear phenotype (Table III). Heat shock significantly increased the frequency of necrotic nuclei (Table III) but there was no association between heat shock and fasting or heat shock and refeeding.

Heterochromatin decondensation - This phenotype was not affected with increasing duration of fasting (Tables I and III). Only in insects fasted for one month did heat shock increase the frequency of this phenotype. No significant differences were observed after general analysis (Table III). On the other hand, refeeding decreased the frequency of heterochromatin decondensation mainly in non-shocked insects one day after a one month fast (Tables II and III).

Normal nuclei – The frequency of nuclei with a normal phenotype decreased significantly with increasing duration of starvation. Heat shock decreased the frequency of this phenotype, especially in specimens fasted for three months (Tables I and III), but there was generally no association between heat shock and starvation. Refeeding after starvation had no effect on the frequency of this phenotype (Tables II and III).

There was a significant decrease in the frequency of nuclei suspected of apoptosis ($p = 0.012$), of necrotic nuclei ($p = 0.017$), and of nuclei with heterochromatin decondensation ($p = 0.000$) in well-fed insects compared with fasted or fasted then refed insects (Tables IV and V).

Discussion

The fact that nearly 50% of the nymphs were alive 80 days after fasting is in agreed with reports showing that blood-sucking hemipterans can withstand long periods of fasting (Sherlock 1979; Mello & Raymundo, 1980; Mello, 1989). Resistance to starvation is of great epidemiological importance because under unfavorable conditions the insects may seek refuge in holes in house walls where they can remain long enough for them to escape from the effects of harmful agents. This behaviour increases the insects' opportunities for reproduction and resettlement (Braga & Lima, 2001).

The large standart deviations observed for some of the nuclear phenotype frequencies indicated considerable individual variations in the length of survival such as seen after the three month. The finding that only the frequency of necrotic nuclei increased with fasting time in *P. megistus* nymphs, suggested that this species may be unable to develop efficient mechanisms of cell survival (heterochromatin unravelling, for instance) such as those seen in *Triatoma infestans* under similar physiological conditions (Mello, 1989; Mello et al., 2001).

The absence of giant nuclei in the Malpighian tubules of fourth instar nymphs of *P. megistus* subjected to a three month starvation differed from a previous report showing that this nuclear phenotype appears after a four and five month starvation in adults and in late fifth instar nymphs of this species (Mello & Raymundo, 1980). These results mean that a longer period of fasting is required to induce such a cell response in *P. megistus* and that nuclear and cell fusions in response to starvation occur only in more advanced developmental phases. This conclusion agrees with the observation that, with exception of adults, the greater tolerance to starvation has been verified with increasing developmental phases (Cabello, 2001). The decrease in the frequency of normal nuclei with increasing fasting suggested that nuclear elimination accompanied cell death during fasting.

The increase in the frequency of apoptosis in insects refed after fasting indicated that feeding after fasting acted as a stressing factor and that no protective mechanism, at least in terms of heterochromatin decondensation, was elicited simultaneously. However, the stress

induced by refeeding was not severe enough to increase the frequency of necrosis (Samali & Cotter, 1996). The decrease in the frequency of almost changed nuclei in well-fed insects compared with fasted and refed specimens reinforces the effect of these variables as stressing factors.

Heat shock did not generally act as a stressing factor in addition to starvation and refeeding since it did not significantly affect the frequency of the various nuclear phenotypes observed. This finding agreed with the resistance of fasted specimens of *T. infestans* to stress factors such as heavy metals and enhanced tolerance to sequential cold shocks (Mello et al., 1995; Campos et al., 2002).

P. megistus is the principal vector of *T. cruzi* in the eastern, southern and some northeastern states of Brazil (Barbosa et al., 2001). Since the ability of this insect to withstand and recover from periods of inadequate or poor nutrition is an important adaptation for the survival and reproduction of the species, and since the frequency and duration of periods of nutritional stress may influence the species' geographic and temporal distribution (Hervant et al., 2001), the data presented here contribute to our understanding of the behavioural responses of *P. megistus* under natural and laboratory conditions.

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Figure legends

Figure 1. Kaplan-Meyer estimates for the survival curves of fully-nourished (dashed line) and fasted (full line) *P. megistus* nymphs

Figure 2. Nuclear phenotypes of Feulgen-stained *P. megistus* Malpighian tubules. A: apoptosis; As: suspected apoptosis; NE: necrosis; N: normal; HD: unraveling heterochromatin. Magnification: a-b: 440x; c-e: 1100x.

Table I. Frequency of nuclear phenotypes in fasted *P. megistus* nymphs subjected to heat shock at 40°C for 1 h.

Fasting period (months)	Time after heat shock (days)	Nuclear phenotypes												
			A		As		NE		HD		N		Total	
		n	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD
1	1 (unshocked control)	3	1.0	1.7	2447.7	1329.2	1270.0	425.0	74.3	106.9	16690.3	1719.4	20483.3	1114.3
	7 (unshocked control)	3	0.3	0.6	2321.3	662.5	621.7	81.5	383.3	377.9	13254.0	1539.9	16580.7	1131.7
	1 (shocked group)	3	0.0	0.0	4193.0	2853.4	1984.0	603.0	1598.3	1791.4	12348.7	3219.7	20123.0	2635.9
	7 (shocked group)	2	0.0	0.0	4690.0	3302.2	1728.5	853.5	849.0	773.6	10802.0	411.5	18069.5	5340.8
2	1 (unshocked control)	3	0.0	0.0	310.7	154.4	799.0	171.5	415.7	311.6	13636.3	3648.4	15161.7	4036.9
	7 (unshocked control)	3	0.0	0.0	99.7	130.2	1284.3	1189.3	190.7	27.4	9902.3	2650.9	11465.7	3524.0
	1 (shocked group)	2	0.0	0.0	166.0	14.1	993.0	927.7	302.5	277.9	9794.0	2295.3	11255.5	2959.2
	7 (shocked group)	3	0.0	0.0	151.3	81.1	990.7	1483.1	153.7	87.1	12529.0	1405.2	14436.0	1681.5

3	1 (unshocked control)	3	3.0	5.2	49.3	18.5	921.3	936.9	411.0	360.6	10685.3	4106.2	12624.0	4162.8
	7 (unshocked control)	2	2.0	2.8	131.0	2.8	4978.0	2153.8	116.0	89.1	10587.0	1145.5	15814.0	3394.1
	1 (shocked group)	4	0.0	0.0	77.8	43.2	1088.8	1411.0	207.2	298.0	8139.8	1610.8	9521.7	2379.0
	7 (shocked group)	2	0.0	0.0	15.0	21.2	1385.5	6.4	350.5	272.2	7856.0	2965.6	9607.0	3265.4

n: number of insects; A: apoptosis, As: nuclei suspected of apoptosis, G: giant nuclei, HD: heterochromatin unravelling, , N: normal nuclei, NE: necrosis, SD: standard deviation, X: arithmetic mean

Table II. Frequency of nuclear phenotypes in refed *P. megistus* nymphs subjected to heat shock at 40°C for 1 h.

Fasting period (months) followed by feeding	Time after heat shock (days)	Nuclear phenotypes												
			A		As		NE		HD		N		Total	
		n	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD
1	1 (unshocked control)	3	2.3	1.5	3270.3	281.3	1172.0	568.4	13.0	11.1	14174.0	1965.9	18630.7	2332.1
	7 (unshocked control)	3	9.0	4.6	2231.3	1931.8	1812.3	1270.3	373.0	292.1	15951.7	1377.8	20377.3	2159.6
	1 (shocked group)	3	0.0	0.0	2693.0	823.6	1654.7	545.4	345.0	254.6	13204.7	766.4	17897.3	1106.2
	7 (shocked group)	3	2.3	2.5	2268.0	1850.6	2154.3	541.4	250.0	197.1	14834.7	1671.1	19509.3	1599.9
2	1 (unshocked control)	4	2.5	4.3	315.2	370.4	933.0	471.4	86.5	73.4	9671.2	1907.4	11008.5	2293.3
	7 (unshocked control)	3	0.3	0.6	765.7	1033.9	740.7	526.4	819.3	945.9	11273.7	1262.2	13599.7	1096.8
	1 (shocked group)	3	1.3	2.3	245.0	243.1	1088.7	1592.6	189.3	246.8	10284.0	1627.6	12949.3	2217.6
	7 (shocked group)	2	0.0	0.0	230.0	233.3	943.5	748.8	447.0	506.3	11032.5	3182.7	12795.0	2361.7

3	1 (unshocked control)	3	2.3	4.0	84.3	71.7	1406.7	2277.2	69.3	18.4	8214.0	2165.8	10357.7	3925.8
	7 (unshocked control)	3	1.7	1.1	138.7	112.5	1811.3	1458.1	149.0	200.0	10003.7	563.7	12104.0	2232.8
	1 (shocked group)	3	0.0	0.0	120.7	96.3	3169.0	847.2	220.7	136.1	10825.0	662.1	14335.3	1086.8
	7 (shocked group)	3	1.0	1.0	520.0	283.1	1751.3	1324.5	248.0	324.8	8055.3	2545.8	11270.0	2940.0

n: number of insects; A: apoptosis, As: nuclei suspected of apoptosis, G: giant nuclei, HD: heterochromatin unravelling, , N: normal nuclei, NE: necrosis, SD: standard deviation, X: arithmetic mean

Table III Analysis of variance (ANOVA) for each nuclear phenotype in *P. megistus* Malpighian tubules of *P. megistus* under various experimental conditions.

Nuclear phenotypes	Effects			
	Heat shock	Duration of Fasting	Refeeding	Time of cytological analysis
A	0.004	0.967	0.005	0.539
As	0.680	0.000	0.563	0.822
NE	0.035	0.000	0.151	0.428
HD	0.115	0.756	0.153	0.287
N	0.05	0.035	0.49	0.462

Effects significant at $p \leq 0.05$ are underlined.

Table IV. Frequency of nuclear phenotypes in fully-nourished, fasted and refed *P. megistus* nymphs subjected to heat shock at 40°C for 1 h.

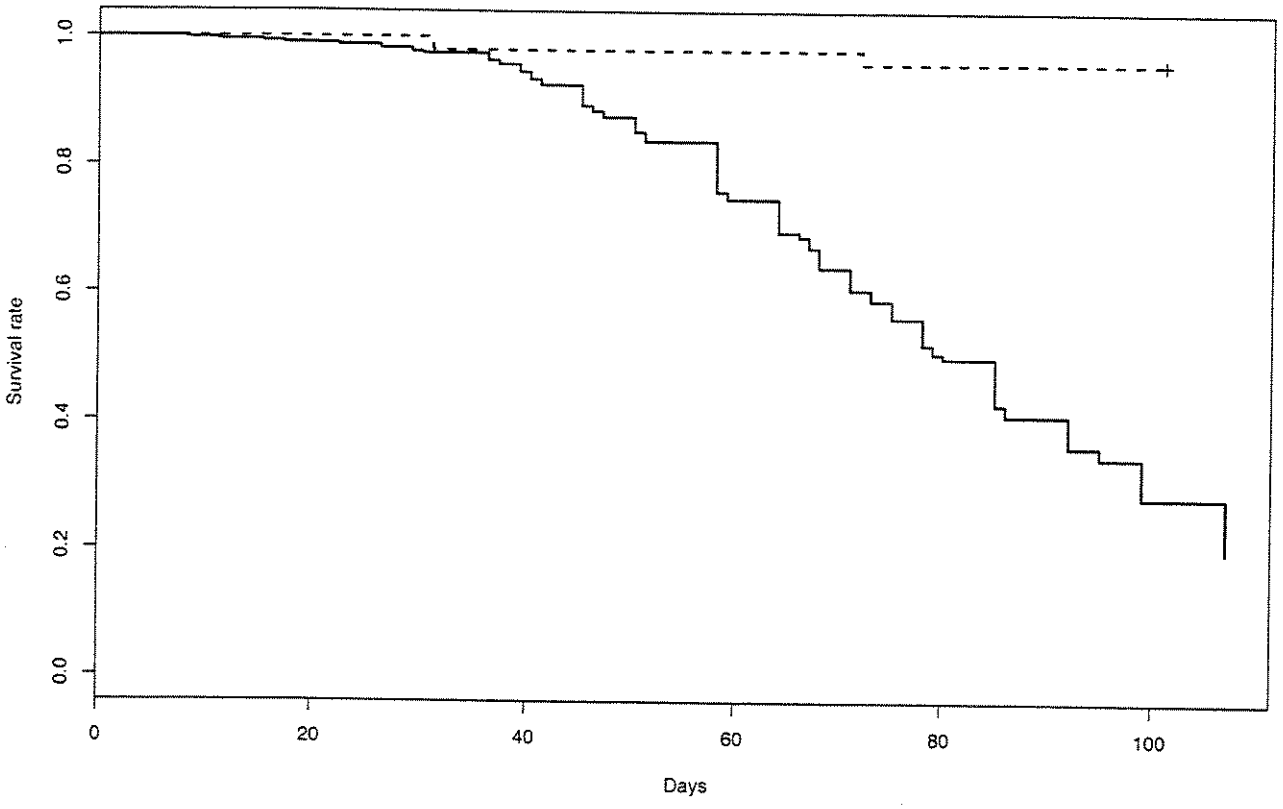
Feeding conditions	Nuclear phenotypes												
		A		As		NE		HD		N		Total	
	n	X	SD	X	SD	X	SD	X	SD	X	SD	X	SD
Fully feel													
Unshocked	13	0.8	2.5	142.8	138.2	797.5	429.8	31.5	19.1	10669.2	2205.2	11640.9	2378.6
Shocked	12	3.3	10.3	115.7	91.9	541.5	375.0	70.5	78.9	10827	2323.8	11558.5	2425.9
Fasted													
Unshocked	17	1.0	2.4	938.1	1225.6	1547.5	1516.9	273.9	263.9	12569.3	3372.5	15327.9	4033.7
Shocked	16	0.0	0.0	1442.9	2460.1	1382.8	972.0	568.0	896.8	10255.8	2655.6	13726.7	4921.5
Fasted + refed													
Unshocked	19	3.0	3.9	1091.1	1430.7	1467.6	1038.8	243.0	439.6	11449.3	3065.4	14170.6	4421.1
Shocked	17	0.8	1.5	1058.8	1318.8	2061.6	940.1	273.7	242.7	11392.7	2735.7	14910.2	3482.8

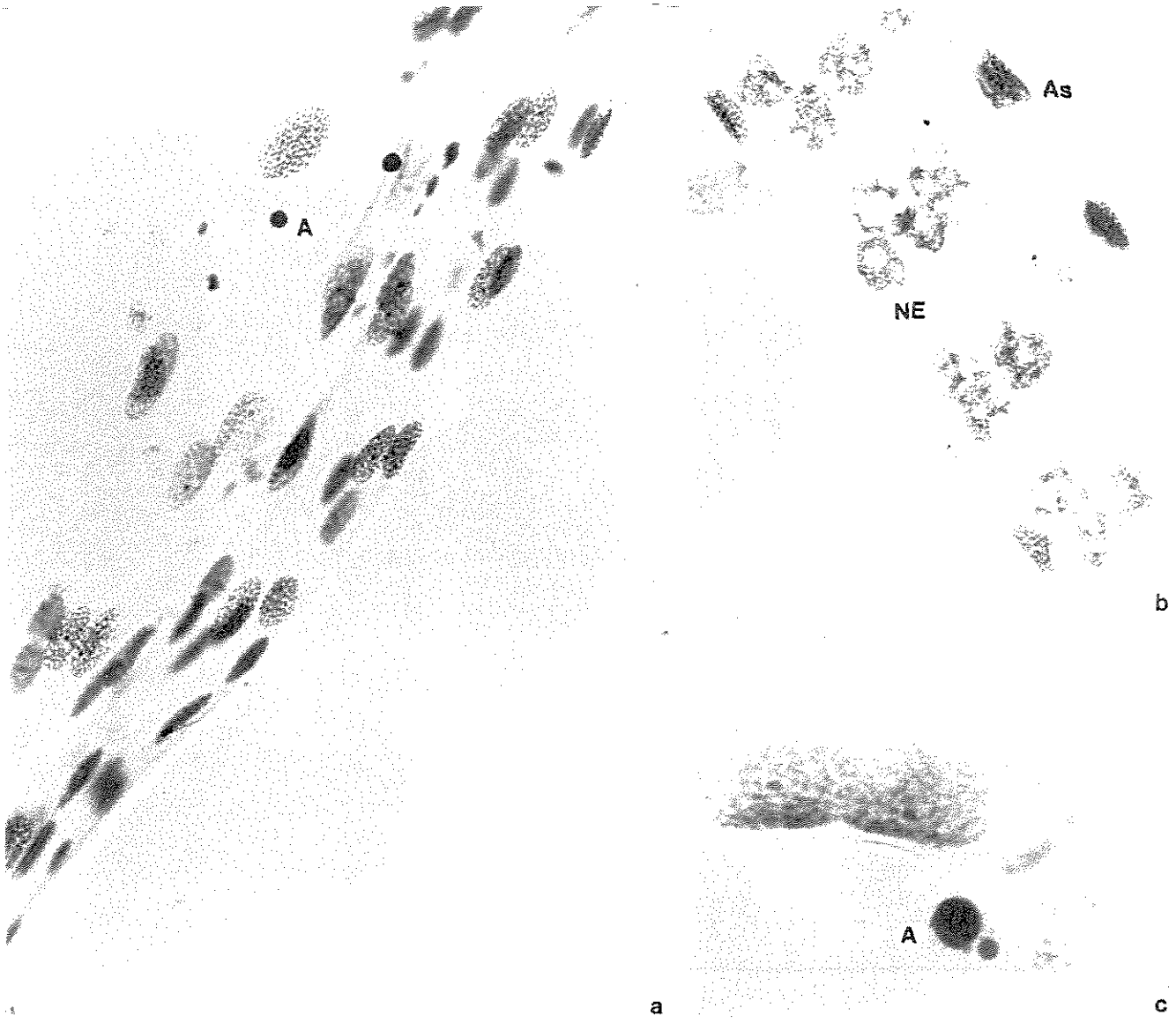
n: number of insects; A: apoptosis, As: nuclei suspected of apoptosis, G: giant nuclei, HD: heterochromatin unravelling, , N: normal nuclei, NE: necrosis, SD: standard deviation, X: arithmetic mean

Table V. Analysis of variance (ANOVA) for each nuclear phenotype under various experimental conditions (fully nourished, fasted, fasted + refed).

	Effects	
Nuclear phenotypes	Nutritional Conditions	Shock
A	0.204	0.116
As	0.012	0.802
NE	0.017	0.284
HD	0.000	0.051
N	0.000	0.199

Effects significant at $p \leq 0.05$ are underlined.


Survival rate
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80
100
Days


A
As
NE
b
a
A
c
N
HD
d
e

Survival and molting rate, nuclear phenotypes, prevalence and intensity of infection, and metacyclogenesis in the blood-sucking insect, *Panstrongylus megistus*, infected with *Trypanosoma cruzi* and subjected to heat shock

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Short title: *T. cruzi* infectivity in *P. megistus*

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Abstract

Survival and molting rate, frequency of nuclear phenotypes, and positivity and frequency rates of *Trypanosoma cruzi* as well as metacyclogenesis incidence were investigated in the blood-sucking insect, *Panstrongylus megistus*, inclusive after heat shock (40°C, 1 h). The strain Y of *T. cruzi* was used. The number of epimastigotes and trypomastigotes was determined in the insect's rectal gland content and the frequency of nuclear phenotypes was determined for the insect's Malpighian tubules. Heat shock did not elicit differences in the insect survival and molting rate in infected as well as in non-infected groups neither affected the prevalence and magnitude of infectivity. Differences between the parasite number at the beginning and at the end of the study were found when shocked and non-shocked insects were compared to each other. Decrease in number of epimastigotes and trypomastigotes indicating changes in multiplication of the parasite and in the metacyclogenesis were induced by heat shock. The factors infection and heat shock, isolatedly, promoted increase in frequency of nuclei suspected of apoptosis, but did not affected the frequency of the other cell death phenotypes. In addition, the heat shock significantly elicited heterochromatin unravelling, a cell survival response to the stress.

Key words: *Panstrongylus megistus*, *Trypanosoma cruzi*, heat shock, metacyclogenesis, nuclear phenotypes

Trypanosoma cruzi, the etiological agent of Chagas' disease, is a flagellate protozoan with variable patterns of virulence, pathogenicity and infectivity in animals (Contreras et al., 1998) This pool of parasites circulating among humans, insect vectors, and wild and domestic animals displays morphological and functional variations in its complex life cycle (Garcia & Azambuja, 1991). Populations of *T. cruzi* also differ in their ability to survive, multiply and differentiate in vector insects (Lana et al., 1998).

Our understanding of the vector-parasite relationship is limited and studies of metacyclogenesis in triatomines have been mainly descriptive. Quantitative studies are difficult to compare because the population density of the parasites is influenced by several factors, and also because metacyclic forms are eliminated in the feces of the vector insect in small amounts (Piesman & Sherlock, 1985; Contreras et al., 1998).

Several factors are important in the parasite-vector interaction. These include the parasite strain and aspects related to the vector species, such as its genetic characteristics and developmental stage (Garcia & Azambuja, 1991). In contrast, neither the number of parasites present in the blood nor the amount of blood ingested are determining factors in the infection of blood-sucking insects (Miles et al., 1975; Neal & Miles, 1977; Mello & Chiarini, 1980). Although many aspects of this relationship have yet to be established (Mello & Chiarini, 1980), *T. cruzi* is an excellent model for studying fundamental biological questions (Souza, 2000).

The factors that regulate metacyclogenesis and maintain high rates of metacyclic forms in insects are unknown. Parasite development may depend on and be controlled by molecular factors in the digestive tract of the insect, which is considered a hostile environment. The release of secretions in the digestive tract, alterations in the epithelial system after blood ingestion, and the excretion and attachment of *T. cruzi* to the surface of the rectal gland epithelium in the insect vector are important factors in this process (Garcia & Dvorak, 1982; Garcia & Azambuja, 1991; Souza, 2000).

Although *T. cruzi* is pathogenic in humans and other mammals, there is no concrete evidence that it is harmful to blood-sucking insects (Lima et al., 1992). According to Tobie (1961), the criteria for assessing pathogenicity in arthropods are poorly defined, with the main criterion being an increase in the mortality rate. In the case of *T. cruzi*, the influence

of infection on the reproductive cycle of triatomines requires further study (Lima et al., 1992).

The use of xenodiagnosis in the chronic phase of Chagas' disease, either as a diagnostic aid or in conjunction with the administration of drugs against trypanosomes, has led to a search for vector species which are more susceptible to *T. cruzi*, and that could serve as experimental models (Silva et al., 1994). The suitability of blood-sucking insects is an important factor in the epidemiology of Chagas' disease (Mello & Chiarini, 1980). The principal criterion for identifying a vector insect as an experimental model is its ability to maintain a high level of parasites after feeding on infected hosts (Szumlewicz et al., 1990). In the case of *T. cruzi*, some of the vector species can potentially limit human and animal infections with *T. cruzi* because they do not favor metacyclogenesis (Szumlewicz & Moreira, 1994).

Panstrongylus megistus (Burmeister) is a reduviid triatomine species with a wide distribution, high rates of infection with *T. cruzi* and an ability to colonize artificial ecotopes (Forattini, 1980). This insect is a native Brazilian species for which domestication is directly related to a reduction in its normal sources of nutrition (Silveira, 2000). *P. megistus* is currently considered the principal vector of *T. cruzi* in the eastern, southern and some northeastern states of Brazil (Barbosa et al., 2001). The efficiency of this species, especially its extraordinary capacity for fast evolution and the rapid multiplication of a few parasites ingested in the chronic phase, are well documented (Lima et al., 1992; Szumlewicz et al., 1988). Other advantages include its occurrence in many parts of Brazil, the ease with which it breeds under changing environmental conditions and its rapid growth, slow locomotion and low mortality (Szumlewicz et al., 1988).

A knowlege of the best conditions for rearing triatomine insects in the laboratory could help to explain many of their responses seen in the wild (Rodrigues et al., 1991; Silva & Silva, 1993; Garcia et al., 1999, 2000a, b; Schmuñis, 2000). The increasing interest in *P. megistus*, that in the past was considered a secondary species, and the epidemiological priorities for new fronts for agricultural occupation (Dias, 2000), make it necessary to understand how this species responds to environmental conditions, especially thermal shocks.

Changes in survival, molting, and nuclear phenotypes have been verified in *P. megistus* after single or sequential shocks (Garcia et al., 1999, 2000 a, b, 2001a, b) and may be similar in specimens infected with *T. cruzi*. In this study, we examined the influence of heat shocks on the prevalence and intensity of infection of *P. megistus* by *T. cruzi* and on the level of metacyclogenesis. Changes in the nuclear phenotypes of infected insects were also analyzed.

Material and methods

Mammalian hosts.

T. cruzi Y strain was obtained from the Institute of Tropical Medicine of the University of São Paulo (USP) and was maintained in the laboratory at Sucen by successive passages in male Swiss mice (21 days old). The mice were inoculated intraperitoneally with 0.1 ml of infected mouse blood containing ~ 10^6 parasites. All mice developed parasitemia and, since the number of parasites inoculated was high, the mice were used 4 days after infection. The number of flagellates in each animal was determined by a modification of Pizzi and Prager's procedure (Andrade, 1974). Blood (5 µl) was collected from mice using a micropipet dipped in liquemine heparin (5.000 I.U./ml). The blood was placed on a glass slide under a coverslip and the number of parasites was determined in 50 microscopic fields (40x) multiplied by the correction factor of the microscope.

Insects.

Two hundred fourth instar nymphs of *P. megistus* (Burmeister) bred in the laboratory at Sucen were fasted for 30 days. A group of 100 specimens were allowed to feed on infected mice and then placed in glass cylinders covered with cheesecloth and fastened with elastic bands. Each cylinder containing eight insects was weighed before and after insect feeding. From this group, 50 nymphs were subjected to heat shock (40°C for 1 h) after two days (group 1). The remaining nymphs were maintained at 28°C, the temperature used to rear *P. megistus* in the laboratory (group 2). After feeding, the few non-engorged specimens were replaced. Another 100 insects were fed on chicken blood.

After 2 days, 50 of these insects were also subjected to heat shock (group 3) and the remaining ones were maintained at 28°C (group 4).

Since short heat shocks (40°C, 1 h) do not affect the insect rate survival and molting (Garcia et al., 1999), these shocks were used to investigate whether infection with *T. cruzi* would have an additional effect on the various parameters investigated. The survival and molting rates were monitored for 45 days after infection. At the end of this period, the proportion of insects positive for *T. cruzi* and the intensity of the infection (Szumlewicz & Muller, 1987) were determined. This period for assessing infection is considered optimal for the examination of insects fed on infected mice (Szumlewicz & Muller, 1987). The intensity or magnitude of the parasite density was determined using the classification of Szumblewicz & Muller (1987), in which the parasite counts in 50 microscopic fields are scored as low (1-5), moderate (6-10), dense (11-100) and very dense (≥ 101).

Insect preparations.

The examination of the infected insects involved sectioning the terminal portion of the insect abdomen and pulling out its rectal gland. This tissue was then placed on a glass slide with 100 µl of PBS. The gland contents were macerated and 5 µl of the homogenate was then placed on a microscope slide and covered with a coverslip. The number of parasites was determined as for the parasite counts in blood (dilution of feces in PBS: 5:100) (Andrade, 1974).

When the insect appeared to be free of parasites, another smear of the same material was examined. Only the rectal gland was used in order to maintain the Malpighian tubules intact for other experiments and also because the rectum contains 2-5 times more parasites than the small intestine (Schaub, 1989). Epimastigotes were distinguished from metacyclic trypomastigotes based on morphology and their motility in a fresh fecal sample viewed with a 40x objective (Piesman & Sherlock, 1985).

In insects positive for *T. cruzi* and in insects fed on chicken blood (groups 3 and 4), the Malpighian tubules were removed and fixed in ethanol: acetic acid solution (3:1, v/v) for 1 min, then rinsed in 70% ethanol for 5 min and subjected to the Feulgen reaction with acid hydrolysis in 4 M HCl at 25°C for 65 min. The Feulgen-stained material was rinsed in

sulfurous and distilled water, air dried, cleared in xylene and mounted in Canada balsam. A Nikon light microscope was used to count the total number of Malpighian tubule epithelial cell nuclei per specimen and to identify the different nuclear phenotypes and their frequencies.

The statistical analyses were done using the Weibull distribution parametric test, the Mantel-Hantzel non-parametric test (influence of stress on survival and molting rates and influence of infection on survival rate), a homogeneity test for subpopulations (influence of stress on the ingestion of parasites), the exact Fisher test (influence of stress on the prevalence and intensity of infection), the chi-square test (influence of stress on multiplication and metacyclogenesis), the likelihood ratio test (influence of stress on parasite numbers at the beginning and at the end of the study), and analysis of variance with stabilization and normalization of variables (influence of stress and infection on nuclear phenotypes) (Kalbfleish & Prentice, 1980).

Results

Influence of stress on the ingestion of parasites. Table 1 shows the number of parasites ingested by insects in each glass cylinder. The chi-square and homogeneity tests showed there was no evidence for rejecting the null hypothesis since there was no difference in the numbers of ingested parasites in shocked and non-shocked insects (P = 0.5596). Heat shock did not alter the prevalence of infection, since $P = 0.41$.

A frequency of 77.7% for *T. cruzi* positivity in non-shocked insects (n = 36) and 71.8% of positivity for shocked insects (n = 39) was verified.

The influence of stress on the magnitude of infection of parasites in 50 microscopic fields showed that there was no significant difference in the intensity of parasites between shocked and non-shocked (Table 2). When low and moderate densities were combined and compared with dense and very dense densities (also combined) there was still no difference between shocked and non-shocked insects (P = 0.085). There was no statistical evidence to reject the assumption of homogeneity in the two groups of treatments.

Influence of stress on the insect survival and molting rates. The insect survival and molting results are presented in Table 3. Heat shock did not alter the survival rate in

infected and non-infected insects (P = 0.671 and 0.380, respectively; distribution of Weibull and the Mantel-Hantzel test) (Fig. 1). This lack of effect was also true of the insect molting rate (P = 0.3370 and 0.142, respectively) (Table 4; Fig. 2).

Influence of the T. cruzi infection on insect survival rate and molting. 99% of the infected specimens survived until 45 days, whereas only 88% of the non-infected insects survived a similar period. In contrast, infection decreased the insect molting frequency from 44.8% in non-infected insects to 26.3% in the infected group. These results were confirmed by the distribution of Weibull and the Mantel-Hantzel test (Table 4, P = 0.035 and 0.001, respectively).

Influence of heat shock on metacyclogenesis. Table 5 shows the number of epimastigotes and trypomastigotes in shocked and non-shocked insects. The chi-square test showed that shocked insects had fewer epimastigotes and trypomastigotes compared to non-shocked insects (P = 0.00001). In heat-shocked insects, the proportion of trypomastigotes also decreased (P = 0.048).

Likelihood ratio test. Since the values for this test were greater than the chi-square limit of 3.84, there was strong evidence of a significant difference between the parasite numbers at the beginning and end of the study. Indeed, there was a decrease in the number of parasites in shocked and non-shocked groups after 45 days. For this test, the death of some insects were considered.

Nuclear phenotypes. The nuclear phenotypes of the Malpighian tubules of *P. megistus* have been previously described (Mello et al., 1986; Garcia et al., 2000a, b). The normal nuclear phenotype in males shows a small heterochromatic body with contains several copies of the Y chromosome (Mello et al., 1986). The other phenotypes are indicative of cell survival (decondensed heterochromatin, nuclear fusion) and cell death (apoptosis, necrosis) responses under stress conditions (Garcia et al., 2000a,b). Tables 6 to 9 show the number of nuclei with specific phenotypes in *P. megistus* specimens with or without *T. cruzi* infection which were or were not subjected to the heat shock.

Both the infection and heat shock significantly elicited the increase in frequency of nuclei suspected of apoptosis (Table 10). These nuclei were assumed to be undergoing apoptosis on the basis of their morphological and deep stainability aspects (Garcia et al.,

2000a, b). Infection with *T. cruzi* increased the frequency of this phenotype from 1.0% to 1.6%, as also did heat shock (from 1.0% to 1.7%). In heat-shocked females, the mean percentage of this phenotype with the heat shock increased from 0.6% to 1.9%, whereas in males it remained close to 1.4%. Infection had a significant effect on males ($P = 0.01$) and heat-shock affected females ($P = 0.01$). In males, the heat shock also induced heterochromatin unravelling, which increased from 0.02% to 0.07%. No single factor or interaction had a significant effect on the other nuclear phenotypes.

Discussion

The frequency of 71.8% for *T. cruzi* positivity in insects subjected to heat shock and 77.7% in non-shocked insects agreed with the value of 91.2% verified by Szumlewicz & Muller (1987) and 98% reported by Alvarenga & Bronfen (1984) for *P. megistus* infected with the Y strain of *T. cruzi*. However, in the present study, heat shock did not cause a drastic fall in infection ($P = 0.412$).

The high percentage of dense positivity observed in this study (68.9% in non-shocked insects) also agreed with the findings of Szumlewicz & Muller (1987) showing 90% positivity, in which over 60% of the insects harbored dense or very dense parasite populations after feeding on animals infected with the Y strain of *T. cruzi*. Heat shock did not significantly change the magnitude of infection in the insect. The individual variations seen in the group exposed simultaneously to the same infection could have been caused by factors such as different capacities of nutrient assimilation by the parasite. Alternatively, the accumulation of a toxic metabolic end product may have caused premature death of the parasite (Szumlewicz & Muller, 1987). All of these factors could result in the spontaneous elimination of parasites or in a reduction in their numbers to levels below detection (Szumlewicz & Muller, 1987). Prolonged starvation of blood-sucking insects has deleterious effects on the multiplication and persistence of their parasites (Szumlewicz & Muller, 1987). On the other hand, the provision of additional blood meals to the insect accelerates parasite multiplication and stimules epimastigote division and subsequent differentiation to

metacyclics (Piesman & Sherlock, 1985; Szumblewicz & Muller, 1987). When insects for xenodiagnosis are maintained for examination at intervals exceeding the first 15 days after feeding on infected blood, they have been fed on chicken blood at two week intervals (Szumblewicz & Muller, 1987). In this study, although the specimens were maintained for 45 days without feeding, this period cannot be considered prolonged starvation since, when fasted for up to 4 weeks, the insects still contain food to be digested (Garcia & Azambuja, 2000). We also used insects without additional feeding on chicken blood, in accordance with methods used at Sucen (Mogi-Guaçu), and to avoid additional stress associated with manipulation or possible drastic changes in temperature once the insects were removed from the insect laboratory.

As reported by Szumblewicz and Moreira (1994), metacyclic forms appeared in the digestive tract of the insects 30-60 days after infection, and on the 45th day, the proportion of epimastigotes varied from 83.2% to 92.9%. After 90 days, there was a dramatic fall in the proportion of epimastigotes which coincided with the increased proportion of metacyclic forms (16.1% to 37.9%). In the rectum, from 4 to 9 weeks after infection, the trypomastigote forms were 3-11% and 15-25%, depending on the *T. cruzi* strain (Schaub, 1989). After this period, the trypomastigotes increased to 30 to 50%, concomitantly with a decrease in the percentage of epimastigotes.

In our study, epimastigotes were also present in a high proportion, generally from 90% to 100% at 45 days after feeding on infected animals. The lower frequencies of trypomastigotes compared to that reported by Schaub (1989), probably reflected the lack of an additional blood meal offered to the insects. Although comparison of well nourished and starved insects has shown that 3 to 4 weeks without feeding does not reduce the rectal total population of *T. cruzi*, metacyclogenesis increases after feeding (Schaub & Losch, 1988).

The statistical analysis proved that epimastigotes and tripomastigotes were present in greater number in insects infected but not heat-shocked, indicating that the multiplication of parasites and metacyclogenesis were affected by heat shock. The influence of temperature on *T. cruzi* in cell cultures, in experimentally infected animals, and in insects infected naturally or in the laboratory is well known (Wood, 1954; Neves,

1971). In the latter, cold temperatures (0°C, 5°C) inhibit the evolution of *T. cruzi*, which, in *Triatoma infestans*, is maintained in blood forms until 35 days. Higher temperatures (36-37°C) for 40 days inhibited the evolution of *T. cruzi*, especially its multiplication, giving rise to a small number of parasites (Neves, 1971).

Lima et al (1992) have shown that in 1st and 2nd instar nymphs of *P. megistus* infected with the strain VLE-9, the number of ecdyses, the development and the mortality remain constant whilst reproduction (egg production, fertility, number of fertile eggs and female longevity) is significantly reduced. In the present study, using 4th instar nymphs and infection with the Y strain of *T. cruzi*, infected insects survived more than non-infected insects, while there was a decrease in their molting rate in association with infection.

The high rate of survival in infected specimens could be a consequence of an increase in heat shock protein production in these insects since one of the main cellular protective mechanisms against stress is the production of heat shock proteins (Lindquist, 1986). These proteins may be produced in response to the presence of the parasite, thereby enhancing the insects' ability to respond to other stressful stimuli.

The decrease in molting rate in infected insects may reflect interference in the hormonal cycle during infection, associated with the nutritional state of the insects.

Heat shock did not decrease the *P. megistus* survival and molting rates, but decreased *T. cruzi* multiplication and metacyclogenesis. The increase in nuclei suspected of apoptosis in infected insects suggested that the presence of the parasite induced this cell death program. The presence of nuclear phenotypes suggestive of cell survival may contribute to this parasite-host interaction. Of the parameters studied, the alterations in the *T. cruzi* multiplication were of great importance. Once the effect of this stressor in the parasite-vector system is known, the manipulation of infected insects during experimentation can be regulated better, by avoiding additional stressors such as changes in temperature, handling, etc.

The changes associated with infection and heat shock, especially the multiplication and metacyclogenesis of *T. cruzi* and the mechanisms of resistance developed by the vector, can contribute to our understanding of the vector-parasite system, epidemiological

patterns, and adequate rearing of *P. megistus* in the laboratory. Such knowledge is important since this species is a very convenient model for use in xenodiagnosis (Szumlewicz et al., 1988; Lima et al., 1992; Pereira et al., 1996).

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Table 1. Concentration of parasites in mouse blood vs. the number of parasites ingested by insects per glass cylinder (1-6: shocked insects; 7-12: non-shocked insects).

Glass cylinder code	Number of parasites per 5µl of blood (x 95.0)	Blood volume sucked by insects in each glass cylinder (µl)	Number of parasites ingested in each glass cylinder
1	880	417	6,972,240
2	657	237	2,958,471
3	835	435	6,901,275
4	664	606	7,645,296
5	2942	321	17,943,258
6	1551	258	7,603,002
7	1798	276	9,428,712
8	1149	382	8,339,442
9	793	739	11,134,513
10	1535	765	22,311,225
11	428	598	4,862,936
12	875	602	10,008,250

Table 2. Influence of heat shock on the intensity of *T. cruzi* infection. Parasite density in 50 microscopic fields after 45 days of infection: low (+): 1-5; moderate (++): 6-10; dense (+++): 11-100; very dense (++++): ≥101.

Heat shock	Magnitude of infection				Number of insects
	+	++	+++	++++	
Yes	13	2	13	0	28
No	6	3	17	2	28

Fisher test: $P = 0.1573$

Table 3. *P. megistus* survival and molting rates 45 days after heat shock. The number of insects used in each case was 50.

T. cruzi infection	Heat shock	Survival		Molting rate	
		N	%	N	%
No	No	45	90	26	52
No	Yes	42	84	18	36
Yes	No	50	100	14	28
Yes	Yes	49	98	12	24

Table 4: Insect survival curves and molting rates compared according to the Weibull distribution.

		Survival			Molting		
Group	Comparison:	Coefficient	Z	*P*	Coefficient	z	*P*
I	S vs. NS	0.087	0.425	0.671	0.203	-0.96	0.337
NI	S vs. NS	- 0.450	-0.875	0.380	0.754	1.47	0.142
T	I vs. NI	2.350	2.110	0.035*	1.080	3.29	0.001*
	S vs. NS	- 0.556	-1.060	0.288	0.432	1.36	0.174
S	I vs. NI	1.870	1.710	0.088**	0.836	1.81	0.069**
NS	I vs. NI	16.810	0.010	0.992	1.300	2.81	0.050*

I (infected, n = 100); NI (non-infected, n = 100); T (total insects; n = 200); S (heat-shocked insects, n = 100), NS (non-shocked insects, n = 100)

* = significant differences at 5% (P <0.05)

** = significant differences at 10% (0.05<P<0.10)

Table 5. Epimastigote and tripomastigote numbers in 50 microscopic fields per specimen. Glass cylinders: 1-6: shocked insects; 7-12: non-shocked insects.

Glass cylinder	Number of parasites		Total
	Epimastigotes	Tripomastigotes	
1	57	0	57
2	111	0	111
3	76	0	76
4	148	1	149
5	38	1	39
6	68	2	70
7	83	8	91
8	216	31	247
9	116	71	187
10	111	4	115
11	131	10	141
12	111	3	114

Table 6. Absolute frequencies of the nuclear phenotypes in Malpighian tubule epithelial cells of *P. megistus* 4th instar nymphs.

	Nuclear phenotypes						
Insect	A	A_s	NE	G	HD*	N	Total
I_1	0	80	975	0	35	14036	15126
I_2	0	144	1373	0	23	9775	11315
I_3	0	228	1017	0	4	9158	10407
I_4	0	89	942	0	24	9269	10324
I_5	0	520	1203	0	50	13604	15337
I_6	0	137	453	0	5	7963	8586
I_7	0	128	281	0	--	11384	11793
I_8	0	170	696	0	--	13499	14365
I_9	0	22	551	0	17	11995	12585
I_{10}	0	120	631	0	33	13418	14202
I_{11}	0	301	453	0	--	13604	14358
I_{12}	0	109	477	0	--	14918	15504
I_{13}	0	30	1606	0	--	9392	11028
I_{13}	0	95	307	0	15	8631	9048
I_{14}	0	296	326	0	36	11277	11935
I_{15}	0	57	1618	0	46	11315	13036
I_{16}	0	104	789	1	--	10216	11114
I_{17}	5	32	804	4	--	9415	10260
I_{18}	0	52	439	0	--	9515	10006
I_{19}	0	75	708	2	--	12516	13301
I_{20}	1	59	282	0	66	11006	11414
I_{21}	9	10	689	1	55	7253	8017

A: apoptosis; A_s: suspected apoptosis; NE: necrosis; G: giant nuclei; HD: heterochromatin unravelling; N: normal nuclei.; *: phenotype detected only in male nymphs; --: female nymphs

Table 7. Absolute frequencies of the nuclear phenotypes in Malpighian tubule epithelial cells of 4th instar *P. megistus* nymphs infected with *T. cruzi*.

	Nuclear phenotypes						
Insect	A	A_s	NE	G	HD*	N	Total
I_1	0	364	718	0	19	8429	9530
I_2	0	206	1010	13	19	14126	15374
I_3	2	510	619	1	1	15457	16590
I_4	3	136	665	0	8	12613	13425
I_5	4	85	1057	2	10	8779	9937
I_6	0	170	448	0	6	10001	10625
I_7	0	82	1008	0	--	11760	12850
I_8	0	72	633	0	--	14053	14758
I_9	0	322	820	0	27	11787	12956
I_{10}	0	272	827	0	41	9665	10805
I_{11}	0	57	431	0	--	12863	13351
I_{12}	0	23	286	0	--	10612	10921
I_{13}	0	93	492	4	23	11330	11942
I_{14}	0	143	333	0	7	12183	12666
I_{15}	4	268	627	0	32	13063	13994
I_{16}	0	102	558	0	8	10500	11168

A: apoptosis; A_s: suspected apoptosis; NE: necrosis; G: giant nuclei; HD: heterochromatin unravelling; N: normal nuclei; *: phenotype detected only in male nymphs; --: female nymphs.

Table 8. Absolute frequencies of the nuclear phenotypes in Malpighian tubule epithelial cells of 4th instar of *P. megistus* nymphs subjected to heat shock at 40°C for 1h.

	Nuclear phenotypes						
Insect	A	A_s	NE	G	HD*	N	Total
I_1	36	23	1387	1	28	10517	11992
I_2	1	103	879	0	18	13291	14292
I_3	0	154	503	0	12	14718	15387
I_4	0	363	458	0	52	13579	14452
I_5	0	54	5267	3	--	13754	19078
I_6	0	147	533	0	--	10187	10867
I_7	0	188	845	0	--	10628	11661
I_8	0	63	896	1	8	11705	12673
I_9	0	45	297	1	--	7134	7477
I_{10}	0	263	551	0	--	8089	8903
I_{11}	0	156	446	0	16	8282	8900
I_{12}	0	154	251	0	--	10890	11295
I_{13}	0	64	340	0	42	11520	11966
I_{14}	0	20	761	0	91	8385	9257
I_{15}	0	57	208	2	219	7669	8155
I_{16}	0	105	174	0	243	8822	9344
I_{17}	0	83	529		--	11713	12325
I_{18}	0	53	169	0	--	8437	8659
I_{19}	0	155	87	1	56	9532	9831
I_{20}	3	126	359	0	61	11904	12453

A: apoptosis; A_s: suspected apoptosis; NE: necrosis; G: giant nuclei; HD: heterochromatin unravelling; N: normal nuclei; *: phenotype detected only in male nymphs; --: female nymphs.

Table 9. Absolute frequencies of the nuclear phenotypes in Malpighian tubule epithelial cells of 4th instar *P. megistus* nymphs infected with *T. cruzi* and subjected to heat shock at 40°C for1h.

	Nuclear phenotypes						
Insect	A	A_s	NE	G	HD*	N	Total
I_1	0	279	200	5	--	7310	7794
I_2	0	934	900	1	--	10404	12235
I_3	0	106	573	0	--	9628	10307
I_4	0	272	930	0	--	10300	11502
I_5	0	224	373	2	34	8659	9292
I_6	0	219	535	0	46	10325	11125
I_7	0	160	246	2	24	7689	8121
I_8	1	78	698	1	49	7000	7815
I_9	0	359	167	0	--	6719	7245
I_{10}	0	109	361	0	--	8783	9253
I_{11}	0	141	335	0	50	8522	9048
I_{12}	2	63	185	2	--	10510	10762
I_{13}	0	5	226	1	140	5742	6114
I_{14}	0	67	205	0	--	6782	7054
I_{15}	0	314	266	0	13	8910	9567
I_{16}	0	210	740	0	--	10207	11157

A: apoptosis; A_s: suspected apoptosis; NE: necrosis; G: giant nuclei; HD: heterochromatin unravelling; N: normal nuclei; *: phenotype detected only in male nymphs; --: female nymphs.

Table 10: Analysis of variance (ANOVA) with two factors (infection with *T. cruzi* and heat shock) for the various nuclear phenotypes. Phenotype frequencies significant at $P<0.05$ are underlined.

Nuclear phenotype	*T. cruzi* infection	Heat shock	Infection x Heat shock
A	0.68	0.82	0.81
As	<u>0.03</u>	<u>0.02</u>	0.11
NE	0.18	0.44	0.95
G	0.37	0.19	0.34
HD (*)	0.76	<u>0.01</u>	0.30
N	0.48	0.64	0.57

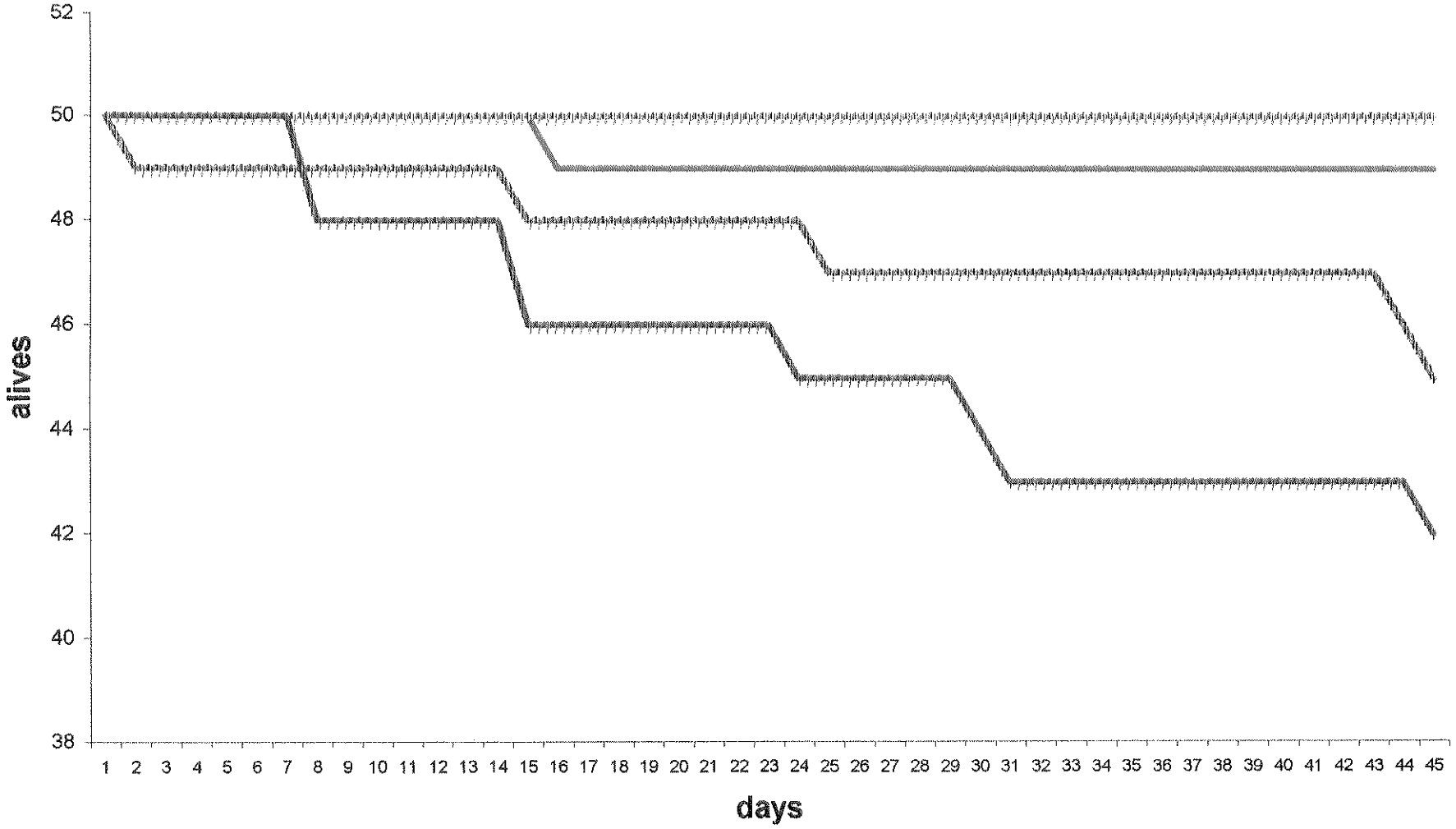
A: apoptosis; A_s: suspected apoptosis; NE: necrosis; G: giant; HD: heterochromatin unraveling; N: normal nuclei.

* phenotype detected only in male nymphs.

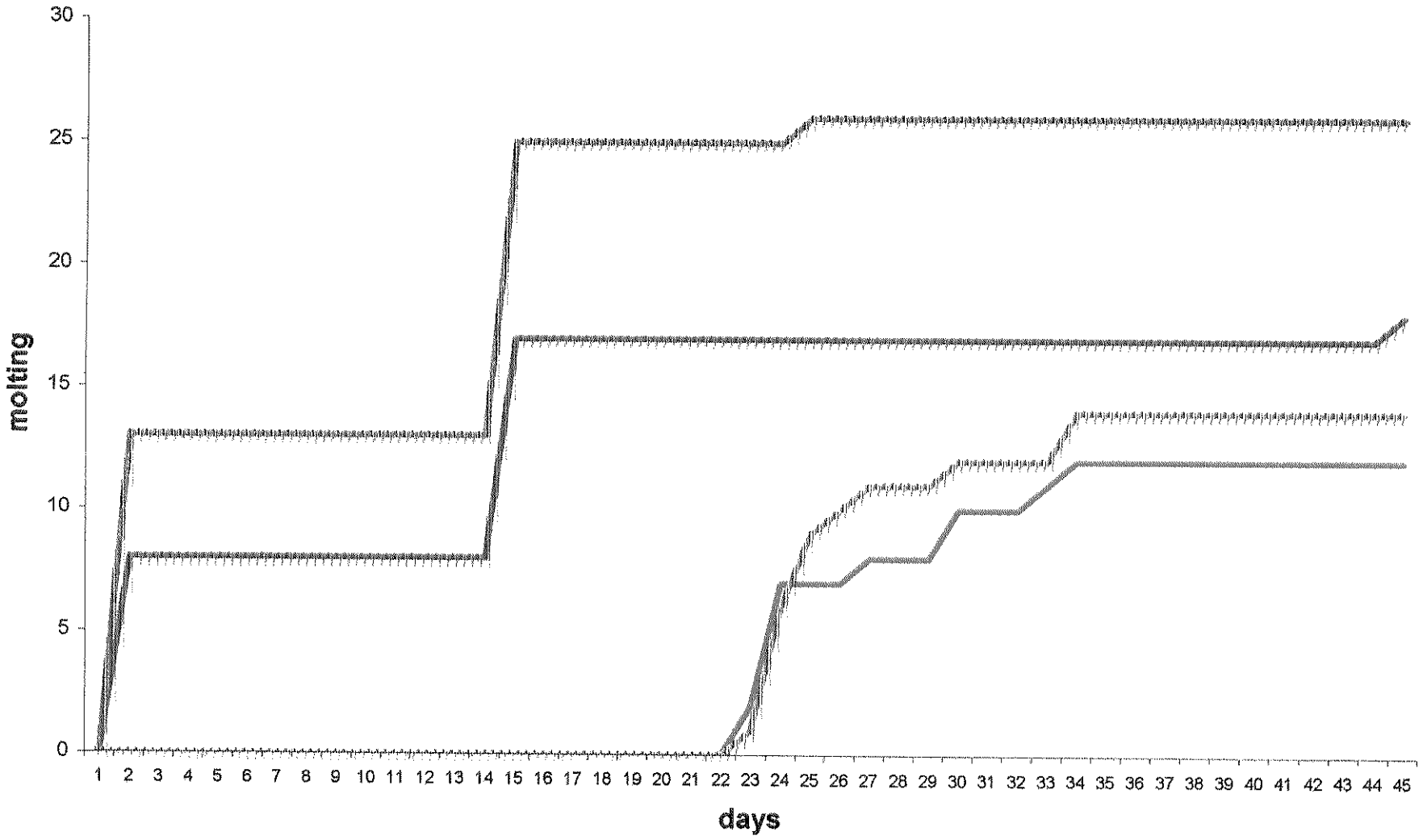
Figure 1. Survival curves for *P. megistus* in the absence or presence of infection with *T. cruzi* and heat shock. Insects infected with *T. cruzi* and subjected to heat shock (green); Insects infected with *T. cruzi* but not shocked (pink); Uninfected insects subjected to heat shock (blue); Uninfected non-shocked insects (brown).

Figure 2. Molting curves for *P. megistus* in the absence of presence of infection with *T. cruzi* and heat shock. Insects infected with *T. cruzi* and subjected to heat shock (green); Insects infected with *T. cruzi* but not shocked (pink); Uninfected insects subjected to heat shock (blue); Uninfected non-shocked insects (brown).

Survival curves of *P. megistus* subjected or not to infection with *T. cruzi* and heat shock



Molting curves of *P. megistus* subjected or not to infection with *T. cruzi* and heat shock



Investigação imunocitoquímica de HSP 70 em túbulos de Malpighi de *Panstrongylus megistus* submetidos a choques de temperatura.

Introdução

É bem conhecido que todos os organismos respondem a diversos tipos de estresse por ativação de um número limitado de genes específicos para a produção de proteínas de choque térmico ou de estresse (HSP), os quais são previamente silentes ou ativas em baixo nível quando na ausência de estresse (Lindquist, 1986; Komatsu et al., 1996). Entre os indutores da resposta ao estresse, além do choque térmico, temos metais tóxicos, álcoois, agentes quimioterápicos, inibidores metabólicos, privação de oxigênio, jejum, hormônios esteróides, prostaglandinas, análogos de aminoácido, agentes anti-oxidantes, infecções viróticas, inflamação, entre outros (Kelley & Schlesinger, 1978; Ashburner & Bonner, 1979; Johnston et al., 1980; Hightower & White, 1981; Nevins, 1981; Burdon et al., 1982; Li & Shrieve, 1982; Sciandra & Subjeck, 1983; Thomas & Mathews, 1984; Ananthan et al., 1986; Lindquist, 1986; Amaral et al., 1988; Welch, 1993). Tem sido sugerido que o aumento em proteínas de choque térmico (HSP) seja o principal fator de proteção celular contra os efeitos do estresse. Porém, em condições de estresse severo, essas proteínas podem ser incapazes de proteger as células e apoptose poderia ser ativada. Com a intensificação ainda maior do estresse, morte por necrose torna-se o modo proeminente de morte celular (Samali & Cotter, 1996).

O número e o tipo de proteínas codificadas varia, com os diferentes organismos e tipos de células, embora predominantemente situem-se nas faixas de 68-110kDa e 15-30 kDa (Burdon, 1986). Entre as várias HSP, as mais estudadas têm sido as da família HSP 70, proteínas altamente conservadas durante a evolução, reportadas em uma ampla variedade de células, desde arqueobactérias, várias espécies de algas a todas as classes de cordados (Lewis et al., 1999). São proteínas sintetizadas sob condições de estresse ou não, que atuam como importantes componentes de vários modelos celulares, podendo também se ligar às cadeias nascentes de polipeptídeos, participando no arranjo conformacional da cadeia protéica, ou alternativamente, minimizando ou solubilizando agregados de proteínas denaturadas resultantes de vários estresses celulares (Lindquist, 1986; Welch, 1990; Krebs & Feder, 1998). Essas HSP parecem, portanto,

envolvidas na estabilização ou eliminação de proteínas indesejáveis ou danificadas (Hahn et al., 1991). Muitos estudos também demonstram um importante papel dessas proteínas na aquisição de termotolerância ou resistência termal (Lindquist, 1986; Lindquist & Craig, 1988; Sanchez & Lindquist, 1990; Mosser & Martin, 1992; Komatsu et al., 1996). Entretanto, apesar de extensos trabalhos na literatura sobre as HSP, não está ainda bem compreendido como essas proteínas conferem termotolerância em diversos organismos (Krebs & Feder, 1998). Um fator que aumenta a dificuldade na compreensão desse mecanismo é a não constatação, por diversos autores, de aumento na concentração de HSP 70 em células termotolerantes ou ausência de linearidade entre alterações na concentração dessa proteína e termotolerância (Fisher et al., 1986; Hatayama et al., 1991; Landry et al., 1991; Krebs & Feder, 1988).

As HSP 70 apresentam a mais ampla atividade específica entre as proteínas de estresse, podendo em princípio serem mais facilmente detectadas. Recentemente, o aumento na atividade específica de HSP 70 dentro de um sistema biológico tem sido usado como um indicador de estresse (Lewis et al., 1999). Algumas vezes, a clara presença ou ausência de indução de HSP 70 pode servir como marcador, podendo ser utilizadas técnicas imunocitoquímicas para localização dessas proteínas. Em outras situações, um aumento gradual na quantidade de HSP 70 leva a necessidade de medição, sendo o Western blotting, slot blotting, radioimunoensaios, enzima ligada a imunoabsorbância (ELISAs), alguns exemplos de métodos utilizados para quantificar proteínas de estresse (Lewis et al., 1999).

Embora proteínas de choque térmico não tenham sido investigadas em triatomíneos, em *P. megistus*, resistência ao choque de temperatura ou aumento de tolerância aos choques seqüenciais são típicos do envolvimento de HSP (Lindquist & Craig, 1988; Sanchez & Lindquist, 1990; Vogel et al., 1997; Garcia et al., 1999, 2000, 2001 a, b), embora outros mecanismos possam estar também envolvidos (Denlinger et al., 1991; Chen & Walker, 1993; Lopez-Garcia & Forterre, 2000).

A resposta ao estresse é uma área de pesquisa de grande interesse, visto constituir um excelente modelo para análise de processos básicos envolvidos no controle da expressão gênica, em que organismos e células diferentes podem responder com uma alteração no padrão de síntese protéica de modos diferentes (Burdon, 1986; Bonato & Juliano, 1987; Amaral et al., 1988). Por sua vez, os triatomíneos também se revelam excelentes modelos de estudo de estresse, já que nos túbulos de Malpighi podemos observar diferentes formas de resistência e morte celular

simultaneamente ocorrendo em resposta ao mesmo (Dantas & Mello, 1992; Garcia et al., 2000 a, b; Mello et al., 2001; Campos et al., 2002).

A compreensão do efeito de estressores na síntese de proteínas de choque térmico em *Panstrongylus megistus* se reveste de particular interesse, visto ser essa espécie, atualmente, o principal vetor do Leste, Sudeste e de alguns estados do Nordeste do Brasil (Barbosa et al., 2001).

Neste trabalho, portanto, procurou-se investigar a presença de HSP 70 por imunocitoquímica, em espécimes de *P. megistus* submetidos ou não a choque de temperatura.

Material e métodos

Ninfas machos de 5o. estádio de *Panstrongylus megistus*, criadas e reproduzidas no insetário da Sucen de Mogi-Guaçu, foram mantidas sem alimento por 7 dias. A distinção sexual foi feita de acordo com Rosa et al. (1992). Parte dessas ninfas foi colocada em pequenos cristalizadores cobertos com morim e submetidos a choque de temperatura de 40°C (1 hora). Essa temperatura de choque foi utilizada com base em trabalhos anteriores de choque térmico e aquisição de tolerância (Garcia et al., 1999, 2000, 2001 a). O grupo controle consistiu em insetos mantidos à 28°C, temperatura normalmente usada para criação dessa espécie no insetário da Sucen e umidade relativa de 80%. Imediatamente após o choque e 3 h, 8 h e 24 h após o mesmo, as ninfas foram dissecadas para retirada dos túbulos de Malpighi destinados ao estudo de localização imunocitoquímica de HSP 70. Esses órgãos foram fixados por 1 hora em solução recém-preparada de paraformaldeído (4% em PBS, pH 7,4) reidratados em tampões de PT (PBS + Triton) 2 vezes por 5 minutos e 1 vez por 30 minutos, respectivamente. O material foi, então, colocado em forno de microondas por 10 segundos, em potência alta, e submetido a bloqueio da atividade de peroxidase endógena incubando-se por 20 min em solução de metanol 70% e água oxigenada 3%. Em seguida, foi reidratado em 1:1 metanol: PBS por 5 min., lavado em PBT (PBS + Triton +BSA (albumina de soro bovino)) pelo menos 5 vezes por 10 min. cada, incubados 30 min em PBT + soro de cabra e com anticorpo primário monoclonal (anti-HSP70 Sigma) na concentração de 1:50 em PBT + soro de cabra, por 12h a 4°C. A partir dessa etapa, o material foi lavado em PBT 3 vezes por 5 min e 4 vezes por 30 min., incubado por 30 min. em PBT + soro de cabra e, em seguida, com anticorpo secundário (anti-camundongo - Sigma) por 1 hora, na concentração de 1:300, lavado 3 vezes por 5 min em PBT, 4 vezes por 30 min cada em PBT, 2

vezes por 5 min. cada em TBS (120 mM NaCl, 20mM Tris, pH 7,5), corado no escuro com 0,5ml (100mM Tris pH 7,5, 0,5mg/ml DAB e 0,03% H_2O_2)de 5 a 10 min., lavados 2 vezes por 1 min. em PT (PBS+ Triton) e contra-corados com verde de metila (Patel., 1994).

Resultados

Em tecidos contendo HSP constitutiva ou induzida por estresse, a coloração imunocitoquímica é demonstrada através de uma marcação marrom resultante da revelação por 3-3'-diaminobenzidina (DAB) após a precipitação de íons obtidos da reação das HSP com peróxido de hidrogênio. Tal marcação foi verificada em tecidos carcinogênicos de ratos, fornecidos pelo Departamento de Patologia Clínica da Faculdade de Ciências Médicas (UNICAMP), quando submetidos ao mesmo procedimento utilizado para túbulos de Malpighi de *Panstrongylus megistus* e que nos serviu como controle para confirmação da especificidade dos anticorpos e da adequação do protocolo.

Nos túbulos de Malpighi de *P. megistus* não foi detectada presença de HSP 70 pela falta de marcação no órgão. Essa resposta foi constatada tanto no material do grupo controle quanto nas diversas situações experimentais, ou seja, logo após ou 3 h, 8 h e 24 h após o choque de 40°C (1h).

Discussão

É conhecido que o número e os tipos de diferentes proteínas HSP podem variar nos diferentes organismos e tipos celulares, embora em todos os casos, as proteínas mais abundantes sejam as HSP 84 e 70 (Burdon, 1986). Os resultados encontrados em *P. megistus* estão de acordo com Singh & Lakhotia (1995), os quais verificaram que contrariamente ao dogma geral de que todas as células ou organismos respondem de modo relativamente uniforme ao choque de temperatura (Schlesinger et al., 1982; Lindquist, 1986; Morimoto et al., 1990), em túbulos de Malpighi de *Drosophila*, coloração imunocitoqúimica não evidenciou HSP 70 antes ou após choque térmico, descartando-se a possibilidade de uma não-indução por auto-regulação, ou seja, inibição pela presença de proteínas constitutivamente presentes da família HSP 70. Singh & Lakhotia (1995) constataram que o choque térmico, embora tenha induzido a síntese de alguns

polipeptídeos, não aumentou quaiquer HSPs, detectáveis por imunocitoquímica, ao contrário do observado em glândulas salivares.

Os resultados para *P. megistus* sugerem que não somente em larvas de *Drosophila*, mas também em *P. megistus*, os túbulos de Malpighi falharam em demonstrar a síntese de HSPs em resposta ao choque térmico. Esta ausência de marcação pode ser resultado de um decréscimo na concentração dessas proteínas, resultado de degradação rápida mediada por ação proteolítica (Singh & Lakhotia, 1995) ou alguma peculiaridade metodológica que tenha sido inapropriada ao material. É conhecido que a resposta ao choque térmico pode ser transitória em alguns organismos ou tipos celulares e sustentada em outros, podendo também variar na amplitude de síntese protéica (Lindquist, 1986).

Em *Drosophila*, além dos túbulos de Malpighi, somente células nos últimos estágios de oogênese, oócitos e embriões pré-blastodérmicos, mostram uma falha na resposta típica ao choque de temperatura em termos de indução de HSP (Zimmermann et al., 1983). Planeja-se para o futuro, repetição do protocolo para confirmação de resultados, bem como investigar-se outros órgãos de *Panstrongylus megistus* visando-se a compreensão dos mecanismos envolvidos na resistência desses insetos a diversas situações de estresse.

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VI- CONCLUSÕES

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Tolerância termal –sobrevivência e mudas

1) *Panstrongylus megistus* desenvolve tolerância após choques térmicos seqüenciais nos quais o primeiro choque é dado a 35 ou 40°C (1h) e 5 ou 0°C (1h), seguindo-se um choque mais severo (40 ou 0°C (12h), respectivamente). A intensidade de tolerância desenvolvida difere dependendo da temperatura do choque, do intervalo entre os choques à temperatura ótima de 28°C e da fase de desenvolvimento do inseto.

2) Expressiva tolerância ao calor nos alados é verificada apenas quando o choque preliminar é dado a 40°C e o intervalo entre choques de 8 h, diferindo da resposta das ninfas para as quais a tolerância a choques quentes se estende até quando o período entre choque é de 24h ou mais (choques iniciais: 35 e 40°C, respectivamente). Enquanto aquisição de tolerância ao frio em ninfas acontece em todas as condições estudadas, nos alados é relativamente eficiente apenas quando o choque preliminar é dado a 0°C e o tempo entre choques ultrapassa 18h. Quando a tolerância difere com o sexo, é maior nas fêmeas.

3) Os resultados de tolerância termal sugerem o envolvimento de proteínas de choque térmico (HSP), embora outros mecanismos possam também estar envolvidos. Essa ação protetora aparentemente perde a eficácia com o desenvolvimento do inseto.

Tolerância termal e fenótipos nucleares

1) Modelos diferentes de alterações nos fenótipos nucleares de túbulos de Malpighi são induzidos em *P. megistus*. A tolerância ao choque quente resulta no decréscimo em apoptose simultaneamente a um aumento nas respostas de sobrevivência celular (fusão nuclear e descompactação da heterocromatina). Choques frios seqüenciais não induzem fusão celular/nuclear e elicitam aumento em necrose com o avanço do tempo pós- choques.
2) As temperaturas de 40 e 0°C são mais efetivas que as temperaturas de 35 e 5°C em elicitar tolerância ao calor e ao frio, respectivamente, considerando-se os fenótipos nucleares.
3) Sugere-se que diferentes choques térmicos ativem diferentes mecanismos de proteção celular contra o estresse em *P. megistus*, o que pode favorecer uma adaptação do inseto a vários ecótopos.

Jejum

1) O jejum prolongado, principalmente após 40 dias, aumenta a taxa de mortalidade de ninfas de 4o. estádio de *P. megistus*. O jejum, seguido ou não por choque térmico, age como um estresse severo em espécimes de 4o. estádio. Os insetos submetidos a essas condições não são aptos a desenvolver de forma significante alguns mecanismos de sobrevivência celular. A ausência de núcleos gigantes é explicada pelo tempo de jejum aplicado, sugerindo-se que períodos de jejum

mais longos sejam requeridos para indução de fusão nuclear/celular.

2) O aumento na frequência de apoptose em espécimes realimentadas, sugere que o fator realimentação possa ter atuado também como fator de estresse, porém, menos severo quando comparado à ação do jejum, na qual se constata um aumento na frequência de necrose. O choque térmico não soma um estresse adicional aos fatores jejum e realimentação.

3) Decréscimo na frequência de fenótipos nucleares alterados em espécimes de *P. megistus* alimentados, em comparação a espécimes em jejum ou realimentados após jejum, reforça a idéia da privação alimentar como um fator de estresse e pode contribuir para o entendimento da biologia da espécie.

Infecção por *Trypanosoma cruzi* e choque térmico

1) A infecção por *Trypanosoma cruzi* influencia a taxa de sobrevivência e de mudas de *P. megistus*, sendo que os insetos infectados apresentam uma sobrevida maior, embora um menor número de mudas do que os não infectados.

2) Em espécimes infectados com *Trypanosoma cruzi* e submetidos a choque térmico (40°C, 1 h), o choque quente não influencia a taxa de sobrevivência e de mudas.

3) O choque térmico não influencia o percentual de insetos positivos à *T. cruzi* nem a magnitude da infecção, mas influencia a proporção geral de epimastigotas e tripomastigotas presentes, ocasionando uma redução na frequência observada e sugerindo interferência não só no processo de multiplicação do protozoário mas também na metaciclogênese.

4) A infecção e o estresse por temperatura, isoladamente, têm um efeito significante

na incidência de núcleos com suspeita de apoptose. O estresse também aumenta a frequência de descompactação de heterocromatina.

5) As alterações induzidas pela infecção em associação ao choque térmico, especialmente concernentes à multiplicação e metaciclogênese do *T. cruzi* e os mecanismos de resistência desenvolvidos pelo vetor, podem vir a contribuir para a compreensão do sistema-vetor-parasita, modelos epidemiológicos e criação adequada desses insetos em laboratório.

VII- REFERÊNCIAS BIBLIOGRÁFICAS

VII- Referências bibliográficas

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